BIO-ECOLOGICAL STUDIES OF LIFE HISTORY TRAITS OF *ANOPHELES GAMBIAE S.L.* IMMATURE STAGES WITH SPECIFIC REFERENCE TO FEEDING PREFERENCE, AND THE INSECTICIDE RESISTANCE STATUS IN SOUTHERN GHANA: IMPLICATIONS FOR MALARIA CONTROL

BY

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DECLARATION

I hereby declare that this submission is my own work towards the MSc and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the university, except where due acknowledgement has been made in the text.

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DEDICATION

This work is dedicated to my parents and mentors Mr and Mrs S. Y Peasah

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ACRONYMS AND ABBREVIATIONS

CAA	Centro Agricoltura Ambiente
CTAB	Cetyl trimethly ammonium bromide
DDE	1,1-dichloro-2,2-bis-p-chlorophenyl ethane.
DDT	Dichlorodiphenyltrichloroethane
GABA	Gamma Amino Butyric Acid
GPS	Global positioning system
GSTs	Glutathione S-transferases
HSD	Tukey's Honest Significant Difference
IAEA	International Atomic Energy Agency
IRAC	Insecticide Resistance Action Committee
IRS	Indoor Residual spraying
Kdr	Knockdown resistance
LLIN	Long lasting insecticide treated net
MACE	Modified Acetylcholinesterase
MFO	Mixed-function oxidase
NSE	Non-specific esterase
OPD	Out–patient cases
PCR	Polymerase chain reaction

PCR-RFLP	Polymerase Chain Reaction Restriction Fragment Length Polymorphism
PUFAs	Polyunsaturated fatty acids
Rdl	Resistance to dieldrin
SINEs	Short Interspersed Elements
SOD	Superoxide dismutase
TAE	Tris-acetate-EDTA
WHO	World Health Organisation

ABSTRACT

Malaria is one of the leading causes of morbidity and mortality in sub Saharan Africa. Currently, malaria vector control measures are faced with setbacks and hence are failing. One of the effective ways of controlling malaria in Africa is the use of long lasting insecticide treated nets (LLINS) and indoor residual spraying (IRS). Over the years the National Malaria Control programs have become focused on strategies targeting the mosquito vectors and hence has scaled up distribution of insecticide treated nets and indoor residual spraying campaigns (WHO, 2012). Yet the robustness and the integrity of LLIN and IRS in vector control is threatened by the development of insecticide resistance to pyrethroids. However, although resistance bioassays exist and are regularly used to assess phenotypic resistance, the fitness of the mosquitoes used and how it affects the results of the bioassays is unknown. The aim of this study is to determine the effect of five different feeding regimes on some life history traits of *Anopheles gambiae* and also profile the insecticide resistance levels in population of *An. gambiae* from the same area. A water bath was used to control larval rearing temperature as the effect of Rabbit pellet (no. 001131 Agrifeed, Kumasi, Ghana.), Fish flakes (Lopis Fish flakes™), Cerelac maize (Nestle®), Cat meal (Purina Friskies®) and Baking yeast on pupation rate, pupal weight , larval developmental time, Larval survivors ship was determined. The insecticide resistance on the urban agricultural setting in Opeibea, Accra was also investigated. Molecular techniques were used to identify mosquito species and screened for *kdr* and Ace-1 mutation. A high pupation rate (21.22%), larval survivorship (24%), pupal weight (2.353mg) and short larval developmental time (8 days) were obtained when the larvae was fed with rabbit pellet. The performance of rabbit pellet and fish flakes on the various life history trait was statistically different from Cerelac and yeast. However, the performance of fish flakes was insignificantly different from the life history traits of larvae

fed with cat meal. Molecular screening for *kdr* and Ace-1 mutation indicated a high occurrence of L1014F *kdr* (109) and Ace-1^R (80%) allele frequency which corresponded with the high phenotypic resistance and the use of insecticide by the famers. Bioassay revealed a high resistance (*RR) *Anopheles gambiae s.s* population at the vegetable farms in Opeibea. The S and M forms of *An. gambiae* s.s. was observed to occur in sympatry at Opeibea with a higher frequency of (78.95%) S form. The farmers use Fenitrothion, Deltamethrin, Emamectin Benzoate, Imidacloprid and Fenvalevrate in growing their vegetables. Rabbit pellets is highly recommended for use in the rearing of *Anopheles gambiae s.s* larvae in the insectary and the level of resistance at the urban agricultural setting in Opeibea indicates that practices such as indiscriminate use of insecticides may be helping in the development of resistance and needs to be controlled and monitored.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Malaria is one of the leading and devastating vector borne diseases in the world with about 3.4 billion people, which is about half of the world's population at risk. Current World Health Organization data estimated that in December 2013, there were about 207 million cases of malaria in 2012 and 627 000 mortalities (WHO, 2013b). Although mortality rates due to malaria have fallen by 42% globally since 2000 and by 49% in the WHO African Region, people living in the poorest countries are still the most vulnerable to malaria. In 2012, 90% of all malaria deaths occurred in African and mostly among children under 5 years of age. Despite the enormous achievement by the Roll Back Malaria Program the prevalence of malaria still remains very high with children being the most vulnerable to infection with deaths occurring every minute. Malaria is endemic in 100 countries with most of them being tropical regions. Globally 80% of malaria cases occur in 17 countries of which most malaria deaths occur in 14 African countries. An estimated annual majority of 1.5-2.5 million death are from these African countries with 90% of cases occurring in Sub-Saharans Africa. Globally 40% of death due to malaria occurs in Nigeria (Africa's most populated country) and The Democratic Republic of Congo (WHO, 2013b). In Ghana, malaria is one of the leading causes of illness and the primary cause of morbidity and mortality, accounting for over 3 million outpatient visits to public health facilities annually for the past ten years. This represents 45 per cent of out-patients cases (OPD) and 33% to 36% in-patient cases, of which 36% to 40% of these OPD cases occur in children under five. It occurs all year round and affects a large proportion of the population in which four persons die out of malaria every hour with two of the four being children under five. In 2009 reported cases attributed to

malaria among children under five years were 48.9% and 11.5% among pregnant women (www.kebaafrica.org 2/06/2014).

Malaria is caused by *Plasmodium* parasites. The protozoan parasite is transmitted when one is bitten by infected *Anopheles* mosquitoes (malaria vectors) which require a blood meal to reproduce. Among the hundred species of *Plasmodium* parasites, there are four of them that are infectious to man and cause malaria. Namely, *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae and Plasmodium ovale,* with *P. falciparum* and *P. vivax* being the most common. *Plasmodium falciparum* is the virulent and most deadly, and has a high prevalence in Africa especially in sub-Sahara Africa. *Plasmodium falciparum* infection has also been implicated in clinical episode that has led to complicated malaria infection and death especially in children under five. The newest member of the group of *Plasmodium parasite* which is infectious to man is *Plasmodium knowlesi*. A species which originally caused malaria among monkeys and occurs in certain forested areas of South-East Asia is now infectious to man (WHO, 2014).

Malaria transmission is mainly through the bites of *Anopheles* mosquitoes and intensity of transmission depends on factors related to the environment, human host, parasite and vector. The population of mosquitoes are affected by climatic conditions. Seasonal changes in rainfall patterns, temperature and humidity affect malaria transmission especially during the peak periods particularly in malaria endemic areas like sub-Saharan Africa (Meyrowitsch *et al*., 2011; WHO, 2014).

Rainfall is recognized as an essential factor influencing malaria transmission. Rainfall creates the necessary conditions which allow creation of mosquito breeding sites as well as high humidity which affect the longevity of adult mosquitoes allowing them to live long to complete its life cycle.

Temperature is also an important factor in the transmission of malaria. Relatively high temperature influence rapid reproduction of *Plasmodium* parasite in the *Anopheles* mosquito, development of larvae and survival rate of adult (Grover-kopec *et al*., 2006; Kelly-Hope *et al*., 2009). The anthropophilic *Anopheles sp.* mainly bite at night and is known to be efficient in transmission of malaria. As such the intensity of transmission and risk associated with effect of the malaria in endemic areas also depends on the immune status of the individual against the disease. Malaria control programs which involve the development and implementation of appropriate strategies to control malaria by interruption of parasite transmission although have been successful in some geographical location and have led to eradication of malaria (Toure, *et al*., 2004). However, in many places malaria control continues to face serious challenges. Over the years, a lot of research work have been done but the application of the research findings to malaria control is faced with some setbacks (Greenwood, 2008). This has made malaria control difficult because most of the interventions are not based on the knowledge of the vector. The most widely implemented vector control strategies are the use of insecticide treated bed nets and indoor residual spraying (Keiser *et al*., 2005; Koffi *et al*., 2013; WHO, 1998) both of which have effectively reduced the transmission of malaria in some areas. Sustainability and effectiveness of this approaches depends on socio-economic political, cultural and as well as the development of insecticide resistance in mosquitoes. Greece reported in 1951 the first cases of resistance to dichlorodiphenyltrichloroethane (DDT) in malaria vectors. This was later followed by reported case in the Islamic Republic of Iran and Turkey. By 1956 there had been case of resistance to dieldrin and other cases had appeared in Asia, the Americas and Africa. In 1960, 43 mosquito species were resistant to one or more insecticides, and this increased to 99 by 1980 (WHO, 2012). Currently resistance to at least one insecticide in mosquitoes have been identified in 64 countries (Duchet *et al*., 2012).

In Africa, current reports indicate the fast spread of metabolic and *kdr* resistance among the M and S forms of *Anopheles gambiae* (Koffi *et al*., 2013; Nwane *et al*., 2013) coupled with extensive use of agricultural insecticide makes it difficult to monitor. Although much work has been done on the mechanism of this resistance, the epidemiological effect on the spread of resistance is not fully understood. Hence scientists fear that, the threat to the efficacy of LLIN and IRS is much greater than expected. Current report indicate that in Ethiopia susceptibility to the four classes of insecticide are lost (Balkew *et al*., 2012; WHO, 2012) whilst in Senegal, increasing resistance pyrethroid and susceptibility to malaria lead to reverberation in morbidity due to malaria (Trape *et al*., 2011). It is estimated that the loss of pyrethroids efficacy, will lead to approximately 120 000 deaths more death annually (WHO, 2012). Thus 55% benefit of vector control would be lost. Resistance can probably be reversed if selection pressure is removed by withdrawing and replacing the insecticide. Unfortunately there have been no introduction of new classes public health pesticide products for wide scale use in more than 30 years (Ranson *et al*., 2011). On the other hand, insecticide resistance management strategies must be effected before the resistance gene becomes establishment in the mosquitoe population or else the resistance status cannot be reversed. In 2010, WHO recommended strategies aimed at delaying resistance and put in place five pillars of collective strategy against insecticide resistance in its 2012 global plan for the management of insecticide resistance (WHO, 2012).

Presently malaria control strategy is gearing towards integrated vector management approach which is a long proven approach, used in vector disease control in the USA. Since the abundance, vector capacities, intensity of transmission, dynamics and fitness of adult mosquitoes also depend on the aquatic stages, strategies aimed at reducing adult vector populations by targeting their aquatic immature stages are now becoming an area of interest (Paaijmans, 2008). In order to improve upon understanding on different nutritional diet, opportunities and optimal natural food

resource for larval development. Some of these approaches have been directed towards larval food requirement. Since larval nutrition affect life history characteristics and adult fitness (Paaijmans, 2008). It is therefore essential to match control methods to the vector biology and ecology (Beier *et al*., 2008; Beier, 1998).

An essential part of this study is the evaluation of current available diet on the market used for rearing of mosquitoes in the insectory at the Noguch Memorial Institiute of Medical Research. All extrinsic factors or variable would be kept constant as much as possible so as to obtain uniformity and prevent interference in the assessment of the performance of diet (Cerelac, Baking Yeast, Rabbit Pellet, Cat Meal and Tropical Fish Meal) on the larvae of *Anopheles gambiae s.s* (Kisumu, laboratory strain) and *Anopheles gambiae s.l* (wild). Mohammed and Chadee, (2011) method of obtaining constant larval rearing temperature using a beaker place in a temperature regulated water bath would be modified and employed. *An. gambiae* was chosen because of its medical importance and would be raised from an already establish colony at Insectary of Noguchi Medical research center. *Anopheles gambiae s.l* (wild) would be obtain from a field colony caught from Opeibea.

Gaining understanding to bio-ecological factors that affects certain life history characteristics of *Anopheles sp*. development, will expand our knowledge of mosquito larval biology and ecology which will lead to better implementation of malaria vector control strategy

The purpose of the study, is to investigate the effect of five feeding regime on some life history characteristics, adult fitness of *Anopheles gambiea* s.l and the susceptibility to insecticide of the Wild *Anopheles gambiae* population at Opeibea urban agricultural farms.

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1.2 Rationale of Study

Much of our understanding of life history trait, physiology, behavior and ecology of *Anopheles* mosquitoes was derived from laboratory experimentations. Most of the insects used in these experiments were reared under artificial conditions and diet in the laboratory (Cohen, 2001).

Fortunately, there is a lot of diet that has been formulated or produced on the market which is used for rearing of insect especially mosquitoes in the laboratory. Larval rearing conditions have a direct and irreversible effect on adult traits therefore a clearly defined diet is a priority (Timmermann and Briegel, 1999; Cohen, 2001; Benedict *et al*., 2009; Puggioli *et al*., 2013).The effect of most of these artificial diets, on some life history characteristics of the larvae are rarely known, since most of these diet are produced for rearing of other animals. A majority of these diets are expensive and not readily available, globally. Some studies have shown that larval food availability and type, influence larval development, survivorship, size of the adult and fitness. According to Benedict *et al*., (2009), evaluating different diets especially for rearing of the immature stages of mosquitoes is normally achieved under experimental conditions. But in recent past, a lot of studies aimed at the nutritional needs of the immature stage of mosquitoes or insects are often looked down upon or neglected (Cohen, 2001). However only few studies have even been directed towards understanding the biology and ecology of these stages (Paaijmans, 2008). The knowledge of locally available larval nutritional diet, quantity and quality needed in *An. gambiae rearing* might be beneficiary in raising quality or fit mosquitoes in the insectary.

The results of this study will help provide information on the effect of diet on the fitness of immature stages of the malaria vector *An. gambaie* s.l.

The extensive use of insecticides by farmers in crop production have been associated with the causes of insecticide resistance in the malaria vector, ever since it was first reported in 1967

(Chandre *et al*., 1999) till now. In Ghana, there is improved access to the distribution and coverage of LLIN in its vector control program. Meanwhile the use of synthetic pesticide for agriculture and domestic pest control is on the increase with a booming market in the sale of this product (Fianko *et al.,* 2011). The situation is further supported by a report from the Ghana Standard Authority of the large scale appearance of fake agrochemicals and substandard insecticide domestic product on the market (www.ghanawb.com 12/05/2014). The *kdr* resistance gene also continues to spread in the malaria mosquito vector. Recent resistance to Carbamate report from Obuasi (Okoye *et al*., 2008), is also an indication of the presence of Ace-1 mutation gene and metabolic resistance mechanism in the *Anopheles species.* The incidence of malaria around urban agricultural setting is relatively high (Asare *et al*., 2004; Klinkenberg *et al*., 2005, 2008) and seems to be on ascendancy in Ghana. There is therefore the need of a study, which will help curb the situation before it gets out of hand.

In order to develop an appropriate, comprehensive and effective implementation of vector control program that will avert insecticide resistance in the malaria vector, there is the need to know the insecticide resistance status of the vector, mechanism of resistance and the type of insecticides involved.

The farmers also used a lot of agrochemical chemical in farming and literature searched revealed that no published work on insecticide resistance had been done at the urban agricultural setting in Opeibea.

Hence, the aim of this study is to determine pyrethroid resistance status of *Anopheles gambiae* in the vegetable farms in the Opeibea area an urban agricultural setting. Result from this study will give information of the types of pyrethroids *An. gambiae* is resistance to. Which will inform the use of appropriate and effective pyrethroids or other class of insecticide for LLIN and IRN intervention vector control program. Hence the data will serve as the basis for evidence base intervention strategy, since

population density of mosquitoe at former site is relatively higher than that Korle-Bu and not much study has been done at Opeibea. The data from resistance studies can also be used in future formulation of malaria vector control strategies in and around urban areas in Ghana.

1.3.1 General objective of study

The main aim of this study is to evaluate the effect of five feeds on life history characteristics (larval developmental time, pupa weight, pupation rate and larval survivorship) of *Anopheles gambiae s.s* (in wild and laboratory ('Kisums') strain), determine the extensive usage of insecticide and resistance development or status in *An. gambiae s.*l wild population colony on urban agricultural farms at Opeibea.

1.3.2 Specific objective

I. To compare the performance of Cerelac, Baking Yeast, Rabbit Pellet, Cat Meal and Tropical Fish meal on larval developmental time, pupa weight, pupation rate and larval survivorship in wild and laboratory bred ('Kisumu' strain) of *Anopheles gambiae* s.l *species* '

II. To investigate resistance status of *An. gambiae* s.l. to the four classes of insecticides approved by the WHO for use in vector control and the types of insecticide used by farmers in growing crops at urban agricultural farms in Opeibea.

III. To identify *An. gambiae species,* M and S forms among the mosquito population in the vegetable farms in Opeibea using molecular characterization methods.

IV. The determination of knock down resistance (*kdr*) and Ace-1 mutation frequencies in *Anopheles gambiae s.s* mosquito population at the urban agricultural farm in Opeibea.

CHAPTER TWO

LITERATURE REVIEW

2.1 Vector Biology of *Anopheles gambiae* s.l

Mosquitoes were the first among insects of medical important to be associated with transmission of disease in 1878. All the mosquitoes that transmit the most human *Plasmodium* parasite belong to the most efficient and widely distributed *Anopheles genera* in the tropics. Out of 422 *Anopheles sp*. only 68 of these species are associated with malaria transmission (Paaijmans, 2008).

Anopheles gambiae complex is the principal and efficient vector in the transmission of *Plasmodium falciparum* (most deadly malaria parasite in sub Saharan Africa) consist of seven siblings with one of the new member of the group being *Anopheles coluzzii* (formerly M form of *Anopheles gambiae* (Coetzee *et al*., 2013) which can be distinguished by molecular methods (Carosi *et al*., 1997; Paaijmans, 2008). These *Anopheles gambiae s.s. (sensu stricto)* mean this is one of the siblings and *Anopheles gambiae s.l. (sensu lato)* which also refers to any of the other siblings.

An. gambiae s.s is a holometabolous (Walker, 2008), anautogenous (Attardo *et al.*, 2005), anthropophilic and endophilic insects or mosquito that usually breeds in aquatic habitat (Esposito and Habluetzel, 1898; Paaijmans, 2008). The selection of breeding site and larva density is affected by high levels of rainfall (Paaijmans *et al*., 2007), predators and competitors (Munga *et al*., 2006). They are most of the time well adapted to humans' settlement with open breeding site which is devoid of little or low aquatic vegetation (Kelly-H ope *et al*., 2009). Hence these habitat are created as a result of human activities and are immediately colonized as soon as they are created. *Anopheles gambiae s.s* mosquitoes copulation in usually after swarming and the adult female *Anopheles* stores the sperms in a spermatheca, so that at each subsequent oviposition the eggs can be fertilized during their transit in the oviduct. Generally mosquitoes feed only on sugar-rich fluids, but females require enriched proteins and lipids for egg maturation (Benedict, 2007) which they obtain from human by blood feeding at dusk or night. A typical characteristic features of a blood fed female is the slender abdomen protrudes or enlarges to accommodate a volume of blood which may even

double the weight of the mosquito itself (1μl to 3μl depending on the species). The adult female can also concentrates the blood whiles excreting urine through a process called diuresis.

Maturation of the eggs occurs within hours after ingesting of blood and the gonotrophic cycle is temperature dependent. In an average day-night temperature of about 23°C, the gonotrophic cycle takes 48 hours. The matured eggs are deposited normally at night in puddles, hallow ponds, borrow pits, brick-pits, tire tracks, ditches, human foot and animal hoof that are filled with small amount of water. *Anopheles* females seek for a suitable source of blood every second to third night. A female mosquito which is experiencing gonotrophic cycle for the first time (primigravidae), usually requires 2 or 3 blood meals (Carosi *et al*., 1997). The initial white eggs are melanized within a few hours and those that fail to melanize or sink do not hatch (Benedict, 2007). The size of *Anopheles* egg is about 0.5mm x 0.2mm, hence it quite small to be seen by the naked eyes. The eggs are distinctively laid (unattached) and possess two air filled lateral floaters which is a distinguishing feature that is not found in *Culex* and *Aedes sp*. The egg normally hatched into larva in two days

The *Anopheles gambiae s.s* larvae possess a head, a thorax, nine abdominal segments and aligned in a straight line at resting position parallel to the surface of the water body. This position might be related to the absence of respiratory siphon (which is the breathing apparatus in the *Culicine*), since the *Anopheles* breathe atmospheric oxygen through two spiracles on the eighth segment of their abdomen (Benedict, 2007; Paaijmans, 2008). The resting position of the adult *Anopheles* body with respect to the substrate surface is one of the distinctive characteristics that is use in identification of *Anopheles* genera.

Anophelines larvae are collector-filterers which feed on aquatic biofilms or microorganism that are found on the surface of water The larvae are usually found just beneath the water surfaces that are usually covered with food particles (organic micro layer). The mouth part of the larva has lateral

palatal brushes that are thought to function as paddles rather than as filters as previously thought (Benedict, 2007). At the surface with their dorsum up and head rotated 180 degrees, food particles are drawn toward the head by the movement of lateral palatal brushes and smaller food particles are directly ingested. The large food particle are masticated or broken into smaller size that can be ingested whilst large food particles that cannot be masticated are discarded (Merritt *et al*., 1996; Benedict, 2007). The larvae undergo 3 successive moults (from 1st stage to 4th stage larvae) that result in corresponding increase in morphology of organism, sizes and amount of the ingested food particle. Generally *Anopheles* larvae feed at the water surface and bottom, but not in the column. The fourth instar larvae moults into a pupa, in which head and thorax are fused under a common envelope and bear the respiratory trumpets (Benedict, 2007). The pupa stage normally lasts for a day or two and does not ingest food. The pupa emerges into adult or *imago*. The duration of the immature life stages of *Anopheles gambiae* is temperature dependent and can take a minimum of 7 days at elevated temperatures of inter tropical regions.

According to Paaijmans (2008) nutrition availability, larval densities and water temperature of the aquatic environment are essential factors that affect growth and development of mosquito larvae. The adult mosquitoes feed on sugar rich fluids as an energy source and are attracted to seedpods of fruits and nectars of flowering (Müller *et al*., 2010). Mosquitoes have been seen ingesting crystallized sucrose by liquefying it with saliva, hence are feed with 10% sugar solution in the laboratory (Benedict, 2007). The females *Anopheles gambiae* also require blood meal for reproduction, hence has a human blood index greater than 0.5. Temperature, nutrition, photoperiod, humidity and physiological condition affect adult longevity or survivorship. For instance under favourable environmental conditions they can live for more than 3 – 4 weeks but in nature, the mean survival ranges from 6 - 9 days.

Most importantly, the environmental conditions of the larval habitat have irreversible direct outcome on life history characteristics which affects adult health and vector capacity of the mosquitoe (Benedict *et al*., 2009).

2.1.1 The effect of temperature, density and, nutrition on larval development of *Anopheles* mosquito

2.1.1.1 The effect of temperature on larval growth and life history parameters of *Anopheles gambiae* s.l

The growth and development of insects are affected by the temperature of their direct environment since insects are poikilothermic (Esposito & Habluetzel, 1997; Mohammed & Chadee, 2011; Paaijmans, 2008). Generally, insects produce limited amount of heat from metabolism, hence temperature dependent body functions or enzymatic metabolic reactions depend external environmental temperature (Paaijmans, 2008). At certain optimal temperature there is an increase in enzyme-catalyzed reactions which correlate to larval growth developmental. Further increase or decrease in temperature affect larval development, lipid storage, body size and lead to death in extreme cases. Exposing mosquitoes to rapid high temperature results in high mortality and resistance of the immature stage decreases again at extreme temperatures as the larval develop from one immature stage to the other (Love and Whelchel, 1957). Briegel and Timmermann, (2001) observed that, *Aedes albopictus* spent 7 days at 32ºC and up to 28 days at 12ºC to develop from first instar to pupation whilst pupal period lasted 2 to 3 day at 32C and 7 to12 d at 12 C. Teneral reserves and body size decrease with increased in temperature. Using a nonlinear model, Delatte *et al*. (2009), calculated the minimal threshold and optimum temperature immature stage development in *Ae. albopictus* to be 10.4ºC and 29.74ºC respectively. The observation was consistent with Mohammed and Chadee (2011), using a water bath to control the larval environment temperature proved that rearing *Aedes aegypti* at constant temperature of 30ºC resulted in relatively high pupation. In *Anopheles gambiae* s.s and its siblings species (*An. dirus* and *An. sawadwongporni*), larval developmental time decreased with increasing temperature, and peaked round 28ºC to 30ºC. Whilst high mortality always

occurred at a lower temperature limit of 12ºC and upper limit of 32ºC. Larval body size and teneral reserve also increased with decreasing temperature. But was high at 28ºC is constant rearing temperature (Bayoh & Lindsay, 2003; 2004; Kirby & Lindsay, 2009; Phasomkusolsil *et al*., 2011).

2.1.1.2 The effect of both temperature and nutrition on mosquito larval development

Despite the enumerated effect, there is an inverse relationship between larval diet, development of immature stage on one hand and temperature on the other hand. Analyses of data involving the study of *Aedes aeypti* shows that, in abundance of diet, size of the adult and larval developmental time from egg to adult stage decreased whilst temperature was increased from the lower to upper limit. Hence the nutritional status of larvae within the habitat is affected by temperature (Tun-lin *et al*, 2000).

2.1.1.3 The effect of larval density on life history traits of *Anopheles gambiae* s.l

The overcrowding of larvae within specific surface area or space and limited food resource affect the development of immature stages. When larval density is high, the population of larvae is more than the food resource. Hence the larvae compete for food which results in early depletion of food resource and nutritious stress. Larval growth in nutritious stress conditions causes decrease in larval survivorship (as it result to cannibalism), weight of the pupa reduces, pupation and prolong duration for larvae to develop from egg to pupae (Terzian & Stahler, 1949; Gleiser *et al*., 2000; Agnew *et al*., 2002; Gimnig *et al*., 2002; Koenraadt & Takken, 2003; Koenraadt *et al*., 2004; Hawley, 2014).

2.1.1.4 The interactive effect of temperature, density and nutrition on life history parameters

The relationship between larvae density and nutrition is unpredictable, especially when none of two factors was limited or constant. The variation in these two factors can result into a tricuspid outcome on the growth and development of the immature stage. When the larva density is decreased and diet increases, the high quantity of food could pollute water and kill the immature stage. Increasing the larval density and decreasing the diet also affect the immature. When the food resource is limited, the larvae competes for it. In the *Anopheles gambiae* s. l. the large size larvae feed on the small size ones (Koenraadt & Takken, 2003; Koenraadt *et al*., 2004), larval survivorship is low, larval developmental rate decreases, and teneral reserves decrease. If both density and diet are increased larvae developmental rate increases (Gilles *et al*., 2011; Jannat & Roitberg, 2013). Conversely there exist a strong interactive relationship between density and temperature effect on *An. gambiae* immature stage (Lyimo *et al*., 1992). Couret *et al*., (2014), also reported that, there is of a strong interactive effect of temperature, diet, and density in development of immature stages of *Aedes aegypti.* The interactive effects of these three factors influence variation in larval developmental time and mortality. Other factors such as turbidity, water level (Paaijmans, 2008), larval competitors (Munga *et al*., 2006) and other extrinsic factors are known to affect growth and development of immature mosquitoes.

2.1.2. Dietary requirement for rearing of *Anopheles* larvae

2.1.2.1 Larval nutrient requirement

Nutrition is an extrinsic and biotic factor that affects the growth and development of the larva and has corresponding effect on the adult. The nutritional availability or constituent affects larval developmental time, larval growth, pupae weight, metabolic storage reserve, and adult health and life history traits.

Mosquito larva feeds on aquatic microbes (detritus, algea and microoganism) to obtain nutrient and accumulate excess nutrient in its body for later utilization (Bond *et al*., 2005). In *Aedes aegypti*, carbohydrate are utilized as energy sources and are synthesized and stored as glycogen (Wigglesworth, 1941) and protein into lipids for growth. (4%) Lipids promotes the growth of the larva when incorporated in the basal liquid media. However, palmitic acid, stearic acid, oleic acids, tripalmitin, tristearin, triolein, lauric acid, myristic acids and oleic acid were observed to be highly toxic at 5% to larval development (Golberg & Meillon, 1948). A lot of mosquitoes larvae cannot synthesize enough cholesterol (sterols) needed for growth (especially metamorphosis) (Wigglesworth, 1941; Golberg & Meillon, 1948), flight and survival (Damiens *et al*., 2012). This might be due to their inability to elongate the C18 polyunsaturated fatty acids (PUFAs), mosquito diets must include C18, C20, and C22 PUFAs (Damiens *et al*., 2012). Generally certain class of polyunsaturated fatty acids, cholesterol derivatives and esters, triglycerides and steroids did not show any beneficial effects when added. In *Aedes aegypti*, the excess fat or lipid are stored in the cells and tissue of the body (Wigglesworth, 1941). Yet, a lot of studies have shown the importance of cholesterol in the diet of insects (Golberg and Meillon, 1948).

Proteins may serve as a source of amino acid, glycogen, lipids and uric acid (when they are metabolized or deaminated (Wigglesworth, 1941). Proteins support larval growth by catalising enzymatic reactions,and oogenesis (Uchida *et al*., 2003), and also serve as energy sources especially during starvation and involved in metamorphic development. An increase dietary protein correlated to increase in larval size, protein stored by larvae (Timmermann & Briegel, 1999; Telang *et al*., 2002) and shortens larval developmental time (Khan *et al*., 2013). A recent study involving *Anopheles stephensi* suggest that, nitrogen availability in the larval diet controlled mosquito size and that teneral reserves (Hood-Nowotny *et al*., 2012).

Salt (NaCl and $CaCl_2$) in small concentration (0.05 to 0.005 M) when added to the growth media of *Aedes aegypti* also resulted into relatively rapid growth and development than when absent. The ions from these are absorbed and involved in electrolytes formation, osmoregulation and nervous function (in the case of Calcium ion) (Trager, 1932).

Apart from lipids, proteins, salts and carbohydrates, other co-factors like vitamins (riboflavin, pyridoxine, nicotinamide, thiamine and Ca-pantothenate) that are involved in metabolic reactions, also support larval growth (Horn & Lichtwardt, 1981). According to Damiens *et al*, (2012), in their search for an effective larval diet for *Anopheles arabiensis*, the addition of a vitamin mix further enriched the quality of the diet.

2.1.2.2 The quantity and quality of larval diet

The form, composition (quality) and quantity of the various nutrients available in a natural habitat or laboratory also affect larval growth and adult traits. Larval diet must always be soluble and ingestible. A non-soluble diets or nutrients on the other hand may result in poor larvae growth since it cannot be absorbed when ingested (Wigglesworth, 1941) . For instance certain species of blue-green algae (Cyanobacteria) and green algae (Chlorococcales) kill larvae because larvae were not able to digest them when ingested. Hence the larvae starve to death (Marten, 2007).

The formulation of a quality diet is essential to the production of effective balance larval diet. Since the constituent of the diet determined the available nutrient for growth of mosquito larvae. In the assessment of several individual diets for rearing of *Anopheles arabiensis* larvae, the combination of two or more dietary components (bovine liver powder, vitamin mix and tuna meal)

in their unrefined state showed the best results in terms of larval survival, developmental rate, and adult size (Damiens *et al*., 2012; Khan, 2010). In a similar study involving Centro Agricoltura Ambiente (CAA) and two International Atomic Energy Agency (IAEA) diet use in mass rearing, *Aedes albopictus* fed on the two IAEA diets resulted in a relatively shorter time to pupation and emergence of adult (Puggioli *et al*., 2013). Khan *et al*. (2013), also observed the fastest growth and development when combined diet of bean, corn, wheat, chickpea, rice and bovine was used in the rearing of *Anopheles stephensi*. The incorporation or mixing of the right amount of various ingredients to obtain a balance diet is very essential to larval nutrition status. This has most often accounted for the differences 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 *et al*., 2005).

Larval diet should provide wide range of nutrients to avoid the risk of deficiencies due to starvation that would affect growth (Timmermann & Briegel, 1999). Nutritional requirements of mosquito larvae are known to include at least 14 essential amino acids (asparagine, arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and valine), sugars, polyunsaturated fatty acids (PUFAs) especially C18, C20 and C22, sterols, nucleotides (for the larval development, survival and adult flight) and a minimal concentration of essential vitamins (Damiens *et al*., 2012) and salt (Ca_2Cl and NaCl) for enzymatic electrolyte and nervous function (Trager, 1932).

The abundance of diet about 16% diet (or 1.6 mg/ml/d) (Gilles *et al*., 2011) in a larval habitat can be beneficial or disastrous to the larval development and might result in death depending on the type of diet and the larva population. In rearing of *Anopheles gambiae* larvae, the quantity of food, as well as the nutritious demand by larvae increases as the larvae develops from one instar to

another. Usually, fourth instar larvae require five times (x5) the quantity of food consumed at first instar. For instance the high quantity of diet in a larva containing few larval population may be poisonous, lead to growth of opportunistic infection, growth of undesired competitors and pollution of aquatic habitat (Gerberg, 1970; Benedict, 2007; Benedict *et al*., 2009). Gilles *et al*. (2011), found the minimum amount of diet needed for larval growth to be 262µg of diet per larva per day. Laboratory studies have shown that survival rate of larvae to pupal and adult stage also accelerated with an increase in the food quantity (Gilbreath *et al*., 2013; Kivuyo *et al*., 2014).

2.1.3 The effect of larval feeding regime on life history trait, susceptibility to insecticide and vectorial capacity in *Anopheles gambiae* s.l

A nutrient rich or well composed larval diet available in right quantities has an effect on life history characteristics and adult trait that affect the vector capacity of the mosquitoes. Larval development, pupa emergence rate, pupal weight, adult size, fecundity, larval survivorship, sex ratio and adult longevity are affected by the dietary regime used in rearing the larvae (Kivuyo *et al*., 2014)

In *Anopheles gambiae* s.l, larval food source affect the developmental rate of larvae and adult development .The use of nutritious diet results in decrease in larval developmental time (that is, the time taken to develop from first instar to adult), pupa emergence rate and relatively fit adult, whilst poorly fed larvae led to increase in developmental rate, delayed pupation (since low growth rate does not stimulate metamorphosis of larvae (Bradshaw & Johnson, 2014) and less fit adult (Damiens *et al*., 2012; Khan *et al*., 2013).

.Also survivorship in both immature stages and adult stage (longevity) of mosquitoes is affected by the type of diet used in feeding. The *Anopheles gambiae* larvae compensate for poor dietary nutritional conditions by feeding on each other (cannibalism) [Koenraadt and Takken, 2003; Jannat *et al*., 2013]. A highly nutrient rich diet reduce larval mortality, prevent cannibalism and enhance

larval survivorship (Damiens *et al*., 2012; Khan *et al*., 2013; Kivuyo *et al*., 2014). The larval environment of mosquitoes is considered to be the controlling factor of adult longevity. Adults' survivorship is affected by the diet used in feeding the larvae stage. Feeding larvae with diet enriched with proteins, carbohydrate, vitamins and other foods source correspond with a relative increase or improvement in longevity of the resulting adult (Puggioli *et al*., 2013).

Hence low larval mortality lead to an increase development rate of larvae, coupled with a short pupa emergence time will results in an increase in the vector population density at a fast rate and more vectors will be available to spread the disease (Beier *et al*., 2008).

Pupal weight adult size and fecundity are another life history parameters that are affected by larval diet. The larva accumulates and stores excess nutrient in the form of lipids, glycogen and proteins, which are carried on to the adult stage (Wigglesworth, 1941; Nishiura *et al*., 2007;). The stored nutrient later serve as teneral reserves that are utilized in metabolic process in the adults (Hahn, 2005). Hence feeding larvae on nutrient rich diet result in high pupa weight and teneral reserve (Timmermann & Briegel, 1999: Kivuyo *et al*., 2014).

The high pupa weight and storage reserves results in the emergence of large and more fit adults that are better adapted to their environment (Agnew *et al*, 2002; Koenraadt, 2008) For instance teneral protein reserves are yolk precursors and act as stimuli for hormonal regulation of egg maturation (Shiao *et al*., 2008: Telang *et al*., 2006) High pupa weight correlated with the emergence of adults with relatively large wings (Bond *et al*., 2005) and reproductive fitness of adult (Okanda *et al*., 2002) that were relatively more fecund (Blackmore & Lord, 2000; Briegel & Timmermann, 2001; Armbruster & Hutchinson, 2002;). Larval nutrition also strongly influenced sex ratio and blood adult feeding of resultant adults (Takken *et al*., 2013; Kivuyo *et al*., 2014).

Finally larvae fed on nutrient rich diet relatively had less larval mortality, high pupa weight, increased larval developmental rate, short pupa emergence time, storage reserves and relatively larger adult size than their conspecifics fed on poor diet (Peck & Walton, 2005; Reiskind & Lounibos, 2009; Damiens *et al*., 2012)

Nutrition also affects the ability of mosquitoes to serve as a vector. Takken *et al*. (2013) observed under the experimental conditions, small-sized *Anopheles gambiae s.s* as a result of poor larval diet, expressed high mortality probably due to *Plasmodium* infections, whilst their conspecific that fed on rich larval diet survived. However, *An. stephensi* was a strong, successful vector irrespective of the nutritional status.

In another study, small sized female *An. arabiensis* which emerged from poor fed larvae were susceptible to DDT intoxication whilst well-fed larvae develop relatively quickly into large adults and were more resistant to DDT. The observed phenomenon was associated with detoxification enzyme activity (Oliver & Brooke, 2013).

Generally the effect of the nutritional status of mosquito larva on adult determines the fitness' status of the adult body and cannot be reversed by enhancing the diet when it grows into an adult. The two observations was due to the differences in the bodies of the adult mosquitoes which depended on the diet the larvae fed on. In *An. gambiae s.s,* the small size adult was not healthy enough to carry out its normal function as vector, supporting the development of *Plasmodium* parasite as a vector and was killed by their presence in poor malnourished body. In the study by Oliver and Brooke (2013), the large adult female body was fit and had teneral reserved. Hence was able to tolerate DDT by the action of detoxification enzyme activity and became more efficient as a vector. Whilst the relatively less fit small adult female body, probably had reduced activity of detoxification enzyme and were therefore more susceptible to DDT.

Although *Anophelines* are less efficient in converting food into biomass (Timmermann & Briegel, 1999), the consequence of larval nutrition on the adult mosquitoes and capacity as a vector, especially in *Anopheles* species cannot be downplayed or overlooked. The relative performance of a particular diet varies from one species to the another (Peck & Walton, 2005; Benedict *et al*., 2009; Huestis *et al*., 2011). Larvae nutritional status go a long way to affect longevity of the resultant adult mosquito (Walker, 2008; Puggioli *et al*., 2013) and determines the amount of blood meal and egg produced (Briegel, 1990; Takken *et al*., 2013).

Nutrition conditions of larva during rearing can have a direct and irreversible effect on adult traits. Conversely Huho *et al*. (2007), reports of relative large the body size and lipid reserves of wild adult males of *Anopheles gambiae Gilies* than their conspecific laboratory strain, when both were fed on the same diet**.**

2.1.4 Effect of Yeast, Fish Flakes, Cerelac, and Rabbit pellet on larval development of *An. gambiae* s.l

Yeasts, have been use in different ways frequently reported from insect habitats and in some cases yeasts have been detected in the insect gut (*Wickerhamomyces anomalus*) (Ricci *et al*., 2011; Cappelli *et al*., 2014) and in other tissues (Wigglesworth, 1941).

The use of yeast as larval diet has resulted in different outcome on the larval development. However the effect of it usage depends on the type of yeast (Brewer yeast (Koenraadt, 2008), baker's yeast (Khan *et al*., 2013), yeast hydralysat (Khan, 2010), yeast proteins (Fellous & Lazzaro, 2010) , an extract or derivative from yeast (Golberg & Meillon, 1948), whole (alive) (Tovar *et al*., 2002) or dead (Trager, 1932), as component in a diet (Puggioli *et al*., 2013) and quantity added (Gerberg, 1970; Peck & Walton, 2005). Khan in his study of assessment of single diet involving *Anopheles arabiensis* observed that 80% to 89 % and 70 to 79% of the larvae

developed in to pupa in 10 and 11 day when Brewer's yeast and Yeast hydralysat were used (Khan, 2010) but another study of *Anopheles stephensi* a combination of baker's yeast did not significantly improve larval survival and this was attributed to scum formation (Khan *et al*., 2013). Conversely, Hood-Nowotny *et al*., (2012) observed none of *Anopheles arabiensis* larvae survived to adult when he fed on hydrolysat yeast only. The difference in the observerd result might be due to the method of preparing and the amount of bakers yeast used. Currently 0.02% of yeast is added to eggs to enhanced hatching and development of larvae in rearing (Gerberg, 1970; Benedict, 2007;) .

The Tertramin fish flakes is the most preferred and widely used in rearing of insect in the laboratory or insectary. Fish flakes was is originally designed for use in feeding fish in aquarium and quite expensive. It is normally used as a Gold standard in studies involving the evaluation of feeds to be used in the insectary (Cohen, 2001; Kivuyo *et al*., 2014). In this study the lopis Fish Flakes was used instead of Tertramin fish flakes since it currently difficult to acquire one from the market.

The use of Cerelac, Rabbit pellet (sheep feed) in rearing mosquitoe in the insectary is not new but little is known about the effects on the larval development. Kivuyo *et al*. (2014) assessed the effect of locally prepared Cerelac mixed with sardine on certain life history trait at various density of *Anopheles gambiae* mosquitoes. The authors observed that the performance of Cerelac was equal or more in same cases than Tertramin fish flakes.

2.2. Insecticide Resistance in *Anopheles gambiae s.l*

The selection pressure exerted on insect populations by the use of synthetic insecticides has resulted in many species of human disease vectors developing resistant mechanisms against, public health insecticides (Nauen, 2007). Unfortunately the emergence and wide spread of mosquito populations capable of withstanding exposure is problematic for implementation of effective control measures.

Operational (field) resistance (Insecticide Resistance Action Committee (IRAC): defined resistance as, a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species (Ranson *et al.,* 2011).

WHO Global Malaria Eradication Programme in the 1950s and 1960s, recorded dieldrin resistance, among most *Anopheles gambiae s.l.* populations in Africa. The first case, involving *Anopheles gambiae s.s.* was observed in 1967 in Bobo Dioulasso, Burkina Faso and was due to the use of DDT on farms. Afterwards it was also observed among *Anopheles arabiensis* in Senegal (Chandre *et al*., 1999). Burkina Faso for that matter in some parts of West Africa, *Anopheles gambiae* resistance to the four major classes of insecticides available for public health has been reported (Djouaka *et al*., 2008; Ranson *et al*., 2009).

2.2.1 Causes of insecticide resistance in *Anopheles gambiae* s.l

The continued use of the same or similar synthetic insecticides to control pests at home, in agricultural and public health coupled with their selective pressure, are said to be the causes and source of insecticide resistance in *Anopheline* mosquitoes (Diabate *et al*., 2002).

Irrigated, urban and other forms of agriculture provide suitable breeding sites for mosquitoes. Hence is characterized by high densities of mosquitoes population in surrounding urban areas or communities as a result of migration from the breeding sites in the farm (Asare *et al*., 2004; Klinkenberg *et al*., 2005). The distribution of the various species of mosquitoes is also affected by these breeding sites. For instance, the M form of *Anopheles gambiae* (now *Anopheles coluzzii* (Coetzee *et al.*, 2013) is said to be typically found in abundance at permanent clean irrigated sites especially in extensive rice cultivation farms whilst the S form is associated within rain-dependent breeding site (Yawson *et al*., 2004). A study by Klinkenberg *et al*. (2008), in vegetable growing areas of Accra, Ghana indicated that, the predominant *Anopheles species* was *Anopheles gambiae s.s.* (which was highly resistance to permethrin) with a relative proportion of molecular forms of 86% S-form and 14% M-form..

In Ghana and other countries, an observed practice in these form of agriculture crop production is the extensive usage of wide range of synthetic agrochemicals and pesticide including insecticide of public health important (Fianko *et al*., 2011). Most of these insecticide are not even applied in accordance with manufactures' instructions (Yadouleton *et al*., 2009). The prolong and extensive use of the similar insecticide, either for IRS, domestic or agricultural purposes exert a selective pressure which favours the rear group (which carry the mutated gene) and will select for those individuals in a population that are able to survive the recommended concentration of the compounds owing to a genetically fixed difference. The selection pressure leads to the

establishment of the resistant population and at 'tipping-point' increase extremely rapidly with time (Nauen, 2007; Azhar *et al*., 2011; Liu, 2011; WHO, 2010, 2012)

Some of these agrochemical or insecticide also leak in to the soil (Fianko *et al*., 2011) and remain as soil residue that inhibit growth at the immature stages leading to a general fitness selection of resistance individuals (Akogbéto *et al*., 2006).

A recent study involving *Anopheles gambiae s.s* at Yaokkoffikro, *kdr*-w mutation allelic frequency increased from 5% to 95% (Koffi *et al*., 2012) whilst M'Bé saw a rise in L1014F *kdr* allelic frequency from 0.05 to 0.33 which correlated with an observed high phenotypic resistance due to the use of pesticide in cotton farms during political crises in Côte d'Ivoire (Koffi *et al*., 2013). Additionally Yadouleton *et al*. (2009) reports of an increase in the development of the vegetable farming coupled with improper application of insecticide, resulted in high selection pressure and emergence of insecticide resistance in *An. gambiae* population in the urban areas of Benin. In another study, Yadouleton *et al*. (2011) observed that the application of synthetic agrochemicals in one farm led to high resistance of *An. gambiae* to permethrin. Whilst bioassay test results of *An. gambiae* from Biological Program (BP) farm (which used only organic and natural fertilizers including animal excreta) gave a high level of susceptibility to permethrin with an average mortality of 94%.

Resistance to insecticide in *An. gambiae* is also due to the use of pyrethroid and other insecticide in IRS, LLIN, domestic coils and aerosols. According to Diabate *et al*. (2002), in the urban area of Bobo-Dioulasso, resistance to both permethrin and DDT was due to insecticide selective pressure from the extensive domestic use of insecticides as sprays or coils. However susceptibility to both permethrin and DDT was relatively high in rice fields and control areas where the use of insecticides was less and hence had limited insecticide selection pressure. Edi *et al*. (2012), Kwiatkowska *et al*. (2013) and Nwane *et al*. (2009) observed that high frequencies of *kdr*, Ace-1^R

alleles and unidentified co-factors worked together in across and multiple resistance mechanism to cause unprecedentedly high levels of phenotypic resistance to all WHO insecticide classes in among *Anopheles gambiae* population on irrigated crop farms. This observation was associated with the extensive use of agricultural insecticide in Côte d'Ivoire, Burkina Faso and southern Cameroon.

A recent study of four localities in Tarkwa, Ghana , indicated the prevalence of high resistance in wild *Anopheles gambiae* colony to pyrethroid (Hunt *et al*., 2011) contrary to what was reported by Kristan *et al*. (2003) who worked in some selected areas of Nigeria and Ghana observed low frequency of *kdr*-type resistance and susceptible *Anopheles gambiae* population despite widespread use of pyrethroids in pest control among the mosquito population in Tarkwa. Yawson *et al*., (2004), indicated very high frequency of *kdr* mutation especially in (98–100%) within the S form but reached a maximum of only 3.38% in *An. gambiae* s.s population in Okyereko in southern Ghana. The authors also observed a sympatric occurrence of M and S molecular forms *An. gambiae s.s* at an irrigation farm in the Ghanaian coastal savanna zone. Later, Achonduh *et al*. (2008) also sampled larvae of *Anopheles gambiae* s.s population in the vegetable farm along the same airport drainage in airport residential areas and observed a high resistance to (11%, 77%, 25%) DDT, deltamethrin and permethrin (respectively) but susceptible to malathion. Whilst there was very high soil residues of organophosphate and pyrethroids after almost 30 years of farming (Odhiambo, 2005; Achonduh, *et al*., 2008).

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Recent studies have indicated, the occurrence of high *kdr* mutation frequency, which is associated with high level of phenotypic resistance to certain class of pyrethroid in *Anopheles gambiae* population in Accra *(*Adasi & Hemingway, 2008; Adeniran *et al*., 2009; Kabula *et al*., 2011).

However Adeniran *et al*. (2009) also reported, moderate resistance to propoxur (cabamate) in *Anopheles gambiae* population at vegetable farms in Korle-Bu.

The evolution of high resistant gene frequencies is more commonly associated with IRS, domestic and agricultural usage of insecticides (Ranson *et al*., 2009). In the urban areas of Accra, Ghana, the high *kdr* frequency and subsequent resistant to pyrethroid in *Anopheles gambiae* was attributed to frequent use of household pyrethroid insecticide especially aerosols or insecticide-impregnated coils (Boakye *et al*., 2009).

2.2.2 Mechanisms of insecticide resistance in *Anopheles gambiae* s.l

The mechanism underlying the resistance of insecticide in insect has been extensively studied over the past years. In mosquitoes, the two major mechanisms by which insects acquire resistance to insecticides are target site insensitivity and increases in the rate of insecticide detoxification or metabolism. Target-site insensitivity results from the mutation of the target proteins that the insecticides act upon and reduces the binding affinity of the protein to insecticides (Narahashi *et al*.1998; 2007), thus lowering the amount of insecticide reaching the target site and enhancing the insect's resistance to insecticides. One of the first target site mutations which was found to confer resistance to pyrethroids and DDT was 'knock-down' (*kdr*) mutation. In *An. gambiae*, *kdr* was due to a non-synonymous mutation in two amino acid changes in the voltage-gated sodium (L1014F) leucine (TTA) with phenylalanine (TTT) *(*Martinez-Torres *et al*., 1998) and (L1014S) leucine (TTA) to serine (TCA) (Ranson *et al*., 2000) in sodium gated channels protein which is the target site for DDT and pyrethroid (Du *et al*., 2009, 2013; Narahashi *et al*., 2007). Hence the reduced binding of insecticide to the sodium channel protein in the cell membrane of the neuron (Ranson *et al*., 2011). Phenylalanine substitution at position 1014 leucine which spread rapidly among the

An. gambiae population was observed to be the main resistance mechanism in earlier cases of resistance to DDT and pyrethroid in the West African (Martinez-Torres *et al*., 1998; Donnelly *et al*., 2009; Koffi *et al*., 2012). Mutation involving changes in amino acid sequence from leucine to serine was initially observed only in the *An. gambiae* S form in the East African population (Ranson *et al*., 2000; Brooke, 2008), but recent studies indicate the occurrence and spread of both mutation in *An. gambiae* S and M form (Chandre *et al*., 1999; Abdoulaye *et al*., 2003; Lynd *et al*., 2005; Etang *et al*., 2006; Santolamazza *et al*., 2008; Namountougou *et al*., 2013; Knox *et al*., 2014). According to Hunt *et al*., (2011) studies in four localities in Ghana, showed *An. gambiae* S form was in abundance and high frequencies of the West African (L1014F) *KDR* mutation was detected with a 100% *Kdr* resistance in some of the communities at Ahafo and Tarkwa. This conferred resistance to all insecticide classes except the organophosphates. Similar observation was also made by Yawson *et al*. (2004).

MACE (modified acetylcholinesterase) and Rdl ('resistance to dieldrin) are also other forms of target site mutation. MACE is a change 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 reduce the ability of both organophosphates and carbamates to inhibit acetylcholinesterase (AChE) in nerve synapses, conferring resistance in *An. gambiae* (Weill *et al.*, 2004; Nauen, 2007; Djogbénou *et al*., 2010; WHO, 2012; Kwiatkowska *et al*., 2013). For instance in Obuasi, a modified acetylcholinesterase conferring carbamate resistance was observed among *An. gambiae*, although there was no evidence in resistance due to *Kdr* mutation (Okoye *et al*., 2008). These results can be used in the planning of an effective malaria control strategy. On the other hand *An. gambiae* mosquitoes larvae carrying the homozygous (G119S mutation) condition are more likely to die during pupation than their susceptible conspecific (Djogbénou *et al*., 2010). Different

mechanism of target site mutation can also act to confer multifactor resistant mechanism. Kwiatkowska *et al*. (2013), observed kdr mutation and acetylcholinesterase gene (Ace-1^R) resistance to organophosphates and carbamates.

Rdl glycine gamma amino butyric acid (GABA) mutation on the other hand, involve the substitution of alanine with serine on GABA receptor gene at codon 296 in the chloride channel leading to resistance to cyclodienes such as dieldrin and other chlorinated hydrocarbons excluding DDT (Narahashi *et al*., 2007; Nauen, 2007).

The mechanism of increased metabolism and detoxification contributes to a decrease in the effective dose of insecticides available at the target site. Metabolic resistance occurs by elevation in the activities of one or more products of three gene families, cytochrome P450s (cytochrome P450-dependent monooxygenases oxidative metabolism), Esterases (ester bond hydrolysis and sequestration) and glutathione S-transferases (GSTs) (conjugations and dehydrochlorination) (Collins *et al*., 2000; Nauen, 2007; Ranson *et al*., 2000). This results in a sufficient proportion of the insecticide being sequestered or detoxified before it reaches the target site to impair the toxicity of the insecticide. The cytochrome P450s, esterases and GST are the primary enzyme families responsible for pyrethroid metabolism in insects (Liu, 2011). The other class of enzyme play a secondary role in insecticide metabolic resistance mechanism (Ranson *et al*., 2011).

In *An. gambiae*, the enzyme families contain large number of groups. A total of 111 P450s, 51 COEs, and 31 GSTs genes have been identified (Djouaka *et al*., 2008; Müller *et al*., 2007).Though their specific role of all the members are not known, these enzyme families have multiple roles in the insect and only a small number are reported to bedirectly involved in insecticide metabolism. Induction, overexpression, amplification and structural modification of the gene may act alone (cross resistance) to metabolize a class of insecticide (Mitchell *et al*., 2012) or with other resistant mechanism

(multiple resistance) to confer resistance to different classes of insecticide (Liu, 2011; Ranson *et al*., 2004, 2011). Conversely metabolic resistance of cytochrome P450 monooxygenases is inhibited by piperonyl butoxide (PBO) (Brooke, 2008; Liu, 2011; Ranson *et al*., 2004; 2000).

P450 enzymes show specificity for either type I pyrethroids (those lacking a cyano group, such as permethrin) or type II pyrethroids (containing an alpha-cyano group, e.g. deltamethrin) (Mitchell *et al*., 2012). Microarray studies have shown that in *An. gambiae* three P450 genes (cyp6m2, cyp6p3 and cyp6z2) which encode for enzymes that are able to bind to pyrethroid found to be repeatedly over expressed in pyrethroid resistant populations but only CYP6P3 and CYP6M2 can metabolize insecticide (David *et al*., 2005; Djouaka *et al*., 2008; Liu, 2011; Mitchell *et al*., 2012; Ranson *et al*., 2011).

Glutathione-S-transferases (GSTs) are soluble dimeric proteins involved in the detoxification of compounds and their up-regulation of GST genes in *Anopheles gambiae* mosquito is implicated in resistance to pyrethroid and other class of insecticides (David *et al*., 2005; Liu, 2011). In another study, the expression of GSTe1 and GSTe2 at elevated levels was responsible for metabolisation of DDT to DDE [1,1-dichloro-2,2-bis-(p-chlorophenyl) ethane] in *Anopheles gambiae* mosquitoes (Ding *et al*., 2005).

The spread of multiple resistance as a result of a cascade of resistance mechanisms is on the increase (Knox *et al*., 2014) and is quite problematic for malaria vector control in Africa (Brooke, 2008; Duchet *et al*., 2012; WHO, 2010, 2012). In Cameroon, multiple resistance mediated by enzyme-based detoxification mechanisms is said to be the cause resistance of reduced susceptibility to DDT, deltamethrin and permethrin in *An. gambiae and An. arabiensis* (Nwane *et al*., 2013).

Also, a torrent of resistance mechanisms including upregulation of cuticular pre-cursor genes, cytochrome P450 genes (CYP6P3 and CYP6M2) and *kdr-west* was observed in resistance to pyrethroids in *An. gambiae s.l* population collected from different locations in southern Benin. (Djouaka *et al*., 2008). The observed phenomenon have been linked to the failure of LLIN. Hence serve as a threat to the success of an effective malaria vector control program.

The situation is not different from Valléedu Kou in Burkina Faso, where the high prevalence of resistance to pyrethroids, DDT and dieldrin is attributed to over expression of cytochromeP450genes (CYP6P3 and CYP6Z2) and acetylcholinesterase coupled with frequencies of L1014F kdr (75%) and Rdl (87%) mutations were observed showing strong correlation with pyrethroids, DDT and dieldrin resistance (Kwiatkowska *et al*., 2013).

In Côte d'Ivoire at M'Bé and Yaokoffikro, oxidases (mixed-function oxidase (MFO)), nonspecific esterases (NSE), glutathione S-transferases (GST), L1014F *kdr* as and the ace-1 G119S were observed in highly resistant DDT, carbamates, pyrethroids and etofenprox strain of *An. gambiae s.s*. (Koffi *et al*., 2012, 2013).

Although much work have not been in Ghana, the few studies suggest a tendency of multiple resistance mechanism. Transcription profiling of pyrethroid resistant *An. gambiae* strains from Odumase, in southern Ghana reveals 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 study observation was similar to Mitchell *et al*. (2012), who's work indicated over expression of P450 gene CYP6M2 in the high DDT-resistant population *An. gambiae s.s*. from Accra. However, recent work done by Mitchell *et al*. (2014) suggest a positive significant interaction between glutathione-S-transferase (Gste2) and voltage

gated sodium channel (Vgsc-1575Y and Vgsc-1014F). The two mechanism act together to confer extreme levels of DDT resistance in wild caught *An. gambiae*.

The phenomenal growth of resistance to insecticides in mosquitoes, ‘‘ has become a classic model or example of evolution occurring within human time scales” (Mitchell *et al*., 2014). Hence it is essential to improve our understanding of causes and mechanisms governing resistance development, in order to develop new and more effective novel malaria vector control strategies.

2.2.3 . Type and mode of action to insecticides

Insecticides used in the control of vector borne disease have specific requirements. Mosquito adulticide should have excellent primary mode action, a rapid knockdown effect and selective toxicology (eg. low toxicity to humans and environment) (Duchet *et al*., 2012; Nauen, 2007). WHO has not approve new mosquito adulticide for the past fifteen years and currently uses four class of insecticide namely, pyrethroids, organochlorines, organophosphates and carbamates. The most preferred and currently used insecticide in IRS and LLINs is pyrethroids. The pyrethroids classes of insecticide consist of α-cypermethrin, bifenthrin, cyfluthrin, deltamethrin, permethrin, λ-cyhalothrin and etofenprox. They have many modes of action on the mosquito vector. Pyrethroids bind to receptor site on voltage-gated sodium channel and inhibit the channel deactivation and inactivation (Du *et al*., 2009). This result in firing (repetitive discharges) and depolarization of the nerve membrane, disrupting the electrical impulse in the insect nervous system leading to continuous nerve excitation, paralysis and death of the vector. Pyrethroids also causes an excito repellency response resulting in hyperactivity, rapid knock-down ,feeding inhibition, shorter landing times and undirected flight, all of which reduce the ability of vectors to bite (Du *et al*., 2013; Duchet *et al*., 2012; Narahashi *et al*., 2007; WHO, 2012). Resistance to

pyrethroid insecticides in mosquitoes is conferred by *kdr* mutation in the voltage gated sodium channel (L1014F and L1014s), over expression of monooxygenases cytochrome P-450 (cyp), Esterases and GSH S-Transferases. Most of the reported cases of pyrethroid resistance by mosquito vector has been reported in sub-Saharan Africa (Knox *et al*., 2014; Nauen, 2007; Ranson *et al*., 2011). These are the chemicals of choice in public health because of their relatively low toxicity to humans, rapid knockdown effect, relative longevity and low cost (Nauen, 2007; WHO, 2012)

The most popular group of insecticide is organochlorines, which has DDT the only member of the class. Organochlorines including DDT was popular in past decade because of its rapid knock down effect and was extensively used in IRS. Like pyrethroids, DDT also modulate voltage- gated sodium channel of the adult mosquito and result in rapid knock down effect. DDT resistance can also be conferred by over expression of monooxygenases (cytochrome P-450), DDT dehydrochlorinase (a GSH S-transferase) and *kdr* mutation in the voltage gated sodium channel (L1014F and L1014S) [Ding *et al*., 2005; Nauen, 2007; Ranson *et al*., 2011]. DDT was also used extensively in agricultural pest control and the eradication campaigns of the 1950s. Due to it harmful effect on the environment, the use of DDT was banned in 2001 except for public health use. The use of organochlorines was implicated in earlier reported cases of resistance in mosquitoes (WHO, 2012).

Organophosphates and carbamates are the most expensive group. Organophosphates class of insecticide consists of a vast array of chemicals but fenitrothion, malathion and pirimiphos-methyl are the only recommended ones by WHO. Members of this class act on the mosquito vector by inhibiting acetylcholinesterase. This prevents breakdown of the neurotransmitter acetylcholine resulting in neuromuscular overstimulation and death. MACE target site mutation in

acetylcholinesterase (glycine-to-serine [G119S]) as well as up regulation of mutation cytochrome P450s gene and Esterase confer resistance in *An. gambiae.* Organophosphates classes of insecticides are and have shorter residual activity (2–3 months when used for IRS) but are relatively expensive.

Carbamates used for IRS vector control are bendiocarb and propoxur. Like Organophosphates, Carbamates also inhibit Acetylcholinesterase, is highly effective with reduced residual activity and expensive.

The four class of public health insecticide are limited and have relatively less target-site diversity, since it has two different modes of action when compared with agricultural sector which have many more modes of action including new chemical agent acting on different target sites (Nauen, 2007; Ranson *et al*., 2011). There is a large array of agrochemicals insecticide including those which have the same target site and mode like that of insecticide used for public health vector control. eg. Imidacloprid and Emamectin benzoate

Imidacloprid is the oldest of neonicotinoids group of insecticides that has been widely used and is less effective than permethrin in control of mosquitoes (Pridgeon *et al*., 2008). It binds with the nicotinic acetylcholine receptor at the post-synaptic membrane and interferes with the transmission of nerve impulses. Paul *et al*. (2006) studies on *Aedes aegypti* suggest that detoxification and metabolism of Imidacloprid involve metabolic (cytochrome P450 monooxygenases) enzyme.

On other hand, Emamectin benzoate is a relatively new semi-synthetic insecticide derived from fermentation of avermectin. Emamectin benzoate act as a chloride channel activator by binding to GABA receptor and glutamate-gated chloride channels resulting in hyperpolarization of neuron. This interfere with transmission of impulses and eventually lead to death of insect (Azhar *et al*., 2011; Carmichael *et al*., 2013).

In spite of the aforementioned limitations, for more than 30 years the public health pesticide market have not seen the emergence of new classes of insecticides for wide usage, due to low investors drive (Nauen, 2007; Ranson *et al*., 2011). Hence the emergence of resistance to the majority of existing class used in public health is a threat to the management of vector control disease (WHO, 2010, 2012).There is therefore the need for an effective monitoring and implementation of integrated vector control management.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1. Study Site and Mosquito Larval Sampling

3.1.1 Study site

The Accra Metropolitan area is the country's biggest, most diverse and most cosmopolitan city with estimated population of over 1.6 million people (Nyamadi & Adamah, 2014). According to the 2010 population and housing census, there about 501,903 in house hold Accra and 3.2% of are engaged in agricultural. Accra is located in the coastal savanna ecological zone, which is characterized by dry climatic conditions. It has two rainfall peaks, the first occurring April to June and the second from September to October. The mean annual rainfall is slightly less than 750 mm and relative humidity of that ranges between 65% and 75%.The lowest mean monthly temperature (about 26 ^{o}C) is recorded during August and the highest (about 30^{o}C) between March and April. There are few streams and ponds which usually dry out during in the dry season.

The vegetable farm is sited close to the airport drainage and stretches from the piece of land behind Alliance Française to CSIR. The airport drainage which start from the airport area to Mamobi Nima, serves as source of water for growing of these vegetables. The farmers also dig bits or trenches which is used in holding water during the raining season. During the dry season the pits are filled with water from the drainage with the aid of a water pump. Hence these pits serves as a water reservoir. Watering of the vegetables is done either by directly pumping water from the drainage with aid of mechanical water pump or the use of watering cans to fetch water from the pits to water the vegetables. These man made pits serves as a breeding site for mosquitoes.

3.1.2 Larval survey

Larvae of wild *Anopheles gambiae* s.l were collected from breeding sites at the vegetable farms in

Opeibea on the premises of Water Research Institute of the Council for Scientific and Industrial Research (CSIR) in the Greater Accra Region of Ghana. The co-ordinates of each collection point at the sites were recorded by global positioning system (GPS). A map of the collection sites is was drawn. All laboratory studies was done at the insectary, Entomology labs, Molecular lab Vestergaard and Parasitology Department at the Noguchi Memorial Institute of Scientific Research.

3.2 Effect of Different Diets on Life History Traits of *Anopheles gambiae*

3.2.1 Establishing wild colony of *Anopheles gambiae* s.l

Anopheles gambiae s.l colony from the wild population from Opeibea was established at the insectary of Noguchi Memorial Institute of Medical Research (NMIMR), University of Ghana Legon. The larvae of *An. gambiae* 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 8cm) containing 1.5L of dechlorinated tab water (resulting in a water depth of 3cm). Tropical fish flakes was grounded using a domestic blender and was double sieved by passing through 425um domestic mesh sieve. The larvae was fed with about 100mg of grounded tropical fish meals twice daily until they grew into pupated. The pupae were collected with the aid of a plastic pipette into a small plastic cup (pupae cup) which was placed in a 30x30x30cm nylon mesh cage. The adult upon emergence were fed on freshly prepared 10%w/v of sugar solution.

The adult female *Anopheles gambiae* mosquitoes were blood fed using a guinea pig, when the adult mosquitoes were three days old. The floor of the 30x30x30cm nylon mesh cage was lined 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 7: 30pm to 5: 30am. In order not to kill or weaken the guinea pig, the subsequent blood feeding was done from

8:00pm to 1:00am and then continued from 3:30am 5:30am. Petri dish lined with wet cotton with a sheet of paper or filter paper cut to fit the circumference of petri dish was used as an oviposition tray. The oviposition tray was positioned in the cage on the second day after blood feeding to obtain eggs.

3.2.2 Laboratory reared mosquitoes

Eggs obtained from laboratory reared *Anopheles gambiae* susceptible sensu stricto (Kisumu strain) colony at the insectary of NMIMR was also used for this study. The colony was established from mosquitoes sampled from Kisumu, Kenya and reared under laboratory conditions perceived as ideal for survival and reproduction. The adult mosquitoes were fed on 10%w/v sugar water solution, ambient conditions of 28ºC-30 ºC, 70%-80% relative humidity and a photoperiod of 12hr:12hr (Light : darkness). Mosquitoes from this colony were used for studies.

3.2.3 Preparation of larval diets

In a preliminary study, Rabbit pellet (no.001131 Agrifeed, Kumasi Ghana.) , Fish flakes (Lopis Fish flakes™), smoked herrings, Cerelac maize (Nestle®), Cat meal (Purina Friskies®) and Baking yeast (SuperBake®) (six food source) were prepared and tested. High larval mortality was observed when the developing larvae were fed on smoked herrings, hence was discarded. Due to the fragile nature of 24 hr old first instar, large quantity of the diet led to death of some larvae. In order to prevent or reduce death at the first instar stage, the protocol was further optimized by reducing the quantity of amount of meal used in feeding the 24 hour old first instar larvae on day one of the experiment.

Rabbit pellet, Lopis Fish flakes and smoked Herring were each blended and sieved twice using a domestic blender and 480um mesh sieve into fine particles. The cat meal was put in the oven at

50^{o}C for an hour so as to reduce the oil content in it. The Baking yeast easily dissolved in water and Cerelac was in a fine particle state already, hence was not blended or sieved, but directly used. Using an AND GH-120 (Sn; 15103038, Japan) electronic balance, either 20mg or 50mg was weighed into small weighing cups which was used in feeding the larvae. In each bowl 0.008 or 0.002g/day/larvae of a particular single diet

3.2.4 Rearing of larvae and experimental design

Fine thermos (F-100, Japan) water bath was used to control the environmental temperature of larva rearing tray (14.5x9.5x4.8cm (650 ml microwave, Safco Dubai)). The water bath was set to 30^{o}C±2. The larva rearing tray was filled with 450ml distill watered and was put in to the water bath. The rearing tray was allowed to acclimatize for four hours and was ensured that the water in the bath covered two thirds (2/3) of the height of the tray. The water level in the bath was kept constant throughout the study. A dozen set of 50ml beaker was filled with 45ml of distilled water. Pasteur pipette was then used to randomly select 25 first day old instar larvae into each beaker and the level of water was adjusted to the 50ml mark (with the aid of pasteur pipette). 25 larvae in each 50ml beaker together with its water was randomly picked from the set and transferred into a 14.5x9.5x4.8cm rearing trays in the water bath, bringing the volume water to 500ml and a depth of 3cm. The rearing procedure was a modified version previously described by Mohammed and Chadee, (2011) The water trough of the thermos (F-100, Japan) bath was replaced with a bigger trough with a longer sleeve which reduced risk of outside interference. The 4x4cm galvanized aluminum mesh in the trough served as a stand which separated the base of the trough from the bottom of the rearing trays. The space between the water trough and rearing tray enable effective circulation of water and even distribution of water temperature in the bath. The laboratory

thermometer was used to check for the same constant temperature in all areas within the trough and the rearing trays. This was employed to ensure that, all the other parameters (temperature and density of larvae) except the treatment (feeds) remained constant as shown in Figure.3.2 below. 20mg of either Rabbit pellet (no.001131 Agrifeed, Kumasi Ghana.), Fish flakes (Lopis Fish flakes™), smoked Herings, Cerelac maize (Nestle®), Cat meal (Purina Friskies®) or Baking yeast (SuperBake®) was added on the first day of the study. The larvae were initially fed with 20mg of a single diet because of the fragile state of the first instar *An. gambiae* larvae. The larvae were then fed with 50mg of the same particular meal every day, from the second day onwards until all the larvae pupated. Each treatment was replicated thrice and the rearing trays was visually inspected twice in a day for pupae. Changes in larvae developmental structure, survival and rearing water was noted. In other not to this disrupt the larvae the rearing water was changed after every 3 days. The pupae were collected into pupal cups containing small amount of distill water and placed in a 30x30x30cm nylon mesh cage. The adult upon emergence were fed with freshly prepared 10%w/v of sugar water solution. The adult were kept under 12hr; 12hr (L: D), temperature range of 26 to 39^0C, 66% to 84% relative humidity and fed on 10%w/v sugar solution until it died. The sugar soaked cotton ball was changed every day.

Figure 3.0 Fine (Thermos F-100, Japan) Water Bath

- **A.** large water through which minimize interference from out side
- **B.** Thermo F-100 water bath with regulator at the top, heather and pump
- **C.** Thermometer used to check for constant temperature in the water bath and rearing trays of water and temperature
- **D.** 4x4 Aluminum wire mesh that serve as a stand and enhanced evenly distribution of water and temperature
- **E.** 650mm (microwave bowl) rearing tray filed with 500ml of distill water and contains the developing larvae.

3.2.5 Determination of life history parameters

Larval developmental time: the larval developmental time was recorded as period (in days), the larvae spent to develop from the first instar stage to pupae under the influence of a specific treatment (feed).

The pupation rate; recorded as the total number of pupae collected per the time (in days) taken to develop from 4th instar larva to the pupation under the influence of particular feed.

Pupal weight; the first five pupae were picked either in groups or individually with the aid of a pasteur pipette and dried on a tissue paper. Their weight was then determine with the aid of AND

GH-120 (Sn; 15103038, japan) electronic balance (as show picture 1 in appendix I). The average weight of the pupae was calculated to obtain the pupal weight.

Larval survivorship: was determined when 3rd or 4th instar larva develop into pupa stage, when they could not developed to pupa, they were recorded as undeveloped larvae and those were died as larval mortality

3.3 WHO Insecticide Susceptibility Tube Assays

Anopheles gambiae larvae was obtain from the same spot in the coordinate map (as shown in fig 3.1 above) from breeding site at vegetable farms in Opeibea. Field collected *An. gambiae* larvae were morphologically identified using the keys of Highton, (1983) and separated or sorted into 27x16x6.5 plastic rearing trays containing 1.5L of dechlorinated water Based on the data and observation from the initial part of this study, the larvae cultures were fed twice daily with rabbit pellet and fish flake until they developed into pupae. The pupae were collected into pupal cups containing small amount of distilled water and was put in (30x30x30cm) nylon mesh cage. The adult upon emergence were fed with freshly prepared 10%w/v of sugar water solution.

Six holding green dotted WHO holding tubes, were lined with six sheets of clean white paper (12 x 15 cm) and fastened into position with a steel spring-wire clip. The slides of each tube was then attached to tubes. Non-blood-fed adult female mosquitoes of 3 to 5 day old were are aspirated (in batches) from a mosquito cage into the six holding tubes through the filling hole in the slide to give five replicate samples of 20 mosquitoes per tube (that is a total of 120 female mosquito). The

mosquitoes were kept in the holding tubes set in an upright position for one hour and any damaged or weaken mosquitoes were removed at the end of one hour.

WHO impregnated papers and tubes was assembled together. Each of the two yellow-dotted control exposure tubes was first lined with oil-impregnated papers which were held in position with steel spring-wire clip. Four of red-dotted exposure tubes were also lined with similar a sheet of insecticide-impregnated paper (to give four replicate) with each of sheet fastened into position with a copper spring-wire clip. The sliding unit was attached and closed. The mosquitoes in the holding tubes were gently transferred into the exposure tubes through the filling hole in the sliding unit. The exposure tubes were set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour (60 minutes). Knockdown (KD) of the 120 *An. gambiae* s.l. susceptibility to insecticide was recorded at 10, 15, 20, 30, 40, 50 and 60 min (as shown in Picture 2 below, refer to appendix I).

At the end of the 1-hour exposure period, the holding tube were attached to the vacant position on the slides unit. With the slide unit opened, the mosquitoes were gently blown into the exposure tubes through the filling hole. The exposure tubes were then detached from the slide units. Eighty percent of the mosquitoes were not knock down after 1-hour exposure. Knock down was therefore observed at an additional 20 min in the holding tubes and recorded. The mosquitoes were fed on a pad of a cotton-wool soaked in 10%w/v sugar solution which was placed on the mesh-screen end of the holding tubes. The adult female *Anopheles gambiae* are maintained in the holding tubes at 26^0C or room temperature and 80% relative humidity for 24 hours (the recovery period).

After 24 hours post-exposure, the number of dead mosquitoes was counted and recorded. An adult mosquito wass considered to be alive if it was able to fly. Mosquitoes were exposed to papers impregnated with the WHO recommended discriminating concentration (v/w) of all four classes

of insecticide. Whilst controls were exposed to non-impregnated papers. The test was done using WHO tubes, test papers and conducted in line with WHO test procedure for insecticide resistance monitoring in malaria vector mosquitoes (WHO, 2013a). At the end of the each bioassay test, the dead mosquitoes were transferred to individual, clearly labelled 1.5 ml microcentrifuge (Eppendorf) tubes containing dry silica gel (separating dead and live mosquitoes into separate tubes). The live mosquitoes were killed by putting them in -80^{o}C freezer for 5 minutes. The mosquitoes were stored in a desiccator for further molecular analyses.

3.4 Questionnaire Survey on Insecticide Usage

The farm was visited on different days to interact with some vegetable farmers and obtain firsthand information on the various types of agrochemicals used on the farm (as shown in Picture 4 of Appendix V). A verbal interview involving questions such as the type of agrochemical especially insecticide used; mode or frequency of application; cost of agrochemical; where they were purchased; the level of educational of farmers; occupation and place of abode; were conducted.

3.5 Molecular Biology Studies

3.5.1 The isolation of genomic DNA of *Anopheles gambiae* s.l.

A total of 138 female mosquitoes samples were randomly selected from female mosquitoes that were stored after exposure to all four class of insecticide during the susceptibility testing. Out of the 138 pooled samples, 7 dead and 10 live was selected from each category of female mosquitoes that were exposed to deltamethrin (0.05%), permethrin (0.75%), lambala-cyhalothrin (0.05%), fenitrothion (1%), propoxur (0.1%) , malathion (5%), and bendiocarb (0.1%). Two (2) dead and ten (10) live mosquitoes were selected from DDT (4.0%) exposed samples whiles 5 live and 1

dead mosquitoes were from cyfluthrin (0.15%). The individual carcass of the whole mosquito were put into an autoclaved 1.5ml microcentrifuge tube and homogenized in 200µl 2% cetyl trimethyl ammonium bromide (CTAB2%) with the aid of a Konte's pestle. The homogeneous mixture was heated at 65^{o}C for 5 min in a Techne (Dri-block) Db-2A (model: FDB02Ad, SN:173105-A) block heater. The microcentrifuge tube was removed from the heating block and 200µl of chloroform added. The content in the tube was mixed by inversion and centrifuged for 5 min at 12000 rpm using Sigma 3-16pk (Sn: 141519, USA) centrifuge, to obtain a two-phase separation of mixture. Acura socorex micropipette was used to pick the upper phase (supernatant) and introduced into another tube. 200µl of isopropanol was then added to the supernatant and mixed well by inversion. This was centrifuged for 15 min at 12000 rpm. In order not to discard the pellet in the bottom, the tube was gently tilted to remove isopropanol and 200 µl of 70% ethanol was then added. This was centrifuged for 5 min at 12000 rpm at room temperature and gently tilted to pour out 70% ethanol (taking care not to discard the pellet at the bottom). The tube was left overnight in an inverted position to dry at room temperature. 20µl of DNAse free water was added, vortexed (to mix) and stored at -20^{o}C (as extracted DNA).The extraction of DNA was done following modified protocol of Wagner *et al*. (1987) and Vestergaard (Datsomo & Okyere, 2015).

3.5.2 PCR Identification of *Anopheles gambiae* s.l complex

A master mix of PCR identification reaction assay was prepared on ice in Captair bio (MA 01967USA) hud for a total of 142 sample. A dilution of 1/40th extracted DNA from a single mosquito was amplified in 25µl PCR mix containing 0.6µl of each 10µM (UN [GTG TGC CGC TTC CTC

GAT GT], AG [CTG GTT TGG TCG GCA CGT TT], AR [AAG TGT CCT TCT CCA TCC TA], AM [GTG ACC AAC CCA CTC CCT TGA]) primer, 12.5µl 2X Green Go Taq (Promega, USA) and DNAse free water. 3µl of 1/40 diluted stock DNA or water (in the case tube negative control) was added to each tube. The Go Taq in the PCR-Mix comprises 1X Green Go Taq (Flexi Buffer), 25mM $MgCl_2$, 0.2mM of each dNTP and 1.25 Units of Go Taq DNA polymerase.

The PCR reaction buffer was prepared in a 3.0 autoclaved microcentrifuge tube for maximum of 50 samples at a time. The PCR tube was put in Tp 650 (Sn E-2275 or D1592, japan) thermal cycle for the amplification reaction. The PCR amplification reaction was initiated at 94^{o}C for 3min to activate DNA polymerase. 35 cycles each consisting of 30sec denaturation at 94 ^{o}C, 30sec annealing at 50^{o}C, and 1min extension at 72^{o}C. Final cycle was extended for 7min at 72^{o}C and held at 4^{o}C.

About 8µl amplicon of PCR product was loaded and electrophoresed in a 2% agarose gels stained with ethidium bromide at 140V for 30min on a Biorad power pac 3000 (SN; 277BR03754) generator. The gel was verified in Toyobo Tm-20 Transilluminator (SN: 102103-003, Japan) and Fas III Ds-30 (SN: 6710530, Japan). Species identification of *Anopheles gambiae* was done using PCR according to established protocol by Scott *et al*. (1993) and Fanello et al., (2002).

3.5.3 Molecular identification of M and S Forms of *Anopheles gambiae* s.s using Sine PCR

SINE master mix PCR assay was prepared in an autoclaved Eppendorf tube for maximum 50 samples at a time for all the 142 samples, in the Captair bio (MA 01967-USA) hud. PCR reactions were carried out in a 25µl reaction which contained 1µl of 10µM each primers (F6.1a and R6.1b) 12.5µl Go Tag, 6.5µl DNAse free water and 4µl of 1/40 diluted stock DNA template extracted from a single mosquito. DNase free water was added in the case of negative control was added to each tube. The thermocycler conditions were initiated at 5min at 95^{o}C and 35 cycles each

consisting of 30sec denaturation at 94ºC, 30sec annealing at 54ºC, 1min extension at 72ºC, final cycle product was extended for 10min at 72ºC and held 4ºC. The resulting products were migrated on 2% agarose gels stained with ethidium bromide. The low and high molecular weight bands corresponding to fragments containing or lacking the targeted SINE200 was analyzed and verified (Santolamazza *et al*., 2008).

3.5.4 Detection of L1014f Kdr Mutation in *An. gambiae s.s*

The L1014F *KDR* mutation in the II domain region of gated channel gene was amplified and detected by use of ADG1 (ATA GAT TCC CCG ACC ATG), ADG2 (AGA CAA GGA TGA TGA ACC), ADG3 (AAT TTG CAT TAC TTA CGA CA) and ADG4 (CTG TAG TGA TAG GAA ATT TA) primers (Martinez-Torres *et al*., 1998). The master mix PCR assay was prepared for maximum 50 samples at a time for all the 142 sample in the Captair bio (MA 01967-USA) hud. The PCR reaction master mix assay contained 4.5µl of DNAse free water, 1µl of 20µM each primer (AGD1, AGD2, AGD3, AGD4) and 12.5µl Go Tag to get a volume of 21µl. 4 µl of 1/40 diluted stock DNA or water (in the case tube negative control) was added to get a total volume of 25µl. The PCR amplification was done with an initial step of 3min at 94ºC followed by 40 cycles each consisting of 30sec denaturation at 94ºC, 1.5min annealing at 48ºC, 2min extension at 72ºC, final cycle product was extended for 5min at 72ºC and held at 4ºC.8ql amplified PCR product was loaded onto 2% agarose ethidium bromide gel and electrophoresed at 140v for 30min. The resulting gel was then verified and photographed. Those samples that did not show any bands was repeated using 4ul of 1/10 or 1/5 diluted stock DNA.

3.5.5 Detection of Ace-1 Mutation

The ace-1 polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) mutation assay for detection of mutations within acetylcholinesterase (AChE) in genes An. gambiae was designed based on the G119S mutation isolated from *An. gambiae* (Weill *et al*., 2004, 2003). Ace-1 PCR assay require two stepwise PCR reaction in which the first step involve the amplification of DNA fragment followed by digestion with *Alu*1 restriction enzyme.

The first step master mix PCR reaction was performed in a total volume of 25µl containing. 9µl of DNAse free water, 1ul of each 10µM (Ex3AGdis and Ex3AGRev) primer, 12.5µl Go Tag and 1.5µl of 1/40 diluted stock DNA or water (in the case tube negative control) The PCR amplification cycling condition began with an initial step of 3min at 94^{o}C followed by 35 cycles each consisting of 30sec denaturation at 94^{o}C, 30sec annealing at 62^{o}C, 30sec extension at 72^{o}C, final cycle product was extended for 5min at 72^{o}C and held at 4^{o}C.

Second step (Restriction enzyme digest)

In the second step, 12.3µl of DNAse free water, 2µl of enzyme buffer, 0.2 acetylated BSA 0.5µl and *AluI* restriction enzyme was added together to get a total volume of 15µl per sample .5µl PCR product from above reaction was added to bring the total reaction volume to 20ql per sample. The reaction mixture in cupped PCR tube and allowed to incubate at 37°C for 4hr in Tp 650 (Sn E2275 or D1592, japan) thermos cycle. The cycling condition was 60min at 37°C for each for 4hours step and a hold at 4^{o}C. PCR product and digestion was carried out for four hours following the protocol of Weill *et al.,* (2003) The final amplified PCR product was electrophoresed and photographed on a 2% ethidium bromide stained agarose gel. Those sample did not show was repeated using 4ul of 1:10 or 1:5 diluted stock DNA.

3.5.6 Gel Electrophoresis

The 2% ethidium bromide stained gel was prepared by dissolving 2.4 g of agarose in 120ml 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 2µl of ethidium bromide. Eight microliters (8µl) amplified PCR product was loaded and migrated at 140v for 30min 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. Four of a sixteen well gel was loaded with PCR DNA samples together with a control and migrated at the same time The gel was verified and photographed in the Toyobo Tm-20 Transilluminator (SN: 102103-003, Japan) connected to a Toyobo Fas III Ds-30 (SN: 6710530, Japan) monitor for printing pictures of electrophoregrams. The results was then read from the electrophoregram and compared with the expected band size. Appendix II provides further information on molecular methods and their expected band size in *An. gambiae* species complex.

3.6 Data Analyses

The evaluation of each diet was accessed by comparing the performance of the five meals on each life history traits parameters were analyzed using ANOVA, followed by Tukey's Honest Significant Difference (HSD) for mean separation and comparison. Pearson Correlation was used to estimate the degree of association or relationship between the various life history parameters

and diets. Log-probit analysis was used to calculate Times for 50% and 90% knockdown (KDT_{50} and KDT_{90}). The genotypic frequencies at the ace-1 locus were compared to Hardy–Weinberg expectations using Chi-square model from SPSS 16.0 and 17.0 for Windows. All the data was analyzed using SPSS 16.0 and 17.0 for Windows

CHAPTER FOUR

RESULTS

4.1 Effects of Different Diets on the Life History Traits

A Total of 525 newly hatched first instar larvae were used in evaluating the effect of rabbit pellet, fish flake and cat meal, cerelac and yeast diet on life history traits (pupae weight, pupation rate , larval developmental time (days) and larval survivorship). Out of these 150 wild strain and 375 were 'Kisumu' laboratory strain.

Two factorial analysis of variance (ANOVA) was used to compare mean differences in a preliminary study in which the two groups of *Anopheles* strain (laboratory breed Kisumu and wild from Opeibea) were fed on rabbit pellet and fish flake. A non-significant difference was observed between the observed life history characteristics; (pupae weight (F = 3.045, df=6, *P*=0.124), pupation rate (F =0.145, df=6, *P*=0.715), larval developmental time (days) (F = 0.304, df=6, *P*=0.598) and larval survivorship [F = 0.304, df=6, *P*=0.598]) of the two strain *Anopheles* (that is, laboratory bred strains (Kisumu) and wild strains from Opeibea). Yet within a strain, significant difference was observed in pupation rate (F =14.913, df=6, *P*=0.006) and Larval developmental time (days) (F =23.014, df=6, *P*=0.002) when fed on different diets alone. There was also, nonsignificant statistical interaction between the type of *Anopheles strain* and diet type (see Table 9.
in Appendix III).

A higher pupal weight (2.353mg) was observed when the pupae was fed on rabbit pellet (fig 4.1). The weight of pupae enhanced by each type of food was significantly (F =13.834, df=6, *P*=0.001) different (as shown in Table 4.1 below).

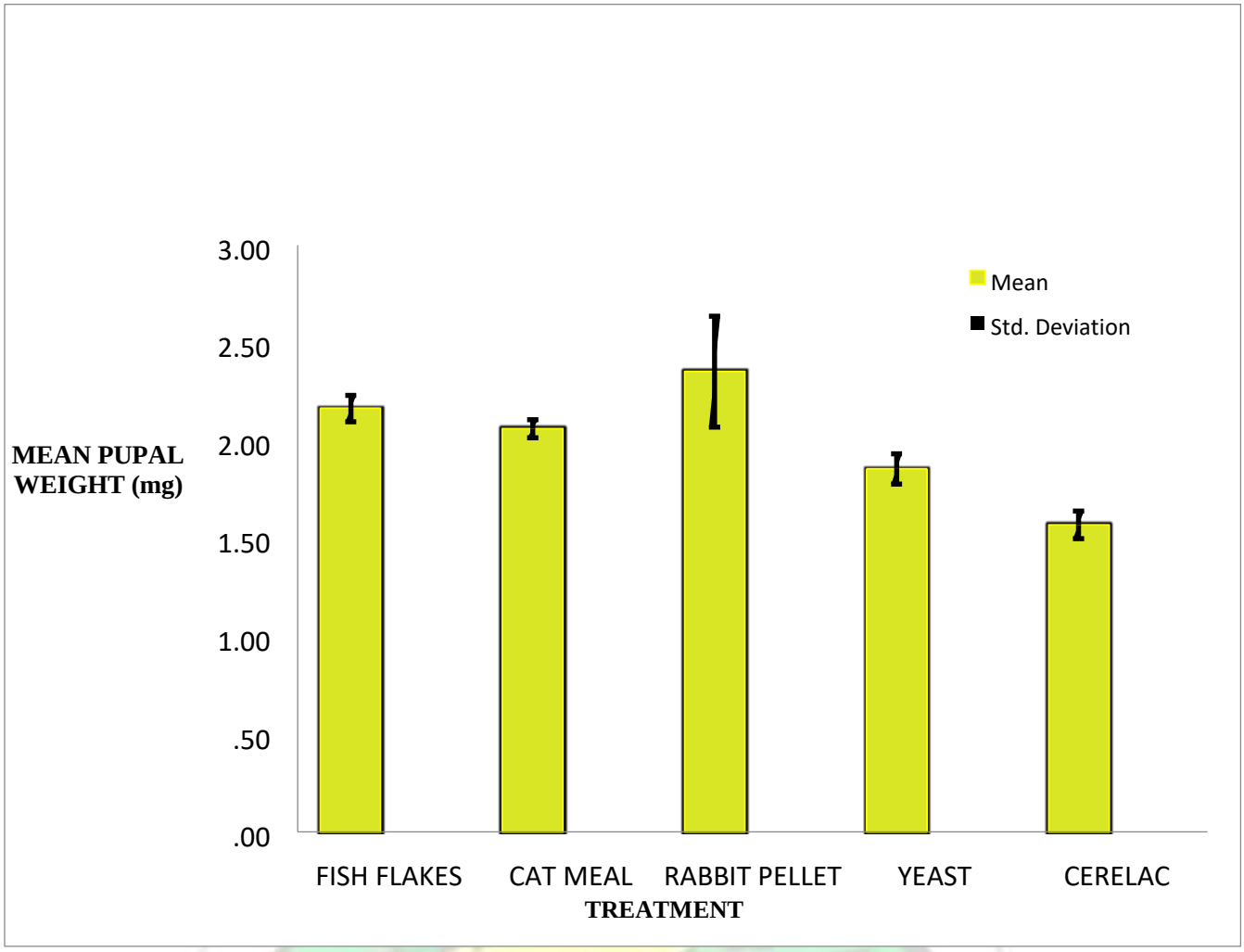

Figure 4.1 Pupal weight (mg) of *An. gambiae s.s.* in different food regimes **Table 4.1** A One-Way Anova table showing the differences in pupal weight of *An gambiae s.s.*

		Sum of Squares	df	Mean Square	F	Sig.
PUPAL WEIGHT(mg)	Between Groups	1.172	4	.293	14.154	0.000
	Within Groups	.207	10	.021		
	Total	1.379	14			

In the above Anova table comparing the differences between the mean Pupal weight of an α value of 0.05 and F=14.154, p value of 0.000. Since the P value is smaller than the set α value, the probability of finding the mean weight as a result of the various

treatment among Population is less and did not occur by chance. Hence the mean pupal weight as a result of the treatments are not equal and there is a significant difference among them.

A Tukey HSD post hoc multiple comparison showed that the differences means weight of pupae whose larvae were fed on rabbit pellet (2.353±0.283) and yeast (1.856 ± 0.076) was significant (*P*=0.012). Pupal weight difference between those fed on Rabbit Pellet (2.353±0.283) and Cerelac (1.570 ± 0.070) was also highly significant (*P*=0.000). But there was no significant difference (*P*=0.865) of that of rabbit pellet (2.353±0.283) with fish flakes (2.240± 0.1). The mean difference of pupal weight of larvae fed on Cat meal (2.062±0.046) and Cerelac ((1.570 ± 0.070, *P*=0.013) and, fish flake and Cerelac (1.570 ± 0.070, *P*= 0.003) varied significantly. The table 10 in Appendix III shows the detailed analyses of Tukey HSD post hoc multiple comparison, of the various life history characteristics and diet.

A higher number of pupae (21.22) was observed in *Anopheles* population fed with Rabbit pellet (Fig 4.2.). The pupation rate in each treatment was significantly different (F =50.448, df=6, *P*=0.000) (Table 4.2).

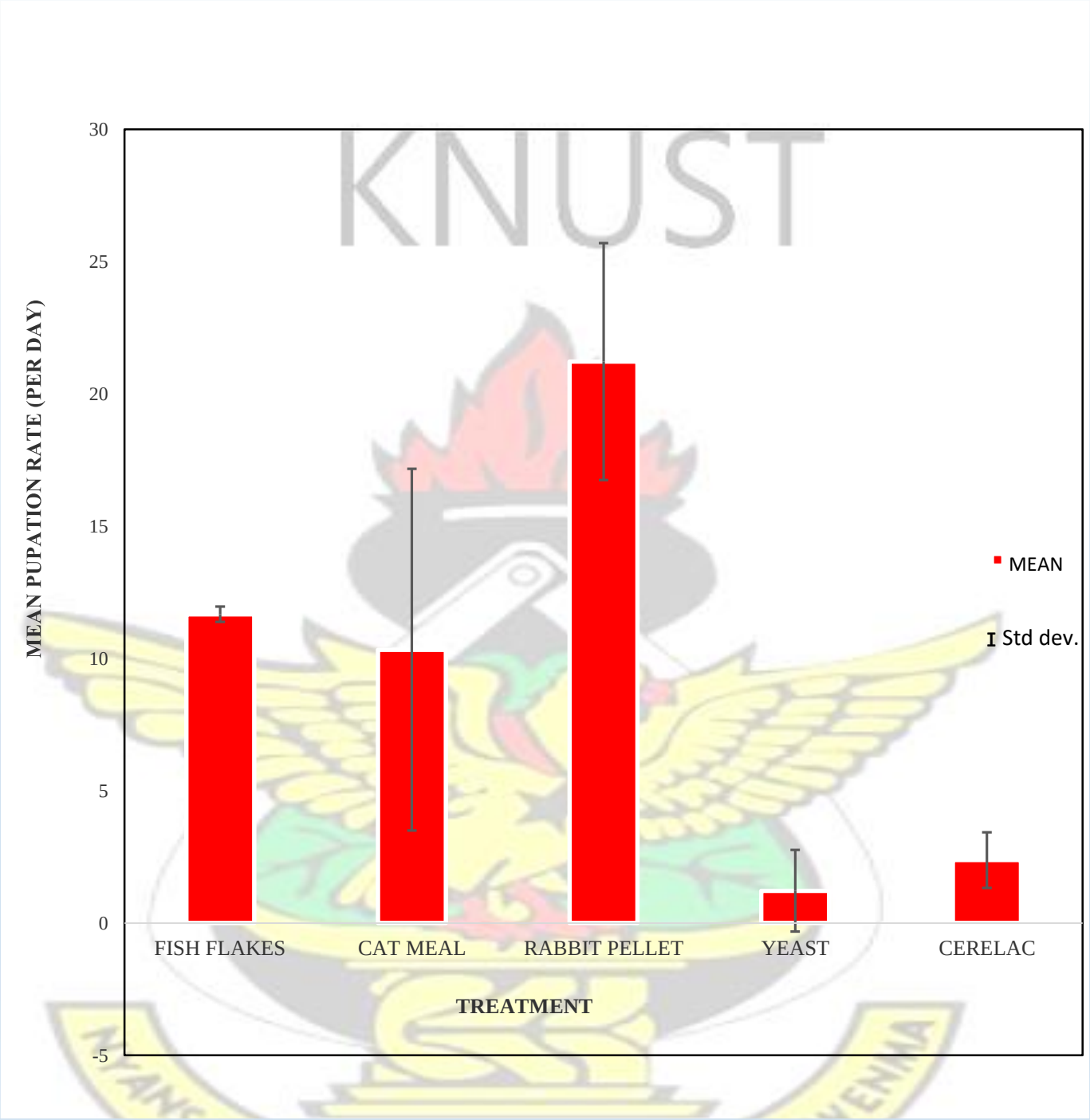

Figure 4.2 Comparing Pupation rates of *An. gambiae s.s.* larvae fed on different food type **Table 4.2** A One-Way Anova table showing the differences in pupation rate of *An. gambiae s.s*

	Sum of Squares	df	Mean Square	F	Sig.

PUPATION RATE	Between Groups	785.904	4	196.476	50.448	0.000
	Within Groups	38.946	10	3.895		
	Total	824.850	14			

From the above Anova table comparing the differences between the mean pupation rate of α value of 0.05 and F= 50.448, p value of 0.000. Since the P value is less than the set α value, the probability of finding the mean weight as a result of the various treatment among population is less and did not occur by chance. Hence the mean pupation rate weight as a result of the treatments are not equal and there is a significant difference among them.

A Tukey HSD Post Hoc multiple comparison was showed that means pupation rate of rabbit pellets (21.222±3.976) was significantly higher than fish flake (11.667±0.289, *P*=0.001), cat meal (10.333±0.289, *P*=0.000), yeast (1.217±1.546, *P*=0.000) and cerelac (2.364±1.049, P=0.000). The difference between the mean pupation rate of fish flake (11.667±0.289) were significantly different from the mean pupation rate of yeast (1.217±1.546, P=0.001) and Cerelac (2.364±1.049, P=0.001). That of cat meal (10.333±0.289) against Yeast (1.217±1.546,) and Cerelac (2.364±1.049), were also found to be significant (P=0.002) (P=0.004) respectively. Whilst the comparison of fish meal with cat meal and yeast with Cerelac were not significant (P=0.916) (P=0.946) respectively at an α=0.005 (see Table 10 in Appendix III).

The shortest time (8) in days it took the larva to develop from the first instar to adult was observed when the larva were fed with rabbit pellet (fig 4.3). The developmental time of the larvae was significantly different (F=114.250, df=6, P=0.001) across each treatment (Table 4.3.)

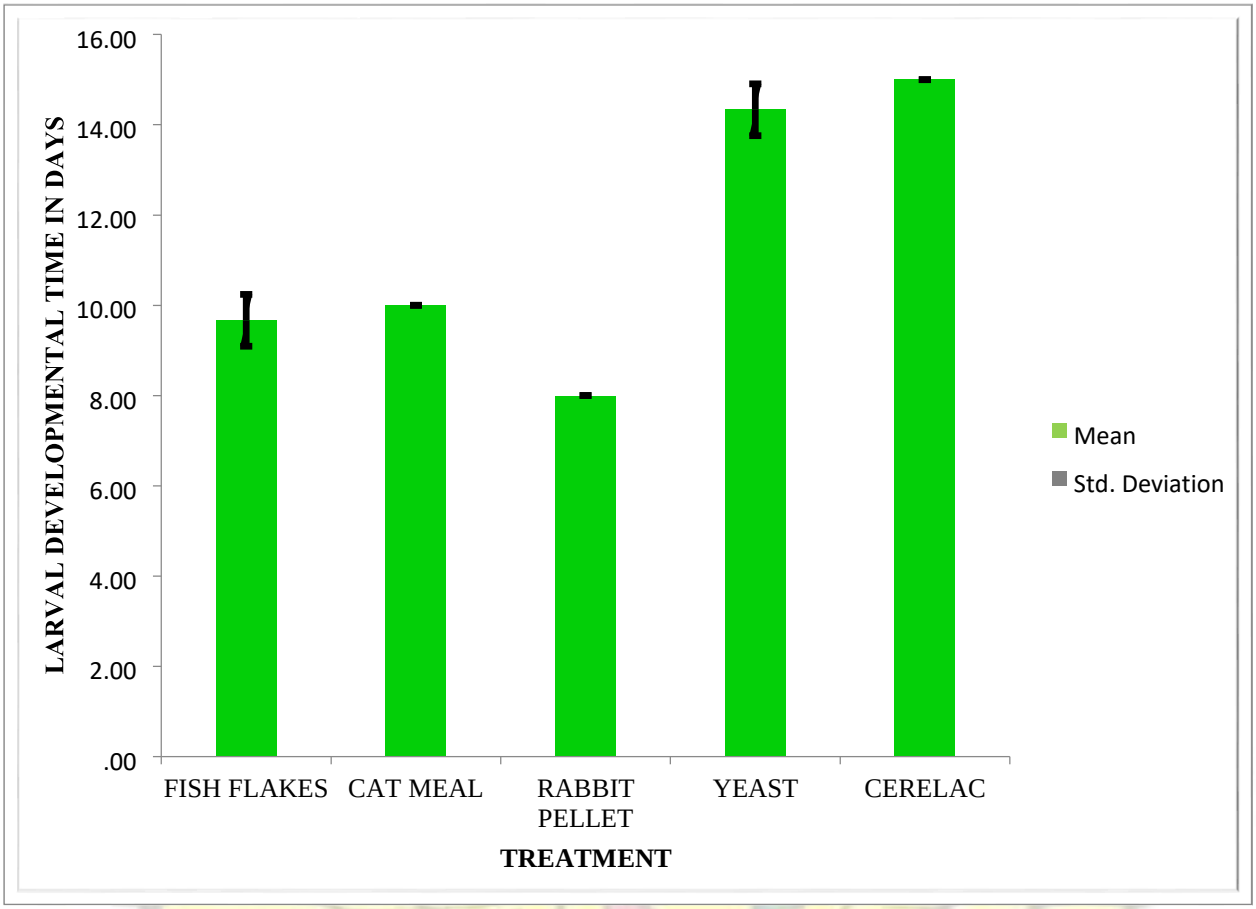

Figure 4.3 Comparison of *Anopheles gambiae* s.s. larval developmental time in different diets **Table 4.3** One-Way Anova of larval developmental time in *An. gambiae s.s*

		Sum of Squares	df	Mean Square	F	Sig.
LARVAL DEVELOPMENTAL TIME (IN DAYS)	Between Groups	122.267	4	30.567	114.250	0.000
	Within Groups	2.667	10	.267		
	Total	124.933	14			

Anova table above, comparing the differences between the mean Larval Developmental Time of α value of 0.05 and F=214.250, p value of 0.000. Since the P value is smaller than the set α value, the probability of finding the mean weight as a result of the various treatment among population is less and did not occur by chance. Hence the mean Pupal Developmental Time as a result of the treatments are not equal and there is a significant difference among them.

Post hoc Tukey HSD multiple comparison revealed that the means larval developmental time of larvae fed on rabbit pellets (8.00±0.000) was a significantly difference from those fed on cat meal (10.00±0.000, *P*=.005), yeast (14.33±0.577, *P*=0.000) and cerelac (15.00±0.000 *P*=0.000). The

mean developmental time of larvae fed on fish flakes (9.00±1.000) also differed significantly from larvae fed on yeast (14.33±0.577, *P*= 0 .000) and cerelac 15.00±0.000, *P*=0.000). There were also a significant difference between the mean larval developmental time of larvae fed on cat meal (10.00±0.000) from that of against yeast (14.33±0.577, *P*=0.000) and cerelac (15.00±0.000, *P*=0.000). That of fish flakes against cat meal (10.00±0.000, *P*=0.200) and yeast against cerelac (0.667±SD, *P*=0.539) was not significant (refer to Table 10 of Appendix III). Hence, larval developmental time was significantly enhanced in larvae fed on rabbit pellet.

A lot of larvae survived (24) when the larvae population was fed with rabbit pellet from the first instar age (fig 4.4). The one way Anova analyses indicated a significant different (F=11.826, df=6, *P*=0.001) in mean larval survivorship of each treatment (table 4.4).

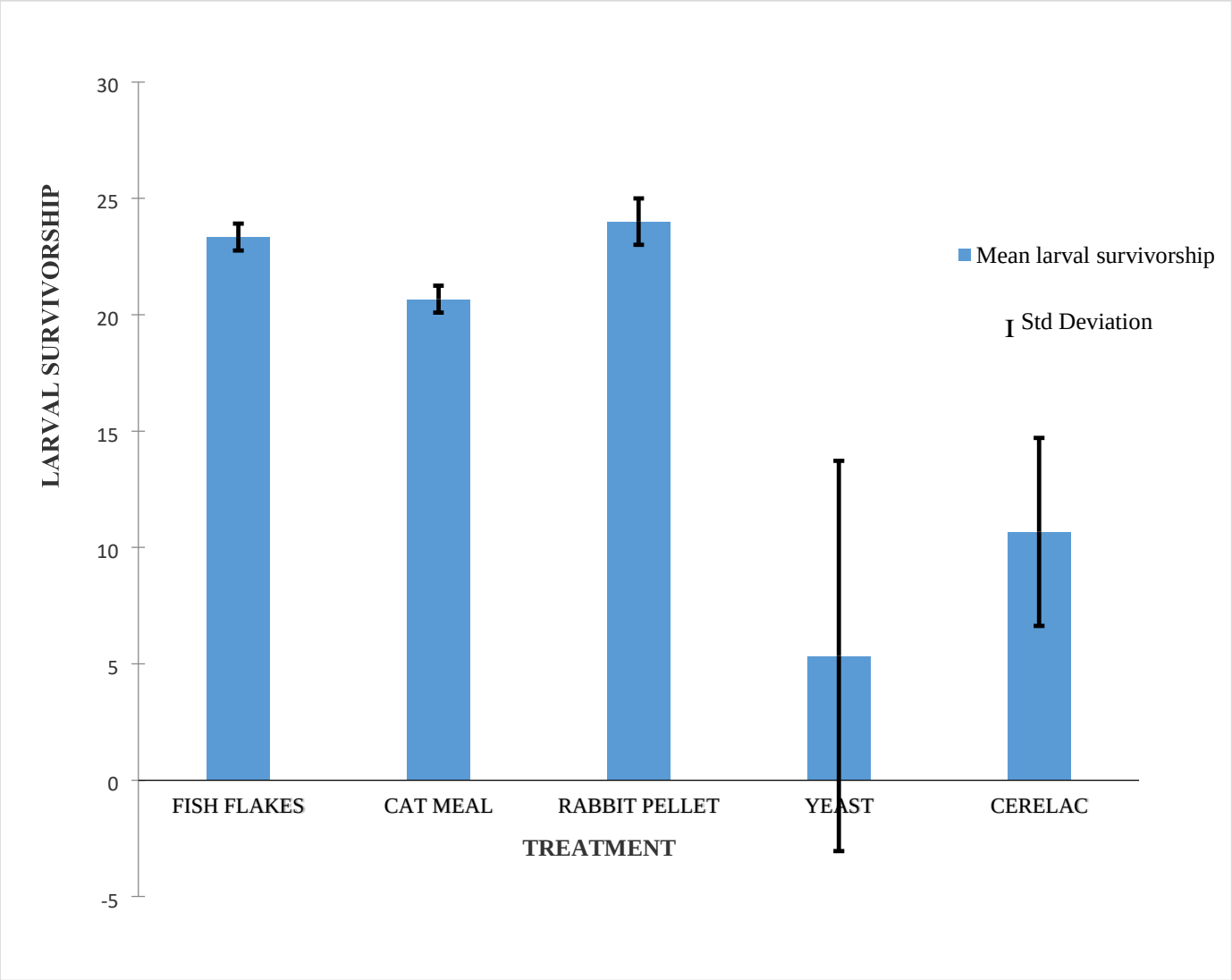

Figure 4.4 Comparison of *An. gambiae* s.s. larval survivorship in five different diet.

Table 4.4. A One-Way Anova table showing the difference in larval survivorship

		Sum of Squares	df	Mean Square	F	Sig.
LARVAL SURVIVORSHIP	Between Groups	835.733	4	208.933	11.826	.001
	Within Groups	176.667	10	17.667		
	Total	1012.400	14			

α value of 0.05

Tukey HSD post hock multiple comparison analyses indicates that mean larval survival when the larvae was fed with fish flasks (23.33±0.577), cat meal (20.67±0.577) and rabbit pellet (24.00±1.000) was significantly different (*P*=0.003, *P*=008 and *P*=002) from the mean survivorship when fed with yeast (5.33±8.386) respectively. A significant number (P=0.027) (P=0.002) of larvae also survived when they fed with fish flakes (23.33±0.577) and rabbit pellet (24.00±1.000) than cerelac (10.67±4.041) (as shown in Table 10 below, see Appendix III).

Correlation analyses indicates a strong association of pupae weight with pupation rate (r (15) =.747, P<0.01), developmental time (r (15) = -.886, P<0.001) and Larval survivorship (r (15) = .741, P<0.01) (refer to Table 11in Appendix III).

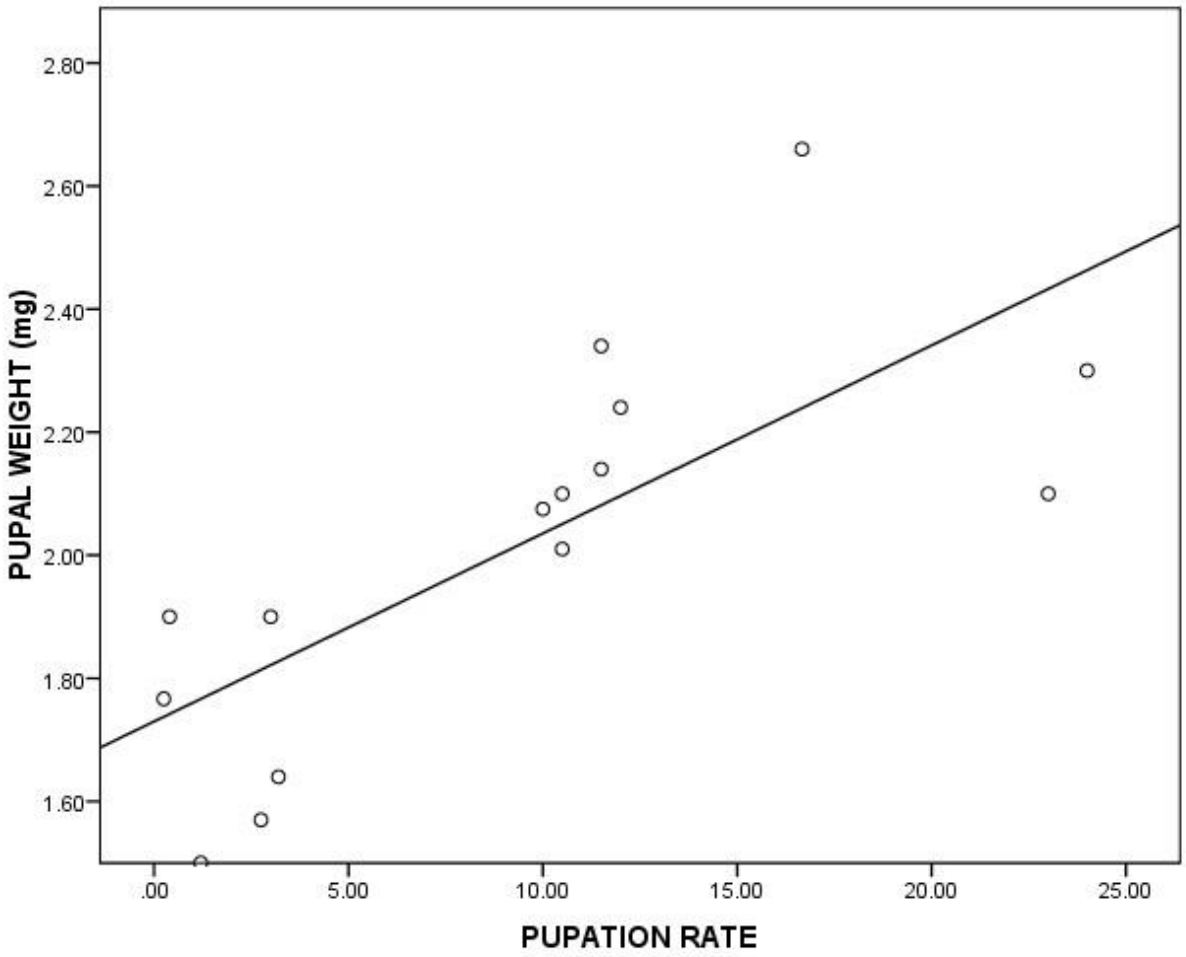

Figure 4.5 Correlation between pupal weight and pupation rate of *An. gambiae* s.s larvae

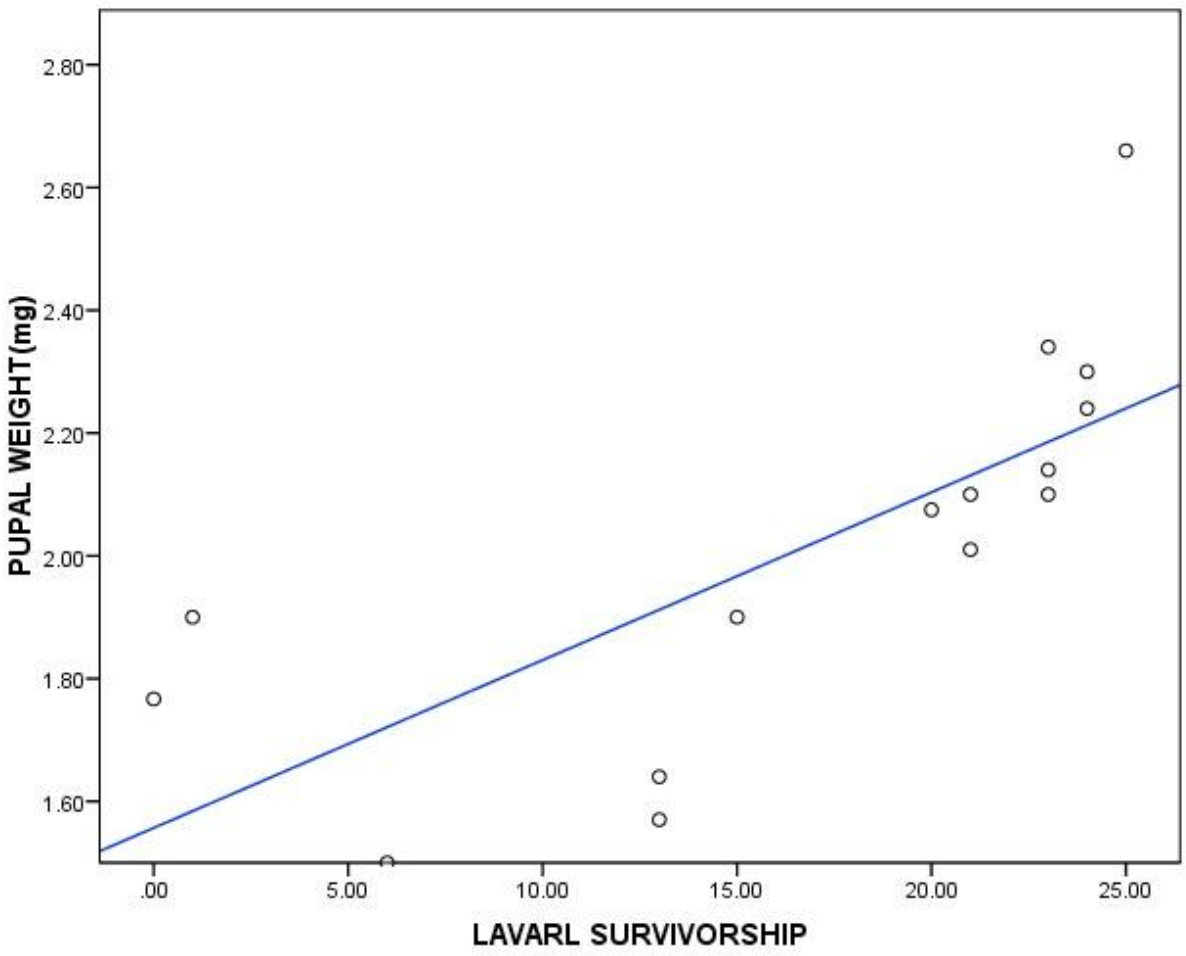

Figure 4.6 Correlation between pupal weight and larval survivorship of *An. gambiae* s.s

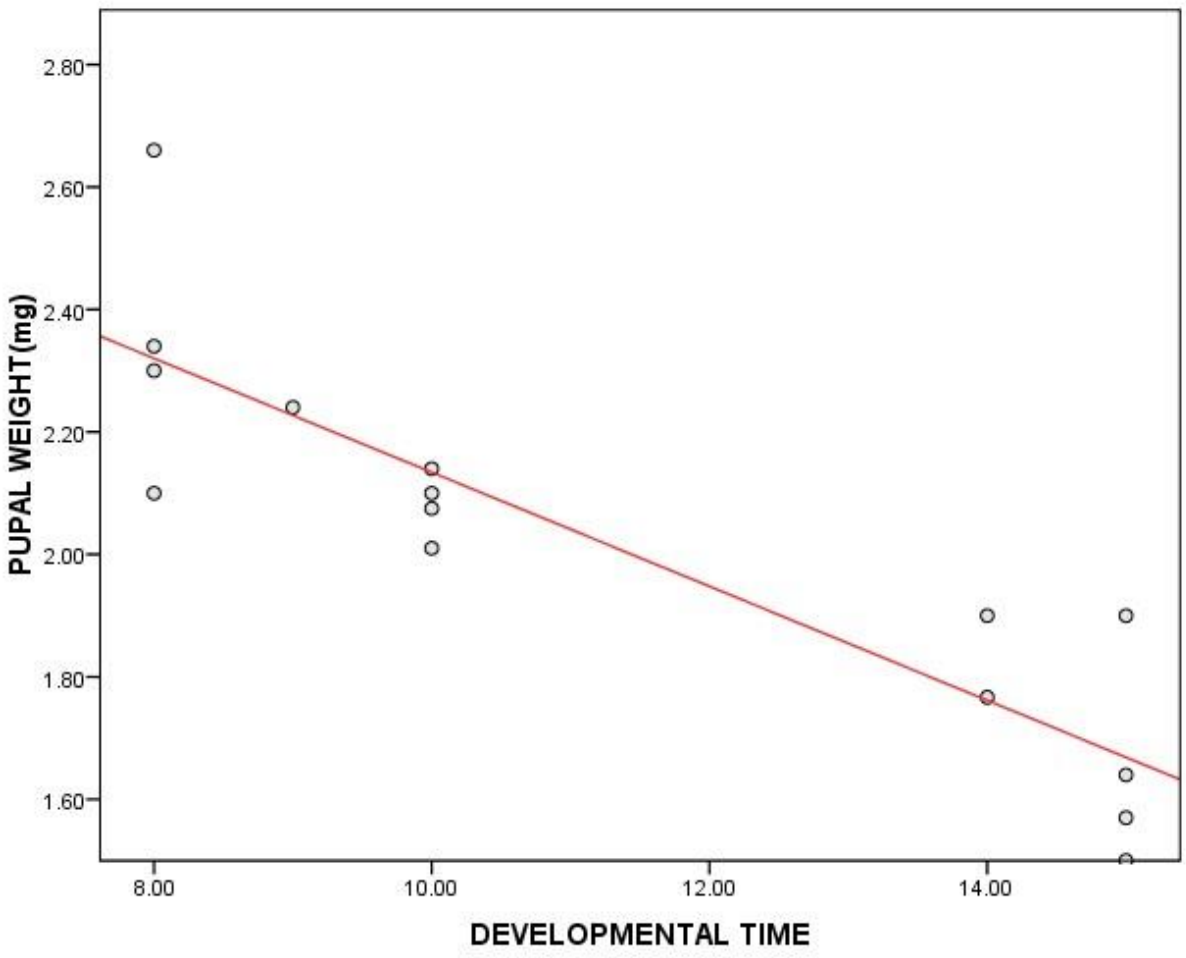

Figure 4.7 Relationship between pupal weight and larval developmental time of *An. gambiae* s.s

4.1.1 Observation of development of the larvae under the influence of the various feeding regime

It was observed that all the five treatment expressed equal effect on the development in terms of growth of the larva from first instar to second instar hence growth rate and size for all the larva at second instar stage was the same. It was however observed that the various feed affected the development of the larva from second instar to fourth instar larva development in difference ways. Cerelac slows the growth and development of the larva from third instar to fourth instar with a lot

of death of the larva before and at fourth instar. Cerelac also formed an oily covering over the surface of the water in the rearing tray.

Yeast on the other hand did affected the development of the larva especially at fourth instar. It was generally observed that it delayed or slowed larva growth from fourth instar to pupae. A lot of the larva got to fourth instar but only few were able to develop into pupae and hence a lot died at this stage. It was also observed that a lot of the larva which died had some part of their bodies missing (see Picture 3. of Appendix IV)

4.2 Susceptibility Study

Of the *An. gambiae* collected from the study site (fig 4.8), a total of 1100 mosquitoes were used for the bioassays. Mortality after 24hr exposures to insecticide in all the classes of insecticide was very low with high KDT_{50} and KDT_{95} values. The number of susceptible female (thus percentage dead after 24hr exposure) exposure to Deltamethrin (0.05%), Permethrin (0.75%), LambdaCyhalothrin (0.05%), Cyfluthrin (0.15%), Etofenprox (0.5%) (Pyrethroid class of insecticide) were

6.25%, 2.25%, 33.75%, 2% and 5% respectively. Those of Fenitrothion (1%), Malathion (5%), DDT (0.4%), Dieldrin (0.4%), Propoxur (0.1%), Bendiocarb (0.1%) was also found to be 16.25%, 47.5%, 2.5%, 7.5%, 16.25% and 21.25% respectively. A relatively high percentage 24hr mortality of 47.5% was recorded when *An. gambiae* s.l was exposed to Malathion (5%). A lowest 24hr mortality of 2% was observed in *An. gambiae* s.l when exposed to cyfluthrin (0.15%).

The estimated mean KDT_{50} and KDT_{95} times was relatively high in all the insecticides. KDT_{50} of Deltamethrin (0.05%), Cyfluthrin (0.15%), Malathion (5%) DDT (0.4%), Dieldrin (0.4%), Propoxur (0.1%) and Bendiocarb (0.1%) was 84.16±16.11, 105±34.607, 117.388±53988,

121.343±174.214, 137.218±0 and 142.468±122.7 respectively. The least estimated KDT_{50} (84.16±16.11) and KDT_{95} (128.41±35) was observed in deltamethrin (0.05%). Whilst the least KD_{60} (2.5) and longest predicted KDT_{50} (121.343±174.214) and KDT_{95} (170.024±369.907) was also observed in DDT. However mean KDT_{50} and KDT_{95} for Permethrin (0.75%), LambdaCyhalothrin (0.05%), Etofenprox (0.5%), Fenitrothion (1%) could not be estimated.

Generally 24hr mortality with in all the insecticides was observed to be very low, although KDT_{50} and KDT_{95} values were high (as shown Table 4.5 below). Hence *An gambiae* population tested in accordance with WHO guidelines were classified to be resistant (RR). In all cases the control mortality was less than 5%, therefore does not require adjustment with Abbot Formula.

Figure 4.8 is a co-ordinate map of each collection point at the sampling site (05º 35.815' N, 000º 11.034' W) (05º 35.796' N, 000º 11.053' W) (05º 35.793' N, 000º 11.072' W) (05º 35.771' N, 000º 11.071' E) (05º 35.761' N, 000º 11.010' W) (05º 35.779' N, 000º 11.044' W) (05º 35.775' N, 000º 11.036' W) (05º 35.779' N, 000º 11.026' W) (05º 35.781' N, 000º 11.018' W) (05º 35.788' N, 000º 11.023' W) (05º 35.788' N, 000º 11.030' W).

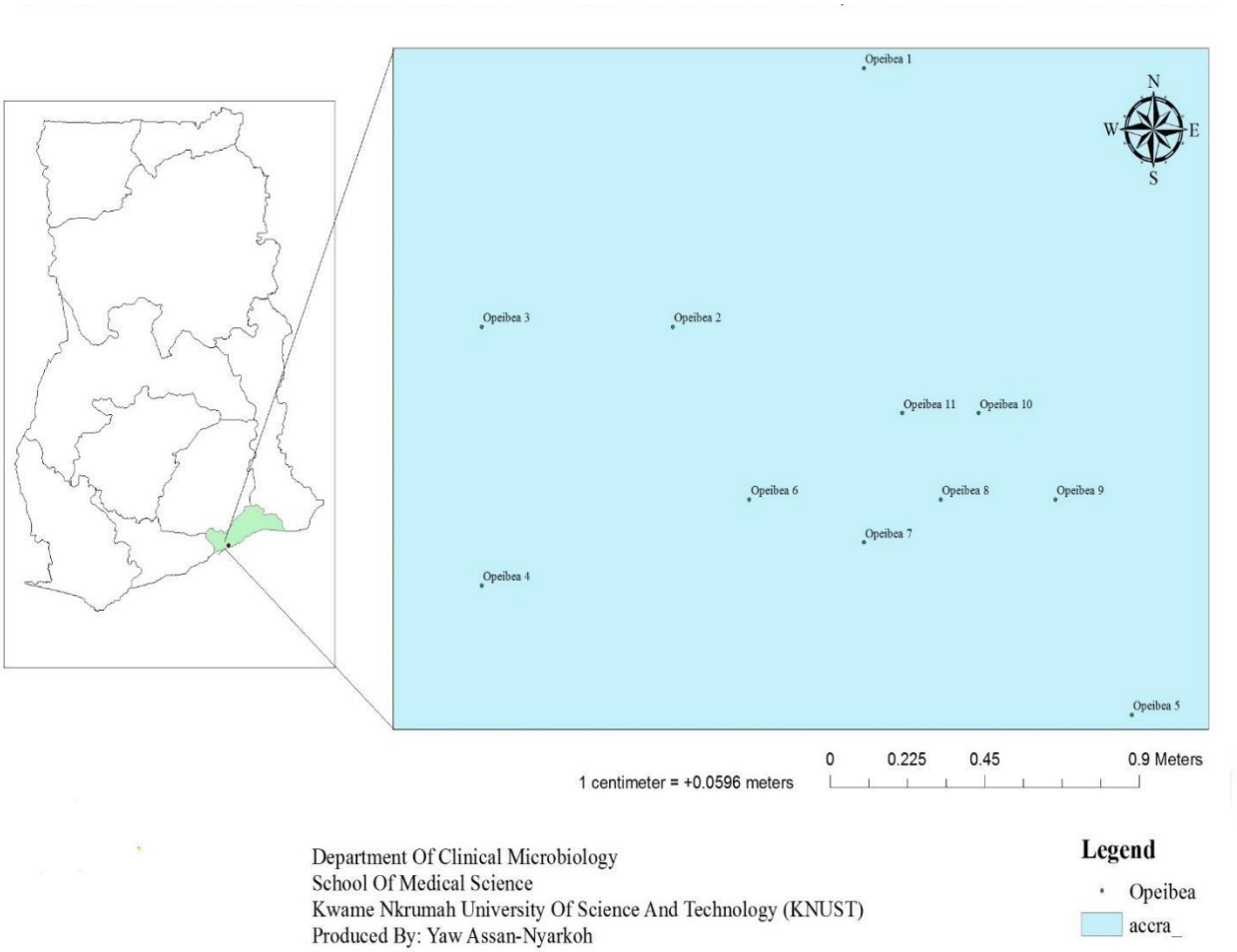

Figure 4.8 A map showing mosquitoes larvae collection sites at the urban agricultural setting at Opeibea in Accra metropolitan area.

Table 4.5 Mortality and knockdown effect of Insecticides on *An. gambiae* from Opeibea

CHEMICAL GROUPING	INSECTICIDE (%)	NUMBER OF FEMALES TESTED (n)	% Average KD_{30min}	% Average KD_{60min}	% MORTALITY 24 HR POST EXPOSURE	KDT_{50} (min; mean ± SD)	KDT_{95} (min; mean ± SD)	STATUS
PYRETHROID	DELTAMETHRIN (0.05%)	80	1.25	22.5	6.25	84.16±16.11	128.41±35.06	RR*
	PERMETHRIN (0.75%)	80	0	0	2.25	-	-	RR*
	LAMBDACYHALOTHRIN (0.05%)	80	0	0	33.75	-	-	RR*
	CYFLUTHRIN (0.15%)	80	0	3	2	105±34.61	163.90±78.57	RR*
	ETOFENPROX (0.5%)	80	0	0	5	-	-	RR*
ORGANOPHOSPHATE	FENITROTHION (1%)	80	0	0	16.25	-	-	RR*
	MALATHION (5%)	80	10	17.5	47.5	117.39±539.88	212.40±878.25	RR
ORGANOCHLORIDES	DDT (0.4%)	80	0	2.5	2.5	121.34±174.21	170.02±369.91	RR*
	DIELDRIN (0.4%)	80	1.25	1.25	7.5	137.22±0	203.633±0	RR*
CARBAMATE	PROPOXUR (0.1%)	80	1.25	7.5	16.25	142.47±122.70	227.32±251.15	RR
	BENDIOCARB (0.1%)	80	1.25	21.25	21.25	-	-	RR

When less than 90% mortality was observed the population was considered 'resistant (RR)'; between 90 and 97% mortality the population was considered to be 'Tolerant (RS)' (or 'suspected of resistance' in the literature (RS)) , when the mortality was above 98% the population was considered 'susceptible'(SS) and (RR*) super resistant.

*Measured by death within 24 h, after 1h exposure to each insecticide. All mosquitoes were resistant according to World Health Organization Classification (<80% dead)

4.3 Insecticide Usage in the Study Sites

A total of 10 farmers in the study site area were pick at random on different visit and interviewed to ascertain the insecticide usage patterns. Several insecticides with different trade names were found to be used in the area. These include 'Attack', Fenitrothion EC, 'Plan D' and Agrocombi. The active ingredient in four of the presentation had Deltamethrin and Fenitrothion, which are among WHO recommend class of insecticides for public health. The mode of action and the target site of the other three (Emamectin Benzoate, Imidacloprid and Fenvalevrate) insecticide have the same target site as carbamates, organophosphates and pyrethroids (as shown in Table 4.6 below). All the farmers apply these pesticide frequently with other agrochemical to improve their yield. Although the type of pesticide used depends on the type of crop, about 70% (7) of the farmer's interview mostly used "Attack and Plan D". The rate of application ranges from once a week to twice a week depending on the type of vegetable and the season. According to the farmers, relatively high amount of insecticide is used when they plant crops like cabbage and other vegetables especially in the dry season. About 40% (3) of the farmers usually apply a mixture of two different types of insecticides to grow crops. The pesticide are usually bought from acgrochemical shops in Accra central or Madina market. Most of the farmers live in either Mamobi or Nima. Some work as laborers, gardeners and watchmen whilst others are not engage in any other work except vegetable farming which provide additional source of income (see Picture 4. of Appendix V).

Table 4.6 Insecticides used by the farmers

PRESENTATION	ACTIVE INGREDIENT
Attack. 250ml	Emamectin Benzoate 1.9% V/W

Fenitrothion Ec 501L	Fenitrothion 50% W/V
Plan D	Deltamethrin 25g/L
Anti ataa	Imidacloprid
Agricombi 250ml/1L	Fenitrothion 30% and Fenvalevrate10%
Kathrine 25SC 250mls/L	Delthamethrine 25g/L

4.4 Molecular Studies

. A total of 137 randomly selected from the morphologically identified *Anopheles. gambiae* s.l. and screened for *kdr* and Ace-1 target site mutation. All the selected mosquitoes species were identified by PCR as *Anopheles gambiae s.s.* . Further molecular analyses by SINE PCR identified 133 into molecular forms of which 105 (78.95%) were found to be S-form and 28 (21.05%) were M-form (*An. coluzzi*). A greater percentage that is 67.62% (71) of the S form was resistant (alive) to insecticide whilst 57.4% (16) of the M-form was susceptible (dead). 108 out of the total sample of 137 had the homozygous *kdr* gene (L1014F) gene occurred in all the samples. Ace-1 Mutation assay indicated that, 67.14 % (47) *Anopheles gambiae mosquitoes had* population to be have RR homozygous Ace-1 gene. Ace-1^R genotypic frequency was found to be 80 % (Table 4.7). The observed ace-1 frequency was significant (p=000) X^2 value of 39.629 as shown in Table 12 of Appendix III).

Fig. 4.9, Fig. 4.10, Fig 4.11 and Fig 4.12 are gel photograph of the species identification, photograph of SINE PCR, identification of KDR by PCR and Ace-1 by the RFLP PCR respectively.

Table 4.7 Molecular characterization, prevalence of Kdr and Ace-1 mutations

Species identification		% S and M Forms population frequency at Opeibea		Status after exposure to insecticide		% L1014F *KDR* frequency (n)	ACE-1 G119S				
							ACE-1 G119s % genotypic frequency (n)			ACE-1 G119S %alle frequency	
Morphological identification of *An. gambiae s.l*	Molecular identification of *An. gambiae* s.s			Dead (Susceptible)	Alive (Resistant)	FF	RR	RS	SS	ACE-1^R	ACE-1^S
1200	137	M	21.05% (28)	57.4% (16)	42.86% (12)	22.22% (24)	10.64 (5)	36.84 (7)	100 (4)	80	20
		S	78.95% (105)	32.38% (34)	67.62% (71)	77.78 % (84)	89.36 (42)	63.16(12)	-		
Total(N)			(133)100	50	83	108	67.14 (47)	27.14(19)	5.71 (4)	100	
N missing			4			1	67				

KDR genotypes FF = homozygous resistant, FL = heterozygous resistant/ susceptible and LL = homozygous susceptible, whilst ACE-1 genotypes RR = homozygous resistant, RS = heterozygous resistant/ susceptible and SS = homozygous susceptible, Ace-1^R G119S= resistant haplotype gene mutation in *Anopheles gambiae*, and Ace-1^S G119S= ^{s} susceptible haplotype recessive gene.

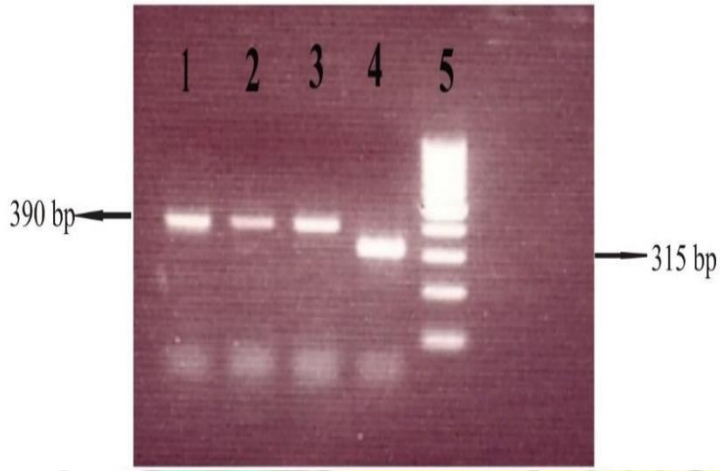

Figure 4.9 Gel electrophoregram of the identification of members of the *An. gambiae* species complex. DNA Bands of molecular species identification of *An. gambiae* after PCR diagnostic test. Lane 1 to 3=bands of *Anopheles gambiae s.s* from field samples. Lane 4= band of known *Anopheles arabiensis* species used as control. Lane 5=1kb DNA ladder.

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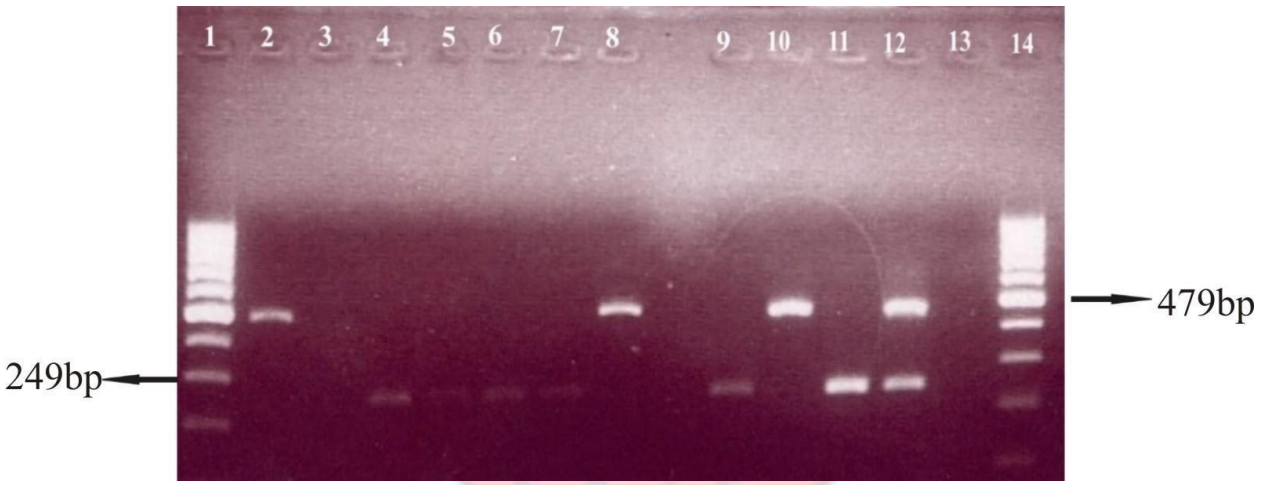

Figure 4.10 Gel picture of DNA bands After SINE PCR, Lane 1and 14 =1.kb DNA leader, lane 2and 8= M form of *Anopheles gambiae*, Lane 4,5,6,7, and 9 =S form, Lane 10= m form control, Lane 11 = S form control, Lane 12= Mm and Ss form hybrid, lane 13 = negative control, Lane 3= did not show

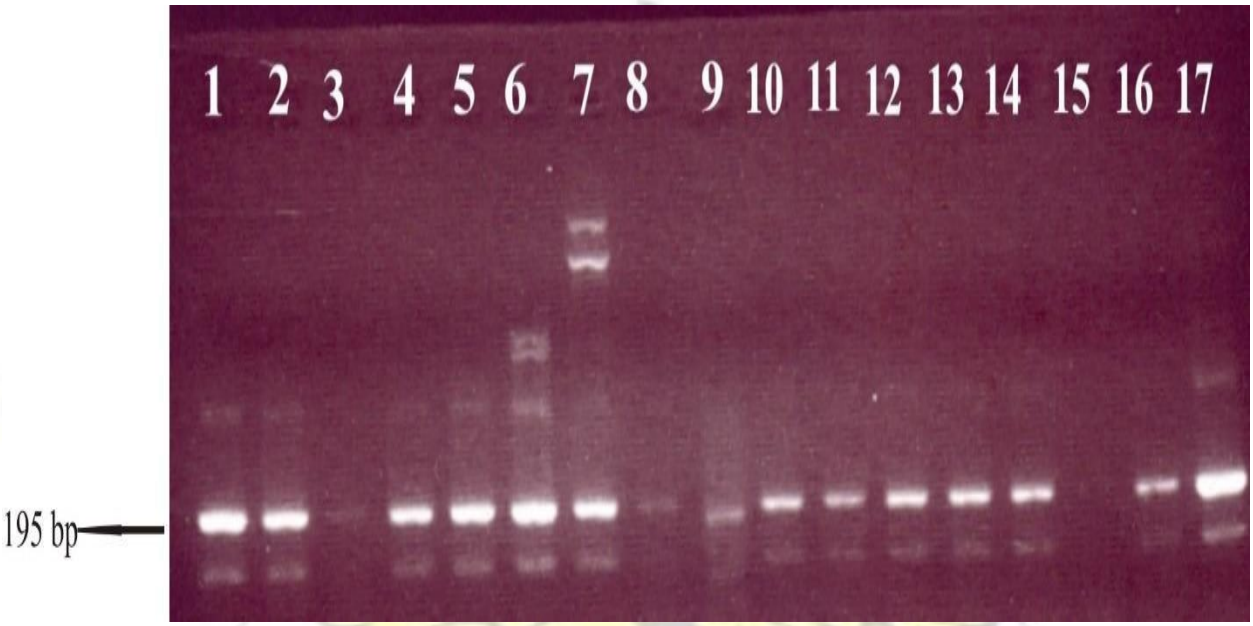

Figure 4.11. A Gel electrophoregram for identification of kdr by PCR-based diagnostic test.Lane 1 to 16 = L1014F homozygote gene mutation in *Anopheles gambiae*, Lane 17 = Control, Lane 15 = was insensitive to PCR detection

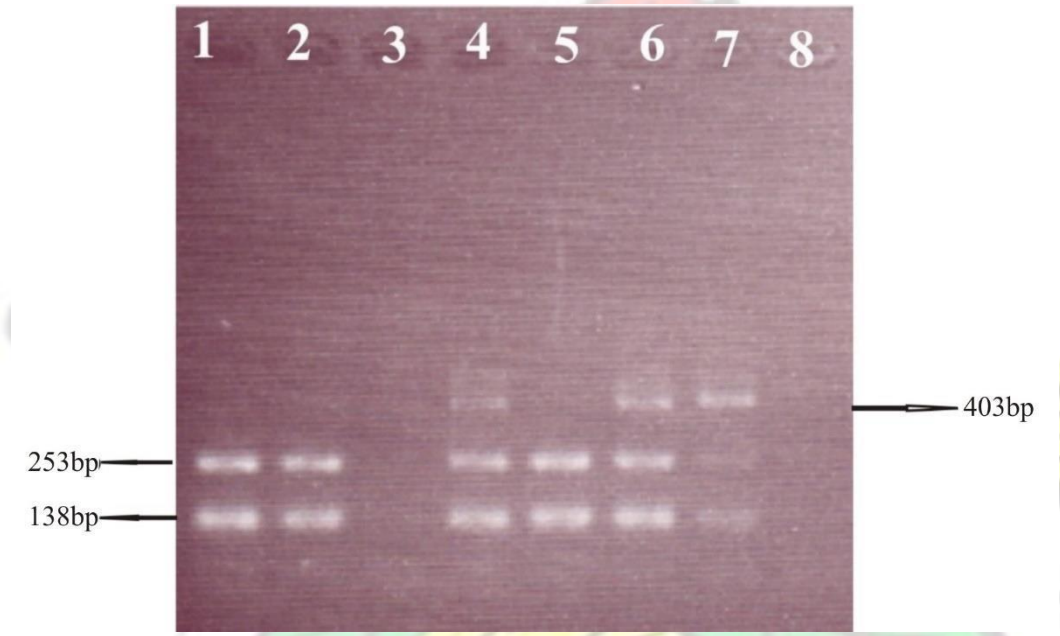

Figure 4.12 Screening Ace-1 G119S mutation by PCR-RFLP based diagnostic test.
Lane 1,2 = RR homozygote resistant gene mutation in *Anopheles gambiae*, Lane 4 = RS heterozygote resistant gene, Lane 5= RR homozygote from control Lane 6= RS heterozygote Control lane 7= SS homozygote recessive control, Lane 8=negative control, lane 3= insensitive to Ace-1 PCR-RFLP

CHAPTER 5
DISCUSSION

The preliminary study in which both wild and laboratory strain of *Anopheles gambiae* were fed on rabbit pellet and fish flakes separately showed that,, the differences between the life history trait were (pupae weight p=0.124, pupation rate p=0.715, larval developmental time (days) p=0.598 and larval survivorship p=0.598) not significant. The observation was however different from an earlier study by Huho *et al*. (2007), who observed significant difference in the energetic reserves, body size and survival in laboratory bred and wild adult *Anopheles gambiae*. When the author fed, both laboratory bred and wild adult mosquitoes from the wild on the same meal. Energetic reserves, body size and adult fitness most often depend on the larval diet or nutrition (Timmermann & Briegel, 1999; Telang et al., 2002; Hahn, 2005; Khan, et al., 2013) and to some extent protein (nitrogen) content of diet (Lang *et al*., 1965). Hence the difference in Huho *et al*., (2007) observation might have risen from the different larval diet and growing condition, since the larvae of the resultant adult mosquitoes were reared in different habitat (Walker, 2008).

The significant (p=0.006 and p=0.002) difference observed in larval developmental time (days) and pupation rate (respectively) within treatment (feed) alone, was because each treatment differently affected pupation rate and larval developmental time of a strain (that is whether Kisumu or wild). This was clearly revealed by the one way Anova analyses of each life history parameter. Also the type of strain of *Anopheles* did not have any effect on the relative performance of each treatment or meals in the mosquitoe. Hence, the observed non-significant interaction between treatment and strain.

The nutritive values of the ingredients present (content) and the refined or defined state of the diets have an impact the aquatic life stage of the mosquitoes. Nutritional requirements of mosquito larvae are known to include at least 14 essential amino acids (asparagine, arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine,

tryptophan, and valine), sugars, polyunsaturated fatty acids (PUFAs), sterols, nucleotides (for the larval development, survival and adult flight), a minimal concentration of essential vitamins (Damiens et al., 2012) and salt (Trager, 1932). In the natural habit, the larvae feed on algae, bacteria, phytoplankton and debris. Which are in their natural state and are not refined. Hence are easily digestible. A highly balanced or nutritious diet that is refined and not digestible in the belly of the larvae of the mosquitoes, always affect the life history parameters (fitness) of the adult mosquito.

The mean pupation rate of rabbit pellet was significantly ($p=0.001$and $p=0.000$) different from that of fish flake and cat meal respectively. Whilst the mean developmental time of rabbit pellet was significantly ($p=0.000$) different from cat meal. It was also observed that, mortality rate was low and development of larvae from the second instar to pupae was relatively faster when the larvae were fed on rabbit pellet. This resulted to the observed high pupation rate. Assessment of the content and nature of the various food regime, rabbit pellet is largely made with some kind of cereal (wheat) in their natural state (that is less refined) and contains the highest percentage of fiber (12%) (refer to Appendix VI). Hence are more easily digestible by the mosquitoe larvae. The addition of methionine, lysine calcium phosphorus and salt by the manufacture, might have enhanced the nutritional capabilities of rabbit pellet since, cereals are reported to have low quantities of certain nutritional, e.g tryptophan and methionine (amino acids). The unrefined nature coupled with the easy digestibility of rabbit pellet and availability of nutrient to the *Anopheles gambiae s.s* larvae reflected in the relative impressive enhancement of all in life history characteristics that was measured. The addition of salt might have led to relative rapid growth and development. Hence the development of larvae fed on rabbit pellet was seen to be relatively faster than fish flakes, cat meal, cerelac and no larval mortality or was less when rabbit pellet was used.

The observation was in line with previous a studies done by (Khan, 2010; Khan *et al*., 2013; Kivuyo *et al*., 2014.), in which cereal based feeds in their natural or less refined state where enhanced by combining or mixing with other natural food ingredients.

Although fish meal out performed cat meal with respect to life history traits, there was no significant difference observed between the two feeds .It was also observed that, each of the two feeds resulted in a relatively quick larval development. But in cat meal, an average of three to four larva did not change into pupa when they reached four instar larva. The two feed relatively are refined and have the same nutritional content except that cat meal contains more nutritional additives and more refined than fish flakes. However as reported in Damiens *et al*. (2012) the presence of the additional additives did not any relative difference in larval life history traits between the two.

On the other hand, the relative performance of cerelac and baking yeast of the various life history characteristics in *Anopheles gambiae s.s* was relatively poor with the exception of pupa weight which was not statically significant (p=0.051) with fish flakes and cat meal feeding regime. The relatively poor performance of life history parameters involving the use of cerelac, was however different from what was reported by Kivuyo *et al*.(2014). Cerelac was the feed with the highest number of refined nutritional ingredient but the fat 10g (10%) content was too high and was less soluble. Hence formed oily scum on the surface of the rearing water., which reduced the availability of nutrients to the developing aquatic stage of *Anopheles gambiae s.s* This resulted in the relatively low pupae weight (1.57mg) and long developmental time (15 days).whilst Kivuyo *et al*.,(2014), used cerelac which was locally prepared following the instruction for preparing cerelac for babies and enhanced the nutritional content by mixing it with grounded Sardine. He observed highest pupation and survival rate in cerelac which strongly correlated with the survival rate when the *Anopheles gambiae s.s* were fed with Tetramin fish flakes. Therefore, the differences

in the two result might be due to the nature of preparation, natural state and the nutrition content of the cerelac used.

Finally yeast (baking yeast) did affect the development of the larvae especially at the third instar to pupation. It was generally observed that it delayed or slowed larva growth from third instar to pupae. A lot of the larvae got to fourth instar but only few were able to develop into pupae. It was also observed that a lot of the larvae which died had some part of their cadavers missing except the head. The relative poor performance of yeast might probably be due to the low nutritional content of yeast. The nutritional demands of the growing larvae increase as the larvae develop. Hence larvae had to result to cannibalism (thus feed on the dead and small nutritionally deprived larvae) to make up for the additional nutrient needed. This explains why although the pupae weight of the larvae fed with yeast was not statically significant ($p=0.051$) from those fed with fish flakes but could not enhance the other life history traits. However the resultant effect of yeast on all the measured life history parameters especially in delaying the larval developmental time was similar to what was reported by Khan *et al*. (2013).

The generally observed, equal expression of the effect of each meal on the development of larvae in terms of growth from first instar to second instar, might have been due to the nutritional requirement of the growing larvae. The quantity of food, as well as the nutritious 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 stage was the same in all feeds, since the growing larva relatively had minimal nutritional requirements. It was however observed that, the various effect off each feed on the development of the larva was structurally evident or seen from third instar to pupation instar larva development in difference ways.

The strong Pearson correlation association of pupae weight with pupation rate (r=747**), developmental time (r=-.886**) and larval survivorship (r=741**) properly indicate that in *Anopheles gambiae s.s* a well balance larval diet which positively enhance the weight of pupae might also improve the larval survivorship, pupation rate and shorten larval developmental.

The finding of this study suggest that different food regimes have varying impact on the some life history characteristics on off the immature stages of *Anopheles gambiae s.s*. The influence of the food source also depended on the type and nutritional content and their nature. The food source effected pupae weight, larval survivorship, pupation rate and larval developmental. In all rabbit pellet had highest positive impact followed by fish flakes and cat Meal. Whilst yeast and cerelac had the relatively least effect on the various life history traits. Understanding larval impact of a larval food on adult life history traits in insectary, is very essential for mass rearing of laboratory colony of mosquitoes in order to obtain fit adult population.. Fitness and vigor of the adult colony should be cross checked any time there is change in the diet so as to have healthy mosquitoes for different the experimental studies. Since the outcome of studies like susceptibility to insecticide in *Anopheles gambiae* s.l population, is affected by the fitness of the adult mosquitoe which also depends on larval diet. Hence the use of Rabbit pellet and fish flakes in the laboratory rearing of the larval culture from Opeibea ensured that relative fit mosquitoes were used in the determination of susceptibility to insecticide.

Discussion with the farmers indicated the use of Emamectin Benzoate, Fenvalevrate, Imidacloprid, Deltamethrin and Fenithrothion class of insecticide. Emamectin Benzoate, Fenvalevrate and Imidacloprid has the same target site and mimic the effect of dieldrin, pyrethroids, carbamates and organophosphate respectively. The use of Deltamethrin, Fenitrothion class of insecticide and three

other insecticide that mimic the effect of carbamates, organophosphates and pyrethroids class of insecticide, might be an indication of the indiscriminate or extensive use of insecticide in urban agricultural farming. Due to the constant and high demands of urban populations, the vegetable farms found in urban settings are active throughout the year and hence, are intensely cultivated. The farmers also find it difficult to expand their farms since there is scarcity of land especially in Accra, where every piece of land, is being use for building purposes or development. In order to meet the market demands by keeping high vegetable productivity and reduce farm loses, in urban areas farmers results to the frequent use of insecticide and other agrochemicals. The farmers' preference to these group of insecticide is due to availability on the market, cost and efficacy against pest. Similarly the indiscriminate used of insecticides in urban agricultural farming was observed in earlier reported studies by Achonduh *et al*., (2008), Yadouleton *et al*., (2009), Djègbè *et al*., (2011), and Koffi *et al*., (2012;)

PCR molecular methods identified all the samples to be *Anopheles gambiae s.s* which were further characterized by SINE PCR. The S and M forms of *Anopheles. gambiae s.s*. were found to occur in sympatry in the study sites, with a relatively higher frequency of 78.95 %(105) S form than 21.05 % (28) M form in the study population. The result is similar in earlier studies reported by Achonduh *et al*. (2005), who also sampled cabbage farms in the Airport residential area along the same drainage which extend from the Airport residential area through Opeibea to Mamobi, Nima and finally join the Odow river before entering the sea. However, there was slight increase in the M population than what Achonduh *et al*. (2008) observed (that is 5% M form), this might be because the farmers usually use irrigated water to cultivate vegetables throughout year. They only rely on rain water during the raining season but most of the time practice irrigation farming by using the drainage water as a source of watering their farm. They dig temporal wells and fill them

with water from the drainage which is latter used to water their farms. Most of this small dug wells serve as a breeding site for mosquitoes especially in the dry season.

The present study also reveal the presence of strong resistance against all the classes of insecticides. Susceptibility test results generally showed high levels of phenotypic resistance (RR*) to all the classes of insecticide. The KDT_{50} and KDT_{95} for Permethrin, Lambda-cyhalothrin, Etofenprox, Fenitrothion and Bendiocarb could not be found because of the low numbers of mosquitoes that was knockdown Although resistance against pyrethroids have already been reported in earlier studies in southern Ghana or Accra (Klinkenberg *et al*., 2005 and 2008; Achonduh *et al*., 2008; Adasi & Hemingway, 2008; Adeniran *et al*., 2009; Boakye *et al*., 2009; Kabula *et al*., 2011) hardly or never has such low numbers of 24HR % mortality and KDT involving Pyrethroids (deltamethrin(6.25%, KDT_{50} =84.16±16.11 and KDT_{95}=128.412±35.064), Lambalacyhalothrin(33.75%) and permethrin(2.25%,)), been reported in the field populations of *An. gambiae* s.l. The highest 24hr mortality of 47.5% was observed with Malathion. The observation was however different from what was reported a decade ago by Achonduh *et al*., (2005) who sampled some farms along the same drainage from the airport Area and observed 100 % 24hr mortality in Malathion while resistance against permethrin and deltamethrin was high. He associated his observation to high amount soil residue of pyrethroids and organophosphates

. Molecular analyses shows high frequency of (100%) L1014F *KDR* and (80%) ACE-1^R mutations in both M and S forms in the population. The continuous use of insecticides containing the same class of active ingredient as deltamethrin or other class of insecticide, that mimic pyrethroids class of insecticide on the farms exerted a selective pressure which favoured mosquito population with the L1014F *KDR* who were able to withstand recommended doses of pyrethroids due to genetically fixed difference. With time, the selective pressure led to the establishment of L1014F *KDR*

resistant population resulting in a general genetic fitness. Hence the observed very high frequency of *kdr* mutation (FF homozygote condition by *kdr* PCR assays), since vegetable farming might have been going on for the past 40 years now (Odhiambo, 2005; Achonduh *et al*., 2008). The. observed 100% occurrence of *KDR* was similar to earlier reports by Hunt *et al*. (2011) at Ahafo and Tarkwa.

Similarly, the use of Fenitrothion and other insecticide which have the same target site as an organophosphate, might have caused an increase in resistant population against 1% Fenithrothion test paper which corresponds with the high occurrence ACE-1^R gene. The presence of the Ace-1 resistant gene in a cross resistant fashion reduced the susceptibility against Malathion ten years after, since they belong to the same class of insecticide. The observation is similar to what happened in M'Bé (Koffi *et al*., 2013) and Yaokoffikro (Koffi *et al*., 2012) in Côte d'Ivoiret. The high significant (p=000) chi square value 39.629 is an indication of theory of panmixia, thus there might be random breeding and free exchange of genes. There is the probability of occurrence of random segregation and independence assortment of genes. Hence the effects of mutations alone, is most of the time not strong enough to cause the genotype frequencies or to deviate from the Hardy-Weinberg model proportions and is more likely to maintain the same frequency of genes from one generation to another. However should the selective pressure exerted as result of the used of Fenitrothion persist, this might lead to a genetic fitness in the population. Although resistant against organochlorids (DDT) and carbamate might be as a result of cross resistance from *Kdr* and AChE target site mutation. It is suggested that the strong resistance against pyrethroids and the other class of insecticide might be as result of combination of cross resistant and multiple resistant mechanism (Mitchell *et al*., 2012, 2014) involving metabolic resistant mechanism. However the

generally high resistant against all class of insecticide conforms to what is already stated in literature by Edi *et al*., (2012) and Nwane *et al*., (2013).

Akogbéto *et al*. (2006), Azhar *et al*. (2011) and Yadouleton *et al*. (2009 and 2011) studies have associated the observed phenomenon of increase insecticide resistance in *An. gambiae* s.l mosquitoes with indiscriminate used of insecticide for agricultural purposes by farmers. Hence the indiscriminate use of pesticide used at the vegetable farm in Opeibea might lead to the increase of incidence of malaria cases in the surrounding communities. The observed high general resistance to pyrethroid and other class of insecticide is a threat to malaria control efforts since it under pinned the notion that resistance to insecticide and incidence of malaria cases is relatively high in rural areas as compared to urban centers. The general high resistance of insecticide has a serious implication for malaria control programmes especially LLIN and IRS.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The relative high pupae weight, pupation rate, short larval developmental time and high larval survivorship observed or enhanced when the *Anopheles gambiae* s.s larvae were fed on rabbit pellet in this study indicated that using rabbit pellet in rearing of *Anopheles* larvae would result in emergence of large numbers of adult that are reproductively fit within the shortest period (that an average of 8 days). Since rabbit pellet and fish flakes enhanced the life history characteristics and health status hence, the use of rabbit pellet and fish flakes in rearing the *An. gambiae* larval population from Opeibea validated the fitness of adult mosquitoes used in the susceptibility studies.

The indiscriminate use of insecticide for agricultural have been largely be the cause of insecticide resistance in mosquitoes. Hence, the high resistance observed status might have been caused by prolong and indiscriminate used of insecticide on vegetable farms. However the resistance to all the classes of insecticide may not have confer by the presence of *KDR* and Ace-1 mutation resistant mechanism alone, as Brooke (2008) puts it, there could be the involvement of other co factors and unidentified mechanism. This might probably, include detoxification enzyme systems based on the relative expression of monooxygenases, glutathione-S-transferases and non-specific esterase. Ace-1R is strongly associated with organophosphate and carbamate resistance, and the absence of 1014F *kdr* homozygotes recessive allele might be attributable to the high fitness cost of the 1014F *kdr* allele in the absence of insecticide.

The findings of this study suggest that, the food regime in insectary have an impact on pupae weight, pupation, larval developmental time, and larval survivorship. The performance of a food regime on the life history characteristics depends nutritional quality (content), availability and natural state (that is refined or not refined).

Also we report the occurrence of high phenotypic resistance status, *KDR* mutation and Ace-1^R resistance which might be associated with the use of agricultural insecticide including those of public health interest on the vegetable farm at Opeibea. Although night blood feeding of wild *Anopheles gambiae s.s* observed in the lab is an indication that the use of LLIN and ITN malaria intervention strategy could be used in the surrounding communities at Opeibea. On the other hand the success of these strategies (LLIN and ITN) is also threatened by the high resistance to pyrethroids.

6.2 RECOMMENDATION

Though the study has evaluated and assessed the effect of five meals on larval development, causes of resistance and the resistance status of *Anopheles gambiae s.s* at Opeibea it is recommended that the study should be expanded and further studies should be carried out to investigate the status of resistance of *An. gambiae* on areas agricultural farms especially in the Urban areas in Ghana. . Further studies should be done involving the various combination of diet tested in this studies. The test of various diet combinations in their unrefined nature especially cereals and legumes should be explored. Baking yeast can be use in hatching of the mosquito eggs but should never alone be used in rearing larvae of *Anopheles gambiae s.s.*

Also, the study should be expanded to test the soil for residue of insecticide at the vegetable farm in Opeibea. Biochemical assay and gene profiling studies can also be done to find out other unidentified resistant mechanism. . Hence, there is the need for studies to monitor the impact of this resistance on malaria vector control activities. In order to help check and prevent it from spreading Integration of the activities of municipal authorities, agriculturalists, health professionals, and communities is essential to reduce the existing impact of malaria and to prevent future increase.

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APPENDICES

Appendix I

Picture 1. Determinations of pupae weights

Picture 2. Susceptibility testing using WHO tube and recommended test papers

Appendix II

Table 5. Lane expected PCR diagnostic for species identification of *An. gambiae s.l*

Species	Band size
An arabiensis	315
An gambiae	390

Table 6. Lane expected PCR diagnostics for molecular forms of *An. gambiae s.s.*

Species identification of molecular forms	Band Size(bp)
Anopheles gambiae s.s. S form	249
Anopheles arabiensis s.s M form	479

Table 7. Lane expected band size after PCR diagnostics for knockdown Resistance (*Kdr*-W) mutations in *An. gambiae*

Genotype	Band Size(bp)
SS (Susceptible)	293
	137(diagnostic band)
RR (Resistant)	293
	195(diagnostic band)
RS (Heterozygous resistant)	293
	195 (resistant band)
	137 (susceptible band)

Table 8. Lane expected PCR diagnostics for Acetylcholinesterase Gene (Ace-1) mutations in *An. gambiae*

Genotype	Band Size(bp)
SS (Susceptible)	403 (diagnostic band)
	138
RR (Resistant)	253(diagnostic band)
	150
	138

RS (Heterozygous resistant)	403 (susceptible band)
	253 (resistant band)
	150
	138

Appendix III

Table 9. A Table of results of a two way Anova factorial analyses showing the difference between strain (laboratory bred and wild *Anopheles gambiae*) and treatment (rabbit pellet and fish flakes).

Dependent Variable	Source	Type III Sum of Squares	df	Mean Square	F	Sig.	R Squared (Adjusted R Squared)
pupa weight	Corrected Model	.214[a]	3	.071	1.276	.355	
	Intercept	49.786	1	49.786	891.797	.000	
	Strain	.170	1	.170	3.045	.124	
	Treatment	.025	1	.025	.454	.522	
	Strain * Treatment	.000	1	.000	.001	.978	
	Error	.391	7	.056			
	Total	52.272	11				
	Corrected Total	.604	10				0.353 (076)
pupation rate	Corrected Model	256.471[a]	3	85.490	8.014	.012	
	Intercept	2752.613	1	2752.613	258.037	.000	
	Strain	1.547	1	1.547	.145	.715	
	Treatment	245.504	1	245.504	23.014	.002	
	Strain * Treatment	.004	1	.004	.000	.985	
	Error	74.673	7	10.668			
	Total	3029.049	11				

	Corrected Total	331.144	10				0 .775 (0 .678)
Larval developmental time (days)	Corrected Model	8.894[a]	3	2.965	5.414	.031	
	Intercept	763.130	1	763.130	1393.541	.000	
	Strain	.167	1	.167	.304	.598	
	Treatment	8.167	1	8.167	14.913	.006	
	Strain * Treatment	.463	1	.463	.845	.388	
	Error	3.833	7	.548			
	Total	816.000	11				
	Corrected Total	12.727	10				0.699 (0.570)
Larval survivorship	Corrected Model	2.348[a]	3	.783	1.430	.313	
	Intercept	6037.796	1	6037.796	11025.541	.000	
	Strain	.167	1	.167	.304	.598	
	Treatment	2.241	1	2.241	4.092	.083	
	Strain * Treatment	.167	1	.167	.304	.598	
	Error	3.833	7	.548			
	Total	6199.000	11				
	Corrected Total	6.182	10				0.380 (0 .114)

Table 10. A Turkey HSD post hock multiple table comparisons showing the differences between means of various treatment

DEPENDENT VARIABLE	(I) TREATMENT	(J) TREATMENT	Mean Difference (IJ)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PUPAE WEIGHT(mg)	Fish Flakes	Cat Meal	.17833	.11748	.574	-.2083	.5650
		rabbit Pellet	-.11333	.11748	.865	-.5000	.2733
		yeast	.38444	.11748	.051	-.0022	.7711
		Cerelac	.67000*	.11748	.001	.2834	1.0566
	Cat Meal	Fish Flakes	-.17833	.11748	.574	-.5650	.2083
		rabbit Pellet	-.29167	.11748	.171	-.6783	.0950
		yeast	.20611	.11748	.447	-.1805	.5928
		Cerelac	.49167*	.11748	.013	.1050	.8783
	rabbit Pellet	Fish Flakes	.11333	.11748	.865	-.2733	.5000
		Cat Meal	.29167	.11748	.171	-.0950	.6783
		yeast	.49778*	.11748	.012	.1111	.8844
		Cerelac	.78333*	.11748	.000	.3967	1.1700
	Yeast	Fish Flakes	-.38444	.11748	.051	-.7711	.0022
		Cat Meal	-.20611	.11748	.447	-.5928	.1805
		rabbit Pellet	-.49778*	.11748	.012	-.8844	-.1111

		Cerelac	.28556	.11748	.184	-.1011	.6722

PUPATION RATE	Fish Flakes	Cat Meal	1.3333	1.6113	.916	-3.970	6.636
		rabbit Pellet	-9.5556*	1.6113	.001	-14.859	-4.253
		yeast	10.4500*	1.6113	.001	5.147	15.753
		Cerelac	9.2833*	1.6113	.001	3.980	14.586
	Cat Meal	Fish Flakes	-1.3333	1.6113	.916	-6.636	3.970
		rabbit Pellet	-10.8889*	1.6113	.000	-16.192	-5.586
		yeast	9.1167*	1.6113	.002	3.814	14.420
		Cerelac	7.9500*	1.6113	.004	2.647	13.253
	Rabbit Pellet	Fish Flakes	9.5556*	1.6113	.001	4.253	14.859
		Cat Meal	10.8889*	1.6113	.000	5.586	16.192
		yeast	20.0056*	1.6113	.000	14.703	25.309
		Cerelac	18.8389*	1.6113	.000	13.536	24.142
	Yeast	Fish Flakes	-10.4500*	1.6113	.001	-15.753	-5.147
		Cat Meal	-9.1167*	1.6113	.002	-14.420	-3.814
		rabbit Pellet	-20.0056*	1.6113	.000	-25.309	-14.703
		Cerelac	-1.1667	1.6113	.946	-6.470	4.136

DEPENDENT VARIABLE	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
DEVELOPMENTAL TIME	Fish Flakes	Cat Meal	-1.000	.422	.200	-2.39	.39
		rabbit Pellet	1.000	.422	.200	-.39	2.39
		yeast	-5.333*	.422	.000	-6.72	-3.95
		Cerelac	-6.000*	.422	.000	-7.39	-4.61
	Cat Meal	Fish Flakes	1.000	.422	.200	-.39	2.39
		rabbit Pellet	2.000*	.422	.005	.61	3.39
		yeast	-4.333*	.422	.000	-5.72	-2.95
		Cerelac	-5.000*	.422	.000	-6.39	-3.61
	Rabbit Pellet	Fish Flakes	-1.000	.422	.200	-2.39	.39
		Cat Meal	-2.000*	.422	.005	-3.39	-.61
		yeast	-6.333*	.422	.000	-7.72	-4.95
		Cerelac	-7.000*	.422	.000	-8.39	-5.61
	Yeast	Fish Flakes	5.333*	.422	.000	3.95	6.72
		Cat Meal	4.333*	.422	.000	2.95	5.72
		rabbit Pellet	6.333*	.422	.000	4.95	7.72
		Cerelac	-.667	.422	.539	-2.05	.72
LARVAL SURVIVORSHIP	Fish Flakes	Cat Meal	2.667	3.432	.932	-8.63	13.96
		rabbit Pellet	-.667	3.432	1.000	-11.96	10.63
		yeast	18.000*	3.432	.003	6.71	29.29
		Cerelac	12.667*	3.432	.027	1.37	23.96
	Cat Meal	Fish Flakes	-2.667	3.432	.932	-13.96	8.63
		rabbit Pellet	-3.333	3.432	.862	-14.63	7.96
		yeast	15.333*	3.432	.008	4.04	26.63
		Cerelac	10.000	3.432	.090	-1.29	21.29
	Rabbit Pellet	Fish Flakes	.667	3.432	1.000	-10.63	11.96
		Cat Meal	3.333	3.432	.862	-7.96	14.63
		yeast	18.667*	3.432	.002	7.37	29.96
		Cerelac	13.333*	3.432	.020	2.04	24.63
	Yeast	Fish Flakes	-18.000*	3.432	.003	-29.29	-6.71
		Cat Meal	-15.333*	3.432	.008	-26.63	-4.04
		rabbit Pellet	-18.667*	3.432	.002	-29.96	-7.37
		Cerelac	-5.333	3.432	.554	-16.63	5.96

Table 11. Correlations analyses of pupae weight, developmental time, pupation rate and larval survivorship

		PUPAE WIEGHT(MG)	PUPATION RATE	DEVELOPMENTAL TIME	LARVAL SURVIVORSHIP
PUPAE WEIGHT(MG)	Pearson Correlation	1	.747**	-.886**	.741**
	Sig. (2tailed)		0.001	0	0.002
	N	15	15	15	15
PUPATION RATE	Pearson Correlation	.747**	1	-.902**	.820**
	Sig. (2tailed)	0.001		0	0
	N	15	15	15	15
DEVELOPMENTAL TIME	Pearson Correlation	-.886**	-.902**	1	-.867**
	Sig. (2tailed)	0	0		0
	N	15	15	15	15
LARVAL SURVIVORSHIP	Pearson Correlation	.741**	.820**	-.867**	1
	Sig. (2tailed)	0.002	0	0	
	N	15	15	15	15

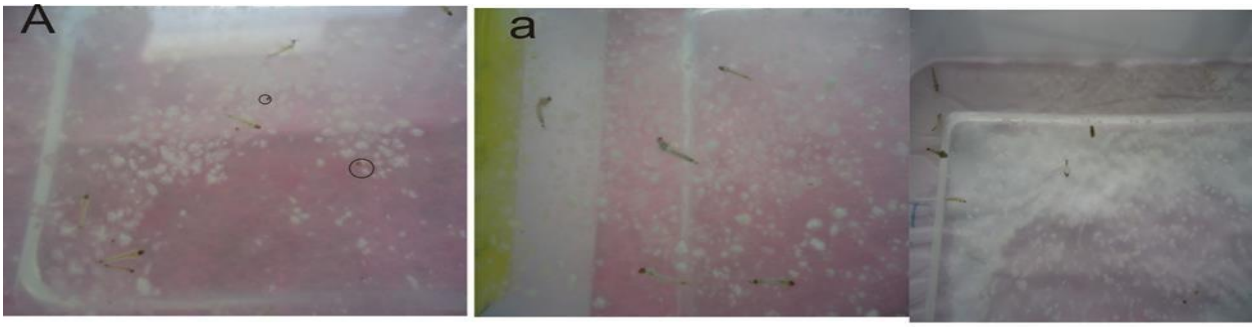
**. Correlation is significant at the 0.01 level (2-tailed).

Table 12 Chi square analyses of Ace-1

	Observed N	Expected N	Residual	ACE_1 Test Statistics	
RR	47	23.3	23.7		
RS	18	23.3	-5.3	Chi-Square	39.629a
SS	5	23.3	-18.3	df	2
Total	70			Asymp. Sig	0

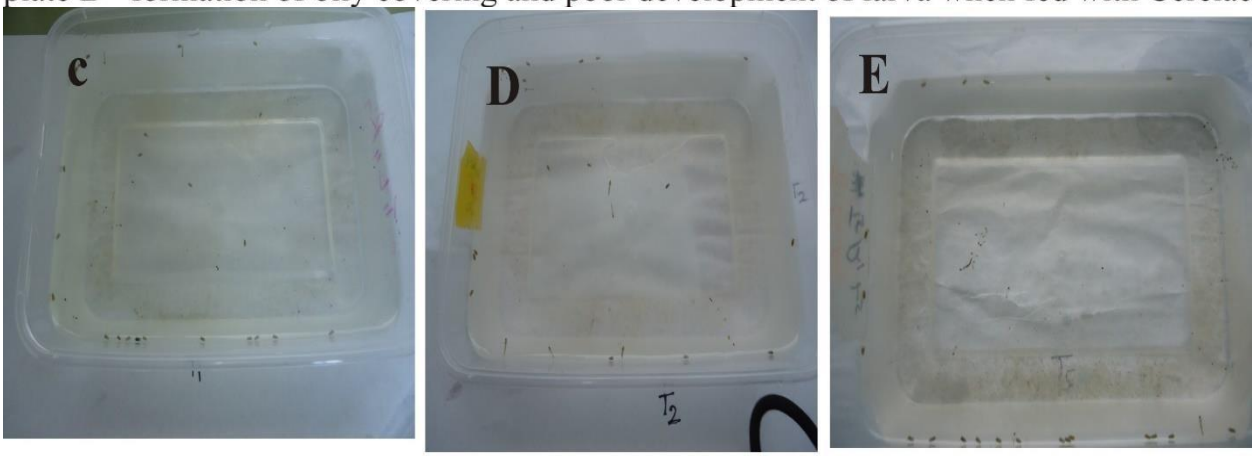
Appendix IV

Picture 3. Pictures of larval development under the influence of various food regime



A,a= larval fed with baking yeast, A= cardaver remains of *anophlese gambiae* larvae ,
a= poor development of larvae

plate B= formation of oily covering and poor development of larva when fed with Cerelac



development of pupae fed with plate C= fish flakes, plate D= cat meal and plate E=rabbit pallet

Appendix V

Picture 4 Findings from the vegetable farm in Opeibea

A=administering the questionnaire
B, C, D= Samples of various types of insecticides
F=Receipt showing the cost of the insecticide

Appendix VI

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))

Poteins crab and cholersrol sterol.

B). Fish Flake (Lopistm Tropical Fish Flakes 20g)

Protein 40% min

Fiber 5% min,

Fat 5% min,

Moisture 12%

Vitamin mix

Soya oil

Flour Fish Oil and Wheat

C). Cat Meal (Purina Friskies 400g)

Protein: 30.0%

Fat content: 10.0%

Crude ash 7,5%

Crude fiber: 2.5

Additives: Nutritional Additives: IU / kg: Vit A: 12500; Vit D 3 : 1000; mg / kg Fe (E1): 145; I (E2): 2.5; Cu (E4): 35; Mn (E5): 16; Zn (E6): 183;Se (E8): 0.25. Taurine: 870 mg / kg.

With dyes and antioxidants.

D). Cerelac (Infant Maize Cereal, Nestle®)/100g

Fat 10g

Protein 15g

Linoleic acid 1.5

Carbohydrates 65

Dietary fiber 4.5g

Sodium 145 mg

Potassium 635mg

Vitamins mix

Iron

Zinc

Mineral 3.0g Calcium and Phosphros

E). 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 VII

Study Work Plan

MONTH/ACTIVITY	SEPEMBER 2013	OCTOCBER 2013	NOVEMBER 2013	DECEMBER 2013	JANUARY 2014	FEBRUARY 2014	MARCH 2014	APRIL 2014	MAY 2014	JUNE 2014	JULY 2014	AUGUST 2014	SEPTEMBER 2015	OCTOBER 2014	NOVEMBER 2014	DECEMBER 2014	JANUARY-2015	FEBRUARY 2015	MARH-2015	APRIL 2015
PROPOSAL WRITING	•	•			•															
ASSESSMENT OF FIVE FEEDS LABORATORY WORK		•	•	•	•	•	•	•	•											
PRELIMINARY STUDY			•	•	•															
LARVAL SAMPLING		•	•	•	•	•				•	•									
SUSCEPTIBILITY TESTING										•	•									
TALKING WITH FARMERS												•	•	•	•					
MOLECULAR WORK																	•	•	•	
WRITING OF THESIS		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•