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INSECTICIDE RESISTANCE STATUS AND MECHANISMS IN *AEDES* MOSQUITOES IN GHANA

BY

ANISA ABDULAI

(10455167)

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DECLARATION FORM

I, Anisa Abdulai, do hereby declare that the work presented in this thesis entitled "Insecticide Resistance Status and Mechanisms in *Aedes* Mosquitoes in Ghana" is my original work and that this thesis has, neither in part nor in whole, been presented to this University or elsewhere for any degree. I further declare that authors whose work was referred to have been duly acknowledged.

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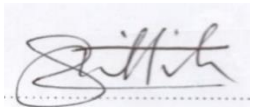
Signature Date

Anisa Abdulai (10455167)

Principal Investigator



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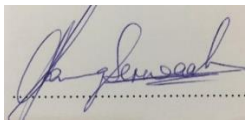
Signature Date

Dr. Simon Kweku Attah

Department of Medical Microbiology (UGMS) (Supervisor)



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Signature Date

Dr. Akua Obeng Forson

Department of Medical Laboratory Sciences (SBAHS)

(Co-supervisor)

DEDICATION

This work is dedicated to my family, supervisors and the insecticide resistance and malaria transmission project (IMAT) for their immense support and contribution to this project. This significant achievement would not have been possible without your love and support.

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LIST OF ABBREVIATIONS

Ace-1	Acetylcholinesterase 1
Ae	*Aedes*
AS- PCR	Allele-specific Polymerase Chain Reaction
Bp	Base pair
Bti	Bacillus thuriengensis
CDC	Center for Disease Control
CYP	Cytochrome P450
CHIKV	Chikungunya virus
DDT	Dichlorodiphenyltrichloroethane
DENV	Dengue virus
DNA	Deoxyribonucleic acid
GPS	Global positioning system
GST	Glutathione-S-transferase
IRS	Indoor residual spraying
Kdr	Knockdown resistance gene
PBO	Piperonyl Butoxide
PCR	Polymerase chain reaction
RT- PCR	Reverse Transcriptase Polymerase Chain Reaction
VGSC	Voltage Gated Sodium Channel
RVFV	Rift Valley Fever
YFV	Yellow fever virus

ZIKV	Zika Virus

ABSTRACT

Background

Aedes mosquitoes are vectors of arboviral diseases such as dengue and yellow fever, which are of major public health concern. Vector control, which relies mainly on the use of insecticides, is the main intervention for arboviral disease control. However, *Aedes* mosquitoes are developing resistance to several classes of insecticides. In Ghana, data about the mechanisms involved in insecticide resistance in *Aedes* mosquitoes is lacking.

Aim of Study

The aim of this study is to investigate the insecticide resistance status of *Aedes* populations and the mechanisms involved in selected urban and suburban settings in Ghana.

Methodology

This is a cross-sectional study carried out in urban sites (Accra and Tema) and suburban sites (Ada and Navrongo) in Ghana. *Aedes* larvae were collected in all the study sites and raised to adults in the insectary. Phenotypic resistance was determined using the WHO susceptibility test. Resistant and susceptible *Aedes* mosquitoes were subjected to morphological identification. Insecticide target site genes were amplified and sequenced to detect mutations conferring genotypic resistance in *Aedes* populations. A synergist assay was also used to determine the involvement of the metabolic enzymes (specifically oxidases) in the phenotypic resistance observed across the sites.

Results

The results showed high phenotypic resistance to Dichlorodiphenyltrichloroethane (DDT) and pyrethroids, with percentage mortalities ranging from 11.3% to 75.8% for those exposed to DDT and 62.5% to 88.8% for those exposed to pyrethroids (deltamethrin and permethrin) in all sites. *Aedes* mosquitoes collected from Tema were found to be resistant

to all the classes of insecticides tested (pyrethroids, organophosphates, organochloride and carbamates). Suspected resistance to carbamate and organophosphates was also detected in some sites. All resistant and susceptible *Aedes* mosquitoes that were morphologically identified were confirmed to be *Aedes aegypti* (100%). High frequency of point mutations at the voltage-gated sodium channel (F1534C and V1016I) were detected in both resistant and susceptible *Aedes aegypti* mosquitoes from all sites. Pre-exposure to Piperonyl Butoxide (PBO) significantly enhanced the susceptibility of *Aedes* to almost all insecticides tested. This may be an indicator that metabolic enzymes (oxidases) may be partially or fully involved in the development of resistance in some *Aedes* populations.

Conclusion

These findings suggest that resistance profiles in *Aedes aegypti* mosquitoes vary across sites in Ghana and seem to be increasing rapidly especially among the pyrethroids and organochlorides (DDT). DDT resistance is widespread across all sites tested. The phenotypic resistance observed are likely to be mediated by multiple resistant mechanisms (genetic and metabolic mechanisms) per the findings obtained. Thus, there is a need to explore ways to effectively control resistant *Aedes* populations to help control arboviral diseases in Ghana.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The *Aedes* mosquito is an important vector for arboviral (arthropod-borne viral) diseases such as Yellow fever, Dengue fever, Chikungunya and Zika virus (Kindhauser *et al*., 2016). The two (2) important species involved in transmission of arboviruses are *Aedes aegypti* and *Aedes albopictus* (Weetman *et al.,* 2018). *Aedes aegypti*, which is the most important of the two (2) species, is found predominantly in urban settings and is the main vector for Dengue fever. *Aedes albopictus* is an extremely invasive species and is spreading rapidly globally. Arboviruses cause serious disease and mortality in humans and have left over four million people with disability (Paixão *et al.,* 2018). Yellow fever virus infects over 200,000 people and causes about 30,000 deaths annually (WHO, 2019). The estimated population at risk of Dengue Fever in Africa is 750 million. Recently, Dengue virus was detected in suspected Malaria and Ebola patients in Ghana (Amoako *et al.,* 2018; Bonney *et al.,* 2018). Also, 21. 6% of children with confirmed malaria in hospitals across Ghana had previous exposure to dengue virus (Stoler *et al.,* 2015).

Currently, there is no registered vaccine for arboviral diseases such as Zika, Rift Valley and Chikungunya. Yellow fever vaccine is widely used worldwide except that funds for regular vaccination are limited (WHO, 2019). A Vaccine for Dengue fever has been recommended by WHO, however, it is not approved for use in all countries and there are reports of vaccination causing severe hemorrhagic fever. There are also no antivirals available for the treatment of arboviruses. Treatment is palliative and mainly to reduce patients symptoms (WHO, 2019, CDC, 2019). Due to these limitations, control and prevention of arboviral infections depend heavily on vector control using insecticides in

combination with larval source reduction and case management. Insecticide classes used in vector control are pyrethroids, organophosphates, organochlorides, and carbamates (WHO, 2009). Pyrethroids are the predominant insecticides for vector control because of their low toxicity and low cost. They are commonly used for indoor and outdoor space spraying to control adult *Ae. aegypti (*Horstick *et al*., 2010). However, the effectiveness of vector control (insecticide based) is threatened by the increasing insecticide resistance in *Aedes* populations worldwide, making insecticide use ineffective and thereby reducing the available alternatives for disease control (Dusfour *et al.,* 2019).

Increasing spread of insecticide resistance is a major challenge that threatens insecticide-based vector control strategies. Resistance of *Aedes* mosquitoes to insecticides has been reported worldwide. Resistance in *Aedes* populations can be mediated by the following mechanisms; target-site mutations (genotypic resistance), metabolic detoxification, reduced penetration of insecticides and behavioural changes (Moyes *et al.*, 2017).

Target site mutations in the domains (II & III) of the voltage-gated Sodium Channel (VGSC) are found to be highly involved in resistance of *Aedes* mosquitoes to pyrethroids (Kawada *et al.*, 2016; Moyes *et al.*, 2017; Granada *et al.*, 2018). Mutations in the VGSC causing resistance to DDT or pyrethroid insecticides are known as Knockdown Resistance (*kdr*) mutations. So far, Eleven (11) *kdr* mutations have been identified in the domains I-IV of VGSC in *Ae. aegypti* (Marcombe *et al.*, 2019). In Ghana, two (2) of these mutations (V1016G and F1534C) have been found to cause resistance to pyrethroids (Kawada *et al*., 2016; Kudom *et al.,* 2019). These mutations differ in their occurrence, their geographical distribution and how they affect phenotypic resistance in mosquitoes. The commonest mutation in *Ae. aegypti* is F1534C (Dusfour *et al*., 2015). This has been found to confer

resistance to permethrin and deltamethrin when it is present with other mutations and is also linked with DDT resistance (Kushwah *et al.*, 2015). The V1016G mutation causes insensitivity to both permethrin and deltamethrin (Moyes *et al.*, 2017). Resistance to organophosphates and carbamates are common in *Aedes* populations but target site mutations in the Acetylcholinesterase gene (Ace-1) do not commonly occur (Marcombe *et al.*, 2009). Ace-1 mutation (G119S) has been found in *Ae. aegypti* and *Ae. albopictus* populations that are resistant to organophosphates (Bisset *et al.*, 2006).

Detoxification enzymes such as oxidases, glutathione-S-transferases (GSTs) and esterases have been implicated in resistance in *Aedes* populations. Cytochrome P450 (Oxidase) overexpression is usually associated with pyrethroid resistance in *Aedes* populations though other enzymes are also involved (Moyes *et al*, 2017). Members of Cyp6 and Cyp9 subfamilies have been associated with resistance of *Ae. aegypti* to pyrethroids (Marcombe *et al.*, 2009). Resistance of *Aedes* mosquitoes to organophosphates has been linked to elevated esterases and GST levels. In both *Ae. aegypti* and *Ae. albopictus*, the alpha-esterases has been found to be the major cause of temephos resistance (Poupardin *et al.*, 2014). Increased GSTs levels are also linked with insecticide resistance in *Aedes* populations (Grigoraki *et al.*, 2015).

1.2 Problem Statement

Epidemics of arboviral diseases such as Yellow fever and Dengue fever have caused a major public health issue in the tropics (Monath, 2001). Yellow fever is an endemic disease in Ghana with major outbreaks occurring in the 1970s in the three northern regions of Ghana (319 cases and 79 deaths) (Appawu *et al.*, 2006). Outbreaks of yellow fever recur every ten to twelve years in the southern part of Ghana (Appawu *et al.*, 2006). Recent

outbreaks occurred in 2016 with 4 cases confirmed by the Ghana health service, 3 in Brong Ahafo region- Jamang South District and 1 case in Volta region- Central Tongu District (www.ghanaweb .com).

Dengue virus has recently been detected in samples from suspected malaria and Ebola children from some parts of Ghana (Amoako *et al.*, 2018; Bonney *et al.*, 2018). There have been outbreaks in neighbouring Burkina Faso and Côte d'Ivoire (WHO, 2017). As at August 2016, Burkina Faso recorded 1,061 cases of Dengue (RDT) positive) out of 1,266 suspected cases reported and a total of 15 deaths as at November 12, 2016 (WHO, 2016). The risk of arboviral outbreak is high, due to the closeness to endemic countries and high density of the vector *Aedes* mosquito in Ghana. Increased movement of people to Ghana from countries where recent arboviral epidemics have been recorded can also facilitate transmission.

Vector control is still the best strategy in the control of these arboviruses. However, resistance of *Aedes* mosquitoes to the insecticides used, is increasing worldwide. Resistance of *Aedes* mosquitoes to various classes of insecticides especially pyrethroids and organochlorides have been reported in Ghana (Kawada *et al*., 2016; Suzuki *et al*., 2016). Despite the fact that resistance is a main threat to control of arboviruses, there is inadequate data on the insecticide resistance status and mechanisms involved in *Aedes* mosquitoes in Ghana.

1.3 Justification

Data on insecticide resistance in *Aedes* mosquitoes worldwide is very patchy with many originating from America and South-East Asia (Moyes *et al*., 2017). In Ghana and Africa,

as a whole, there is also paucity of data on insecticide resistance in *Aedes* mosquitoes (Weetman *et al*., 2018). Few studies done in Burkina Faso, Cameroon and Central African Republic have proven that *Aedes* populations have developed resistance to currently used insecticides especially, pyrethroids and organochlorides (Ngoagouni et al., 2016; Kamgang et al., 2017; Badolo *et al.,* 2019). Earlier studies done in Ghana, confirmed resistance of *Aedes* populations to DDT and pyrethroids (Kawada *et al.*, 2016; Suzuki *et al.*, 2016). Also, studies done in neighbouring countries where outbreaks of dengue fever have been reported, have also confirmed resistance to Carbamates and Pyrethroids (Badolo *et al*., 2019). Though insecticide resistance is rapidly increasing among African *Aedes* populations, very little information about the mechanisms involved in resistance of *Aedes* mosquitoes in Ghana is available. Understanding the insecticide resistance mechanisms of *Aedes* mosquitoes is crucial for fortifying existing control strategies against arboviral vectors. Therefore, it is important to investigate the mechanisms available for the development of insecticide resistance in *Aedes* populations in Ghana. The purpose of this study is to investigate the insecticide resistance status and its mechanisms in *Aedes* mosquitoes in selected sites across Ghana to help improve pre-existing vector control measures to prevent and control future outbreaks of arboviral diseases.

1.4 Aim of Study

- To investigate the insecticide resistance status of *Aedes* populations and the mechanisms involved in Ghana.

1.5 Specific Objectives

- The specific objectives of this study are to investigate, in the sites across Ghana:

  i. The diversity of *Aedes* species

  ii. The role of phenotypic and target-site resistance in *Aedes* mosquitoes.

  ii. The role of metabolic resistance and the impact of piperonyl butoxide (PBO) on the insecticide susceptibility status of *Aedes* mosquitoes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Aedes* Mosquitoes as Vectors of Arboviruses

Aedes mosquitoes originated from the tropical and subtropical zones, but is currently found globally except in Antarctica (Huang *et al.,* 2019). These mosquitoes are uniquely identified by the black and white marks on their bodies and legs. They are active and bite only during the daytime unlike other mosquitoes (Weetman *et al*., 2018). Their biting periods are early mornings and evenings (before dusk). *Aedes aegypti* is the main vector for majority of these arboviruses; however other African *Aedes* species have been found to be competent in disease transmission and are increasing in their range. An example is *Aedes albopictus* which was first transported into Africa about 30 years ago (WHO, 2014; CDC, 2016). Adaptations in some *Aedes* vectors have resulted in serious consequences, as these vectors are becoming epidemiologically significant in the transmission of some arboviruses. For example, a change in amino acid led to the transmission of chikungunya virus by *Ae. albopictus* in many areas in Africa (Weetman *et al*., 2018).

2.1.1 *Aedes aegypti*

Aedes aegypti is recognised by its white marks on its legs and a mark (like lyre) on the top of its thorax. This mosquito is from Africa, from a generalist, zoophilic tree-hole breeder (Brown *et al*., 2014; Weetman *et al.*, 2018). The mosquitoes mostly feed at dusk and dawn and in indoors, shady or cloudy areas (Scott and Takken, 2012). Outside Africa, *Aedes aegypti* prefers to feed on humans indoors and can survive and lay its eggs in a clean water in artificial containers (Mcbride *et al.,* 2014). However, *Ae. aegypti* in Africa breed in artificial containers close to human settlement and in ancestral sylvatic habitat (i.e. natural containers eg rock pools and tree holes) (Powell & Tabacknick, 2013).

Two morphological subspecies of *Aedes aegypti* have been described in Africa, that is, *Ae. aegypti aegypti* and *Ae. aegypti formosus*. *Aedes aegypti aegypti* is light in colour with pale scales on the first abdominal tergite and is strongly anthropophilic, while *Ae. aegypti formosus* is darker and inhabits forested habitats (Crawford *et al.,* 2017). Evidence have shown that *Ae. aegypti formosus* is rapidly inhabiting urban areas (Powell, 2016). The two subspecies were initially distinguished based on colour differences. However, the West African *Ae. aegypti aegypti* with pale scales seems to be genetically alike to the *Ae. aegypti formosus* than *Ae. aegypti aegypti* from other tropical continents (Powell and Tabechnick, 2013). Thus, some scientists classify all African *Aedes* as *Ae. aegypti formosus*, while others still use the original morphological identification (Sylla *et al.,* 2013).

Aedes aegypti is known to be the major vector of dengue viruses globally, mainly due to its competence as a vector and its anthropophilic nature as compared to *Ae. albopictus* (Lambrechts *et al.,* 2010).

2.1.2 *Aedes albopictus*

Aedes albopictus (Asian tiger mosquito) is the most invasive species in temperate regions. *Aedes albopictus*, unlike *Ae. aegypti*, is mainly exophagic (feed outdoors) and feeds both on humans and animals (Paupy *et al*., 2009), but it has been found to possess a strong anthropophilic nature similar to *Ae. aegypti* (Delatte *et al*., 2010).

In recent times, *Aedes albopictus* has spread extensively from Asia to Africa, Americas and Europe through the used tire trade (Weetman *et al.,* 2018). *Aedes albopictus* was first described in Cameroon by Fontenille and Toto (2001). In Cameroon, another study conducted by Simard *et al* (2005) discovered that *Ae. albopictus* was only in the south of the country while *Aedes aegypti* is widespread across the country. Another study in Central

Africa Republic showed that *Aedes albopictus* was more prevalent as compared to *Ae. aegypti* at all sites where both species were found to be sympatric (Kamgang *et al.*, 2013). However, some cities in Gabon have reported high densities of *Aedes albopictus* (Pages *et al*., 2009; Paupy *et al.*, 2010).

Aedes albopictus is able to survive in diverse habitat types. Studies in several African counties such as Cameroon and Central Africa Republic revealed varied breeding sites, from natural sites (such as tree holes and coconut shells) to artificial containers (such as water storage containers and used tyres) (Ngoagouni *et al.*, 2015; Kamgang *et al.*, 2017). *Aedes albopictus* is the main factor for the spread of chikungunya virus since 2004. Also, this increasing spread will increase the risk of transmitting arboviruses in other areas outside urban areas (Weetman *et al.*, 2018).

2.1.3 Other *Aedes* Species

Other *Aedes* species have been found to be very important in arboviral transmission in Africa. This is because they are involved in transmission of sylvatic arboviruses and form a link between human transmission and sylvatic transmission cycles (Haddow *et al*., 1948). In Africa, *Aedes africanus* is known to be the main sylvatic vector of the yellow fever virus. *Aedes furcifer* and *Ae. luteocephalus* transmit sylvatic dengue viruses to animals and can also be transmitted to humans by the bites of *Ae. furcifer* (Hanley *et al*., 2013). These two species belong to *Aedes simpsoni* complex. Other members of this complex include *Aedes bromeliae, Aedes taylori, Aedes metallicus, Aedes opok, and Aedes vittatus* (Haddow *et al.*, 1948). Majority of these species inhabit forested areas and rural areas. Thus, they are not necessarily a threat to urban areas unless there is a drastic change in their habitats, allowing them to adjust to different environments and hosts (Weaver & Reisen, 2010).

2.2 Arboviruses and Diseases

Arboviruses are viruses transmitted by arthropod vectors and they pose a significant public health problem worldwide (Weaver *et al.,* 2018). Arboviral diseases are caused by a wide range of RNA viruses and their life cycle involves both a host (birds or mammals) and a vector. There are about 250 arboviruses distributed worldwide; out of this number, at least, 80 cause human disease (Jones *et al.*, 2020). Prior to transmission, arboviruses replicate in an arthropod vector (e.g. mosquitoes, sandflies). Majority of arboviruses are either with the *Flavivirus* genera, *Alphavirus* genera or *Bunyavirus* (Huang *et al.,* 2019). The competence of these viruses is based on factors such as urbanization and genetics. Examples of arboviral emergence associated with urbanization, travel and trade are; Dengue virus (DENV), Chikungunya virus (CHIKV), Yellow fever virus (YFV), Zika virus (ZIKV) and Rift valley fever virus (RVFV) (Weaver & Reisen, 2010).

Yellow fever was introduced from Africa to other places in the 1600s and spread with the slave trade (Bryant *et al.*, 2007). It is endemic in the tropical areas of Africa and continues to cause outbreaks in Africa (Braack *et al.,* 2018). Some of the epidemics had extensive impact such as the yellow fever outbreaks in Ethiopia between 1960 and 1962 (Mutebi and Barret, 2002). These outbreaks killed about 30,000 people. Another typical example occurred in Nigeria, in the 1990s, where over 21,000 cases of yellow fever were reported (Mutebi and Barret, 2002). Ghana also reported a major yellow fever outbreak in the 1970s where 319 cases with 79 deaths occurred (Appawu *et al.*, 2006). In 2015, Angola also reported an outbreak with more than 3,552 cases with 355 mortalities (Grobbelaar *et al*., 2016).

Dengue fever is a health concern globally, however, its epidemiology and effect of public health is uncertain. Outbreaks of dengue fever was reported in many countries in Africa. About 750 million people in Africa are at risk of dengue fever (Weetman *et al.*, 2018). Thirty four (34) African countries are endemic for dengue fever (Amarasinghe *et al*., 2011). Dengue virus has recently been detected in samples from suspected malaria and Ebola children from some parts of Ghana (Amoako *et al.*, 2018; Bonney *et al.*, 2018). Also, there have been outbreaks in neighbouring Burkina-Faso and Côte d'Ivoire (WHO, 2017).

2. 3 Control of *Aedes* Mosquitoes

Control of arboviral diseases heavily depends on vector control since vaccines are not available for the control of most of the viruses involved (WHO, 2012). Control of yellow fever can be done by mass drug administration. However, there are no vaccines currently available for some of the arboviruses such as Zika virus and Chikungunya. The current vaccine for dengue fever doesn't provide full serotype protection. There are also concerns with its safety for mass administration (WHO, 2019). There are also no antivirals available for the treatment of arboviruses. Treatment is palliative and mainly to reduce patients symptoms (WHO, 2019). Reducing the burden of arboviral diseases worldwide still heavily relies on controlling the vector or avoiding the human to vector contact (Roiz *et al.*, 2018). Previously, well implemented vector control strategies proved effective for control of arboviruses. Majority of the *Aedes* control studies are focused outside Africa (Jaenisch *et al.*, 2014). A review of current literature revealed a total of 41 trials on the effectiveness of vector control, but none was from Africa (Bowman *et al.*, 2016). In Africa, surveillance and response strategies for arboviruses like dengue may not be present or are usually under developed. Implementing an effective surveillance plan for *Aedes*-borne diseases like dengue fever and yellow fever are of top priority for reducing dengue virus transmission.

However, initial signs for arboviruses transmission within communities isn't dependable. Also, the indices for checking *Ae. aegypti* populations are not accurate (Weetman *et al*., 2018). Many are of the view that because Malaria eradication is at the centre of African healthcare, thus it may be possible to have an integration of *Aedes* vector control with Malaria vector control.

Current strategies being employed in vector control are the use of insecticides to control adult populations and larval source management. However, the extent to which these control strategies have helped reduce and control arboviral disease spread is not properly understood due to inadequate data (Weetman *et al.*, 2017; Archee *et al.*, 2019).

2.3.1 Adult *Aedes* Control

The use of chemical-based insecticides form an integral part of vector control for most vector borne diseases (Moyes *et al.*, 2017). Historically, the use of these chemical-based insecticides for the control of *Aedes* mosquitoes had many success stories particularly, the *Ae. aegypti* eradication programmes carried out in South America in the 1960s, but insecticide control strategies were never replicated in Africa (Chan, 2016). Insecticides have different target sites within the mosquitoes. The mostly used insecticides target the voltage-gated sodium channels (organochlorides and pyrethroids). Organophosphates and carbamates insecticides inhibit the acetylcholinesterase enzyme leading to the death of mosquitoes (WHO, 2017).

Though in Africa there is the possibility of merging *Aedes* control with that of malaria control, some of the interventions employed in the control of adult malaria vectors may not be useful for *Aedes* control due to variations in the biology of the vectors involved. A

typical example is the use of the long-lasting insecticide nets (LLINs) which mainly target nocturnal biters and zooprophylaxis (diversion of disease carrying insects from humans to animals which targets domestic animal-biters). Although this example may be of some relevance to the *Ae. albopictus*, it does not apply to *Ae. aegypti* because of its anthropophilic nature, (Bonizzoni *et al.*, 2013; Weetman *et al*., 2018).

Control of adult *Aedes* vectors with insecticides are applied either as residual surface treatments or as space spraying (WHO, 2017). Indoor residual spraying (IRS) involves the use of chemical insecticides which are long lasting on the walls and roofs of houses and animal shelters to kill adult vectors that land or rest on these surfaces. This is done by using insecticides suitably pyrethroids and organophosphates (WHO, 2017). In Africa, IRS used for malaria vector control may prove to be effective in reducing Adult *Aedes* populations. This is because IRS focuses on indoor resting behaviour common to *Anopheles gambiae*, *Anopheles funestus* and *Aedes aegypti* (Weaver & Reisen, 2010). Indoor residual spraying has not been extensively used for control of *Aedes*, However, it is a good approach for control especially in places where *Ae. aegypti* is responsible for transmission (Bowman *et al*., 2016; Vazquez-Prokopec *et al.*, 2017).

Space spraying is recommended by WHO for emergencies to suppress a continuing arboviral epidemic. It helps to massively reduce infective adult *Aedes* mosquitoes in affected areas to reduce viral transmission during the outbreak or epidemic (WHO, 2017). Space spraying, if done early, helps to reduce the intensity of viral transmission. It helps to save time to ensure an effective long-term vector control (Faraji, 2016). In some previous studies, the use of curtains treated with long-lasting insecticides for the control of *Aedes* indoors were successful but more recent studies have been unsuccessful mainly because of

the type of housing and the increase in pyrethroid resistance in *Ae. aegypti* in the tropics (Toledo *et al.,* 2015). The story is not different in Africa due to increasing resistance in *Aedes* populations (Kawada *et al.,* 2016; Kamgang *et al*., 2017).

2.3.2 Larval Source Management

Larval source management in *Aedes* control is aimed at targeting mosquitoes at their larval stages in order to decrease adult population densities (WHO, 2012). Larval control involves different aspects including larviciding, environmental management, source reduction and biological control (Roiz *et al.,* 2018). Larvicides such as temephos and *Bacillus thuringiensis* (Bti) are widely used to treat *Ae. aegypti* larval habitats (WHO, 2017). Source reduction also mainly targets artificial containers to reduce *Aedes* vector population. This intervention may be very beneficial in Africa especially in targeting *Aedes* larvae in urban and semi urban areas to help reduce the densities of *Aedes* mosquitoes that breed in containers and, for that matter, arboviral transmission (Bowman *et al.*, 2016).

Several studies in Africa have showed high larval indices of *Aedes* mosquitoes, which can be exploited to control the vector. A recent study by Ferede *et al.* (2018) on the distribution of larval habitats in residential cities in Ethiopia reported a container index of 32.9, with the majority of *Aedes* habitats being discarded tires (57.5%). In Ghana, studies have reported higher larval indices in some sites suggesting that the larval densities are sufficient enough to promote an arboviral disease outbreak (Appawu *et al.,* 2006; Suzuki *et al.,* 2016). A recent study by Kudom (2019) in Cape Coast, also reported higher larval indices compared to that reported by Appawu *et al.* (2006) from the Northern region of Ghana which is similar to reports by Appawu *et al.* (2006) and Suzuki *et al.* (2016).

It is important to involve community-based approaches in *Aedes* control. Community members can be educated on how to identify potential and existing breeding habitats to destroy them. Biological control methods (using fish, copepods etc) are generally acceptable and can be used for treating large and permanent breeding sites. However, according to Olliaro *et al*. (2018), current evidence is not enough to determine its efficacy for control of dengue fever.

2.3.3. Challenges with Vector Control

Vector control is a major control strategy in the control of vector-borne diseases. However, several challenges have been observed with the use of insecticides for vector control. Some of the challenges that reduce the effectiveness of vector control strategies include insecticide resistance, variation in mosquito behavior, presence of behavioral avoidance and impact of environmental changes on mosquito ecological traits (Benelli *et al.*, 2017). Insecticide resistance is one of the major challenges affecting vector control especially in the arboviral diseases because arboviral control heavily depends on vector control with the use of insecticides (Kawada *et al.*, 2016).

2.4 Insecticide Resistance in *Aedes* Mosquitoes

Usage of insecticides and larval source reduction are important tools for arboviral vector control. However, control has been threatened by increasing insecticide resistance (Kasai *et al.*, 2014). Insecticide resistance (Phenotypic Resistance) is defined as an innate ability of mosquitoes to survive insecticides at a dose that susceptible mosquitoes of the same species would have usually being killed under the same conditions (Dusfour *et al.*, 2019). The occurrence of insecticide resistance is mediated by natural selection under the control of biological, genetic, and environmental factors (Ffrench-Constant, 2013). Resistance to

DDT is very common and has been detected in every country tested. *Ae. aegypti* and *Ae. albopictus* resistance to pyrethroids are more sporadic but it has been confirmed in *Ae. aegypt*i from Western, Central and Eastern parts of Africa (Weetman *et al.*, 2018).

Studies done in Cameroon by Kamgang *et al.* (2017) revealed that both *Aedes aegypti* and *Aedes albopictus* were resistant to 0.05% deltamethrin, 0.1% bendiocarb and 4% DDT, but were fully susceptible to malathion. Also, a recent study done by Kamgang *et al*. (2020) in the Democratic Republic of Congo revealed susceptibility of *Aedes* mosquitoes to organophosphates (temephos and fenitrothion), and Suzuki *et al*. (2016) also found out that *Aedes* populations in some sites in Accra, Ghana were resistance to DDT (4%) and lambdacyhalothrin (0.05%). Also, high resistance to DDT and possible resistance to permethrin were observed in all mosquito colonies collected in selected sites in Ghana (Kawada *et al.*, 2016).

Clearly, resistance in *Aedes* populations across Africa is spreading rapidly. This fast spread of insecticide resistance can reduce the effectiveness of insecticide-based vector control policies.

Data on the prevalence and mechanisms of insecticide resistance in different geographical areas are very crucial to help inform national vector control programmes in choosing the most effective insecticide for different settings (Moyes *et al.*, 2017; Weetman *et al.*, 2018).

2.5 Mechanisms of Resistance in *Aedes* Mosquitoes

Several mechanisms have been confirmed to mediate insecticide resistance in mosquitoes including *Aedes* mosquitoes. Insecticide resistance mechanisms in mosquitoes include decreased penetration of insecticides into the insect (cuticular resistance), mutations within the target site genes for insecticides (target-site mutations) and elevated enzymatic biodegradation or sequestration (metabolic resistance) (Moyes *et al.*, 2017). Cuticular

resistance is caused by changes of the insect cuticle leading to hardening of the cuticle (David *et al*., 2013). Target-site mutations (also referred to as genotypic resistance) and metabolic resistance are known to be the 2 major resistance mechanisms in *Aedes* mosquitoes.

2.5.1 Genotypic Resistance

The target sites for chemical insecticides are proteins that are involved in the activities of the nervous system of the mosquito. Mutations in these target sites affect the binding of insecticides to its target site, thus leading to resistance (Moyes *et al.*, 2016). Insecticides such as pyrethroids and organochlorides target the voltage-gated sodium channel while insecticides such as carbamate and organophosphates target the acetylcholinesterase enzyme (AChE). Target-site mutations that affect all insecticide classes are common in insects (Davies *et al*., 2007).

Acetylcholinesterase resistance is common in *Aedes* but AChE mutations do not occur extensively (Grigoraki *et al*., 2015). The detection of a change of glycine to serine at codon 119 is the most widespread mutation associated with resistance in mosquitoes specifically *Anopheles* spp. and *Culex pipiens*. However, there have been reports of resistance to organophosphates in *Ae*. *aegypti* and *Ae*. *albopictus* (Bisset *et al*., 2006; Lee *et al*., 2014). The G119S mutation was also recently detected in a population from India (Muthusamy *et al*., 2015).

Mutations in the voltage gated sodium channel are also called Knockdown Resistance. They are very common in *Ae*. *aegypti*. So far, 10 mutations in VGSC domains II and IV have been detected (Moyes *et al.*, 2017). The frequency of these mutations vary from one area to another. These mutations also differ in the extent to which they affect the outward display of resistance (phenotypic resistance) in *Aedes* mosquitoes. The most common

mutation (1534C) in *Ae. aegypti*, causes resistance to pyrethroids when in combination with other mutations. F1534C mutation is also associated with DDT resistance (Kushwah *et al*., 2015). The V1016I/G mutation has also been confirmed to be strongly associated with pyrethroids resistance. The V1016I and V1016G mutations have been reported in different geographical locations. V1016I is found in the Americas whiles V1016G is found in Asia (Moyes *et al.*, 2017).

These mutations have been detected in *Aedes* populations from some parts of Africa. A study by Badolo *et al.* (2019) from Burkina Faso revealed that the 1534C *kdr* mutation was almost constant in semi-urban and urban areas, however, it was less frequent in the rural areas. They also detected the 1016I *kdr* mutation at a lower frequency. The mutation V1016I has also been found in Ghana at a lower frequency in comparison to F1534C (Kawada *et al.*, 2016; Moyes *at al.*, 2017). Kudom (2019) detected F1534C kdr-mutation (allele frequency of 35%) in *Aedes* populations in the central region of Ghana. However, some studies carried out in Africa did not reveal any kdr mutation among resistant *Aedes* populations suggesting that other mechanisms may be involved. Examples of such studies by Kamgang *et al.* (2017) and Ngoagouni *et al.* (2016) in Congo and the Central African Republic have reported similar findings. Several studies have revealed that the 1016I/1534C haplotype was greatly linked with pyrethroid resistance in increasing frequency and range (Dusfour *et al.*, 2015). V1016I increases the extent of resistance to pyrethroids resistance caused by F1534C rather than directly causing resistance in *Aedes* populations. Only F1534C and I1011M mutations seem to have the ability of causing resistance directly (Du *et al.*, 2013). Some mutations such as D1763Y with V1016G, G923V with I1011M, and T1520I appear at a lesser frequency and are mostly in

combinations with mutations at positions 989, 1016, and 1534 of the voltage-gated sodium channel (VGSC) gene. However, their effects on resistance are unclear (Moyes *et al*., 2017).

A new mutation, V410L in the VGSC gene of resistant *Aedes aegypti* strains from Brazil was first discovered by Haddi *et al*. (2017). This mutation alone or in combination with F1534C has drastically reduced insecticide sensitivity resulting in pyrethroid resistance (Haddi *et al*., 2017). In Africa, it was first reported in Angola and the Madeira Island by Ayres *et al.* (2020).The study also found an important link between phenotypic resistance and both the V410L and V1016I in the Angolan population (Ayres *et al.*, 2020).

2.5.2 Metabolic Resistance

Metabolic resistance is mediated by the enhanced activity of metabolic enzymes that participate in the metabolism, sequestration, and excretion of insecticides (Moyes *et al.*, 2017). The main enzymes involved are those in the cytochrome P450 monooxygenases (P450S), glutathione s-transferases (GSTs), and esterases gene families. Also, other gene families may play a slight role in resistance (Hemingway *et al*., 2004). Metabolic resistance is widespread in mosquitoes. It has been found to cause resistance to all insecticides used for public health and agriculture purposes (Liu *et al*., 2015).

Cytochrome P450s are responsible for the metabolism of variety of compounds. Its increased activity is mostly linked to pyrethroids resistance in mosquitoes, although other enzymes may be involved (Kasai *et al.*, 2014). Several studies globally and in Africa have linked increased activity of mixed function oxidases (including cytochrome p450's) in *Ae. aegypti* and *Ae. albopictus* samples to the reduced susceptibility to DDT and pyrethroids (Dusfour *et al.*, 2015; Ngoagouni *et al.*, 2016; Kudom, 2019). Ngoagouni *et al.* (2016) reported that the activity of detoxifying enzymes were more in most *Aedes* populations

from the Central African Republic than in the susceptible *Ae. aegypti*, showing increased resistance to DDT and deltamethrin.

Members of cytochrome P450 genes, especially CYP6 and CYP9 subfamilies, have been associated with resistance because of the overexpression observed in resistant strains as compared to susceptible ones (Moyes *et al.*, 2017). Four (4) of the CYP genes are mostly consistently expressed in resistant populations and they are CYP9J10, CYP6BB2, CYP9J26, and CYP6J28. These four (4) highly expressed genes have been proven to metabolize pyrethroids and also confer pyrethroid resistance when expressed transgenically in Drosophila (Pavlidi *et al.*, 2012; Stevenson *et al.*, 2012).

The mechanism of action of cytochrome P450s in pyrethroid metabolism is well understood, however, other enzyme families may be involved. For instance, esterases have been found to metabolize pyrethroids (Chandor-Proust *et al*., 2013). Other detoxification genes are also implicated in resistance of *Ae*. *aegypti* to deltamethrin especially *GSTE2* and *GSTE7* (Lumjuan *et al.*, 2011). This finding is similar to that determined by Kudom (2019) in a study conducted in Ghana, which found that the GSTs and cytochrome P450s activities were elevated in *Ae. aegypti* than in the *An. gambiae Kisum*u (reference susceptible strain). Other enzymes that may have the ability to cause resistance include adenosine triphosphate (ATP)-binding cassette transporters and sulfotransferases. More studies is needed to determine the involvement of each in the detoxification pathway (Moyes *et al.*, 2017).

2.5. 3 Other Resistance Mechanisms

Other resistance mechanisms that are not majorly implicated in resistance in *Aedes* mosquitoes include cuticular resistance and behavioural resistance. Cuticle thickening is implicated as one of the causes of insecticide resistance. This resistance involves the reduction of the insecticide that gets to the target site as a result of modifications of

chemical composition of the cuticle (Kasai *et al.,* 2014). Insects with cuticular resistance depict resistance level about 3 times more than that of susceptible insects. It is among the least understood mechanisms though its occurrence with other resistance mechanisms will increase in insecticide resistance level tremendously, particularly in pyrethroid-resistant *Ae. aegypti* (David *et al.,* 2010).

Behavioral resistance is defined as a change in the resting and feeding behaviour of mosquitoes to reduce contact to insecticides (Chareonviriyaphap *et al.,* 2013). Some studies in which *Ae. aegypti* were exposed to pyrethroids showed that mosquitoes tend to escape from areas with insecticides on contact as compared to leaving the toxic area before contact with a treated surface (Paeporn *et al.,* 2007; Amelia-Yap *et al.,* 2018). Behavioural resistance to insecticides may be a crucial factor affecting the effectiveness of vector control against mosquito-transmitted diseases but there is scarcity of information about this mechanism (Chareonviriyaphap *et al.,* 2013).

2.6 Impact of Insecticide Resistance on the Control of *Aedes* Mosquitoes

Studies have showed that insecticide resistance has its impacts on the control of *Aedes* mosquitoes in the field. Some studies have associated insecticide resistance in immature *Aedes* populations with reduced treatment efficacy or residual efficacy to larvicides such as temephos (Dusfour *et al.,* 2019). Also, some studies have showed that resistance to pyrethroids in *Ae*. *aegypti* decreases the effectiveness of space spraying (Marcombe *et al.,* 2009; Marcombe *et al.,* 2011). Also, cages containing *Ae*. *aegypti* were not killed after treatment with pyrethroids, while susceptible mosquitoes were easily killed. Subsequent genetic studies detected the involvement of a combination of different resistance mechanisms, including target-site mutation and metabolic enzymes (Marcombe *et al.,* 2012). Serious interventions using insecticides were employed after a dengue

epidemic hit Northern Brazil from 2010 to 2011. However, there was a rapid increase in resistance to pyrethroids and this was thought to be the cause of the failure of deltamethrin treatments (Maciel-de-Freitas *et al.*, 2014).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Design and Sites

This was a cross-sectional study. Mosquito samples were collected in all study sites. The study was carried out in four (4) different localities covering urban and sub-urban sites in Ghana. The urban sites are Accra and Tema while the sub-urban sites are Ada Foah and Navrongo. Urban sites were chosen to target urban species such as *Aedes aegypti*, which is more adapted to urban environments. Suburban sites were also chosen to know the species available within the transition from urban to rural and the level of resistance amongst these species.

Accra is the capital city of the Greater Accra Region. It lies within latitude 5° 33' North and longitude 0° 12' west. It has a high annual humidity (77.4% relative humidity), but has low precipitation (monthly average of 46.7 mm in 2012–2013). The average annual rainfall, which falls primarily during Ghana's two rainy seasons is about 730 mm. Rainy season begins from April to mid-July and the minor season is in October. This site was chosen because of the abundance of breeding habitats for the mosquitoes. Within Accra, *Aedes* larval samples were collected from Legon and Korle-bu. Legon was chosen because of its residential status to know the level of resistance in that area. Korle-bu was also chosen because of the presence of agricultural farms within that environment. This site was chosen to know the resistance level within areas that have pesticide usage.

Tema, also in the Greater Accra region and the capital of the Tema Metropolitan District, is located 25 kilometres east of Accra. It is also called the harbour city because of the presence of Ghana's largest seaport there. It lies within latitude 5° 40' North and longitude

0° 0’ west. Tema has a tropical climate with an average rainfall of 750 mm. Within this site, *Aedes* larval samples were collected from Tema community 2. This site was chosen due to its proximity to the port to target *Aedes albopictus*, which is known to be transported through tyre trade to Africa.

Ada Foah is a coastal town in the Greater Accra region which can be found within the coastal savannah zone in Ghana. It is the capital of the Ada East district and lies within latitudes 5°45’ south and from longitude 0°20 west. In the dry season, the climate is typically tropical savannah with temperatures ranging between 23ºC and 33ºC. The rainfall average of 750 mm is recorded yearly. This site was chosen because of the abundance of larval habitats as well as the high risk of transmission of arboviruses due to tourist activities.

Navrongo is the capital of the Kassena-Nankana District in the Upper East Region of Ghana. It is found within the North-Eastern part of Ghana. The district lies within coordinates 10°53'5"N and 01°05'25"W. It has a typical Sahel savannah landscape with grassland and shrubs. It has an average temperature of 25°C and 19% humidity. This site was chosen because of its high risk of transmission due to its proximity to neighbouring Burkina Faso where arboviral outbreaks have been reported. Therefore, knowing the resistance status of *Aedes* mosquitoes in this site will aid in the control of future outbreaks.

Figure 3.1: Map of Ghana showing the study sites. Urban sites (yellow) and suburban sites (red).

3.3 Larvae Collection and Rearing

Aedes larval samples were collected from *Aedes* habitats and oviposition traps in the various study sites. Sampling was done using a plastic bowl and ladle. Global Positioning System (GPS) coordinates and the characteristics (land use type and vegetation) of the habitat were documented and the collected larvae were transported to the insectary.

In the insectary, the larvae were transferred into well labeled bowls containing dechlorinated water which is essential for their growth. Larvae were fed daily on fish meal to ensure their proper development. The larvae were raised into adults in the insectary. Once the adults emerge, they were kept in large cages for future bioassays.

3.4 Determination of Mechanisms of Insecticide Resistance in *Aedes* Mosquitoes

In determining the insecticide resistance status of the *Aedes* mosquito populations, WHO bioassays were done to confirm the presence of phenotypic resistance in the mosquito populations. Genotyping was done to identify specific point mutations that confer resistance in *Aedes* populations. Piperonyl butoxide (PBO) synergist assays were also performed to establish the role of metabolic enzyme, cytochrome p450's in the development of resistance.

3. 4.1 Who Insecticide Susceptibility Assays

WHO Susceptibility tests for adult mosquitoes was performed using WHO bioassays (WHO, 2016). This was done to detect the resistance profile of the populations of *Aedes* mosquitoes collected against five (5) insecticides, namely, pyrethroids (permethrin (0.75%) and deltamethrin (0.05%), organochloride (DDT (4%), organophosphates (pirimiphos-methyl (0.25%) and carbamates (0.1% bendiocarb).

Clean white sheets impregnated with insecticides were rolled into a cylinder shape and inserted into 4 holding tubes. Two oil impregnated sheets were also inserted into 2 holding tubes (controls). (A total of 6 replicates were used for the bioassays.) Adult mosquitoes (2 to 5 days old) were aspirated into the holding tubes (about 20 to 25 mosquitoes per tube) and then transferred into exposure tubes (contains insecticides). The tubes were then closed and the exposure tubes were placed in a vertical position up to an hour (60 mins). Within this period, the number of knockdown mosquitoes was recorded at time points 10, 15, 20, 30, 40, 50 and 60 mins. At the end of the 60 minutes, any mosquitoes that is dead or cannot fly were removed from the tubes. After exposure for 1 hour, mosquitoes were placed back into the holding tubes. Mosquitoes were feed on 10% sugar water that was soaked with cotton wool by placing the cotton wool on the wire gauze end of the tubes.

Mosquitoes were kept in the holding tubes for 24 hours at 27°C ± 2°C temperature and 75% ± 10% relative humidity. After 24-hours, dead and alive mosquitoes were counted and recorded. A mosquito was classified as alive if it was able to fly irrespective of the number of legs remaining. A mosquito was also classified as dead if it was knocked down and unable to stand or fly irrespective of the number of legs or absence of wings (WHO, 2016).

3. 4.2 Morphological Species Identification

Resistant and susceptible *Aedes* mosquitoes from the WHO bioassays were morphologically identified using identification keys by Huang *et al*., (2004), to distinguish the *Ae. aegypti* and the other *Aedes* species.

3.4.3 DNA Extraction

DNA was extracted using the DNeasy tissue Kit (QIAGEN Inc., USA). Whole *Aedes aegypti* mosquitoes were ground and lysed in 180 µl of ATL buffer using a pestle and 20 µl of proteinase K added to the mixture in an eppendorf tube. The ATL tube was then vortexed for 5-10 seconds and placed on a 56°C heat block. The solution was incubated for about 1 to 3 hours. During incubation of the ATL solution, a premix solution of 250 µl of AE buffer and 200 µl of 100% ethanol was prepared. The contents of the mixture were vortexed for 5 to 10 mins. Four hundred microlitres (400 µl) of the premix solution was added to the ATL tube and the contents mixed by vortexing for 5–10 seconds. The mixture was transferred into a DNeasy Mini spin column in a spin tube and centrifuged at 8000 rpm for 1 minute. Afterwards, the spin column was removed and placed in another spin tube. Five hundred microlitres of AW1 buffer was pipetted into the DNeasy Mini spin column in the spin tube and centrifuged at 8000 rpm for 1 minute. After that, the spin column was placed into a new spin tube and 500 µl of AW2 buffer was pipetted into the spin column. The spin column together with the tube was then centrifuged at 14000 rpm for 3 minutes.

After centrifugation, the spin column was removed and placed into a new spin tube. One hundred (100) microliters of the AE buffer was pipetted into the spin column and the mixture then incubated at room temperature for 1 minute. The spin column together with the tube was centrifuged at 8000 rpm for 1 minute and the spin column removed from the spin tube. The spin tube which contains the DNA was stored and refrigerated at a temperature of -80℃ for subsequent genotypic resistance determination.

3.4.3 Genotypic Resistance Determination

The frequency of mutations in the VGSC that confers resistance to *Aedes aegypti* mosquitoes was determined using Linss *et al.,* (2014) protocol. Genotyping was done using allele specific PCR. A total of 249 resistant *Aedes aegypti* mosquitoes were genotyped at 1016 position of the VSGC gene using allele-specific PCR (AS-PCR). Two hundred and twenty-three (223) resistant *Aedes aegypti* mosquitoes were also genotyped at the 1534 position of the VGSC gene. (Table 3.1). Also, 160 susceptible *Aedes aegypti* mosquitoes across the study sites were also genotyped at both 1016 and 1534 positions respectively. A total master mix of 12.5 µL was prepared using GoTaq Green Master Mix kit (Promega), 0.5 µL of genomic DNA, 0.24 µM of the common reverse primer, 0.12 and 0.24 µM of the forward primers (1534 Cys kdr and 1534 Phe). The polymerase chain reaction was run at a denaturation time of 95°C for 30 secs, annealing time of 54°C for 40 secs and an extension time of 72°C for 45 secs in a total of 32 cycle runs. PCR products were loaded on a 3% agarose gel stained with ethidium bromide and run on a gel electrophoresis tank at 100V for 45 minutes. The gels were then visualized under a UV transilluminator.

Table 3. 1 Primers for Allele Specific PCR

Target Primers	Forward (Sequence 5' -3')	Reverse (Sequence 5' -3')	Amplicon Size	Reference
1016 Val+	## ACAAATTGTTTCCCACCCGCACCGG	GGATGAACCGAAATTGGACAAAAGC	80	Linss *et al.*, (2014)
1016 Ile kdr	#ACAAATTGTTTCCCACCCGCACTGA	GGATGAACCGAAATTGGACAAAAGC	100	Linss *et al.*, (2014)
1534 Phe+	#TCTACTTTGTGTTCTTCATCATATT	TCTGCTCGTTGAAGTTGTCGAT	90	Linss *et al.*, (2014)
1534 Cys kdr	##TCTACTTTGTGTTCTTCATCATGTG	TCTGCTCGTTGAAGTTGTCGAT	110	Linss *et al.*, (2014)
AaEx31	TCGCGGGAGGTAAGTTATTG	GTTGATGTGCGATGGAAATG	350	Linss *et al.*, (2014)
long 5'-tail	GCGGGCAGGGCGGCGGGGGCGGGGCC			Linss *et al.*, (2014)
short 5'-tail	GCGGGC			Linss *et al.*, (2014)

+ = wild-type specific primer, kdr = specific primer, # = short 5′tail attached, ## = long 5′tail attached

3.5 Metabolic Resistance Determination

3.5.1 PBO Synergist Assay

Piperonyl butoxide (PBO) synergist assay was performed to establish the role of cytochrome p450's in the development of resistance in the *Aedes* mosquitoes. Piperonyl butoxide inhibits the activity of cytochrome P450 enzymes. This synergist assay was performed as previously described by Kamgang *et al.* (2017). Papers impregnated with PBO were used for the assays. Four (4) replicates of 20 female *Aedes* mosquitoes each were pre-exposed to 5% PBO for one (1) hour. After which, the mosquitoes were immediately exposed to insecticides for another one hour. Two control tubes were included in the set up for testing. Mortality rates were recorded during the 60 min period as stated earlier. The mosquitoes were then transferred into holding tubes and monitored for 24 hours and the mortality rates recorded.

3.6 Ethical Consideration

Ethical approval was obtained from the Ethics and Protocol Review Committee (EPRC) of the College of Health Sciences (CHS) of the University of Ghana with protocol identification number: CHS-Et/M2 -4.13/2019-2020

3.7 Data Management and Statistical Analysis

Data was entered into excel 2016 and the results presented in the form of graphs and tables. WHO insecticide susceptibility tests and PBO synergist tests were analyzed using the WHO criteria (WHO, 2016). Mosquitoes are classified as susceptible if the mortality rate of 98% to 100%; mortality rate of 90% to 97% is classified as suspected resistance and a

mortality rate of below 90% signifies that the mosquito population is resistant. Chi-square analysis was used to compare significance in phenotypic resistance observed across study sites. The allelic genotypic frequencies were calculated using the Hardy-Weinberg formula below:

$$\text{Allelic frequency} = \frac{2RR + RS}{2n}$$

Where RR is the number of homozygote mutants, RS is the number of heterozygotes and n is the total number of mosquitoes analyzed.

CHAPTER FOUR

4.0 RESULTS

4.1 Insecticide Resistance in *Aedes* Mosquitoes

4.1.1 Phenotypic Resistance of *Aedes* to Insecticides

Aedes mosquitoes sampled from four (4) different sites in Ghana were tested for resistance against insecticides within the four (4) major classes of insecticides in use. These insecticides include pyrethroids (deltamethrin and permethrin), organophosphate (pirimiphos methyl), carbamates (bendiocarb) and organochlorides (DDT). The intensity of resistance observed in the urban sites, Accra and Tema varied from what was observed in the suburban sites, Ada Foah and Navrongo.

Results from the WHO bioassays revealed that *Aedes* populations from all the study sites were resistant to DDT (11.3% to 77.3%) with the highest resistant site being Tema. Results from the study sites showed varying resistance to pyrethroids, deltamethrin and permethrin **(Table 4.1)**. Mosquitoes showed resistance to permethrin in Tema (82.5%), Accra (71.3%), Ada Foah (82.5%) and Navrongo (88.8%). Also, the mosquitoes showed resistance to deltamethrin in Tema (62.5%), Accra (81.3%), Ada Foah (83.8%) and Navrongo (78.8 %). Suspected resistance to carbamates and organophosphates (90.1% to 97.5%) was detected in some study sites **(Table 4.1)**.

The mosquitoes showed resistance to bendiocarb (carbamate) in Tema (80%) and suspected resistance in Accra (97.5%), (χ_2. = 400, df = 4, P= 0.00). Also, *Aedes* mosquitoes were resistant to organophosphate, pirimiphos-methyl in Tema (85%), suspected resistance in Ada Foah (90.1%), Navrongo (93.8%) and susceptible in Accra (100%) (χ_2. = 800, df = 8, P= 0.00). These are shown in **figure 4. 1** below.

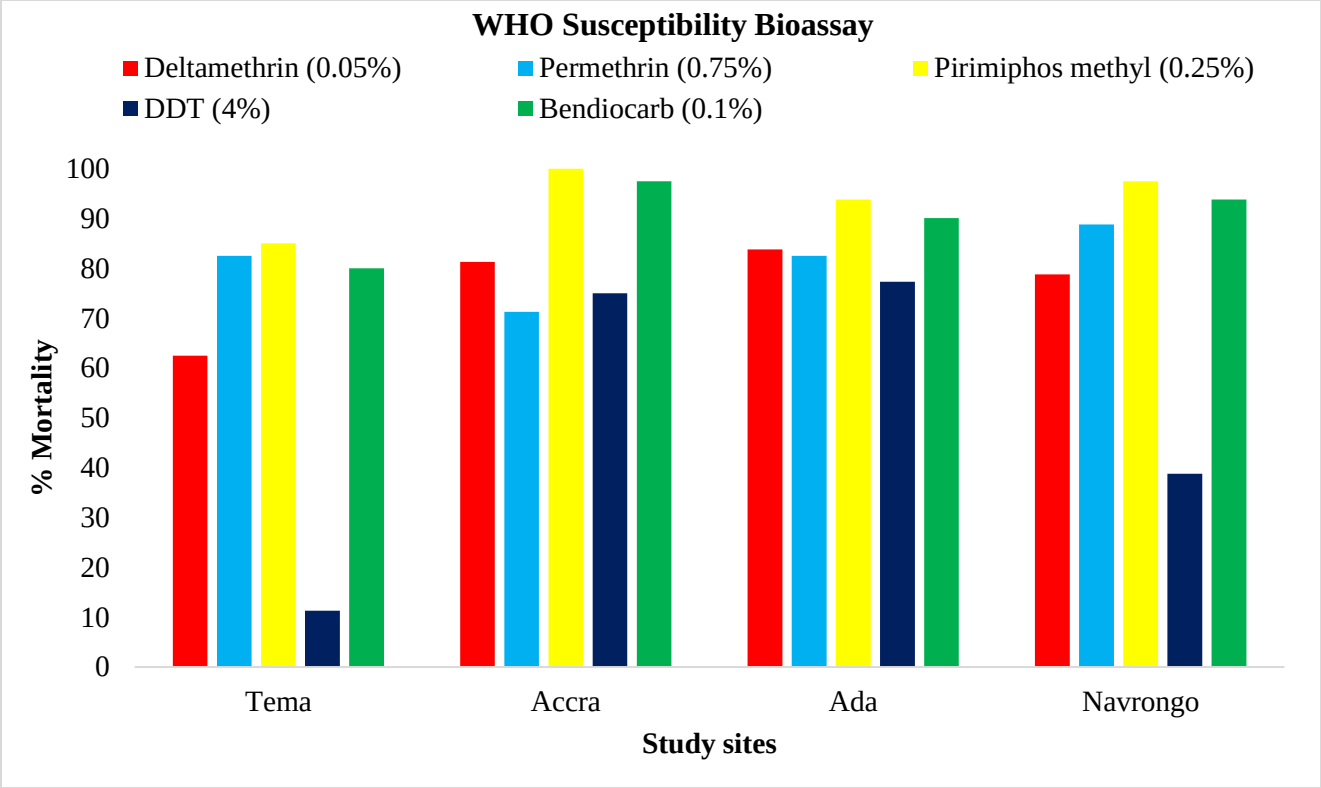

Figure 4.1: 24-hr mortalities of *Aedes* mosquitoes exposed to different insecticide classes

4. 2 Morphological Species Identification of Resistant and Susceptible Aedes mosquitoes

A total of four hundred and nine (409) resistant and susceptible *Aedes* mosquitoes from all four study sites subjected to morphological identification using taxonomic keys were found to be *Aedes aegypti* (100%). No *Aedes albopictus* or *Aedes luteocephalus* was detected.

4.3 Genotypic Resistance of *Aedes aegypti* to Insecticides

Resistant and susceptible *Aedes aegypti* mosquitoes were subjected to allele-specific PCR to detect the point mutations (F1534C and V1016I) in the voltage-gated sodium channel gene. From the results obtained, high frequencies of the two mutations were found in *Aedes aegypti* mosquitoes across the study sites.

F1534C mutations were detected at high frequencies across all sites: 363 homozygous mutants, 6 homozygous wildtype and 14 heterozygous mutations out of a total of 383 resistant and susceptible *Aedes aegypti* mosquitoes genotyped. **Table 4.1** shows the number of genotypes and frequencies of the F1534C mutation in the voltage-gated sodium channel gene of resistant *Aedes aegypti* mosquitoes collected from four different sites across Ghana. Homozygous frequencies of F1534C mutations were extremely high in all sites; Accra (100%), Tema (100%), Ada Foah (100%) and Navrongo (98.6%). No heterozygote mutation of F1534C was found in the resistant *Aedes* mosquitoes in any of the sites. Only one phenotypically resistant *Aedes* aegypti mosquito from Navrongo and Kumasi had homozygote wild-type genotype. Homozygous frequencies of F1534C mutations in susceptible *Aedes aegypti* mosquitoes were also high in almost all sites, Accra (65%), Tema (95%), Ada Foah (95%) and Navrongo (97.5%) (**Table 4.3**). Allelic frequencies among resistant samples were also slightly higher than susceptible samples across the sites. A total of 141 homozygote mutant, 14 heterozygotes mutant and 6 homozygote wildtype genotypes was detected.

V1016I mutations were detected at high frequencies across all sites: 334 homozygous mutants, 33 homozygous wildtype and 42 heterozygous mutations out of a total of 409 resistant and susceptible *Aedes aegypti* mosquitoes genotyped. **Table 4.2** shows the number of genotypes and frequencies of the V1016I mutation in the voltage-gated sodium

channel gene of resistant *Aedes aegypti* mosquitoes collected from four different sites across Ghana. Homozygous frequencies of V1016I mutations were extremely high in all sites; Tema (80.6%), Accra (100%), Ada Foah (91.5%) and Navrongo (94.4%). Heterozygote mutations of V1016I were detected in 9 resistant *Aedes aegypti* mosquitoes from Tema. A total of ten (10) mosquitoes had the homozygote wild-type genotype. Allelic frequencies of resistant *Aedes aegypti* samples were significantly high as compared to those of susceptible samples (**Table 4.3** & **Table 4.4**). Homozygote frequencies of the V1016I mutations were also relatively high among susceptible *Aedes aegypti* mosquitoes, Tema (67.5), Accra (50%), Ada Foah (60%) and Navrongo (82.5%) (**Table 4.4**). A total of 104 homozygote mutants, 33 heterozygote mutants and 23 homozygote wildtype genotypes were recorded.

Table 4.1: Number of genotypes and frequencies of the F1534C mutation in the voltage-gated sodium channel gene of resistant *Aedes aegypti* mosquitoes

Collection Place	Total	1534F			Homozygous %	Allelic %
		F1534C	F1534C/+	+/+		
Tema	68	68	0	0	100	100
Ada Foah	47	47	0	0	100	100
Navrongo	72	71	0	1	98.6	99
Accra	36	36	0	0	100	100
Total	**223**	**222**	**0**	**1**		

Table 4.2: Number of genotypes and frequencies of the V1016I mutation in the voltage-gated sodium channel gene of resistant *Aedes aegypti* mosquitoes

Collection Place	Total	1016V			Homozygous %	Allelic %
		V1016I	VI1016/+	+/+		
Tema	96	84	9	3	80.6	92%
Ada Foah	47	43	0	4	91.5	91%
Navrongo	70	68	0	2	94.4	97%
Accra	36	35	0	1	100	97%
Total	**249**	**230**	**9**	**10**		

Table 4.3: Number of genotypes and frequencies of the F1534C mutation in the voltage-gated sodium channel gene of susceptible *Aedes aegypti* mosquitoes

Collection Place	Total	1534F			Homozygous %	Allelic %
		F1534C	F1534C/+	+/+		
Tema	40	38	0	2	95	95
Ada Foah	40	38	0	2	95	95
Navrongo	40	39	0	1	97.5	98
Accra	40	26	14	0	65	83
Total	**160**	**141**	**14**	**5**		

Table 4. 4: Number of genotypes and frequencies of the V1016I mutation in the voltage-gated sodium channel gene of susceptible *Aedes aegypti* mosquitoes

Collection Place	Total	1016V			Homozygous %	Allelic %
		V1016I	VI1016/+	+/+		
Tema	40	27	2	11	67.5	70%
Ada Foah	40	24	4	12	60	65%
Navrongo	40	33	7	0	82.5	91%
Accra	40	20	20	0	50	75%
Total	**160**	**104**	**33**	**23**		

4.3 Metabolic Resistance in *Aedes* Mosquitoes

4.3.1 PBO Synergist Assay

Four hundred (400) *Aedes* mosquitoes from each of the four (4) study sites were used to perform PBO synergist assays to determine the involvement of major metabolic enzymes Oxidases in the development of resistance in *Aedes* mosquitoes from Ghana. Results obtained from these assays showed that oxidases may be involved in the development of resistance in *Aedes* mosquitoes across the study sites.

For *Aedes* populations in Tema, pre-exposure to PBO synergist increased the mortality rates to the insecticides, deltamethrin, DDT and bendiocarb only but not permethrin and pirimiphos-methyl **(Figure 4.2)**. The mortality rate for deltamethrin increased from 20% to 50%, while that of DDT and bendiocarb increased from 15% to 90% and 75% to 95% respectively due to the introduction of the synergist, PBO. The combination of PBO to insecticides, permethrin and pirimiphos methyl reduced from 95% to 75% and from 5% to 0 respectively.

In Accra, mortality rates of *Aedes* mosquitoes exposed to pyrethroids, deltamethrin increased from 80% to 90%, permethrin from 70% to 80% and bendiocarb from 95% to 100%. Decreased mortality rate was observed from 75% to 50% after pre-exposure to PBO synergist **(Figure 4.3)**. Synergist-insecticide combinations increased the mortality rates of *Aedes* mosquitoes from Ada Foah to all insecticides tested as compared to insecticides alone. For the pyrethroids, deltamethrin and permethrin, the mortality rates increased from 75% to 100% and 80% to 95% respectively **(Figure 4.5)**.

In Navrongo, mortality rates due to permethrin increased from 60% to 100%, deltamethrin from 75% to 100% but showed a decrease in the case of DDT from 40% to 10% after pre-exposure to PBO synergist **(Figure 4.4)**.

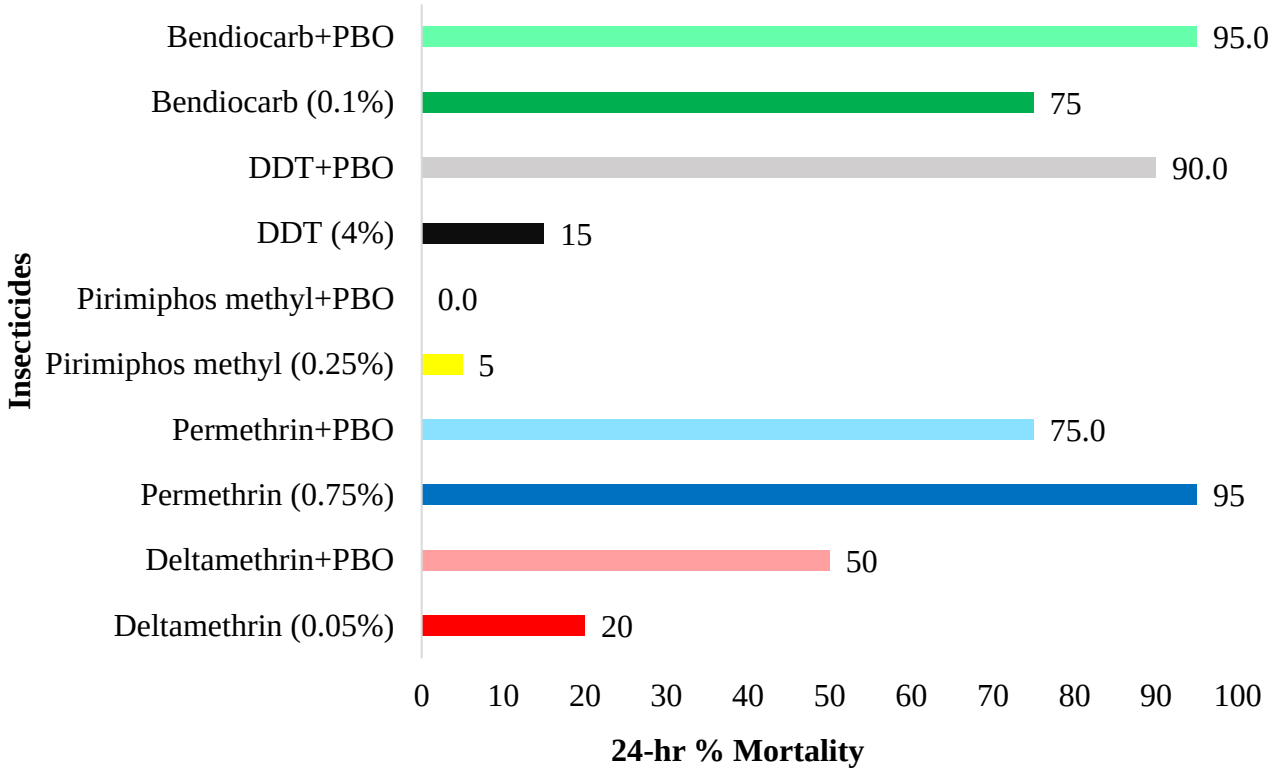

Figure 4. 2: Synergistic effects of PBO on the insecticide susceptibility status of *Aedes* populations from Tema

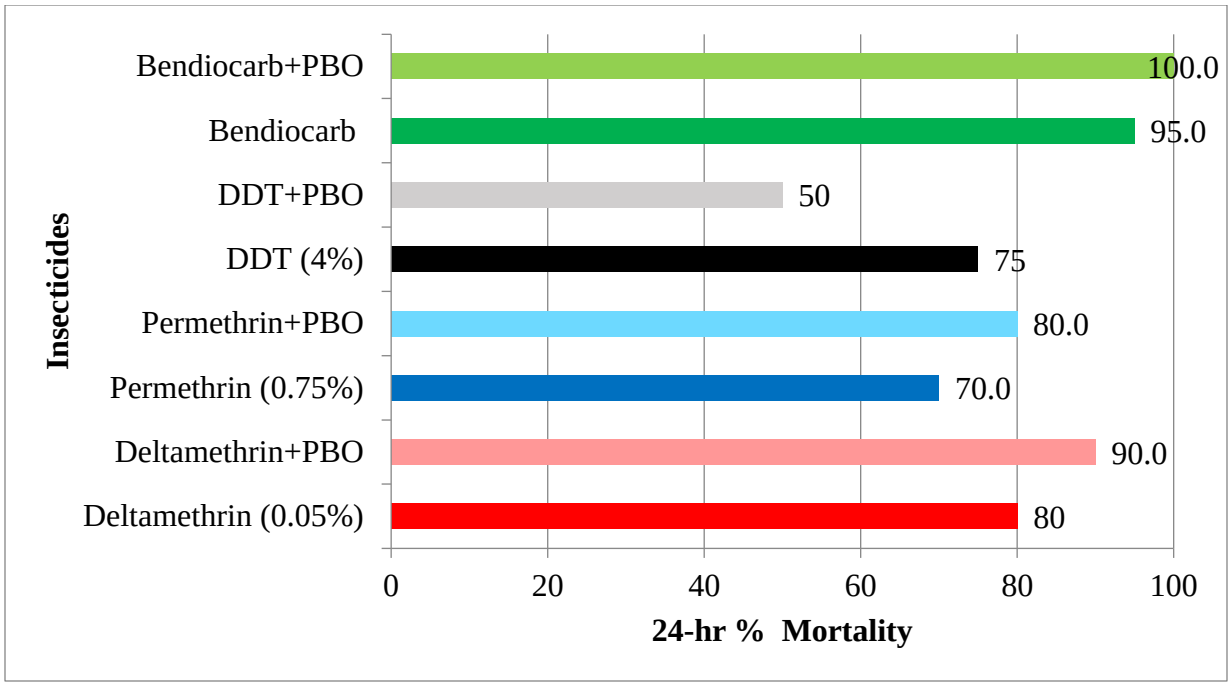

Figure 4.3: Synergistic effects of PBO on the insecticide susceptibility status of *Aedes* populations from Accra, Ghana.

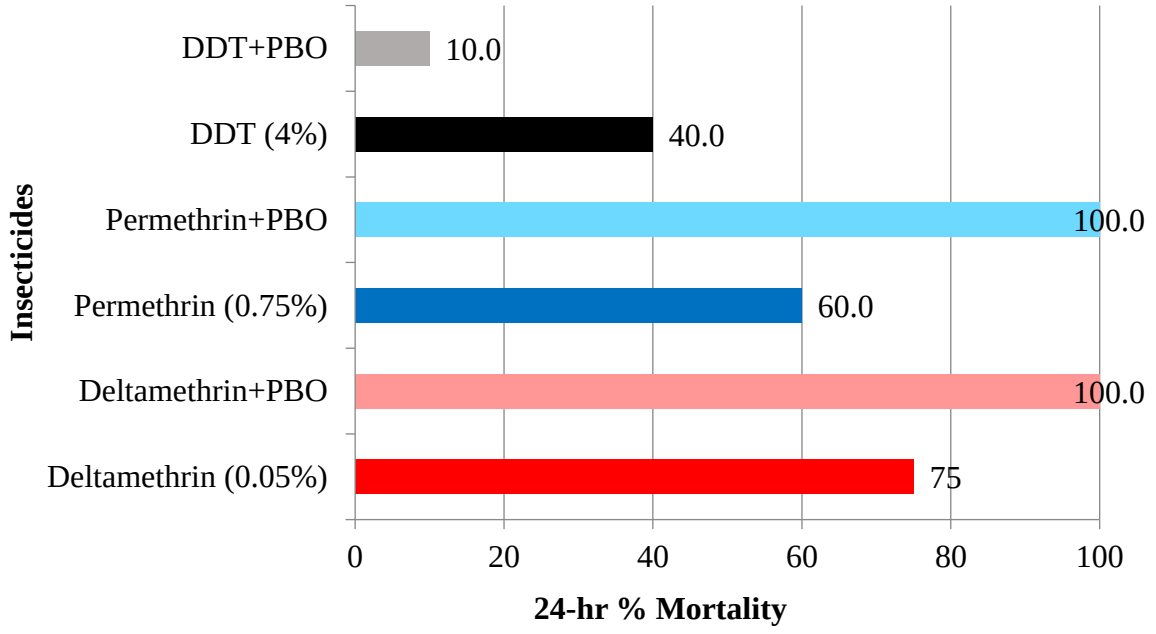

Figure 4.4: Synergistic effects of PBO on the insecticide susceptibility status of *Aedes* populations from Navrongo, Ghana.

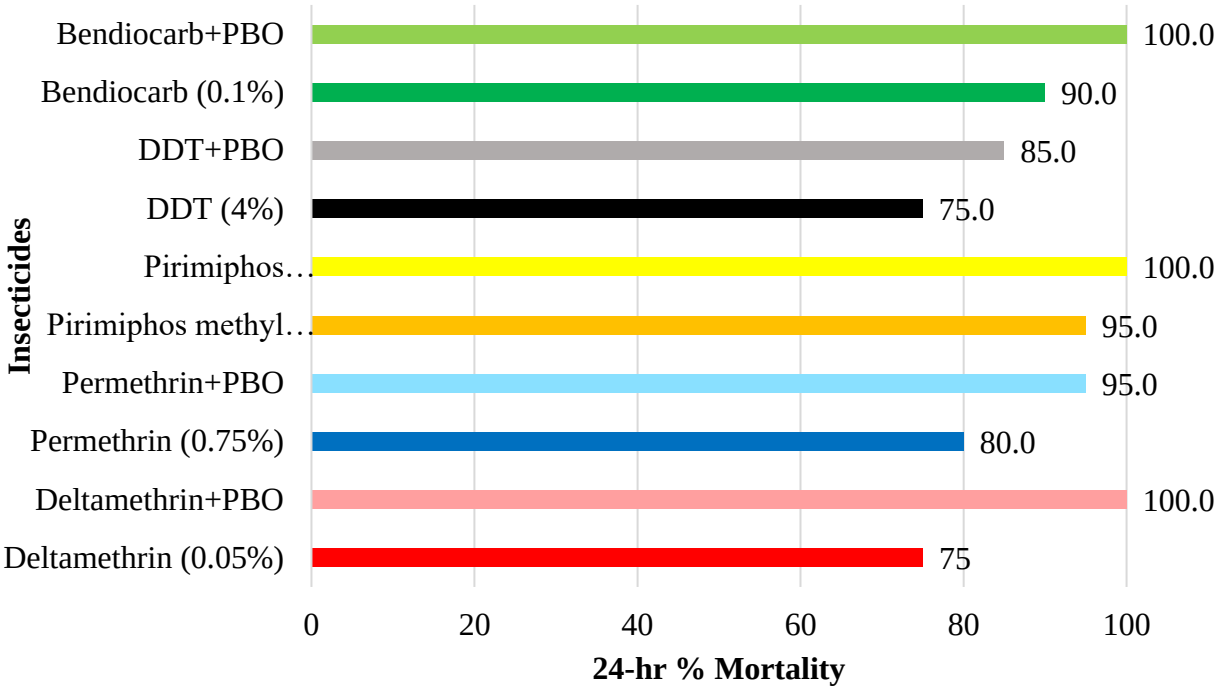

Figure 4.5: Synergistic effects of PBO on the insecticide susceptibility status of *Aedes* populations from Ada, Ghana.

CHAPTER FIVE

5. 0 DISCUSSION

Insecticide resistance in *Aedes* mosquitoes is a major concern for arboviral disease control worldwide. Main resistance mechanisms that have been implicated in resistance in *Aedes* mosquitoes are the genetic resistance and metabolic resistance. This study presents the insecticide resistance status and mechanisms of *Aedes* mosquitoes sampled from various study sites between the periods of November, 2019 to July, 2020.

Morphological identification on *Aedes* mosquitoes from bioassays was performed. Mosquitoes were confirmed to be *Aedes aegypti*. No *Aedes albopictus* or *Aedes luteocephalus* was detected. *Aedes aegypti* is the main vector for dengue viruses globally due to its high vector competence and highly anthropophilic as compared to that of *Aedes albopictus* (Lambrechts *et al*., 2010). *Aedes aegypti* is also the main vector for yellow fever in urban settings. Thus, high densities of this vector increases the risk of transmission of these arboviruses (Ngoagouni *et al.*, 2016).

Overall, the levels of resistance of *Aedes* mosquitoes to major insecticides used for public health varied across study sites. All *Aedes* mosquitoes across the four (4) sites tested were found to be resistant to deltamethrin (0.05%), Permethrin (0.75%) and DDT (4%). DDT resistance was widespread across of sites tested. Susceptibility and suspected resistance to organophosphates and carbamates was observed in all sites except Tema. High Frequencies of the KDR mutations F1534C and V1016I were detected in resistant and susceptible mosquitoes genotyped. Piperonyl butoxide (PBO) also significantly enhanced the susceptibility of *Aedes* mosquitoes to almost all insecticides tested.

The resistance level of DDT was relatively higher in all sites as compared to those for the pyrethroids (permethrin and deltamethrin). This is likely to increase the rate of cross

resistance to pyrethroids in the near future, since organochlorides (eg DDT) and pyrethroids share the same target site (VGSC) within the mosquito. Thus, any target site mutation conferring resistance to DDT is likely to also cause resistance to the pyrethroids. This was similar to results obtained by Kawada *et al.* (2016), who found samples obtained across various sites in Ghana exhibiting resistance to the pyrethroids and DDT. Also, similar findings were observed by Badolo *et al.* (2019) in Burkina Faso and Kamgang *et al.* (2017) in Cameroon.

The World Health Organization (WHO) recommends the use of insecticides such as organophosphates, carbamates and pyrethroids for the control of arboviral diseases such as yellow fever and dengue fever. However, the trend of insecticide resistance among *Aedes* populations across Ghana and Africa is rapidly increasing. In 2016, Suzuki and other scientists accessed the insecticide susceptibility status of *Aedes aegypti* in some sites in Accra, Ghana. Comparing their study to this one, the resistance level of *Aedes* mosquitoes to the pyrethroids and DDT has increased, affirming the fact that resistance seems to be increasing as time progresses. The use of organochlorines and pyrethroid pesticides by farmers may be one of the causes of the resistance being observed. Ada Foah for instance, is a farming community, thus there is a high use of pesticides for farming purposes. Also, the indirect impacts of LLINs and insecticides for malaria vector control may have added to the development of DDT and pyrethroid resistance in *Aedes aegypti* populations in Ghana (Kristian *et al.*, 2003; Kudom *et al.*, 2012).

There has been varied reports on the resistance level of *Aedes* mosquitoes in urban and sub urban across the world. Some of these reports were made by Li *et al.* (2018) in China and Badolo *et al*. (2019) in Burkina Faso. Li *et al.* (2018) reported high resistant rates in urban

populations as compared to suburban and rural *Aedes* populations. Pyrethroid resistant levels were slightly higher in urban sites (Accra and Tema) as compared to suburban sites (Ada and Navrongo). This slight variation in resistance level is still unclear but can be attributed to the influence of environmental factors such as pollution. This finding is in agreement with that determined by Badolo *et al.* (2019) from a study conducted in Burkina Faso, where higher resistance to pyrethroids was observed in urban sites. High resistance in urban areas is very alarming for arboviral vector control since there is an increased risk of transmission due to the high densities of *Aedes aegypti* and high populations of peoples within urban settings (Weetman *et al.,* 2018). Reports from Tema indicated resistance to all insecticides tested with DDT showing the highest resistance level. Although insecticide resistance is thought to be mainly result because of the selection pressure caused by insecticides used for vector control, other environmental factors also play a significant role in resistance. One significant factor is the presence of anthropogenic pollutants in urban, agricultural or industrial areas which may affect mosquito's responses to pyrethroid insecticides and how they select their resistance mechanisms (Suwanchaichinda & Brattsten, 2002; Nkya *et al.*, 2013; WHO, 2018).

Tema is an urban site, a harbour town with high industrial activities which potentially could influence the resistance development in the *Aedes* mosquitoes. The influence of the chemical and waste products from the site should be investigated because interactions between industrial pollutants such as heavy copper and the expression of *Aedes* genes encoding detoxification enzymes have been established (Poupardin *et al.,* 2008; David *et al*., 2010). Microarray and RT-qPCR analyses conducted by Poupardin *et al.* (2008) revealed that industrial pollutants were linked to the overexpression of some CYP genes

including those involved in resistance to pyrethroid. Across the study sites, *Aedes* mosquitoes exhibited suspected resistance and susceptibility to organophosphates (pirimiphos methyl) and carbamate (bendiocarb) except in Tema where they were found to be fully resistant to the two (2) insecticides. Resistance seems to be developing rapidly and a lot more action should be done to slow it down.

Chemical insecticides target key proteins involved in the functioning of the mosquito nervous system. Mutations in these target sites for insecticides have been linked to phenotypic resistance observed in *Aedes* mosquitoes worldwide (Kamgang *et al.*, 2017; Moyes *et al.*, 2017, & Badolo *et al.*, 2019). Knockdown resistance (KDR) mutations, F1534C and V1016I have been implicated in resistance. F1534C is the most widespread mutation in *Ae. Aegypti* conferring resistance to deltamethrin, permethrin and DDT. While V1016I mutation has distinct geographical distribution. It has been detected in the Americas but in low frequencies in Ghana (Kawada *et al.*, 2016; Moyes *et al.*, 2017). The detection of these kdr mutations in *Aedes* mosquitoes from Ghana was first reported by Kawada *et al* (2016).

In this study, we detected high frequencies of the F1534C and V1016I mutations in both resistant and susceptible *Aedes* mosquitoes genotyped. Across the sites, the F1534C mutation was more widespread as compared to the V1016I mutation. Both the allelic and homozygotes frequencies were significantly higher for the F1534 mutation as compared to V1016I mutation in both resistant and susceptible *Aedes* mosquitoes genotyped. High homozygote and allelic frequencies observed in susceptible samples, especially for the F1534C mutation, suggesting that multiple resistance mechanisms may be involved in the

resistance observed in the *Aedes* mosquitoes, since the two mutations are almost fixed across both susceptible and resistant groups of the *Aedes* mosquitoes genotyped.

Metabolic resistance arises from overexpression of enzymes involved in the metabolism, sequestration and excretion of insecticides. Metabolic enzymes such as Cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), and esterases have been implicated in resistance of *Aedes* mosquitoes worldwide (Moyes *et al.,* 2017). Cytochrome P450s especially members of the CYP6 and CYP9 subfamilies, have been mostly associated with resistance of *Aedes* mosquitoes to pyrethroids, though other metabolic enzymes are also involved (David *et al.,* 2012). Piperonyl butoxide is a synergist that inhibits cytochrome P450 monooxygenase enzyme activity. The effect of PBO on the insecticide susceptibility of *Aedes* populations across the sites was investigated in this study. The study revealed that *Aedes* mosquitoes that were pre-exposed to PBO before exposure to the insecticides had a significant enhancement in their susceptibility from almost all the study sites. This signifies that metabolic enzymes may be either partially involved or fully involved in the resistance observed. For the urban sites (Tema and Accra), metabolic enzymes may be partially involved in resistance since the increase in mortality observed for the synergist-insecticides combinations were still below the 98% mortality rate threshold for susceptibility (WHO, 2016).

The results obtained for the synergist assays of *Aedes* mosquitoes from Ada, showed an increase in mortality to all insecticides tested after pre-exposure to PBO, signifying the involved of metabolic enzymes, either partially of fully. However, for the suburban site (Navrongo) metabolic enzymes may be fully involved in pyrethroid resistance, since there was a total restoration of susceptibility after pre-exposure to PBO. Results obtained for

PBO assays provide a ray of hope for arboviral vector control especially in endemic areas with high resistance among the vector populations. PBO can be incorporated in insecticide combinations to increase susceptibility of *Aedes* mosquitoes to insecticides used for control.

This study shows that moderate to high phenotypic resistance among Aedes populations across the sites tested. Phenotypic resistance observed in *Aedes* mosquitoes across the sites tested may be a combined effect of the multiple resistant mechanisms (i.e. genotypic and metabolic mechanisms). The findings also suggest the use of synergists such as PBO in insecticide combinations for arboviral vector control in Ghana as it helped in minimising resistance in most of the *Aedes* populations to the insecticides tested. Thus, this data will help inform vector control policies for the control of *Aedes* mosquitoes.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATION

6.1 Conclusion

Moderate to high levels of phenotypic resistance was observed in all the sites tested with Tema reporting resistance to all five insecticides tested. The levels of resistance observed in urban areas (Tema and Accra) were slightly similar to those of suburban sites (Navrongo and Ada). However, the resistance level of DDT was relatively higher in all sites as compared to those for the pyrethroids (permethrin and deltamethrin), showing that resistance of *Aedes* populations to DDT is widespread. Across the study sites, *Aedes* mosquitoes exhibited suspected resistance and susceptibility to organophosphates (pirimiphos methyl) and carbamate (bendiocarb) except in Tema from where the mosquitoes were fully resistant to the two insecticides.

Target sites mutations (F1534C and V1016I) previously implicated in resistance in *Aedes* mosquitoes worldwide were detected in both phenotypically resistant and susceptible *Aedes* mosquitoes at high frequencies. Thus, suggesting that these mutations may be fixed in the populations especially the F1534C mutation. Pre-exposure to the synergist, PBO increased the susceptibility of *Aedes* mosquitoes to the insecticides tested in almost all sites. These findings suggest that multiple resistance mechanism may be involved in the resistance observed and that this resistance may be as a result of both genotypic and metabolic resistance mechanisms.

6.2 Limitations

Based on an initial preliminary larval survey, rural sites were not included in the study because of difficulty in getting enough larvae. This is because *Aedes aegypti* is mostly

adapted to urban environment. Thus, achieving high number of larvae for the bioassays was challenging. Also, Confirmatory tests such as RT-PCR and metabolic assays were not performed to know which specific metabolic enzyme is mediating the resistance observed.

6.3 Recommendations

This study investigated the insecticide resistance status of *Aedes* populations and the mechanisms involved in Ghana. Based on the findings, it is recommended that

- Synergists should be incorporated into insecticide formulations to increase susceptibility of *Aedes* mosquitoes within the areas studied especially in Tema where high resistance was detected.
- More work should be done on *Aedes* resistance by increasing the number of sites in the different categories (urban, sub-urban and rural sites) so as to determine the extent of the resistance in *Aedes* mosquitoes in Ghana to help inform vector control strategies for arboviral control in Ghana.
- The role of metabolic enzymes in the development of resistance should be further investigated using RT-PCR to determine the expression profile of major metabolic genes implicated in resistance. Also, enzymes assays should be performed to determine the level of enzymatic activity of metabolic enzymes in *Aedes* populations. These two methods are confirmatory tests to establish the involvement of metabolic enzymes in resistance.

- The level of insecticide resistance among the different *Aedes aegypti* sub species (*Aedes aegypti aegypti* and *Aedes aegypti formosus*) should be determined to help inform vector control policies for arboviral diseases.

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APPENDICES

Appendix I: WHO Insecticide Susceptibility Bioassay Form and larval sampling form

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. Exposed						

Number knocked down after exposure in minutes.

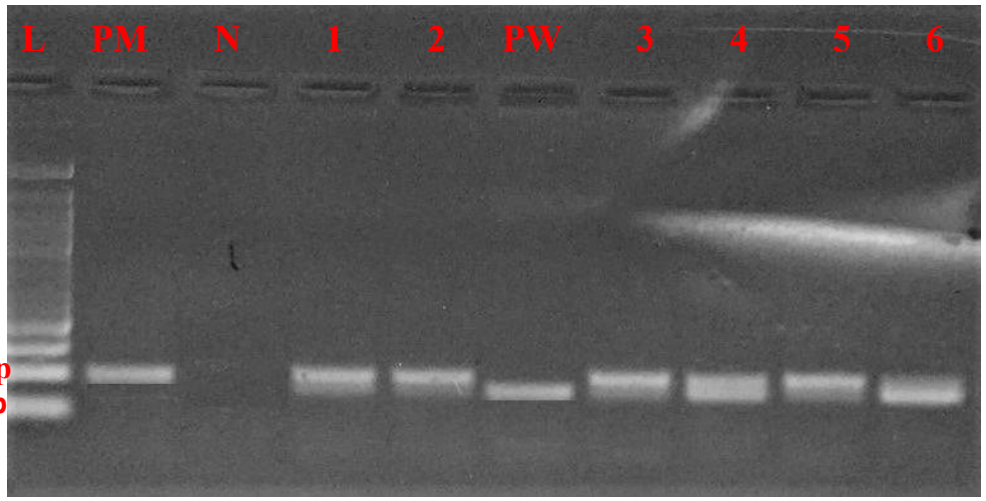
Time/minutes	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
10						
15						
20						
30						
40						
50						
60						

Number of dead/alive mosquitoes at the end of holding period (24 hours)

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. dead						
No. alive						

Appendix II: Gel Electrogram showing Knock down mutations (Kdr) in Aedes mosquitoes subjected to allele-specific PCR.

Gel 1: Shows the PCR Products of **1016** position of the VGSC representing the **genotypes homozygote mutant (HM- 100bp), Heterozygotes (HT- 100bp &80bp) and homozygote wildtype (HW- 80bp) genotypes.**



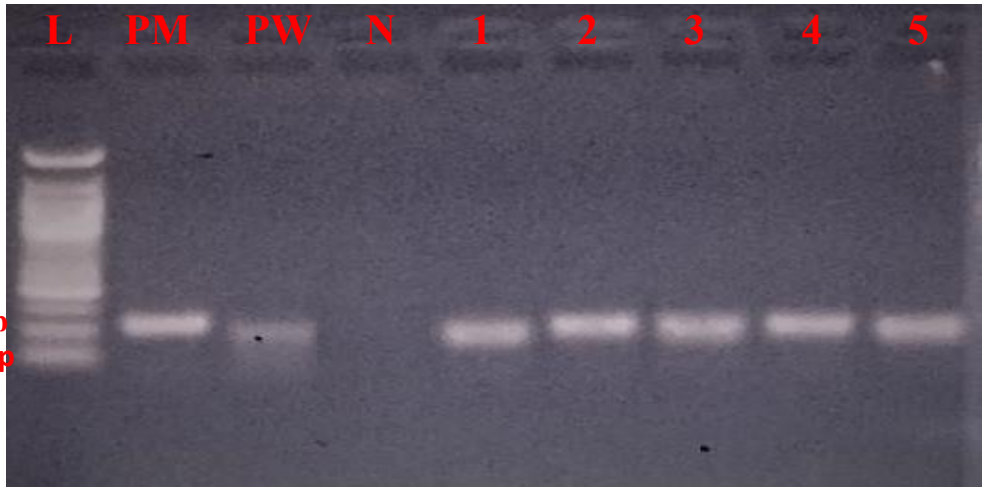
L- 50bp Ladder

PM- Positive control for mutant

PW- Positive control for wildtype

N- Negative Control

Gel 2: Shows the PCR Products of the 1534 position of the VGSC representing the genotypes homozygote mutant (HM- 110bp), Heterozygotes (HT- 110bp &90bp) and homozygote wildtype (HW- 90bp) genotypes.



L- 50bp Ladder

PM- Positive control for mutant

PW- Positive control for wildtype

N- Negative Control

Appendix III: Ethical Clearance Certificate for the study

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.: ....EPRC/MAR/2020..... March 03, 2020

Miss. Anisa Abdulai
Department of Medical Microbiology,
University of Ghana Medical School
Korle Bu.

ETHICAL CLEARANCE

Protocol Identification Number: ***CHS-Et/M2 –4.13/2019-2020***

FWA: 000185779 **IORG: 0005170** **IRB: 00006220**

The College of Health Sciences Ethical and Protocol Review Committee (EPRC) on March 03, 2020 unanimously reviewed and approved your re-submitted research protocol.

Title of Protocol: **"Insecticide Resistance Status and Mechanisms in Aedes Mosquitoes in selected Sites across Ghana"**

Principal Investigator: **Miss. Anisa Abdulai**

This approval requires that you submit six-monthly review report(s) of the study to the Committee and a final full review report to the EPRC at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study before, during and after implementation.

Please note that any significant modification(s) to this project/study must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the EPRC within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till March 04, 2021.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: .....
Professor Andrew Anthony Adjei
Chair, Ethical and Protocol Review Committee

cc: Provost, CHS
Dean, UGMS
Head, Department of Medical Microbiology,

• P. O. Box KB 52, Korle Bu, Accra, Ghana. • Telephone: +233 (0) 306 665103/4 • Fax: +233 (0) 302 660762
• Email: administration@chs.edu.gh / provost@chs.edu.gh • Website: www.chs.ug.edu.gh