EFFECTS OF LARVAL DIET ON THE FITNESS AND INSECTICIDE RESISTANCE PROFILE OF \textit{Anopheles gambiae} s.l. (Diptera: Culicidae) FROM OPEIBEA AREA IN THE GREATER ACCRA REGION, GHANA.

BY

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COLLABORATING DEPARTMENTS: ANIMAL BIOLOGY AND CONSERVATION SCIENCE AND CROP SCIENCE
DECLARATION

I hereby certify that this thesis is the result of a research undertaken by me, Chukwu, Maureen Amuche, towards the award of Master of Philosophy in Entomology in African Regional Postgraduate Programme in Insect Science (ARPPIS), University of Ghana, Legon. This thesis has not been submitted, either in part or in full, for any other degree and all references to other people’s work have been duly acknowledged.

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This work is dedicated to God, the Commander-in-Chief of my life for His grace and to my wonderful mother, siblings and my best friend for their prayers, care, love and support throughout the period of this study.
ACKNOWLEDGEMENTS

My sincere gratitude goes to All-knowing and Compassionate God whose steadfast love is new every morning in my life. His mercies, faithfulness and grace made this research work a success.

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<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>df</td>
<td>Degree of freedom</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>ACTs</td>
<td>Artemisinin-based combination therapies</td>
</tr>
<tr>
<td>ARCGIS</td>
<td>Aeronautical Reconnaissance Coverage Geographical Information System</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl Trimethyl Ammonium Bromide</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GHS</td>
<td>Ghana Health Service</td>
</tr>
<tr>
<td>GMT</td>
<td>Greenwich Mean Time</td>
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<tr>
<td>GPS</td>
<td>Global Positioning System</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>s.s.</td>
<td>sensu stricto</td>
</tr>
<tr>
<td>sddH₂O</td>
<td>Sterile double distilled water</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate EDTA</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-1-hydroxymethyl-1, 3propanediol</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WHOPES</td>
<td>World Health Organisation Pesticides Evaluation Scheme.</td>
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ABSTRACT

Malaria is a life-threatening disease and is hyper-endemic in Ghana. The *Anopheles gambiae* complex is established to contain the main vectors that transmit malaria parasite, *Plasmodium falciparum* in Sub-Saharan Africa and it has been implicated as the main vector of malaria in Ghana. Vector control remains the most effective measure to prevent malaria transmission. This can largely be achieved by the use of insecticides. However, the effectiveness of insecticide-based vector control is threatened by the emergence of resistance in *Anopheles* mosquitoes to the insecticides used. Nutrition is an extrinsic and biotic factor that affects the growth, life cycle and other life history characteristics of the larva, which has a corresponding modulating effect on the fitness of the adult. Fitness is also known to affect the ability of insects to respond to stress. Hence, the aim of this study is to assess the effect of larval nutritional status on fitness and insecticide resistance status of *Anopheles gambiae s.l.* Mosquito larvae and pupae were collected from Opeibea area in the Greater Accra Region of Ghana using the dipping method and reared to the adult stage. Mosquitoes were further molecularly identified to sibling species of the *Anopheles gambiae* complex by polymerase chain reaction (PCR) assay following standard protocols. A total of 208 adult females were identified. *An. gambiae s.s.* was predominant (205) followed by *Anopheles coluzzii*, formerly M form (2) and hybrids of the two species (1) Eggs obtained from the established colony of wild population of the Opeibea strain and reference population of the Kisumu strain from the Laboratory were reared at two diet regimes, rabbit pellet and baking yeast. Insecticide susceptibility was carried out on the adult females using four different insecticides selected from different insecticide classes. The susceptibility test results showed that *An. gambiae* populations in Opeibea were resistant to all the four insecticides (0.05 % Deltamethrin, 4 % DDT, 5 % Malathion and 0.1 % Bendiocarb) tested. The mean adult body size (wing length) of the mosquitoes fed with baking yeast (2.89 ± 0.0072 mm) and (2.89 ± 0.0068 mm) for
Kisumu and Opeibea strains respectively was significantly higher (p < 0.001) as compared with that of rabbit pellet (2.84 ± 0.0072 mm) and (2.81 ± 0.0076 mm) for Kisumu and Opeibea strains respectively. However, rabbit pellet had the highest positive impact on larval survivorship and higher pupal weight in a preliminary study, which was evident in the adult response to susceptibility assay. For both Kisumu and Opeibea strains, baking yeast had a non-significant (p > 0.05) lower KDT\textsubscript{50} and KDT\textsubscript{95} when compared to rabbit pellet for all the insecticides except for Malathion where the reverse was the case. However, for Kisumu, DDT and Deltamethrin there was significant (p < 0.001) difference in the KDT\textsubscript{50} and KDT\textsubscript{95} for both larval diets. The baking yeast for both Kisumu and Opeibea strains consistently required higher time than its rabbit pellet-fed counterpart in order to produce the same level of knockdown. Fitness and resistance in both Kisumu and Opeibea strains were positively correlated (correlation = 1 and p > 0.05). Rabbit pellets is highly recommended for use in the rearing of Anopheles gambiae s.s. larvae in the insectary. The results from this study indicate the importance of larval diet in determining the insecticide resistance status of mosquito vectors. This is very useful in the formulation of future vector control strategies as well as getting a standard larval diet that gives the best fitness for routine monitoring of insecticides resistance.
CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 INTRODUCTION

Diseases transmitted by mosquitoes for instance filariasis, malaria and a surfeit of viruses intensely impact public health and characterize a key problem in relations to worldwide economy and development (WHO, 2011). The global burden of LF consists of at-risk population of 947 million people in 54 countries.

Malaria is of great importance when diseases transmitted by vectors are considered in the world today with an estimated 3.2 billion people at risk, 88 % of which are from the African region (WHO, 2015). In 2015, an estimated 429 000 malaria deaths were recorded accounting for more death than any human disease transmitted by vectors (WHO, 2015). Over 20 % gross national product of the African continent for the past 15 years has been reduced by malaria alone (IRAC, 2011). According to Carter et al. (2005), economists consider malaria to be liable for progression drawback of up to 1.3 % yearly in a number of African countries.

*Plasmodium* parasites are the causative agent of malaria, a life-threatening disease. Six parasite species namely *Plasmodium falciparum* Welch, *Plasmodium vivax* Grassi and Feletti, *Plasmodium malariae* Laveran, *Plasmodium ovale* Stephen, *Plasmodium knowlesi* (zoonotic) and *Plasmodium cynomolgi* (zoonotic) are established to cause malaria in humans (Ta et al., 2014; Mueller et al., 2007; Singh et al., 2004). In Ghana, although *P. malariae* and *P. ovale* have been reported; *P. falciparum* remains the principal species accounting for up to 80 to 90 % of the malaria cases (Asenso-Okyere and Dzator, 1997).

The *Anopheles gambiae s.l.* is proven to comprise the core vectors that spread malaria parasite, *Plasmodium falciparum* in Sub-Saharan Africa. In Ghana, *Anopheles gambiae* has been implicated as the main vector of both lymphatic filariasis and malaria, with malaria
responsible for about 38% of all outpatient cases and hospital deaths in children under five years and 36% of all admission in Ghana (PMI, 2014). As a result of its hyper-endemicity in the country, everyone is at jeopardy of being infected with pregnant women, children below five years of age and non-immune visitors being at the greatest risk.

The parasite, with the exemption of some cases of transmissions associated with transplacental and blood transfusion (Murphy and Breman, 2001), is exclusively transmitted to the human host through bites of an infected female *Anopheles* mosquito which occurs mainly between dusk and dawn. Approximately 490 *Anopheles* mosquito species including sibling species are known. Out of 60–70 species that can transmit malaria worldwide, about 30 of them are vectors of main significance (Sinka et al., 2012). Members of the *Anopheles gambiae s.l.* and the *Anopheles funestus* group are the most effectual vectors in Sub-Saharan Africa, including Ghana (Afrane et al., 2004; Appawu, 2005). Among these complexes, the *An. gambiae* is more widespread in tropical Africa (Coetzee et al., 2000) and throughout Ghana with higher vectorial capacity. Among these sibling species, only *An. arabiensis*, *An. melas* and *An. gambiae s.s* have been recorded in Ghana with *An. gambiae s.s* as the most predominant (Appawu et al., 1994; Okoye et al., 2005; de Souza et al., 2010).

Timely diagnosis and quick treatment; avoidance, early identification and containment of epidemics; control of vector; and reinforcing local competences in basic and applied research are the four basic technical elements of the WHO Global Malaria Control Strategy. Vector control continues to be the best efficient strategy to preclude transmission of malaria. This can be achieved principally by the usage of indoor residual spraying (IRS), biological control, long lasting insecticide nets, environmental manipulation and larviciding (WHO, 2005). These vector control interventions are efficient in precluding morbidity and mortality due to malaria in a series of epidemiological sceneries, decreasing masses and infection of malaria vectors, reducing global broadcast as well as protecting all individuals within a community.
Insecticides play a major role in reducing malaria transmission. Insecticides of different classes have been continuously expended ever since 1920s, however most present-day control programmes are principally reliant on synthetic pyrethroids, which are the single insecticides recommended or suggested by WHO for ITNs (WHO, 2006). However, the efficacy of insecticide usage in control of the vector is jeopardized by emerging *Anopheles* mosquitoes insecticide resistance in both ITNs and IRS. Right from 2010, 60 out of 78 countries gathering data on resistance monitoring, had recorded resistance to a minimum of one insecticide, and 49 had recorded resistance to two or above insecticide classes in one vector population (WHO, 2015). Currently, pyrethroid efficiency is endangered by the upsurge of resistance in target populations.

Resistance to insecticide is a growing setback encountered by communities which require insecticides to effectively keep in check veterinary, agricultural and medical arthropod pests. Resistance in several insects is spread to all main insecticides classes. From 1947 when the initial incidence of resistance to DDT was reported, the frequency of insecticide resistance has intensified per annum at a threatening speed. It has been evaluated that there are over 580 arthropods species that are resistant in the world today to more than one insecticides (Sparks and Nauen, 2015).

Existing malaria vector control generally depend on two key components: indoor residual spraying (IRS) and Long-lasting insecticide treated bed nets (LLINs) which focus only aerial adult stage leaving out the aquatic immature stages.

Nutrition is an extrinsic and biotic factor that affects the growth, life cycle and other life history characteristics of the larva, which has a resultant effect on the fitness of the adult. In mosquitoes, carbohydrates are stored as glycogen (Wigglesworth, 1941) that are utilized as energy sources (Bond *et al.*, 2005), while proteins support larval growth by catalyzing enzymatic reactions and oogenesis (Uchida *et al.*, 2003).
It has been reported that dietary resources in larval habitations define adult mosquito body size, yet studies have not really been carried out to determine if mosquito larval diets on have an influence on the insecticide resistance profile of the emergent adults.

Insecticide resistance profile of *Anopheles gambiae* and resultant resistance mechanism might be compromised by the larval nutritional status. An enhanced comprehension of the influence of larval diets on insecticide susceptibility status could go a long way in getting a standard diet to be used in mosquito rearing during insecticide resistance monitoring and testing which is a key factor in managing insecticide resistance. The wing length of mosquitoes has been used as an important attribute in determining its body size, this is due to the fact that it is simple to determine and is directly linked with body mass in many species (Nasci, 1990).

Widespread usage of insecticides in agriculture and the scale-up of insecticide-based malaria vector control throughout the preceding era seem to have enacted a fundamental part in the advent and swift increase of insecticide resistance on the continent. Resistance, particularly to pyrethroids insecticides and DDT, in *An. gambiae s.l.*, ensues through Africa. In recent times, resistance to Carbamate (CA) insecticides (bendiocarb and propoxur) and Organophosphates (Ops) (fenitrothion and malathion) has also been recorded. In 2014, 75% of the countries examined for insecticide resistance identified resistance in all main vectors of malaria (WHO, 2015). Evidence has shown that resistance to pyrethroid insecticides has become a threat to successful vector control of malaria (Strode *et al.*, 2014). This menace has become more conventional than presumed following a latest methodical reevaluation on the effect of this resistance on the efficiency of insecticide treated bed-nets, thereby drawing attention to the non-specificity of most studies which covers the associations between vector control flop and insecticide resistance.

The Global Plan for Insecticide Resistance Management in Malaria Vectors (GPIRM) (WHO, 2012) offers standard guideline for management of resistance to insecticide particularly
anywhere it arises. Tactics for averting and handling resistance to insecticide is very crucial in order to certify and safeguard the few insecticides available for use in the control of vectors. Insecticide resistance monitoring through routine surveillance and its mechanisms ought to be characterized where possible as an element of this tactic (WHO, 2012). Data on resistance mechanisms in Anopheles mosquitoes can be gathered using quite a lot of methods available varying in complexity and price tag.

1.2 Rationale

The development of insecticides resistance by Anopheles put at risk the aim of reducing transmission of malaria, in an arms race stuck between vector populations and malaria control programmes. Pyrethroid efficiency is now endangered by the upsurge of resistance in target populations. Effective insecticide resistance management is an imperative component in sustaining the worth of valuable insecticides and depends on early detection of insect populations that are resistant in order to balance insecticides selections.

In Ghana, insecticide resistance by An. gambiae s.l. has been extensively studied (Adasi, et al., 2000; Adeniran et al., 2009; Achonduh et al., 2007; Yawson et al., 2004). Even though the mechanisms by which insecticides become less active are related across all vector taxa, individual resistance glitch is potentially distinctive and may encompass a multifaceted form of resistance foci (Brogdon and McAllister, 1998). It is quite vital to prolong monitoring varying trends of resistance and explicate the various resistance mechanisms employed by these mosquitoes to confer resistance to insecticides which is vital in planning and embarking on resistance management strategies and developing better insecticides for vector control.

Since the current malaria control strategy is gearing towards integrated vector management that targets all developmental stages, the understanding of the effect of different nutritional diets used for larval development on adult resistance status will expand our knowledge of
mosquito larval biology and ecology which will lead to better implementation of malaria vector control strategy. The effect of most of these artificial diets, on some life history characteristics of the larvae are rarely known, since relative performance of a particular diet also varies from one species to the another (Huestis et al., 2011).

Study done on the effect of five different diets on some life history characters of immature stages of *Anopheles gambiae s.l.* by Assan-Nyarkoh et al. (Unpublished) observed that out of the five different diets used to rear the mosquito larvae which included rabbit pellet, fish flake, cat meal, baking yeast and cerelac maize; those fed with rabbit pellet resulted in a fitter adult on emergence compared to the other diets used whilst those fed with baking yeast resulted in a less fit adult on emergence. This project is a follow-up study to that, taking rabbit pellet as the high diet and baking yeast as the low diet.

Development of an effective Insecticide Resistance Management (IRM) strategy will require accurate insecticide resistance data of insect vectors. The purpose of IRM is to avert or defer the development of resistance to insecticides, or to assist reclaim susceptibility in insect pest populations in which resistance has previously occurred. This study will assess the impact of larval nutritional status on the insecticides resistance profile of *Anopheles gambiae s.l.*

### 1.3 Objectives

#### 1.3.1 Main Objective

This study is aimed at determining the effects of two different larval diets on the fitness and insecticides resistance profile of adult *Anopheles gambiae s.l.* from Opeibea area in Accra, Ghana.
1.3.2 Specific Objectives

1. To determine the species composition of malaria vectors in the study area.

2. To determine the effect of two larval diets on the fitness of *An. gambiae s.l.* and standard Kisumu species.

3. To determine the resistance profile of *An. gambiae* in relation to adult fitness based on their larval diets as compared to the standard Kisumu species.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 VECTOR-BORNE DISEASES

Diseases transmitted by vectors are liable for over 17% of appraised worldwide load of all parasitic and infectious diseases, instigating over 1 million deaths per annum (WHO, 2014). Vector-borne diseases are ailments initiated by parasites and pathogens in human populaces that are transmitted by vectors. The vectors enact an important phase in the conduction of the illness from animal hosts to humans or from one human to another. There is a plethora of parasitic, viral, bacterial and rickettsia diseases transmissible by arthropod vectors. Annually there are over 700,000 deaths from diseases transmitted by vectors such as malaria, onchocerciasis, Chagas disease, human African trypanosomiasis, dengue, schistosomiasis, Japanese encephalitis, leishmaniasis and yellow fever globally (WHO, 2017). Diseases transmitted by vectors are one of the ultimate contributors to human and livestock mortality and ill health in the world today particularly in tropical settings and beyond. These diseases are associated with hardship and human gehenna, as several people who endure infection are left always debilitated, disfigured, maimed, or blind. One sixth of the ailment and debility globally is attributable to vector-borne diseases, with over half the world’s population presently projected to be at risk of these diseases (WHO, 2014).

More than a billion people, primarily in developing countries, are now at the risk of contracting such diseases like Japanese encephalitis, relapsing fevers, malaria, leishmaniasis, yellow fever, onchocerciasis, trypanosomiasis, plague, dengue, lymphatic filariasis and various rickettsial diseases. They result in avertable, curable ill-health and death, and economic adversity for afflicted communities, and are a severe impairment to economic growth (WHO, 2008). The underprivileged sectors of the world and least-developed countries
are most affected. These diseases influence urban, peri-urban and rural communities but increase commonly among communities with poor living settings mostly deficiency of contact to suitable housing, safe drinking water and sanitation. Globalization of travel and trade, unintended urbanization and environmental challenges like weather variation are taking a substantial influence on transmission of disease of late.

Vector-borne diseases greatly constrain socioeconomic status and development in countries with the highest proportions of infection, several of them are situated in the tropics and subtropics. These infections are accountable for enormous economic losses both in terms of loss of yield and health-care costs, particularly in countries that cannot really meet the expenses. An econometric prototypical for malaria recommends that countries with serious malaria have earnings levels of only one third of those that do not have malaria.

Diseases transmitted by vectors exact key public health load on developing countries such as West Africa because many of them are endemic to the region. Dispersal of vector-borne diseases are established through multifaceted dynamic of ecological and societal factors. Mosquitoes are the best-known disease vector. Others include certain species of ticks, lice, dipteran flies (sand flies, black flies, biting midges), fleas, bugs, freshwater snails and copepods.

2.2 Lymphatic filariasis: A Global Burden

There are about 856 million people in 52 countries at risk of Lymphatic filariasis according to WHO (2017). Lymphatic filariasis is a vector-borne parasitic transmittable disease initiated by three types of parasitic nematodes; *Brugia malayi*, *B. timori* and *Wuchereria bancrofti*. *Wuchereria bancrofti* is predominant with 90 % cases, which is endemic in the tropics, including in sub-Saharan Africa. It is one of the 17 Neglected Tropical Diseases (NTDs) that commonly affect people of the lower economic class living in more or less inaccessible
communities. Due to its alarmingly high prevalence in developing countries, lymphatic filariasis remains one of the utmost important transmittable diseases worldwide and constitutes a serious public health issue in tropical regions.

Lymphatic filariasis comprises most of the world’s filarial infection. It is passed on to humans through the bites of infective female mosquito vectors belonging to the genera *Anopheles, Culex, Mansonia* and *Aedes* depending on the geographic region (Bockarie *et al.*, 2009). In West Africa, the mosquito *An. funestus* and *An. gambiae s.l.* are vectors of lymphatic filariasis and malaria. Humans are the only reservoir host of the lymphatic filariasis parasite in Africa. Over 120 million individuals are presently infected with lymphatic filariasis, and approximately 40 million of them are disfigured and incapacitated by the disease. Lymphatic filariasis affects over 25 million men with genital disease and over 15 million people with lymphoedema (WHO, 2014). About 65 % of the global at-risk population is found in the WHO South-East Asia Region, 30 % in the African Region, of which most of these people are poor and living in remote areas (WHO, 2015) and the rest in other tropical areas. About 512 million people in sub-Saharan Africa are at threat of being infected, with nearly 40 million men at risk of developing hydrocoele (WHO, 2002), whereas about 28 million are already infected. In addition, one billion people giving 20 % of the world’s population were estimated to be at threat for infection. Lymphatic filariasis is prevalent in 32 of the world’s 38 least developed countries. The disease features among the world’s foremost causes of permanent and long-term disability; it is in fact the second leading cause of disability worldwide (WHO, 1995).
2.2.1 Lymphatic Filariasis Burden in Ghana

In Ghana, the distribution of lymphatic filariasis reveals a pattern of endemicity separated by a continuous area of non-endemicity. Brancroftian filariasis remains the sole filarial infection in Ghana transmitted by *Anopheles* mosquitoes. The disease is prevalent in 61 out of the 216 districts in 10 regions of Ghana. It is an incapacitating disease which has hostile consequences on efficiency of infected persons and socioeconomic development of prevalent countries.

In Ghana, higher prevalence of LF is found in the Northern part of the country compared to the Southern part. The population at risk of contracting the disease is 11,587,953. *Anopheles gambiae* s.s., *An. melas*, *An. pharoensis* and *An. funestus* are the incriminated vectors of lymphatic filariasis (Appawu *et al*., 2001; Amuzu *et al*., 2010; Dzodzomenyo *et al*., 1999). However, a recent study by Ughasi *et al*., (2012) also revealed *Mansonia* species as vectors. Nevertheless, other species of mosquitoes co-exist with these vectors, though they have not been incriminated in transmitting lymphatic filariasis in Ghana and the rest of West Africa.

2.3 Malaria: A Global Burden

Malaria, which is transmitted by mosquitoes, is the best-known and the leading killer among vector-borne diseases. A disease causing the most critical public health crises worldwide is malaria and is existing in nearly one hundred countries and jeopardizes half of the world’s population especially in sub-Saharan Africa. In 2015, an appraised 3.2 billion people were at risk of malaria with up to 214 million cases of malaria globally, 88 % of which were found in WHO African region and leading to 438 000 deaths (WHO, 2015). It is a principal cause of morbidity and mortality in many developing countries especially in sub-Saharan African
countries where the burden of the disease is high and malaria control is one of the greatest challenges. Malaria has a substantial impact on the health of infants, young children and pregnant women worldwide. It also plays a role in malnutrition of children (NIAID, 2011).

Malaria parasite that causes the most severe cases of malaria are *Plasmodium falciparum* and *P. vivax*. Amongst these, *P. falciparum* is more predominant in Africa, and has been found to be most accountable for malaria deaths. However, *P. vivax* has a broader topographical spread than *P. falciparum*, this is due to the fact that it has adaptive features to thrive in mosquito vectors ability to endure variations in temperature, altitudes and in colder climates (WHO, 2015).

### 2.3.1 Malaria Burden in Ghana

Malaria is widespread and persistent in all parts of Ghana, with seasonal discrepancies that are more noticeable in the north. Ghana’s whole population of nearly 25 million is at risk of malaria infection. It is responsible for 33 % of hospital death in children under 5 years, 38 % of all outpatient cases and 36 % of all hospital admission (PMI, 2014). In the northern part of the country, it accounted for nearly a quarter of deaths in children under five years (Binka *et al.*, 1994, 1995). Between 3.1 and 3.5 million annual cases of clinical malaria are recorded in public health amenities, of which 900,000 cases are in children under five years and 3,000 - 4,000 result in in-patient deaths (PMI, 2012).

### 2.4 Key Malaria Vectors

There are more than 3,000 species of mosquitoes of which about 100 species are vectors of human disease. Malaria is spread by different infected female *Anopheles* mosquito which is
subject to the geographical area and region. Female *Anopheles* which can pass on malaria must be able to ingest the parasites, foster their maturation to the infective stage and must be able to survive until the next blood meal. The principal vectors of malaria in Sub-Saharan Africa are the *An. gambiae* complex and the *An. funestus* complex are, with *An. gambiae* complex being the predominant (Gillies and Coetzee, 1987).

The *Anopheles gambiae* complex is established to comprise the core vectors spreading malaria parasite, *Plasmodium falciparum* in Sub-Saharan Africa (WHO 2000) and comprises of eight morphologically indistinguishable sibling species. They are: *An. gambiae sensu stricto* Giles which consist of molecular "M form" named *Anopheles coluzzii* Coetzee and Wilkerson and the "S form" which retained the name *Anopheles gambiae* Giles, *An. arabiensis* Patton, *An. bwambae* White, *An. quadriannulatus* Theobald, *An. amharicus* Hunt, Wilkerson and Coetzee (formerly designated *An. quadriannulatus* species B due to its close similarity to *An. quadriannulatus*), *An. merus* Donitz and *An. melas* Theobald (Gillies and Coetzee, 1987; Coetzee *et al.*, 2013). These sibling species can only be distinguished by chromosome banding patterns, allozyme analysis, chromatography of hydrocarbons with DNA probes and species-specific polymerase chain reaction (Gillies and Coetzee, 1987; Hunt *et al.*, 1998). *Anopheles gambiae* complex have a wide range of biological attributes (Gillies and de Meillon 1968) and are ecologically diverse, reportedly feeding indoors or outdoors on humans or cattle, resting indoors or outdoors, and their larvae were established in a wide-ranging habitation such as saline water, rice paddies, hoof prints, rain pools, and mineral springs (Coetzee *et al.*, 2013).

The sibling species consists of freshwater, mineral water and saltwater breeders (Davidson *et al.*, 1967). The freshwater breeding species include the *An. amharicus, An. gambiae s.s., An. quadriannulatus* and *An. arabiensis*. The two saltwater breeders are the *An. merus* for the East African species and *An. melas* for the West African species, while *An. bwambae* is the
only mineral water breeder. These species differ in relative occurrence and distribution from one geographical location to another; their distribution is subjective to dominant climatic conditions.

*Anopheles melas* is confined to the West African coast while *An. merus* is restricted to the islands and East African coast, and also observed in the Natal in the South and North of Somalia (Diop et al., 2002). *Anopheles quadriannulatus* was first reported in Onderstepoort, South Africa and was considered to be a highland species because of its distribution in Ethiopia (White, 1985). However, the Ethiopian population was shown to be a separate species based on cross-mating and chromosomal studies (Hunt et al., 1998) and has formally being assigned a new name, *An. amharicus* (Coetzee et al., 2013). *Anopheles bwambae* has been reported from the geothermal hot springs in the Semliki forest of Uganda's Bwamba County where it was found breeding in mineral water springs (White, 1985). *Anopheles arabiensis* and *An. gambiae s.s.* are highly widespread and the best effectual disease transmitting sibling species of the *An. gambiae s.l.* in Africa (Coetzee et al., 2000). While *An. gambiae s.s.* is usually predominant in tropical environments, *An. arabiensis* appears to be found in drier areas and predominate in arid savannas. In areas where *An. arabiensis* and *An. gambiae s.s.* co-exist, there are huge heterogeneities in densities, with the former predominating throughout the arid season and the latter becoming further copious in the wet season (Coetzee et al., 2000).

Three sibling species namely *An. melas, An. arabiensis* and *An.gambiae s.s.*, have been reported in Ghana, with significant variations in their capability to transmit malaria. *Anopheles melas* is more prolific than the other members and tends to show significant outdoor feeding where people slept on mats (Tuno et al., 2010). This species has been reported in the coastal zone of Southern Ghana but has not been implicated in malaria transmission (Appawu et al., 2001). *Anopheles arabiensis* has been found in the Western
Region, Southern coastal zone and Northern savannah zone of Ghana (Kristan et al., 2003; Kelly-Hope et al., 2006). *Anopheles gambiae s.s.* on the other hand, has been detected in almost every ecological zone of Ghana. For example, studies by Adasi et al., (2000), Appawu et al. (2001), Kabula et al. (2011), Tchouassi et al. (2012) and Yawson et al. (2004) recorded *An. gambiae s.s.* in the coastal savannah and coastal forest zone in the Greater Accra Region of Ghana. *Anopheles gambiae s.s.* has also been recorded from the strand and mangrove zones of the Central Region as well as the rainforest zone of the South-Western Region of Ghana (Kristan et al., 2003). In the rainforest, middle belt or central part of Ghana, this species has been reported (Afrane et al., 2004; Yawson et al., 2004; Stiles-Ocran et al., 2007; Dery et al., 2010), and it is the most prevalent species in the malaria transmission in the country (Appawu, 2005).

However, reports in West Africa have made available proof that within the *An. gambiae s.s.* designated as Bamako, Forest, Mopti, Bissau and Savanna, there are as minimum as five forms of chromosomes (Bryan et al., 1982; Coluzzi et al., 1985, Fanello et al., 2003). Three of these forms namely the Forest, the Savanna and the Mopti have been reported in Ghana, (Appawu et al., 1994). Further analysis into the complexity of *An. gambiae s.s* revealed genetic differences in the non-coding sequences of the ribosomal DNA (rDNA), distinguishing between the Mopti chromosomal form and Bamako/Savanna populations (Favia et al., 1997; della Torre et al., 2001).

Hence, the concept of molecular forms of *An. gambiae s.s.*, using the terms M and S was first introduced by della Torre et al. (2001) to describe such populations, with the M form coinciding with Mopti chromosomal forms and S form coinciding with the Savannah/Bamako chromosomal forms only within the boundaries of Mali and Burkina Faso while outside these boundaries, the linkage is broken. With the recent studies by Coetzee et
al. (2013) which was centered on bionomical and molecular evidence, the *An. gambiae* molecular M form is now known as *An. coluzzii*, while the S form still maintains the *An. gambiae*. In Ghana, both molecular forms have been reported, with the S form prevailing in the middle belt, while the *An. coluzzii* predominated in the northern and coastal Savannah belts (de Souza *et al.*, 2010). The S form is mostly dependent on temporary breeding sites and rainfall, whilst *An. coluzzii* is better acclimated to permanent breeding sites such as rice fields, reservoirs, drainage ditches and irrigation plains (Diabaté *et al.*, 2005; de Souza *et al.*, 2010).

### 2.5 Control of Malaria

Malaria is an avertable and treatable disease and up till now still has an overwhelming effect on worldwide livelihoods and health of people. Malaria control is a structured effort to conduct suitable anti-malaria measures to realize the best possible enhancement in the health of any population afflicted by malaria or exposed to an intensified peril of its renaissance. The goal of malaria control is to avert mortality and decrease morbidity and social and economic losses, through the progressive enhancement and consolidation of local and national capabilities (WHO, 1993). The three pillars of the World Health Organisation Global Technical Strategy for Malaria 2016 – 2030 includes safeguarding universal access to malaria prevention, vector control, diagnosis and treatment; accelerate efforts towards elimination and attainment of malaria-free status; and transform malaria surveillance into an essential intervention.

Increased efforts in malaria control are dramatically decreasing the malaria burden in several places. Between 2000 and 2015, malaria death rates among populations at threat dropped by 60 % globally among all age groups, and by 65 % among children under 5 (WHO, 2015). It is
assessed that 57 countries out of the 106 which endured the disease in 2000 has decreased proportion of >75% for its occurrence of malaria in 2015 (WHO, 2015), some of these countries include Algeria, Botswana, Namibia, Rwanda, South Africa, Swaziland, Argentina, Brazil, Mexico, Morocco, Georgia, Bangladesh and China. Chemotherapy and vector control are basic interventions of the current global strategy to fight malaria.

2.5.1 Chemotherapy

Early diagnosis and prompt treatment of malaria decreases disease and averts deaths. It also contributes to decreasing malaria transmission. Malaria is controlled by the use of prophylactic and chemotherapeutic drugs against the parasite. The best and most effective chemotherapeutic drugs available for treatment, particularly for \textit{P. falciparum} malaria, the major form in Africa and the deadliest, is artemisinin-based combination therapy (ACT) which is a WHO recommended treatment policy for \textit{falciparum} malaria in all countries facing resistance to monotherapies, such as chloroquine, sulfadoxine/pyrimethamine and amodiaquine (WHO, 2001). Artemisinin-based combination therapies are manufactured by combining compounds from the \textit{Artemisia annua} plant with several antimalarial partner drugs. ACTs are believed to defer the evolution of drug-resistant strains of the disease. They are also dynamic against gametocytes, the sexual stage of the parasite cycle, efficiently decreasing disease transmission.

In order to supplement these, extra treatment suggested to particular endangered individuals in places where the disease is known to be endemic is intermittent preventive treatment in infancy and pregnancy. Chemoprophylactic drugs comprises of sulfadoxine-pyrimethamine (SP) for pregnant women, seasonal malaria chemoprevention (SMC) with amodiaquine plus SP (AQ+SP) for children (WHO, 2015), proguanil and atovaquone (Wells \textit{et al.}, 2009). However, development of resistance by \textit{Plasmodium} parasite which has led to resurgence of
malaria infection in places where it was previously eliminated is the major challenge of the use of drugs for malaria prevention and infection (NIAID, 2011).

2.5.2 Vector Control

Vector control is an integral element of the Global technical strategy for malaria 2016 - 2030 and remains the most effectual measures to prevent malaria transmission. Vector control interventions, aim to decrease human-vector contact by decreasing the lifespan of female mosquitoes and in so doing reduce the burden of malaria (Curtis and Towson, 1998). The focus of vector control against malaria mosquitoes includes firstly, safeguarding humans from bites of infected Anopheles mosquito; secondly, decreasing strength of transmission of malaria at community stage through decreasing the density, longevity and human-vector contact of the indigenous mosquito vector population (WHO, 2011). The puissant and mostly utilized interventions are control measures directed towards adult mosquitoes which includes indoor residual spraying and long-lasting insecticidal nets (WHO, 2011). Other vector control interventions that can be employed against malaria vectors include larval control, source reduction and environmental management (NIAID, 2011). To maximize the impact of vector control against malaria, principles of integrated vector management are inevitable. It strives for the development of efficacy, cost–efficiency, environmental soundness and sustainability of disease vector control with the utmost aim of averting vector-borne diseases transmission.

2.5.2.1 Challenges of Vector Control

The major and particular challenges of vector control are resistance to insecticides by vector, residual, transmission while outdoors among others. Malaria vectors have heterogeneous behaviours varying across species and within species which is subject to the topographical
situation, with all the discrepancies demonstrating behavioural flexibility. With an intensive use of interventions such as IRS, and ITNs, malaria mosquito behaviours in a population might be selected, resulting in: species shift, as recorded in Kenya where rising coverage of LLINs led to an intensifying proportion of *Anopheles arabiensis*, substituting progressively *Anopheles gambiae*. Study in South-eastern Tanzania has shown that great utilization of ITNs can dramatically alter vector populations so that strong, principally indoor transmission has been interchanged by greatly lowered residual transmission, a greater ratio of which occurs outdoors. The heterogeneity in mosquito biting and resting behaviour requires specific targeted interventions. *Anopheles* vectors in not less than 64 countries that are endemic to malaria worldwide were detected to be resistance to as minimum as an insecticide and it persists with increase in Latin America, South East Asia and Africa. Quality assurance of existing and new vector control products and equipment is crucial for sustained efficacy and safety of vector control interventions.

### 2.6 Biology of *Anopheles* Mosquitoes

*Anopheles* mosquitoes like other dipterans are holometabolous insects having four life stages; egg, larvae, pupae and adult. The adult stage is the only terrestrial stage while the remaining three (3) stages are aquatic. These aquatic forms take place in a wide array of breeding sites, but most species have a preference for clean, unpolluted water. Most of them have been found in freshwater or saltwater marshes, rice fields, mangrove swamps, the edges of streams and rivers, grassy ditches, and small, temporary rain pools. Several species prefer habitations with vegetation, but others choose habitats with none (Foster and Walker 2009). A number of them breed in open, sun-lit pools and stagnant pools close to human dwellings, while others are found only in shaded breeding sites in forests. A small number of species breed in tree holes or the leaf axils of some plants.
Anopheles mosquitoes have short development time which lasts for 5 – 14 days depending on the species and ambient temperature. In the wild, adults usually survive around one to two weeks even though they can survive up to a month in confinement. Male and female adult mosquitoes feed on nectar from plants, but only the females feed on blood from vertebrates, where they acquire nutrients for maturation of their eggs (Foster and Walker 2009). After mating and taking a blood meal, a gravid Anopheles female lays 50 - 200 eggs individually and directly on the water, with each egg having floats on both sides (Foster and Walker 2009). Anopheles eggs are not resistant to drought and can emerge within 2 - 3 days, but emergence may stay longer in colder climates taking up to 2 - 3 weeks. Larvae are filter feeders, usually feeding on bacteria, yeasts, algae, protozoa, pollens and other microorganisms on the water surface; they also lie parallel in order to breathe in air through spiracles (Service, 1980). Rates of larval growth are influenced by environmental factors such as temperature, photoperiodicity, food supply, degrees of overcrowding and the species (Service, 1993). Larvae develop through four instars within 6 - 9 days before pupating. Pupae are non-feeding, mobile stage that uses the paddle at the end of their abdomen to quickly move through their aquatic habitat (Foster and Walker 2009). The mosquito’s adult body is formed during the pupal stage. They breathe through the paired respiratory trumpets. In the tropics, pupal stage usually last for 2 - 3 days, but can be as short as 26 hours at 30 ° C (Service, 1993) with male larvae generally pupating before the female.
During emergence, the adult ingests air to expand the abdomen, splitting open the dorsal surface of the cephalothorax and the mosquito emerges with the head emerging first. The newly emerged adult expands its wings, separates and cleans its head appendages before taking flight (Kettle, 1992). Females usually mate few hours after emergence or sometimes much sooner but males do not mate until their genitalia have rotated 180° (Service, 1993). In most species, males form large swarms which the females fly into to mate. Most males habitually die after mating. Females have need of blood meal for ovarian development, followed by maturation and oviposition (Gillies, 1955). On full development of the eggs, they are laid and the females resume host seeking. Most *Anopheles* are nocturnal in their activities, thus emergence from pupae, mating, blood feeding and oviposition normally occur in the evenings, at night or in the morning around sunrise. Most *Anopheles* species are not completely exophagic or endophagic, exophilic or endophilic, but a mixture of these extremes of behavior. Similarly, *Anopheles* feed exclusively on either man or animal; most feed on both man and animals but the degree of anthropophily and zoophilic varies according to species (Coluzzi et al., 1999). Owing to their short development time as well as their
preference for developmental habitats near human dwellings, *Anopheles gambiae* are considered effective vectors of human malaria, as well as lymphatic filariasis.

### 2.7 Effects of Larval Nutrition on Resultant Adult Fitness

The rapidity and duration of larval development is influenced by a series of extrinsic and intrinsic factors (Christophers, 1960; Clements, 1999) but one of the most important extrinsic drivers is nutrition. Other factors such as temperature, turbidity, water level (Paaijmans, 2008) and larval competitors (Munga *et al*., 2006) are well-known for their effect on the growth and development of immature mosquitoes. Nutrition is an extrinsic and biotic factor that affects larval growth and development and has consistent influence on the adult. The nutritional constituent has an impact on larval growth, larval developmental time, pupae weight, metabolic storage reserve, and adult health and life history traits. It has been recorded that mosquito larvae reared at extreme temperatures and under food stress situations grow into small adults and are likely to experience high death rate (Reisen *et al*., 1984; Siddiqui *et al*., 1976). On the contrary, larvae reared at extreme temperatures and fed at an optimum rate grow into large adults (Tun-Lin *et al*., 2000). More so, dietary resources in larval habitats determine the size of adult mosquitoes and most often accounted for the differences in developmental fitness of the emerged adult (Peck and Walton, 2005).

In order to acquire nutrient and accumulate excess nutrient in its body, mosquito larva feeds on aquatic microbes for later utilization (Bond *et al*., 2005).

The body size of a mosquito depends on intrinsic genetic factors, larval nutrition and temperature, thus, understanding the effects of these factors on mosquito population helps in developing a good mosquito control program. In *Aedes aegypti*, carbohydrate which are made and stored as glycogen is used as energy sources (Wigglesworth, 1941) while protein is
stored as lipids for growth. (4 %) Lipids when incorporated in the basal liquid media can increase larva growth. Proteins assist in maintaining larval growth by enzymatic reactions, and oogenesis (Uchida et al., 2003), and also serving as energy sources particularly during starvation. An increase in larval size and protein stored by larvae is associated to increase in dietary protein (Timmermann and Briegel, 1999; Telang et al., 2002) and shortens larval developmental time (Khan et al., 2013).

Other co-factors like vitamins (riboflavin, pyridoxine, nicotinamide, thiamine and Ca-pantothenate) that are involved in metabolic reactions, also support larval growth (Horn and Lichtwardt, 1981). In the search for an effective larval diet for Anopheles arabiensis, Damiens et al., (2012), noted that the quality of the diet was enhanced by adding a vitamin mix. The mixing of the right quantity of different components to gain balance diet is very necessary to larval nutrition status. This is usually responsible for the dissimilarity in larval growth and fitness in mosquitoes reared in their natural habitat and laboratory reared mosquitoes of the same species, with latter being healthier than the former (Wotton et al., 1997; Peck and Walton, 2005). In rearing of Anopheles gambiae larvae, the nutritious needs as well as the amount of food taken by larvae increases as the larvae develops from one instar to another. Normally, fourth instar larvae need five times (x5) the amount of food used up at first instar.

The usage of high nutritious diet decrease’ larval developmental time and mortality, increase pupation rate and pupal weight, prevent cannibalism, and enhance adult survivorship (Damiens et al., 2012; Gilbreath et al., 2013; Khan et al., 2013; Kivuyo et al., 2014) emergence of large winged adults of An. gambiae that are fit for reproduction (Okanda et al., 2002) and are better adapted to their environment (Koenraadt, 2008). Whilst poorly feed of larvae results in increase in developmental rate (Terzian and Stahler, 1949; Gleiser et al., 2000; Agnew et al., 2002; Gimnig et al., 2002; Koenraadt and Takken, 2003; Koenraadt et
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Recent studies have shown that larvae reared under a low food schedule took extra time to develop, had a poorer development success and resulted in smaller adults than were their high food counterparts. (Vantaux et al. 2016a and b; Takken et al., 2013). Therefore, the fitness of an adult mosquito is estimated largely by larval nutrition, body size and nutritional reserves at emergence (Briegel, 1990). This fitness is measured by wing length, with relationship between the length of the wing and its body mass being inconstant, differing between different species of mosquitoes, strain and rearing background (Nasci, 1990).

2.7.1 Effect of Yeast and Rabbit Pellet on Larval Development of An. gambiae s.l.

Yeasts use in mosquito rearing have been employed in various ways and has usually been reported from insect habitats. In some instances, yeasts (Wickerhamomyces anomalus) have been discovered in the insect gut (Ricci et al., 2011; Cappelli et al., 2014) and in other tissues (Wigglesworth, 1942).

The usage of yeast as larval diet has given rise to various outcome on the larval development. Although, the effect of its use is dependent on the type of yeast used, other components in a diet (Puggioli et al., 2013) and quantity added (Gerberg, 1970; Peck and Walton, 2005). It could either be brewer yeast (Koenraadt, 2008), yeast proteins (Fellous and Lazzaro, 2010), baker’s yeast (Khan et al., 2013), an extract or derivative from yeast (Golberg and Meillon, 1948), yeast hydralysat (Khan, 2010), dead (Trager, 1936) or whole (alive) (Tovar et al., 2002). A study on the assessment of single diet involving Anopheles arabiensis observed that
80 - 89 % and 70 – 79 % of the larvae developed into pupa in 10 and 11 days respectively when Brewer’s yeast and Yeast hydralysat were used (Khan, 2010). Also, Hood-Nowotny et al., (2012) observation noted that none of Anopheles arabiensis larvae survived to adult when fed with hydralysat yeast only. while another study of Anopheles stephensi a combination of baker’s yeast did not significantly advance larval survival and this was attributed to scum formation (Khan et al., 2013). The discrepancies in the observed results could probably be because of the preparation process and the quantity of baker’s yeast utilized. Presently, 0.02 % of yeast is being added to eggs for facilitating hatching and development of larvae while rearing (Gerberg, 1970; Benedict, 2007).

2.8 Effect of Larval Nutritional Regime on Adult Fitness and Susceptibility to Insecticide

Factors affecting the fitness of adult female mosquitoes includes the availability and quality of blood meals, nutrition during larval development, and ambient conditions. This fitness is mostly estimated using nutritional reserves and body size. Fitness and susceptibility of mosquitoes to insecticides may perhaps decrease the vector’s nutritional status. It has been recommended that resistance to insecticides might influence the condition of these mosquitoes (McCarrroll et al., 2000) and resistance to insecticides is linked to a wide-ranging of pleiotropic impact on various main life-history attributes of mosquitoes such as behavior and longevity. A study by Takken et al., (2013), showed that mosquitoes that were reared with high and low diets developed into adults of diverse sizes which were similar amongst An. gambiae and An. stephensi. Females fed with low-diet had fewer lipid, glycogen and protein when they emerged as adults than those fed with high diet, thereby producing significantly different size classes of mosquitoes (p < 0.001).
Larval diet of *Anopheles* also affects the ability of adults mosquitoes to serve as a vector (Takken *et al.*, 2013), resistance to insecticides (Oliver and Brooke, 2013), strongly influences the sex ratio and blood feeding of resultant adults (Takken *et al.*, 2013; Kivuyo *et al.*, 2014). Resistance to insecticides is often associated with energetic and fitness costs. In vectors such as mosquitoes, such fitness costs include decrease in preimaginal survival, adult size, longevity, and fecundity. A recent study showed that small sized female *An. arabiensis* which emerged from poor fed larvae were susceptible to DDT intoxication, whilst well-fed larvae develop relatively fast into big adults and were more resistant to DDT. The observed phenomenon was associated with detoxification enzyme activity (Oliver and Brooke, 2013).

### 2.9 Techniques in *Anopheles* Species Identification

#### 2.9.1 Morphological identification

In the morphological identification of *Anopheles* species, there are a number of systematic procedures designed which can be used, including unrelated species with varying specificity. These procedures are mostly based on the universal characters which entails the forms and structures used as diagnostic characters that cut across geographical zones for a particular species. Also, they are applicable in the field situations where they are used in sorting out samples prior to the use of any other techniques, if needed. Established identification methods of the anopheline mosquitoes include the use of morphological characters described by Gilles and de Meillon (1968) and later improved upon by Gillies and Coetzee (1987). These morphological keys are still the most widely used methods for taxonomic and systematic studies of anophelines, but due to the evolution of species groups and complexes within the anopheline taxa, morphological features are becoming less useful for the identification of sibling species of the vectors.
2.9.2 Molecular identification of the sibling species

Due to the evolution of sibling species of anopheline mosquito complexes which are morphologically indistinguishable species but reproductively distinct, morphological methods of identification is now supplemented with more alternative specific method for identification. Some of these methods that have been employed compasses chromosome banding patterns on the giant polytene chromosomes (cytotaxonomy), isoenzyme analysis using electrophoresis, cuticular hydrocarbon analysis and synthetic DNA probes but with a lot of limitations. The discovery of species-specific polymerase chain reaction (PCR) employs the variation in the DNA sequences among specific species of the mosquito genome (Collins et al., 1987).

2.9.2.1 Bases of Polymerase Chain Reaction (PCR)

This is a DNA–based method used in studying mosquito populations in vitro. It allows the identification of all developing phases of insects and with the polymerase chain reaction, infinitesimal amount of template DNA is needed for analysis (Sambrook et al., 1989). This method is developed for the enzymatic production of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Saiki et al., 1985). PCR reaction involves three steps of repeated cycles- heat denaturation of the template DNA to separate the two strands, followed by annealing of the primers to the complementary DNA sequence at a lower temperature, and extension of the new DNA strands by the Taq polymerase which is done using the free nucleotides in the reaction mixture. Reaction constituents include the DNA polymerase enzyme known as Taq polymerase, which is added at the beginning of the reaction, the deoxynucleotide triphosphates (dNTPs), two oligonucleotide primers, DNA template and the reaction buffer. The reaction is automated by programmable thermal cycling devices. A PCR protocol by
Scott et al. (1993) is currently and widely used in the identification of sibling species of the *An. gambiae* complex. The method utilizes species-specific nucleotide sequences in the intergenic spacer (IGS) regions of the ribosomal DNA (rDNA).

### 2.9.2.2 Bases of Restriction Fragment Length Polymerization (RFLP)

Restriction Fragment Length Polymerization (RFLP) is a technique that takes the advantage of variations in homologous DNA sequences thereby affording a means of discerning between similar DNA samples at the species or strain level. Hence, it is used in distinguishing the *An. gambiae* molecular forms; the M form *Anopheles coluzzii* and the S form *An. gambiae* because of its specificity.

The PCR-RFLP protocol described by Favia et al. (1997) is employed in differentiating the molecular forms within the *An. gambiae s.s.* In this assay, the individual mosquitoes are first identified by PCR following the protocol described by Scott et al. (1993). Then, further separation of *An. gambiae s.s.* species are into their molecular forms either by Favia et al., (1997) or Favia et al., (2001) methods which are all established on the existence of substitutions of two nucleotides within the rDNA region amplified.

Recently, a new method for simultaneous identification of species of the *An. gambiae* complex and molecular forms of *An. gambiae s.s.* was developed by Fanello et al. (2002). This method, which involved both protocols described by Favia et al. (1997) and Scott et al. (1993), is centered on the study which shows that inside the *An. gambiae*-specific fragment amplified lies the 5’-GCG/C-3’ restriction site for *Hha* 1 enzyme for the S form but not in the *An. coluzzii* (Favia et al., 1997). It is therefore, likely to digest this fragment to distinguish *An. coluzzii* and S forms.
2.10 Evolution of Insecticides Resistance in *Anopheles gambiae*

Vector control is still indispensable in combating most arthropod borne diseases and malaria is not an exception especially in endemic foci. The use of insecticides has been the cornerstone of control programmes for malaria, which is largely achieved through Long Lasting Insecticide-treated Nets (LLINs) and indoor residual spraying (IRS). World Health Organization approved twelve insecticides belonging to four different classes; pyrethroids, organochlorine, carbamates and organophosphate for use in IRS programs. Six insecticides belonging to pyrethroids are the only recommended insecticide class that can be used for LLINs. In addition to the limited insecticides available for vector control, these same insecticide classes have been widely embraced in Africa for agricultural purposes, leading to extra selection pressure on mosquito vectors as ground water contaminated with insecticides infiltrates larval habitations (Ranson *et al.*, 2009). These intensified contact, besides the use of just one class of insecticides has unavoidably brought about the development of insecticide resistance by *Anopheles* species.

According to WHO (2013), insecticide resistance is the development of an ability in a strain of some organisms to tolerate doses of a toxicant which would prove lethal to a majority of individuals in a normal population of the same species. Insect resistance to DDT developed in the 1940s, with the first definite study conducted on the *Culex molestus* mosquitoes in 1947 in Italy. Resistance to the organochlorines (DDT and dieldrin) was first reported in African malaria vectors in the 1950s and 1960s (Brown, 1958; Hamon *et al.*, 1968). In 1955, it was reported in the *An. gambiae* species in Nigeria. Pyrethroid resistance was detected in African malaria vectors in 1993 (Elissa *et al.*, 1993). Afterward, resistance has been reported in over 500 insects, 50 of which transmit malaria parasites in humans. Likewise, studies in countries from South, East, West and Central Africa have shown populations of *Anopheles gambiae* complex that have pyrethroid resistance and *Anopheles funestus* in South Africa,
Mozambique and Ghana (Ndjemai et al., 2009; Munhenga et al., 2008; Protopopoff et al., 2008; Awolola et al., 2009). Recently, carbamate and organophosphate resistant populations of *An. gambiae* have been reported in West Africa.

The standard WHO susceptibility test has long been recognized as the primary method by which insecticide resistance in malaria vector population can be detected and identified (WHO, 2013). According to the protocol, mosquito samples are exposed to a series of different insecticides using insecticide-impregnated papers with a single diagnostic dose for each insecticide. After the exposure of mosquitoes to insecticide impregnated papers, they are held in the absence of insecticide-impregnated papers for 24 hours before mortality is recorded. The tests are performed using non-blood fed females aged 2 to 5 days post-emergence either derived from larval collections or F1 progeny of wild-caught female mosquitoes. Male mosquitoes however, are usually not recognized in monitoring insecticide resistance since they are usually small in size and more fragile than females; with control mortalities (WHO, 2013). This method has been widely used in the field with satisfactory results in detecting insecticide resistance for monitoring purposes.

The evolution of resistance is influenced by many factors (Georghiou and Taylor, 1986). These include genetic factors such as the number and frequency of resistance alleles in the insect population, fitness cost and relative dominance of the characters; biological factors including the insects life history parameters, the fitness of the heterozygous and homozygous resistant phenotypes and initial population size; ecological factors including isolation, mobility and migration; operational factors comprising previous choice of insecticides, characteristics of an insecticide in use, its methods of application, chemical nature, and proportion of population exposed to selective doses, dosage of insecticide taken up by exposed insects and the developmental phase of the mosquito selected; and reproductive
factors containing the proportion of surge and variations in population size (Georghiou and Taylor, 1986; Tabashnik, 1990).

2.11 Resistance Mechanisms

Insecticides resistance occurring in many insect vectors have diverse mechanisms. Although mechanisms of insecticide resistance are same across all vector taxa, every resistance difficulty is actually exclusive and could entail multipart array of resistance foci. There are two major types of resistance mechanisms which are responsible for the widespread levels of resistance. They are; target site resistance that is those facilitated by alterations at the action site of the insecticide and metabolic resistance, those instigated by upsurges in the degree of insecticide metabolism (WHO, 2013). Others include behavioural resistance that lessens an insect's contact with toxic compounds or lets an insect to endure in a surroundings not suitable to the greater part of other insects (Liu et al., 2006); and physiological modification mechanisms which allows insects to live on after exposure to deadly quantities of a toxicant by reduced permeation of insecticides, amplified sequestration/storage of insecticides, and quicker excretion of insecticide. Multiple resistance and cross resistance also complicates the management of insecticide resistance. Different insecticide classes sharing similar mode of action may develop cross resistance. It arises when an insect develop resistance to one insecticide without prior exposure due to its resistance to a previous insecticide. Hence, mosquito vectors having pyrethroid resistance due to the fact that they have the $kdr$-resistant gene most likely will have DDT resistance. Similarly, vectors that are resistant to organophosphate due to the $Ace-1$ alteration could also cause carbamate resistance (WHO, 2013). When cross resistance is present however, it limits selection of insecticide substitute in conditions with resistance. Multiple resistance occurs when insects develop resistance to several compounds by expressing multiple resistance mechanisms. The different resistance mechanisms can combine to provide resistance to multiple classes of insecticides.
2.11.1 Target Site Resistance

The pyrethroids and the organochlorine insecticides particularly DDT target the voltage-gated sodium channel on the insects’ neurons. Insecticide binding delays the closing of the sodium channel prolonging the action potential and causing repetitive neuron firing, paralysis and eventual death of the insect. Target-site resistance occurs when there are modifications in the sensitivity of insecticide target due to non-silent point mutations (Hemingway and Ranson, 2000). These changes take place through mutation at the protein receptor which the insecticide intends to strike. After this alteration, the proposed target site of the receptor in the insect will not bind to the insecticide, thereby leaving the insect either unaffected, or less affected by the insecticide. With pyrethroids and DDT, the mutation arises in the sodium channel receptors, bestowing what is frequently denoted as knockdown resistance (kdr) in reference to the ability of insects with these alleles to withstand prolonged exposure to insecticides without being ‘knocked-down. For carbamates and organophosphates, Ace-1 resistance mutation happens in the acetyl-cholinesterase (AChE), a neurotransmitter and protein (Liu et al., 2006).

Knockdown resistance (kdr) has been linked with a single point mutation in the gene encoding the voltage-gated sodium channel known as the para-type sodium channel gene in several insect species and more than 20 unique para-type sodium channel mutations have been isolated as being involved in reducing sodium channel sensitivity to insecticides or neurotoxins (Soderlund and Knipple, 2003; Liu et al., 2006). There are two different types of kdr mutations found in Anopheles gambiae, kdr associated with a mutation which results in a leucine to phenylalanine substitution in the transmembrane segment 6 of domain II of the para-type sodium channel (L1014F) and the second mutation causes a leucine to serine substitution (L1014S) at the same amino acid position (Williamson et al., 1996). In many
West African countries, the predominant kdr mutation in An. gambiae populations is the leucine to phenylalanine substitution (L1014F) and so, it is termed kdr west (kdr-w), whilst in East African populations the leucine to serine substitution (L1014S) termed kdr east (kdr-e) is more common (della Torre et al., 2001; Yawson et al., 2004) Studies have reported the existence of species heterozygous for both the kdr-w and kdr-e alleles (Verhaeghen et al., 2006).

Besides the presence of kdr mutation in resistant strains of An. gambiae, studies have indicated its presence in resistant strains of An. stephensi (Enayati et al., 2003), An. arabiensis (Diabate et al., 2004), Anopheles sacharovi (Luleyap et al., 2002), Culex pipens (McAbee et al., 2003), Culex quinquefasciatus (Xu et al., 2005), Aedes aegypti and Aedes albopictus (Kamgang et al., 2011). The knockdown resistance (kdr) type of the target-site insecticide resistance mechanism is known to be prevalent in some parts of Ghana including the Greater Accra Region (Adasi et al. 2000; Adeniran et al, 2009; Kabula et al., 2011) and the Ashanti Region (Yawson et al., 2004). For instance, kdr frequencies of 88 % and 91 % were observed at Dodowa and Accra respectively in the Greater Accra Region (Adasi and Hemingway, 2008). Achonduh et al. (2008) also reported kdr allele frequency of 65 % for Korle-Bu and 95 % for Airport, all in the Greater Accra region. Also, a kdr frequency of 100 % was observed in An. gambiae s.s. in Kumasi in the Ashanti Region (Yawson et al., 2004). However, high kdr frequency have been recorded in the S-form than in the M-form (Chandre et al., 1999; Yawson et al., 2004), although both are exposed to heavy pyrethroid/DDT pressure.

Other forms of target site mutations are modified acetylcholinesterase (MACE) and Rdl (‘resistance to dieldrin). MACE is a mutation in the amino acid sequence of glycine to serine at position 119 in the catalytic domain of the acetylcholinesterase (AChE) gene (thus G119S mutation) which decrease the ability of both carbamates and organophosphates to hinder
acetylcholinesterase (AChE) in nerve synapses, conferring resistance in An. gambiae (Djogbénou et al., 2010; Kwiatkowska et al., 2013). For instance, Kwiatkowska et al. (2013), observed kdr mutation and acetylcholinesterase gene (Ace-IR) resistance to organophosphates and carbamates. Also in Obuasi, an altered acetylcholinesterase conferring carbamate resistance was detected among An. gambiae, although there was no evidence in resistance due to kdr mutation (Okoye et al., 2008).

2.11.2 Metabolic Resistance

Metabolic resistance begins to occur when great activities of one or more enzymes leads in an increased sequestration/detoxification of the adequate proportion of the insecticide than normal before it gets to the target site to impair the toxicity of the insecticide. There are three enzyme systems for vectors of malaria which are known to be involved in insecticide detoxification: the esterases, the mono-oxygenases (mixed functions oxidases, MFOs) and the glutathione S-transferases (GSTs) (WHO, 2013). Hemingway (2007) noted out that these three groups of enzymes provide the first line of protection against xenobiotics in most organisms. Their link in resistance is normally detected through the sudden rise in the typical metabolites produced. According to Hemingway et al. (1998), the production of these excess enzymes may be achieved through non-restricted mechanisms including gene amplification to increase gene’s copy number, and gene expression through modification in the promoter region or mutations in the trans-acting regulatory genes.

The carboxyl esterases (ChEs) are responsible for organophosphates and carbamates, GSTs for organochlorines and pyrethroids, and MFOs for most insecticide classes, mostly in conjunction with other enzymes. Increased activities of these enzymes have been linked to resistance development due to the fact that most insecticides can be detoxified by at least one
of these enzymes in insects (Vulule et al., 1999). The cytochrome P450s are the primary enzyme family responsible for pyrethroid metabolism in insects. There are 111 P450 enzymes in *Anopheles gambiae* and, as in other insects, only a small number of these enzymes are capable of detoxifying insecticides. DDT resistance in *Anopheles* species is frequently associated with increased levels of GSTs activity leading to significant DDT detoxification (Penilla et al., 1998; Hargreaves et al., 2003) Studies have shown elevated levels of cytochrome P450 activities in pyrethroid resistant mosquitoes. They have been found to be linked with permethrin resistance in *An. albimanus* (Brogdon et al., 1999), *An stephensii* (Enayati et al., 2003), *Aedes aegypti* and *An gambiae* (Vulule et al., 1999). Mixed function Oxidases have been demonstrated as a major mechanism of pyrethroid resistance in *Culex quinquefasciatus* and *An. funestus* (Brooke et al., 2001). Martinez-Torres et al. (1998) also reports that organophosphate resistance in *Anopheles subpictus*, a vector of malaria in Sri Lanka, is linked to increased monooxygenase titres and higher insecticide metabolic rates. It was the “mono-oxygenase” resistance in Kwa-zulu Natal that enabled the *An. funestus* population there to become highly resistant to pyrethroids, forcing the national malaria control program to return to the use of DDT.

Biochemical studies in both *Anopheles stephensi* and *An. arabiensis* showed that the toxicity of malathion to resistant strains was greatly increased by the use of esterase-inhibiting synergists, thus implicating a hydrolytic resistance mechanism.

The majority of reports of DDT resistance in *Anopheles* species indicate that it is GST based (Prapanthandara et al., 1995). In some cases, there is cross resistance between this DDT resistance mechanism and some organophosphates. For example, GST-based DDT resistance in *An. subpictus* acts as a secondary resistance mechanism for fenithrothion and is maintained in linkage disequilibrium with a monooxygenase-based organophosphate resistance mechanism in this species in Sri Lanka by organophosphate insecticide resistance pressure.
Even though only a few studies have been done in Ghana, it is suggested that there is possibility of multiple resistance mechanism. In Southern Ghana, Transcription profiling of pyrethroid resistant *An. gambiae* strains from Odumase, shows over-expression of cytochrome P450s CYP6Z2 and CYP6M2 along with a members of a sigma class GSTs (GSTS1-2) and the superoxide dismutase (SOD) gene family (Müller *et al.*, 2007). This observation was related to that of Mitchell *et al.* (2012), whose work showed over-expression of P450 gene CYP6M2 in the high DDT-resistant population of *An. gambiae s.s.* from Accra. A study by Mitchell *et al.* (2014) recommend that there is a positive significant interaction between Glutathione-S-transferase (GST2) and voltage gated sodium channel (1575Y and 1014F). Severe heights of resistance of DDT in wild populations of *An. gambiae* is caused by the two mechanism which work jointly.
CHAPTER THREE

3.0 MATERIALS AND METHODOLOGY

3.1. Study Area

The study area was within Accra metropolis, in the Greater Accra Region of Southern Ghana. It is regarded as the largest, most varied and most multiethnic city in Ghana with appraised population of over 1.6 million people (Nyamadi and Adamah, 2014). There are approximately 501,903 households in Accra according to the 2010 population and housing census, and 3.2 % of them are engaged in agricultural activities. It has a dry climatic conditions and two rainfall climaxes ranging from April to June and September to October. Accra is described as the coastal savanna ecological zone. The annual mean rainfall is somewhat less than 750 mm and relative humidity of that ranges between 65 % and 75 %. The least mean monthly temperature which is approximately 26 °C and the maximum approximately 30 °C is documented during August and amid March and April respectively. There are a small number of ponds and streams, regularly drying out all through the dry season.

Vegetable farms where cabbage and the like are grown were sited in Opeibea area close to the airport drainage and stretches from behind the Alliance Française to the Council for Scientific and Industrial Research (CSIR). Hence, Opeibea was chosen because of its relevance as an urban agricultural site. The airport drainage which runs from the airport area to Maamobi-Nima, functions as source of water for growing of the vegetables. The farmers also dig trenches which they used in collecting and storing water during the raining season. During the dry season, water from the drainage with the aid of a water pump are used to fill the trenches. Hence, these trenches works as a water reservoir. The vegetables are watered
either by direct pumping of water from the drainage with assistance of mechanical water pump or the practice of using watering cans to fetch water from the trenches to water the vegetables. Mosquitoes use these trenches made by the farmers as their breeding sites. The co-ordinates of each sampling site at the Opeibea area were recorded using Global Positioning System (GPS) and a customized map of the breeding sites was created using the ArcGIS lite version 10.1. A map of the sampling sites is shown in Figure 3.1.

Figure 3.1: Map showing the sampling sites of *Anopheles gambiae* s.l. at Opeibea area.

### 3.2 Mosquito Sampling and Rearing

#### 3.2.1 Larval Survey

*Anopheles* mosquito larvae and pupae were collected from the trenches (Figure 3.2) at the vegetable farms in Opeibea area with a ladle into plastic containers using dipping method. Identification of the *Anopheles* larvae were based on a characteristic resting position, which
has the body parallel to the surface of the water and lying below the surface film. Furthermore, the larvae and pupae from each sampling sites were poured into well labeled plastic containers with perforated lids to allow proper aeration and then transported to the insectary at Noguchi Memorial Institute for Medical Research (NMIMR) for rearing.

Figure 3.2: Sample collection sites at Opeibea A: An open trench in the vegetable farm with sampling container and long ladle for larval collection. B: Trench containing water used to irrigate the vegetables.

3.2.2 Insectary Rearing of Mosquito Larvae

Mosquito larvae collected from the breeding sites were transferred into well-labeled mosquito rearing plastic bowls with a large surface area covered with netting material. The batches were examined and non-mosquito species were picked and discarded with the aid of a hand-sucking pipette. In cases where larvae of Culicines were present, they were recognized by their angular position on the water surface and were also removed from the larval bowls. The larvae of *Anopheles* collected from the field were identified using morphological features described by Highton, (1983). They were then placed into plastic rearing trays (36 x 21 x 8 cm) having 1.5 L of dechlorinated tap water (giving a water depth of 3 cm). The larvae were fed once a day on finely ground fish meal. Pupae were picked and separated daily from the
larval bowls into small plastic cups placed in 30 x 30 x 30 cm adult mosquito cages (Figure 3.3) for emergence. The pupae were transferred into small plastic cups and placed in labeled adult mosquito cages made of cubical metal frames and wrapped with fine mesh material for adult emergence. Relative humidity of 80 ± 10 % and temperature of 25 ± 2 °C.

Figure 3.3: Mosquito cages for pupae incubation and maintaining of emerged adults. A: Kisumu strain feed with rabbit pellet and yeast respectively. B: Opeibea strain feed with yeast and rabbit pellet respectively.

3.3 Ethical Clearance

Approval and ethical clearance for the study was obtained from the Animal Experimentation Department of NMIMR to allow the use of the guinea pigs for blood feeding the adult mosquitoes. Also consent and approval were sought from the volunteers.
3.4 Determination of the Effect of Different Diets on Fitness Level of *Anopheles gambiae* s.l.

3.4.1 Establishing Wild Colony of *Anopheles gambiae* s.l.

*Anopheles gambiae* s.l colony from Opeibea (wild population) was established at the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana Legon insectary. Adult males and females were kept together in two different cages and provided with 10 % glucose solution presented in a cotton wool which was changed at two days’ interval. For colony continuance, female mosquitoes were blood-fed on a human arm and guinea pig respectively. The blood feeding was done simultaneously for the two different mosquito cages. The floor of the cage (30 x 30 x 30 cm) was covered with a white sheet and the guinea pig was put in a restrainer which was then placed in the cage. The cage was then placed in the dark. The first blood feeding was done in the night from 5:00 pm to 7:00 pm. Subsequent blood feeding was done from 7:30 pm to 1:00 am. The oviposition tray was made from petri dish covered with wet cotton and lined with a sheet of paper or filter paper cut to fit the circumference of petri dish. This oviposition tray was placed in the cage on the second day after blood feeding to acquire eggs. Eggs laid on the moist filter paper were transferred into larval bowls for hatching.

**Figure 3.4:** A: Mosquito cage with restrained guinea pig during blood feeding. B: Oviposition tray containing eggs.
3.4.2 Laboratory Reared Mosquitoes

Eggs obtained from laboratory-reared insecticide-susceptible *Anopheles gambiae sensu stricto* (Kisumu strain) colony at the insectary of Vestergaard-Frandsen Laboratory (VFL) at NMIMR were used to establish a colony. The colony was established from mosquitoes sampled from Kisumu, Kenya and reared under laboratory ambient conditions of 28 - 30 °C, 70 – 80 % relative humidity and a photoperiod of 12 hr:12 hr (Light: darkness) for survival and reproduction. Cotton wool soaked with 10 % glucose solution was presented to the adult mosquitoes in a 30 x 30 x 30 cm cage. After several trials without success to get sufficient eggs from F1 generation from the Opeibea strain to carry out the study, F5 generation of the Opeibea strain were used for the study on fitness and susceptibility assay.

3.4.3 Preparation of Larval Diets

Rabbit pellet was blended and sieved twice using a domestic blender and 480 um mesh sieve into fine particles. The finely powdered baking yeast (Angel Yeast®) was directly used because it easily dissolved in water.

3.4.4 Rearing of Larvae and Experimental Design

Eggs collected on filter papers (Figure 3.4) from the adult mosquito cages after oviposition were rinsed into larvae bowls holding dechlorinated water at 27 °C and a 20mg yeast added to catalyse the hatching process. After 24 hours’ period, the hatched first larval instars were divided into two groups with one population fed with rabbit pellet and the other population on baking yeast (Angel Yeast®) respectively. Fine thermos (F-100, Japan) water bath was used to control the water temperature of larva rearing tray measuring 14.5 x 9.5 x 4.8 cm. The water bath was set to 30 ± 2 °C. The larva rearing tray was filled with 400 ml dechlorinated
water and was placed in the water bath. The rearing tray was allowed to acclimatize for four hours and it was ensured that the water in the bath covered two thirds \((\frac{2}{3})\) of the depth of the tray. The water level in the bath was kept constant throughout the study. Forty (40) first instar larvae were randomly selected using Pasteur pipette into each 200 ml beaker and the level of water was adjusted to the 50 ml mark, then transferred into the rearing trays in the water bath, bringing the volume of water to 450 ml. The water came to a final volume of 500 ml and a depth of 3 cm by the addition of extra 50 ml of dechlorinated water again. The rearing technique designated by Mohammed and Chadee, (2011) was used with slight modification. Thermos (F-100, Japan) bath water trough was substituted with a bigger trough with a longer sleeve to minimize interference. Galvanized aluminum mesh (4 x 4 cm) which was used as a stand for the rearing bowl was placed in the trough such that the base of the trough is separated from the bottom of the rearing trays (Figure 3.5). In between the water trough and rearing tray is a space to allow effective circulation of water and even distribution of water temperature in the bath. Temperature within the trough and the rearing trays was measured using laboratory thermometer. This was done to ensure that the temperature and density of larvae except the treatment (diet), remained constant.

The larvae were initially fed with 20 mg of a single diet (either Rabbit pellet or Baking yeast (Angel Yeast®)) for the first and second days of the study because of the fragile state of the first instar \(An.~gambiae\) larvae. From the third day onward they were fed with 50 mg of the same particular diet every day, until pupation. Each treatment was replicated 31 times for the Kisumu and the Opeibea strains and the rearing trays were visually inspected once in a day for pupae. Changes in larvae developmental structure, survival and the level of rearing water were noted. The rearing water was changed after every 4 - 5 days. The pupae were collected into 200 ml pupal cups containing 50 ml of dechlorinated water and placed in a 30 x 30 x 30 cm nylon mesh adult cages in the insectary at Noguchi Memorial Institute for Medical
Research (NMIMR). Upon emergence the adults were fed with freshly prepared 10 % glucose solution which was changed every two days interval. They were maintained until ready to be used for the bioassays. The rearing set up was conducted in the Microbiology Laboratory of the Department of Animal Biology and Conservation Sciences (DABCS). The rearing room temperature and relative humidity were recorded daily and it ranged between 28 to 30 °C and 70 to 82 % respectively.

![Figure 3.5: Larvae rearing set up. A: Large water trough which minimize interference from outside (top view). B: Thermo F-100 water bath with regulator at the top, heather and pump. C: 750 ml (microwave bowl) rearing tray filled with 500 ml of distilled water and contains the developing larvae and sitting on top of the 4 x 4 Aluminum wire mesh stand to enhance even distribution of water and temperature. D: Thermometer used to check for constant temperature in the water bath and rearing trays. E: Side view (larvae rearing set-up).](image)

3.5 Determination of the Adult Fitness using the Wing Length

A total of 4960 freshly hatched first instar larvae were used in evaluating the effect of rabbit pellet and baking yeast on the fitness of the emerged adult. Out of these, 2480 were of the Opeibea strain while the remaining 2480 were of the Kisumu strain. Half (1240) of each of
the mosquitoes strains were fed with either rabbit pellet or baking yeast. The fitness parameter was the adult body size which was the estimated adult wing length (mm). Both wings of 1040 adult female mosquitoes were measured for each larval diet (n = 520 per treatment) resulting in a total of 2080 pairs of wings for both strains.

The fitness of the adult mosquito which is measured from its body size was appraised using the wing length (mm). Wing length is well-known to be positively correlated with body size in mosquitoes (Nasci, 1990; Lyimo and Takken, 1993). After the WHO susceptibility assay, the mosquitoes were transferred into paper cups and life mosquitoes were frozen for easy removal of the wings. The mosquito wings were detached and fixed on glass microscope slides using clear nail lacquer, after which the image was captured using Leica dissecting microscope camera (EZ4D-57577426-0000-P) at magnification of 30 times. Wing measurements in millimeter (mm) were taken from the tip of the wing (excluding fringe) to the distal end of the alula (Figure 3.6) using LAS EZ Leica microsystems software version 3.3.0 (Leica Application Suite). Both wings were measured and their mean length used as a determinant of mosquito size. The bodies of the mosquitoes were individually placed in Eppendorf tubes and frozen.
3.6 WHO Insecticide Susceptibility Assays

3.6.1 Insecticide Susceptibility Test

A total of 2480 (1040 Kisumu and 1040 Opeibea) adult female *An. gambiae s.s.* of 2 to 5 days old raised in the insectary were used for the WHO susceptibility bioassay. Of these, 360 female mosquitoes each were exposed to doses of different insecticides while 160 female mosquitoes were used as control. The insecticides used were 0.05 % Deltamethrin (pyrethroid), 5 % Malathion (organophosphate), 0.4 % DDT (organochlorine) and 0.1 % Bendiocarb (carbamate). This was done for each larval diet and for both strains.

Susceptibility assay was carried out to estimate the mosquito vector population that was physiologically resistant to the insecticides selecting one insecticide from each of the four major classes of insecticides recommended for controlling malaria vectors. The susceptibility of *Anopheles* species obtained from the field-collected larvae (Opeibea strain) and the

**Figure 3.6:** *Anopheles gambiae* wing showing x, the measured wing length.
laboratory-reared Kisumu strain were tested using the procedure designed by the World Health Organization (2013) for testing insecticide resistance in the field.

Non-blood fed adult female *An. gambiae*, aged 2 to 5 days old were transferred using aspirator from the mosquito cage into paper cups with mesh screen cover. Twenty (20) mosquitoes were transferred to a special plastic holding tube lined with insecticide-free paper. A plastic tube lined with filter paper impregnated with mineral oil (used as control) was connected with the holding tube and twenty mosquitoes were transferred to the tube through a hole in the slide between the 2 tubes; the same number of mosquitoes was transferred to plastic tube with 0.05 % deltamethrin-impregnated filter paper. The slide was closed and the exposure and control tubes were allowed to stand upright for 60 to 80 minutes depending on the rate of knockdowns. The filter papers impregnated with mineral oil and deltamethrin were held in place by silver and copper rings respectively. Separate sucking tubes were used to transfer mosquitoes to the exposure and control tubes to avoid contamination. The number of mosquitoes knocked down (KD) were recorded at intervals of 5 minutes for the first 20 minutes, followed by a 10-minute interval till the 60th minute. After exposure, the mosquitoes were moved into a holding tube and fed on 10 % glucose solution. For Opeibea strain, the number of mosquitoes knocked down after the 60th minute which were less than 80 %, were noted, and then further exposed for 20 minutes in the same tube. Mosquitoes were maintained in the holding tubes for 24 hours (the recovery period) and at the end of this period, the number of dead mosquitoes were counted and documented. Mosquitoes were considered to be alive if they were able to fly, regardless of the number of legs remaining. Any knocked-down mosquitoes, whether or not they have lost legs or wings, were deemed moribund and were counted as dead. The experiment was conducted at a temperature of 27 ± 2 °C. Mosquitoes killed by contact with the Deltamethrin insecticide-impregnated paper and those killed in the control tubes at the end of the recovery period (24
hours post exposure) were counted. The experiment was replicated in order to estimate percentage (%) mortality in the exposure and control tubes. The same experimental procedure was repeated for 5 % Malathion, 0.1 % Bendiocarb and 4 % DDT impregnated filter papers to define the susceptibilities of the mosquitoes to these insecticides treated papers. Four (4) replicates and two (2) controls (Figure 3.7) were conducted for all the four classes of insecticides. This procedure was carried out for both Kisumu strain and Opeibea strains of *Anopheles gambiae* mosquitoes.

![Insecticide susceptibility tests for adult mosquitoes using the WHO protocol. A: Adults fed with baking yeast. B: Adults fed with rabbit pellet.](image)

**Figure 3.7:** Insecticide susceptibility tests for adult mosquitoes using the WHO protocol. **A:** Adults fed with baking yeast. **B:** Adults fed with rabbit pellet.

### 3.7. Species Identification

#### 3.7.1 Morphological Identification

Collected mosquitoes after each sampling were sorted out and morphologically identified using taxonomic keys described by Gillies and Coetzee (1987) and Gillies and de Meillon (1968).
Mosquito larvae were identified as *Anopheles* mosquito species based on the fact that they lie parallel to the water surface and do not possess siphon (WHO, 2003).

Adult *Anopheles* mosquitoes were identified using the equal length of palps and proboscis, with the banding and speckling on the legs, presence of markings on the palps, the presence of block patterns of scales along the costa margin of the vein on the wings. *Anopheles gambiae* complex were identified by the presence of anal vein coloration with three white spots, five pale spots on the costal margin of the wings, a white speckled on the tibia ornamentation and dark apical fringe.

### 3.7.2 Molecular Identification of *An. gambiae s.l.* Sibling Species

#### 3.7.2.1 Isolation of Genomic Deoxyribonucleic Acid (DNA) Extraction

A total of 4960 emerged F1 adult mosquitoes were reared. This included 2480 adult female mosquitoes that were used for the WHO susceptibility assay. Out of these, genomic DNAs were extracted from 208 adult female *An. gambiae s.l.* for molecular analysis of their sibling species. This comprised of 80 (40 from each treatment) adult female *An. gambiae s.l.* from Kisumu strain and 108 (64 from each treatment) from Opeibea strain. In the Kisumu strain 10 adult female *An. gambiae s.l.* each were selected from the 4 classes of insecticides they were exposed to, while for Opeibea strain 16 (8 alive and 8 dead) adult female *An. gambiae s.l.* each were selected taking into consideration the 4 classes of insecticides.

A total of 208 adult females mosquitoes were randomly selected from the female mosquitoes that were stored after exposure to all four classes of insecticide during the susceptibility testing. Out of the 208 pooled samples, 8 dead and 8 alive were selected from each category of female mosquitoes that were subjected to Deltamethrin (0.05 %), Malathion (5 %),
Bendiocarb (0.1 %) and DDT (4.0 %) making it a total of 128 (64 for each treatment) female mosquitoes for Opeibea strain. For Kisumu strain, 10 mosquitoes each were selected from the four class of insecticides with a total of 40 for each treatment. Extraction of genomic DNA from whole body of *Anopheles* mosquito without the wings was done using the Cetyl Trimethyl Ammonium Bromide (CTAB) method, as explained in Wagner *et al.* (1987) with slight modification.

The extraction buffer (2 % CTAB) was prepared using a mixture of 1 M Tris HCl pH 8.0, 0.5 M EDTA, 1.4 M NaCl, 2 % cetyl trimethyl ammonium bromide (Appendix I) which were vortexed and autoclaved. The individual carcass of the whole mosquito was put into an autoclaved 1.5 ml microcentrifuge tube and homogenized in 200 μl of 2 % cetyl trimethyl ammonium bromide (2 % CTAB) with the aid of a Konte’s pestle. The homogenate was heated at 65 °C for 5 minutes with heating block. An estimate of 200 μl of chloroform was then added to the mixture, mixed per inversion and centrifuged at 12,000 rpm for 5 minutes. After this, the supernatant was moved into a new 1.5 ml Eppendorf tubes. 200 μl of isopropanol was added and mixed per inversion to the supernatant to precipitate the DNA. This was then centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded, taking care not to dislodge the DNA pellet at the bottom. 200 μl of 70 % ethanol was added to the pellet and centrifuged at 12,000 rpm for 5 minutes. Again, the supernatant was discarded, the tubes were opened and inverted over a dry paper towel and left overnight at room temperature to dry by evaporation. The DNA pellets were reconstituted in 20 μl of DNAse free water, left to stand for one hour. After which it was vortexed and centrifuged at 12,000 rpm for 2 minutes and stored as stock at -85 °C until ready to be used.

The extracted DNA samples were used as DNA template for subsequent molecular species identification.
3.7.2.2 PCR Identification of Sibling Species of the *An. gambiae s.l.*

The polymerase chain reaction (PCR) amplification protocol of Scott *et al.* (1993) and Fanello *et al.*, (2002), designed for the species identification of single specimen of *An. gambiae s.l.* was employed with slight modification. The DNA extracted from whole female mosquito samples using the process described above were used for the PCR analysis. The amplification process used one universal primer and three species-specific primers each of 20 bases (Table 3.1). The universal primer designated UN anneals to the same position on the ribosomal DNA (rDNA) of each of the five species of *An. gambiae s.l.* namely, *An. gambiae s.s.*, *An. arabiensis*, *An. melas*, *An. merus* and *An. quadriannulatus*. The other three primers used in the amplification process were species specific and were the reverse primer GA, which anneals to the *An. gambiae s.s* template; AR, which anneals to the *An. arabiensis* template; and QD, which anneals to the *An. quadriannulatus* template. The ME primer, which anneals to both the *An. merus* and *An. melas* templates the was in the protocol of Scott *et al.* (1993) but was not used in this work as *An. gambiae s.s*, *An. arabiensis* and *An. melas* are the only *An. gambiae s.l.* reported in Ghana (Appawu *et al.*, 1994, Appawu, 2005; Kristan *et al.*, 2003; Yawson *et al.*, 2004).
Table 3.1: Species-specific primer sequences of *An. gambiae s.l.* with respective melting temperatures (Tm) and expected band sizes of amplified DNA products (Scott *et al.*, 1993).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5' to 3')</th>
<th>Tm (°C)</th>
<th>Base pairs (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UN</td>
<td>GTG TGC CCC TTC CTC GAT GT</td>
<td>58.3</td>
<td>468</td>
</tr>
<tr>
<td>GA</td>
<td>CTG GTT TGG TCG GCA CGT TT</td>
<td>59.3</td>
<td>390</td>
</tr>
<tr>
<td>AR</td>
<td>AAG TGA CCT TCT CCA TCC TA</td>
<td>47.4</td>
<td>315</td>
</tr>
<tr>
<td>ME</td>
<td>TGA CCA ACC CAC TCC CTT GA</td>
<td>57.2</td>
<td>464</td>
</tr>
<tr>
<td>QD</td>
<td>CAG ACC AAG ATG GTT AGT AT</td>
<td>42.7</td>
<td>153</td>
</tr>
</tbody>
</table>

The master mix for PCR identification reaction assay was prepared on ice in Captair bio (MA 01967-USA) hud. A mastermix of 20 μl was prepared for each sample to be run for PCR. This mixture contained 12.5 μl of Green GoTaq® (Promega, USA), 0.6 μl of 10 μm of each of the oligonucleotide primers, 4.6 μl PCR-grade water and 0.5 μl of the extracted DNA. The GoTaq in the PCR-Mix comprises 1X Green GoTaq (Flexi Buffer), 25 mM MgCl₂, 0.2 mM of each dNTP and 1.25 Units of GoTaq DNA polymerase. The mixture was thoroughly mixed and then centrifuged briefly for 20 seconds before being placed in a thermocycler. The temperature profile/cycling conditions for the PCR amplification were 95 °C for 3 minutes (initial melt) followed by 35 cycles of 95 °C for 30 seconds (denaturation), 54 °C for 30 seconds (annealing), 72 °C for 1 minute (extension), and a final cycle of 72 °C for 5 minutes. For each set of reactions, a negative control, which had no DNA template, was used. The
amplification reactions were conducted using a polymerase chain reaction Express Thermal Cycler (MJ Research Inc., USA).

3.7.2.3 Gel Electrophoresis

Polymerase chain reaction (PCR) products were analyzed using gel electrophoresis. The 2 % Ethidium bromide-stained 2 % agarose gel was prepared by dissolving 3.0 g of agarose in 150 ml of 1X TAE (Tris-acetate-EDTA) buffer with the aid of a Bosch microwave (Multi air flow GR-3493QF). This was allowed to cool and stained with 2 μl of Ethidium bromide. Eight microliters (8 μl) of each amplified PCR product was loaded into the gel well created by the comb and electrophoresed in 1x Tris Acetate EDTA (TAE) buffer at 140 V for 1 hour on a Biorad power pac 3000 (SN: 277BR03754) generator. The 2 % agarose gel did not require any loading buffer since the flexi Buffer already contained a green loading dye that give the GoTaq its green colour. The gel was visualized and photographed over an ultraviolet Polaroid (UVP) dual intensity trans-illuminator using a Toyobo Tm-20 Transilluminator (SN: 102103-003, Japan) and Fas III Ds-30 (SN: 6710530, Japan). The siblings of the An. gambiae complex were identified using the PCR products obtained by comparing their mobility with a standard 100 bp ladder (Sigma, USA).

3.7.2.4 Identification of An. gambiae s.s. Molecular Forms

The identification of An. gambiae S form and An. coluzzii was done using Short Interspersed Elements-polymerase chain reaction (SINE-PCR) protocol of Santolamazza et al., (2008) with slight modifications.
The genomic DNA extracted from whole female mosquito samples using the process described above were used for the PCR analysis. The amplification process used two species-specific primers each of 20 bases (Table 3.2). The primers designated F6.1a and R6.1a were designed to amplify across the element. The approach utilized was to design specific primers pairs in the flanking regions of SINE200 insertions within M and S An. gambiae speciation islands, where a higher degree of form-specific genetic differentiation was expected.

SINE master mix PCR assay was prepared in an autoclaved Eppendorf tube in the Captair bio (MA 01967-USA) hud. A total reaction mix of 25 μl each were prepared for the samples to be run for PCR; containing 12.5 μl of Gotaq®, 1.0 μl of 10 μm of each of the oligonucleotide primers, 6.5 μl PCR-grade water and 4 μl of the extracted DNA. The mixture was thoroughly mixed and then centrifuged briefly for 20 seconds before being placed in a thermocycler. The temperature cycling conditions for the PCR amplification were 95 °C for 5 minutes (initial melt) followed by 35 cycles of 94 °C for 30 seconds (denaturation), 59 °C for 30 seconds (annealing), 72 °C for 1 minute (extension), and a final cycle of 72 °C for 10 minutes. For each set of reactions, a negative control (DNAse free water), which had no DNA template was used. The amplification reactions were conducted using an Express Thermal Cycler (MJ Research Inc., USA). were then run through using an Ethidium bromide stained 2 % agarose gel, the PCR products were run and then, visualized under ultraviolet light illumination as described in section 3.7.2.3.

**Table 3.2:** Sequences of primers used in the *An. gambiae s.s.* with their respective expected band sizes of the amplified DNA products (Santolamazza et al., 2008).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’ to 3’)</th>
<th>Expected Band size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F6.1a</td>
<td>TCGCCTTAGACCTTGCGTTA</td>
<td>479</td>
</tr>
<tr>
<td>R6.1a</td>
<td>CGCTTCAAGAATTCGATAC</td>
<td>247</td>
</tr>
</tbody>
</table>
3.8 Data Entry and Analysis

The data collected was entered into a Microsoft Excel spreadsheet and imported into GENSTAT programme version 9 and PAST software version 2.17c for analysis.

The observed difference in mean wing length of adult of larvae fed on rabbit pellet and baking yeast that was within and among the different strains were analyzed using T-test for independent two samples. Pearson Correlation was used to estimate the degree of relationship between the diets. All statistical tests and generalizations were done by assuming 95 % confidence interval and 5 % level of significance.

Insecticide susceptibility status was defined based on the WHO criteria for characterizing susceptibility, where susceptibility is defined by 24-hour post-exposure mortality greater than 98 % and resistance is defined by 24-hour post-exposure mortality less than 80 %. Regression probit analysis (Finney, 1971) was used to analyze dose/response association insecticide susceptibility tests results. Also linear log-time probit model using EPA probit analysis program, version 1.5 was used to estimate knockdown times (KDTs), that is time in minutes at which 50 % and 95 % of the mosquito populations assayed were knocked down.
CHAPTER FOUR

4.0 RESULTS

4.1 Species Composition in the Study Area

PCR amplification for mosquito species identification was successful for all the 208 *An. gambiae* s.l. processed, 104 from each population. All identified species were *An. gambiae* s.s. based on their DNA band size which was 390 base pairs for both Kisumu strain and samples collected from the study site (Figure 4.1).

![Ethidium bromide-stained 2.0 % agarose gel electrophoregram of PCR amplified rDNA sequences of An. gambiae s.s.](image)

**Figure 4.1**: Ethidium bromide-stained 2.0 % agarose gel electrophoregram of PCR amplified rDNA sequences of *An. gambiae* s.s. Lane M: 100 bp ladder; Lanes 1-22: *An. gambiae* s.s., Lane 23: *An. gambiae* s.s. positive control, Lane 24: *An. arabiensis* positive control and Lane 25: negative control.
4.1.1 *Anopheles gambiae s.s.* Molecular Forms Identified in the Study Area.

SINE-PCR method was used to further analyzed the identified *An. gambiae s.s.* so as to determine their molecular forms. Out of the total, 2 were identified as *An. coluzzii* forms, 1 was hybrid and 205 were *An. gambiae s.s.*

For Kisumu strain, all the analyzed 104 *An. gambiae s.s.* were only *An. gambiae* S forms, *An. coluzzii* forms were not found. However, for Opeibe strain, 2 were *An. coluzzii* (formerly M form), 1 was a Hybrid while 101 were *An. gambiae s.s.* (Figure 4.2).

![Species composition of both Kisumu and Opeibea.](image)

**Figure 4.2:** Species composition of both Kisumu and Opeibea.

Identification was done by comparing their band sizes with that of the 100 bp molecular weight ladder (Lane M). *Anopheles coluzzii* were characterized by a single fragment of 479 bp.
bp, the *An. gambiae s.s.* by 249 bp (Figure 4.3) while the hybrid (M/S forms) have two fragments 249 bp and 479 bp (Figure 4.4).

**Figure 4.3:** Ethidium bromide-stained 2.0 % agarose gel electrophoregram of rDNA_PCR bands for *An. gambiae s.s.* molecular forms Lane M: 100 bp ladder; Lanes 1-11, 13-19, 21-25: molecular S forms of *An. gambiae s.s.*, Lanes 12 and 20: *An. coluzzii*; Lane 26: negative control; Lane 27: *An. coluzzii* positive control; Lane 28: *An. gambiae s.s.* positive control; Lane 29: Hybrid positive control.

**Figure 4.4:** Ethidium bromide-stained 2.0 % agarose gel electrophoregram of rDNA_PCR bands for *An. gambiae s.s.* molecular forms. Lane M: 100bp ladder; Lanes 1-8, 10-19: *An. gambiae s.s.*, Lane 9: hybrid (m/s form).
4.2 Fitness level of emerged *Anopheles gambiae s.s.* Species

T-test for independence of two samples was used to compare mean wing length of the two groups of *Anopheles* strain (Kisumu and Opeiba n = 1040 adults mosquitoes per strain) which were fed on rabbit pellet and baking yeast (520 larvae per diet per strain). A statistically non-significant difference (p = 0.738) was observed between the mean wing length among adults of the two strains fed as larvae on baking yeast (2.89 ± 0.0072 mm) and (2.89 ± 0.0068 mm), (Appendix II), while a statistically significant difference (p = 0.009) was observed in the wing length of adults fed as larvae on the rabbit pellet (2.84 ± 0.0072 mm) and (2.81 ± 0.0076 mm), in the Kisumu and Opeiba strain respectively (Figure 4.5, Appendix III).

![Figure 4.5](image)

**Figure 4.5:** Mean wing length of *Anopheles gambiae s.s.* fed on the two different diets (baking yeast and rabbit pellet).
There was statistically significant difference \( p < 0.001 \) in the mean wing length. For Opeibea strain, the mean wing length for adult mosquitoes that emerged from larvae fed with rabbit pellet was \( 2.81 \pm 0.0076 \text{ mm} \) while the mean length for baking yeast was \( 2.89 \pm 0.0068 \text{ mm} \), \( p < 0.001 \) (Appendix IV).

For Kisumu strain, the mean wing length for adult mosquitoes that emerged from larvae fed with rabbit pellet was \( 2.84 \pm 0.0072 \text{ mm} \) while the mean length for baking yeast was \( 2.89 \pm 0.0072 \text{ mm} \), \( p < 0.001 \) (Appendix V).

### 4.2.1 Fitness level using pupal weight

A higher mean pupal weight \( (94.67 \pm 8.229 \text{ g}) \) was observed when pupae were fed on rabbit pellet while pupae fed with baking yeast had a lower mean pupal weight \( (56.67 \pm 3.283 \text{ g}) \). The mean weight of pupae enhanced by each type of food was significantly \( (df=5, p=0.002) \) different (as shown in Appendix IX).

### 4.2.2 Observations on development of the larvae under the influence of the two feeding regime

It was observed that the two different treatments expressed equal effect on development in terms of growth of the larva from first instar to second instar hence growth rate for all the larvae at the second instar stage were the same. It was however observed that the various feed affected the development of the larvae from second instar to fourth instar larval stages in different ways. The development of larvae from second instars to pupae was observed to be moderately faster when fed with rabbit pellet than baking yeast.

Baking yeast did affect the development of the larva especially at fourth instar. It was also observed that an average of one to two pupa could not emerge to adult stage and mortality was high when larvae were fed with yeast which could be attributed the formation of oily
scum on the surface of the rearing water (see Appendix VI) thereby, preventing influx of oxygen for respiration and reducing the availability of nutrients.

### 4.3 Insecticide Susceptibility Status of *An. gambiae* s.s. Species

In overall, deltamethrin had the highest knockdown (KD60) and 24 hours’ mortality rate (MT24), while the insecticide with the lowest knockdown and 24 hours’ mortality rate was Bendiocarb.

For the Kisumu strain adult mosquitoes from larvae fed on baking yeast; Deltamethrin recorded the highest knockdown rate of 100 %, followed by 93.8 % for DDT, 91.3 % for Malathion and the lowest was 57.5 % for Bendiocarb (Figure 4.6).

**Figure 4.6:** Susceptibility assay for adults which emerged from larvae fed with baking yeast (Kisumu strain).

With the rabbit pellet; Deltamethrin, Malathion and Bendiocarb also had the highest 24 hours’ mortality rate of 100 % while 98.8 % for DDT had the lowest. Deltamethrin recorded
the highest knockdown rate of 100 %, followed by 88.8 % for Malathion, 57.5 % for DDT and the lowest was 46.3 % for Bendiocarb (Figure 4.7).

![Knockdown and Mortality Graph]

**Figure 4.7:** Susceptibility assay for adults which emerged from larvae fed with rabbit pellet (Kisumu strain).

For the Opeibea strain the adult mosquitoes from larvae fed on baking yeast, the observed 24 hours’ mortality rate for Deltamethrin, Malathion, DDT and Bendiocarb were 87.7 %, 82.5 %, 34.1 % and 21.3 % respectively. Deltamethrin recorded the highest knockdown rate of 71.6 %, followed by 23.2 % for DDT, 7.5 % for Malathion and the lowest was 5.0 % for Bendiocarb (Figure 4.8). With those fed on rabbit pellet, the observed 24 hours’ mortality rate for Deltamethrin, Malathion, DDT, and Bendiocarb were 86.3 %, 74.1%, 26.8 % and 20.0 % respectively. Deltamethrin recorded the highest knockdown rate of 61.3 %, followed by 15.9 % for DDT, 6.2 % for Malathion and the lowest was 3.8 % for Bendiocarb. (Figure 4.9).
Figure 4.8: Susceptibility assay for adults which emerged from larva fed with baking yeast (Opeibea strain).

Figure 4.9: Susceptibility assay for which adults emerged from larva fed with rabbit pellet (Opeibea strain).
4.3.1 Knockdown Times of *An. gambiae* s.s. after one hour of Exposure

The estimated times at which 50 % and 95 % of the Kisumu strain of *An. gambiae* population exposed to the four different insecticides were knocked down are given in Table 4.1. 0.05 % Deltamethrin acted faster than the other insecticides in knocking down 50 % and 95 % of *An. gambiae* mosquitoes. This was followed by 0.4 % DDT, 5 % Malathion and then 0.1 % Bendiocarb. For adult mosquitoes from larvae fed on baking yeast and rabbit pellet, the different responses to Deltamethrin and DDT were statistically significant (p < 0.05). Here, differences between values were considered significant (p < 0.05) if the respective 95 % confidence intervals did not overlap. Whilst for Malathion and Bendiocarb there was no statistically significant difference because there was an overlap between the confidence intervals for the KDT$_{50}$ and KDT$_{95}$ of both baking yeast and rabbit pellet.
Table 4.1: Knockdown times in hours (with 95 % confidence intervals) of Kisumu strain of *An. gambiae s.s.* to four different insecticides tested.

<table>
<thead>
<tr>
<th>Insecticides</th>
<th>Diets</th>
<th>No. exposed</th>
<th>Slope ± SE</th>
<th>KDT&lt;sub&gt;50&lt;/sub&gt; (hours)</th>
<th>95 % C.I KDT&lt;sub&gt;50&lt;/sub&gt; (hours)</th>
<th>95 % C.I KDT&lt;sub&gt;95&lt;/sub&gt; (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 % Deltamethrin</td>
<td>Baking Yeast</td>
<td>80</td>
<td>5.25 ±0.41</td>
<td>0.2</td>
<td>0.22 – 0.25a</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>Rabbit Pellet</td>
<td>80</td>
<td>4.43 ±0.30</td>
<td>0.31</td>
<td>0.29 – 0.34b</td>
<td>0.74</td>
</tr>
<tr>
<td>0.4 % DDT</td>
<td>Baking Yeast</td>
<td>80</td>
<td>4.93 ±0.33</td>
<td>0.46</td>
<td>0.43 – 0.48c</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>Rabbit Pellet</td>
<td>80</td>
<td>3.90 ±0.37</td>
<td>0.83</td>
<td>0.76 – 0.92d</td>
<td>2.19</td>
</tr>
<tr>
<td>0.1 % Bendiocarb</td>
<td>Baking Yeast</td>
<td>80</td>
<td>3.35 ±0.31</td>
<td>0.80</td>
<td>0.76 – 0.90d</td>
<td>2.49</td>
</tr>
<tr>
<td></td>
<td>Rabbit Pellet</td>
<td>80</td>
<td>3.33 ±0.37</td>
<td>1.01</td>
<td>0.90 – 1.18d</td>
<td>3.15</td>
</tr>
<tr>
<td>5 % Malathion</td>
<td>Baking Yeast</td>
<td>80</td>
<td>7.06 ±0.56</td>
<td>0.62</td>
<td>0.59 – 0.65e</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>Rabbit Pellet</td>
<td>80</td>
<td>6.16 ±0.93</td>
<td>0.53</td>
<td>0.45 - 0.61ce</td>
<td>0.98</td>
</tr>
</tbody>
</table>

KDTs with the same alphabet in the same column are not significantly different.

For Opeibea strain in Table 4.2, there was no statistical significant (p > 0.05) difference between response of adult mosquitoes fed as larvae on baking yeast and rabbit pellet to the four different insecticides tested. There was an overlap between the confidence intervals of both KDT<sub>50</sub> and KDT<sub>95</sub> in all cases. However, in both Kisumu and Opeibea strains it was observed that baking yeast had a lower KDT<sub>50</sub> and KDT<sub>95</sub> when compared to rabbit pellet for all the insecticides except Malathion. Hence, there is significantly faster induction of knockdown by the insecticide for those fed on baking yeast than those fed on rabbit pellet.
Table 4.2: Knockdown times in hours (with 95 % confidence intervals) of Opeibea strain of *An. gambiae s.s.* to four different insecticides tested.

| Insecticides | Diets       | No. exposed | Slope ± SE | KDT<sub>50</sub> (hours) | 95 % C.I KDT<sub>50</sub> (hours) | KDT<sub>95</sub> | 95 % C.I KDT<sub>95</sub> 
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 % Deltamethrin</td>
<td>Baking Yeast</td>
<td>80</td>
<td>3.24 ±0.62</td>
<td>0.57</td>
<td>0.58 – 0.88a</td>
<td>1.79</td>
<td>1.13 – 5.67ab</td>
</tr>
<tr>
<td></td>
<td>Rabbit Pellet</td>
<td>80</td>
<td>3.47 ± 0.51</td>
<td>0.69</td>
<td>0.43 – 0.77a</td>
<td>2.07</td>
<td>1.43 – 4.37a</td>
</tr>
<tr>
<td>0.4 % DDT</td>
<td>Baking Yeast</td>
<td>80</td>
<td>2.99 ±0.51</td>
<td>1.70</td>
<td>1.33 – 2.72b</td>
<td>6.03</td>
<td>3.49 – 17.93abc</td>
</tr>
<tr>
<td></td>
<td>Rabbit Pellet</td>
<td>80</td>
<td>2.66 ±0.53</td>
<td>2.20</td>
<td>1.56 – 4.68b</td>
<td>9.18</td>
<td>4.41 – 47.79bc</td>
</tr>
<tr>
<td>0.1 % Bendiocarb</td>
<td>Baking Yeast</td>
<td>80</td>
<td>1.82 ± 0.64</td>
<td>7.3</td>
<td>2.7 – 2347.7b</td>
<td>58.3</td>
<td>9.4 – 1027878.3c</td>
</tr>
<tr>
<td></td>
<td>Rabbit Pellet</td>
<td>80</td>
<td>1.99 ±0.93</td>
<td>8.1</td>
<td>2.49 – ∞b</td>
<td>54.66</td>
<td>6.83 – ∞c</td>
</tr>
<tr>
<td>5 % Malathion</td>
<td>Baking Yeast</td>
<td>80</td>
<td>2.76 ±1.19</td>
<td>4.03</td>
<td>1.87 – 26565.22b</td>
<td>15.85</td>
<td>3.97 – 164676334 .93abc</td>
</tr>
<tr>
<td></td>
<td>Rabbit Pellet</td>
<td>80</td>
<td>2.82 ±1.09</td>
<td>3.59</td>
<td>1.83 – 380.81b</td>
<td>13.71</td>
<td>3.96 – 86598.38abc</td>
</tr>
</tbody>
</table>

KDTs with the same alphabet in the same column are not significantly different.

For both Opeibea and Kisumu strains, there was no overlap in the confidence intervals between adults from larvae fed on baking yeast and rabbit pellet showing that there is statistical significant difference between the two strains.
4.3.2 Mortality Rates of *An. gambiae s.s.* after 24 hours Exposure

The classification of resistance or susceptibility status of *An. gambiae* mosquitoes from the study sites for the four insecticides was based on the criteria of WHO (2013). Figure 4.11 shows the average mortality rates of *An. gambiae s.s.* from the study sites after 24 hours post-exposure to the four concentrations of insecticides. Tested mosquitoes samples from the study area were highly resistant to Bendiocarb (average mortality of 20 %), as compared to the rest (with average mortality ranging from 30.5 % for DDT, 78.3 % for Malathion to 87 % for Deltamethrin. No corrected mortality was made because the control mortality was below 5 % in all the four insecticides used.

For Kisumu strain adult mosquitoes from larvae fed on baking yeast and those fed on rabbit pellet; Deltamethrin, Malathion and Bendiocarb had the highest 24 hours’ mortality of 100 % while 98.8 % for DDT was the lowest.

The Opeibea strain adult mosquitoes from larvae fed on baking yeast had 24 hours mortality of 87.7 % for Deltamethrin and Bendiocarb had the lowest mortality of 20 %. Whilst those fed on rabbit pellet had 24 hours mortality of 86.3 % for Deltamethrin and Bendiocarb had the lowest mortality of 20 %.
Figure 4.10: Overall average 24 hours mortality for four (4) insecticides for both Kisumu and Opeibea strain.

### 4.4: Relationship between Fitness and Phenotypic Resistance

Pearson correlation was used to measure the association between fitness and phenotypic resistance. For the Kisumu strain, the correlation analysis indicates a strong but not significant association between fitness and phenotypic resistance ($r = 0.915$, $R^2 = 84\%$, $P = 0.085$) and ($r = 0.889$, $R^2 = 79\%$, $P = 0.111$) respectively for those adults fed as larvae with baking yeast and rabbit pellet. For Opeibea strain, the correlation analysis indicates a strong but not significant association between fitness and phenotypic resistance ($r = 0.811$, $R^2 = 66\%$, $P = 0.188$) and ($r = 0.751$, $R^2 = 56$, $P = 0.249$) respectively for adults from larvae fed on baking yeast and rabbit pellet (refer to Appendix III). Generally, there is strong positive correlation and strong but not significant association with $R^2$ greater than 50% in all cases for both Kisumu and Opeibea strains. Hence there is inconclusive evidence about the significance of the association between fitness and phenotypic resistance.
4.5: Resistance Ratio of the *An.gambiae* s.s. from the Study Area.

Resistance ratio (RR) were estimated by dividing the mortality of the resistant (Opeibea) strain by the mortality of the susceptible Kisumu strain. When RR of the four different insecticides to Opeibea strain were compared to the reference population (Kisumu strain), the result showed that resistance ratio for adults that emerged from larvae fed with baking yeast were 0.88, 0.83, 0.35 and 0.2 for Deltamethrin, Malathion, DDT and Bendiocarb respectively. Also, for adults that emerged from larvae fed with rabbit pellet, the result showed that their resistance ratio was 0.86, 0.74, 0.27 and 0.2 for Deltamethrin, Malathion, DDT and Bendiocarb respectively. Hence, the level of resistance of Deltamethrin, Malathion, DDT and Bendiocarb insecticides in Opeibea strain was greater than in the Kisumu strain.
CHAPTER FIVE

5.0 DISCUSSION

5.1 Fitness of Adults fed as Larvae on Different Diets.

The body size of field populations of several mosquito species varies considerably (Nasci and Mitchell, 1994). An overall comparison of the mean wing length (mm) was made between Anopheles strains from Kisumu and Opeibea which were fed with rabbit pellet and baking yeast during their larval stage. There was no significant difference observed between the mean wing length among the two strains for baking yeast (p = 0.738). This result confirmed earlier work by Okoye et al., (2007), who observed that certain fitness components such as fecundity, larval and pupal survival did not differ significantly between resistant and susceptible strains of An. funestus.

A significant difference was observed for those adults fed as larvae on the rabbit pellet (p = 0.009) among the two strains. This observation was confirmed to the study by Huho et al. (2007), who also observed significant difference in the energetic reserves, body size and survival in laboratory-bred and wild adult Anopheles gambiae when fed on the same meal.

Within the strains, the mean wing length of adult mosquitoes that emerged from larvae fed with baking yeast was significantly different (p < 0.001) from that of adults reared as larvae on rabbit pellet for both Kisumu and Opeibea strains. This could be due to the fact that the adult fitness, body size and energetic reserves frequently rely on the nutritional constituent or larval diet (Timmermann and Briegel, 1999; Telang et al., 2002; Khan, et al., 2013) and to some point protein components of the diet (Lang et al., 1965).

The nutritional constituents present and the state of larval diets influences the aquatic life stage of the mosquitoes. Mosquito in its larval stage is known to need at least 14 essential
amino acids which includes proline, serine, threonine, glycine, histidine, asparagine, leucine, lysine, methionine, arginine, isoleucine, phenylalanine, tryptophan, and valine. Sugars, sterols, polyunsaturated fatty acids (PUFAs), nucleotides are required for larval development, survival and adult flight, with a little concentration of essential vitamins (Damiens et al., 2012) and salt (Trager, 1936). When found in the natural habit, the mosquito larvae feed on debris, algae, phytoplankton and bacteria in their natural form, therefore are easily digestible. The mean wing length of adults from larvae fed on baking yeast was significantly higher (p < 0.001) than those fed on rabbit pellet. However, larvae fed on rabbit pellet better survived when compared to those fed on baking yeast. Also, in a preliminary study pupal weight of larvae fed on rabbit pellet was significantly higher (p < 0.001) than that fed on baking yeast. It was observed that an average of one to two pupae could not emerge to adult stage and mortality rate was high when larvae were fed with baking yeast. The mortality could be attributed to the formation of scum on the surface of the rearing water thereby, prevented easy flow of oxygen for respiration and reduced the availability of nutrients to the developing larvae of An. gambiae s.s. This corroborated with the findings by Khan et al., (2013) who also observed that the addition of baker’s yeast did not lead to any improvement in the measured parameters and attributed the scum formation observed in yeast-added diets to larval mortality and delayed pupal emergence.

The mortality was reasonably low for mosquito larvae which were fed with rabbit pellet and their development from second instar larval to pupae was faster compared to when the larvae were fed with baking yeast. This accounted for an observed high pupation rate. Valuation of the components of the different food regime and its nature, indicated that the rabbit pellet is basically made with wheat in their natural state and consist the highest percentage of fiber (12 %) (refer to Appendix VI). Hence, they are further easier to digest by the mosquito larvae. The development of larvae which fed on rabbit pellet was observed to be relatively more
rapid than those fed on the baking yeast with minimal larval mortality. The observation confirms studies done by Khan, (2010); Kivuyo et al., (2014) and Khan et al., (2013), in which feeds which essential ingredient is cereal in their natural or not refined form were enriched by adding a mixture of other natural food ingredients.

Equal expression of the effect of each meal on the development of larvae in terms of growth from first instar to second instar was generally observed. This could be due to the nutritional requirement of the growing larvae. The quantity of food, as well as the nutrients demand by a growing larva increases to about five times (x5) the quantity of food consumed at first instar to fourth instar stage. Hence growth rate and size for all the larvae at second instar was the same in all diets, since the growing larva relatively had minimal nutritional requirements. However, it was observed that the various effects of each diet on the development of the larva was structurally evident or seen from third instar to pupation in difference ways.

The findings of this study suggest that the two different diets have varying impact on some fitness components of the immature stages of *Anopheles gambiae s.s*. some of these components were wing length, larval survivorship and pupal weight. The influence of the food source also depended on the type, nutritional content and their nature.

### 5.2 Species Composition of the *Anopheles gambiae* complex

Malaria continues to be a main public health menace in Sub-Saharan Africa as the most effective vector and hyper-endemic in Ghana. della Torre et al. (2001) opined that *An. gambiae s.l.* has become more anthropophilic. In the present study, *An. gambiae s.l.* was the major vector collected. This discovery was coherent with earlier studies which showed that this species was the major malaria vector in Greater Accra Region of Ghana (Appawu, 2001;
The success of this species as a malaria vector could include the fact that they are highly anthropophagic and are adapted extensively to a range of environmental conditions (della Torre et al., 2001; Kabula et al., 2011).

Molecular identification of the sibling species of *An. gambiae* found in the area studied revealed *An. gambiae s.s.* as the only member present. This observation was similar to earlier studies in some communities in the Kpone-on-Sea of Coastal Ghana (Tchouassi et al., 2012), in Kintampo in the middle belt of Ghana (Dery et al., 2010) where *An. gambiae s.s* was the only sibling species of *An. gambiae s.l.* found elsewhere in Ghana and Africa at large.

The *An. coluzzii* and *An. gambiae s.s.* were found to occur in sympatry in the study sites, with a relatively high frequency of 98.56% of *An. gambiae s.s.* and lower frequency of *An. coluzzii* 0.96%. The observation then confirmed earlier studies described by Achonduh et al. (2008), who also sampled vegetable farms within the Airport Residential area along the same drainage which stretches from the Airport Residential area through Opeibea to Maamobi, Nima and finally united with the Odaw River before entering the sea. (*An. coluzzii* is well-known to be connected with flooded/irrigated sites, characterized by extensive rice cultivation, whereas the *An. gambiae s.s.* is typically found in rain-dependent breeding sites (Diabate’ et al., 2005; de Souza et al., 2010)). Thus, *An. gambiae s.s.* could have predominated because the farmers usually used irrigated water to cultivate vegetables throughout the year. They however depend majorly on rain water during the raining season and sometimes in the airport drainage during the dry season. They dig trenches which is filled with water whenever rain falls and is later used to water their farms. These trenches serve as a breeding site for mosquitoes thereby serving as rain-dependent breeding sites for the S forms.
One (1) hybrid M/S form (0.48 %) was identified in the study area. This was supported by previous studies that indicated low level of hybridization between *An. gambiae* s.s. and *An. coluzzii* at frequencies ranging from 0.26 % (della Torre *et al*., 2001) to 0.71 % (Taylor *et al*., 2001).

### 5.3 Insecticide Susceptibility Status of *An. gambiae*

The insecticide susceptibility test on the populations of *An. gambiae* in the Opeibea area revealed high levels of resistance to the four insecticides tested, which were selected from four different classes of insecticides. Bases for characterizing insecticide susceptibility result were on WHO criteria, where susceptibility is described as mortality of 98-100 % 24 hours post-exposure; suspected resistance as mortality between 90-97 % and resistance as mortality less than 90 % 24 hours post-exposure (WHO, 2013). In the study, mortality less than 90 % were observed in all cases for the Opeibea strain while mortality of 98-100 % were observed for the Kisumu strain which was expected for the fact that Kisumu is the control susceptible laboratory strain. Such low mortalities for the Opeibea strain which suggest strong resistance rates in the *An. gambiae* populations in this study are consistent with the levels of insecticide resistance reported from previous studies in the Greater Accra Region of Ghana (Adeniran *et al*., 2009; Kabula *et al*., 2011; Adasi and Hemingway, 2008). For both diets, resistance to Deltamethrin was high with mortality of 87.7 % and 86.3 % for baking yeast and rabbit pellet respectively. This observation was in line with earlier studies in the Greater Accra Region of Ghana by Achonduh *et al*., (2008) which reported that resistance against pyrethroids. In contrast to this, Kristan *et al*. (2003) reported that *An. gambiae* s.s. remained susceptible to Permethrin and Deltamethrin despite the use of pyrethroids for agricultural intents in South-Western Ghana.
The observed high general resistance to pyrethroid and other classes of insecticide is a risk to malaria control efforts since it underpinned the belief that resistance to insecticide and prevalence of malaria cases is reasonably high in rural areas when likened to urban centers. The wide-ranging high resistance of insecticide has a severe consequence for malaria control programmes particularly Long Lasting Insecticide-treated Net (LLIN) and Indoor Residual Spraying (IRS).

The *An. gambiae s.s.* population in Opeibea area revealed high resistance to DDT with mortality of 34.1% and 26.8% for adults from larvae fed on baking yeast and rabbit pellet respectively. This finding follows a similar trend with other studies (Achonduh *et al.*, 2008; Hunt *et al.*, 2011). The use of DDT in agriculture in Ghana has been abolished since the 1970s and its use in vector control has not been reported by any study after its ban, thus its persistent resistance could be as a result of either illegal use in pest control (Avicor *et al.*, 2011) or its accumulation and long persistence in the environment. In some cases, cross-resistance can occur between pyrethroids and DDT due to their shared mode of action.

Bendiocarb, on the other hand, revealed very high resistance status in the Opeibea strain with the same mortality of 20.0% for both adults from larvae fed on baking yeast and rabbit pellet. This is similar to what was reported by Hunt *et al.*, (2011). This carbamate has been extensively used in agricultural pest control over the years. Since it has similar mode of action with organophosphates, one of which has shown resistance on *An. gambiae s.s.* in this same study area, Hence, may contribute to the development of cross resistance.

Malathion had a mortality of 82.5% and 74.1% for adults fed as larvae on baking yeast and rabbit pellet respectively which indicates high resistance of the Opeibea strain. The observed resistance pattern in this study was similar to the study on *An. gambiae s.l.* in Southern Ghana (Hunt *et al.*, 2011) which pointed out that a population from Ahafo was resistant to
malathion. It was however, contrary to research findings of Achondhu et al., (2008) which showed susceptibility status of *An. gambiae* of Airport and Korle-Bu areas to Malathion, an organophosphate.

Generally, the development of insecticide resistance may be attributed to insecticide usage in the area. The present study did not establish insecticide usage pattern in the study areas but it is known that farmers who cultivate vegetables make use of Deltamethrin and other insecticides that mimic the effect of carbamates, organophosphates and pyrethroids classes of insecticides throughout the growing season to control a wide range of agricultural pests. Studies conducted in the Greater Accra Region in Southern Ghana (Adasi et al., 2000) and in the Ashanti Region of the middle belt of Ghana (Stiles-Ocran et al., 2007) have established a positive correlation between development of resistance in *An. gambiae s.l.* and insecticide use.

The observation in the present study showed that the adults both for Kisumu and Opeibea strains fed as larvae on baking yeast had a lower KDT$_{50}$ and KDT$_{95}$ when compared to those fed on rabbit pellet for all the insecticides except Malathion. This corroborates the earlier study by Kulma et al., (2013) who observed that larvae nutrition had an effect on the expression of insecticide-resistance even though the effect was minimal. Although the confidence intervals overlap did not highlight any significant differences in KDT$_{50}$ and KDT$_{95}$ between any of the baking yeast and rabbit pellet cohorts, the baking yeast in both Kisumu and Opeibea strains consistently required higher time than its rabbit pellet counterpart in order to produce the same level of mortality.
5.4: Relationship between Fitness and Phenotypic Resistance

The strong positive Pearson correlation between fitness and resistance in both Kisumu and Opeibea strains was not significant, so the evidence is inconclusive. Therefore, in *Anopheles gambiae s.s.* a well-balanced larval diet could positively enhance the fitness might also improve the resistance. This might be because of the significance of resistance for the survival of mosquitoes. The resistance mechanism usually involves intensified production of metabolic enzymes and probably or presumably utilizes extra resources. It was observed that the resistance of both Opeibea and Kisumu strains was affected by larval diet. This observation partly contrast with what was reported from an earlier study by Kulma *et al.*, (2013) who observed that the resistance of ZANU (resistant) mosquitoes was not affected by larval diet, whilst Kisumu mosquitoes were less resistant when fed less as larvae. This probably suggest that both strains of Opeibea and Kisumu are very sensitive to environmental variation. Hence, in order to appreciate the expression of insecticide resistance better understanding of environmental variation is inevitable.
CHAPTER SIX

6.0 CONCLUSION, LIMITATIONS OF THE STUDY AND RECOMMENDATIONS

6.1 Conclusion

In this study, *An. gambiae s.s.* was identified to be the major malaria vector of the *An. gambiae s.l.* in Opeibea area. *An. gambiae s.s.* and *An. coluzzii* were sympatric at the study site but *An. gambiae s.s.* was predominant and only one hybrid of the molecular forms was observed. On the impact of larval diet on fitness, the results indicated that adults fed as larvae on baking yeast had the highest mean adult body size (wing length). However, adults fed as larvae on rabbit pellet had the highest positive impact which was evident in the pupal weight in a preliminary study, larval survivorship and adult response to insecticides resistance assay.

The results obtained in the present study on insecticide resistance showed that *An. gambiae s.s.* populations in Opeibea area were resistant to the four insecticides selected from each class of insecticides. The evolution of resistance in the *An. gambiae s.s.* in this study area could partly be due to the recent widespread use of pyrethroids for agricultural and domestic insect control purposes and partly due to cross-resistance developing from insecticides with similar mode of action.

Understanding the impact of larval diet on adult fitness in insectary, is very essential for mass rearing of laboratory colony of mosquitoes in order to obtain fit adult population. Fitness and robustness of the adult colony should be properly considered any time there is change in the diet so as to have fit mosquitoes for various experimental studies particularly in insecticide resistance monitoring. Since insecticide susceptibility of *Anopheles gambiae s.l.* population had an impact on the fitness of the adult mosquito fed on various diets. Consequently, the
usage of rabbit pellet in the laboratory rearing of the mosquito larval will aid to make sure that relative fitter mosquitoes are used in the determination of susceptibility to insecticide.

6.2 Limitations and Recommendations of the Study

Fitness was measured using wing length and pupal weight. Other fitness components such as fecundity, pupation rate, adult longevity and larval survivorship were not measured and thus, should be explored in assessing fitness in further studies.

It is recommended that in order to associate the level of resistance between different studies or sites, larval diets need to be considered. Resistance mechanisms associated with resistance were not tested in this research. It may be necessary to evaluate over time if fitness level of the mosquito vectors could be influence the resistance profile of the species. Future investigations at understanding resistance of insecticide expression should consider the impact of environmental variation. Such facts would significantly advance our perception of evolution of resistance and could advice on strategies for vector control initiatives.
REFERENCES


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Detection of the East and West African _kdr_ mutation in _Anopheles gambiae_ and _Anopheles arabiensis_ from Uganda using a New Assay based on FRET/Melt Curve analysis. _Malaria Journal._ 5: 16.


APPENDICES

Appendix 1

Molecular Biology Techniques

Preparation of Standard Solutions used in Molecular Biology Studies.

Standard solutions used were prepared using sterile double distilled water (sddH20) and autoclaved where appropriate at 121 lb/sq. for 15 minutes (Hirayama autoclave, Tokyo). The solutions include:

a. Solutions for Deoxyribonucleic Acid (DNA) Extraction

Extraction Buffer: 1 M Tris HCl, pH 8.0, 0.5M EDTA, 1.4 M NaCl, 2 % cetyltrimethyl ammonium bromide.

Chloroform: Stock solution was used as ordered from Sigma-Aldrich USA.

Isopropanol: Stock solution was used as ordered from Sigma-Aldrich USA.

70 % alcohol: 70 ml of absolute alcohol was added to 30 ml of sterile double distilled water making a volume of 100 ml. The solution was then stored at room temperature.

PCR Primers

Primers used were diluted as specified by the Manufacturers, Eurofins Genomics, USA. 117
b. Solutions for Electrophoresis

10 X TAE Buffer: 242 g Tris Base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH was adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000 ml with sddH20.

Ethidium bromide, EtBr (10 mg/ml): 1 g of EtBr was completely dissolved in 100 ml sddH20 and stored in a dark bottle in the dark at room temperature.

2 % Agarose Gel

This was prepared by dissolving 3 g of the Agarose powder in 150 ml 1× TAE buffer and microwaved to dissolve the powder and then cooled under running tap water. Then 2.0 μl (10 mg/ml) EtBr was added and the beaker stirred to mix thoroughly. The solution was then poured in a gel mould (Mini gel system, BIORAD, USA) with combs of <1 mm and left for about 15 minutes to solidify. The combs were removed and wells created in which samples were loaded.

DNA molecular weight size marker: The 100 bp molecular weight size marker was obtained from sigma, USA and was diluted according to the manufacturer’s recommendations and used as standard for the experimentation. For the 100 bp ladder, the first band size is 100 bp, the next ones measure 200, 300, 400, 500, 600, 700, 800, 900 and 1000 base pairs.
Appendix II

T-test for Independent Two Samples for Baking Yeast (among Strain)

Analysis of the wing length between the adults fed as larvae on baking yeast for both Kisumu and Opeibea Strain.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Minimum</th>
<th>Mean</th>
<th>Maximum</th>
<th>Values</th>
<th>Missing</th>
</tr>
</thead>
<tbody>
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<td>Kisumu_BY</td>
<td>2.82</td>
<td>2.891</td>
<td>2.94</td>
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<td>0</td>
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<tr>
<td>Opeibea_BY</td>
<td>2.84</td>
<td>2.894</td>
<td>2.96</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

Two-sample t-test

**Variates:** Kisumu Strain_Baking Yeast (BY), Opeibea_Baking yeast (BY).

Test for equality of sample variances

Test statistic $F = 1.13$ on 23 and 23 d.f.

Probability (under null hypothesis of equal variances) = 0.78

**Summary**

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<thead>
<tr>
<th>Sample</th>
<th>Size</th>
<th>Mean</th>
<th>Variance</th>
<th>Standard deviation</th>
<th>Standard error of mean</th>
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<tbody>
<tr>
<td>Kisumu Strain_BY</td>
<td>24</td>
<td>2.891</td>
<td>0.001251</td>
<td>0.03538</td>
<td>0.007221</td>
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<tr>
<td>Opeibea Strain_BY</td>
<td>24</td>
<td>2.894</td>
<td>0.001112</td>
<td>0.03335</td>
<td>0.006808</td>
</tr>
</tbody>
</table>

Difference of means: -0.00333

Standard error of difference: 0.00992
95% confidence interval for difference in means: (-0.02331, 0.01664)

Test of null hypothesis that mean of Kisumu Strain_BY is equal to mean of Opeibea Strain_BY

Test statistic $t = -0.34$ on 46 d.f.

Probability = 0.738
Appendix III

T-test for Independent Two Samples for Rabbit Pellet (among Strain)

Analysis of the wing length between the adults fed as larvae on rabbit pellet for both Kisumu and Opeibea Strain.

<table>
<thead>
<tr>
<th>Identifier</th>
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<tbody>
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<td>Opeibea_RP</td>
<td>2.76</td>
<td>2.814</td>
<td>2.94</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

Two-sample t-test

**Variates:** Kisumu Strain_Rabbit Pellet (RP), Opeibea Strain_Rabbit Pellet (RP).

Test for equality of sample variances

Test statistic $F = 1.13$ on 23 and 23 d.f.

Probability (under null hypothesis of equal variances) = 0.77

**Summary**

<table>
<thead>
<tr>
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<th>Variance</th>
<th>Standard deviation</th>
<th>Standard error of mean</th>
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</thead>
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<tr>
<td>Kisumu Strain_RP</td>
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<td>2.843</td>
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<td>2.814</td>
<td>0.001399</td>
<td>0.03741</td>
<td>0.007636</td>
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</tbody>
</table>

Difference of means: 0.0287

Standard error of difference: 0.0105
95% confidence interval for difference in means: (0.007646, 0.04985)

Test of null hypothesis that mean of Kisumu Strain_RP is equal to mean of Opeibea Strain_RP

Test statistic $t = 2.74$ on 46 d.f.

Probability $= 0.009$
Appendix IV

T-test for Independent Two Samples (Opeibea Strain)

Analysis of the wing length between the adults fed as larvae on baking yeast and those fed on rabbit pellet.

<table>
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<td>2.814</td>
<td>2.940</td>
<td>24</td>
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</tr>
</tbody>
</table>

Two-sample t-test

**VARIATES:** Rabbit pellet_wing length (WL), Baking yeast_wing length (WL).

Test for equality of sample variances

Test statistic $F = 1.26$ on 23 and 23 d.f.

Probability (under null hypothesis of equal variances) = 0.59

Summary

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<td>Rabbit pellet_WL</td>
<td>24</td>
<td>2.814</td>
<td>0.001399</td>
<td>0.03741</td>
</tr>
<tr>
<td>Baking yeast_WL</td>
<td>24</td>
<td>2.894</td>
<td>0.001112</td>
<td>0.03335</td>
</tr>
</tbody>
</table>

Difference of means: -0.0800

Standard error of difference: 0.0102
95% confidence interval for difference in means: (-0.1006, -0.05941)

Test of null hypothesis that mean of Rabbit pellet_WL is equal to mean of Baking yeast_WL

Test statistic $t = -7.82$ on 46 d.f.

Probability $< 0.001$
Appendix V

T-test for Independent of Two Samples (Kisumu Strain)

Analysis of the wing length between the adults fed as larvae on baking yeast and those
fed on rabbit pellet.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Minimum</th>
<th>Mean</th>
<th>Maximum</th>
<th>Values</th>
<th>Missing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baking yeast_WL</td>
<td>2.820</td>
<td>2.891</td>
<td>2.940</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit pellet_WL</td>
<td>2.790</td>
<td>2.843</td>
<td>2.940</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

Two-sample t-test

Variates: Rabbit pellet_wing length (WL), Baking yeast_wing length (WL).

Test for equality of sample variances

Test statistic $F = 1.01$ on 23 and 23 d.f.

Probability (under null hypothesis of equal variances) = 0.98

Summary

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size</th>
<th>Mean</th>
<th>Variance</th>
<th>Standard deviation</th>
<th>Standard error of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit pellet_WL</td>
<td>24</td>
<td>2.843</td>
<td>0.001239</td>
<td>0.03520</td>
<td>0.007185</td>
</tr>
<tr>
<td>Baking yeast_WL</td>
<td>24</td>
<td>2.891</td>
<td>0.001251</td>
<td>0.03538</td>
<td>0.007221</td>
</tr>
</tbody>
</table>

Difference of means: -0.0479

Standard error of difference: 0.0102
95% confidence interval for difference in means: (-0.06842, -0.02741)

Test of null hypothesis that mean of Rabbit pellet_WL is equal to mean of Baking yeast_WL

Test statistic t = -4.70 on 46 d.f.

Probability < 0.001
Appendix VI

Baking Yeast Rearing Bowls containing Water with Oily Scum Formation on the Surface and Dead Larvae
Appendix VII

Nutritional Content of Diets

A). Baking Yeast

Yeast fat 7% (Palmitic, oleic and linoleic acids, lauric acids, arachidic acid ergosterol and that ergosterol (Maclean and Thomas, 1920))

Proteins crab and cholesterol sterol.

B). Rabbit Pallet (Agrifeed By Agricare, Kumasi Ghana)

Calcium 1.5 % Salt 1 %

Protein 16 % min Lysine 0.6 %

Fat 5 % max Vitamin Premix

Fiber 12 % max Mould inhibitor
## Appendix VIII

### Correlation Table for Relationship between Fitness and Phenotypic Resistance

<table>
<thead>
<tr>
<th>Diets</th>
<th>Fitness</th>
<th>Phenotypic Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kisumu Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baking Yeast</td>
<td>Fitness</td>
<td>Pearson Correlation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sig. (2 tailed)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.915</td>
</tr>
<tr>
<td></td>
<td>Sig. (2 tailed)</td>
<td>0.085</td>
</tr>
<tr>
<td>Phenotypic Resistance</td>
<td>Pearson Correlation</td>
<td>0.915</td>
</tr>
<tr>
<td></td>
<td>Sig. (2 tailed)</td>
<td>0.085</td>
</tr>
<tr>
<td>Rabbit Pellet</td>
<td>Fitness</td>
<td>Pearson Correlation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sig. (2 tailed)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.889</td>
</tr>
<tr>
<td></td>
<td>Sig. (2 tailed)</td>
<td>0.111</td>
</tr>
<tr>
<td>Phenotypic Resistance</td>
<td>Pearson Correlation</td>
<td>0.889</td>
</tr>
<tr>
<td></td>
<td>Sig. (2 tailed)</td>
<td>0.111</td>
</tr>
<tr>
<td><strong>Opeibea Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baking Yeast</td>
<td>Fitness</td>
<td>Pearson Correlation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sig. (2 tailed)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.811</td>
</tr>
<tr>
<td></td>
<td>Sig. (2 tailed)</td>
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</tr>
<tr>
<td>Phenotypic Resistance</td>
<td>Pearson Correlation</td>
<td>0.811</td>
</tr>
<tr>
<td></td>
<td>Sig. (2 tailed)</td>
<td>0.188</td>
</tr>
<tr>
<td>Rabbit Pellet</td>
<td>Fitness</td>
<td>Pearson Correlation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sig. (2 tailed)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.751</td>
</tr>
<tr>
<td></td>
<td>Sig. (2 tailed)</td>
<td>0.249</td>
</tr>
<tr>
<td>Phenotypic Resistance</td>
<td>Pearson Correlation</td>
<td>0.751</td>
</tr>
<tr>
<td></td>
<td>Sig. (2 tailed)</td>
<td>0.249</td>
</tr>
</tbody>
</table>
Appendix IX

T-test for Independent Two Samples

Analysis of the mean pupal weight between the pupae fed as larvae on baking yeast and those fed on rabbit pellet.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Minimum</th>
<th>Mean</th>
<th>Maximum</th>
<th>Values</th>
<th>Missing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baking yeast_WL</td>
<td>26.00</td>
<td>56.67</td>
<td>79.00</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit pellet_WL</td>
<td>80.00</td>
<td>94.67</td>
<td>102</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

Two-sample t-test

Variates: Rabbit pellet_Pupal weight (PW), Baking yeast_pupal weight (PW).

Test for equality of sample variances

Test statistic $F = 6.28$ on 5 and 5 d.f

Probability (under null hypothesis of equal variances) = 0.07

Summary

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size</th>
<th>Mean</th>
<th>Variance</th>
<th>Standard deviation</th>
<th>Standard error of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit pellet_WL</td>
<td>6</td>
<td>56.67</td>
<td>406.3</td>
<td>20.16</td>
<td>8.229</td>
</tr>
<tr>
<td>Baking yeast_WL</td>
<td>6</td>
<td>94.67</td>
<td>64.7</td>
<td>8.04</td>
<td>3.283</td>
</tr>
</tbody>
</table>

Difference of means: - 38.000

121
Standard error of difference: 8.859

95% confidence interval for difference in means: (-57.74, -18.26)

Test of null hypothesis that mean of Rabbit pellet_PW is equal to mean of Baking yeast_PW

Test statistic $t = -4.29$ on 10 d.f

Probability = 0.002