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SPECIES COMPOSITION AND RISK OF TRANSMISSION OF *AEDES*-BORNE ARBOVIRUSES AROUND THE MOLE GAME RESERVE IN NORTHERN GHANA

BY

JOANNITTA JOANNIDES

(10701105)

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DECLARATION

I hereby certify that this dissertation is the outcome of research undertaken by me, Joannitta Joannides, towards the award of Master of Public Health from the School of Public Health, University of Ghana, Legon. This dissertation has not been submitted, either in part or in full, for any other degree and all references to the work of others have been duly acknowledged.

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JOANNITTA JOANNIDES

(STUDENT)

_____

DR. SAMUEL K. DADZIE

(SUPERVISOR)

_____

DR. MAWULI DZODZOMENYO

(SUPERVISOR)

DEDICATION

I hereby dedicate this dissertation to my beloved parents, Mr. George Joannides and Mrs. Joyce Joannides for their words of encouragement and support.

I also dedicate this research work to all upcoming research scientists in the field of public health entomology.

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ABSTRACT

Aedes-borne viral diseases mainly Dengue (DEN), Zika (ZIK) and Chikungunya (CHK) have contributed to mortality and morbidity in the world especially in Africa. There have been major outbreaks recorded in West Africa. Ghana has not recorded any outbreak of these diseases but the country is at risk of future outbreaks due to its proximity to West African countries where outbreaks have been recorded. This study assessed the risk of transmission of Dengue (DENV), Chikungunya (CHKV) and Zika (ZIKV) viruses in Larabanga and Mole Game Reserve area in Savannah Ghana. The immature and adult stages of *Aedes* mosquitoes were collected from Larabanga and Mole Game Reserve area. There was a significant ($P<0.001$) number of mosquitoes collected during the rainy season than the dry season. A total of 1,930 *Aedes* mosquitoes were collected during the rainy season and morphologically identified. Of these, 1,915 (99.22%) were *Aedes aegypti* and 15 (0.22%) were *Aedes vittatus*. During the dry season, 27 *Aedes aegypti* mosquitoes were collected. A total of 415 *Ae. aegypti* mosquitoes were molecularly identified to subspecies level of which *Aedes (Ae) aegypti aegypti* was the predominant subspecies. Both *Ae. aegypti aegypti and Ae aegypti formosus* exist in sympatry in the area. All *Aedes* pools (75) were negative for DENV, ZIKV and CHKV when examined by RT- PCR. Three Larval indices namely House Index, (HI) percentage of houses positive for *Aedes* larvae or pupae, Container Index, (CI) the percentage of containers positive for *Aedes* larvae or pupae and Breteau Index, (BI) the number of positive containers (with larvae and/or pupae per 100 inspected houses) were assessed as a measure for risk of transmission. The HI, CI and BI for both sites were as follows; Mole game reserve (HI,42.1%, CI, 23.5% and BI, 100 for rainy season and 0 for all indices for dry season) and Larabanga (39%, 15.5% and 61 for rainy season and 2.3%, 1.3% and 2.3 for dry season). The spatial distribution of *Aedes* breeding sites

in both areas indicated that *Aedes* larvae were breeding in areas with close proximity to humans. Information about the species composition and the potential role of *Aedes* mosquitoes in future outbreaks of the diseases that they transmit is needed to design efficient surveillance and vector control tools.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Three *Aedes*-borne viral diseases, Dengue, Zika , and Chikungunya were known to contribute minimally to global mortality and morbidity (Wilder-smith *et al*., 2017). However, over the past five (5) decades the occurrence of these diseases have increased exponentially (Paixão, Teixeira, & Rodrigues, 2017). Amongst these three diseases, Dengue infection has become the most dangerous and most rapidly spreading infection worldwide (Bhattacharya, Maitra, Ganguly, Bhattacharya, & Sinha, 2013). It is an acute febrile illness caused by a single stranded RNA virus belonging to the flavivirus genus which includes Yellow fever and Zika virus (Johnson, 2008). An individual infected with the Dengue virus (DENV) would exhibit symptoms such as muscle and joint pain, nausea, vomiting, rash and headaches (Gubler, 1997). There are four serotypes (DENV-1, DENV-2, DENV3 and DENV4) of the virus and DENV-2 is the most virulent (Murray, Quam, & Wilder-Smith, 2013). DENV was first isolated in Japan in 1943, by the inoculation of serum of patients in suckling mice (Dhra, Bhandari, Kumar, & K, 2017). In the 1980's, countries in the American region experienced major epidemics of Dengue. Before 1980, there was very little information on the distribution of DENV in Africa. However, major epidemics have occurred in West and East Africa since then. All four serotypes have been implicated for these major epidemics in Africa (Were, 2012a).

Zika virus (ZIKV) infection which is closely related to Dengue virus has received a striking public health attention. This is due to its association with microcephaly and other neurological

disorders such as the Guillian Barre syndrome in babies born to infected mothers (Sharma & Lal, 2017). Research shows that 1 out of 4 individuals infected with the virus show mild symptoms such as mild fever, skin rash or headache, muscle and joint pains (Barzon, Trevisan, Sinigaglia, Lavezzo, & Palu, 2016). The Zika virus has been around for a long time. It was first identified in 1947 in monkeys in Uganda while scientists were monitoring Yellow fever in the Zika forest in Uganda. Zika was later detected in humans in 1952 but there were no outbreaks at the time (Johnson, 2008; WHO, 2019). In 2007 the first major outbreak of Zika occurred in the Pacific Island of Yap (WHO, 2019). Apart from transovarial transmission and transmission through the bite of an infected *Aedes* mosquito, a person can get infected through blood transfusion and sexual contact (CDC, 2018b).

Chikungunya (CHKV) which means "disease that bends up the joints" in Tanzanian Makonde dialect is a febrile illness caused by the Alphavirus from the Togaviridae family (Bonthius & Bale, 2017). There are three circulating genotypes of the virus. These are the Asia, West Africa and East/Central/South Africa genotypes (Zeller, Bortel, & Sudre, 2016a). The very first outbreak was reported in Tanzania in 1953. Since then, a few outbreaks and sporadic cases were reported mainly in Asia and Africa. About 80% of cases reported are symptomatic. These symptoms usually occur after an incubation period of 1-12 days (Burrell, Howard, & Murphy, 2017). Most people with chikungunya infection experience severe joint pains (arthralgia) which may lead to disabilities (Patterson, Sammon, & Garg, 2016a).

The viruses that cause Dengue, Zika and Chikungunya are transmitted by mosquitoes of the *Aedes* species specifically *Aedes aegypti* and *Aedes albopictus*. All three viruses are transmitted in forest cycle (zoonotic) which involves non-human primates and arboreal mosquitoes (Weaver & Reisen, 2010). Nonetheless, the domestication and the spread of the vectors outside their

native range have caused a spillover from the zoonotic transmission cycle to urban transmission cycle involving human primates. Among the *Aedes* species, *Aedes aegypti* is known to be the most efficient vector in the transmission of these viruses (Weetman *et al*., 2018). They are efficient vectors because they have learnt to live their entire life cycle in close proximity to humans. They also feed solely on human blood even in the presence of other mammals as humans are the most available and stable source of blood (Powell & Tabachnick, 2013) .The *Aedes aegypti* species is highly susceptible to all three viruses and are efficient in propagating these diseases because during the intake of a single blood meal, they bite several humans hence transmit the virus to multiple hosts (Wilder-smith *et al*., 2017). *Aedes albopictus*, also known as the "Asian tiger" mosquito, has become an important vector in many regions. As it complements the role of *Aedes aegypti* in a lot of places, *Aedes aegypti* is most common inside of the house while *Aedes albopictus* presents an outdoor risk. The spread of this invasive species of *Aedes* mosquitoes is increasing rapidly. It has become effective in transmitting some of these *Aedes*-borne viruses especially CHIKV. This is due to the E1-A226V mutation in the Eastern/Central/South African genotype which has conferred an enhanced transmission of the Virus in *Aedes albopictus*(Zeller *et al*., 2016a).

Humans also contribute to the spread of these diseases and their vectors. In middle and low income countries, uncontrolled urbanization is one of the driving factors of *Aedes*- borne diseases (Wilder-smith *et al*., 2017). The uncontrolled urbanization in these countries is usually accompanied by poor housing conditions, poor sanitation, poor drainage systems and lack of good water supply leading to the storage of water in containers which serve as good breeding sites for *Aedes* mosquitoes (Overgaard *et al.,* 2017). Furthermore, the increase in human travel

and globalization speeds up the introduction of *Aedes*-borne viruses to new location (Liang, Gao, & Gould, 2015).

With the absence of vaccines for Dengue, Zika and Chikungunya infections, vector control is the most viable approach to controlling these diseases (CDC, 2018a; Patterson *et al*., 2016a). Some examples of these control measures are the use of insecticides, repellents and wearing of protective clothing to avoid the bite of these mosquitoes. There is however the possibility of *Aedes* mosquitoes building resistance to these insecticides which will lead to the increase in disease burden. There can also be community-based campaigns in middle and low-income countries to reduce mosquito populations breeding near human settlements. Long term steps can also be taken towards reducing vector populations through the delivery of better housing and provision of better solid waste management.

1.2 Problem Statement

Aedes-borne viral diseases especially Dengue (DENV), Chikungunya (CHKV) and Zika (ZIKV) are the most important and the most rapidly spreading mosquito- borne viral diseases in the world (Patterson, Sammon, & Garg, 2016b). About 50% of the world's population lives in dengue endemic countries. According to the World Health Organization (2012), 50-100 million dengue infections occur in endemic countries with a record of 20,000 deaths each year. In Africa, Dengue has been reported in 34 countries including Togo, Burkina Faso, Côte d'Ivoire, Gabon and others with their capital cities being the most severely affected (Tarnagda *et al*., 2018; Were, 2012b)

Zika (ZIKV) virus infection on the other hand has circulated in Africa and Asia after its discovery in Rhesus monkeys in the Zika forest of Uganda in 1947 (Plourde & Bloch, 2016).

Approximately 80% of reported cases are asymptomatic. WHO recently declared this disease to be a "Public Health Emergency of International Concern"(Patterson, Sammon, & Garg, 2016, pp657) due to the increase of reports of prenatal microcephaly and Guillain Barre syndrome (GBS) in endemic areas. There have been major outbreaks of ZIKV infection in Latin America and the disease has also been detected in Gabon (Grard *et al*., 2014; Patterson *et al.*, 2016a).

Yellow fever (YF) outbreaks had occurred in Ghana in recent years (Halstead, 1980; Monath, 1986). However, the presence of a vaccine against YF has substantially mitigated the risk and outbreaks. Ghana has previously not recorded outbreaks of Dengue and Zika virus infections but the country is at risk due to its proximity to West African countries where recent outbreaks had occurred (Segbefia, 2015). There is no vaccine for DENV and CHIKV, although some vaccines are currently under evaluation for ZIKV.

Recent studies have established previous exposure to Dengue virus among some diagnosed malaria patients in some urban areas in Ghana (Stoler *et al.*, 2015). The most recent study confirmed the presence of Dengue virus in the blood of two children in the Greater Accra region. Further investigations showed that these children had not travelled outside the country. There is therefore a possibility that the infection was acquired locally (Amoako *et al*., 2018). Local transmission may also mean that they may have acquired the infection from the bite of an infected *Aedes* mosquito. There is however very limited information on the distribution, abundance and risk of transmission of *Aedes*-borne arboviruses such as DENV, CHKV and ZIKV in the country. This study will investigate the species composition and assess risk of transmission of arboviral diseases within the Mole Game Reserve.

1.3 Justification

The Mole Game Reserve located near Damongo in Savannah Ghana has been previously described as a high-risk area for transmission of viral hemorrhagic fevers because of the presence of high population of *Aedes aegypti* in the Damongo area (Appawu *et al*., 2006). The southern part of Ghana has been found to be a low risk area (Suzuki *et al*., 2016). The Damongo and Mole Game Reserve area have been a hotspot for Yellow fever outbreaks in recent times. The area is also a tourist site that receives many visitors from different parts of the world including some areas endemic for DENV, CHKV and ZIKV. The presence of travelers in and around the game reserve where there are primates that serve as reservoir of these viruses makes it a potential area for future outbreaks of DENV, ZIKV and CHKV. It is therefore highly imperative to conduct entomological studies in this area to better inform the relevant authorities about possible outbreaks and plan control strategies against the vectors.

1.4 Conceptual framework

The transmission of Dengue and Zika virus is dependent on the presence and abundance of *Aedes* mosquitoes. There are a variety of factors that affect the species composition and abundance of these mosquitoes in an area. First and foremost, climatic factors such as rainfall and temperature affect the availability of breeding sites which may increase or reduce vector populations (Helmersson, 2018). Secondly, non- climatic factors such as global trade, migration and urbanization may influence species abundance and composition. Increase in global trade of used tires contaminated with eggs of aedes mosquitoes into the country facilitates the spread of new species into new areas (Paul, 1998). These mosquitoes may also be infected which may increase the risk of transmission of these viruses. Increase of urbanization may cause a shift of *Aedes* species from forest areas to areas where there are large populations of people as some

species of aedes mosquitoes prefer human blood to animal blood for reproduction. Some of the above non-climatic factors such as urbanization (unplanned) have resulted in poor sanitation, poor house hold design and prolong storage of water (socio-economic factors) which have led to the reproduction and spread of *Aedes* mosquitoes in the country(Khan, Khan, & Amin, 2016). Entomological studies which involve determining the species composition and abundance of Dengue and Zika vectors in the country, detection of viruses in these vectors together with serological studies will help in assessing the countries risk of a possible outbreak which will lead to mortality and morbidity in humans. This study focused on entomological studies of *Aedes* mosquitoes around the Mole game reserve.

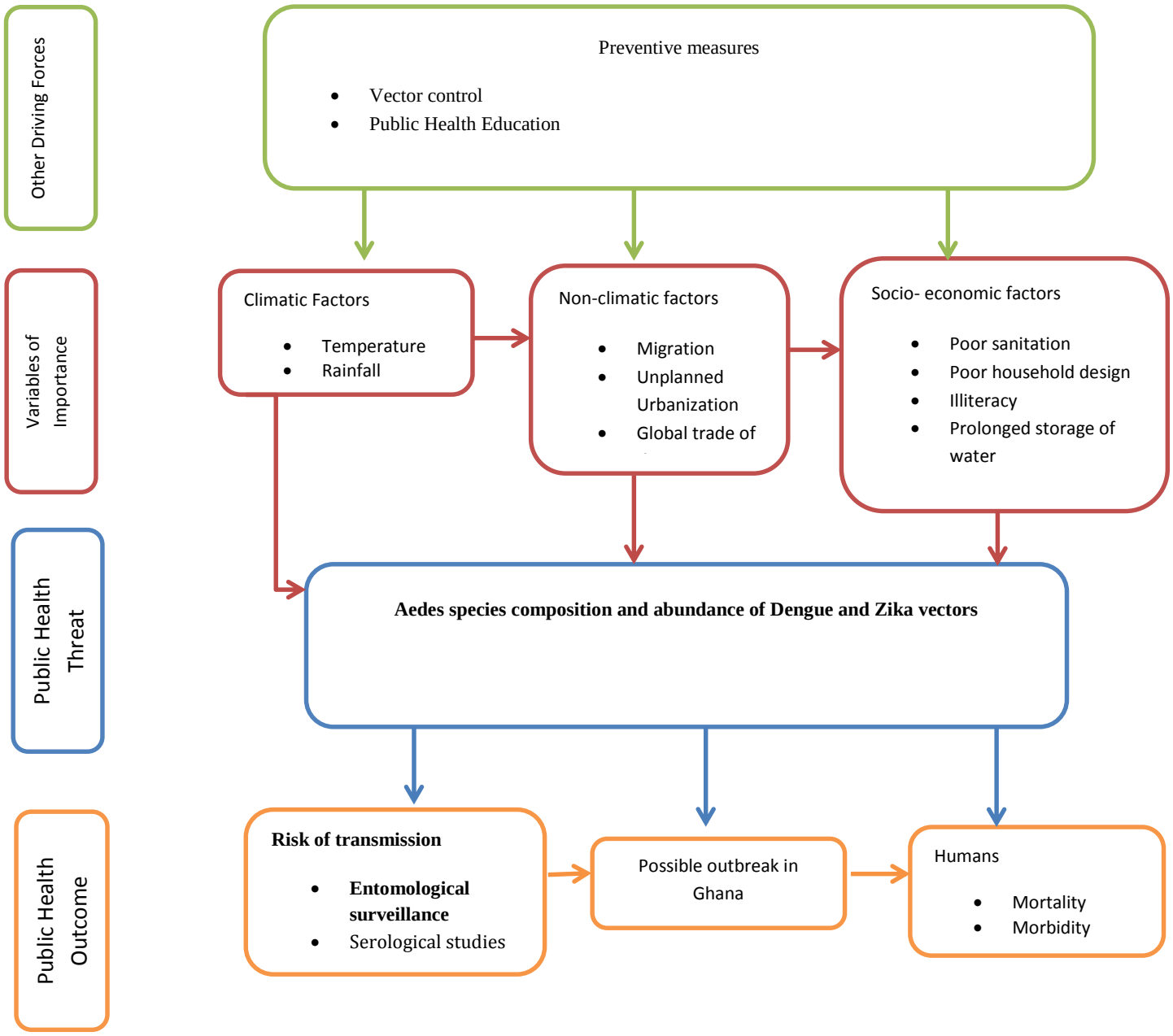

Figure 1. Conceptual framework of factors that affect the species composition of Aedes mosquitoes and the risk of transmission of Aedes-Borne Arboviruses

1.5 Objectives

1.5.1 General Objective

To assess the risk of transmission of *Aedes* borne viral diseases around the Mole game reserve in Savannah Ghana.

1.5.2 Specific Objectives

1. To determine the species composition and density of *Aedes* mosquitoes in the study areas.
2. To determine the transmission risk of DENV, CHKV and ZIKV in the study areas.
3. To determine virus infectivity rates of *Aedes* mosquitoes in the areas.
4. To spatially map out the distribution of *Aedes* mosquitoes around the study areas.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Burden of Dengue, Chikungunya and Zika in the World

In recent years Dengue, Zika and chikungunya viruses have spread across the world. Yellow fever which is also an important *Aedes*-borne virus has been of great public health importance. However, with the presence of a vaccine, outbreaks of this disease have reduced (Patterson *et al*., 2016b). The presence of these viruses in various countries has been attributed to the presence of the major vectors *Aedes aegypti* and *Aedes albopictus*. Dengue and Zika are *Flaviviruses* from the family *Flaviviridae* while chikungunya is an *Alphavirus* from the Family *Togaviridae* (Burrell *et al*., 2017; Johnson, 2008).

There are four genetically related viruses (DEV-1, 2, 3 and 4) that cause Dengue. One or more of these viruses have been detected in countries across the continent. All serotypes have been detected in Africa and are maintained between non-human primates and arboreal mosquitoes (Were, 2012b). A close relation of Dengue serotype two has been detected in two children in Ghana even though there have not been outbreaks of the disease in the country (Amoako *et al*., 2018). Burkina Faso which is neighboring to Ghana recorded major outbreaks in 2016 and Dengue serotype 2 was detected (Tarnagda *et al*., 2018). Zika virus after its isolation in Africa has emerged in less than eighty countries, including the Americas and the Caribbean (Morens & Fauci, 2017). The ability of the virus to harm unborn babies has caught the attention of infectious disease researchers. In Angola, about 72 babies have been born with microcephaly between 2017

and 2018 and this follows the detection of a highly dangerous strain of the virus in early 2017 (Hackett, 2018). Zika cases in Africa are largely under reported. Four patients in Guinea Bissau who experienced symptoms for severe malaria were tested positive for Zika Virus(CDC, 2017a). Usually Dengue, Zika and Chikungunya have similar symptoms as malaria and most cases that have been detected in Africa are of people presumed to be infected with Malaria. Most outbreaks of Chikungunya have occurred in Africa and Asia. A large epidemic of the disease occurred on the coast of Kenya in 2004. The virus has now spread to India and Southeast Asia. Countries in West and East Africa has also experienced small out breaks (Zeller, Bortel, & Sudre, 2016b).

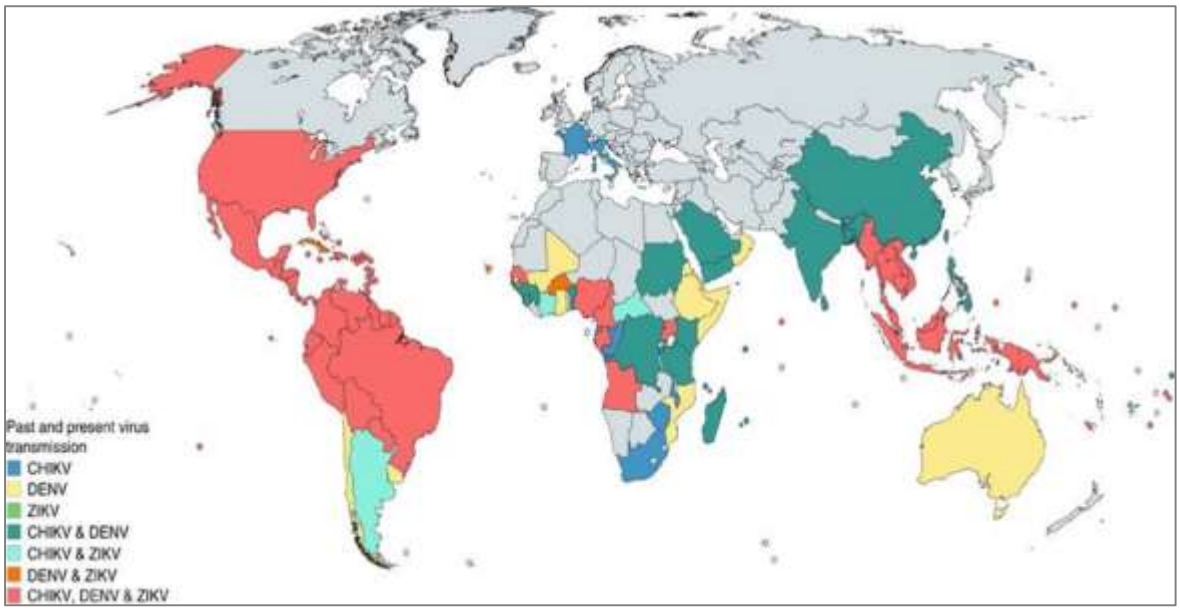

Figure 2: Global Distribution of DENV, CHKV and ZIKV.

2.2 *Aedes* Mosquitoes

Aedes is a genus of mosquitoes that belongs to the subfamily culicinae (Service, 1980). These mosquitoes were initially found in tropical and subtropical areas, but now they are found on all continents with the exception of Antarctica. There are about 700 identified species in the world

and most of these species have been spread largely by human activity. Adult *Aedes* mosquitoes are visually distinctive because of their typically black body, noticeable black and white scales on the thorax and abdomen and alternating black and white bands on their legs (Rueda, 2004). Females are further distinguished by the shape of the abdomen, which commonly comes to a point at its tip, and by their maxillary palps (sensory structures associated with the mouthparts), which are shorter than the proboscis (Rogers, 2019). Unlike other mosquitoes, they are active and bite during the daytime. Adult female *Aedes* mosquitoes are hematophagous. During the intake of a blood meal they may inflict harm to their host by introducing pathogens mostly viruses. These include *Aedes agypti* and *Aedes albopictus* which are involved in the transmission of Dengue virus, Zika virus, West Nile virus and the others to humans (Weetman *et al*., 2018). Other species involved in transmission of these viruses between non-human primates are *Aedes Africana, Aedes Vittatus, Aedes luteocephalus* and many others.

2.3 Life cycle of *Aedes* Mosquitoes

The Life cycle of mosquitoes is very important in the control of these organisms. Stages in the life cycle are targeted and disrupted. Like all mosquitoes, *Aedes* mosquitoes go through four stages in their life cycle. The first three stages which are the egg, larval and pupal stage occur in water. Female *Aedes* mosquito requires a blood meal to develop her eggs. The proteins and iron that is present in blood aides with egg development. It is at this point where females infected with a pathogen in this case viruses pass on the virus to their host. One's a female acquires a blood meal, she finds a warm moist place to rest and wait for egg development. Eggs are usually laid singly on damp soil, tree holes that hold water, plant axils, clay pots, rain water that have collected in plastic containers and cans (Bashar, Rahman, Nodi, & Howlader, 2016). Unlike other mosquitoes, the eggs of *Aedes* mosquitoes are able to withstand desiccation for long

periods and this adaptation makes it difficult to control *Aedes* population (Faull, Webb, & Williams, 2015). This means that even as the other developmental stages are being eradicated, the dormant but viable eggs that remain in containers bring back populations of *Aedes* mosquitoes. It is this adaptation that enables them to survive cold winters and other adverse climatic conditions hence their survival on all continents. Once conditions required are available, the eggs hatch into larvae in less than a day. The larvae live in water, naturally hanging upside down at an angle from the surface of the water. In this position the larvae are able to breathe using a short thick respiratory siphon that takes up oxygen from the surface of the water. *Aedes* larvae wiggle to the bottom of the water to feed or when the water is disturbed. They usually feed on organic solid or liquid matter such as strands of macroalgae, leaves, dead invertebrates which can be of their own kind and other microscopic organisms in their aquatic habitats. After four stages of larval development which takes four days, the last stage develops into pupae. The pupal stage is a mobile stage that lasts for two days. The pupae do not feed and respond to stimuli. They also take in oxygen from the surface of the water through their respiratory trumpets. Adults then emerge from the pupal case on the surface of the water. Newly emerged adults stay on the surface of the water for a while to dry their cuticle and expand their wings and legs. Within two to three days of emergence, adult mosquitoes mate and the females take up a blood meal to develop their eggs to continue the cycle.

Figure 3: Life cycle of *Aedes* mosquito (Dengue Patrol, 2015).

2.4 Distribution of *Aedes* mosquitoes

Aedes mosquitoes are found across all continents and the spread of *Aedes* species to new regions has been greatly due to human activities. The two *Aedes* species responsible for the transmission of *Aedes*-borne diseases to humans are present in all continents. *Aedes aegypti* is native to Africa. The ancestral form of *Aedes* aegypti was a zoophilic tree-hole mosquito named *Ae. aegypti formosus* (Kraemer *et al*., 2015). Due to the increase in human population and encroaching of the native land of these species, which has led to the domestication of *Aedes* species forming a subspecies named *Ae.aegypti aegypti* (Powell & Tabachnick, 2013). *Aedes*

albopictus is native to South East Asia, spreading to all five continents. The rapid spread of *Aedes albopictus* has been caused mostly by the distribution and sales of used tires across countries (Kamgang *et al.*, 2018).

2.5 Sampling methods for *Aedes* species

There are several methods that can be employed in the sampling of *Aedes* species. Sampling methods usually depend on the aim of the study. All the developmental stages of *Aedes* mosquitoes can be sampled using different methods. Adult *Aedes* mosquito sampling involves the use of baited traps such as Bio-agent sentinel trap (BG-sentinel trap), CDC light trap, and sticky traps (WHO, 2016). Some of these traps use a combination of attractive visual and olfactory cues. Collection is also done using mechanical aspirators for collecting resting mosquitoes and Human Landing catches which is the oldest technique used in collecting adult mosquitoes (CDC, 2017b). These methods however require highly skilled individuals and it is labor intensive. For collecting immature stages, ovitraps are used to collect *Aedes* eggs (CDC, 2017b). Larvae and pupae are sampled using dipping method. This involves the use of ladles, pipettes and buckets to collect from water holding containers and from stagnant water collection. Among these the use of adult traps is expensive. In detecting the presence of *Aedes* mosquitoes in areas with low infestation and unproductive larval survey, ovitrap is the best option (WHO, 2016).

2.6 Identification of *Aedes* mosquitoes

Various species of *Aedes* mosquitoes are identified morphologically using taxonomic keys unique to the geographical regions (Rueda, 2004). The use of this method however is time consuming and requires highly skilled entomologists to identify species reliably (Batovska, Blacket, Brown, & Lynch, 2016). Also the use of morphological keys may be challenging in

identifying samples with destroyed morphological features due to improper handling during collection and storage. Molecular techniques have also been developed to identify *Aedes* species. It is the most effective and reliable method in identifying species of mosquitoes. This method is DNA based and involves the use of Polymerase chain reaction (PCR). Here, specific regions of the genomic material unique to various species are amplified using primers. For instance, Ballinger-Crabtree *et al*., (1992) established a Random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) method that permits identification of *Ae*. *aegypti* subspecies. DNA barcoding is another molecular method that has become popular for the identification of animal species (Soni, Bhattacharjee, & Khan, 2018). This method is based on the concept that species has a unique genetic identity. A DNA barcode is a short standardized sequence of DNA that can be used as a genetic maker for species identification. The marker being used for *Aedes* species identification is known as the mitochondrial cytochrome c oxidase subunit 1 (COI) gene (Chan *et al*., 2014).

2.7 Medical and Economic importance of *Aedes* mosquitoes

Aedes mosquitoes have become of great medical and economic importance. Infected Female Aedes mosquitoes transmit viruses to their host during the intake of a blood meal. *Aedes* species specifically *Aedes aegypti* and *Aedes albopictus* have been implicated for the transmission of Dengue virus, Chikungunya virus, Yellow fever virus and Zika virus (Patterson *et al.*, 2016a). Disease caused by these viruses has been known for a long time and are reappearing causing high mortality and morbidity in many parts of the world (Gould, Pettersson, Higgs, Charrel, & Lamballerie, 2017).

2.8 Transmission and life cycle of *Aedes*-borne viruses

The cycle of *Aedes*-borne viruses naturally involves a vector and an invertebrate host. Upon ingestion of the virus by a female Aedes mosquito, the virus replicates in the midgut and in the salivary glands (Kuno & Chang, 2005). Females remain infected throughout their life cycle and are able to pass on the virus to their offspring which is known as transovarial transmission. The virus is then transmitted from one vertebrate host to another through the bite of an infected female *Aedes* mosquito also called the sylvatic cycle. *Aedes* species involved in this transmission cycle are *Aedes africanus*, *Aedes Vittatus* and *Aedes luteocephalus*. The invertebrate host also serves as an amplification Host. Primary vertebrate hosts are mostly species of wild animals and birds. The natural zoonotic cycle involving mosquitoes and vertebrate host did not include humans. Transmission to humans was not effective. However, due to the high viremia in humans infected with these viruses, there is effective transmission between humans and mosquitoes without the inclusion of an amplification host. This transmission cycle is known as the urban cycle. *Aedes aegypti* and *Aedes albopictus* are the primary vectors in this cycle as they live in close proximity to humans.

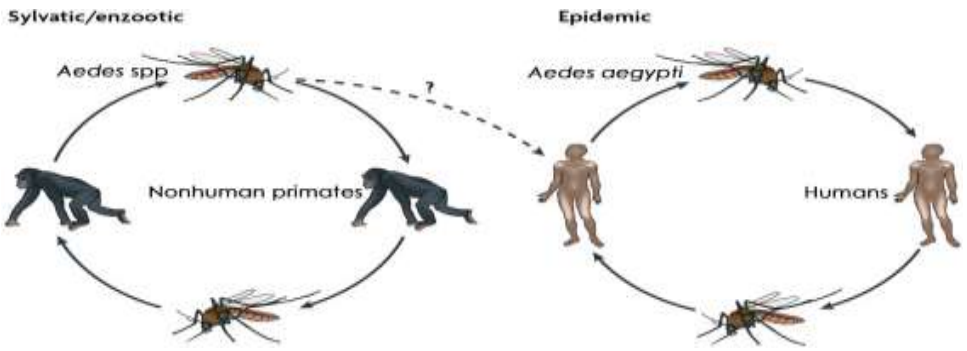

Figure 4: Sylvatic/ enzootic and epidemic transmission of dengue virus (Whitehead, Blaney, Durbin, & R, 2007)

2.9 Molecular detection of *Aedes*-borne viruses

Reverse transcriptase Polymerase chain reaction (RT-PCR) is a simple, sensitive and rapid method that has been used for the detection of infectious diseases in laboratories (Pabbaraju *et al*., 2016). It is now used in the detection of *Aedes* born viruses in human clinical samples and in mosquitoes (Suwanwong, Moungkote, Wiwanitkit, & Soogarun, 2010). This method involves the amplifications of gene regions of a genome by the use of primers (Deubel *et al*., 1990). These PCR methods vary somewhat in terms of the amplified gene regions of the genome, in the ways they detect RT-PCR products, and the virus typing methods. Primers have been developed to amplify the 5'-UTR region of Dengue (serotypes1-4), Envelope gene region of Zika virus and the nSP1 region of Chikungunya virus. Some studies have demonstrated that nucleotide sequencing of gene fragments amplified by RT-PCR can be used as a fast method of genetic classification of *Aedes*-borne viruses such as dengue virus serotypes (CDC, 2017c).

2.10 Control and prevention of *Aedes*-borne viral disease

Among the *Aedes* born viral diseases, Yellow fever is the only disease with an effective vaccine (Weetman *et al*., 2018). While the development of Dengue, Zika and Chikungunya vaccine is underway, the prevention and control of these diseases is dependent on vector control and limiting human vector contact. In limiting human vector contact, the use of personal protective strategies such as wearing protective clothing and the application of repellent are used. Efforts are been made to reduce vector populations around human settlements through the use of larvicides and biological control agents (CDC, 2017b)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3. 1 Study Sites

The study was carried out in and around the Mole National Park, in Damango, West Gonja District. Samples were collected from within the game reserve, around the quarters of the game reserve and in Larabanga which is 44km away from the game reserve (Figure 5).

Figure 5: Map of Ghana showing the study sites.

3.1.1 Mole game reserve and Mole Quarters

The Mole game reserve is a guinea savanna ecological zone characterized by prolonged dry season and a short rainy season covering about five months with an annual rainfall of about 1000mm. The game reserve has a latitude and longitude of 9˚30' 0" N 2˚0' 0" W and it is located at an elevation of 200 meters. The game reserve covers an estimated 4,577 square kilometers and harbors different species of animals including elephants, antelopes, chimpanzees, birds and about 400 other species. It attracts a great number of tourists both nationally and internationally. This site was selected based on the potential of zoonotic infections and also previous histories of outbreaks of Yellow fever and suspected viral haemorrhagic fevers which are caused by flavivirus.

3.1.2 Larabanga

Larabanga (9˚ 13' 0" N 1˚ W 51' 0") is a village also in the West Gonja district. It is 44km away from the mole game reserve. The village is known for its Sahelian mosque which is the oldest mosque in all of Ghana and if possible West Africa. Tourists who visit the game reserve usually visit the Larabanga mosque due to its proximity. The population for Larabanga as of 2010 is 3,602 and there are about 467 households in Larabanga (Dramani & Mahama, 2014)

3.2 Study Design

A cross-sectional entomological study was carried out in and around the Mole Game Reserve in the Savannah Region of Ghana. Each of the study sites were divided into four clusters and larval survey was carried out in selected houses within clusters to assess the risk of transmission of, DENV, CHKV and ZIKV. This was done by collecting *Aedes* larvae and adults as well as counting the number of houses and containers positive for *Aedes* larvae. Quantitative data was collated to determine the species composition and larval indices. Mosquito survey was carried

out in October 2018 representing the rainy season and in March 2019 representing the dry season.

3.3 Sampling of adult *Aedes* mosquitoes

Adult *Aedes* mosquitoes were sampled in and around the game reserve by using Biogents sentinel traps (BG) (Figure 6) as well as sweep nets to capture *Aedes* mosquitoes resting on vegetation outdoors. The BG-traps consist of an attractant and a fan which attracts mosquitoes into a catch bag. The trap was set outdoor from 6:00am to 6:00pm. The adult mosquitoes were grouped according to sex and put in 2ml vials and labelled with information on place and date of collection after which they were immediately stored in liquid nitrogen to keep the integrity of the genetic material.

All samples were transported to Noguchi Memorial Institute for Medical Research within 24 hours and stored in -80C freezer for further analysis.

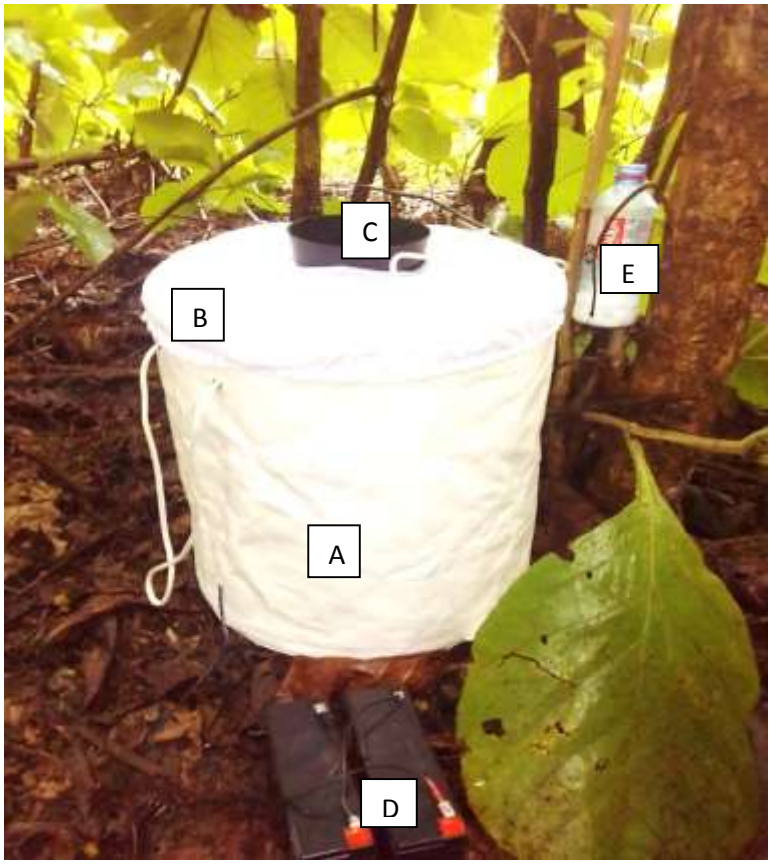

Figure 6: A picture of a BG-sentinel trap set up for Aedes adult sampling in the Mole game reserve.(Parts of the trap: A. main body; B. Top cover; C. intake funnel; D.Battery; E. Bottle containing dry ice)

3.4 Survey for immature stage of *Aedes* larvae and estimation of larval indices

Larvae were collected from selected houses and potential breeding sites in and around the Mole Game Reserve as well as in Larabanga using ladles, dippers, pipettes and buckets.

Houses and containers inside and around houses were inspected for the presence of *Aedes* larvae (Figure 7). The data from the larval survey was used to calculate the larval indices which are a surrogate marker for the risk of transmission of DENV, ZIKV and CHKV. Three of these indices were assessed namely House Index (percentage of houses positive for larvae or pupae), Container Index (the percentage of containers positive for *Aedes* larvae or pupa) and Breteau Index (the number of positive containers (with larvae and/or pupae) per 100 inspected houses).

Larvae collected were grouped and stored in appropriately labelled falcon tubes containing RNA-later to keep the integrity of the genetic material and also to prevent decomposition that will change morphological characteristics.

Figure 7: Houses and containers being inspected for the presence of *Aedes* larvae in Mole Game Reserve area and Larabanga.

3.6 Geo-Reference of sample collection sites

The sites where the *Aedes* samples were collected and the houses that were inspected for immature stages of *Aedes* larvae were Geo-referenced using Geographical positioning system (GPS) and the data spatially displayed on a map.

3.7 Identification of *Aedes* mosquitoes

3.7.1 Morphological Identification

Adult and larvae of *Aedes* mosquitoes collected were morphologically identified to species level in the NMIMR-VNVL entomology laboratory using morphological identification keys (Rueda, 2004) and a dissecting microscope .

Fundamental features used in identifying adult *Aedes* mosquitoes to the species level include the colour or markings on the scutum of the thorax, the hind tarsomeres and the abdomen. Adult *Aedes* mosquitoes were identified using their black and shiny white or silver colour, black and white or silver shiny markings and patterns on head, legs, thorax and abdomen which are peculiar to *Aedes* species (Figure 8).

For the larvae, fundamental features such as the length of the siphon, the shape of the comb scales and length of spines on the comb scales were used to identify to species level (Figure 9)

After identification, the legs of the adults were detached from each adult mosquito and separately stored in well labeled 1.5ml micro centrifuge tubes for molecular identification. The last abdominal segment of each larva was also detached and placed in well labelled micro centrifuge tubes for molecular identification. The remaining body parts were grouped into pools of 30 by species, sex and point of collections and stored for viral detections.

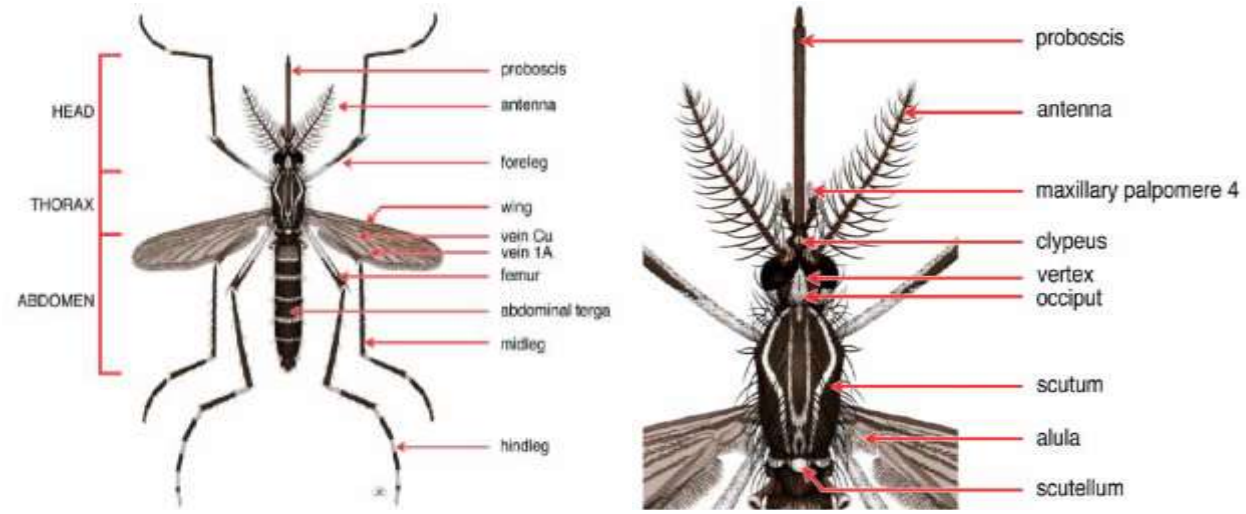

Figure 8: Dorsal view of adult female mosquito-Aedes (Stegomyia) aegypti (Rueda, 2004, p.10)

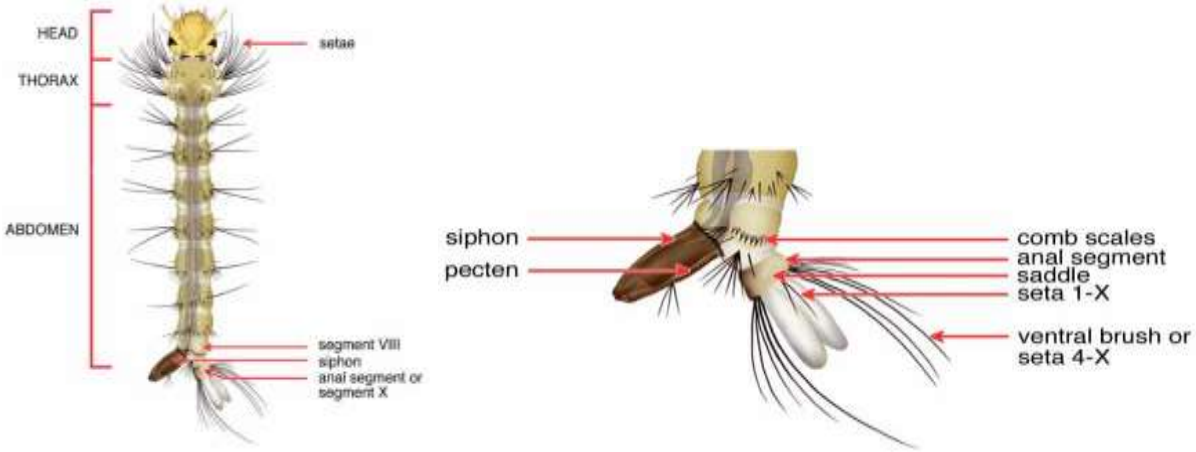

Figure 9: Dorsal view of mosquito larva (segments VIII and X, lateral view)- Aedes (stegomyia) albopictus (Rueda, 2004, p.12-13).

3.7.2 Molecular Identification

The random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) as described by Ballinger-Crabtree, Black, & Miller (1992) was used to identify and differentiate *Aedes* mosquito species. DNA was extracted from the legs and wings and from the last abdominal

segment of the larvae using the Qiagen kit as described by the manufacturer. The random regions of genomic DNA were amplified using a 10 base pair primer B3.

3.7.2.1 DNA extraction

DNA extractions were done from the legs of adult *Aedes* mosquitoes and from the last abdominal segment of the *Aedes* larvae using the Qiagen Kit. Samples were placed in well labelled 1.5ml micro centrifuge tubes. According to the manufacturer's protocol, 180ul of animal tissue lysis buffer (ATL) were added to the tubes and homogenized using clean pestles. A volume of 20ul of proteinase K was added and mixed by vortexing. Tubes were incubated at 56˚C while shaking for 15minutes to ensure complete lysing of the cells. After 15minutes of incubation, 200ul of Buffer AL was added to the samples which were mixed by vortexing and incubated at 70˚C for 10 minutes. To the samples, 200ul of absolute ethanol was added and mixed per vortexing for 15 seconds. After mixing, samples were briefly centrifuged and the mixture was then carefully applied to the QIAamp Mini spin column without wetting the rim and centrifuged at 8000rpm for 1 minute. The spin column was then transferred to a clean 2ml collection tube and the tube containing filtrate was discarded. A volume of 500ul of Wash buffer one (AW1) was added to the QIAamp Mini spin column and centrifuged at 8000 rpm for 1 minute. The QIAamp mini spin column was then transferred to a clean 2ml collection tube and the collection tube containing the filtrate was discarded. Another 500ul of Wash Buffer two (AW2) was added to the QIAamp Mini spin column and centrifuged at 14000rpm for 3 minutes. The filtrate in the collecting tube was discarded after centrifugation. The QIAamp Mini spin column was transferred to a clean 1.5ml Eppendorf tube which was well labelled and 200ul of Elusion buffer (AE) was added. Samples were incubated at room temperature for 1 minute and then centrifuged at 8000rpm for 1 minute. The tubes were closed and stored at -20˚C to be used for further analysis.

3.7.2.2 Species identification using (RAPD-PCR)

Random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) involved amplification of random segments of *Aedes* mosquito DNA using a 10 base pair primer B3 (5' - CATCCCCCTG-3'). Table 1 shows the master mix for the reaction.

Table 1: PCR master mix component and mosquito DNA template

Reagents	**1 Reaction(ul)**
DNAse free water	13.2
10x Reaction Buffer	2
50uM MgCl2	0.6
10uM DNTP's	0.5
10uM B3 primer	0.5
5uM Taq Polymerase	0.2
DNA template	3
Total Volume	20

The master mix was briefly vortexed and centrifuged, and 17ul was into each well in the 96 wells plate. 3ul of thawed DNA was added to each well. The plate was sealed and placed in a thermocycler and allowed to run using the reaction steps shown in table 2.

Table 2: Reaction steps for PCR for species identification.

Step	Cycles	Temperature	Time
1	1	94˚C	4minutes
2		94˚C	1minutes
3	45	35˚C	1minutes
4		72˚C	2minutes
5	1	72˚C	5minutes

3.7.2.3 Agarose gel electrophoresis and visualization of PCR products

The PCR products were separated by electrophoresis on a 1% agarose gel in 0.5% Tris-acetate Ethylenediaminetetraacetic acid buffer (TAE) (Serva Electrophoresis, Heidelberg, Germany) stained with Ethidium Bromide. Each well was loaded with 10ul of the PCR product and 1ul of blue/orange 6XDNA loading dye (Promega, Madison, USA) and allowed to run for 1 hour 20 minutes at 100 volts. The agarose gel was visualized using a photo-spectrometer.

3.8 Processing of mosquitoes for DENV, CHIKV and ZIKV

Adult and larvae of *Aedes* mosquitoes collected were grouped into pools of 10 by place of collection, sex and species. RNA was extracted from these pools using the Viral RNA mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA were amplified using Trioplex Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT- PCR) to detect the 5'-UTR region of Dengue (serotypes1-4), Envelope gene region of Zika virus and the nSP1 region of Chikungunya.

3.8.1 RNA extraction

Pools of mosquitoes were homogenized using clean pestles. In a 1.5 ml micro centrifuge tube, 560 ul of prepared buffer AVL containing carrier RNA and 140ul of homogenized mosquito tissue supernatant was added. The contents were vortexed briefly for 15 seconds to ensure effective lysis and incubated at room temperature for 10 minutes for complete viral particle lysis. After incubation, the tubes were centrifuged for 10 seconds at 8000 rpm to remove drops from the inside of the lid. 560ul of absolute ethanol (96 -100%) was added to the sample mixture, then mixed by pulse-vortexing for 15 seconds and centrifuged for 10 seconds at 8000 rpm to remove drops from inside the lid.

Following centrifugation, 630ul of the sample mixture were transferred to the QIAamp mini-column placed in a 2ml collection tube. The column was centrifuged at 8000 rpm for one minute. The collection tube with the filtrate was discarded and replaced with a clean 2 ml collection tube. The final volume of sample mixture was transferred to the column, centrifuged at 8000 rpm for one minute after which the collection tube with filtrate is discarded and replaced with a clean 2ml collection tube.

The bound nucleic acid was washed by adding 500 ul of buffer AW1 to the column and centrifuged at 8000rpm for one minute. The collection tube with the filtrate was discarded and replaced with a clean collection tube. For the second wash, 500 ul of buffer AW2 was added and centrifuged at maximum speed (approximately 13200 rpm) for 3 minutes. The collection tube with the filtrate was discarded and replaced with a clean 1.5 ml micro centrifuge tube. RNA was eluted by adding 60 ul of buffer AVE equilibrated to room temperature to the column. Column contents were incubated at room temperature for one minute, then centrifuge at 8000 rpm for 1 min. RNA extracted was immediately stored at -80˚C.

3.8.2 Real-time PCR for the detection of DENV, CHIKV and ZIKV

The Trioplex Assay for Dengue/ Chikungunya/ Zika using AgPath-ID RT-PCR kit was used to detect DENV, CHIKV and ZIKV. The viruses were detected from the extracted RNA following the protocol by CDC (2017). Table 3 shows the master mix for the reaction.

Table 3: RT-PCR master mix component and mosquito RNA template

Reagents	**Volume/ tube (µl)**
Nuclease-free water	0.5
2x RT Buffer	12.5
DENV Mix	0.5
CHIKV Mix	0.5
ZIKV Mix	0.5
Enzyme Mix	0.5
Volume of Master Mix	**15**
Template RNA	5
Total Reaction Volume	**20**

The fast plates were then prepared as well as a plate map and 15ul of the master mix was added to the appropriate wells. 5ul of nuclease-free water was added to the well designated for non-template control. The plate was placed on a cool rack and moved to the hood designated for sample addition. 5 µl of test RNA was added to the wells followed by the positive controls according to the plate map. The plate wells were covered with optical cap and placed on ABI 7500 Fast real-time machine. The standard 7500 and the right detectors for each target were selected. Table 4 and 5 shows the targets and detectors and the reaction steps respectively.

Table 4: Detectors and their corresponding targets for RT-PCR reaction.

Detector Name	Reporter Dye	Quencher Dye
DENV	FAM	NONE
CHIKV	VIC	NONE
ZIKV	Texas Red	NONE
RP	FAM	NONE

Table 5: RT-PCR reaction steps for virus detection.

		Temp/ Time	Number of cycles
Stage 1	Reverse Transcription	50°C / 30 mins	1
Stage 2	Hot start	95°C / 2 mins	1
Stage 3	Denaturation	95°C / 15sec	
	Annealing and extension	60°C / 1 min	45

3.9 Data Processing and Analysis

3.9.1 Data processing

Data on morphological identification, molecular identification, virus infectivity and larval indices were recorded and collated in Microsoft Excel 2010 and exported as csv files to Stata 15 for cleaning and analysis.

3.9.2 Data Analysis

The proportions of mosquitoes by species, season and site were calculated using Fishers exact test.

Three indices were used to assess *Aedes* mosquito density in the various collection sites. These were House index (HI), Container index (CI) and Breteau index (BI). HI was expressed as the percentage of houses infested with *Aedes* larvae; CI as the percentage of containers infected with larvae or pupae; and BI as the number of positive containers per 100 inspected houses. The risk of transmission of *Aedes*-borne viruses at the study site was estimated using the WHO criteria. For the WHO criteria, an area where BI, HI, and CI exceed 50, 35 and 20 respectively, the risk of *Aedes*-borne viruses is considered to be high; BI between 5 and 50, the density of *Ae. aegypti* is considered to be sufficient to promote an outbreak of *Aedes*-borne viral disease; an area where BI, HI and CI are less than 5, 4 and 3 respectively; it is considered to be unlikely for *Aedes*-borne virus transmission to occur.

3.10 Geographic Information System Analysis

The data on the distribution and density of *Aedes* species collected in and around the Mole Game Reserve were analyzed with the GPS coordinates of the corresponding study site. A spatial map of houses positive of *Aedes* larvae and breeding sites were constructed using the version 10.6 of ArcView geographical information system (GIS) software package.

3.11 Quality Control

3.11.1 Sample Collection and *Aedes* Identification

Training was carried out prior to the commencement of the study. It involves morphological identification of *Aedes* species for a period of one week. Each *Aedes* mosquito that was morphologically identified during the study was confirmed by two senior scientists from the NMIMR entomological laboratory.

3.11.2 Data Entry and Analysis

After data entry was done, two research assistants were made to cross check the sample collection forms and laboratory note books to prevent omissions and double entry of data.

3.12 Ethical Clearance

This study is part of a larger project entitled ‘Surveillance of Arthropod-borne viruses in Ghana based on virome analysis of hematophagous arthropods’ with ethical clearance obtained from the Noguchi memorial Institute for Medical Research Ethical Review Board (NMIMR-IRB)-073/24/15.

CHAPTER FOUR

4.0 RESULTS

4.1 Mosquito collection

4.1.1. Mosquitoes collected during rainy season and dry season.

A total of 1957 *Aedes* mosquitoes were collected from the study sites. During the rainy season, a total of 1930 *Aedes* mosquitoes were collected from the study sites of which 879 were adult *Aedes* mosquitoes and (1051) were *Aedes* larvae (Table 6). Fishers exact test indicated that *Aedes* mosquitoes collected during the rainy season were significantly higher than *Aedes* mosquitoes collected during the dry season ($P < 0.001$). For the dry season, a total of 27 adult *Aedes* mosquitoes were collected from both sites with 96.29% collected from Larabanga and 3.70% from Mole quarters (Table 6).

4.1.2. Species composition of *Aedes* mosquitoes collected from both sites.

Majority of *Aedes* species identified during the dry and rainy season from both sites were *Aedes aegypti*. 99.16% *Aedes aegypti* and 0.84% *Aedes vittatus* were identified from mosquitoes collected from Mole quarters in the rainy season and all the *Aedes* mosquitoes collected from Larabanga were identified as *Aedes aegypti*. *Aedes aegypti* was the only species identified in both sites during the dry season (Table 7). A total of 415 *Aedes aegypti* mosquitoes from the rainy and dry season were identified to subspecies level. All the *Aedes aegypti* mosquitoes collected from Larabanga during the rainy and dry season were identified as *Aedes aegypti aegypti*. *Aedes aegypti formosus* (9) were identified in the Mole quarters

Table 6: *Aedes* mosquitoes collected from Mole Game Reserve area and Larabanga during the rainy and dry season

Community	*Aedes* mosquitoes collected					
	Rainy season			Dry season		
	Larvae N (%)	Adult N (%)	Total	Larvae N (%)	Adult N (%)	Total
Mole Game Reserve(Quarters)	914(86.96)	872(99.20)	1786	0	1(3.70)	1
Larabanga	137(13.04)	7(0.80)	144	0	26(96.29)	26
Total	1051	879	1930	0	27	27

Table 7: Number of *Aedes* species identified for Mole Game Reserve and Larabanga during the rainy and dry seasons.

	Aedes species collected					
	Rainy season			*Dry* season		
Study site	*Aedes aegypti* N (%)	*Aedes vittatus* N (%)	**Total N (%)**	*Aedes aegypti* N (%)	*Aedes vittatus* N (%)	**Total N (%)**
Mole game reserve (Quarters)	1771 (99.16)	15(0.84)	**1786**	1(100)	0	**1**
Larabanga community	144 (100)	0	**144**	26(100)	0	**26**
Total	**1915**	**15**	**1930**	**27**	**0**	**27**

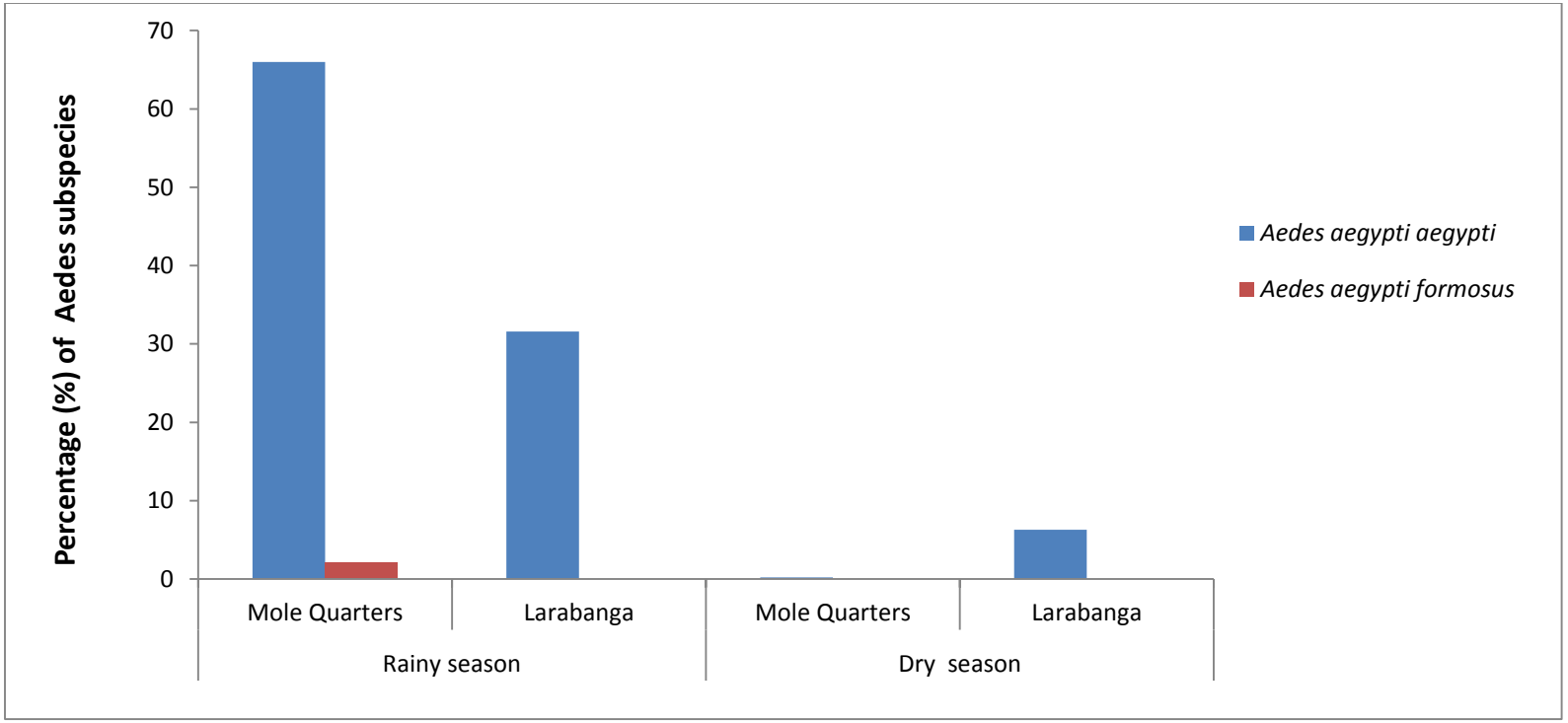

Figure 10: Proportion of Aedes subspecies identified for the dry season and rainy season in Mole Game Reserve and Larabanga

4.2 Larval indices estimation from household surveys

A total of 167 households (44 in Larabanga and 123 in Mole quarters) were inspected during the rainy season and 209 (86 in Larabanga and 123 in Mole quarters) during the dry season. Table 8 indicates the three larval indices estimated from the study areas as well as the World Health Organization (WHO) threshold for potential risk assessment of *Aedes aegypti*-transmitted VHF's. All three indices were high during the rainy season than the dry season. Observed larval indices were higher in Mole quarters compared to Larabanga. All larval indices (HI, CI and BI) for Mole during the rainy season were above the WHO threshold. HI and BI were above the WHO threshold for Larabanga during the rainy season (Table.8). During the survey, 242 containers were observed of which 18.2% were positive for *Aedes* larvae during the rainy season. 220 containers were inspected during the dry season of which 2.3% were positive for *Aedes* larvae. Overall, 45.5% of households were positive for *Aedes* larvae in Mole game reserve area and 36.4% for Larabanga during the rainy season. 25% of containers inspected in Larabanga during the dry season were positive for *Aedes* larvae. There were no positive containers observed in Mole game reserve area during the dry season. Containers and breeding sites that was inspected included earthenware pots (Fig 11), Plastic barrels, metal drums, poly-tank, reservoir, jerry cans, basin, car tires (Fig 12), stagnant water.

Table 8: *Aedes* mosquito larval indices and WHO threshold for transmission risk of *Aedes*-borne viral diseases in Mole Game Reserve and Larabanga

Larval indices for study areas	Rainy season	Dry season	WHO Threshold for transmission risk of VHF's
Mole Quarters			
House index	42.1*	0.0	4-35 or above
Container index	23.5*	0.0	3-20 or above
Breteau index	100.0*	0.0	5-50 or above
Larabanga			
House index	36.4*	2.3	4-35 or above
Container index	15.5	1.3	3-20 or above
Breteau index	56.8*	2.3	5-50 or above

(*) indicates areas with High VHF transmission risk indices

Figure 11: Earthenware pots breeding *Aedes* larvae and pupae in Larabanga.

Figure 12: Car tires outdoor where *Aedes* mosquitoes were breeding in Larabanga.

4.3 Viral detection

Overall 75 pools consisting of 1957 *Aedes* mosquitoes were tested. These included both males and females. There were 66 pools for *Aedes aegypti* and 2 pools for *Aedes vittatus* for the rainy season. *Aedes aegypti* (7 pools) collected in the dry season were also used for detection of *Aedes*-borne Arboviruses. All pools were negative for DENV, ZIKV and CHKV.

4.4 Spatial map of Households positive for *Aedes* larvae and breeding sites.

The GPS coordinates of positive households and breeding sites close to these houses were used to construct four maps. Two maps for Mole Quarters and Two maps for Larabanga, representing the rainy season and the dry season.

4.4.1 Map of Households positive for *Aedes* larvae and breeding sites in Mole Quarters during rainy and dry season

The general map of Mole Game reserve sites was drawn at a scale of 1km due to the spread of some of the households. However, sections of the map were drawn to clearly show the different households. The positive households were denoted with red dots while the breeding sites close to the households were shown as yellow dots (Fig.14 and Fig 16). In the rainy season houses positive for *Aedes* larvae were clustered just around the breeding sites. It was observed that most of the breeding sites (in yellow dot) were extremely close to households. There were no positive houses and breeding sites during the dry season (Fig16).

4.4.2 Map of Houses positive for *Aedes* larvae and breeding sites in Larabanga during the rainy and dry season

The general map of Larabanga sites was drawn at a scale of 0.8km due to the spacing out of some of the households. Sections of the map were drawn to clearly show the study sites. The

positive households were pictorially denoted with red dots while the breeding sites close to the households were shown as yellow (Fig.13 and Fig.15). It was observed that breeding sites were close to a household during the rainy season. In the dry season there were no breeding sites observed however, there were a few households positive for *Aedes* larvae.

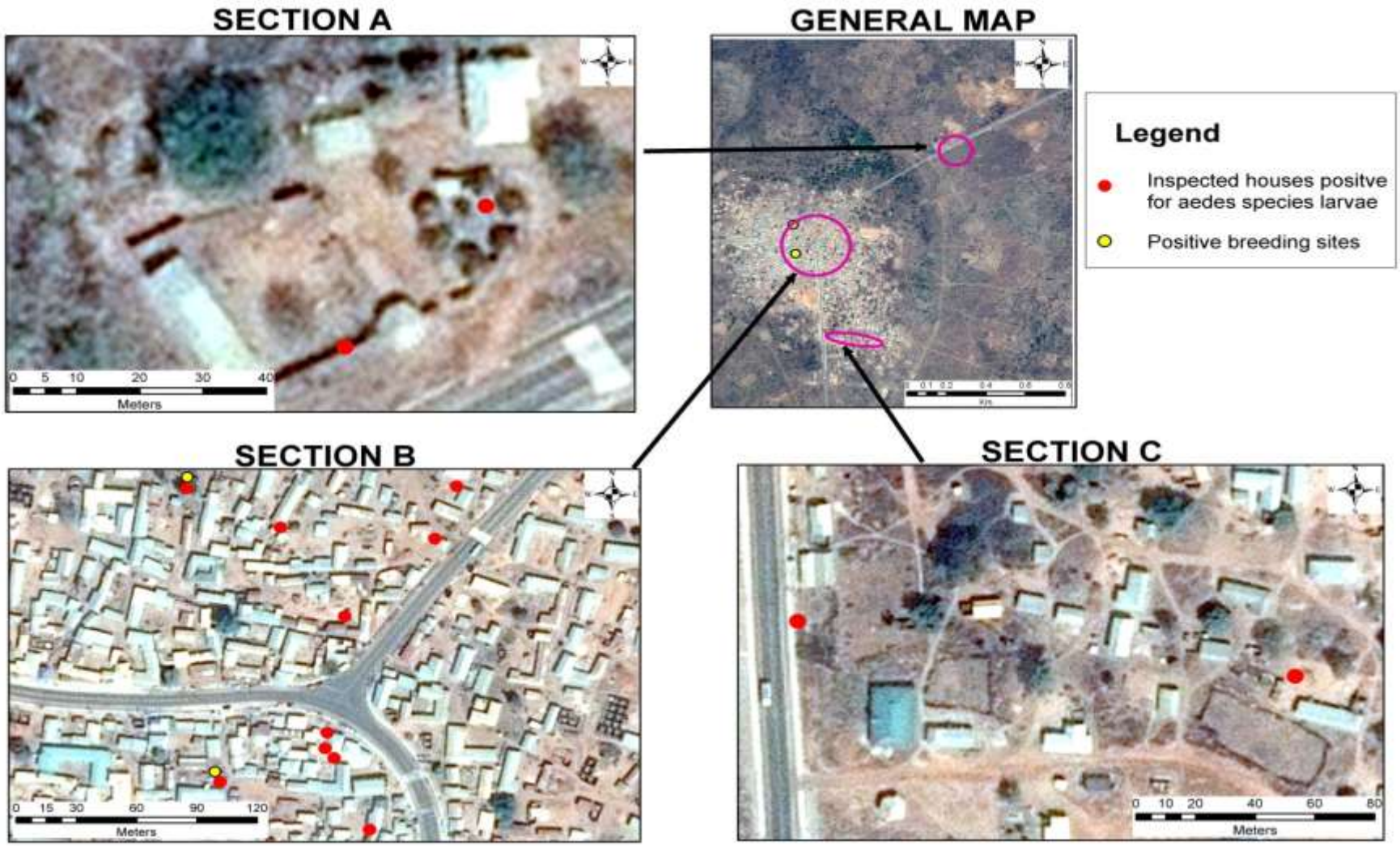

Figure 13: Spatial map of houses positive for *Aedes* larvae in relation to breeding sites of *Aedes* mosquitoes and human habitation in Larabanga during the rainy season.

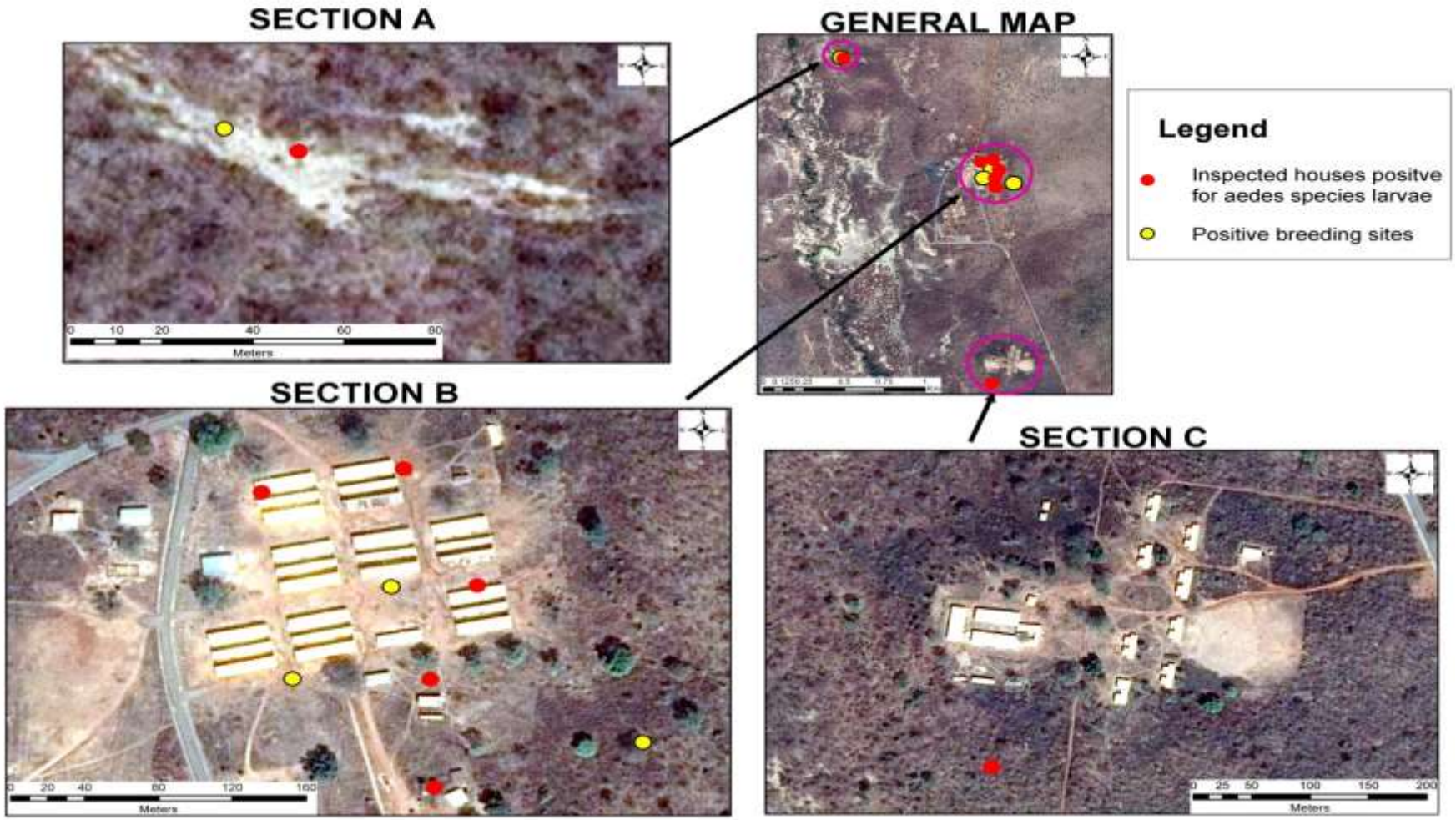

Figure 14: Spatial map of houses positive for *Aedes* larvae in relation to breeding sites of *Aedes* mosquitoes and human habitation in Mole Game Reserve during the rainy season.

Figure 15: Spatial map of houses positive for *Aedes* larvae and breeding sites of *Aedes* mosquitoes and human habitation in Larabanga during the dry season.

Figure 16: Spatial map of houses positive for *Aedes* larvae and breeding sites of *Aedes* mosquitoes and human habitation in Mole Game Reserve during the dry season.

CHAPTER FIVE

5.0 DISCUSSION

Aedes mosquitoes are responsible for the transmission of viral diseases such as Dengue, Chikungunya, Yellow fever and Zika across the World. This genera of mosquitoes have already been established in Ghana. *Aedes* species, such as *Ae. vittatus, Ae. aegypti, Ae. simpsoni, Ae. africanus, Ae. luteocephalus, Ae. metallicus* and *Ae. furcifer-taylori* have been identified in various regions of the country (Appawu *et al*., 2006; Chukwemeka, 2017; Suzuki *et al*., 2016). With *Aedes aegypti* being the most common species identified, it has been implicated for the transmission of Yellow fever in the country. The presence and abundance of *Aedes* mosquitoes in an area can favor the transmission of viruses. Their survival and multiplication is dependent on varying factors. *Aedes* mosquitoes are known to breed in both natural and artificial containers in close proximity to human settlements. They require the right amount of humidity and temperature to develop. However, their eggs have the ability to withstand harsh weather conditions by staying dormant and reviving when conditions are favorable (Faull *et al*., 2015).

In Ghana, Dengue and chikungunya antibodies were detected in suspected dengue and chikungunya febrile patients that suggest the presence of DENV and CHKV in the country. However, none of the patients were positive for the viruses (Manu *et al.*, 2019). A study carried out in the Ashanti region and Brong Ahafo region revealed the presence of dengue virus IgG (Narkwa *et al*., 2016). These studies show the need to understand the dynamics of *Aedes* populations to formulate efficient control strategies and potentially prevent future outbreaks.

5.1 Species composition and abundance of *Aedes* mosquitoes across study site

In this study, mosquitoes were collected from the Mole quarters and from Larabanga during the rainy and dry seasons. Larval sampling using the dipping method yielded high numbers of *Aedes* mosquitoes during the rainy season. The BG-Sentinel traps were less effective in this area during the rainy season because the traps were disrupted by rain. The traps collected a few mosquitoes during the dry season as well. Studies have shown that the trap colour and location significantly affects the number of mosquitoes collected. In this study white BG-traps were used which are not as effective as black BG- traps. They have been found to collect a significant number of mosquitoes compared to white traps as *Aedes* mosquitoes are attracted more to highly attractive visual features such as black and white stripes, checkerboard patterns, broad black surfaces (Iyaloo, Facknath, & Bheecarry, 2017). There were high numbers of *Aedes* mosquitoes collected during the rainy season. The abundance of *Aedes* populations is controlled by rainfall because it supports the development of additional breeding sites, hatching of eggs, growth of vegetation cover and cool shaded environment for the development of immature stages and high relative humidity(Rozilawati, Zairi, & Adanan, 2007). *Aedes* mosquitoes collected during rainy and dry season in the study sites were predominantly *Aedes aegypti*. This may be due to the presence of discarded water holding containers and water storage containers around the human settlement which is the preferred site for *Aedes* aegypti female mosquitoes to lay their eggs (Ndenga *et al*., 2017).This is however contrary to findings of a study conducted by Dom, Madzlan, Hasnan, & Misran (2016) where *Aedes albopictus* was the most predominant species. *Aedes aegypti* are also known to live in close proximity to humans and they prefer human blood even in the presence of other animals (Powell & Tabachnick, 2013). This is evident in the spatial maps for the sites (Fig 11 and 12), which shows the proximity of major breeding sites (represented by

yellow dots) to houses positive for *Aedes* larvae (represented by red dots). This characteristic increases the potential of transmission of Arboviruses among humans. There was significantly high collection of *Aedes Aegypti* mosquitoes during the rainy season than in the dry season for both sites. During the rainy season, conditions such as humidity and temperature are favorable for the proliferation of *Aedes aegypti* mosquitoes (Althouse *et al*., 2015; Konan *et al*., 2013). Furthermore, the high numbers of *Aedes aegypti mosquitoes* recorded during the rainy season may be due to the hatching of eggs laid by these mosquitoes during the dry season. These species of mosquitoes have their eggs being able to withstand desiccation and only hatch when conditions are favorable (Reinhold, Lazzari, & Lahondère, 2018). The presence of this species throughout the rainy and dry seasons can be attributed to favorable climatic conditions, availability of artificial breeding sites and traditional practices and behavior of residents to store water. The presence of the major vector for the transmission of DENV, CHIKV and ZIKV in the study areas indicates the possibility of future outbreaks.

The second *Aedes* species observed was *Aedes vittatus*. This species was only identified in the Mole game reserve during the rainy season. This species are usually predominant in forest and savanna areas which is a characteristic of the Game Reserve area (Sudeep & Shil, 2017). The *Aedes vittatus* has been incriminated as a vector for yellow fever in various parts of Africa (Diagne *et al*., 2015; Sudeep & Shil, 2017; Weetman *et al.*, 2018). DENV, ZIKV and CHKV have been isolated from this species indicating that it has the potential to replicate and transmit these viruses experimentally (Diagne *et al*., 2015).

The *Aedes aegypti* mosquitoes identified in this study were further identified to the subspecies level. *Aedes aegypti aegypti* (*Aaa*) was the dominant subspecies identified in both study sites during the rainy and dry seasons. It is known as the "domesticated" form of *Aedes aegypti* and it

is closely associated with and dependent on human habitats. This may be the reason for their presence in both sites. However, the other subspecies, *Aedes aegypti formosus* (*Aaf*) was identified only in the Mole game reserve. *Aaf* are the ancestral African type of *Aedes aegypti* that prefer nonhuman mammals as a blood source (Powell, Gloria-soria, & Kotsakiozi, 2018). The game reserve is hence a suitable place for their survival due to the presence of a wide variety of nonhuman mammals. These species were collected from breeding sites which were close to human settlements in the game reserve area (Fig 12). Their presence in these areas may be due to the presence of monkeys and warthogs around human settlements.

5.2 Risk of transmission of DENV, ZIKV and CHKV in the study sites

In assessing the risk of transmission of DEN, ZIKV and CHIKV, water holding containers around the study sites were surveyed for the presence or absence of immature stages of *Aedes* mosquitoes. The presence of water holding containers around the study sites allows the breeding of *Aedes* larvae thereby increasing the population of *Aedes* mosquito and the associated risk for arbovirus transmission (Kampango *et al*., 2018). During the rainy season the larval indices HI, BI and CI for Mole game reserve were above the threshold values for the WHO criteria, but higher than those of a similar study in Damango, Savannah Ghana (Appawu *et al*., 2006) where the HI, BI and CI were 87.7, 180.9 and 44.8 respectively based on the WHO criteria for risk of transmission. These high observed values for Mole Game Reserve indicates a high risk of transmission of Dengue, Chikungunya and Zika. For Larabanga, there were high values recorded for HI and BI during the rainy season. However, CI was within the threshold values. In the dry season, the larval indices for both sites were extremely low compared to the rainy season. These observations are in agreement with other studies (Chukwemeka, 2017; Fofana, Michel, Beugré, Yao-acapovi, & Lendzele, 2019). However, contrasting results were observed by Appawu *et al*

(2006) where it was observed that larval indices were higher in the dry season compared to the rainy season in the Savannah region of Ghana. In the dry season, the pipes in Mole and Larabanga are opened two days in a week and most water storage containers are cleaned regularly and thus discarding *Aedes* eggs in the containers. This may have reduced the numbers of *Aedes* mosquitoes in the area hence low larval indices. The individuals in Larabanga also relied on water from the dam which was accessed daily due to the higher demand for water during the dry season. Therefore, water was not stored for longer periods during this season. In the Mole quarters area, larval indices were low during the dry season. It was observed that *Aedes* larvae were mostly found in outdoor containers than in indoor containers .This has also been observed in some studies (Chareonviriyaphap, Akratanakul, Nettanomsak, & Sommawan, 2001). Individuals preferred storing water indoors rather than outdoors because the monkeys in the game reserve area disturbed their water collections and items when stored outdoor. Other studies showed *Aedes* mosquitoes breeding indoor rather than outdoor (Kuning, Luemoh, & McNeil, 2003; Rozilawati *et al*., 2007) and this may be because water containers stored outdoor are well covered, preventing *Aedes* mosquitoes from laying their eggs inside.

In this study, discarded car tires had high positivity rate for *Aedes* larvae compared to the other containers inspected during the rainy season followed by earthen ware pots. However, it was observed that the earthen ware pots were the most positive for *Aedes* larvae during the dry season. Discarded tires usually collect water and tend to harbor *Aedes* larvae without interruption. This makes it an ideal place for *Aedes aegypti* mosquitoes to breed undisturbed. Earthen ware pots are usually used to store drinking water. Due to the cool temperature, humidity and reduced light, it serves as a suitable environment for *Aedes* mosquito breeding (Ferede *et al*., 2018).

5.3 Detection of DENV, ZIKV and CHKV in *Aedes* mosquito samples.

All pools of *Aedes* mosquitoes from Mole game reserve and Larabanga analyzed for DENV, ZIKV and CHKV were negative. This is in accordance with the report from Appawu *et al* (2006) where all *Aedes* mosquitoes collected from various sites in the Savannah region of Ghana were negative for flaviviruses. Research shows that African *Aedes aegypti* are less competent vectors for flaviviruses such as DENV, ZIKV than *Aedes aegypti* in the other continents (Black IV *et al*., 2002; Bosio, Beaty, & Black IV, 1998). Their susceptibility to these viruses can be influenced by various biotic and abiotic factors. For instance, African *Aedes aegypti* are able to breed in both domestic environment and in the wild. However, larvae breeding in a domestic environment maybe exposed to bacterial communities which are different from that in the wild, potentially resulting in differences in vectorial capacity between *Aedes aegypti* breeding in two ecotypes (Lambrechts *et al*., 2017). Previous studies also indicated that the susceptibility of African *Aedes* aegypti and its ability to transmit these viruses (vector competence) depends on specific pairings of mosquito population and viral isolate (Diallo *et al*., 2008; Vazeille *et al*., 2013). This specificity has been widely confirmed in other host pathogen systems (Lambrechts, 2010).

Aedes aegypti formosus was one of the subspecies identified in the study area. Studies on the vector competence of these subspecies show that they are more refractory for DENV especially DENV serotype 2 (Diallo *et al*., 2008). This may be the reason why these viruses were not detected and possibly why there are no outbreaks of these diseases in Ghana.

5.4 Spatial distribution of *Aedes* breeding sites relative to houses positive for *Aedes* larvae in Larabanga and Mole game reserve (Quarters).

During the raining season, breeding sites identified were close to households in the study areas. *Aedes aegypti* mosquitoes prefer breeding close to human settlements. The rainfall developed more breeding sites around the human settlements making it an ideal place for development. As these mosquitoes emerge, female *Aedes* mosquitoes move into households where they can access a blood meal to develop their eggs. Engorged females then look for a warm moist place to rest and lay their eggs. The presence of water holding containers around these households serve as a suitable place for them to lay their eggs. In the dry season, most of these breeding sites were dried up. However, there were some households positive for *Aedes* larvae. These household had water holding containers available and most of these containers had water stored in them for a long time.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this study, *Aedes Aegypti* and *Aedes vittatus* were the two species identified around the Mole game reserve. The predominant species of *Aedes* mosquito identified in the study sites were *Aedes aegypti* which were documented in the rainy season and dry season. Among the *Aedes aegypti* species identified, majority were *Aedes aegypti aegypti*. The risk of transmission for DENV, ZIKV and CHKV in the study areas as shown by the larval indices were high during the rainy season than the dry season. This study found that discarded tires and earthen ware pots were the preferred breeding habitats for these species. The study also showed that *Aedes* mosquitoes were breeding in close proximity to human habitation. All *Aedes* mosquito pools were however negative for DENV, ZIKV and CHKV.

6.2 Recommendations

Based on the findings in this study, it is recommended that there should be national surveillance programs implemented to identify all *Aedes* species present and virus infection so as to prevent an outbreak in the near future. This will also aid in formulating effective control measures.

Water storage containers should be well covered to prevent access by mosquitoes and a proper implementation of tire disposal through public health education.

There is the need to monitor the movement of animals especially monkeys and baboons from within the game reserves to human settlements, since the existence of *Aedes vittatus* can promote sylvatic transmission. Serological studies can also be done to assess the infectivity of non-human primates found around human settlements and in the Game Reserve.

There should be further studies to assess the competence in *Aedes* mosquitoes in Ghana and its neighboring countries to access the risk of infection and their role in virus transmission and outbreak.

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APPENDICES

Appendix I: Larval survey for *Aedes* mosquitoes in Mole game reserve (Quarters) and Larabanga during the rainy season and dry season.

	Mole game reserve(quarters)		Larabanga	
	Rainy season	**Dry season**	**Rainy season**	**Dry season**
Households inspected	123	123	44	86
Positive households, N (%)	56(45.5%)	0	16(36.4%)	20(25%)
Containers inspected	81	64	161	156
Positive containers, N (%)	19(23.5%)	0(0%)	25(15.5%)	5(3.2%)

Appendix II: Collection of Adult *Aedes* Mosquitoes in Mole Game Reserve (Quarters)

Plate1: Setup of BG-Trap for *Aedes* adult collection in the Mole Game Reserve (quarters)

Appendix III: *Aedes* Mosquito Sampling Form

ID No	Community	Breeding place or site	Coordinates			Type of Trap	Stage	Species
			Long.	Lat.	Elevation			
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								