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BASELINE SUSCEPTIBILITY OF MALARIA VECTORS TO CLOTHIANIDIN IN THE NORTHERN REGION OF GHANA

BY

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DECLARATION

I, Cosmos Manwore-Anbon Pambit Zong, authenticate that the work presented in this thesis is the result of my own research undertaken in the Department of Medical Microbiology and the US President's Malaria Initiative (PMI) Vectorlink project under the supervision of Prof.Yaw Assare Afrane and Dr. Simon Kwaku Attah (Department of Medical Microbiology, University of Ghana), and that all references cited in this work have been properly acknowledged.

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LIST OF ABBREVIATIONS

IRS	Indoor Residual Spraying
LLINs	Long-Lasting Insecticidal nets
WHO	World Health Organization
Vgsc	Voltage-gated Sodium Channels
Ace	Acetylcholinesterase
PCR	Polymerase Chain Reaction
An.gambiae s.l	*Anopheles gambiae sensu lato*
An.gambiae s.s	*Anopheles gambiae sensu stricto*
An	*Anopheles*
Min	minutes
US PMI	United States Presidents Malaria Initiative
NMCP	National Malaria Control Program
IVCC	Innovative Vector Control Consortium
USAID	United States' Agency for International Development
EPA	Environmental Protection Agency
P	*Plasmodium*
IPTp	Intermittent Preventive Treatment in pregnancy
ITN	Insecticidal Treated Nets
ACTs	Artemisinin Combination Therapies
DDT	Dichlorodiphenyltrichloroethane
PQ	Pre-Qualification
Ache	Acetly cholinesterase

NACHR	Nicotinic Acetylcholine Receptors
Kdr	Knockdown resistant genes
NSE	Non-Beta Esterase
GST	Gluthathione Transferases
RH	Relative Humidity
°C	Degree Celsius
DNA	Deoxyribonucleic Acid
ul	Microliters
uN	Universal
AA	*Anopheles arabiensis*
AM	*Anopheles melas*
Sec	Seconds
V	Voltage
Bps	Base pairs
SS	Homozygous susceptible
RS	Heterozygous resistant
RR	Homozygous resistant
Rev	Reverse
Dir	Direct
IBM	International Business Machines Corporation
CHS	College of Health Sciences
EPRC	Ethics and Protocol Review Committee

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ABSTRACT

The emergence and spread of insecticide resistance coupled with the widespread distribution of malaria vectors in Ghana makes malaria control a cumbersome task. Currently in Ghana and most of the West African countries, malaria vectors have developed resistance to all four (4) classes of insecticides. Therefore, a new insecticide, clothianidin, which has a different mode of action, has been deployed for Indoor Residual spraying (IRS) in Ghana since March 2021. A detailed understanding of current phenotypic susceptibility to clothianidin used for IRS and the effect of this on mosquito mortality, as well as underlining resistance mechanisms is vital to inform management strategies.

The aim of this study was to document the sub lethal effects of clothianidin on malaria vector susceptibility and longevity.

This was a cross-sectional study. WHO susceptibility bioassays were conducted to determine phenotypic susceptibility of *Anopheles gambiae* complex to clothianidin whilst cone bioassays were conducted to determine the effect of exposure on mosquito mortality. Conventional PCR was done to discriminate the sibling species of the *Anopheles gambiae* complex and also to detect target-site mutations.

WHO susceptibility bioassay results showed significantly effective but prolonged mortality of mosquitoes. Mortality rates recorded with the insecticide demonstrated a delayed effect with optimum mortality requiring up to 144hrs, six (6) days. The mortality rate for wild *Anopheles* mosquitoes exposed in WHO cone bioassays for 5-min, 24hrs after was 63%, showing resistance. Mortality increased to 97% after 120hrs, indicating possible resistance but after 144hrs, mortality of 100% was reached indicative of vector

susceptibility. Mosquitoes exposed for 10-min had a mortality of 72% after 24hrs.This increased to 97% after 96hrs indicating possible resistance, but 120hrs was taken to reach full susceptibility of 100%. After 24hrs, mosquitoes exposed for 20-min reached 82% mortality, which increased to 97% after 96hrs indicative of possible resistance, this increased slightly to 98% after 120hrs showing susceptibility. A 95% mortality was reached for mosquitoes exposed for 30-min after 24hrs, thus giving an indication of suspected resistance, however, after 48hrs mortality reached was 98% suggesting susceptibility

The PCR results revealed that *Anopheles gambiae s.* was the most abundant malaria vector accounting for 63% followed by *Anopheles coluzzii* also accounting for 25%, with *Anopheles arabiensis* accounting for 9% of the samples. There was high allele frequency of Kdr west (L1014F) in all vector species identified (0.89-0.94) with *Ace-1* occurring in moderation (0.32-0.44).

In this study, it was found that malaria vectors were susceptible to clothianidin. There were also a lot of resistant mutations in wild *Anopheles* mosquitoes. The most abundant malaria vector species was *Anopheles gambiae s.s*. Continued surveillance for susceptibility of malaria vectors to clothianidin should be conducted yearly to monitor possible resistance.

CHAPTER 1

1.0 INTRODUCTION

1.1 Background

Malaria continues to be serious public health problems in many parts of the world, particularly in Sub-Saharan Africa. According to the WHO, there were 229 million cases of malaria worldwide in 2021, with the WHO African region accounting for nearly over 94% of all malaria cases and fatalities (WHO, 2020). Indoor Residual spraying (IRS) has been one of the most important methods in the fight against malaria since its discovery in 1939 (Dengela *et al.,* 2018). In the 1960s, WHO endorsed a large-scale application of IRS as a main malaria vector control tool in order to minimize the rise in malaria burden (Dengela *et al.,* 2018). Other measures such as long-lasting insecticide treated nets (LLINs), mosquito larvae control, and the construction of mosquito-proof structures, were also utilized to augment the impact of IRS (Dengela *et al.,* 2018; Desalegn *et al.,* 2018). Factors such as insecticide resistance, logistical constraints and issues with finance and sustainability compelled many countries to discontinue its use (Dengela *et al.,* 2018; Agossa *et al.,* 2018).

In 2006, the WHO re-recommended IRS as a frontline strategy for reducing local malaria transmission in a variety of epidemiological settings (Oxborough *et al*., 2016). As a result, several nations, particularly those in the WHO African region, have adopted and expanded IRS programmes, thanks to funding from the US Presidents Malaria Initiative (PMI) and the Global fund (Oxborough *et al*., 2016). It was later reported that IRS

reduced the incidence and prevalence of malaria by 14% and 16% in 2000 and 2015 (Oxborough *et al.,* 2016) respectively.

Since 2008, the National Malaria Control Program (NMCP) of Ghana, in collaboration with the US Presidents Malaria Initiative (PMI), has been implementing IRS in several districts in northern Ghana to reduce the burden of malaria. IRS is known to work mainly by killing female *Anopheles* mosquitoes resting indoors (endophilic) after taking a blood meal (Dengela *et al*., 2018).

The efficacy of IRS is determined largely by multiple factors such as: vector species composition and their resting behavior (WHO, 2016); the spray quality, residual bio-efficacy of the insecticides sprayed (Dengela *et al*., 2018); spray coverage, the formulation of insecticides (Agossa et al., 2018); the type of spray surfaces (Desalegn *et al*., 2018) as well as the timing of the spray campaign (Dengela *et al*., 2018). Additionally end user/beneficiary acceptance, attitudes, perceptions and practices could also affect the effectiveness IRS (Opiyo & Paaijmans, 2020).

Malaria vector resistance has been documented across Sub- Saharan Africa to the four (4) insecticide classes recommended by the WHO for IRS (WHO, 2020). This needs the development of new IRS insecticides that do not exhibit any cross-resistance to present insecticides (Ngufor *et al*., 2017), thus vital for more impactful malaria vector control programmes.

Clothianidin is the first insecticide with a novel mode of action to be recommended by the WHO for use in IRS in over 40 years (IVCC, 2020). With the increasing emergence of resistance in mosquitoes to most of the current insecticides used for IRS, clothianidin will remain the new insecticide of choice for most IRS programmes. Clothianidin

however, is a relatively slow-acting insecticide, unlike the pyrethroids, organophosphates, carbamates and organochlorides (Oxborough *et al.*, 2019), requiring between 72 and 168 hours to assess its efficacy (Agossa *et al.*, 2018; Oxborough *et al.*, 2019).

1.2 Problem Statement.

The current tools for vector control (LLINs and IRS) are insecticide-based and resistance to the commonly used insecticides is increasing in many parts of Africa, including Ghana, and is hampering vector control (WHO, 2020). These vector control tools rely solely on the use of insecticides (Agumba *et al*., 2019). The intensive application of insecticides for vector control has imposed selection pressure on mosquito populations resulting in resistance to the four (4) insecticide classes. Reports from several studies in Ghana and other West African countries suggest there exist resistance mechanisms against current insecticides (Ranson *et al*., 2011; Baffour-Awuah *et al*., 2016). Resistance to insecticides is widespread both geographically and across vector species (WHO, 2020) and may encompass physiological or behavioral changes. Maintaining the efficacy of control programmes requires effective resistance management strategies to reduce selection for resistance. In response to insecticide resistance concerns, the WHO recommends the introduction of new insecticide classes with novel mode of action and has since approved clothianidin, a neonicotinoid as the new insecticide class for malaria vector control interventions.

The PMI vectorlink project that undertakes IRS for Ghana Malaria Control Programme deployed fluroda fusion (clothianidin active ingredient) from March 2021, for IRS in

Northern Ghana. This study was undertaken to provide baseline data on malaria vector susceptibility to clothianidin. The data obtained would be built on for future studies.

1.3 Justification.

Malaria is endemic and unending in Ghana, with seasonal fluctuations being more prominent in Northern Ghana (Oduro *et al*., 2015). Malaria infection affects the entire population; however, the rate of transmission varies depending on the ecological zone. The most effective malaria control tools have been vector control interventions; unfortunately, the level of protection provided by these methods is gradually declining due to the excessive use of insecticides that appears to be driving insecticide resistance. This current development is alarming and requires an urgent and concerted effort to help fight this insecticide resistance. Therefore, constant monitoring and surveillance of the susceptibility of malaria vectors to clothianidin is required in order to monitor its efficacy.

1.4 Aim of Study.

The aim of this study was to document the sub lethal effects of clothianidin on *Anopheles gambiae s.l* susceptibility.

1.5 Specific Objectives.

1. To determine susceptibility status of *Anopheles. gambiae s.l* to clothianidin on sprayed wall surfaces and the possible resistant genes.
2. To determine the effect of different exposure times to sprayed IRS insecticide (Fludora Fusion) on *Anopheles gambiae s.l* mosquito mortality.

CHAPTER 2

2.0 LITERATURE REVIEW.

2.1 Burden of Malaria.

The WHO estimated that global malaria cases would be approximately 241 million cases in 2020, and in 85 endemic countries, increasing from the initial 227 million in 2019 (WHO, 2021), with the WHO African region being the most burdened, accounting for 96% of malaria cases and deaths worldwide (WHO). Again, 11.6 million (34%) pregnant women in WHO African region were at risk of getting infected with malaria, with the highest prevalence of risk occurring in West Africa (38%). Malaria mortality in children under five (5) reduced from 87% in 2000 to 77% in 2020 (WHO, 2021). Ghana is responsible for 2% of global malaria cases and 3% of deaths (WHO, 2020). Malaria is endemic and perennial in the country and more marked seasonal variations occur in Northern Ghana (NMCP, 2019). Malaria transmission in the country is less intense in urban settlements as compared to rural areas throughout the country (Awine *et al.,* 2017). The most important malaria vector species in Ghana are the *Anopheles gambiae* and the *Anopheles funestus* complexes. Currently, there are eight (8) *Plasmodium* species known to infect humans: *P. falciparum, P. malariae, P. vivax, P. ovale wallikeri, P. ovale curtisi, P. knowlesi, P. cynomolgi* and *P. simium* (Mourier *et al*., 2021). *Plasmodium falciparum* has been linked to more than 97% of total malaria cases in the country with *P. malariae* and *P. ovale* occurring as co-infections (WHO, 2020).

2.2 Malaria Vectors and Transmission in Ghana.

There are over 400 different species of *Anopheles* mosquitoes but only about 30 of these species are of significant importance (WHO, 2020). *Anopheles gambiae s.l* and *Anopheles funestus s.l* have been identified as the important malaria vectors in Ghana (Baffour-Awuah *et al*., 2016; Awine *et al*., 2017). *Anopheles coluzzii* is the predominant vector species identified in the coastal zone together with *Anopheles melas*, which is adapted to living in salty environments (Kudom, 2015). In the forest zones, *Anopheles gambiae s.s* and *Anopheles coluzzii* are the most abundant vector species (Chabi *et al.,* 2016; Hinnie *et al.,* 2021). However, in the savannah zones, *Anopheles gambiae s.s* and *Anopheles coluzzii* are the most abundant (Appawu *et al.,* 2001) together with *Anopheles arabiensis*, usually in lesser numbers (Hinnie *et al*., 2021). It has been established that malaria vector population density varies significantly across different geographical locations and also influenced by a number of environmental factors which include; climate, rainfall pattern and vegetation (Appawu *et al*., 2001).

The parasite, the vector, the human host, and the environment all play key roles in the transmission of malaria. In regions where the vector lifespan is longer (as parasites take ample time to fully develop into infective stages) and where they prefer to feed on humans rather than animals, transmission is more intense (Sibanda *et al.,* 2011; Ibrahim *et al.,* 2014; Dabire *et al.,* 2015;Fossog *et al.,* 2015). Malaria transmission varies significantly amongst the three ecological zones. In comparison to the other two zones, the forest zone has a higher parasite prevalence (Awine *et al*., 2017; Hinnie *et al*., 2021). Malaria frequency in the Guinea savanna zone increases during the single rainy season (June-October). Malaria parasite prevalence, on the other hand, peaks twice a year in the

forest and coastal zones (May-June/October–November), which coincides with the bi-modal rainfall pattern.

2.3 Malaria Interventions in Ghana.

With financing from the US Agency for International Development (USAID), the US President's Malaria Initiative (PMI), and the Global Fund to Fight AIDS, Tuberculosis, and Malaria, the National Malaria Control Programme has achieved significant progress in reducing the country's malaria burden. This was made feasible by the introduction of new malaria control interventions following the implementation of new policies. The use of artemisinin-based combination therapy (ACT) as a first-line treatment for clinically uncomplicated malaria is one of the intervention options.

All health facilities in the country are expected to deliver quick and effective ACT treatment, with approximately 90% of all patients with uncomplicated malaria expected to be treated properly with ACTs (NMCP, 2020). The policy also aims to ensure that about 90% of parents and caregivers can recognize early onset of malaria, and that approximately 90% of children under the age of five (5) will receive appropriate ACT within 24 hours of commencement (NMCP, 2020). Sulfadoxine-pyrimethamine (SP)-based intermittent preventative therapy for malaria in pregnancy (IPTp) was also implemented. IPTp is expected to be used by almost all pregnant women, and at least two (2) doses are required (NMCP, 2020).

Another part of malaria control that has been in use since before independence is malaria vector control. Indoor residual spraying (IRS) and the use of long-lasting insecticidal treated-nets are the two most common vector control methods (LLINs). The NMCP began distributing insecticidal treated nets across the country in 2002, and later launched

Indoor Residual Spraying in northern Ghana to supplement the impact of ITNS use. The malaria control policy's particular aims were to guarantee that every home in the country had at least one insecticide-treated net, and that approximately 80% of the general population slept beneath ITNs (NMCP, 2020; Nash *et al*., 2021). The percentage of children under the age of five (5) and pregnant women sleeping under treated nets would rise to almost 85% from present levels. Again, the IRS would cover approximately 90% of all structures within the targeted districts (NMCP, 2020).

2.4 Insecticide Resistance

Principal malaria vector control interventions are over-reliant on the use of insecticides. This incessant insecticide application has led to vector resistance to all four (4) classes of insecticides (pyrethroids, carbamates, organophophates and organochlorides). This widespread vector resistance in many parts of Africa, which includes Ghana, appears to be hampering malaria vector control (WHO, 2020). As part of the measures to help curb the menace of insecticide resistance, the WHO recommends the introduction of new insecticide classes for use in vector control interventions (IRS and LLINs). This is to help minimize the spread of vector resistance in order to maintain the efficacy of vector control programmes.

The PMI IRS project in northern Ghana sprayed pyrethroids (alpha-cypermethrin and deltamethrin) between 2008 and 2012 (Oxborough *et al.,* 2019). In response to insecticide resistance concerns and the need to maintain programme efficacy, the programme switched from pyrethroids to pirimiphos-methyl, an organophosphate. The programme continued to spray pirimiphos methyl for over six years since the vectors remained susceptible and also due to unavailability of alternative

replacement. However, in 2018 SumiShield 50WG, which contains clothianidin as an active ingredient from the neonicotinoid class, received World Health Organization (WHO) prequalification as a new IRS product and Ghana EPA approval as an IRS product (WHO, 2020). With the availability of a new IRS product, the PMI IRS project commenced the spraying of clothianidin since March 2021 as part of the insecticide resistance management strategies in selected districts that had sprayed pirimiphos-methyl for over six years. Reports of insecticide resistance to pirimiphos-methyl in some communities compelled the National Malaria control programme to switch to a new insecticide class, in order to ensure programme efficiency. The project introduced a second clothianidin-based insecticide Fludora Fusion developed by Bayer Crop Science for IRS, in 2020 (IVCC, 2020). Fludora Fusion, which combines two insecticides, clothianidin and deltamethrin, and is the first dual mode of action IRS product to obtain WHO pre-qualification (WHO, 2020).

Neonicotinoids insecticides were first developed for agricultural application in the 1990s, and they quickly became the world's most widely used insecticides against a wide range of economically important crop pests. Clothianidin is a metabolite of thiamethoxam, another neonicotinoid insecticide (Ngufor *et al.*, 2017; Agossa *et al.*, 2018). Clothianidin is primarily an insecticide that targets agricultural chewing pests, but is also used in households to suppress malaria vectors through tarsal contact. This insecticides target insect nicotinic acetylcholine receptors, a novel mode of action for malaria vector control that makes cross-resistance through existing mechanisms implausible (Uragaya *et al.*, 2018).

Because this class of insecticides has a lower affinity for vertebrate nicotinic acetylcholine receptors than insect nicotinic acetylcholine receptors, they have low toxicity to mammals and are thus a potential for public health use (Ngufor *et al.,* 2017; Oxborough *et al.,* 2019). It is also known to have a delayed mode of action, hence the WHO has made modifications to the holding period in order to assess its slow mode of action (Oxborough *et al*., 2019).

2.4.1 Mechanisms of Resistance.

Target-site mutations, metabolic resistance, reduced insecticide penetration and behavioral resistances have all been identified as resistant mechanisms behind observed phenotypic resistance in malaria vectors.

2.4.1.1 Target-site Mutation.

Knockdown resistance (kdr), which occurs when point mutations in sodium channel genes in the mosquito nervous system result in cross-resistance to DDT and pyrethroids, is mediated by target site mutations (Santolamazza *et al*., 2008; Pluess *et al*., 2010Rohani *et al*., 2014). In a wild type, these mutations induce phenylalanine to replace leucine at locus 1014 of the sodium channel gene, resulting in L1014F, or serine to replace leucine, resulting in L1014S (Stump *et al*., 2004;Rozilawati *et al*., 2005; Santolamazza *et al*., 2008). Ace-1 mutation is also induced by a single amino acid substitution at locus 119, in Ache 1 catalytic region, where glycine is replaced with serine (G119S)(Hemingway, 2000; Santolamazza *et al*., 2008; Ojuka *et al*., 2015).

A third sodium channel mutation, N1575Y discovered in Central and West Africa. At codon 1575, an asparagine to tyrosine mutation occurs in the linker domains between the voltage gated sodium channels, an asparagine to tyrosine mutation, which occurs at

codon 1575(Lindsay *et al*.,1998; Jeschke *et al*., 2011; Edi *et al*., 2017). There is suspicion that N1575Y would improve L1014F mediated pyrethroid resistance because it is on the same L1014F haplotype (Jones *et al*., 2012; Edi *et al.*, 2017). Mutations in the *Ace-1* gene render Acetyl cholinesterase insensitive to carbamates and organophosphates, as well as other altered target site resistance.

2.4.1.2 Metabolic Resistance.

Mosquito vectors overexpress detoxifying enzymes in reaction to xenobiotics, resulting in metabolic resistance. Non-beta esterases (NSE) are overexpressed as a result of organophosphate and carbamate selection pressure, whereas cytochrome P450-dependent monooxygenases are overexpressed as a result of DDT and pyrethroid selection pressure (Lengeler et al., 2000; Fonseca-gonzalez *et al*., 2019). An increase in Glutathione-S- transferases (GST) aids in the detoxification of organophosphates, DDT and pyrethroids (Hemingway & Ranson, 2000; Wondi *et al*., 2011). Overexpression of these enzymes is caused by the gene amplification or changes in the trans-acting regulatory element or the promoter region (Vuvule *et al.*, 1999; Hemingway & Ranson, 2000; Lindblade et al., 2006).

2.4.1.3 Reduced Penetration of Insecticide.

Insecticide penetration is reduced due to the thickening of the insect cuticle, which results in lower rates of insecticide absorption. This mode of resistance gives detoxifying enzymes enough time to break down the chemical (Sibanda *et al.,* 2011; Simon-Delso *et al.,* 2015). In pyrethroid-resistant mosquitoes, however, cuticular thickness is greater in susceptible mosquitoes, according to studies (Awolola *et al*., 2009).

2.4.1.4 Behavioral Resistance.

The WHO defined behavioral resistance in 1957 as the ability to escape a dose that has lethal implications and is linked to the unsettling aspect of some insecticides that cause mosquitoes to avoid sprayed surfaces. According to studies, vector biting preferences have shifted from indoor to outdoor, as well as behavior from night biting to early night biting, in insecticide treated homes (Awolola *et al.,* 2009).

CHAPTER 3

3.0 MATERIALS AND METHODS.

3.1 Study Design.

This study was a cross-sectional one. *An*opheles mosquito larvae and pupae were collected from the study site and raised into adults in the insectary at the Vectorlink Project facility in Tamale, Northern region. This is a project that is part of the US President's Malaria Initiative, Ghana and undertakes Indoor Residual Spraying for the National Malaria Control Programme of Ghana.

3.2 Study Site.

This study was conducted in a rural community, Kpalsogu (09°24'27" N 00°51'12" W) in the Kumbungu District sprayed with Fludora Fusion (with clothianidin as an active ingredient. The rainfall pattern in this community is unimodal from May to November. The average yearly temperature of 28°C appears to be ideal for *Anopheles* larval development, however temperatures sometimes exceed 42°C. Water is collected during the rainy season and stored in dugout dams and other water reservoirs for agricultural operations during the dry season. During the rainy season, these dams flood, resulting in the formation of several of swamps that are ideal breeding habitats for *Anopheles* mosquitos. Mosquitoes breed in the water that is diverted from dams to farmland via channels. Most of these dugout dams dry up during the severe dry season, forming small, temporary open water pools that are also ideal breeding habitats for *Anopheles* mosquitoes.

Figure 3.1: Map of Ghana showing the position of the study site in the Northern Region.

3.3 Immature mosquito sampling.

Using standard 350-ml dippers and hand pipettes, *An*opheles mosquito larvae and pupae were collected from their natural breeding habitats and placed in plastic bowls. To avoid gathering sibling species, immatures were taken at random from separate breeding locations. Geographical positioning coordinates and characteristics of the habitats (land use and vegetation) were documented. The larvae and pupae were transported to the PMI vectorlink project insectary where they were raised to become adults. Larvae were fed with ground fish meal (Tropical Fish Food Flakes) and under typical conditions (26 ± 2 °C; $80\% \pm 10\%$ relative humidity (RH) with 12 h: 12 h light/dark cycle). Upon pupation, individuals were removed and moved to cages and allowed to emerge as adults. Emerged adults were maintained in cages with 10% sucrose solution soaked in clean white cotton wool.

3.4 WHO Susceptibility Bioassays.

Insecticide susceptibility tests were conducted according to established WHO protocols, with modifications made to the holding period (WHO, 2020). Before this experiment, mosquitoes were held for one (1) hr to check for fitness, weak and unfit (broken wings or legs or unable to fly) mosquitoes were removed. A total of 120, 3-5 days old female *Anopheles* mosquitoes were exposed to filter papers impregnated with clothianidin at 13.2mg (2%) active ingredient for 60 min in four (4) replicates of 20 mosquitoes (80). Two additional replicates of 20 mosquitoes (40) were used as negative control (untreated papers). Knockdown scores were recorded at intervals of 10-min during the 60-min exposure period. After exposure, mosquitoes were transferred back to clean holding tubes and provided with 10% sucrose solution. Mortality was scored after 24hrs recovery period. Mosquitoes that survived after 24hrs were monitored for delayed mortality for an extra 168hrs (7 days). Tests were conducted under controlled environment (temperature of 27°C±2 °C and relative humidity of 75%±20% following the standard World Health Organization protocol. A positive control experiment was conducted using susceptible Kisumu strain following the same procedure.

3.5 WHO Cone Bioassays.

Cone bioassays were conducted in four (4) different houses (2 with cement wall surfaces and 2 with mud wall surfaces). The unfed female (3-5 day old) adults emerging from the field-collected larvae were exposed to the walls of houses sprayed with clothianidin. Three cone assays were carried out in each house from May to July. In each selected room a total of 40 mosquitos were exposed for 5, 10, 20, and 30 mins and collected in holding cups as 4 replicates of 10 mosquitoes per cup (WHO 2016). The knocked down and alive mosquitoes were recorded after every 10 mins for 60 mins and mortality observed at intervals of 24hrs post exposure, up to 168hrs (7 days) post exposure.

To avoid the possibility of the control mortality increasing due to fumigant effect of the sprayed formulations, the control tests were setup in unsprayed rooms with similar conditions (relative humidity and temperature) as the rooms being tested. After exposure, mosquitoes were held in holding cups and provided with 10% sugar solutions and maintained under standard insectary conditions (temperature 27 °C±2 °C and relative humidity of 75%±20%). The Kisumu susceptible strain was used as a control for all assays.

3.6 Polymerase Chain Reaction (PCR) Amplification for Mosquito species Identification.

Before doing PCR to further discriminate into sibling species, sub samples of wild *Anopheles gambiae s.l* mosquitoes from the field population were identified morphologically. Individual mosquito legs were cut into PCR tubes to be used as DNA templates for the PCR reactions. The PCR reaction mixture consisted of 0.6µl universal primer (µN) (5'-GTG TGC CGC TTC CTC GAT GT-3'), 0.6µl each of the species-specific primers for *Anopheles gambiae s.s* (R 6.1a) (5'-TCG CCT TAG ACC TTG CGT TA-3'), *Anopheles coluzzii* (R 6.1b) (5'-CGC TTC AAG AAT TCG AGA T). Each PCR tube containing mosquito leg DNA template received 25µl of the reaction mixture. The PCR reaction was carried out in a thermal cycler, with a single phase of 95°C for 3 minutes followed by 54°C for 30 seconds. The reaction was then exposed to 35 cycles of 72°C for 1 minute and 54°C for 20 seconds. The final phase was a 5-minute extension at 72°C. On a 2% agarose gel stained with Ethidium bromide, the PCR products were run (Biotium, Hayward, California, USA). After 45 minutes of operation at 100V, the gel was photographed and visualized under ultraviolet light. Band fragments of 260bps for *Anopheles gambiae s.s*, 500bps for *Anopheles coluzzii*, 315bps for *Anopheles arabiensis*, and 464bps for *Anopheles melas* were expected from the PCR result(Scott, 1993). For the gel electrophoresis, a 100 bps DNA ladder of standard size was employed as a control. The specificity of *Anopheles gambiae s.l* primers were checked by using 8 samples of *Anopheles gambiae s.s* positive controls obtained from the IMAT insectary facility, at Korle-Bu Accra.

3.7 Detection of Voltage-Gated Sodium Channel and Acetylcholinesterase Mutations.

The methods according to Martinez *et al.*, (1998) and their primer sequence were utilized to detect target-site mutations namely, Kdr west (Vgsc-L1014F), Kdr-east (Vgsc-L1014S), and Ace-1 (G119S) in *Anopheles gambiae s.l* mosquitos exposed in WHO tube bioassays. To serve as DNA templates, individual mosquito legs were cut into PCR tubes. The reaction mixture for analyzing the L1014F resistant allele constituted of 0.6µl of AgD1 (5'-ATA GAT TCC CCG ACC ATG-3'), 0.6µl of AgD3 (5'-AAT TTG CAT TAC TTA CGA CA-3'), 0.6µl of AgD2 (5'-AGA CAA GGA TGA TGA TGA ACC-3'), 4.5µl of nuclease free water, and 7.5µl of Dream Taq. A total of 15.0µl of the reaction mixture was pipetted into each PCR containing mosquito leg DNA template. The PCR products were expected to produce fragments of 293bps and 137bps for homozygous susceptible (SS), and three fragments of 293bps, 195bps and 137bps for heterozygous resistant (RS) and finally fragments of 293bps and 195bps for homozygous resistant (RR)

The reaction mixture composition for analyzing L1014S resistant allele were primers; 0.6µl AgD1 (5'-ATA GAT TCC CCG ACC ATG-3'), 0.6µl AgD2 (5'-AGA CAA GGA TGA TGA ACC-3'), 0.6µl AgD4 (5'-CTG TGA TGA TAG GAA ATT TA-3'), 0.6µl AgD5 (5'-TTT GCA TTA ACT TAC GAC T-3'), 0.6µl AgD6 (5'-TTT GCA TTA ACT TAC GAC T -3'). A 5.0µl of Dream Taq and 2.0µl of nuclease free water. A total of 10.0µl was pipetted into each PCR tube containing mosquito leg DNA template. The digestion products resulting from the PCR process were expected to produce fragments of 293bps and 137bps for homozygous susceptible (SS), 293bps, 195bps and 137bps for heterozygous resistant (RS) and 293bps and 195bps for homozygous resistant (RR).

Denaturation was performed at 95°C for 3 minutes, followed by annealing in 35 cycles (95°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds). For a 5-minute extension, the temperature was set to 72 °C. The PCR products were put into wells of a 2 percent agarose gel stained with Ethidium bromide after completion. The gel was operated at 100V for 45 minutes, then photographed and visualized under ultraviolet light.

Similarly, analyzing G119S resistant allele was performed as described by Weil *et al*., (2000). The reaction mixture constituted; 0.4µl of Ex3Agdir (5'-GAT CGT GGA CAC CGT GTT CG-3'), 0.4µl of Ex3Agrev (5'-AGG ATG GCC CGC TGG AAC AG-5'), 4.2µl of nuclease free water, and 5.0ul of Dream Taq made up the reaction mixture. A total of 15.0µl of the reaction mixture was pipetted into Mosquito leg DNA templates in PCR tubes. An initial denaturation at 94°C for 3 minutes was followed by 35 cycles of annealing (94°C for 30 sec, 62 °C for 30 sec, 72°C for 20 sec) and a final extension at 72°C for 5 minutes. The restriction enzyme *Alu* 1 was used to digest the PCR products, which consisted of 3.9µl nuclease-free water, 1.0µl cut smart buffer, 0.1µl *Alu* 1, and 5.0µl PCR products. The digestion resulted in a 403-bps fragment for susceptible homozygous mosquitoes (SS) and two 253 bps and 150 bps fragments for homozygous resistant mosquitoes (RR). Heterozygous mosquitoes (RS) exhibited both sensitive and homozygous resistant bands.

3.8 Scientific and Ethical Approval.

Scientific and ethical approval was sought 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/M.1-5.5/2021-2022

3.9 Data Management and Statistical Analysis.

The results were displayed in tables and graphs. Statistical Package for Social Sciences (IBM) and Microsoft Excel were used for statistical analysis. Frequency distribution was used to determine the most abundant *Anopheles gambiae* s.l species. The Hardy-Weinberg equilibrium equation was also used to compute the allele frequencies for resistant genotypes. The WHO standards were used to calculate insecticide susceptibility status. Mosquitoes knocked down during the 60-min exposure to clothianidin treated papers were expressed as percentages and expressed in a time series graph. A test population was considered susceptible if it had a mortality of 98% or higher; if it had a mortality rate of 90% to 97% was considered as possible resistance; and if it had a mortality rate of less than 90%, the test population was indicative of resistance. The daily mortality counts were expressed as percentages and expressed in a graph with target lines indicating levels of mosquito susceptibility, suspected resistance and resistance. Mortality rates of mosquitoes exposed in cones on cement and mud wall surfaces were computed in Microsoft Excel and expressed in a bar graph.

CHAPTER 4

4.0 RESULTS.

4.1 WHO Susceptibility Bioassay

Exposure of wild *Anopheles* mosquitoes to clothianidin after 30 mins resulted in 2% of mosquitoes being knocked down. After 60 mins of exposure, 13% were also knocked down. For the susceptible Kisumu strain, 51% of mosquitoes were knocked down within 30 mins of exposure, however, after 60 mins of exposure 78% were knocked down. Clothianidin was observed to have a stronger knockdown effect on susceptible Kisumu strain than on wild *Anopheles* mosquitoes. This is shown in figure 4.1 below.

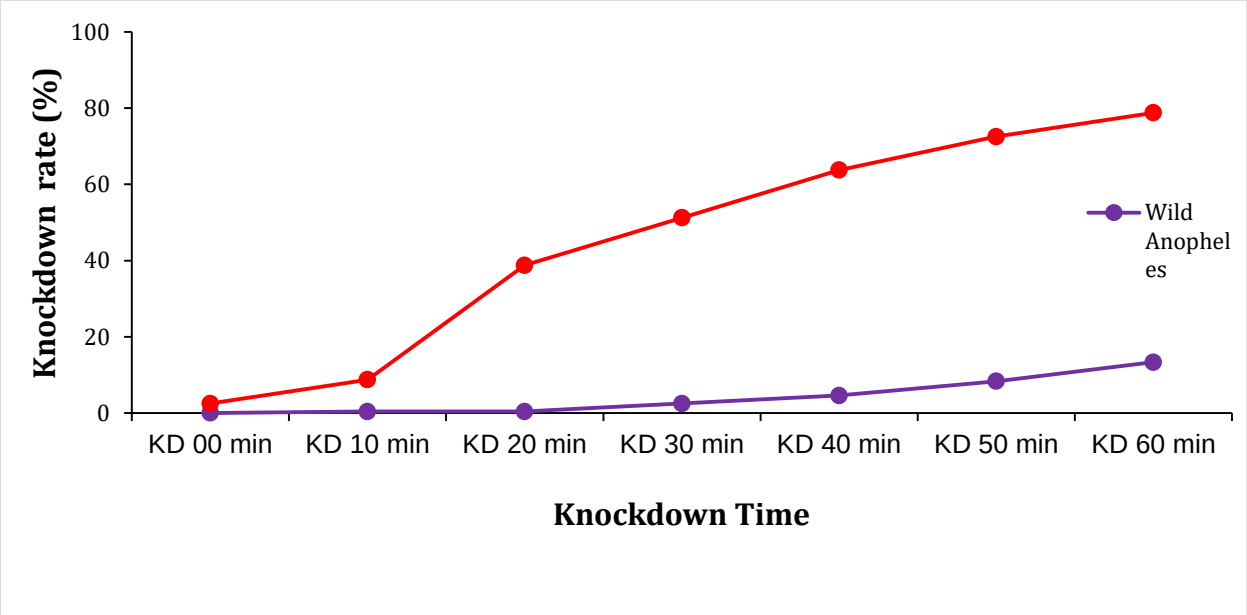

Figure 4.1 Knockdown effect of clothianidin on wild *Anopheles* mosquitoes and Kisumu strain.

Daily mortalities: After 24 hours, mortality rate for wild *Anopheles* mosquitoes was 56%, which showed resistance to the insecticide. This increased to 90% after 96 hours (4days), indicating suspected resistance in the mosquitoes. Mosquito mortality reached 99% after 120 hours (5 days) indicating susceptibility. For the positive control susceptible Kisumu strain, after 24 hours (1 day), mortality was 99% indicative of susceptibility and finally showed 100% full susceptibility after 48 hours (2 days). These are shown in the figure 4.2 below.

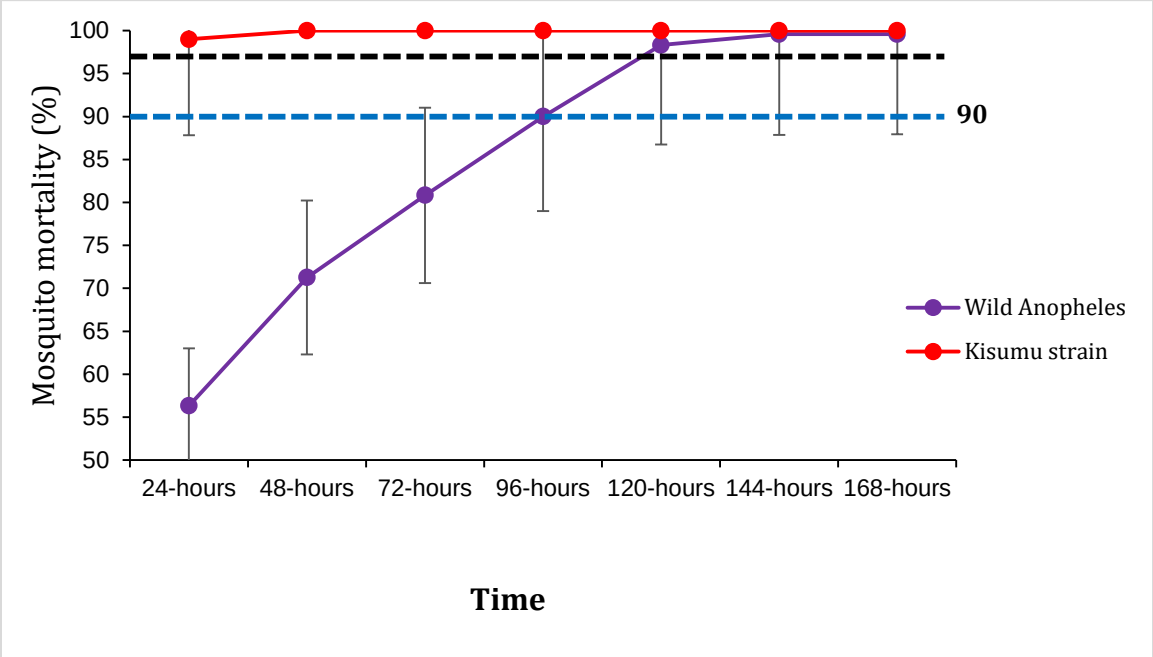

Figure 4.2 Delayed mortality rates of wild *Anopheles gambiae s.l* and susceptible Kisumu strain following 60-min exposure to clothianidin. Error bars indicate 95% confidence interval

4.2 Effect of exposure to clothianidin using WHO cone bioassays

The mortality rate, after 2 4 hours (1day), for wild *Anopheles* mosquitoes exposed in WHO cone bioassays for 5-min, was 63% showing resistance. This increased progressively to 97% after 120 hours, giving an indication of possible resistance. However, after 144 hours (6 days), mortality of 100% was reached indicative of vector susceptibility.

Mosquitoes exposed for 10-min had a mortality of 72% after 24 hours (1 day) showing resistance, which increased to 97% after 96 hours (4 days) indicating of possible resistance. A total of 120 hours (5 days) was taken to reach 100% showing full susceptibility.

After 24 hours (1 day), mosquitoes exposed for 20-min reached 82% mortality indicating resistance, which increased to 97% after 96 hours (4 days) indicative of possible resistance, this increased slightly to 98% after 120 hours (5 days) indicating mosquitoes have achieved susceptibility.

A 95% mortality rate was reached for mosquitoes exposed for 30-min after 24 hours (1 day), thus showing an indication of possible resistance, however, after 48 hours (2 days), 98% mortality was reached indicative of susceptibility. These are shown in figure 4.3 below

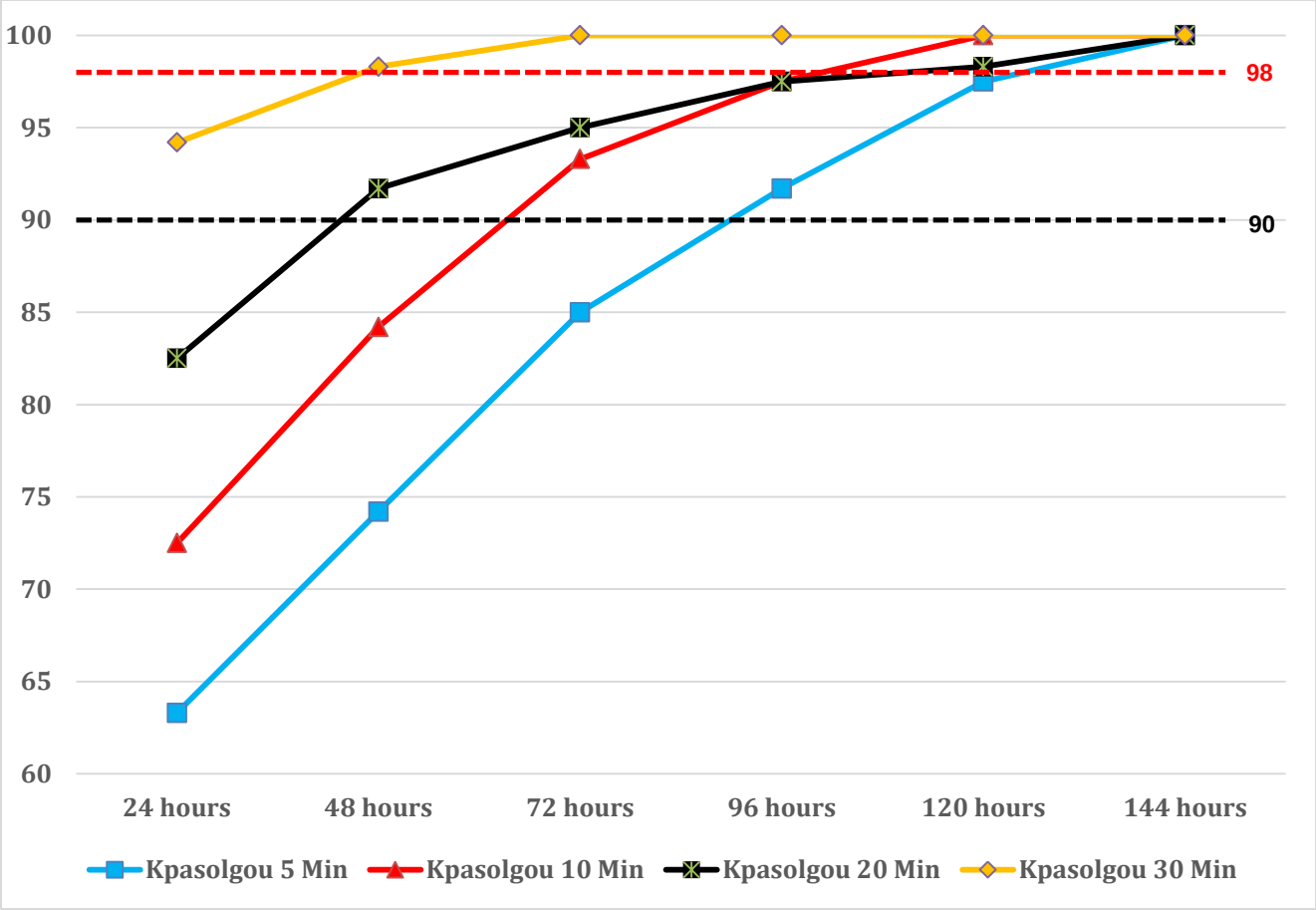

Figure 4.3 Survival curve of daily mortalities of alive mosquitoes 24 hours post wall exposures for a range of exposure times. Error bars represent 95% confidence interval.

Mosquitoes exposed on mud wall surfaces for 5-min reached a mortality rate of 60% after 24 hours and became susceptible after 144 hours. Similarly, mosquitoes exposed for 10-min recorded a mortality rate of 62% after 24 hours but became susceptible after 120 hours. Again, mosquitoes exposed for 20-min recorded a mortality rate of 80% after 24 hours and became susceptible after 96 hours. Finally, mosquitoes exposed for 30-min reached full susceptibility (100%) after 48 hours. However, mosquitoes exposed on cement wall surfaces for 5-min after 24 hours recorded a mortality rate of 72% but became susceptible after 120 hours. For the mosquitoes exposed for 10-min, after 24 hours of exposure recorded a mortality rate of 77% but became susceptible after 96 hours. Mosquitoes for 20-min recorded mortality rate of 85% after 24 hours but became susceptible after 48 hours. Finally, mosquitoes exposed for 30-min became susceptible after 24 hours.

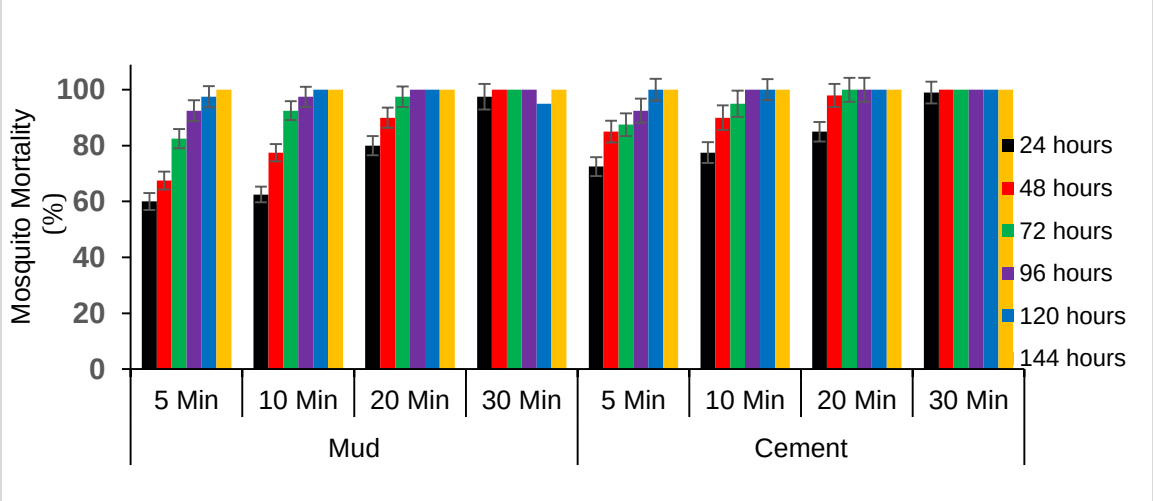

Figure 4.4 Mortality rates of wild *Anopheles gambiae* s.l exposed on mud and cement wall surfaces.

4.3 Species Composition.

Sub samples of *Anopheles gambiae s.l* exposed to the WHO tube bioassays from field collections were processed by conventional PCR to differentiate the sibling species. This revealed that *Anopheles gambiae s.s* was the most abundant accounting for (63%), followed by *Anopheles coluzzii* (25%), with *Anopheles arabiensis* accounting for (9%), (3%) did not amplify. These are shown in the table 4.1 below

4.4 Frequency of resistant alleles (Kdr and Ace-1) among *Anopheles gambiae s.l* populations from Northern Ghana.

The vgsc L1014F occurred in very high frequencies across all vector species identified whereas Ace-1 mutation occurred in moderation. The allele frequency of vgsc L1014F in *Anopheles gambiae s.s* was 0.89 (P=0.32), whilst *Anopheles coluzzii* had an allele frequency of 0.92 (P=0.66). Similarly, *Anopheles arabiensis* was also observed to have an allele frequency of 0.94 (P=0.86). The Ace-1 G119S mutation was also observed in all species of *Anopheles* mosquitoes identified with frequencies of 0.37 (P=0.0001) in *Anopheles gambiae s.s*, 0.32 (P=0.019) in *Anopheles coluzzii* and 0.44 (P=0.016) in *Anopheles arabiensis*. The vgsc L1014S mutation was not detected in any of the vector species. These are shown in the table 4.1 below.

Table 4.1 Frequency of resistant alleles (Kdr and Ace-1) and species composition of *Anopheles gambiae s.l* populations from Northern Ghana.

Species	Species Composition	*Vgsc*				*Ace-1*		
		Locus 1014			P-	Locus 119		P-
		L1014	L1014F	L1014S	value	G119	G119S	value
Anopheles gambiae s.s	63	0.11	0.89	0.00	0.32	0.63	0.37	0.0001
Anopheles coluzzii	25	0.08	0.92	0.00	0.66	0.68	0.32	0.019
Anopheles arabiensis	9	0.06	0.94	0.00	0.86	0.56	0.44	0.016

Note: if < 0.05= significant departure from Hardy-Weinberg equilibrium.

CHAPTER 5

5.0 DISCUSSION

Malaria vectors in Ghana have developed resistance to all four (4) classes of insecticides that threatens the sustainability of malaria vector control programmes in the country. The widespread insecticide resistance highlights the need to introduce new insecticide classes with diverse modes of actions in key malaria vector control interventions. This could potentially minimize the effects of insecticide resistance and also ensure that malaria vector control interventions maintain their efficiency by keeping mosquito population under control. In this study, there was the detection of insecticide resistant markers in high frequencies in the malaria vectors; however, they showed phenotypic susceptibility to clothianidin.

WHO susceptibility test results demonstrated significantly effective but protracted mortality of the wild *Anopheles gambiae s.l* unlike the unlike the neurotoxic insecticides; pyrethroids, carbamates, organochlorides and organophosphates which are quick-acting and require only 24 hours for optimum mortality to be reached. In the cone bioassays, mosquitoes exposed for relatively longer minutes required fewer days to become susceptible. Generally, irrespective of the exposure time, mosquitoes required a maximum of six (6) days to reach full susceptibility. Test Anopheles mosquitoes exposed according to WHO's standard protocol of thirty (30) minutes exposure time required forty-eight (48) hours to become susceptible. The duration of exposure of mosquitoes in cones was a determinant in the timing of mosquito mortality. The neonicotinoids act on the nicotinic acetylcholine receptors (nAchR) of insects

causing stomach poison and acute contact toxicity (Ngufor *et al.,* 2017; Oxborough *et al.,* 2019), the last-mentioned suspected to be responsible for mosquito delayed mortality (Oxborough *et al.,* 2019). It was also observed that mosquitoes exposed on cement wall surfaces recorded slightly higher mosquito mortality than those exposed on mud wall surfaces. Previous studies in determining factors which affect residual efficacy of insecticides revealed that, insecticides sprayed on cement wall surfaces have a high residual activity but a short residual life as compared to mud wall surfaces which also gives a relatively low residual activity but with a longer residual life. A more likely explanation is the difference in absorption of insecticides by these wall surfaces (Ibrahim *et al.,* 2014; Oxborough *et al.,* 2016).

WHO susceptibility and cone bioassays showed that mosquitoes were phenotypically susceptible to clothianidin, the new insecticide used in IRS in northern Ghana. However, the malaria vectors harbored high frequencies of Kdr and Ace-1 mutations. This was an interesting finding in this research study, and signifies that, vector control interventions with clothianidin could drastically reduce the population of malaria transmitting mosquitoes, and probably, the prevalence of malaria in northern Ghana. In 2019, Oxborough *et al*., (2019) conducted a study to assess the susceptibility of malaria vectors to clothianidin in sixteen (16) African countries, which included Ghana. The findings from this study showed that, malaria vectors across these countries were susceptible to clothianidin, thus confirming our findings. It was also observed in this study that, clothianidin demonstrated a slow mode of action, often requiring a maximum of seven (7) days for optimum mortality to be reached. This observation emphasizes the need to assess delayed mortality, in order to avoid an underestimation of the efficacy of the insecticide

and also to avoid false reportage of malaria vector resistance (Oxborough *et al.,* 2019; Agumba *et al*., 2019).

The most abundant malaria vector species identified from this study was *Anopheles gambiae s.s*, followed by *Anopheles coluzzii* with *Anopheles arabiensis* occurring in lesser numbers. The findings from this study is similar to findings by Hinnie *et al*., (2021), who also reported the presence of these vector species in the Sahel Savannah zone of Ghana, with the predominant species being *Anopheles gambie s.s* and *Anopheles coluzzii*, which sometimes vary in abundance in the different communities within the Sahel savannah zone of the country.

The kdr mutations detected occur in the voltage gated sodium channels and confer resistance to mosquitoes against pyrethroids and DDT. Resistance to carbamates and organophosphates is mediated by the Ace-1 mutations. The introduction of clothianidin for IRS in northern Ghana from the year 2021 offers a new pathway of action in controlling resistant mosquito population. Since mosquitoes have become resistant to the current four (4) classes of insecticides. The occurrence of cross-resistance is less likely as clothianidin acts on nicotinic acetylcholine receptors, which is different from the target-sites of the four (4) insecticide classes (Oxborough *et al*., 2019). Ngufor *et al*., (2017) conducted a study to assess the susceptibility of pyrethroid-resistant A*nopheles* mosquitoes to a mixture of clothianidin and deltamethrin. The findings from this study showed that, these resistant mosquitoes were susceptible to clothianidin, thus corroborating the absence of cross-resistance. Clothianidin therefore promises to be a very effective insecticide for public health use against resistant *Anopheles* population.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion.

In this study, it was found that malaria vectors were phenotypically susceptible to clothianidin that was used for IRS in northern Ghana for malaria vector control. The duration of mosquito exposure to clothianidin in cones played a significant role in the time required to reach susceptibility. The wild mosquitoes also carried resistant mutations including the kdr allele L1014F and Ace-1 mutation. The most abundant malaria vector species collected from larval habitats in northern Ghana was *Anopheles gambiae s.s* followed by *An. coluzzii* and *An. arabiensis* were found in small numbers. The introduction of clothianidin as a key malaria vector control interventions could reduce local malaria transmission in areas with resistant mosquito population.

6.2 Recommendations.

1. This is the first time clothianidin is being used for IRS in Ghana, and the results of the study show that malaria vectors are phenotypically susceptible to it. Yearly surveillance of susceptibility of clothianidin in malaria vectors in northern Ghana is recommended.
2. It is recommended that clothianidin should be rotated with other insecticides as an insecticide resistance management strategy

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8.0 Appendices

Appendix I WHO Susceptibility Bioassay Form (1)

Time/minutes	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control2
0						
10						
20						
30						
40						
50						
60						

Holding period/Days	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
1						
2						
3						
4						
5						
6						
7						

Appendix II Cone Bioassay Form

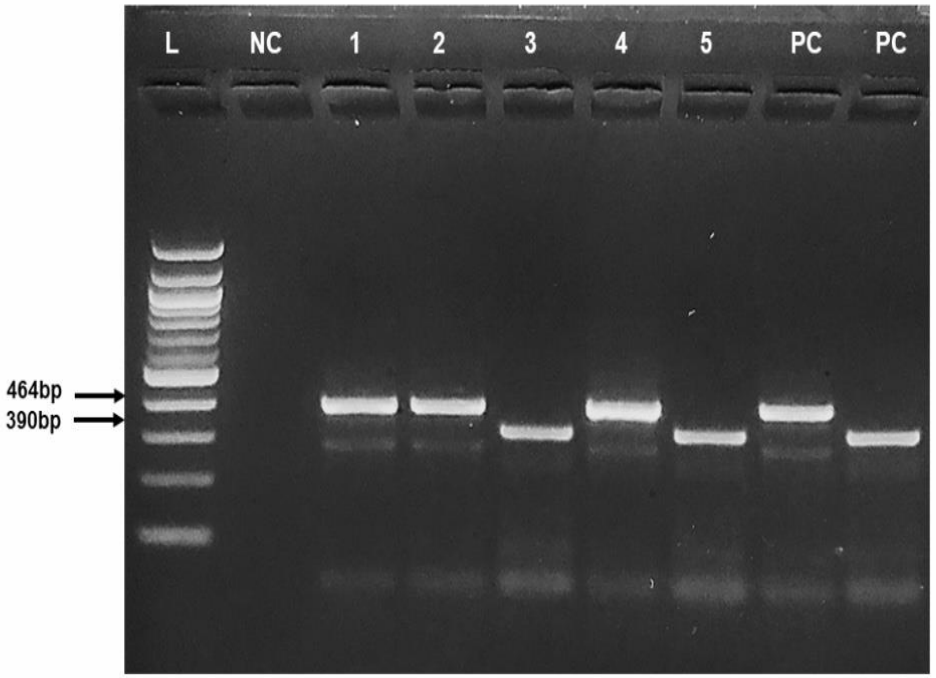
Knock Down

Time/minutes	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Co+/ Kisumu	Co/ Wild	Co-/ Kisumu
0							
10							
20							
30							
40							
50							
60							

Daily mortalities

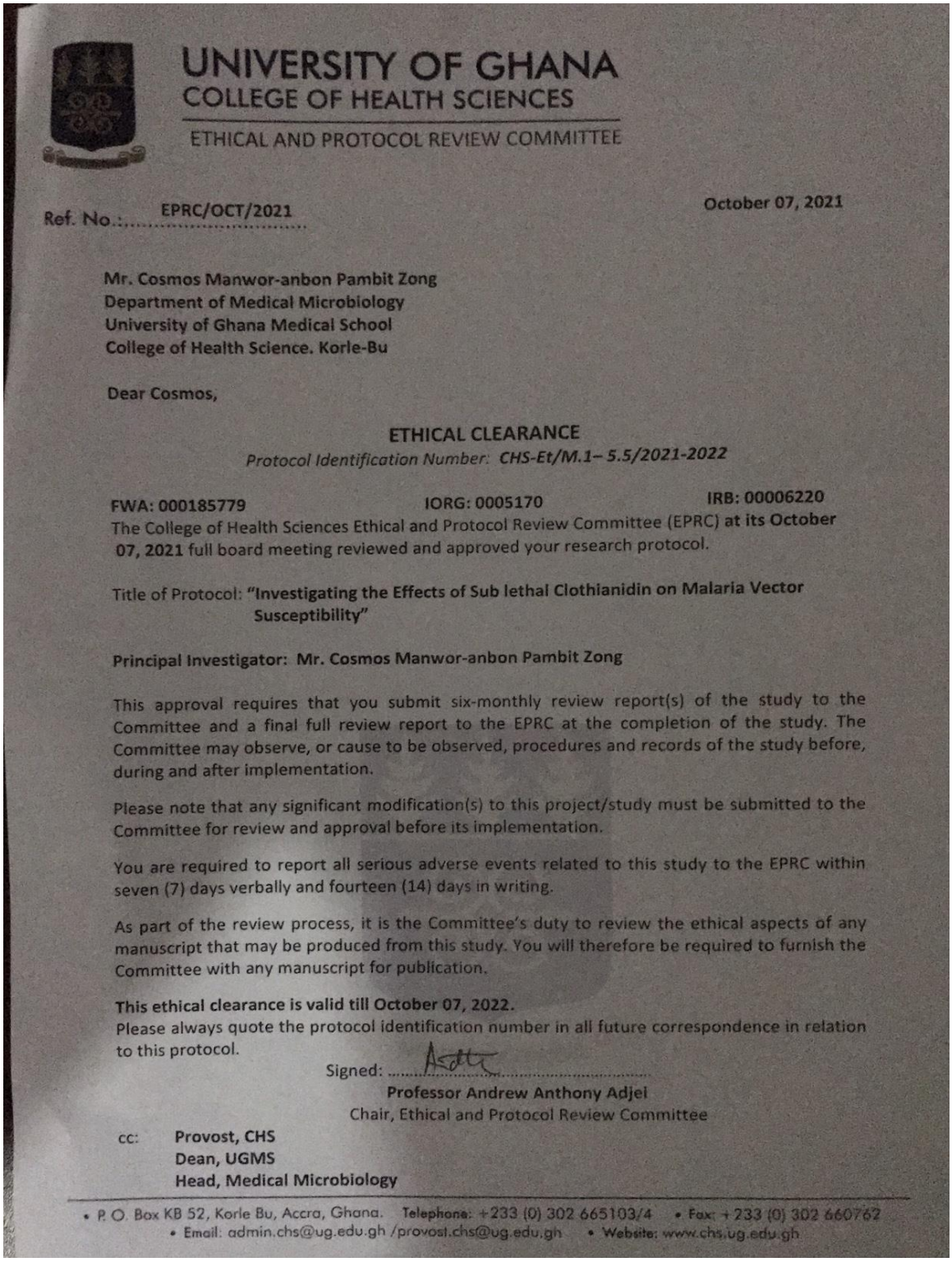
Holding period/Days	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Co+/ Kisumu	Co/ Wild	Co-/ Kisumu
24							
48							
72							
96							
120							
144							
168							

Appendix III: Agarose gel showing PCR amplification of Anopheles gambiae complex



L= DNA LADDER, NC = NEGATIVE CONTROL, PC= POSITIVE CONTROL
(2% Agarose gel image showing PCR amplification of *Anopheles gambiae complex;*
An. gambiae = 390bp, *An. arabiensis* = 464bp)

Appendix IV Ethical clearance form



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

Ref. No.: EPRC/OCT/2021

October 07, 2021

Mr. Cosmos Manwor-anbon Pambit Zong
Department of Medical Microbiology
University of Ghana Medical School
College of Health Science. Korle-Bu

Dear Cosmos,

ETHICAL CLEARANCE

Protocol Identification Number: ***CHS-Et/M.1– 5.5/2021-2022***

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

The College of Health Sciences Ethical and Protocol Review Committee (EPRC) **at its October 07, 2021** full board meeting reviewed and approved your research protocol.

Title of Protocol: **"Investigating the Effects of Sub lethal Clothianidin on Malaria Vector Susceptibility"**

Principal Investigator: Mr. Cosmos Manwor-anbon Pambit Zong

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 October 07, 2022.
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, Medical Microbiology

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