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Approval Sheet

Assessment of the Prevalence, breeding sites and Insecticide Resistance among Mosquitoes of Medical Importance in two selected cities in Sierra Leone.

By

Christopher Sandi

Master of Public Health

Hubert Department of Global Health

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Dr. Deborah A. McFarland

Committee Chair

_____

Dr. Dabney P. Evans

Committee Member

_____

Dr. Rebecca S. Levine

Committee Member

Abstract Cover Page

Assessment of the Prevalence, breeding sites and Insecticide resistance among Mosquitoes of Medical Importance in two selected cities in Sierra Leone.

By

Christopher Sandi
Higher Diploma in Community Health and Clinical Studies
Njala University
2010

Thesis Committee Chair: Deborah A. McFarland, PhD, MPH

An abstract of
A thesis submitted to the Faculty of the Rollins School of Public Health of Emory University in partial fulfillment of the requirements for the degree of Master of Public Health in Hubert Department of Global Health
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ABSTRACT

The prevalence of arboviral diseases transmitted by *Aedes aegypti* mosquitoes in West Africa has increased in recent years, with outbreaks of Dengue, Yellow Fever, and Chikungunya reported throughout most of the region. However, little basic entomological information is known about the vector in the region, which limits the ability to prevent and control its spread and hence transmission of the associated viruses. Therefore, we performed an entomological survey of *Ae. aegypti* in Sierra Leone, where *Ae. aegypti* has not been studied in recent decades. Between June and August 2017, we conducted egg, immature, and adult sampling of *Ae. aegypti* in two locations in Sierra Leone (Bombali and Bo Districts). Eggs were collected using ovitraps to estimate densities and then used to rear adults in the insectary. Lab-reared females were tested for resistance to six different insecticides as well as for the prevalence of knock-down resistance (kdr) mutations. Household surveys for immatures were conducted to identify common breeding sites and calculate standard *Aedes* infestation indices. Wild-caught adult females were collected from houses using traps and aspirators and were later tested for arboviruses. The overall mean eggs per trap was 23.7 while 58 adult females were captured from 194 houses in total. Immature indices were high in general across both sites, ranging from 13-17 for the container index, 18-61 for the house index, and 65-153 for the Breteau index. Tires and bottles were the most common breeding sites, accounting for 44% and 20% (respectively) of all positive container types. Adults reared from eggs in both locations were 100% susceptible to deltamethrin and resistant to permethrin (53% mortality). Evidence of higher kdr allele frequencies among permethrin-resistant mosquitoes was found in Bombali. Testing of adult mosquitoes for flaviviruses and chikungunya was negative, however the sample size was small. Recommendations arising from

these findings are to focus on tires and bottles for clean-up campaigns, continue arboviral surveillance in mosquitoes, and use deltamethrin rather than permethrin if chemical control measures begin for *Ae. aegypti* in either location.

ASSESSMENT OF THE PREVALENCE, BREEDING SITES AND INSECTICIDE RESISTANCE AMONG MOSQUITOES OF MEDICAL IMPORTANCE IN TWO SELECTED CITIES IN SIERRA LEONE, WITH A FOCUS ON *AEDES AEGYTI*

Christopher Sandi

Thesis submitted to Hubert Department of Global Health

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MASTER OF PUBLIC HEALTH

In

Infectious Diseases

Deborah A. McFarland, PhD, Chairperson

Dabney P. Evans, PhD, MPH

Rebecca S. Levine, PhD, MPH

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TABLE OF CONTENTS

DEDICATION

I dedicate this to the Almighty God, who guide and never leave me in making this research, to my Father who is now late; may his gentle soul rest in peace. As he had made me to be whom I am today. I wouldn't have never been able to reach this stage if it were not for the support you have been given to me when you were alive. And to each member of this group for making this research possible. I will also want to dedicate this work to my son, who has been with me during my tough times in doing this write up.

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND AND IMPORTANCE

Mosquitoes of medical importance are those that transmit disease in humans and are therefore considered important in human epidemiology. These include *Anopheles*, *Culex* and *Aedes*. These mosquitoes differ in terms of the diseases they transmit, their feeding patterns and breeding sites. The *anopheles* transmit Malaria and feed between sunset and sunrise. They tend to feed on people and cattle, rather than other warm-blooded creatures. They will fly farther than a mile in search of a bloodmeal, and their breeding sites are typically in clean and unpolluted water sources and fresh or salt water. Examples are: edges of streams/rivers, puddles, temporary rain pools, grassy ditches, rice fields, swamps, marshes. Culex transmit Lymphatic Filariasis (LF) and various Encephalitis Viruses such as West Nile Virus. They also feed primarily between sunset and sunrise. They are considered aggressive and persistent biters. They feed on birds, but they will also feed on people and other animals. They will fly more than a mile in search of a bloodmeal. Their breeding sites are in polluted or dirty water, and either fresh or salt water. Examples include: gutters, sewage, ditches, barrels, and ground water. *Aedes* are known for transmitting viral diseases such as Dengue, Yellow Fever, Zika, and Chikungunya. They feed day and night, and several of the species are considered particularly troublesome because they prefer to feed on people. They usually fly no more than 200 meters in their lifetimes. Some species breed exclusively in and around human homes, so they are sometimes considered domestic mosquitos. Their breeding sites are usually fresh water with minimal pollution, such as: artificial containers, tires, barrels and buckets, toilet tanks, water jars for house pets, flower pots and bases, and many types of discarded

items (bottles, cans, jars, plastic containers, vehicles, appliances, and machinery) (MosquitoWorld (2017).

1.2 VECTOR SURVEILLANCE

Mosquito based-surveillance is a critical component in quantifying human disease risk and in mitigating mosquito-borne disease outbreaks, by determining local vector presence and abundance as well as pathogen prevalence within vectors. In the absence of effective vaccines for many mosquito-borne diseases, their prevention and control depend on the reduction of mosquito populations and minimizing vector-human contact. Mosquito surveillance provides the basis for making decisions that guide these vector control interventions.

Aedes aegypti is the primary mosquito vector for the viruses: Yellow Fever, Dengue, Zika, and Chikungunya; which are priority pathogens in West Africa. An explosive Yellow Fever outbreak in Angola and Democratic Republic of Congo (DRC), an ongoing Dengue outbreak in Burkina Faso, and the spread of the American Zika strain into Guinea-Bissau, Cape Verde, and Angola were observed in 2016. Surveillance methods used for *Aedes* are similar to surveillance methods used for other vectors (including *Anopheles* and *Culex*, for malaria and filariasis respectively), so improvements in *Aedes* surveillance will improve vector surveillance and control in general, under an Integrated Vector Management (IVM) framework.

1.3 ISSUES OF INSECTICIDE RESISTANCE

Recent research in insecticide resistance among mosquitoes has confirmed strong suspicions that wide scale use of a single class and related classes of mosquito insecticides has given rise to resistance in several predominant malaria vector species, including, but not limited to *Aedes Aegypti* (WHO, 2017c). Resistance has been reported in several malaria endemic countries, with

pyrethroids resistance being the most common in sub-Saharan Africa (Ranson, N'Guessan, et al., 2011). This resistance may be due to increase in selection pressure caused by the use of pyrethroids in all the approved Long Lasting Insecticide Treated Nets (LLITNs) and in most Indoor Residual Spraying (IRS) programs worldwide (Bhatt et al., 2011). The pyrethroids used include deltamethrin and permethrin compounds. In some regions, malaria has been reported to be on the rise even after a significant decline of malaria cases in the previous years. This rise was initially associated with insecticide resistance as a result of the continued use of LLITNs. (Snow, Amratia, Kabaria, Noor, & Marsh, 2012)

However, Viana et al. recently observed that continuous exposure to insecticides against insecticide resistant mosquito population reduces malaria transmission (Viana, Hughes, Matthiopoulos, Ranson, & Ferguson, 2016). With the appearance and rapid spread of the West African Knock Down Resistance (Kdr) mutation and the recent increase in the frequency of the East African kdr mutation in mosquitoes, regular monitoring is required within different vector population in a locality. This will help elucidate the mechanism of resistance and thus lead to downstream implementation of improved and effective vector control strategies and ultimately a decline in malaria incidence. The compromised mode of action should be well understood to allow for the introduction of a new class of insecticide with a different mode of action against the malaria vectors. It is important however also to understand that further extension of vector control intervention poses a threat to an increase in vector resistance. Reducing reliance on a single intervention or a single insecticide is a major objective of any resistance management policy.

1.4 GAPS IN SURVEILLANCE AND MONITORING OF RESISTANCE

There are no recent published studies on *Aedes* in Sierra Leone and limited research on *Aedes* in Africa. In 1967 a study was conducted in two West African countries to document the distribution, density, and seasonal prevalence of the *Aedes aegypti* mosquitoes.(Surtees, 1967) There is scientific evidence to show that *Aedes-borne* diseases are in circulation in Africa. For example, in 2009, Sierra Leone had two cases of Yellow Fever from two communities in Bo District, and in 2011, Sierra Leone had two cases of the same illness in Bonthe District. Bonthe was one of two districts that did not receive vaccine coverage (WHO, 2017f).

The proposed study provides information on the current status of susceptibility or resistance to insecticides on mosquito vectors in Bombali and Bo Districts after the scaling up of vector control interventions in these areas from the Ministry of Health and Sanitation (April- June 2017) (Ministry_of_Health_and_Sanitation, 2016).

1.5 PURPOSE

The purpose of this study is to determine the prevalence, breeding sites and insecticide resistance among mosquitoes of medical importance with specific reference to *Aedes Aegypti.*

1.6 RESEARCH QUESTIONS

1. What is the prevalence (abundance and density of eggs, immature, and adults) of mosquitos of medical importance, with a focus on *Aedes Aegypti* in two cities in Sierra Leone (Bombali and Bo)?
2. What are the primary breeding sites in and around human homes in urban and peri-urban areas?
3. Are *Aedes aegypti* resistant to any of insecticides? If so, what are the primary resistance mechanisms?
4. Are any *Aedes-borne* viruses (Dengue (DEN), Zika (ZIK), Chikungunya (CHIK), Yellow Fever (YF)) detectable in field-collected adult females?

1.6.1 Null Hypotheses

1. *Aedes* mosquitoes are not prevalent in Sierra Leone (Bombali and Bo)
2. *Aedes* mosquitoes do not breed around human homes in urban and peri-urban areas
3. *Aedes* mosquitoes are not Resistant to insecticides in Sierra Leone
4. There are no viruses detected (DEN, ZIK, CHIK, YF) in any of the field-collected adult females *Aedes* mosquitoes.

1.6.2 General objective

To assess the prevalence, breeding sites and insecticide resistance among mosquitoes of medical importance in two selected cities in Sierra Leone with focus on *Aedes Aegypti*.

1.6.3 Specific objectives

1) Locate and describe breeding habitats (types of containers) and densities of immature stages (larvae and pupae) of *Ae. aegypti*. Calculate appropriate indices of infestation (house, container, and breteau).
2) Collect *Aedes* eggs using ovitraps. Measure egg densities (eggs per trap). Eggs will also be used to rear adult mosquitoes to measure insecticide resistance using the CDC bottle bioassay.
3) Collect adult mosquitoes using BG Sentinel traps. Measure adult densities (adult females per trap). Test adults for viruses.
4) Compare human disease indicators for Yellow Fever, Dengue, Chikungunya, and/or Zika (if available) to entomological indices.
5) Extract DNA from mosquito samples, conduct PCR analysis to detect genes related to insecticide resistance, and perform sequence analyses on samples with unusual results.

This study also provides information on the parasite infection rates of the vector species that acted as hosts for the parasites (Dengue virus, Yellow Fever virus, Chikungunya virus and Zika virus) in Makeni and Bo. This information can be used to implement future vector control strategies.

1.8 DEFINITION OF TERMS

ENCEPHALITIS: swelling of the brain tissue (WHO, 2018b).

EPIDEMIOLOGY: the study of the distribution and determinants of health-related states or events in a specified population, and the application of this study to the control of health problems. (WHO, 2018c)

INSECTICIDE: A chemical used specifically to kill or control the growth of insects (Mosquitoes) (Wikipedia, 2018a).

KNOCK DOWN RESISTANCE: describes cases of resistance to diphenylethane (e.g. DDT) and pyrethroid insecticides in insects (Mosquitoes) and other arthropods that result from reduced sensitivity of the nervous system caused by point mutations in the insect population's genetic makeup.

MUTATION: Is the permanent alteration of the nucleotide sequence of the genome of an organism, virus, or extrachromosomal DNA or other genetic elements (Wikipedia, 2018b).

OVIPOSITION: It is the process of laying eggs.

PATHOGEN: A biological agent that causes disease or illness to its host. (ScienceDaily, 2017)

PREVALENCE: The proportion of mosquitoes in a population who have a particular disease or attribute at a specified point in time or over a specified period of time. (CDC, 2012)

SURVEILLANCE: Ongoing, systematic collection, analysis, and interpretation of health-related data essential to the planning, implementation, and evaluation of public health practice (CDC, 2017b).

VECTOR: disease-causing pathogens

CHAPTER TWO
LITERATURE RELEVANT TO THE STUDY

2.1 Introduction

The objective of this chapter is to review the relevant literature on the prevalence, breeding sites, behavior, and insecticide resistance among mosquitoes of medical importance. Specific attention is given to *Aedes Aegypti* and other moquitoes of medical importance in Sierra Leone. We consider current methods of resistance management and upcoming methods of resistance management which may come into widespread use in the future.

Aedes aegypti and *Aedes albopictus* are the primary vectors of viral diseases such as Dengue, Chikungunya, Yellow Fever, and Zika. Together, these viruses represent a rapidly increasing burden of morbidity and mortality and a major public health issue worldwide. Of even greater concern is the fact that both mosquito species have vastly increased their range in the past 50 years, putting millions of people at risk of infection (WHO, 2017a). Both types of mosquitoes are found in Sierra Leone, where they constitute a major threat, especially to unvaccinated populations.

The most effective method to prevent mosquito-borne infections is vector control, which is largely achieved through residual indoor spraying and insecticide-treated bed nets. However, resistance to insecticide compounds has been on the rise in recent years. A seroprevalence study was conducted on vector-borne diseases (Chikungunya, Dengue, and Malaria) in Bo District in Southern Sierra Leone in 2012-2013, and it revealed that Chikungunya has a very high prevalence (39%) compared to Malaria (24%) (Dariano et al., 2017).

To inform vector control efforts it is important to examine the breeding sites and prevalence of mosquitoes. In Sierra Leone it is unknown if these mosquitoes are resistant to any insecticides. If resistance is found, the data collected in this study will be used to inform the Ministry of Health on vector control strategy.

2.2 Mosquitoes of medical importance

2.2.1 Life cycle

The mosquito is a holometabolous insect, meaning that it goes through a complete metamorphosis with an egg, larva, pupa, and adult stage. The immature stages are found in water-filled habitats, mostly in artificial containers closely associated with human dwellings and often indoors. The adult life span can range from two weeks to a month depending on environmental conditions (Abreu, Morais, Ribeiro, & Eiras, 2015; Zettel & Kaufman, 2015). *Aedes* mosquitoes are visually distinctive because they have noticeable black and white markings on their body and legs. Unlike most other mosquitoes, they are active and bite only during the daytime. The peak biting periods are early in the morning and in the evening before dusk (Zettel & Kaufman, 2015).

Only the female bites for blood, which she needs to mature her eggs. Flight range studies suggest that most female *Ae. aegypti* may spend their lifetime in or around the houses where they emerge as adults and they usually fly an average of 400 metres. This means that people, rather than mosquitoes, rapidly move the *Aedes* viruses within and between communities and places (WHO, 2017a). To find a host, these mosquitoes are attracted to chemical compounds emitted by mammals, including ammonia, carbon dioxide, lactic acid, and octenol (Zettel & Kaufman, 2015).

2.2.2 Habitat and its impact on disease transmission

Aedes aegypti comes in three polytypic forms: domestic, sylvan, and peridomestic. The domestic form breeds in urban habitats, often around or inside houses. The sylvan form is a more rural form, and breeds in tree holes, generally in forests, and the peridomestic form thrives in environmentally modified areas such as coconut groves and farms (Zettel & Kaufman, 2015). Each of these species has a particular ecology, behavior and geographical distribution (Gratz,

2004). In Asia and the Americas, *Ae. aegypti*, also known as "urban" or "domestic" is the predominant vector, while sylvatic transmission by *Ae. formosus*, the forest-dwelling subspecies, predominates in some African settings (Urdaneta-Marquez & Failloux, 2011).

2.2.3 Aedes as a vector of human diseases

The *Aedes aegypti* mosquito is the main vector that transmits the viruses that cause Dengue, Yellow Fever, Zika, and Chikungunya. These viruses are transmitted to human beings through the bites of infected female *Aedes* mosquitos, which contract the virus while feeding on infected human blood (WHO, 2017a). Within the mosquito, the virus infects the mosquito mid-gut and subsequently spreads to the salivary glands over a period of 8-12 days. After this incubation period, viruses can be transmitted to humans during subsequent probing or feeding. The lifespan of an adult *Ae. aegypti* is two to four weeks depending on environmental conditions. Additionally, *Ae. aegypti* mosquitoes are the primary vehicle for spreading Zika, the virus that has been associated with the neurological birth defect microcephaly, and with Guillain-Barre syndrome (Zettel & Kaufman, 2015).

Dengue infection rates are higher outdoors and during daytime, when these mosquitoes bite most frequently. However, *Ae. aegypti* breed indoors and are capable of biting anyone throughout the day. The indoor habitat is less susceptible to climatic variations and increases the mosquitoes' longevity (Zettel & Kaufman, 2015). Dengue outbreaks have also been attributed to *Aedes albopictus*, *Aedes polynesiensis* and several species of the *Aedes scutellaris* complex.

2.2.4 Aedes species and Human Impact

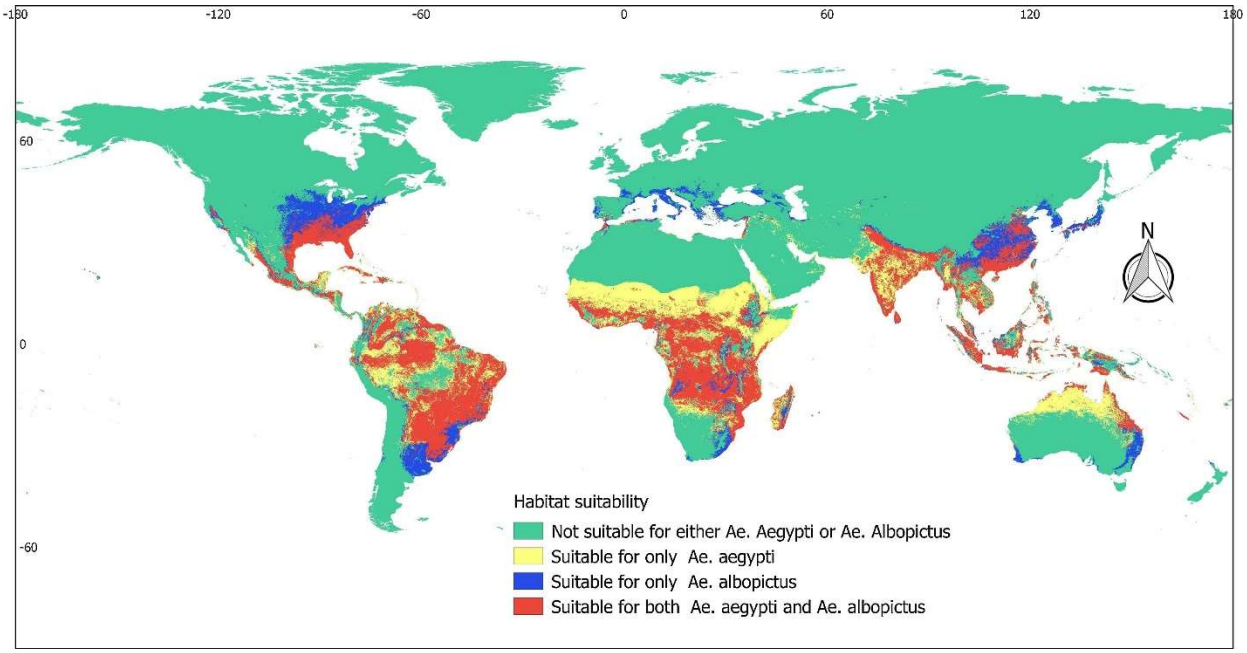
Ae. aegypti and *ae. albopictus* are vectors for both endemic diseases such as Dengue and Yellow Fever and emerging diseases such as Chikungunya and Zika. These diseases are becoming increasingly important due to the wide geographical range of their vectors and the high disease burden they cause. The past few decades have seen a large increase in distribution and public health impact of these arboviruses due to the widespread distribution of their vectors paired with

increases in trade and travel (Leta et al., 2017). Dengue infections alone have increased by a factor of 30 in the past 50 years and now account for 20,000 deaths each year. Even with a highly effective vaccine, Yellow Fever accounts for another 30,000 estimated deaths annually (WHO, 2017a). Since 2015 there have 200,000 confirmed cases of Zika, and more than half of these cases were in Brazil. Over 2,500 children that were born had a confirmed congenital syndrome that is linked with Zika virus, and most of these cases also came from Brazil (PAHO/WHO, 2017). In 2016, there were 146,914 laboratory confirmed cases of Chikungunya recorded within the Americas (WHO, 2018a).

2.3 Global distribution of *Aedes Aegypti* and other species of interest

Because it can breed in a variety of habitats and environments, *Aedes* can be found in urban, suburban, and peri-rural areas. *Aedes* species can transmit viruses in a vertical or transvenereal manner in nature, which explains why these viruses maintain endemic levels in areas where they are prevalent (Knudsen, 1995). *Aedes aegypti* and *Aedes albopictus* can now be found on all continents, including North America and Europe (Kraemer et al., 2015). Where the two species overlap (**see Figure 1**), they are often in competition for breeding sites and *Ae. Albopictus* has displaced *Ae. Aegypti* in certain areas (Gratz, 2004).

Figure 1: Range of Ae. aegypti and Ae. albopictus habitats



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Aedes albopictus is the second most important vector of dengue after *Ae. aegypti*. Combined, these two-species put 2 billion people in tropical and sub-tropical regions at risk of infection from Dengue. *Ae. albopictus* is primarily a forest species that has become adapted to rural, suburban and urban human environments. During the past 50 years, *Aedes albopictus* has expanded its range beyond Asia due to the international tire trade by laying its eggs in rainwater collected in these tires ((Rodhain, 1996), (Gratz, 2004)). The eggs can withstand very dry conditions (desiccation) and remain viable for many months in the absence of water. The European strain of *Aedes albopictus* can even undergo a period of reduced development (diapause) during the winter months (WHO, 2017a).The eggs of *Ae. aegypti* also exhibit this ability and can be viable for over a year in a dry state, which allows the mosquito to re-emerge after a cold winter or dry season, depending on climate (Zettel & Kaufman, 2015).

Ae. aegypti's distribution has also increased in the past two to three decades worldwide, and it is

considered to be among the most widespread mosquito species. *Aedes* mosquito populations have also been able to adapt for persistence in warm temperate climates. Such populations have been identified in parts of west and south of Sierra Leone (Kraemer et al., 2015).

2.4 Breeding sites and behavior of *Aedes Aegypti* and other species of interest

Aedes aegypti and *Aedes albopictus* larvae are commonly sampled at the same breeding sites, suggesting competition for habitats. Preferred sites of oviposition (laying eggs) include used tires, tin and plastic containers, abandoned car parts, brick holes, dead leaves on the ground, tree holes, and rock pools (Simard, Nchoutpouen, Toto, & Fontenille, 2005). After taking a complete blood meal, females produce on average 100 to 200 eggs per batch; however, the number of eggs produced is dependent on the size of the bloodmeal. Females can produce up to five batches of eggs during a lifetime. A smaller bloodmeal produces fewer eggs. Eggs are laid on damp surfaces in areas likely to temporarily flood and are placed at varying distances above the water line (Zettel & Kaufman, 2015).

Ae. aegypti favor stagnant water with a minimum of decaying organic detritus and have demonstrated a significant preference for areas that have increased access to food and decreased threat of predation. Studies have shown that certain chemicals emanating from bacteria in water containers stimulate the female mosquitoes to lay their eggs, and mosquitoes can discern between unfiltered water and filtered water in which bacteria once lived. They are particularly motivated to lay eggs in water containers that have the correct amounts of specific fatty acids associated with bacteria involved in the degradation of leaves and other organic matter in water (Simard et al., 2005).

Ae. aegypti females practice “skip oviposition” behavior, laying eggs at several different breeding sites, usually between four and six, but as many as 12 have been observed experimentally and in the wild. Typically, a “favorite” site receives up to 40% of the eggs. It has been hypothesized that

skipping oviposition may be used to reduce competition for food or for predator avoidance (Abreu et al., 2015). It was originally believed that *Ae. aegypti* females typically travel 50-100 meters to deposit eggs, but recent research suggests that this may be a gross underestimate, and may require current vector control strategies to be modified (Reiter, Amador, Anderson, & Clark, 1995). When highly productive or preferred containers are unavailable, females may travel up 840 meters or more in search of oviposition sites over a course of several days (Reiter et al., 1995).

In tropical climates, eggs may develop in as little as two days; in colder climates, egg development can take up to one week. Larval development is also temperature dependent. The larvae pass through four instars, spending a short amount of time in the first three, and up to three days in the fourth instar. Instars are the stages of larval development, and there are five (5) stages, with the first instar emerging from the egg as the smallest stage and the instar being the biggest stage and the last one before pupation. Males develop faster than females; males generally pupate earlier. If temperatures are cool, *Aedes aegypti* can remain in the larval stage for months as long as the water supply is sufficient (Zettel & Kaufman, 2015).

2.5 Core Vector Control Methods

Mosquito control is currently the best method for disease prevention. This primarily includes source reduction, pesticide spraying or "fogging", larvicide, or the use of insecticide treated bednets.

The WHO Pesticide Evaluation Scheme (WHOPES) currently recommends 15 insecticide compounds and formulations belonging to four chemical classes: pyrethroids, organophosphates, organochlorines, and carbamates. The primary means by which these are deployed are indoor residual spraying (IRS) and insecticide treated bednets. Vector control programs need to select insecticides for a given area on the basis of the residual efficacy of the insecticide, cost, safety and the type of surface to be sprayed and in consideration of up-to-date information on insecticide

resistance (WHO, 2017e). The World Health Organization outlined some of their key priorities to improve on vector surveillance, capacity building, good coordination and to integrate those action in other sectors such as Ministry of Housing and Country Planning, Ministry of Health, Ministry of Finance etc. They do these by creating four (4) pillars; 1) Strengthen inter- and intra-sectoral action and collaboration, 2) Engage and mobilize communities, 3) Enhance vector surveillance, and monitoring and evaluation of interventions, and 4) Scale up and integrate tools and approaches (WHO, 2017b)

The most commonly deployed insecticides used to control mosquito populations worldwide are pyrethroid compounds, including permethrin. Pyrethroids are used in insecticide-treated bed nets, coils, sprays, and IRS. They work by binding to and disrupting voltage-gated sodium channels (Hemingway, Hawkes, McCarroll, & Ranson, 2004).

2.6 Insecticide resistance in *Aedes Aegypti* and other species of interest

Insecticides have been used as a means of vector control for several decades, and consequently, mosquitoes have developed mechanisms of resistance. Several mechanisms of resistance have been documented in diverse mosquito populations in Africa, Asia, and the Americas (Hemingway et al., 2004). Mosquito behavior changes to avoid exposure to insecticides such as DDT and permethrin include shorter times spent in houses and changes in preferred biting times. Physical means of resistance have also been observed- for example, thicker or altered cuticles reduce insecticide penetration. Many species have also been observed to overproduce non-specific carboxylesterases as a response to organophosphates and carbamates, which work as acetylcholinesterase inhibitors.

Many important vector species, such as *Aedes* and *Anopheles*, which transmit malaria, have developed so-called “knock-down resistance” (kdr) in response to pyrethroid and organochlorine exposure (Liu, 2015). Both classes of insecticide target the voltage-gated sodium channel, which

consists of four domains with six-transmembrane helices each. Point mutations, which cause changes in the structure of the sodium channel, in the S6 hydrophobic domain II have been found in almost all resistant strains. These changes in the structure of the sodium channel prevent insecticide compounds from binding effectively. Thus far, over 20 unique amino acid sequence polymorphisms affecting the primary structure of the sodium channel have been documented (Liu, 2015).

2.7 Managing insecticide resistance

Resistance has been detected in all major vector species and to all classes of insecticides and has been recorded in more than 60 countries worldwide (WHO, 2015). However, many countries do not routinely carry out insecticide resistance testing, which means that our understanding of the scale of insecticide resistance is incomplete (WHO, 2015).

Insecticide resistance, especially against pyrethroids, which are the major class of chemical insecticide used on all approved long-lasting insecticide-treated nets (LLITNs) and in most IRS programs worldwide has been confirmed in some parts of sub-Saharan Africa in mosquitoes. If such resistance were to spread it would threaten the sustainability and operational impact of Integrated Vector Management (IVM) programs (Ranson, N'Guessan, et al., 2011). IVM is a way by which the resources that are used to control the vectors are used judiciously. Using IVM will help improve on the efficacy, cost-effectiveness, ecological soundness and sustainability of vector control for multiple diseases. One of the main goals of IVM is to prevent the transmission of vector-borne diseases such as Malaria, Dengue, Lymphatic Filariasis, Onchocerciasis, Leishmaniasis, Schistosomiasis, Trypanosomiasis (sleeping sickness and Chagas disease), Zika, Yellow Fever and Chikungunya (WHO, 2017d).

Pyrethroid resistance is now widespread in *Anopheles gambiae,* the major vector for malaria in sub-Saharan Africa. This resistance may compromise malaria vector control strategies that are currently in use in endemic areas. Novel combinations of different insecticides across insecticides classes have shown promise in the fight against resistance. For example, the combination of a pyrethroid-treated bed net and carbamate-treated plastic sheeting has proven effective against mosquito populations which have shown resistance to either of these insecticides alone (Djenontin et al., 2009). The WHO Global Plan for Insecticide Resistance Management in malaria vectors (GPIRM) has also recommended the use of insecticide rotations for IRS (WHO, 2015).

New research is looking into the use of a bacterium called *Wolbachia* as a method of biocontrol. Studies show that invasion of *Ae. aegypti* containing the endosymbiotic bacteria allows mosquitos to be resistant to the certain arboviruses such as Dengue Fever and Zika virus strains currently circulating (Moreira et al., 2009).

2.8 Gaps in the literature

This literature review reveals that very little about the *Aedes* vectors is known in Africa or West Africa, and virtually nothing about its prevalence in Sierra Leone. However, a recent workshop on *Aedes* surveillance in West Africa revealed that in the past five years, almost every West African country has experienced an outbreak of an *Aedes*-borne disease (Personal_Communication, 2017). This tells us that the vector and its pathogens are both prevalent and circulating in the region, Therefore, in order to have a stronger public health response capability regarding the vector and the diseases it transmits, further research should be conducted to help inform preparedness and decisions. The study herein is one such attempt to provide insight to the situation concerning *Aedes* and *Aedes*-borne pathogens in Sierra Leone.

CHAPTER THREE
RESEARCH METHODOLOGY

3.1 Preamble

This chapter consists of the systematic description of procedures the researcher employed in conducting the research. The purpose of this study was to conduct a general entomological survey of *Ae. aegypti* in two districts (Bombali and Bo), in Sierra Leone as shown in **Figure 2**.

Figure 2: Map of study area (https://www.ezilon.com/maps/africa/sierra-leone-physical-maps.html)

NOTE: Red circle indicates study areas

Following the survey and collection of mosquito samples, several analyses were performed to contribute to the understanding of vector transmission of *Aedes*-borne diseases (Zika, Dengue, Yellow Fever, and Chikungunya) in Sierra Leone.

The field work was conducted in selected areas in Bo and Bombali District where mosquito samples were collected. The bioassay experiments were conducted at the Insectary at the US Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia.

Additional laboratory tests (KDR, and viral testing) were also carried out in CDC laboratories, for all the study areas.

3.2 RESEARCH DESIGN

The researcher used quantitative methods. The general methodology adopted is a descriptive analysis. The use of quantitative (Descriptive Design) methods involves the direct observation of mosquitoes in the areas sampled as well as the collection of samples through trap-setting.

3.3 SAMPLING

This study was an observational study and specifically, a cross-sectional study. Sample collection was done between June 26, 2017, just before the commencement of the short rainy season in Sierra Leone and continued through August 4, 2017, immediately after the end of the short rains. During the short rainy season, discarded containers retain water but do not receive daily flushing from the heavy rains, so it is the ideal time to conduct immature sampling.

Simple random sampling was used to collect as many adult mosquitoes and eggs of mosquitoes as possible. Eggs were a particular focus because one of the study aims for insecticide resistance testing requires a large number of *Aedes* mosquito eggs: at least 100 female mosquitoes, per district per insecticide. The sampling sites were selected from previously identified areas by the Ministry of Health and Sanitation (MOHS). These sites were selected as target districts which had

previously been used for an *Anopheles* vector insecticide resistance project. (Government_of_Sierra_Leone, 2016).

3.3.1 *AEDES AEGYPTI* EGG COLLECTION

Eggs of *Aedes aegypti* mosquitoes were collected from Ovitraps, **Figure 3,** placed outside randomly selected houses in the designated areas. Houses were selected either randomly among those who were at home during the time of the survey or by selection of the local chiefs. The Ovitraps were set with germination paper labeled with the date, location, and house ID number, which was clipped to a black plastic cup. Hay infusion water was then added to the Ovitraps, which were positioned in areas that are attractive to the mosquitos and left there for 3-4 days. After 3-4 days traps were retrieved from the houses and the germination papers were placed in a humidified box and brought to the Sierra Leone MOHS insectary in Freetown. Once in the insectary, the papers were hung to dry so that the eggs would dry in a controlled fashion and remain viable for later hatching. Germination papers containing eggs were kept for the CDC Bottle Bioassay, where they were later used for rearing in the Insectary at CDC Atlanta to perform insecticide resistance tests.

Figure 3: Ovitrap set outside a house in Bombali District, Sierra Leone

Photo credit: Rebecca Levine, Sierra Leone, 2017

3.3.2 ADULT MOSQUITO COLLECTION USING PROKOPACKS (BACK PACK ASPIRATORS) AND BIOGENT (BG) SENTINEL TRAPS

A Prokopack is a hand-held mechanical aspirator that uses pressure from a battery to suck adult mosquitoes into the cup fitted on the aspirator as shown in **Figure 4**. This collection method targets adult mosquitoes that are resting inside homes (usually after taking a blood meal). It collects all kinds of mosquitoes, and a high proportion of them are usually blood-fed. Sorting has to be done immediately in the laboratory to discriminate against the different mosquito species and to remove other insects that might have been trapped alongside the mosquitoes. Prokopack was done instead of mouth aspiration as the aspirator collected mosquitoes that were not visible or were far from the reach of the collector. Each house had a separate cup with a unique bar code number label, and sorting was done in the field insectary immediately after collection.

Figure 4: A staff member using the Prokopack (Backpack Aspirator)

Photo credit: Rebecca Levine, Sierra Leone, 2017

The BG trap, as shown in **Figure 5,** uses a 12-volt battery to help the fan in the trap to draw in mosquitoes. This trap targets host-seeking adult mosquitoes, and as such it is baited with a human-scented, non-toxic lure, which serves as an attractant to the mosquitoes. This trap was set inside the selected houses and left to run for 24 hours and retrieved the following day. When the battery

dies, the trap closes on its own, which prevents the mosquitoes already inside from coming out from the trap.

Figure 5: A staff member assembling the BG Sentinel Trap

Photo credit: Rebecca Levine, Sierra Leone, 2017

Adult mosquitoes collected from the field using the two methods were placed in a freezer to euthanize them immediately upon returning to the field laboratory. After separating males and females, the mosquitoes were separated by species. Only the female *Aedes* mosquitoes were preserved in the field by washing them with 99% ethanol and then storing them in tubes with *RNA Later*. The mosquitoes were preserved in tubes labeled with the location and date of collection and were pooled if they were caught in the same location on the same date, with a maximum of five individuals per pool. The preserved mosquitoes were then shipped from Sierra Leone to Atlanta, where they were then stored at -80°C. At the CDC lab in Atlanta, specimens were tested for all flaviviruses and one alphavirus.

3.3.3 LARVAL AND PUPAL SURVEYS

We did larvae and pupae surveys in and around selected homes, garages, University hostels, and convenience stores by looking inside and outside of homes for potential breeding sites for mosquitoes as shown in **Figure 6**. Since the study focused on *Aedes* mosquitoes, we mainly

concentrated on searching for artificial containers that *Aedes* prefer to breed in, but we also checked all-natural breeding sites that were found on properties as well. When we found such sites, we collected the water using large pipettes (turkey basters) and placed the water in clean white bowls to identify which immature stages were present and to get a rough estimate of the numbers present at each stage. We also took this as an opportunity to show the immature mosquito stages to the residents of the homes and to explain what they are and to encourage them to always discard water if they see such things inside it and to try to eliminate areas of stagnant water which the mosquitoes use as breeding sites. House index (HI) was calculated using percentage of houses infested with larvae and/or pupae, Container index (CI) was also calculated using percentage of water-holding containers infested with larvae or pupae. Breteau index (BI) also was measured using number of positive containers per 100 houses inspected. Pupa index (PI): number of pupae per 100 houses inspected (WHO, 2018d).

Figure 6: Identifying Larvae and Pupae in the communities

Photo credit: Festus Pessima, Sierra Leone, 2017

3.3.4 DATA MANAGEMENT AND ANALYSIS

Data were entered directly in the field on mobile tablet devices (Samsung Galaxy Tab A) using the Epi Info Mobile app for Vector Surveillance (CDC, 2017a). Data from the field and the insectary were synced to a cloud daily. Indices were calculated directly from the app's automated analysis function. Maps and charts were also created this way. Data were exported into Microsoft Excel (Microsoft_Corporation, 2016). Average adult female and eggs were calculated.

3.4 LABORATORY ANALYSIS

3.4.1 Rearing

In the laboratory at CDC Atlanta, eggs collected from both Bombali and Bo District were raised for resistance testing. These were considered generation zero (F0) because they were raised from eggs laid by wild mosquitoes. First, the eggs were hatched by putting the germination papers in water, waiting for them to hatch, feeding the larvae, waiting for pupae to emerge, picking pupae, and then putting the pupae in cages so that they become adults. These mosquitoes were raised in the insectary at CDC at a temperature of 28° C. As adults they were fed on sugar (corn syrup) by soaking cotton wool in the sugar and putting the cotton wool on top of the cages, and Human blood that was collected from the blood bank was also used to feed the female Mosquitoes as shown in **Figure 7** using a Hemotek. The feeding with human blood was done weekly. Although it would have been preferable to do the resistance tests on the F0 generation, it was not possible in this case. We needed more eggs for the resistance tests than we had available from the F0 eggs, so that is why we raised an F1 generation. Once blood fed, males and females mated in the cages and, small Ovitraps were placed in the cages to get another generation of eggs. This

generation of eggs is called the First Generation (F1), since they were the first to be laid by laboratory-raised mosquitoes as compared to wild mosquitoes.

Figure 7: Feeding *Aedes aegypti* Mosquitoes using Hemotek

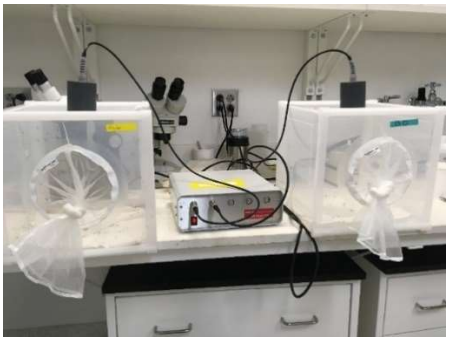


Photo credit: Christopher Sandi, Atlanta, USA 2017

3.4.2 Resistance Testing

The F1 mosquitoes were tested for insecticide resistance using the standard CDC bottle bioassay (Brogdon, 2012). Briefly, the CDC bottle bioassay test is performed by coating four bottles with the insecticide being tested while leaving one bottle coated only with ethanol as a control. After allowing enough time for the insecticides to completely dry, 100 female mosquitoes in total are used with 20 mosquitoes per bottle. Then the bottles are checked every 15 minutes up to 30 minutes (45 minutes for DDT). Those mosquitoes that die after the time limit are the susceptible ones and those that do not die are resistant ones. The test is considered valid if all the control mosquitoes remain alive after the time limit. The insecticides tested are as follows, insecticide class listed in parentheses: permethrin (pyrethroid), deltamethrin (pyrethroid), alphacypermethrin (pyrethroid), bendiocarb (carbamate), malathion (organophosphate), and DDT (organochlorine). After the resistance test was completed for all six (6) insecticides, both

resistant, susceptible and the control mosquitoes were put in separate tubes and stored in -80° C to be used for the KDR test. The data were recorded on a standard paper form and later entered to an excel sheet.

Figure 8: Coating bottles for Resistance testing

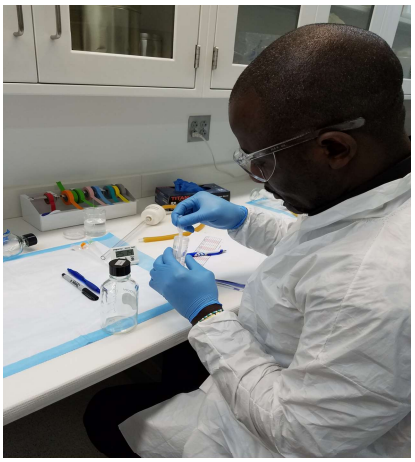


Photo credit: Rebecca Levine, Atlanta-USA, 2017

3.4.3 Knock Down Resistance (KDR) Testing

The other activity that was done in the laboratory was extracting DNA from mosquito samples for Polymerase Chain Reaction (PCR) analysis in order to detect genes related to insecticide resistance. The genes detected can help us identify the molecular mechanism that the mosquito is using to develop insecticide resistance. We are looking for mutations in codons 1016 and 1534, which are two well-studied mutations that are associated with KDR in *Aedes* (Saavedra-Rodriguez et al., 2007). In codon 1016, the wild-type genotype is homozygous Valine/Valine (V/V), while mutants are heterozygous Valine/Isoleucine (V/I) or homozygous Isoleucine/Isoleucine (I/I). In codon 1534, the wild-type genotype is homozygous Phenylalanine/Phenylalanine (F/F), while mutants are heterozygous Phenylalanine /Cystine (F/C) or homozygous Cystine / Cystine (C/C).

Kdr testing was done by using the mosquitoes tested with the three pyrethroid insecticides (Permethrin, Deltamethrin, Alpha-cypermethrin) during the resistance testing, and includes both the resistant and susceptible mosquitoes from the bottle bioassay, but not the control mosquitoes. During the extraction process, each mosquito was put in a separate tube and immersed into 25µl of extraction reagent. The tubes were then put into a thermocycler to heat at 95^{o} C for 30 minutes and allowed to cool to room temperature (12^{o} C). Then 25µl of stabilization reagent was added to each of the tubes, and vortexed.

To proceed to the real-time PCR with the already extracted DNA, master mixes for mutations 1016 and 1534 were prepared by multiplying the number of DNA samples used by two (2). This is done to make a replicate of each sample, as shown in **Tables 1 and 2**. After getting the master mix for each of the mutations (1016 and 1534), 19µl of the master mix and 1µl of the DNA were placed into each well of the PCR plate (BioRad MLL9651) for the 1016 mutation while the same thing was done for the 1534 mutation, but using18µl master mix and 2µl DNA.

After the samples and master mix were placed in the PCR plates, the plates were put in the centrifuge to run at 4000 rmp for 2 minutes, and then removed and brought to the real-time PCR (RT-PCR) thermocycler. PCR was performed according to the methods described by Saavedra-Rodriguez et al 2007 (Saavedra-Rodriguez et al., 2007). The data were stored in the RT-PCR computer and later transferred to a standard paper form and excel spreadsheet.

Table 1: Master Mix for Mutation 1016

		1 rxn	18
Master Mix	ddH_2O	6	108
	iQ SYBER mix	10	180
	Primer Val1016f	1	18
	Primer Ile1016f	1	18
	Primer Ile1016r	1	18
	Total	19	342
	DNA	1	1

Table 2: Master Mix for Mutation 1534

		1 rxn
Master Mix	ddH20	7.15
	iQ SYBER mix	9
	Primer Cys1534+	0.65
	Primer Phe1534+	0.6
	Primer 1534-	0.6
	Total	**18**
	DNA	2

3.4.4 Virus Testing

The adult female *Aedes* mosquitoes collected from the field were tested for the presence of viruses in the laboratory. The samples were tested for all flaviviruses, including: Dengue (DENV), West Nile (WNV), Yellow Fever (YFV), and Zika (ZIKV). The samples were also tested for one alphavirus, Chikungunya (CHIKV). These are all of the major *Aedes*-borne arboviruses that are of human public health concern. For the viral testing, RNA was first extracted from the samples as shown in **Figure 9** using an Arcturus PicoPure RNA Isolation Kit (Thermo Fisher Scientific Baltics UAB, Lithuania), since the viruses of interest were all RNA viruses. The extracted RNA was then run through into a tape station to ensure the quality of the extractions. After the quality of the extractions was confirmed, Superscript III one step RT-PCR (Invitrogen, Carlsbad, CA, USA) was performed using the flavivirus-specific primers (Kenney, Solberg, Langevin, & Brault, 2014) . The components used for the master mix for template synthesis and sequencing are shown in **Table 3** and a cycling set-up for the thermal-cycler in table 4 (Kuno, Chang, Tsuchiya, Karabatsos, & Cropp, 1998). RT-PCR products were visualized by gel electrophoreses.

Table 3: Components for the Master Mix

Invitrogen Superscript III Reaction set up	Total 50 µl/ each reaction
2×reaction mix	25 µl
RNA template	5 µl
10 uM Forward Primer: TACAACATGATGGGAAAGAGAGAGAA	1 µl
10 uM Reverse Primer: AGCATGTCTTCCGTGGTCATCCA	1 µl
Enzyme	2 µl
DdH_2O	16 µl

Table 4: 40 Cycles step 3-5 RT-PCR

CYCLE STEP	TEMP Celsius	Time
1. cDNA synthesis	55	30 min
2. Denaturation	94	2 min
3. Initial denaturation	94	15 sec
4. Anneal	53	30 sec
5. Extend	68	1 min
Final extension	68	5 min

Figure 9: Extracting RNA from field collected Mosquitoes

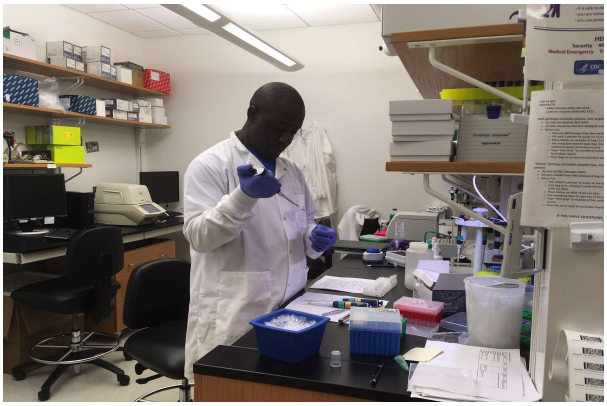


Photo credit: Nsa Dada, Atlanta-USA, 2017

3.5 ETHICAL CONSIDERATIONS

Prior to sample collection in and around homes, verbal consent was obtained from the Ministry of Health officials at the national level and the district medical officers at the district level. At the local level, town chiefs, area leaders and house heads or their representatives also gave their verbal consent before any sampling procedures were undertaken.

Human involvement in the sample collection was not invasive. The role of house head was limited to accepting that mosquitoes would be collected from their houses and providing some very brief demographic and contact information. Field workers who assisted in the collection of mosquitoes were trained before they could go to the field to ensure that they acquired good data collection techniques along with good communication skill as demonstrated in **Figure 10**.

Figure 10: Training both MOHS and field staffs

Photo credit: Rebecca Levine, Sierra Leone, 2017

3.6 LIMITATIONS AND DELIMITATIONS

It would have been ideal to collect samples from all the 14 Districts in Sierra Leone, but it is often not possible to conduct such a comprehensive study in any country. The Ministry of Health and Sanitation had already selected four sites they called "*sentinel sites*" for the study prior to our arrival to the country, and it would have been ideal to be able to collect samples in all four of the sentinel sites, However, due to limited time and financial constraints, samples were collected in just two of the sentinel sites. It would have also been important to do all the laboratory work within the country. But because there is no laboratory for entomology within Sierra Leone, samples needed to be shipped to the United States for analysis. Despite these difficulties, the findings of this study are applicable to Sierra Leone.

I was not able to rear the eggs collected directly from the field in Sierra Leone to do the resistance testing in the field lab there. This is because I did not have enough eggs from the field to produce enough females for all the resistance tests (since at least half of the eggs collected would have been males). It would have been ideal to perform the resistance tests on females raised from wild-caught eggs (F0 generation) in the Sierra Leone field lab, but since there we not enough females, I had to bring the eggs back to Atlanta and rear them there and then use the eggs laid by those mosquitoes (F1 generation) for the resistance tests.

I was not able to compare human disease indicators for Yellow Fever, Dengue, Chikungunya, and/or Zika to entomological indices because the Ministry of Health and Sanitation has no data in relation to these diseases.

CHAPTER 4

RESULTS

4.1 INTRODUCTION

This section of the study details the results from the data collected from the field and the laboratory. The descriptive data are presented in the form of tables and graphs and organized according to the objectives of the study.

4.2 *AEDES AEGYPTI* EGG COLLECTION

In Bombali District, 112 Ovitraps were set over the course of 21 days. The resulting average egg density was 189 eggs collected per trap. In Bo District, 219 Ovitraps were set over the course of 21 days. The resulting average egg density was 4.1 eggs collected per trap.

4.3 ADULT MOSQUITO COLLECTION USING PROKOPACKS (BACK-PACK ASPIRATORS) AND BIOGENT (BG) SENTINEL TRAPS

For Bombali District a total of 53 collections were made using the Back-pack aspirator, and 102 collections were made in Bo District. The results for the total number of adult female mosquitoes collected in Bombali and Bo District respectively were 887 (Average of 16.7 adult females per collection) and 295 (Average of 2.9 adult females per collection). Blood fed mosquitoes totaled 326 (37%) from Bombali and 205 (69%) from Bo District. Looking at the collection by species, in Bombali District, *Anopheles* there were 92 adult females, 741 *Culex* adult females, and 54 *Aedes*

Aegypti adult females. Blood fed mosquitoes for this District were; *Anopheles* 47 (51.1%), *Culex* 279 (37.7%), and no *Aedes aegypti* blood fed mosquitoes.

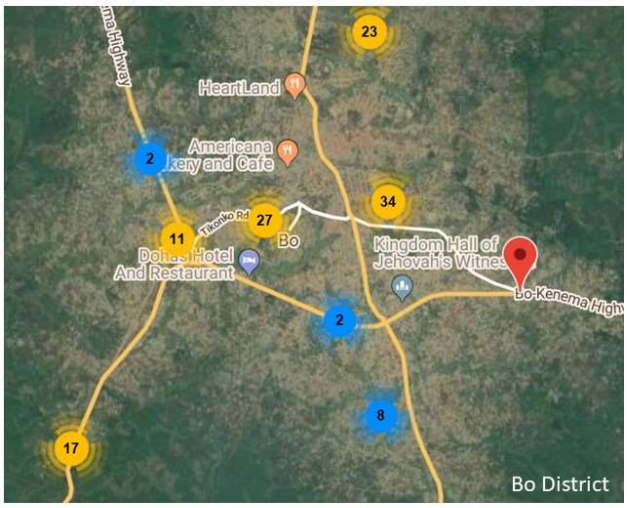
For the collection by species in Bo District, there were 100 *Anopheles* adult females, 191 *Culex* adult females, and 4 *Aedes Aegypti* adult females. The blood fed mosquitoes for this District were; *Anopheles* 88 (88%), *Culex* 113 (59.2%), and *Aedes aegypti* 4 (100%).

In Bombali District, 23 BG Sentinel traps were set over the course of 21 days. The resulting average adult female density was 0.04 adult females collected per trap. Considering the collections by species, *Anopheles* and *Culex* had an average of 0.7 adult females per trap, and *Aedes Aegypti* had an average of 0.13 adult females per trap. In Bo District, 6 BG Sentinel traps were set over the course of 21 days. The resulting average adult female density was 0.17 adult females collected per trap. Considering the collections by species, *Anopheles* and *Culex* had an average of 0.3 adult females per trap, and *Aedes Aegypti* had an average of 0.0 adult females per trap.

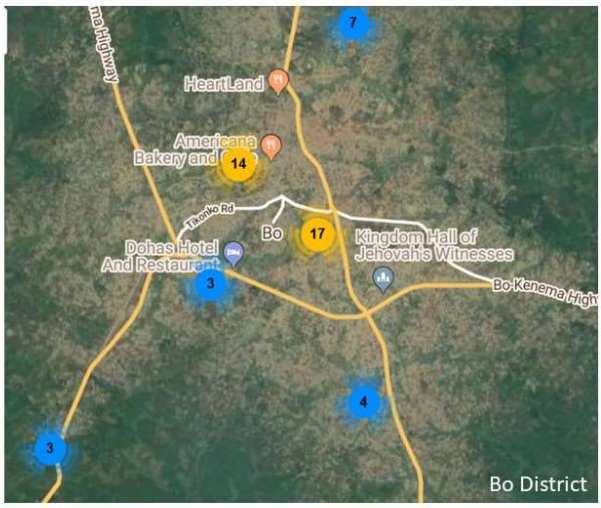
4.4 LARVAL AND PUPAL SURVEYS

In Bombali District, 61 houses were surveyed for larvae/pupae over the course of 21 days, with 32 being found positive for larvae/pupae. The sites that were sampled are shown in **Figure 11C** with the sites that were positive shown in **Figure 11D**. In Bo District, 124 houses were surveyed for larvae/pupae over the course of 21 days, with 48 being found positive for larvae/pupae. The sites that were sampled are shown in **Figure 11A** with the sites that were positive shown in **Figure 11B**. Larvae and pupae were not brought back to the lab for sex determination or species identification, and instead, the standard indices of House, Breateu, and Container were calculated for immatures, which are shown below.

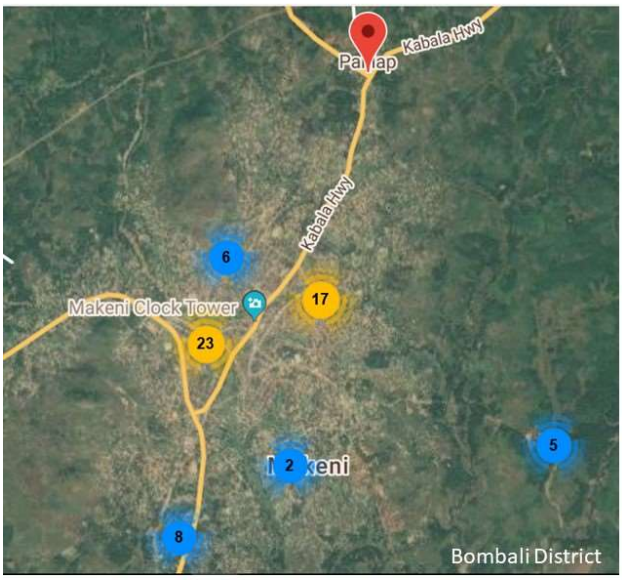
Figure 11: Survey areas with Positive sites for Larvae/ or Pupae



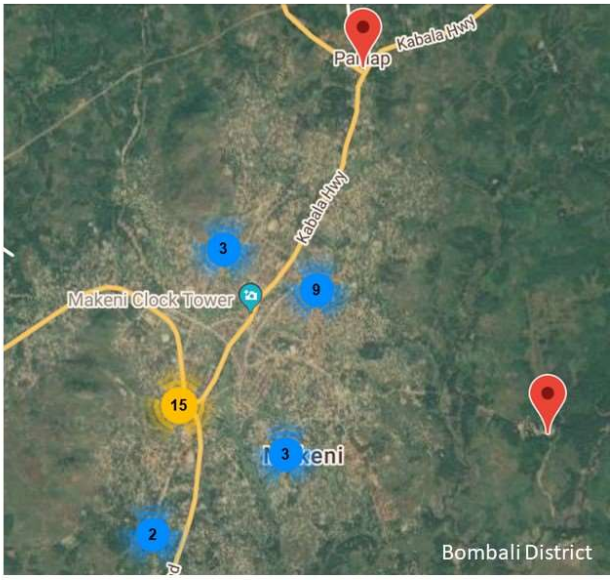
A: All sites sampled



B: Sites that were positive for larvae and/or pupae



C: All sites sampled



D: Sites that were positive for larvae and/or pupae

The Breteau Indices showed that Bombali Sebora Chiefdom in Bombali District had the highest index (153), followed by Kakua Chiefdom in Bo District (110), followed by Makari Gbanti Chiefdom in Bombali District (91), and Tikonko Chiefdom in Bo District (65). In general, the higher indices came from Bombali District as compared to Bo District.

The Container Indices showed that Kakua Chiefdom and Tikonko Chiefdom both in Bo District had the highest indices (17%), followed by Makari Gbanti Chiefdom in Bombali District (14%), and Bombali Sebora Chiefdom in Bombali District (13%). In general, the higher indices came from Bo District as compared to Bombali District, but indices in all Districts were very similar.

House Indices showed that Bombali Sebora Chiefdom (61%) and Makari Gbanti Chiefdom (55%), both in Bombali District had the highest indices, and Kakua Chiefdom (43%) and Tikonko Chiefdom (18%), both in Bo District. The higher indices came from Bombali District as compared to Bo District.

The Pupae Indices showed that Bombali Sebora Chiefdom in Bombali District (13) had the highest indices (1528), and Makari Gbanti Chiefdom in Bombali District (1068), followed by Kakua Chiefdom in Bo District (643), and Tikonko Chiefdom in Bo District (147). In general, the higher indices by far came from Bombali District as compared to Bo District.

The Pupae per Container Indices showed that Bombali Sebora Chiefdom in Bombali District (3.67) had the highest indices, followed by Makari Gbanti Chiefdom in Bombali District (3.22), Kakua Chiefdom in Bo District (1.97), and Tikonko Chiefdom in Bo District (0.53). In general, the higher indices came from Bombali District as compared to Bo District, but all the Pupae per Container indices are below the outbreak threshold for this index (set for Dengue Virus) and are shown in **Table 5.**

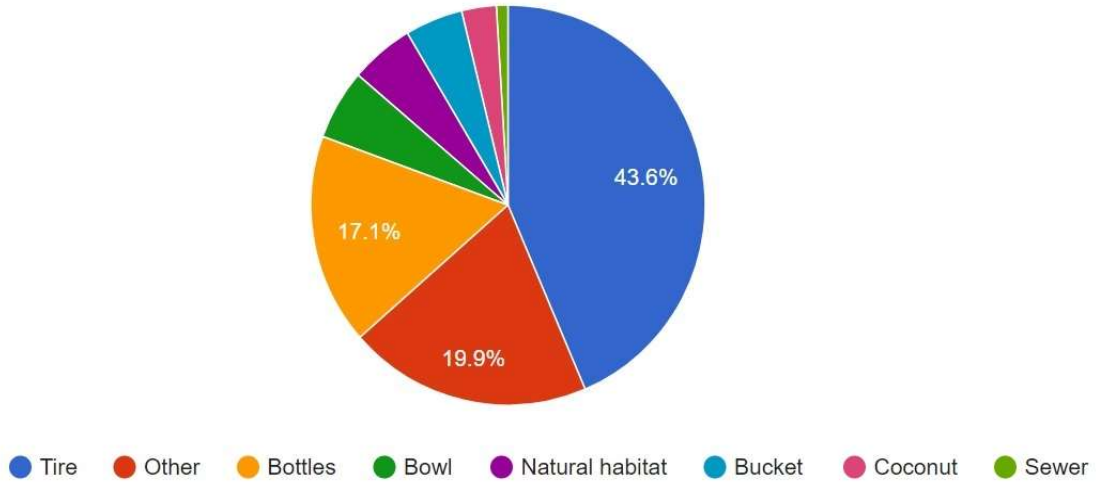
Table 5: Showing Indices for Bombali and Bo District

Index	Bombali District		Bo District	
	Bombali Shebora Chiefdom	Makari Gbanti Chiefdom	Kakua Chiefdom	Tikonko Chiefdom
Breteau	153	91	110	65
Container (%)	13	14	17	17
House (%)	61	55	43	18
Pupae	1528	1068	643	147
Pupae per Container	3.67	3.22	1.97	0.53

We examined the types of containers that were positive for larvae or pupae found during the house surveys, to know which types can be considered to be most productive. As shown in **Figure 12**, the most clearly productive type of container was tires, with 92 tires, or 43.6% of all positive containers, found to be holding larvae or pupae. After tires, the other types of important containers were "other," with 19.9% of the total positive containers, and bottles, with 17.1% of the total positive containers. The types of containers that were classified as "other" include objects like abandoned vehicles, abandoned machines, pit, bathtubs, broken cups, broken cool man, kittle, tanks, broken bottles used as security over the top of a perimeter fence, domestic water collection drum standing outside, water Miller tank, pools of stagnant water, and deep open pits with water.

The container type producing the smallest percent of positive containers were sewers with only 2 being found positive, or 0.9% of all positive containers.

Figure 12: A pie chart showing the Types of Containers found during house surveys, that were positive for the presence of larvae/pupae.



4.5 **Insecticide Resistance Testing**

We performed CDC bottle bioassays on adult females raised from field-captured eggs to determine insecticide resistance status against six different insecticides in mosquitoes from both Bombali and Bo Districts. All bioassays had a diagnostic time of 30 minutes, except for DDT which had a diagnostic time of 45 minutes. Mosquitoes are considered fully susceptible when 98-100% die after exposure, potential for resistance developing when between 80-97 % die after exposure, and resistant when less than 80% die after exposure.

In Bombali, our results showed that *Aedes aegypti* mosquitoes are fully susceptible to Deltamethrin (pyrethroid), Alpha-cypermethrin (pyrethroid), Malathion (organophosphate), and DDT (organochlorine), with each of these insecticides causing 100% mortality in the

mosquitoes. There is a possibility of resistance developing to Bendiocarb (carbamate), as there was 95% mortality in the mosquitoes after being exposed. The mosquitoes were resistant to Permethrin (pyrethroid), as there was only 52.5% mortality in the mosquitoes after being exposed. These results are shown in Table 6.

Table 6: Percent Mortality per Insecticide in Bo and Bombali District

Insecticide	**Percent Mortality**	
	Bo	**Bombali**
Deltamethrin	100	100
Bendiocarb	100	95
Malathion	92.5	100
Alphacypermethrin	97.5	100
Permethrin	52.5	52.5
DDT*	68.75	100

NOTE: * Shows a diagnostic time of 45 minutes

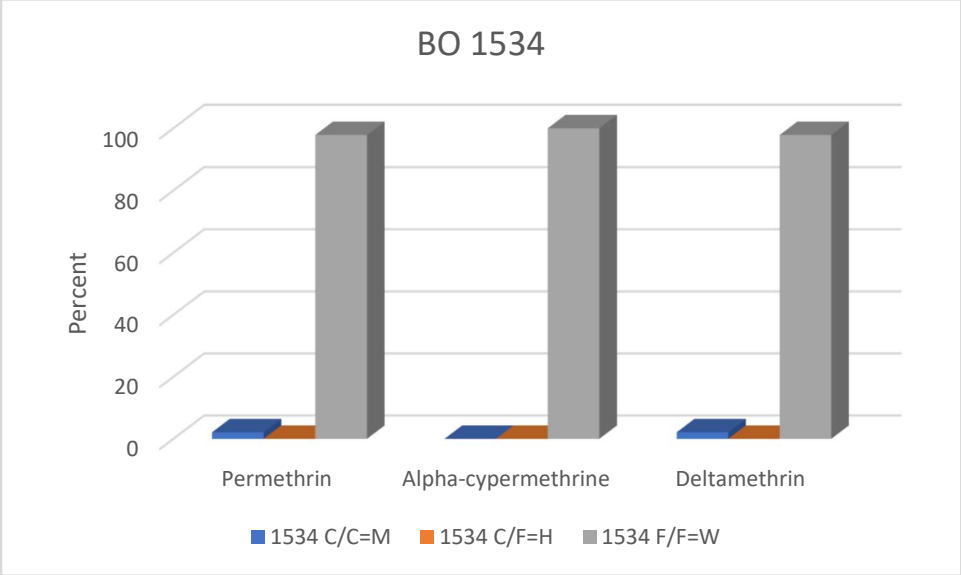
4.6 **Knock Down Resistance (KDR) Testing**

We performed KDR testing on all the mosquitoes used in the bottle bioassays tested against the three pyrethroid insecticides (Permethrin, Deltamethrin, and Alpha-cypermethrin) to know whether resistance to these insecticides was due to mutations in two genes (1534 and 1610) known to cause KDR in *Aedes aegypti*.

Looking at the 1534 KDR gene in Bo District shows that 100% of the mosquitoes that were exposed to Alpha-Cypermethrin had a wild type (F/F) genotype, and 97.9% of the mosquitoes exposed to each of Permethrin and Deltamethrin had a wild type (F/F) genotype. There was no heterozygote (F/C) gentotypes in mosquitoes exposed to any of these three insecticides

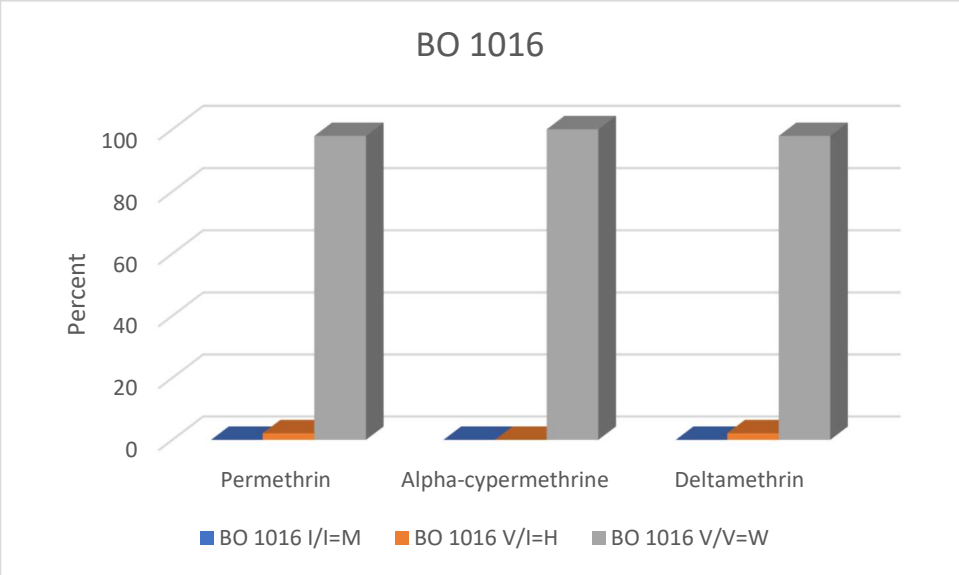
(Permethrin, Alpha-cypermethrin, Deltamethrin). 2.13 % of the mosquitoes exposed to each of Permethrin and Deltamethrin had a mutant (C/C) genotype. This is shown in **Figure 13**.

Figure 13: 1534 KDR GENE IN BO DISTRICT



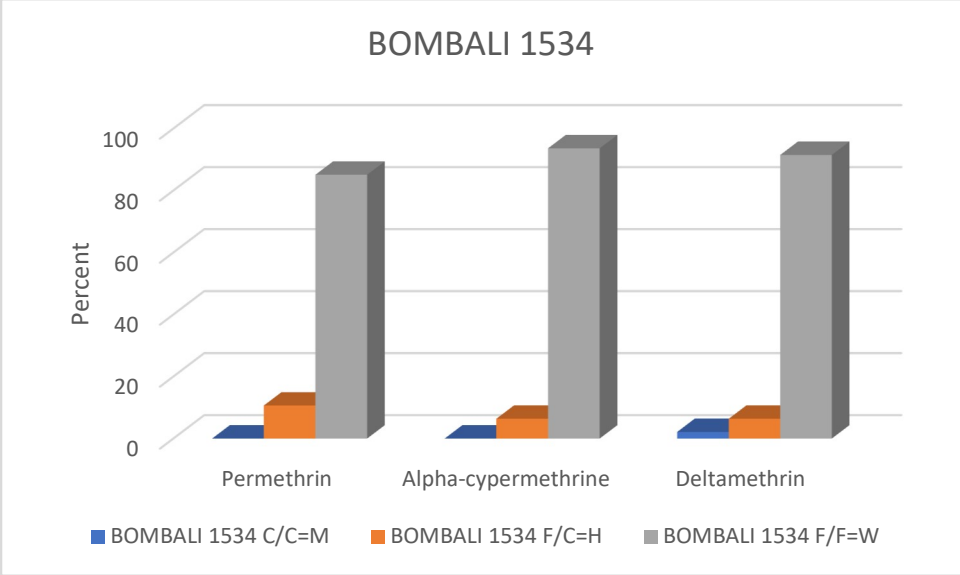
Looking at the 1016 KDR gene in Bo District shows that 100% of the mosquitoes that were exposed to Alpha-Cypermethrin had a wild type (V/V) genotype, and 97.9% of the mosquitoes exposed to each of Permethrin and Deltamethrin had a wild type (V/V) genotype. It also showed that 2.13 % of the mosquitoes exposed to each of Permethrin and Deltamethrin had a heterozygote (V/I) genotype. There were no mutant genotypes (I/I) in mosquitoes exposed to any of these three insecticides (Permethrin, Alpha-cypermethrin, Deltamethrin) as shown in **Figure 14**.

Figure 14: 1016 KDR GENE IN BO DISTRICT



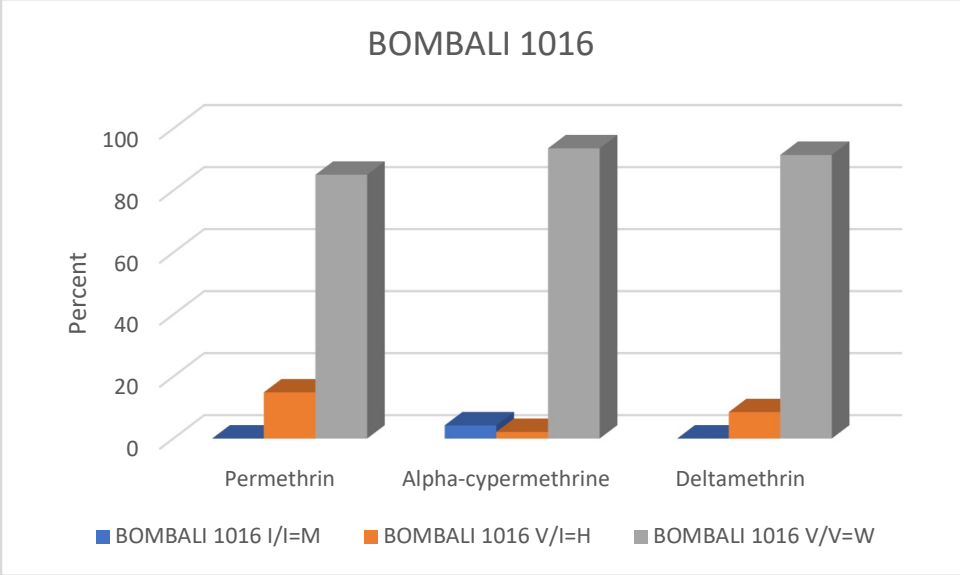
Looking at the 1534 KDR gene in Bombali District shows that 100% of the mosquitoes that were exposed to Alpha-Cypermethrin had a wild type (F/F) genotype, and 97.9% of the mosquitoes exposed to each of Permethrin and Deltamethrin had a wild type (F/F) genotype. Of the mosquitoes exposed to each of Permethrin and Deltamethrin, 2.3% had a heterozygote (F/C) genotype. There were no mutant genotypes (C/C) in mosquitoes exposed to any of these three insecticides (Permethrin, Alpha-cypermethrin, Deltamethrin) as shown in **Figure 15**.

Figure 15: 1534 KDR GENE IN BOMBALI DISTRICT



Looking at the 1016 KDR gene in Bombali District shows that 93.6% of the mosquitoes that were exposed to Alpha-Cypermethrin had a wild type (V/V) genotype, 91.5% of the mosquitoes exposed to Deltamethrin had a wild type (V/V) phenotype, and 85.1% of the mosquitoes exposed to Permethrin had a wild type (V/V) genotype. Of the mosquitoes that were exposed to Permethrin, 14.9% had a heterozygote (V/I) genotype, 8.5% of the mosquitoes exposed to Deltamethrin had a heterozygote (V/I) genotype, and 2.1% of the mosquitoes exposed to Alpha-cypermethrin had a heterozygote (V/I) genotype. Of the mosquitoes exposed to Alpha-Cypermethrin, 4.26% had a mutant (I/I) genotype as shown in **Figure 16.**

Figure 16: 1016 KDR GENE IN BOMBALI DISTRICT



PCR to detect KDR genes1016 and 1534 was performed on 282/600 (47%) mosquitoes tested for resistance to the pyrethroids deltamethrin, permethrin, and alpha-cypermethrin in Bo and Bombali Districts using the CDC bottle bioassay. Of the 1200-adult female *Ae. aegypti* reared in the insectary for these bioassays, 141 were tested for each of the 1016 and 1534 genes. These results are shown in **Table 7**.

The mutations appear to be evolving with higher frequency in Bombali. This is consistent between all the insecticides and at both loci. The most obvious association between higher frequency of mutation and phenotype is in permethrin-resistant mosquitoes, and the pattern holds true at both loci. The association is strong enough to result in a similarly higher frequency of mutant alleles in resistant mosquitoes across all the pyrethroids in Bombali compared to all the susceptible mosquitoes in Bombali. The 1534 locus seems to have a slightly higher frequency of mutation compared to the 1016 locus. This is true for the absolute values of the frequencies (i.e.:

bigger numbers) but also in the differences in frequencies between resistant and susceptible mosquitoes.

Table 7: Summary of data relating permethrin, deltamethrin, and alpha-cypermethrin resistance phenotype to kdr genotype per District. Yellow indicates a frequency between 5% and 7%, orange indicates a frequency between 7% and 10% and red indicates a frequency greater than 10%.

				V1016I					F1534C				
District	**Insecticide**	**Phenotype**	**n**	**V/V**	**V/I**	**I/I**	**Freq. I**	**95% CI**	**F/F**	**F/C**	**C/C**	**Freq. C**	**95% CI**
Bo	Alphacypermethrin	Susceptible	45	45	0	0	0	N/A	45	0	0	0	N/A
		Resistant	2	2	0	0	0	N/A	2	0	0	0	N/A
		Total	47	47	0	0	0	N/A	47	0	0	0	N/A
	Deltamethrin	Susceptible	47	46	1	0	0.011	0.003	46	0	1	0.021	0.006
		Resistant	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		Total	47	46	1	0	0.011	0.003	46	0	1	0.021	0.006
	Permethrin	Susceptible	24	24	0	0	0	N/A	24	0	0	0	N/A
		Resistant	23	22	1	0	0.022	0.009	22	0	1	0.043	0.018
		Total	47	46	1	0	0.011	0.003	46	0	1	0.021	0.006
	Total	Susceptible	116	115	1	0	0.004	0.001	115	0	1	0.009	0.002
		Resistant	25	24	1	0	0.02	0.008	24	0	1	0.04	0.016
		Total	141	139	2	0	0.007	0.001	139	0	2	0.014	0.002
Bombali	Alphacypermethrin	Susceptible	47	44	1	2	0.053	0.015	44	3	0	0.032	0.009
		Resistant	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		Total	47	44	1	2	0.053	0.015	44	3	0	0.032	0.009
	Deltamethrin	Susceptible	47	43	4	0	0.043	0.012	43	3	1	0.053	0.015
		Resistant	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		Total	47	43	4	0	0.043	0.012	43	3	1	0.053	0.015
	Permethrin	Susceptible	23	21	2	0	0.043	0.018	21	1	1	0.065	0.027
		Resistant	24	19	5	0	0.104	0.042	19	4	1	0.125	0.05
		Total	47	40	7	0	0.074	0.021	40	5	2	0.096	0.027
	Total	Susceptible	117	108	7	2	0.047	0.009	108	7	2	0.047	0.009
		Resistant	24	19	5	0	0.104	0.042	19	4	1	0.125	0.05
		Total	141	127	12	2	0.057	0.009	127	11	3	0.06	0.01

Total	Alphacypermethrin	Susceptible	92	89	1	2	0.027	0.006	89	3	0	0.016	0.003
		Resistant	2	2	0	0	0	N/A	2	0	0	0	N/A
		Total	94	91	1	2	0.027	0.005	91	3	0	0.016	0.003
	Deltamethrin	Susceptible	94	89	5	0	0.027	0.005	89	3	2	0.037	0.008
		Resistant	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		Total	94	89	5	0	0.027	0.005	89	3	2	0.037	0.008
	Permethrin	Susceptible	47	45	2	0	0.021	0.006	45	1	1	0.032	0.009
		Resistant	47	41	6	0	0.064	0.018	41	4	2	0.085	0.024
		Total	94	86	8	0	0.043	0.009	86	5	3	0.059	0.012
	Total	Susceptible	233	223	8	2	0.026	0.003	223	7	3	0.028	0.004
		Resistant	49	43	6	0	0.061	0.017	43	4	2	0.082	0.023
		Total	282	266	14	2	0.032	0.004	266	11	5	0.037	0.004

4.7 **Virus Testing**

All adult female *Aedes aegypti* captured from the field using prokopacks or BG Sentinels were preserved for viral testing. This amounted to nine (9) samples, eight (8) of which were collected from Bombali District and one (1) which was collected from Bo District. Each sample was tested for all Flaviviruses (including Dengue, Yellow Fever, and Zika) and the Alphavirus (Chikungunya) using RT-PCR. Figure 17 shows the gel resulting from the Flavivirus test. Since no band appeared in the sample lanes or the negative control, but a band appeared in the positive control, the test is confirmed to have worked and no Flaviviruses were detected in any samples. Figure 18, shows the PCR graph result from the Chikungunya virus test. The red lines indicate the two positive controls. The flat blue lines indicate the two negative controls, while the blue triangles represent the mosquito samples. Because the positive controls amplified, and the negative controls and samples did not amplify, the test is confirmed to have worked and Chikungunya virus was not detected in any samples.

Figure 17: Gel showing result for Flavivirus testing

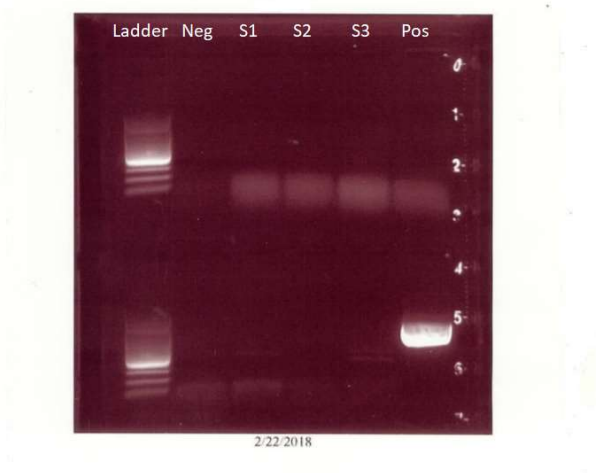
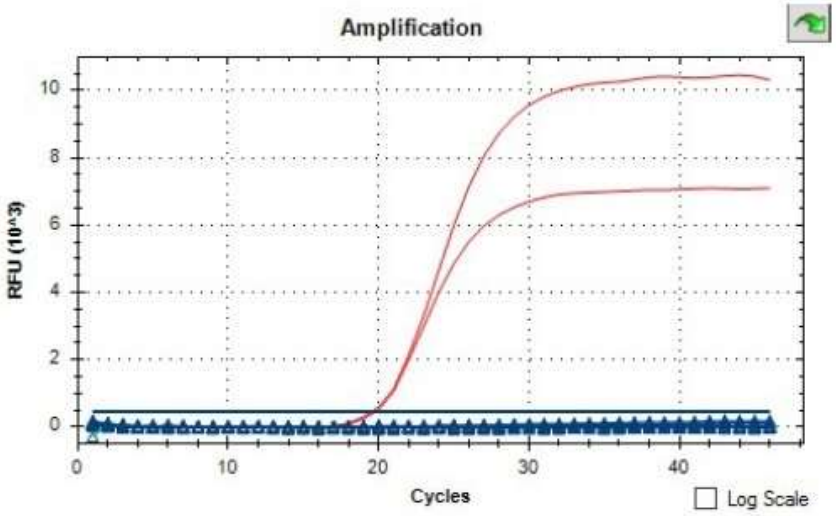


Figure 18: Graph from RT-PCR showing result for Chikungunya Virus Testing



CHAPTER 5

5.0 Discussion, Conclusion, and Public Health Implications

5.1.0 Discussion

We examined the prevalence, breeding sites, and insecticide resistance status of *Aedes aegypti* and other mosquitos of interest in two districts in Sierra Leone.

5.1.1 *Vector Prevalence*

Bombali District showed evidence of greater vector density in almost all metrics used. In egg density from Ovitraps, Bombali had 189 eggs per trap compared to Bo, which had 4.1 eggs per trap. Bombali's egg density was greater by a factor of 46. The difference in density of adult females collected was less pronounced but there was still a five-fold greater density of adult female mosquitoes in Bombali as compared to Bo (16.7 vs. 2.9 per collection). Despite this, Bo had a much greater proportion of blood-fed mosquitoes (69% vs 37%). This was likely due to the fact that collection procedures for female mosquitoes were not uniform across the two sites. The Prokopak, which was used indoors where mosquitos rest after feeding (Maia et al., 2011),was used more in Bo than in Bombali. And the BG Sentinel, which is used indoors where mosquitoes are seeking a host before feeding, was used more in Bombali than Bo.

Differences in vector species prevalence were also seen in adult females, although *Culex* was the predominant species at both sites. In Bombali District, more than 6% of adult females were *Aedes*, whereas less than 2% of adult females in Bo were *Aedes*. At both sites, *Anopheles* had a greater proportion of blood-fed adult females than *Culex*. Differences in overall mosquito prevalence as well as species prevalence may be due to the higher level of sanitation in Bo

district or lower building density, limiting the amount of preferred breeding sites, particularly for *Aedes* which is considered a domestic species (Kamgang et al., 2010).

There was greater variation in the larval and pupal surveys, although Bombali sites generally also had higher standard indices for larvae and pupae. Overall, Bombali's Breteau index was 52.5 compared to Bo's, which was 38.7. Container indices were higher for Bo, meaning that although a greater proportion of households were positive for larvae/pupae in Bombali, there was a greater proportion of positive containers in Bo, possibly due to less containers being available for breeding due to better sanitation practices. And, for the house index, it shows that Bombali had a very high percentage compared to Bo. Almost all the indices measured were above outbreak thresholds set for dengue virus.

5.1.2 *Breeding Sites*

Overwhelmingly the most common positive containers were tires, accounting for nearly half of all positive containers, with bottles and "others" accounting for an additional 36%. No other container types accounted for a meaningful share of positive containers. Tires as a preferred mosquito breeding site are well documented in the literature and are largely responsible for the geographical expansion of *Aedes* species in the past 50 years (Gratz, 2004).

5.1.3 *Insecticide Resistance*

Mosquitoes in both Bombali and Bo showed 100% susceptibility to the pyrethroid deltamethrin, indicating that this is an insecticide that would likely be effective against *Aedes* mosquitoes at both locations. On the other hand, mosquitoes at both sites showed resistance to two classes of insecticides and possible resistance to two additional classes of insecticides. Resistance testing showed that in Bombali, there was resistance to permethrin (a pyrethroid) and possible developing resistance to bendiocarb (a carbamate). In Bo, there was resistance to both permethrin (a pyrethroid) and DDT (an organochlorine) and possible developing resistance to malathion (an organophosphate). The results showing resistance to permethrin were supported with kdr genotyping, where permethrin-exposed mosquitoes had the highest proportion of kdr mutations. This was true across both Bo and Bombali sites, and for both mutations (1016 and 1534) studied. However, Bombali had a greater proportion of both types of mutants than Bo and the frequency was higher in 1534 than 1016.

5.1.4 *Virus Testing*

All nine pooled samples were negative for both flaviviruses (including Dengue, Yellow Fever, and Zika) and Chikungunya. However, it is difficult to draw conclusions about the prevalence of these viruses in the vector population because of the incredibly small sample size. Live arboviruses are found very rarely in *Aedes* mosquitoes, even during a known outbreak and with large sample sizes (Elizondo-Quiroga et al., 2018), so with small sample sizes like we had and no outbreak, it was highly unlikely that we would have found an infected mosquito.

5.2 Limitations

Collection method of adult female mosquitoes differed between sites. This was due to security reasons for the batteries we were using for the BG traps. More indoor collections were performed in Bo with the Prokopack, likely resulting in the higher proportion of blood-fed adult females in this district. The Prokopacks are also quite sensitive to variations in collection success based on the individual users. BG Sentinel traps were used for both indoor and outdoor collection and were the more commonly used method in Bombali. Differences in methodology may have also resulted in variations in the proportion of the different species collected.

Species was not determined in the larval and pupal surveys, which means we did not get information on the preferred breeding site for each type of vector or the proportion of different species found in different containers and locations. Although *Aedes* are the only major vector that prefers to breed in artificial containers in and around homes, like the ones we sampled, we cannot say for sure that the larvae and pupae we found were in fact *Aedes*. Thus, some of the indices we calculated could be also counting immatures from other species and not just *Aedes*. Furthermore, while we hypothesize that many of the differences in vector density and species composition between the sites were due to differences in sanitation and the density of buildings, we did not assess sanitation or building as part of the study, so we cannot say for sure.

In terms of insecticide resistance, the tests were performed on F1 adult females rather than F0s. Although the F1 mosquitoes were only one generation in colony in the lab, there still could have been some small evolutionary changes in the population that was reared completely in the lab as compared to wild-caught eggs or adults, which could have changed the results. Additionally we were only able to look at a snapshot of resistance from the six weeks of this study as compared to

looking over time, which would give a more accurate resistance profile. For the kdr testing, only two common genotype mutations were tested, but other mutations have been documented, so there may be other mutations present in the population that we did not test for.

Finally, as previously mentioned, a very small number of mosquitoes were tested for viruses due to the few wild *Aedes* females that we were able to capture in the field. As such, all samples were negative, but it is impossible to draw any meaningful conclusions on the prevalence of these viruses in the *Aedes* population with such a limited sample.

5.3 Public Health Implications

Larval source management, including the removal or covering of open containers, has been shown to be an effective strategy in vector control of mosquitoes (Tusting et al., 2013). This was a very clear factor in the high larval and pupal indices recorded in both Bombali and Bo Districts, especially with regard to tires. Control efforts need to focus on garages, dump sites, and other areas where tires are left in the open. Almost all indices measured were above threshold levels for dengue outbreak, leaving human populations in these areas vulnerable to infection if an outbreak of the virus occurs.

Evidence of pyrethroid resistance in both Bo and Bombali could pose a problem in control efforts, depending on which pyrethroid is used and against which vector species, since this is the only class of insecticide that is approved for use in IRS. Pyrethroid resistance has already led to the failure of some malaria control efforts in African countries, forcing a switch back to DDT (Ranson, N'Guessan, et al., 2011). Although Sierra Leone is not presently using IRS for any type of vector control, if and when it begins (as expected in the near future due to the initiation of PMI activities in late 2018), the results presented here suggest that using permethrin would be

largely ineffective against *Aedes*, since resistance was found at both locations. However, using deltamethrin could be quite effective against *Aedes*, as 100% susceptibility was found at both locations. Moreover, some resistance to organophosphate, organochlorine, and carbamate insecticides was also recorded for *Aedes* at the study sites, suggesting that there may be cross-resistance that differs by site and/or shifting resistance patterns in different geographic locations. Managing effective insecticide use with these complex patterns of insecticide resistance among vectors in different locations will require more consistent surveillance of mosquito populations and coordination of efforts to control different vectors. Combinations of different insecticide classes for IRS and ITNs are an option that should be considered. However, like any combination therapy, it comes at the risk of accelerating cross-resistance (Hemingway et al., 2016). Regardless, because of the complexities of managing insecticide resistance, it seems clear that we cannot rely on a single, static approach and that insecticides must be used in conjunction with larval source management and other methods.

Finally, Sierra Leone's high prevalence of malaria, in combination with its weak capabilities of detecting non-malarial febrile illnesses, indicates that surveillance of *Aedes* vectors and subsequent virus testing could serve as the only method of alerting health authorities to an arbovirus outbreak.

5.4 Conclusions

The large variability across sites suggests that further research into factors influencing vector populations will be necessary to inform future vector control efforts. The predominance of tires as breeding sites confirms what is known in the literature; efforts to prevent standing water from accumulating in tires may be an effective strategy to combat *Aedes* and other vectors. The high indices recorded for larvae and pupae in homes also indicates that larval source management will

be necessary to bring populations down to below outbreak threshold levels. Moreover, insecticide resistance and knockdown mutations were seen at both sites, indicating that resistance patterns will have to be consistently monitored to focus IRS and ITN programs.

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