COMPARATIVE BIOLOGY AND REPRODUCTIVE BEHAVIOUR OF A LABORATORY-ADAPTED REDCO STRAIN OF *ANOPHELES GAMBIAE* GILES (DIPTERA; CULICIDAE) AND WILD POPULATIONS OF THE SAME SPECIES.

A THESIS PRESENTED TO THE;

DEPARTMENT OF NUCLEAR AGRICULTURE AND RADIATION PROCESSING,

UNIVERSITY OF GHANA

BY

(EMMANUEL BARFI, 10442653)

BSc. ENTOMOLOGY AND WILDLIFE (CAPE COAST), 2011

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PHILOSOPHY

IN

RADIATION PROCESSING AND ENTOMOLOGY

JULY, 2015

DECLARATION

This thesis is the result of research work undertaken by Emmanuel Barfi in the Department of Nuclear Agriculture and Radiation Processing, School of Nuclear and Allied Sciences, University of Ghana, under the supervision of Dr. Michael Osae and Dr. Charles Emmanuel Annoh. I hereby affirm that apart from references which have been cited, this work is a result of my own research and that it has not been presented in part or whole for any other degree in this University or elsewhere.

……. Date…….

EMMANUEL BARFI

(STUDENT)

DR. MICHAEL OSAE

(SUPERVISOR)

……. Date…….

DR. CHARLES EMMANUEL ANNOH

(SUPERVISOR)

……. Date…….

DEDICATION

I dedicate this work to everybody who has lovingly played a part in my upbringing.

ACKNOWLEDGEMENTS

All acknowledgements are given to the Lord God for making this thesis work a reality, and also granting me life free of charge. My special recognition and thanks go to my supervisors, Dr. Delphina A. M. Adabie-Gomez, Dr. Charles Emmanuel Annoh and Dr. Michael Osae. I am genuinely grateful to Dr. Michael Osae for the professional guidance, support in knowledge transfer, laboratory ethics, and field work coaching in both motivation and in kind. It was exciting having those free, and educative car rides to my experimental site. Your humility juxtaposed with intellectual brainstorming during both laboratory and field works were impeccable. I am also very thankful to Dr. Delphina A. M. Adabie-Gomez and Dr. Charles Emmanuel Annoh for their time, in painstakingly reviewing this work, correcting both grammatical and scientific errors in my work, coaching me for long hours before each presentation and the explicit consultancy they freely offered me. I appreciate your motivation and strict but adorable parental assistance you gave me. Sincere thanks to my family, the Barfi-Berko families and to my family friends. My gratitude goes to Dr. Maxwell Billah for his humor, generous and intellectual mentorship. I am thankful for making me have access to his stereoscope for my morphometric studies and also training me on how to correctly use the Lecia application suite software and the stereoscope. Sincere accolade to my alacrity and smart laboratory assistants; Diana Graham, Mavis Bossman, Norbert Seyrah Avevor and Kwabena Buabeng for their support and assistance during both laboratory and field work. My next heartfelt appreciation goes to the staff of Radiation Entomology and Pest Management Centre (REPMC), Ghana Atomic Energy Commission (GAEC) for their family unity and support they shared with me. Special thanks to Mr. Simon Yeboah, Mr. Francis Apaata, Mr Albert Nkumsah and Mr. Fredrick Abruaquah for giving me what I needed for the work to be realism.

Lastly, I am thankful to the Head of Department of Nuclear Agriculture and Radiation Processing Dr. Harry Amoatey. I am grateful to Dr. Adotey, lecturers at the University of Ghana, Legon, for their ubiquitous coordination, motivation and leadership role in making this work possible. I am thankful to all lecturers of the Department for their role and criticism at seminar presentations. To all who might have contributed in diverse ways to make this work possible, I say God bless you abundantly.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS/ACRONYMS/ SYMBOLS

ANOVA	Analysis of Variance
BSI	Body Size Index
BNARI	Biotechnology and Nuclear Agriculture Research Institute
CRA-IAEA	Coordinated Research Activities
CIMMYT	Centro Internacional de Mejoramiento de Maiz Trigo
CDC	Center for Disease Control
CRD	Complete Randomized Design
DNA	Deoxyribonucleic Acid
ECDC	European Center for Disease Prevention and Control
GAEC	Ghana Atomic Energy Commission
GSS	Ghana Statistical Service
HEG	Homing Endonuclease Genes
IIT	Incompatible Insect Technique
Iso	Isolated
IVM	Integrated Vector Management
ITNs	Insecticide Treated Nets

IRS	Indoor Residual Spray
LR	Laboratory-adapted REDCO
LAS	Lecia Application Suite
mm^2	Squared millimeter
MR4	Mosquito Research 4
ml	milliliters
mm	millimeter
NaCl	Sodium Chloride
NS	Non Significant
PBS	Phosphate Buffer Solution
REPMC	Radiation Entomology and Pest Management Center
SIT	Sterile Insect Technique
spp.	Species
SPSS	Statistical Package for the Social Sciences
SAM	Scientist Against Malaria
WR	Wild REDCO
WHO	World Health Organization

WSI	Wing Size Index
µm	micro meter
♀	Female sex
♂	Male sex

ABSTRACT

The sterile insect technique involves mass rearing of male insects for sterility purpose. This heavily relies on male fitness and genetic compatibility of laboratory-adapted male insects and the wild to ensure successful competition with their male counterpart in the wild. Uniform environment in the laboratory as compared to the wild conditions might lead to genetic drift which might lead to reduced sexual competitiveness, fitness, morphological changes or changes in the sexual behaviour of mosquitoes. This work investigated the sexual compatibility, morphometry and sexual behaviour of laboratory-adapted strain and wild strain of *Anopheles gambiae* under laboratory conditions. These measurements were done by observing swarm formation, genitalia rotation, percentage insemination, fecundity, fertility, wing length, wing width, thoracic width, body length, body size index and wing size index. Morphometric studies of laboratory-adapted and wild strain of *Anopheles gambiae* were carried out by observing the wing length, body length and thoracic length under Lecia 4D stereoscope in order to find out variations in the body size between the two strains. The results showed significant difference between thoracic width and wing length between the laboratory-adapted strain and wild strain. Indices such as body size index and wing length index also showed significant difference between the two strains; laboratory-adapted REDCO strain (BSI 4.45 ± 0.10, p = 0.010 ; WSI 1.92 ± 0.07, p = 0.026) and wild REDCO strain (4.08 ± 0.10, p = 0.010 ; WSI 1.73 ± 0.04, p = 0.026). Body length of laboratory-adapted male mosquitoes (4.24 ± 0.05, p = 0.462) was not significantly different from its thoracic width, wing length, and wing width. The wild strain on the other hand had significant difference between its body length (4.19 ± 0.04, p = 0.462), thoracic width (0.096 ± 0.02, p = 0.002) and wing length

(2.99 ± 0.03, p = 0.050). In the mating experiment, egg production in each of the crosses (W♂ x W♀; 594.00 ± 20.00, p = 0.00), (L♂ x L♀ ; 108.00 ± 119.00, p = 0.00) and (237.00 ± 40.00, p = 0.00) showed significance in egg production. The results suggest that sizes of the two strains differ and also laboratory-adapted strain produced the highest number of eggs.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The sterile insect technique (SIT) and genetically engineered insects releases are part of insect pest control strategies which have mass rearing of insects as starting points. The gene pool of these colonized insects is limited as a result of selection and genetic bottlenecks that have come about from long term rearing of the insects (Baeshen *et al.*, 2014 ; Dyck *et al.*, 2005). The fitness of these colonized strains are at stake due to these genetic changes (Benedict *et al.*,2009; Howel and Knols, 2009; Reisen, 2003). These genetic changes as well as changes in behaviour resulting from isolation could lead to sexual incompatibility. Therefore using comparative biology as a tool for measuring the extent of the changes caused by colonization of the laboratory strain relative to its wild strain will prove effective. This study involves comparative biological studies to determine the extent to which prolonged colonization has changed the REDCO strain. It will focus on The comparative biology, which will involve the morphometric, developmental and reproductive biology as tools to compare any differences between the two strains.

In the World Health Organization (WHO) report, (2012 ; 2013) about 3 billion people of the world population, especially Africa and Asia, stood the risk of getting malaria disease in 2012. This had resulted in very high mortality rates especially in children below the age of 5 years and pregnant women. In West

Africa, including Ghana about 90 percent of malaria deaths happen every year (Klinkenberg *et al.*, 2008 ; Rueda, 2008). But this has been reducing due the success of integrated vector management (IVM) method.

According to a review executed in 2015 by WHO, deaths resulting from malaria disease has declined by 47 % worldwide, 54 % in African countries under the auspices of WHO, and death rates in children dropped by 58 % since the year 2000. Out of the 198 million records in malaria in 2013, about 584,000 resulted in deaths. It is extrapolated that an infant dies of malaria every minute in Africa. Travellers from non-malaria endemic areas are highly vulnerable since their body is devoid of immunity to the parasite (WHO, 2015).

Malaria is hyper-endemic in Ghana and accounts for over three million outpatient visits every year in health institution records (Obala, *et al.*, 2012). The Ghana Health Service reported that 20 - 30.3 % of infants below the age of 5 years face mortality and about 5.98 % mortality occur in pregnant women. The above statistics derived from Ghana Ministry of Health (2008) and Ghana Health Service (2014) indicates loss in productivity through the decrease in man power in the nation.

1.2 Problem Statement

There have been several current control measures for malaria. These control measures can be grouped into Rapid Disease Diagnosis, Chemotherapy and Vector Control. Rapid disease diagnosis involves the early detection and correct diagnosis of malaria and treatment of the disease by professional health personnel,

which involves the right prescription and dosage of drugs to malaria patients so as to get rid of the *Plasmodium* parasite in the populace. This has not proved effective due to inadequate accessibility to health care and the dilemma of self-medication which causes resistant *Plasmodium* strains. Large scale prophylaxes have proved effective but have some dangerous lapses on drug resistance as indicated by (Killeen *et al*., 2002).

According to White *et al.,* (2011), malaria vector control is also employed in reducing mosquito population by killing the aquatic immature stages and the adult mosquitoes. Larvae and pupae can be controlled through the use of larvacide, pupacides, and environmental management. The use of chemical insecticide spray at breeding sites and our homes or the use of repellents on our body, sleeping under Insecticide Treated Nets (ITNs), and indoor residual spray (IRS) on walls of buildings control adult mosquitoes (WHO, 2006). The chemical control methods are faced with the problem of resistance (Baeshen, *et al.,* 2014).

The resistance and other problems associated with insecticide use have led to new trends in autocidal vector control methods, including sterile insect technique (SIT) and the use of genetically modified mosquitoes. The sterile insect technique is a highly potential tool of mosquito control and is currently being used in an *Anopheles arabiensis* pilot control program in Northern Sudan (Dyck *et al.,* 2005 ; Muhenga *et al.,* 2011). This was due to the high success of the SIT mosquito programs piloted in Pensacola, Florida in the 1960's to control *Aedes spp*, and in El Salvador for the control of *Anopheles albimanus* (CRA-IAEA, 2013). Both of which required mass rearing of mosquitoes in the laboratory or a rearing facility.

According to Baeshen *et al.*, (2014), certain laboratory factors such as temperature uniformity, nutrition, overcrowding and inbreeding lead to shortening of sperm length, increase in testes size and decrease in accessory gland size. In addition, long term rearing of mosquitoes for several generations can lead to genetic homogeneity resulting from genetic isolation and natural selection. The laboratory reared mosquitoes might not perform well when released into wild populations. There is also a high probability of timidity, modified sexual and courtship skills of laboratory reared ones which might render it incompatible with the aggressive wild strain. In the 2011 article by Muhenga *et al.*, significant fitness loss was said to be highly probable in the laboratory reared mosquitoes (over 90 generations) and may reduce genetic variation, since a more diversified gene pool indicates fitness. The loss or reduction in variation can be associated with population bottle-neck, founder effects or likely homogenous laboratory conditions. The genes responsible for search and location of food, and swarm markers under wild conditions could be lost or modified due to active disuse of those genes. The reasons being that, they do not actively search for food and female mating partners during colonization, which might make survival and fitness of male mosquitoes reared for many generations less likely in wild conditions. This could adversely affect mass rearing of any species of insects being targeted for control using autocidal method. There is the need to compare *Anopheles gambiae* Giles (Diptera; Culicidae) reared in the laboratory for over 90 filial generations with the first filial generation of the wild population, both of which originated from breeding sites at REDCO a residential area in Madina and

its environs in the Greater Accra Region of Ghana to observe any difference in their morphometry, biology and reproductive behaviour.

1.3 Justification

There is the need for this study to be undertaken, since long term colonization and its associated parameters such as temperature, nutrition and inbreeding may affect the biology and certain behavioural parameters of the *Anopheles gambiae* Giles (Diptera; Culicidae) which might affect competitiveness in the wild during field releases. Studies done by Mwangangi *et al.*, (2004) showed that longevity had significant correlation with body size. The bigger the size of the mosquito, the longer it lived. Bigger male mosquitoes also attracted female mosquitoes better than small sized mosquitoes. This is to determine whether *Anopheles gambiae* Giles (Diptera; Culicidae) reared over several generations, which might be used as test subjects for insecticide studies and other experiments truly simulates what happens under field conditions. Muhenga *et al.*, (2011) compared MALPAN laboratory *Anopheles arabiensis* colony reared over 20 years with its wild strain collected at Malahlapago. The two populations were found to show significant difference in reproductive and physiological fitness. I am investigating and replicating it on *Anopheles gambiae s.s*, Giles reared over 90 generations to its wild strain.

This study seeks to find out if rearing of *Anopheles gambiae s.s*, Giles significantly affects their morphometry and reproductive biology. Whether the laboratory adapted strain is a good candidate for SIT programmes.

1.4 Main Objective

- To determine whether prolonged colonization has any significant effect on the biology and reproductive behaviour of Anopheles gambiae Giles (Diptera; Culicidae).

1.5 Specific Objective

- To determine differences in the morphormetrics and developmental biology of laboratory adapted colony and the wild population
- To assess effect of prolonged colonization on the sexual compatibility of the laboratory adapted colony and the wild population.
- To determine variation in mating of the laboratory adapted colony and the wild populations.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria vectors and classification

Anopheles spp. are the principal vectors of malaria. Many species of *Anopheles* mosquitoes are distributed globally. *Anopheles culicifacies* are mostly found in the south Asian plains. *Anopheles fluviatiles* and *Anopheles minimus* are geographically situated in riverine valleys while *Anopheles stephensis* are dominant in the arid and semi-arid plains and urban areas. In Ghana and other African countries the main vectors of malaria are 3 species of *Anopheles*, namely, *Anopheles gambiae, Anopheles arabiensis* and *Anopheles funestus*. These species have been known to have an evolutionary relationship with the *Plasmodium* parasites. However, in other places such as China, *Anopheles sinensis* and *Anopheles anthropophagus* are the vectors of malaria. In Central and South America, *Anopheles darling* and *Anopheles pseudopunctipennis* are the main malaria vectors. These evolutionary related *Anopheles spp.* have evolved in the tropical-savanna zones and have mastered the spread of mutant *Plasmodium* parasites (Coluzzi, 1999).

Mosquitoes belong to the Order Diptera, and the Infraorder Culicomorpha (Reinert *et al.*, 1997 ; Reinert *et al.*, 2009). Mosquitoes under the Family Culicidae are grouped into two subfamilies, which are Anophelinae and Culicinae as reported by Harbach in 2004. The subfamily Anophelinae consist of three

genera; *Anopheles* (cosmopolitan), *Bironella* Theobald (Australasian) and *Chagasia* Cruz which is neotropic. Most of the anopheline species are found in the genus *Anopheles* which is made up of six subgenera. Based on Harbach's work in 2013 and a work done by the New Zealand BioSecure Entomology Laboratory (2007), the total number of *Anopheles* species is approximated to be 465. This value keeps on alternating since about 13 % of the 537 species published in SAM, (2015) are yet to be named. About 70 are malaria vectors of humans and 40 of them are being given a status of important vectors (Amani *et al.*, 2014).

Anopheles is the most studied among the mosquito genera. This is due to its negative health effects on humans and domestic animals. Mosquitoes in comparison with other insects have affected man in all aspects of life and have intricately been woven into human evolution. *Anopheles* genera are classified based on internal and external structures. The external structures are the shape of scales and distribution of scales on the thorax and abdomen, wing spots, head of the adult mosquito, larval and pupal anatomy of the *Anopheles* mosquito (Harbach, 1994). Currently, the classification of the male *Anopheles* is based on the number and positions of specialized setae found on the gonocoxites of the aedeagus (Harbach, 2013). The internal structures are currently based on chromosome structure and DNA sequencing. About 465 - 484 of *Anopheles* species are currently fully studied, and have been divided among six genera, with the largest of the genera being *Anopheles*, *Celia*, and *Nyssorhynchus.* These changes in classification of *Anopheles* were made to delete multiplicity, duplicity,

erroneous classification and to ensure clarity (Harbach, 1994). *Celia, Kerteszia, Baimaia, Nyssorhynchus* and *Stethomyia* have been re-assigned subgenera status with 14 other genera used as synonyms of *Anopheles, Celia* or *Nyssorhynchus* (Harbach, 2013). The genus *Anopheles* is comprised of 182 species and are found in all zoogeographic regions in the world. *Celia* subgenus is found in the Australasian, Oriental, Afro-tropical regions and sometimes Palearctic regions, and is made of 227 species. *Kerteszia* (Neotropic region) has 12 species. *Nyssorhynchus* (dominantly Neotropical and with little occurrence in Nearctic regions) has 30 species, and *Stethomyia* (Neotropical regions) consist of 5 species (Harbach, 2013). These classifications were based on the external and internal structures.

Anopheles spp. are the only species that transmits all the four protozoal parasites that cause malaria namely *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale,* and *Plasmodium malariae. Anopheles* mosquitoes do not only transmit malaria but are also vectors of Bancroftian filariasis (*Wuchereria bancrofti*), whose vectors are *Anopheles gambiae s.l.* and *Anopheles funestus.* Reuda reported in 2008 that *Anopheles gambiae* Giles is part of the most efficient vectors in the spread of this malicious filarial disease. Malayan filariasis (*Brugia malayi*) is caused by *Anopheles sinensis.* Brugian filariasis (*Brugia timori*) is also spread by *Anopheles barbirostris.* Ross river virus, Cache valley virus and O'Nyong Nyong virus are spread by *Anopheles amictus, Anopheles quadrimaculatus,* and *Anopheles gambiae* respectively. *Dirofilaria immitis* and *Dirofilaria repens* (Dog heart worms) are disease causing filarial organisms also

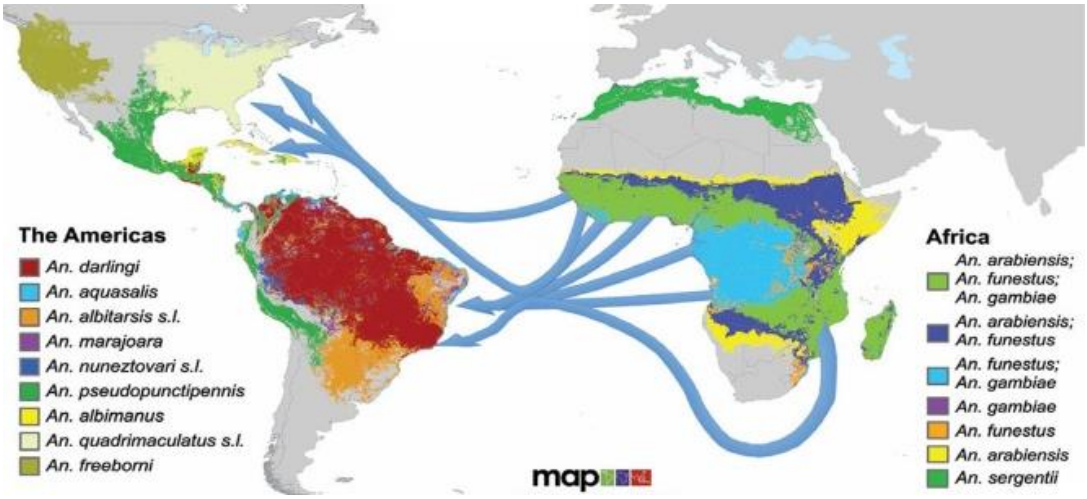
transmitted by the *Anopheles spp.* Research has shown some viral isolates from several *Anopheles spp.* These are the Kowanyama virus (*Anopheles amictus* and *Anopheles annilipes*), Mapputta virus (*Anopheles amictus* and *Anopheles annulipes*), Trubanaman virus (*Anopheles annulipes*), Sindbis (*Anopheles meraukensis*), Eubenangee virus (*Anopheles farauti*) and Bovine ephemeral fever (*Anopheles bancroftii*).

Due to the stubborn nature of the *Anopheles spp,* and their threat to life, medical and economic importance, there have been several conventional measures, such as cultural control, biological control and chemical control used in to reduce their population. These control methods are proving to be less effective due to high cost in implementing them on a large scale or development of resistant gene. This evasive behaviour of *Anopheles* mosquitoes has led to a paradigm shift to new cutting edge genetic control methods. Genetic control methods involve the sterile insect technique or other genetically engineered mosquito technologies (Howell and Knols, 2009 ; Lees *et al.*, 2014 ; Muhenga *et al.*, 2011 ; Nolan *et al.*, 2011). These genetic control strategies can be successful only if male mosquitoes are mass reared under artificial laboratory conditions, treated then released to achieve control purpose. The problems which have been found by other researchers concerning this method are low fitness level, altered behaviour and reduced competition of colonized mosquitoes, since this method involves mass rearing and colonization of mosquitoes. It has been found that colonized male mosquitoes have shorter sperm tail, smaller accessory gland size and abnormally enlarged testes. These changes in the male mosquitoes reared under laboratory conditions

for long periods have been linked to reduce fitness and competitiveness when compared to their wild brothers. Therefore it is suspected that these changes affect their biology and behaviour which might flaw any genetic control programme (Howell and Knols, 2009 ; Lees *et al.*, 2014 ; Muhenga *et al.*, 2011 ; Nolan *et al.*, 2011).

2.2 Geographic distribution

Anopheles mosquitoes are cosmopolitan, and are dominant in the tropics and sub-tropical zones and also distributed in the temperate climates (Figure 2.1). The population distribution of local *Anopheles species* is seasonal and is directly proportional to the rainy season, making distribution relatively at its peak during heavy rainfall and lower during the drier periods in the year. (Sinka, 2013). *Anopheles spp.* are endemic in the sub-Saharan Africa and is known as the *Anopheles* belt (Figure 2.1).



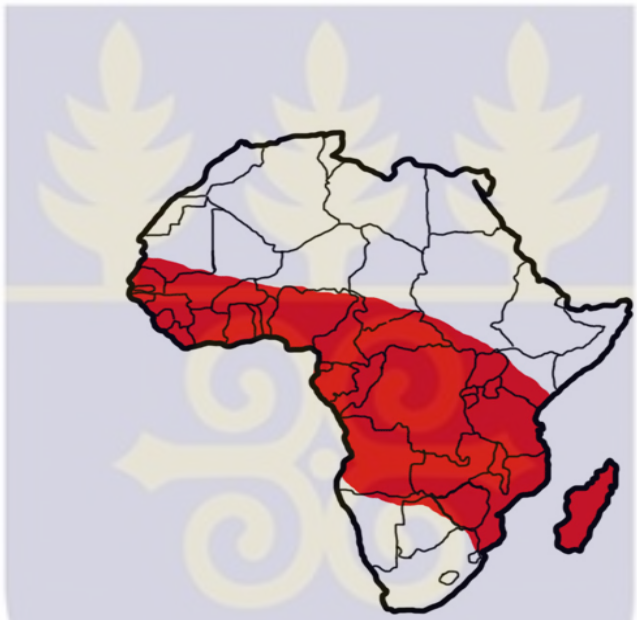


Figure 2.1 World-wide and sub Saharan distributions of *Anopheles spp.*

(Guelbeogo and Ayala, 2013; White and Kaufman, 2014).

2.4 Comparative biology and mating behaviour

Comparative biology takes into consideration the functional and evolutionary biology of an insect, which combines to give an in-depth understanding of how the structural component of the insect work. It also shows how the biotic and physical environment of the insect interacts with time and ecological conditions. SriKumar and Bhat, (2012) on their study on the comparative biology of the tea

mosquito bug (*Helopeltis spp.*) placed oviposition, adult survival rate, longevity, egg production, developmental time and sex ratio under comparative biology. Also, sexual maturity, morphometry, time to complete life cycle and egg production per female are all classified under comparative biology of an insect. The sexual behaviour on the other hand focuses on how male mosquitoes form swarms, and the relationship between egg production and number of male mosquitoes which formed swarms.

2.5 Genitalia rotation

Genitalia rotation is the physiological changes that exhibited by the male mosquito as a sign of sexual maturity. Dahan and Koekemoer (2013) indicated that these changes are visible few hours after adult emergence. During this process the terminalia either turn in at most 45^{o} intervals clockwise or counter clockwise direction till sexual maturation is reached at 180^{o} full rotation. The inability to start rotation or complete genitalia rotation is an indication of sexual immaturity in male mosquitoes. Male mosquitoes that are able to complete the genital rotation at a faster rate stand the chance of showing high reproductive performance. The male genitalia is made of an aedeagus and other external genitalia structures such as subgenital plate, claspers, styli and other structures which help in mating (Meyer, 2005). The prominent part is the gonocoxites which are sclerotized non lobed structures bearing the basimere, basistyle, coxite, and claspers as illustrated in figures 2.2.

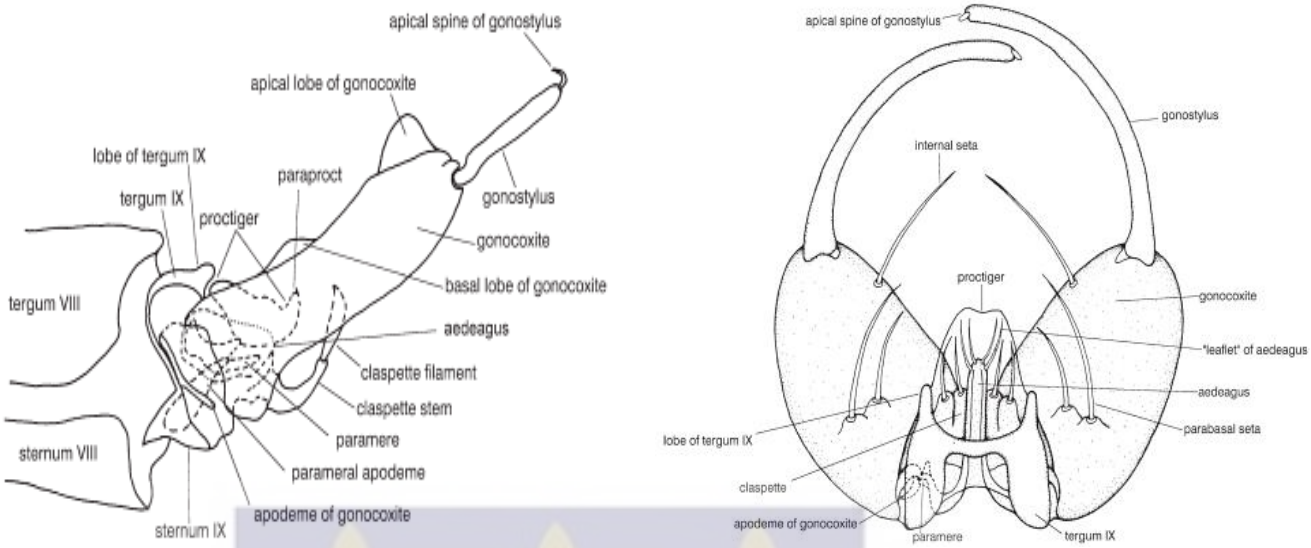


Figure 2.2 Structure of the aedeagus with gonocoxite bearing claspers and other post genital structures
(Becker *et al.,* 2010).

The claspers are movable since they form a moveable joint with the apex of the gonocoxites, thus helping in hooking to female during mating. Male genitalia is a modification of the ninth abdominal segment with the tenth and eleventh segments reduced into post genital structures on the ninth segment. A complete genitalia rotation turns the sterna, terga and genitalia upside down, which brings the aedeagus on top of the anus. This makes insertion of the aedeagus into the female's genital opening possible during copulation (Becker *et al.*, 2010).

Dahan and Koekemoer (2014) conducted an experiment in which batches of male *Anopheles funestus* fed with 10 % sucrose solution were picked and killed by freezing at -20 ^{o}C from 2 to 48 hours at hourly intervals. The mosquitoes were kept under laboratory conditions of 18 ^{o}C to 29 ^{o}C and 74 - 80 % rh respectively. The rate of genitalia rotation was examined based on different degrees of genitalia rotation listed below. In figure 2.3 each stage shows the degree of rotation with

the white arrows showing the position of the seventh and eighth pleura. When it is in a straight line, and the claspers face down, it shows the final stage of genitalia rotation (Oliva *et al.*, 2010; Oliva *et al.*, 2011).

As illustrated in Figure 2.3, stage 0 shows no rotation in the first two hours, stage 1 has a rotation of about 45^{o} which occurs often within the first six hours. Stage 2 are dominant between six to fourteen hours after emergence and have their genitalia rotating within the range of 45^{o}- 90^{o}. Between six to twenty four hours, stage 3 has 90^{o}- 135^{o} genitalia rotation. At age fourteen to forty eight hours, stage 4 has full rotation of 180^{o}. The white arrows indicate the position of the 7^{th} and 8^{th} pleura when it is in line with the ventral-facing claspers; it shows the final stage of genitalia rotation. The duration of complete rotation varies from species to species, with some *Aedes spp.* taking from 40 - 96 hours to complete rotation. In-depth research into rate of genitalia rotation of anopheline mosquitoes have not yet been done, but limited observations show that at least 24 hours rotation period is required before *Anopheles arabiensis, Anopheles gambiae s.s* and *Anopheles stephensis* become sexually matured to embark on mating.

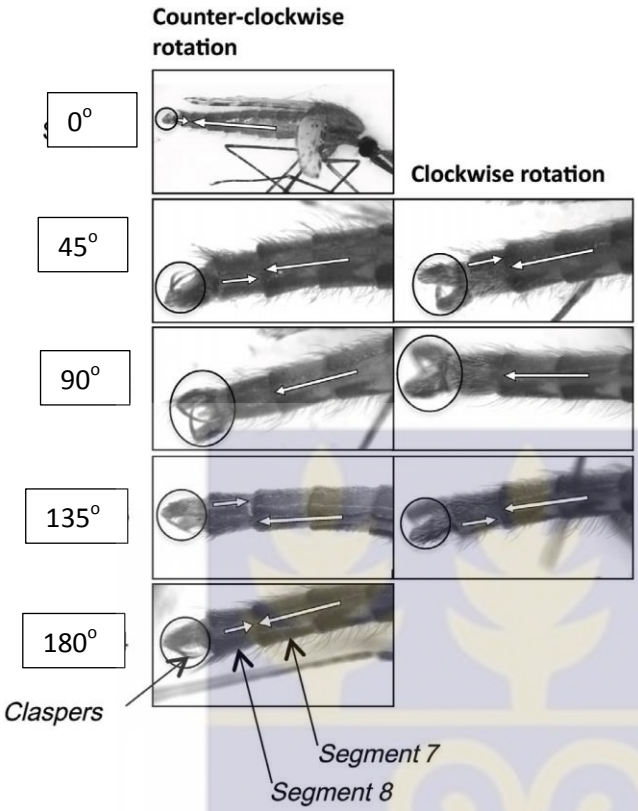


Figure 2.3 Lateral view of the clockwise and counter clockwise rotation of male *Anopheles arabiensis* genitalia, and the various stages of inversion (Oliva *et al.,* 2011).

Genitalia rotation in mosquito species *Anopheles arabiensis* and *Anopheles gambiae* require at least 24 hrs to fully mature sexually (Oliva *et al.*, 2011). A 2x2 contingency was used by them to compare the rates of rotation between the laboratory adapted and wild populations of *Anopheles arabiensis*. The p-value was calculated using Fisher exact test in GraphPad Prism vs.-6 and the statistical significance set to 5 % level. The results of the laboratory adapted and wild populations of *Anopheles arabiensis* showed that at 2 hours, genitalia had not rotated in the two populations. More than half of the male population of both the wild and laboratory-adapted male mosquitoes had reached their second stage at 14

hours after emergence, and between 20 - 48 hours, almost all had reached full rotation. Research showed that the difference between genitalia rotation of the laboratory adapted and wild mosquito populations at each age were not significant ($p > 0.05$; Fisher exact test). On the other hand, when the same experiment was conducted under 23 - 29 ^{o}C ± 1 ^{o}C and 74 % rh conditions, there were significant differences in each age between the laboratory adapted and wild populations. The experiments of Oliva *et al.*, (2011), showed that wild males had slower genitalia rotation than laboratory males. At 17 hours post emergence, 96 % of laboratory males had completed rotation of genitalia, whereas 0 % of the wild males had completed their genitalia rotation. At 11 hours post-emergence stage, 42 % of the laboratory males had completed their genitalia rotation, which suggests that sex separation at adult stage should be done earlier than 11 hours so to ensure the virginity of the female mosquitoes. According to Oliva *et al.*, (2011), the males were separated from the females between in not more than 18 hours after emergence, but this has been changed to 12 -16 hours after further experiments has shown earlier sexual maturation than previously proposed.

Mosquitoes like many Dipterans are not sexually matured right after emergence. Male mosquito genitalia are found in the 8th to 10th abdominal segments. At the tip of the last abdominal segment is a pair of claspers bearing claws which help males to grasp females during mating. Their genitalia need to go through a complete rotation of 180^{o} to be ready to mate. Genitalia rotation is made possible by the action of two cross muscles that oppose each other in action, which can either be in the clock wise or anti-clockwise direction. Female mosquitoes, on the

other hand, are usually unreceptive to males during the first 30 - 60 hours; however, they sometimes allow copulation without insemination within the period. The male genitalia rotation can be seen by observing the relative position of the abdominal claws, the claspers, the pleura and the basistyles (Figures 2.2). The claws and claspers may either face ventrally (downwards), laterally (side-ways) or dorsally (upwards) at different stages of the genitalia rotation designated as stages 0, 1, 2, 3 and 4. Stage 0 and Stage 4 represent no rotation or complete rotation respectively. The pleura of segments 7 and 8 are continuous and both sides of the pleuron must be observed on the 7^{th} and 8^{th} segments to justify the fate of rotation or make an informed decision. The other stages have the basistyles (Figures 2.2) perpendicular or at an angle to pleuron. Virgin male mosquitoes kept in cages were aspirated and killed when their respective ages were reached (Oliva *et al.*, 2011).

At age 0 - 3.5 hours, all of the laboratory-adapted mosquitoes recorded no genitalia rotation. About 25 % were in stage 1 genitalia rotations when recorded at 4.5 hours after emergence. From 14 hours upwards, 100 % males had their genitals rotating to at least 135 ^{o}. More than 90 % of the laboratory-adapted population had reached full rotation and after 14 hours. Between 24 - 168 hours all laboratory reared male mosquitoes had full rotation. In the wild population 40% had reached complete rotation at 23.5 hours. Genitalia rotation in laboratory-adapted population was significantly different from that in wild population. The significant speed in genitalia rotation in the laboratory-adapted population is associated to natural selection, genetic drift (neutral evolution), and inbreeding.

These factors have been found to cause variation, short maturation period and modify sexual behaviour in laboratory-adapted mosquitoes.

2.6 Life cycle of *Anopheles* mosquito

Anopheles mosquito exhibits complete metamorphosis; egg, larvae, pupae and adult stages (Figure 2.4). It can take about 10-27 days to reach the adult stage (Cross, 2004 ; Glyshaw and Wason, 2013 ; Rios and Connelly, 2013 ; White and Kaufman, 2014) depending on the species and the ambient temperature. Only the adult stage which is an intermittent parasitic ectoparasite is not aquatic. Female *Anopheles* mosquitoes enter swarms formed by male mosquitoes, where they form mating pairs for nuptial to take place. Swarming behaviour shows fitness of male mosquitoes, since one round of swarming routine can make use of about 50% of energy reserves (Howell and Knols, 2009). This means that the most competitive males are energetic and are able to mate with more than one female. Some male mosquitoes are denied mating which can be associated to sexual immaturity and lower fitness. Female mosquitoes who have successfully mated obtain blood meal, preferably from humans and a single female can lay from 100 to 300 eggs in their life time (Glyshaw and Wason, 2013). In ambient temperature, the percentage egg hatch can be as high as 80 %.

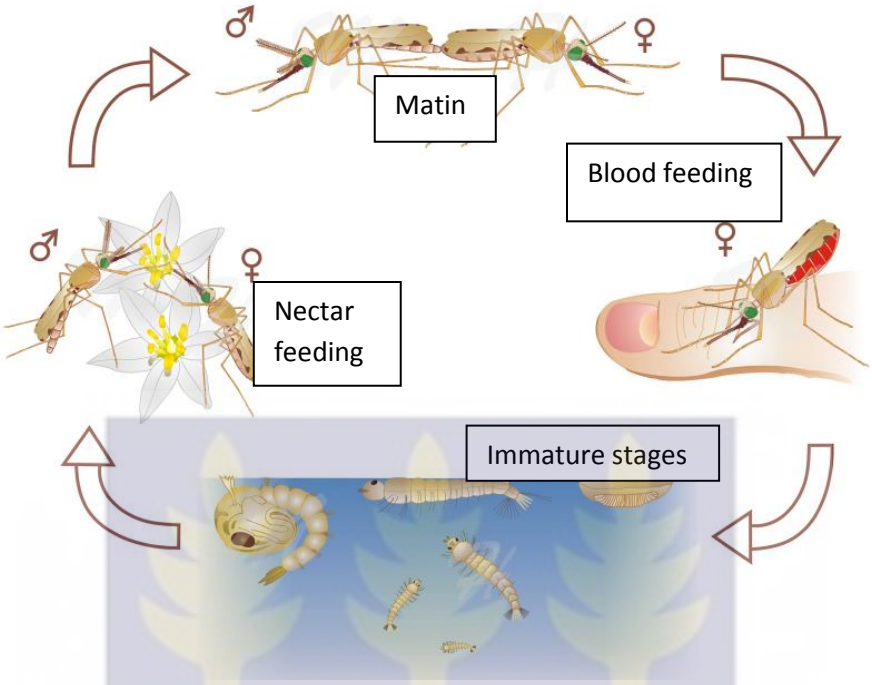


Figure 2.4 Life cycle of *Anopheles spp.* mosquito (www.biographix.cz)

2.7 Oviposition and biology of eggs

2.7.1 Breeding sites

The female *Anopheles* mosquito is attracted to slow moving fresh water found in puddles, pools, fresh water marshes, flooded areas, ditches, road ruts, pot-holes, rice fields, irrigated areas, clean drainages, house hold water containments (Chinery, 1995). The eggs are also found in water accumulated in cans, hollow coconut shells, hollow car tyres, deep car tracks, foot and hoof print depressions and burrows in trees, due to enough oxygen present in their clean water and possibly security provided for survival of larvae (Khaemba *et al.*, 1994; Minakawa *et al.*, 2001). *Anopheles gambiae* has shown a possible evolutionary

adaptation to certain levels of pollution in water bodies due to urbanization as reported by Klinkenberg *et al.,* (2008) and special case studies in Cote d'Ivoire and Cameroon (Antonio-Nkondjio *et al.*, 2011; Matthys *et al.*, 2006; Omlin *et al*, 2007,). In Abeokuta, Nigeria, research showed that *Anopheles gambiae* now existed in sympatry with *Culex spp* which is known for its affinity to contaminated water (Adeleke *et al.*, 2008).

2.7.2 Oviposition of eggs

According to the Centre for Disease Control, CDC (2012), adult females of *Anopheles* mosquitoes can lay an average of 100 eggs per oviposition. Eggs are laid singly and directly on water surface to reduce desiccation (Figure 2.5).

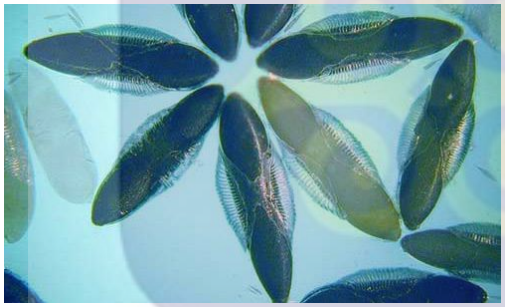


Figure 2.5 Eggs of *Anopheles spp*. in water with visible lateral floats (Rios and Connelly, 2007).

Eggs of *Anopheles* have length and width of about 371.5µm, and 141.0µm respectively. The egg has length to width ratio of about 2.634µm and usually canoe or surf-shaped (Figure 2.6). The eggs are not stuck together, they have fairly pointed anterior and posterior ends (ECDC, 2014; Glyshaw and Wason, 2013; Malhotra *et al.*,2000; Sehrawat, 2014). The mean length of 100 specimens was 596 ± 8.35µm within a range 530 - 700 and the width taken at the broadest

point was 196 ± 1.73µm also in the range of 180 - 210). Also the length/width ratio is 3.0 4 ± 0.04 with range 2.6 - 3.5 µm (Malhotra *et al.*, 2000). They have their upper surfaces usually flattened, and the lower surfaces are convex shaped.

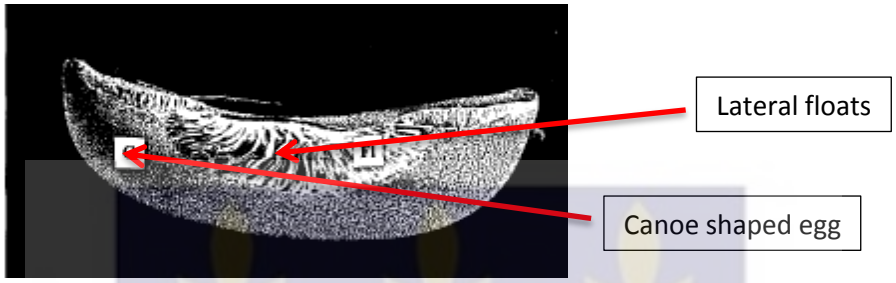


Figure 2.6 Lateral view of canoe shaped egg with lateral floats on its side (Malhotra *et al.*, 2000).

The wall of the egg-shell is made of the inner wall known as the endochorion which tans after drying. The outer wall (exochorion) has a transparent material which might have frills, bosses and floats on either side (Sehrawat, 2014). Some species such as *Anopheles sacharovi* Fa'vr most often lack lateral floats, but at certain cold conditions eggs develop rudimentary or vestigial floats (ECDC, 2014; Romi, *et als.*, 2002). Most eggs often hatch after 24 hours, but during adverse conditions they might take 3 – 7 days. Eggs need contact with water in other to survive or else it will not hatch. *Anopheles* eggs take about 1 - 3 days in the warm, humid tropic regions, and longer time in harsh, colder and temperate environments.

2.8 Larval stage

2.81 External morphology

The next phase in the mosquito life cycle is the larval stage, which requires solely a water source to survive. The length of *Anopheles gambiae* larvae at their 1st instar stage is < 1 mm and at the 4th instar it is 7.5 mm. They most often mimic the colour of their breeding sites. Larvae vary in colouration from yellowish, to greyish-dark or sometimes green. The larvae have prominent dark coloured head, large thorax and segmented abdomen with hairs on their body. In contrast to *Culex* and *Aedes* mosquitoes, *Anopheles* larvae lack respiratory siphon and for this reason they lie parallel to the surface of the water to breathe through spiracles at the posterior end of the 8th abdominal segment (Figure 2.7, left specimen) (Meyer, 2005 ; Ponlawat and Harrington, 2009).

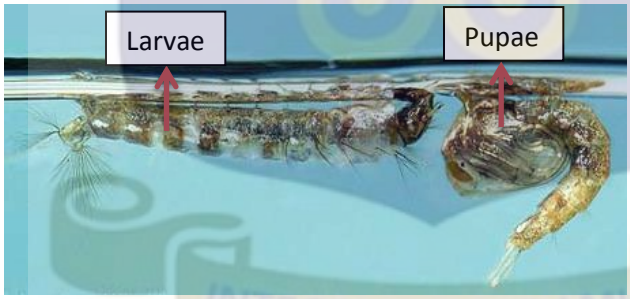


Figure 2.7 Horizontal positions of larva and pupa of *Anopheles spp.*

(Rios and Connelly, 2007)

2.8. 2 Feeding habit of the larvae

The *Anopheles* larvae have a pair of relatively large mouth brushes used for collecting food items such as phytoplanktons and zooplanktons. During their first two instars they feed on dissolved food. The 3rd and 4th instar larvae feed on a lot

of spend most of algae, bacteria, and other microorganisms so that they store enough energy for the transformation and development that occur at the pupal stage, which is a non-feeding stage. They take about 7 - 14 days for larvae metamorphose into pupal stage.

2.9 Pupal stage

The third phase of metamorphosis is the pupal stage which is also aquatic. Its lateral view is comma-shaped. The cephalothorax which is made up of the fusion of head and thorax are exaggerated in size as compared to the rest of the body (Figure 2.7, right specimen). Although the pupae are active, they do not feed but use stored food at the larval stage in their body. They breathe through a pair of respiratory trumpets on the dorsal side of cephalothorax. Adult mosquito emerges from pupal stage after 24 to 48 hours (Cross, 2004).

2.10 Adult stage

The adult stage is the end of metamorphosis and the only non-aquatic and parasitic stage. After emergence from puparium, adults wait for an average period of 48 hours for sexual maturation to occur. In males, this is made evident by either the clock-wise or counter clock-wise rotation of the male genitalia. The adult mosquitoes like any insect has three body parts namely head, thorax and abdomen (Figure 2.8). They have a pair of wings with scales covering the veins and margins. Presence or absence of dark spots on wings can be used to identify mosquito species. Antennae may be bushy or brush-like (plumose) in the males

and pilose in the female mosquitoes. Their mouthparts form a long piercing and sucking proboscis. *Anopheles* adult mosquito rests with the abdomen positioned obliquely on resting surfaces during feeding or digestion of blood meal, whereas other species keep their bodies parallel to the surface, which makes them easy to identify when sitting on the skin (Meyer, 2005 ; Rutledge *et al.*, 2005).

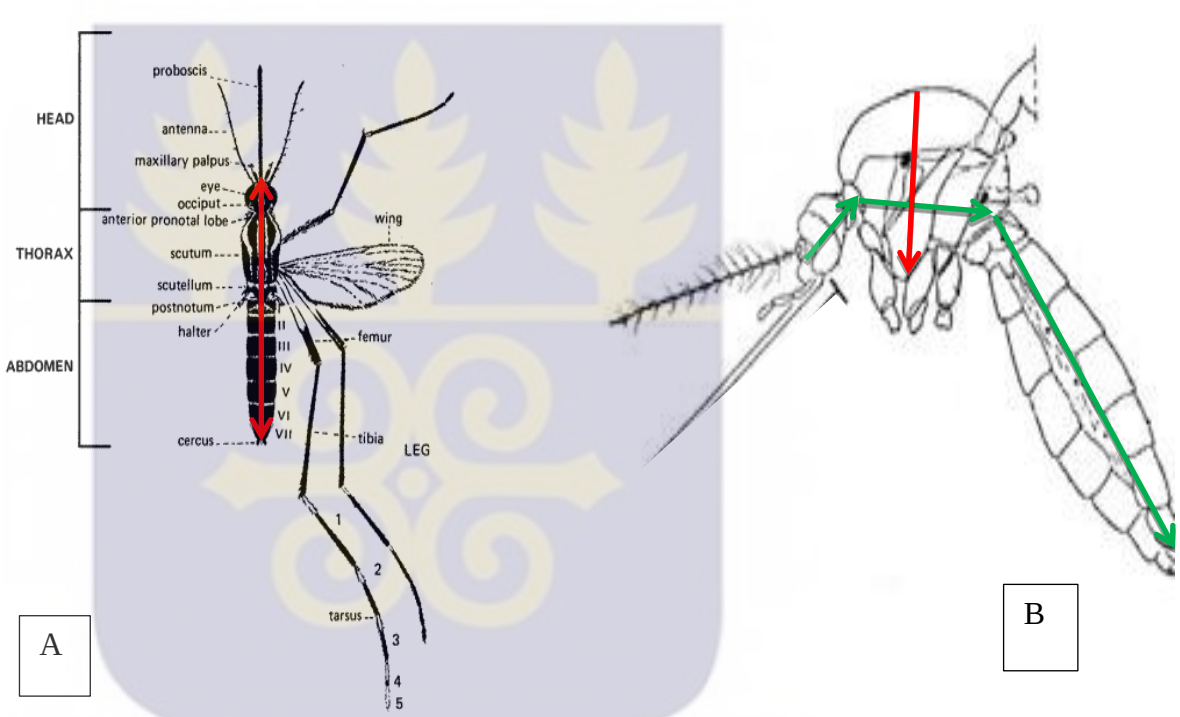


Figure 2.8 Measurement of body length and thoracic depth of a mosquito (http;//www.cdc.gov; Silva *et al.*, (2012)).

The thorax of mosquitoes resembles an upside down pyramid. The meso-notum which is the basal region is rounded off, and the coxae form the apex. The four sides of the coxae are formed from the two pleurae of both the anterior and posterior of the thorax. The legs are converged around the mid-coxae region. Due to the complicated nature of finding body size of mosquitoes in the sixties, it is a fact now that wing length is the most reliable, accurate and easiest way to

determine the size of mosquitoes since wing length is directly proportional to mosquito size.

2.11 Structure of *Anopheles gambiae* wing

Wing length starts from the knuckle or root of the wing, or base of the costa to the most pointed tip of the wing, with exemption to the hair fringes along the edge of the wings (Figures 2.9). Female *Aedes* wings are narrower than males, with proportions of 4 wing length ; 1 wing width and 4 wing length ; 3 wing width respectively. Female wings are also longer than the males, 3.5 mm and 2.5 mm respectively. Wing length is also equal to weight of the mosquito but with higher uncertainty. However, the wing length cannot be used to equate body weight of gravid and well-fed female mosquitoes. An unfed mosquito has an average abdominal length and body size equal to its wing length. However, Carron, (2007) indicated that wing length was longer in males than in females but vice versa in wing width based on his research on *Ochlerotatus caspius* mosquito. In Carron's conclusion, it was ascertained that wing size or surface or area as estimated by multiplying wing length by wing width had a positive and significant relationship with the body weight. Yeap *et al*. (2013) also proved that wing length and centroid sizes were much correlated to body size, whereby centroids are the distances between certain landmarks located on the wings (Figure 2.11).

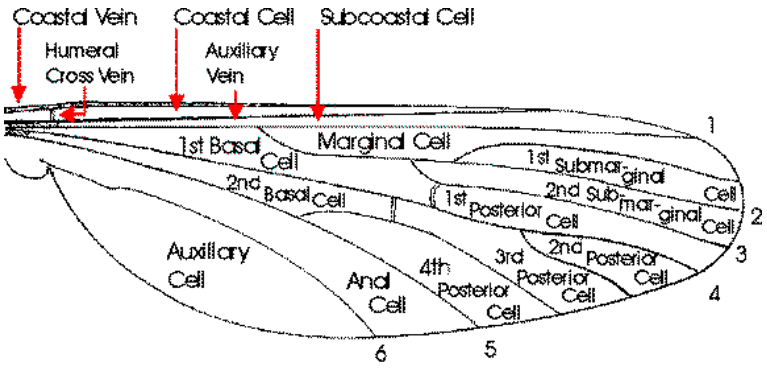


Figure 2.9 Labelled mosquito wing from the point of attachment of the thorax to the wing tip
(http;//pestech.com/wp-content/uploads/2013/03//ad5.gif)

2.12 Spermathecae structure

During copulation, the spermatophores from the male are deposited at the base of the spermathecal duct which emanates from the female bursa copulatrix. After the insemination, blood taken in by the female is used for developing the ovaries and eggs and triggers the release of sperms from the spermathecae (Figures 2.10).

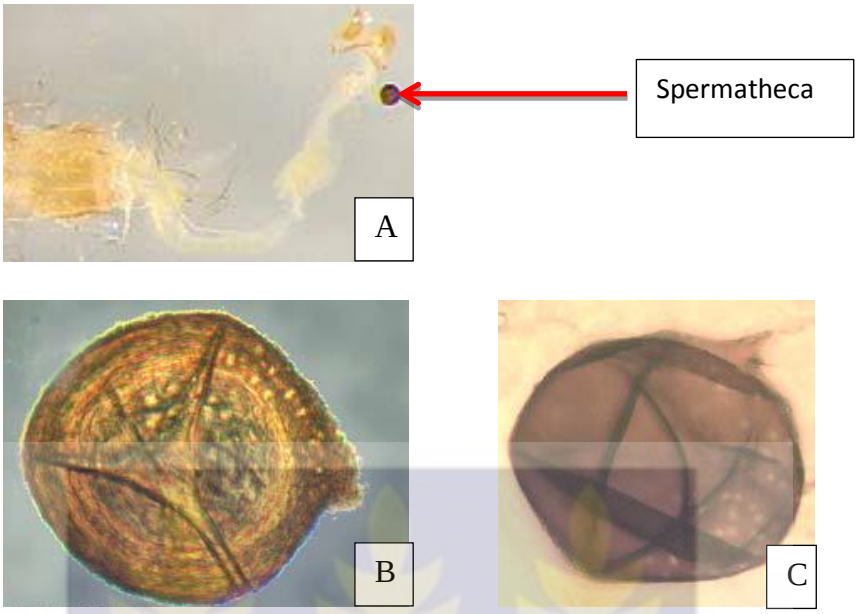


Figure 2.10 Spermathecal structure of *Anopheles gambiae*.

(Benedict, 2007)

A; Spermatheca attached to some internal organs after being removed from the eighth abdominal segment. B; An opaque coloured fully inseminated spermathecae with sperms swimming in concentric rings. C; An uninseminated spermathecal showing transparency.

The spermatozoa swim into the space in the spermathecal reservoir. This mostly occurs in sandflies *Phlebotomus papatasi* Scopoli (Diptera; Psychodidae). A sign typical of full spermathecae is presence of mating plugs. In fully inseminated mosquitoes, the spermathecae are opaque (Figures 2.10 B) and show sperms swimming in concentric rings Whereas uninseminated spermathecae is fairly transparent in nature as shown in Figure 2.10 C (Benedict, 2007; Ilango, 2005). Female mosquitoes often mate once and store sperms in their spermathecae which are located in the ectodermis, between the eighth and ninth abdominal segments. More detailed work has been done on *Aedes* spermathecae than on *Anopheles* species, however they share some similarities. The reason being that, both species have gland secreting cells, spermathecal duct and a reservoir, whose shape is

spherical and resembles more or less a miniature golf ball. Along the walls of the spermathecal reservoir of anophelines are glandular cells whose secretions nourish, keep and ensure viability of stored sperms (Pascini *et al.*, 2013).

2.13 Morphometry of male *Anopheles gambiae* mosquitoes

Morphometrics is a quantitative and statistics based method for taxonomically analyzing body parts of an organism using measurements. Morphometry is a tool which is very essential in assigning species into different taxa and classes (Tibayrenc, 2007). This is because it has the ability to measure phenotypic alterations in same species. The two independent factors which are used in morphometric studies are shape and size (Demirci *et al.*, 2012). The shape of a typical insect wing as illustrated in (Figure 2.11- B), uses cutting edge geometric equipment and scanning technology to translate key landmarks on the insect wings to form an image of the insect wing (Demirci *et al.*, 2012 ; Lorenz *et al.*, 2012). Figure (2.11 – A) on the other hand is an example classical morphometry, which makes use of wing length and width to determine the size of the insect wing.

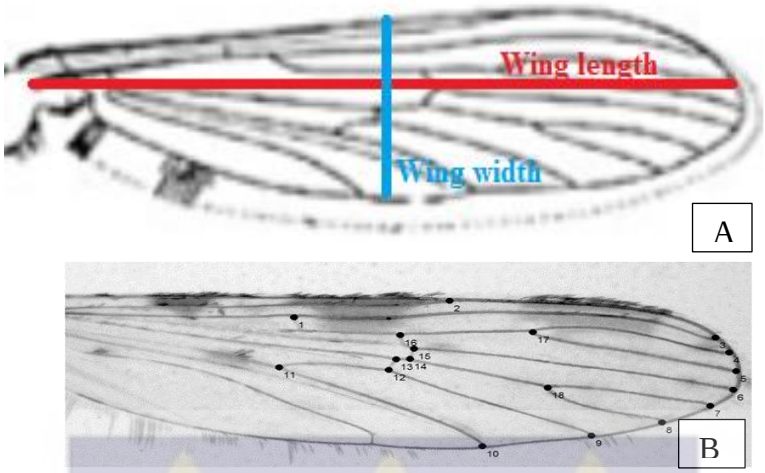


Figure 2.11 Morphometric measurement of the mosquito wing.

Virginio *et al.,* (2015).

Although the two morphometric methods relies on each other, it can successfully be used independently to study hereditary, genetic and evolutionary differences in populations. Also, morphometrics especially geometric morphometrics have been successfully used in studying sexual dimorphism in insects (Tibyrenc, 2011). In classical morphometry (Figure 2.11 - A), wing length is used to quantify the value of body size and dry weight of mosquitoes since they are directly proportional to each other.

Length of *Aedes aegypti* is measured morphometrically from the proximal end of the proboscis to the rear of the abdomen (Figure 2.8 A). Most often in determining the body depth of the mosquito, one might face difficulties since it has a hunch back and wedged thorax. It is often measured dorso-ventrally from tergum to sternum and errors are bound to occur when mosquito samples dry up (Figure 2.8 B). The length of the thorax was also measured from the scutellum to the further most anterior point of the thorax (Yeap *et al.,* 2013). Body size has

being proved to affect longevity. The reason being that, the larger the body size, the higher the possibility of stored food such as glycogen which is used to generate energy (Benitez, 2013). Dujardin (2011) indicated that the size of mosquito is based on assumption, thus not accurate entirely. The longest axis of the wing from the point of attachment to the thorax to the tip of the wing is used as the wing length, which correlates with body size of the mosquito. According to Klingenberg (2002), environmental variations cause changes in the phenotypic makeup of mosquitoes, which might even result in natural selection. This can affect certain developmental pathways which might result in changes in structures found in intraspecific organisms. One possible way of quantifying any of such changes structurally is through morphometrics.

Morphometry is a reliable method to analyze the phenotypic parameters such as wing length, wing width, thoracic width and length, and body length, for both wild and long term colonized mosquito strains since morphometry was successfully used to show intraspecific variation in *Aedes aegypti* in the same locality (Sendaydiego *et al.*, 2013). Morphometric studies proved to be a success when it was used to investigate the effect of local adaptation and genetic divergence in Triatominae (Hemiptera; Reduviidae) and Phlebotominae (Tibayrenc, 2007). Morphometry was again used to identify sexual dimorphism in 10 culicid species (Virginio *et al.*, 2015). These successes were possible because wing morphology, and wing length showed strong correlation with insect weight and size (Sendaydiego *et al.*, 2013). However, morphometric studies on insects can give in accurate results when done on individual or few insect populations

(Tibayrenc, 2007). Sometimes changes could occur in either wing shape or wing length or both. An experiment done on *Aedes aegypti* female mosquitoes reared over 10 generations can show significant difference in wing shape but no significant change in wing length. The advent of image processing tools have taken morphometry to a different pedestal level, since landmarks, outline, texture and patterns can be well analyzed (Dujardin, 2011). Some researchers use centroid size, thorax length but not thorax width to calculate the wing size to thorax ratio and coefficient of variation used to compare variations in the morphometric parameters of each strain. A higher ratio means it has a lower wing load, which means it has higher survival probability (Yeap *et al.*, 2014).

2.14 Iso-female egg production

Iso-female egg production represents the number of eggs produced by one female in one gonotrophic cycle. Recent studies shows that the larger the size of the female mosquito, the more eggs it can lay. A single mated female mosquito which is well fed with blood was put in small vials for single oviposition. Little film of water and egging paper is used as oviposition site. Eggs obtained from iso-female egg production experiments proved that a single female mosquito can lay between 100 – 200 eggs per gonotrophic cycle (Benedict, 2007).

2.15 Swarm formation and sexual behaviour

Male mosquitoes swarm by converging during the dark cycle of the 12 hours light ; dark cycle in the laboratory and dusk and dark in the natural environment (Lees

et al., 2014). Swarm formation is also called mating aggregation. This mating aggregation aerially is executed by males purposely to search for females. Females are attracted to the swarm, and once in, the males meet and mate with females in the air. The mating pairs leave in-copula. Mating is able to last for about 17 seconds. During mating, male *Anopheles gambiae* dope their sperm packages with a steroid hormone known as 20E (20-hydroxyecdysone), a nuptial flight gift found in the mating plug which ignites egg-laying behaviour and increases the eggs produced by female by a percentage between 20 - 25%. This 20E hormone is also responsible for the increase in the number of *Plasmodium* parasites in the female *Anopheles gambiae* and extends the longevity of the *Plasmodium* parasites (Gabrieli *et al.,* 2014; Milius, 2015).

The mating swarm provides a mechanism for reproductive isolation of varying molecular forms of same species, of which there are about five types forming a complex, with molecular forms M and S being very common ones (Castello, 2014). Both forms appear to be in the same swarm, but mating activities of these forms are not yet known thus no hybridization can occur. The M-molecular form of *Anopheles gambiae* is now known as *Anopheles coluzzii* (Sawadogo *et al,* 2013). In the wild, males are found swarming 2 - 3 m up high above bushes and shrubs. During the dusk period, swarming starts 2 minutes before sunset in areas occupied by humans and one minute in unsheltered areas. It takes 5 minutes for a number of males to reach their swarming peak and after about 8 minutes pairing was at its zenith. Swarming occurs more at dusk than dawn, with swarming sound being a major factor of attracting females. The effective swarming makes the

transfer of genes from transgenic insects to wild population or transfer of non-viable sperms into wild mosquito populations. Swarming speed of *Anopheles gambiae* is within 1 - 4ms^{-1} and appears as streaks or often very difficult to see.

CHAPTER 3

3.0 GENERAL MATERIALS AND METHOD(S)

3.1 Study sites

The study was done at REDCO residential area and the mosquito insectary located at the Radiation Entomology and Pest Management Centre (REPMC) under the Biotechnology and Nuclear Agricultural Research Institute (BNARI) of the Ghana Atomic Energy Commission (GAEC), Kwabenya - Accra. Figure 3.1 Map of Ghana Atomic Energy Commission where the laboratory-adapted strains are kept and its original collection point at REDCO.

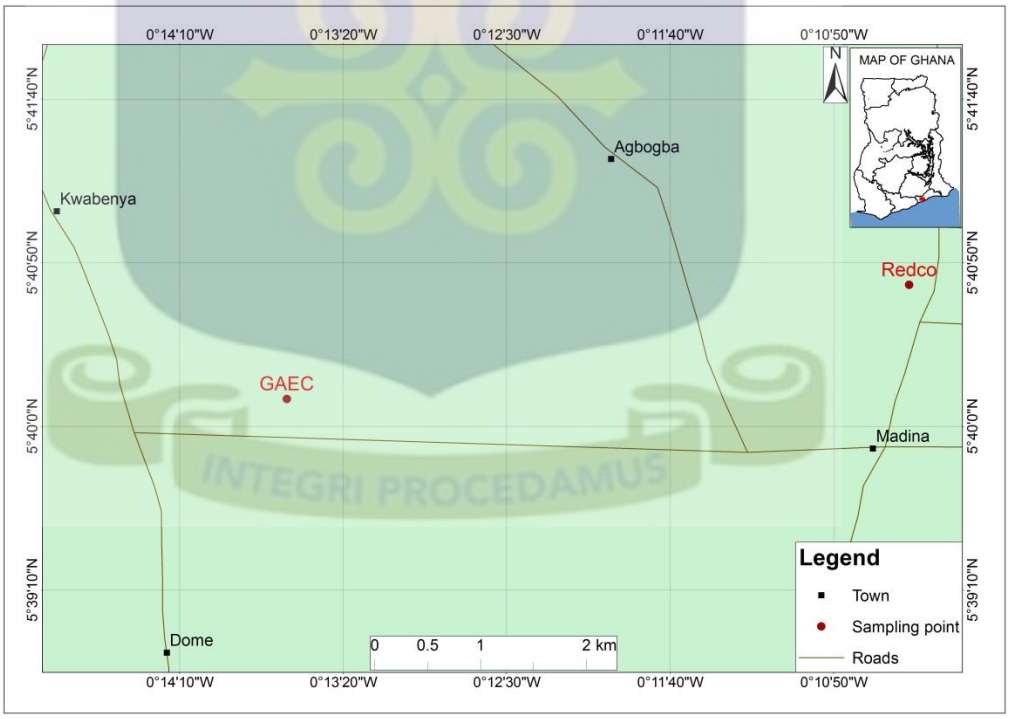


Figure 3.1 Map showing REDCO and GAEC where the immature stages of the wild strain were collected and reared.

The insectary containing the colonized REDCO strain is found in the Dome – Kwabenya constituency while REDCO is in Madina – Abokobi constituency, both in the Ga – East Municipal Assembly at the north of Greater Accra region. The experimental site has coordinates Latitude 5^{o} 32' 60 N and Longitude 0^{o} 13' 0 W, and an altitude of 58 m above sea level. It has a land area of 166 sq km (Figure 3.1). The temperature of the two study sites ranges from 24.7 ^{o}C in August and 28 ^{o}C in March. The study sites have an annual rainfall ranging from 730-1,140 mm. The daylight hours are uniform and the sites have an average relative humidity of 80 %. The study sites are classified as urban and peri-urban with Madina, Dome, Taifa and Haatso being surrounding communities (GSS, 2012). Mechanic industries, service, commerce and small scale agriculture are the four major economic activities in the two sites. Stagnant gutters in these vicinities have provided breeding sites for mosquitoes. The environs of REDCO have pockets swamps and open gutters which serve as breeding sites for *Anopheles* mosquito.

3.2 Experimental set-ups

Each of the experiment was done in three replicates including control set-ups. The experimental design used was completely randomized design, because all experiments were done in the laboratory which has uniform conditions. The laboratory-adapted REDCO strain is over ninetieth filial generation (F 90) generations. The wild strain on the other hand was reared to the F1generation only throughout the whole experiment. Mosquitoes used were all subjected to the same

laboratory conditions. In the genitalia rotation experiment, duration of 1 – 48 hours was used in the laboratory-adapted strain. This helped obtain a baseline data which helped limit the duration of the wild strain experiment to 1 – 24 hours.

3.3 *Anopheles gambiae* colony initiation and maintenance

3.3.1 Source of mosquito and colony management

The laboratory adapted *Anopheles gambiae* strain used in the study were all originating from REDCO, a suburb of Madina near Accra and colonized for over 90 generations over a five year period. The wild strain was obtained from REDCO and reared to the f1 generation before use. The mosquito colony was maintained in the Radiation Entomology and Pest Management Centre (REPMC) under the Biotechnology and Nuclear Agricultural Research Institute (BNARI) of the Ghana Atomic Energy Commission (GAEC) under controlled environmental conditions of temperature 27 ± 2 ^{o}C, relative humidity of 70 ± 5 % and light cycle of 12 hours light ; 12 hours darkness. The light – darkness cycle was mechanically controlled by a 24 hour time switch. Larvae were reared in larval trays with 40 cm x 30 cm x 7 cm (length x breadth x height) dimensions (Figure 3.8).

The 1st instar larvae were fed with 2 % baker's yeast slurry for the first two days after hatching and subsequently fed with aquarium powdered fish meal (Lopis Goldfish ^{TM}, Martin and Martin (Pty) Limited, Kempton Park, South Africa). The originally flaked fish meal were blended into fine powder and passed through a 224 µm mesh during feeding to ensure sparse but even distribution of the fish

meal in the larval tray. The main ingredients of the Lopis Goldfish ™ food was high protein fish meal, wheat flour, lecithin, vitamin C, mono calcium, phosphate, fish oil, soya oil, antioxidant, anti-fungal agents, minerals and vitamin mix. The nutritional composition of Lopis Goldfish ™ food are 30 % protein, 3 % fat, 5 % fibre and 12 % moisture, making it the ideal source of nourishment for rearing mosquitoes in the laboratory. They were reared in clean water medium and fed with powdered fish meal till they pupated. The pupae formed in both wild and laboratory-adapted strains were collected and place in separate cages (Figure 3.2) for emergence. Both adult sexes that emerged were kept for 3 – 5 days in adult holding cages to facilitate mating. On the fifth day, the female mosquitoes were blood fed to ensure ovarian development and egg production. An oviposition cup was put into cages belonging to each strain 24 hours after blood feeding for egg collection (Figure 3.3).

Figure 3.2 Adult cage rearing cages.

Figure 3.3 Eggs of Anopheles gambiae in oviposition cup with some mosquitoes drowned in it.

3.3.2 Laboratory conditions

Laboratory conditions were kept at a constant temperature and relative humidity of 27 ^{o}C and 70 % respectively. This was made possible with the aid of an air conditioner and a humidifier. The humidifier was used especially during the dry season; this is due to the extreme dryness accompanied with the harmattan. Increase in dehydration in the mosquitoes increased mortality but decreased egg production in the mosquito colonies.

Cleanliness in the laboratory was high. Wood powder, dust and dead mosquitoes were swept from shelves and floor each morning. Fungal and bacterial growth on the floor was greatly reduced to the barest minimum by mopping floor of the laboratory each morning with 1 % bleach solution. Every used test tubes, measuring cylinders, Pasteur pipettes, aspirators, vials, egg dishes, incubation bowls, larval trays, larval tray nets for covering larval trays and cages were all washed with detergent, rinsed properly, and bleached in 1 % bleach solution for 20 minutes to decontaminate them, each time after use. Laboratory coats and gloves were washed each time for personal protection and to reduce

contamination. Cob webs were removed occasionally from shelves and the laboratory to avoid spider pest menace. Ants were effectively prevented from climbing mosquito shelves and/or entering the laboratory by smearing wholesome amount of petroleum gel on shelves' stand and vantage entry points into the laboratory, such as the floor of the door post (Onyido *et al.*, 2009).

3.3.3 Feed preparation for 1 st instar larvae

2 % yeast solution for first instar larvae was prepared by measuring 1 g yeast powder into a 50ml measuring cylinder with the aid of a spatula. 20 ml of distilled water was added to it using a wash bottle, it was closed with a cork and shaken thoroughly till a uniform mixture was achieved. More distilled water was added to reach the 50 ml mark. 15 ml of yeast solution (slurry) was pipetted into each larval tray.

3.3.4 Feed preparation for 2^{nd} – 4^{th} instar larvae.

Flaked gold fish meal was blended into very fine particles using a blender. The particles were fine enough to pass through ≈1000 – 1400 µm sized sieves. The blended fish meal was emptied into containers having ≈1000 – 1400 µm sized mesh as its covering. This enables food to easily sift through it when shaken, and allow little and even distribution of feed when feeding the larvae. Blended fish meal was kept in cool dry place in the laboratory until needed.

3.3.5 Preparation of 20% glucose solution for adult feed

20 % glucose solution was prepared by measuring 10 g of glucose using an electric balance into a 50 ml beaker. Distilled water from a wash bottle was used

to dilute it till it reached the 50 ml mark. It was gently swirled to allow fast dissolving rate. Cotton wool were rolled into lump-sized balls, put into small vials and soaked with about 15 ml of glucose solution each. This was repeated every two days, when there was change of sugar solution feed.

3.3.6 Blood feeding

Adult female mosquitoes were starved overnight, to make sure they have the urge to feed on blood. Blood feeding was done in the dark phase of the twelve hours cycle. The human arm was gently placed on the netted upper side of the cage as shown in Figure 3.4. The arm was left there quietly, not wavering or shaking it for 20 - 25 minutes, to ensure no disruption during feeding. An enlarged abdomen signaled well-fed female mosquitoes (Figure 3.5). Arms were removed after feeding period; blood stains in cage netting were cleaned. Cotton wool balls soaked with sugar solution were provided, so that mosquitoes get energy to carry out other activities. Blood feeding of each sample was done once to prevent infestation of the malaria parasite to the human host.

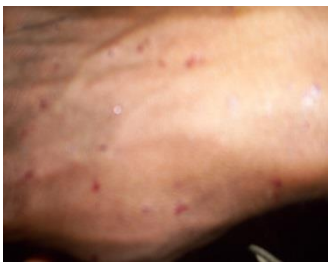


Figure 3.4 Female *Anopheles gambiae* mosquitoes blood feeding and hand showing blood stains.

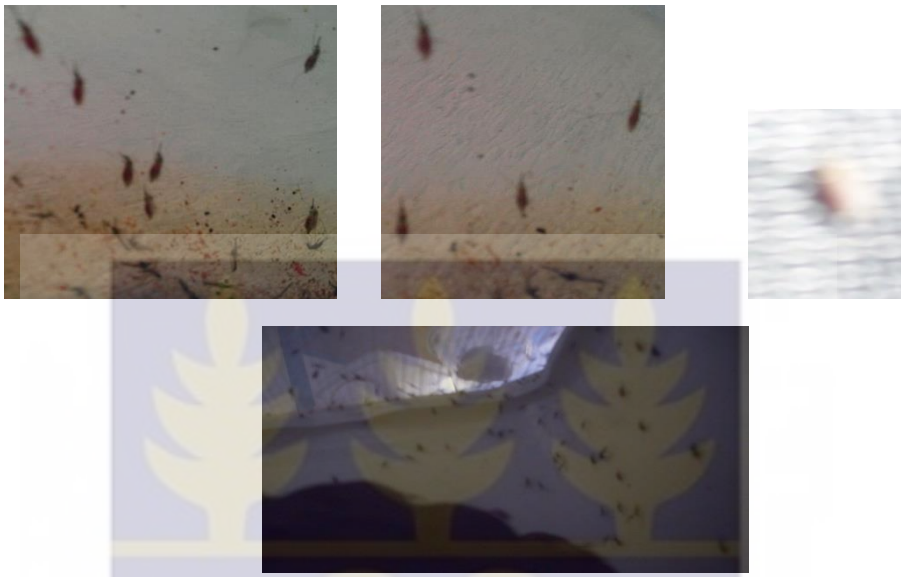


Figure 3.5 Fully engorged adult female *Anopheles gambiae* mosquitoes with red coloured abdomen.

3.3.7 Egg collection

A plastic egg collection cup with 3 cm x 4 cm (diameter x height) lined with a strip of filter paper of 4 cm width and 10 cm length, containing 30 ml of distilled water was put in each rearing cage as oviposition cups 24 hours after blood feeding. The inner lining was to provide a wet medium and prevent eggs from dehydration.

3.3.8 Estimation of number of eggs

Eggs collected from each oviposition cup were viewed, counted, and their number estimated with the aid of a dissecting microscope of 400 x magnification (Figure 3.6).

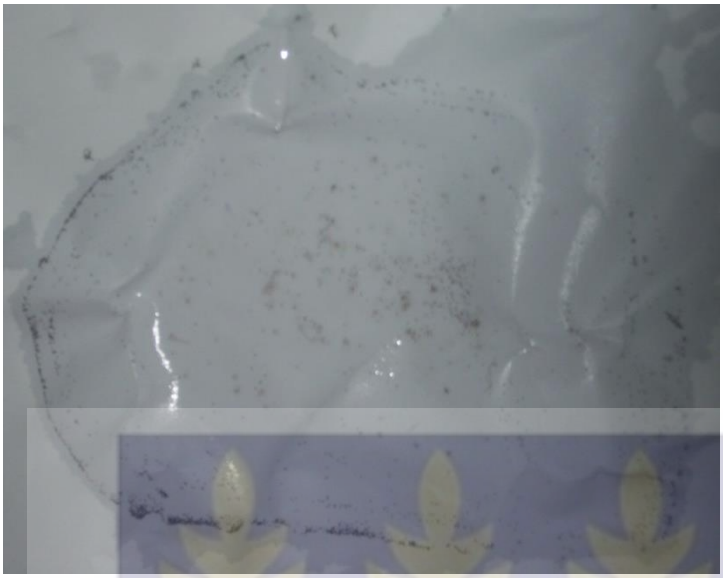


Figure 3.6 Eggs on filter paper about to be counted.

3.3.9 Incubation of eggs

After estimating the number of eggs, the eggs are washed from the filter paper into incubation bowls (Figure 3.7). It is left for a maximum incubation period 48 hours under ambient temperature of 27 ± 2 ^{o}C before they were transferred into the larval trays.

Figures 3.7 Eggs of *Anopheles gambiae* set up in incubation trays for hatching.

3.4 Rearing of *Anopheles gambiae* larvae

Each larval tray of dimensions 40 cm x 30 cm x 7 cm (length x breadth x height) was filled with 1000 ml of distilled water (Figure 3.8) and 10 ml of 2 % concentration of baker's yeast solution (w/v) was poured into each tray to serve as a source of nourishment for the 1st instar larvae for two days. The incubation bowls and oviposition cups were rinsed thoroughly with distilled water to ensure that every larvae and unhatched egg is washed into the tray.

Figure 3.8 Rearing of all larval stages of *Anopheles gambiae* to pupation.

Figure 3.9 Evenly distributed larvae in larval tray to prevent overcrowding.

Trays were well labelled to ensure easy and accurate record keeping and identification. An average of 300 larvae was kept in each tray to ensure even distribution of larvae as in Figure 3.9. The larval tray were covered with white netted clothes to prevent other insects which might be in the insectary from laying their eggs in it, to prevent spillage and also to prevent pupae from flying away in case they emerge in the tray. After the second day of feeding the larvae with slurry yeast solution, the larvae are fed with just a sprinkle of fish meal. They were not fed the next day, but after the fourth day, larvae were fed until they all reached pupation.

3.4.1 Pupal incubation

Pupae were picked using pasteur pipette into incubation cups. Cups containing pupae in 50 ml distilled water were placed in adult rearing cages of dimensions (30 x 30 x 30) cm. They were incubated at ambient conditions and temperature 27 ± 2 ^{o}C and relative humidity 70 ± 5 %. Each cage contained at most 700 pupae. A

5 ml vial containing cotton balls soaked in 10 % glucose solution was put in each cage to provide a source of energy for emerged adults.

3.4.2 Adult emergence

Most of the pupae emerged as adults after 24 hours. Sex separation was immediately done for experiments that involved virgin male and female mosquitoes by aspirating the sexes into different containments by observing the antennae and tip of abdomen, to eliminate the chances of mating among freshly emerged adult mosquitoes.

3.5 Raising mosquito colonies

3.5.1 Rearing laboratory colonies

The 98th (F 98) generation of *Anopheles gambiae* already being reared in the laboratory where starved by denying them of 20 % glucose solution overnight. They were then blood-fed using hand feeding method. Fully engorged females *Anopheles gambiae* were ensured by observing the sizes of their abdomens. After blood feeding, they were given 15 ml of 20 % glucose solution soaked in cotton wool. After 24 hours, filter paper lined cups containing thin film (15 ml) of distilled water were placed into each cage containing about 700 mosquitoes to serve as oviposition cups. Thin film of water was used since female mosquitoes lay their eggs in water. The wet filter paper prevented the eggs from sticking to the cup and dehydrating. Little water was used in the oviposition cups to reduce drowning of mosquitoes. Oviposition cups were collected two days later, and using a pair of feather weight forceps, few drowned mosquitoes were removed

from them, to avoid decay and microbial growth. The oviposition cups containing about 300 egg were emptied into 15 cm x10 cm x5 cm transparent tray, which contained 250 ml each of distilled water for incubation. The incubation bowls were loosely covered with lids to reduce the rate of evaporation. Wash bottle was used to wash eggs stuck to the sides of the incubation bowls to ensure that all the eggs were in contact with water needed for hatching. The emptied egg dishes were reset and two successive eggs were collected. The eggs hatched after 3 - 4 days, and they were transferred into 40 cm*30 cm*10 cm larval trays, each containing an average of 300 larvae. These larval trays were covered with ≈ 1000 – 1400 µm sized nets to serve as a barrier with the outside. The mosquitoes were blood fed multiple times in order to get more progenies for each generation to get adequate samples for replications. The labels on each larval tray bore the name of the species, place of collection, filial generation, date of larval setup, and whether laboratory or wild collection.

First instar larvae in each tray were given 2.5 ml of 2 % slurry yeast solution. They were spread-fed after every two days until they reached the third instar stage, after which they were fed daily till they reached pupation. Care was taken to make sure that the larvae were not over-fed and to ensure that incidence of algal bloom did not occur, neither were they under-fed to reduce death from starvation. Pupae were singly picked using a Pasteur pipette into clean plastic pupae bowls, counted, recorded and put into 30 cm*30 cm*30 cm cages (Figure 3.2). This was done till all the larvae emerged as pupae. Sugar solution was setup in each cage to make food available for emerging adults. Pupal bowls were taken

from cages after emergence to avoid drowning of adults. Adults were blood fed and the whole life process was repeated to obtain successive generations. Prophylaxis was taken by me to reduce the risk of acquiring malaria.

3.5.2 Field collection mosquitoes

Larvae and pupae of *Anopheles gambiae* were scooped from gutters containing clear and less polluted water using short ladles and meter long scoops into plastic bowls. They were sieved to remove large debris. Since field collections contained both larvae and pupae, the pupae were collected using a Pasteur pipette when few, but swirling method was used when they were many (Figures 3.10 and 3.11). In the swirling method, field collections containing both larvae and pupae were poured into a round bottom flask. It was gently swirled till spinning was intense and left to settle. The larvae settled below, leaving the pupae afloat. Pupae were gently poured through a funnel to separate them into another container. They were counted and recorded. A total of 700 pupae were set up into cages, each bearing name of species, date of pupal setup, place of collection, and wild population written on the label. The larvae were also setup in larval trays, which also bore the name of species, date of larval setup, place of collection, and wild population written on the label. Larvae were not fed with yeast slurry this time, but given powdered fish meal till all pupated. Pupae were collected into cages bearing similar labels except date of setup. All the adults were fed with 20 % glucose solution, and the female mosquitoes were blood fed, till eggs were collected. The eggs collected went through the same life cycle as the laboratory reared

mosquitoes to obtain first filial generation. For the wild populations, only members of the first filial generation were used for the experiments.

Figure 3.10 Field collection of immature stages of *Anopheles gambiae*.

Figure 3.11 Sorting out of immature stages from debris.

CHAPTER FOUR

4.0 EFFECT OF PROLONGED COLONIZATION ON THE BIOLOGY OF THE REDCO STRAIN OF *ANOPHELES GAMBIAE . S . S.*

4.1 Background

During the process of colonization, relatively few of the wild adults are captured, and only few are able to reproduce readily. As such, laboratory adapted strains, which are usually the starting point of all release programs such as SIT and genetic control strategies, start off with a limited gene pool. Whiles in colony, the strain may further go through at least one, and possibly several selective sweeps and genetic bottlenecks (Baeshen *et al.,* 2014). These genetic changes associated with colonization have the potential to affect the competitiveness and fitness of a candidate released strain (Benedict *et al.,* 2009; Howel and Knols, 2009; Reisen, 2003). In extreme cases, the genetic changes coupled with behavioural changes resulting from confinement could lead to sexual incompatibility. Comparative biology is an effective tool for determining the extent to which colonization has changed a strain relative to its wild con-specifics.

Comparative biology looks at the functional and evolutionary biology that integrate to give a deeper understanding of the function of an organism's structural make-up. It also shows how the biological and physical environment of the organism interacts with respect to time and ecological conditions. SriKumar and Bhat, (2012) in their study on the comparative biology of the tea mosquito bug (*Helopeltis spp.*) considered oviposition, adult survival rate, longevity, egg

production, developmental time and sex ratio as key parameters. Sexual maturity, morphometry, time to complete life cycle and egg production per female are all classified under comparative biology.

The REDCO strain was colonized in 2008 and has been in colony for about 100 generations. During this period, the population has gone through several bottlenecks, resulting in near homogeneity of homozygotes based on the knockdown resistance gene (kdr) (Osae, unpublished). Inbreeding is thought to negatively affect fitness by increasing the frequency of homozygotes at the expense of hererozygotes (Wright *et al*., 2008). Negative effects can occur either through the accumulation of deleterious recessive alleles leading to unfit homozygotes - the partial dominance hypothesis, or through the loss of favourable heterozygotes - the overdominance hypothesis (Charlesworth and Charlesworth, 1987; Charlesworth and Charlesworth, 1999).

This aspect of the project aims at using comparative biological studies to determine the extent to which prolonged colonization has changed the REDCO strain. It will focus on the morphometric, developmental and reproductive biology as tools to compare any differences between the two strains. The hypothesis is; colonization has not significantly changed the REDCO strain in colony from the wild population.

4.2 Preparation of wild and laboratory-adapted mosquitoes for genitalia observation

Five 30 cm x 30 cm x 30 cm cages were used, each containing 700 mosquitoes. Thirty (30) adult male mosquitoes were mouth aspirated from the adult cages into three separate transparent cups immediately after emergence to ensure their virginity. The transparent cups were labeled from 1 hour to 48 hours, hourly intervals for laboratory reared mosquitoes and 1 hour to 24 hours hourly intervals for wild male mosquitoes. Thirty (30) male mosquitoes were put into each transparent cup, covered with fine nets. The male mosquitoes were left on shelves till their designated hour(s) were reached. They were killed by freezing after their respective ages were reached, which are 1-48 hours (laboratory-adapted strain) and 1-24 hours (wild strain). For the laboratory reared mosquitoes, it was done at hourly intervals till the 48th hour. Each replicated three times. The wild mosquitoes were done from 1 hour to 24 hours. Five crystals of silica gel were wrapped in pieces of filter paper and put into each cup containing the dead specimens, to reduce hydration and to prevent moulding of specimen.

4.2.1 Observation of genitalia rotations

The genitalia of each sample were observed under a binocular microscope at 10 x magnification. The images in Figure 3.20 and the classification in Figure 3.43 were used as reference points for classifying genitalia into the following categories;

Table 4.1 Degrees of genitalia rotation and the directions they face.

Stage	Degree of rotation	Features used either clockwise or anticlockwise direction
0	0	claspers face dorsal with no rotation of segments
1	45^{o}	claspers faced acutely lateral
2	90^{o}	claspers faced perpendicularly lateral
3	135^{o}	claspers faced obtusely ventral
4	180^{o}	claspers faced ventral

Observation was done by either the lateral view, posterior or ventral view due to the posture at which mosquito died (Table 4.1 and Figures 4.1 and 4.2). Photographs of the various genital orientations were taken using a stereoscope, connected to a computer.

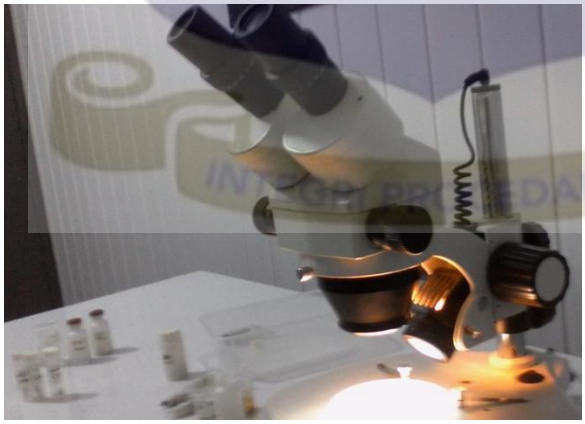


Figure 4.1 Observation of the genitalia rotation of male *Anopheles gambiae* under a dissecting microscope.

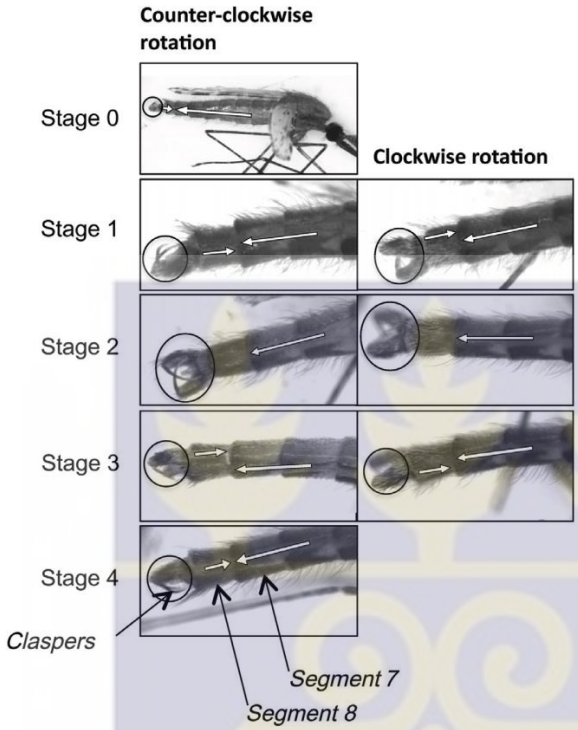


Figure 4.2 Genitalia rotation key of mosquitoes

(Oliva *et al.*, 2011)

4.3 Morphometric studies

Mosquitoes were carefully positioned on a slide, so that the whole length of the mosquito from the tip of the head to the tip of the abdomen is clearly seen and observed under a stereoscope connected to computer running the software of the Lecia stereoscope (LAS EZ 2.0, Leica Application Suite software). Photographs were taken for both the right wing and the body for each male mosquito. From the computer screen, the width of the thorax and the length of the abdomen were

measured by dragging the cursor from end to end, which automatically calibrated the dimensions (Figure 4.3).

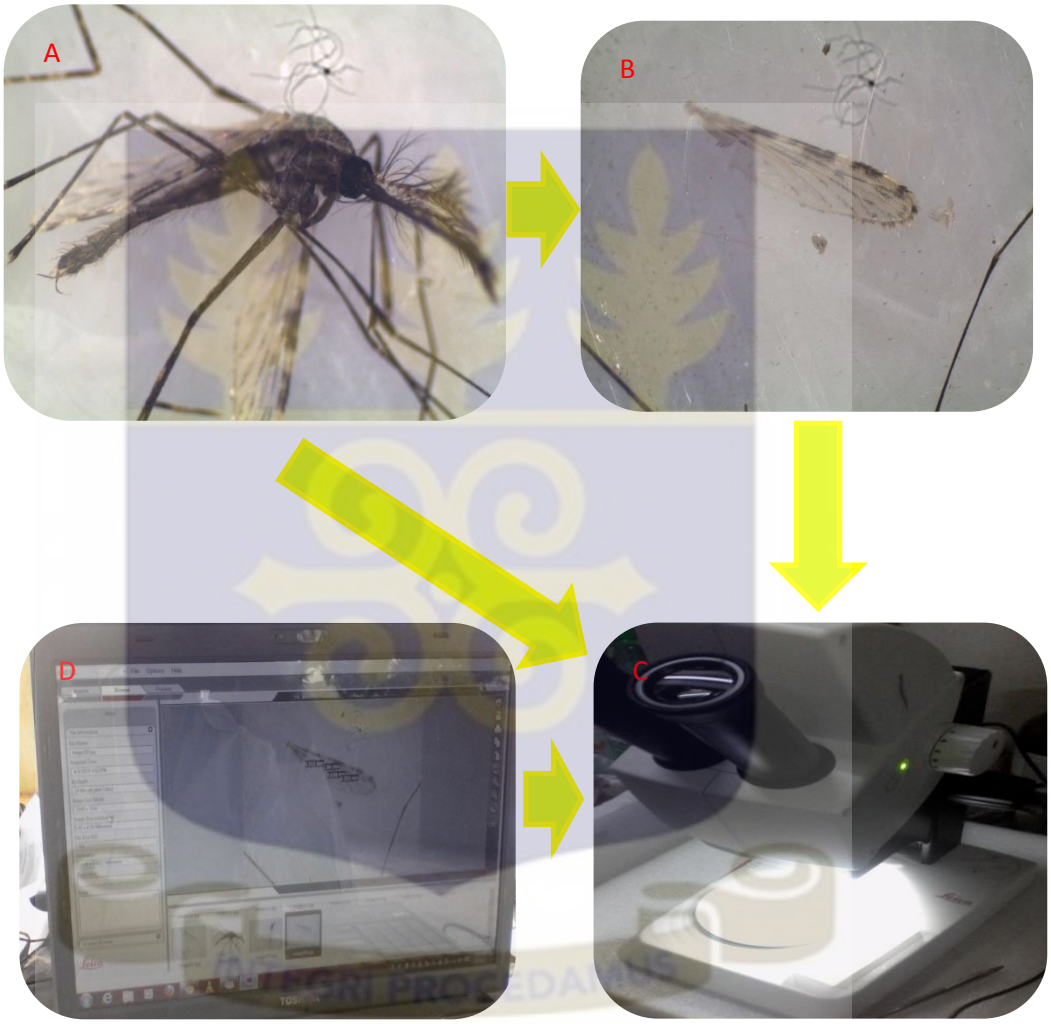


Figure 4.3 Process of wing dissection and morphometric studies.

A; Male mosquito to be mounted B; Right wing dissected C; Analyses of specimen and D; Stereoscope capture

The right wings of all the samples were gently removed from the thorax using feather-light forceps and a dissecting knife, and the wing lengths measured

through the same procedure. Whole wings from wing tip to thorax joint were used. The wing lengths and wing width were used to estimate the size of the mosquito. The body lengths of the mosquitoes were also measured from the proximal end of the head to the distal tip of the abdomen. The wing width and wing length was used to estimate the wing size index and the body size index was also calculated using body length and thoracic width of mosquitoes. One hundred male mosquitoes each from the wild REDCO and laboratory adapted REDCO strains were observed and recorded.

4.4 Duration of life cycle

Twenty life cycle durations were taken from egg to adult stage for the wild mosquitoes and the laboratory mosquitoes. Ten egg incubation cups each for both the laboratory adapted mosquito strain and the wild strain were set up and their respective larval trays also set up after the eggs hatch. The total days it took for completion of the life cycles were recorded in days. The days taken for all eggs to hatch, days taken for 1st instar larvae to reach the pupal stage and from late pupal stage to reach adult stage were recorded.

4.5 Data analysis

The experimental design used was the completely randomized design (CRD) with three replications. The data acquired was analyzed using the Statistical Package for Social Sciences (SPSS) version 16.0. A 'T' - test was done for the morphometric work and the mean separation between the two strains checked for

any significant difference. A p – value of 5% was used as a standard for statistical significance level. The analyzed data were illustrated in figures or graphs. Chi square test was used to check for significance in genitalia rotation and correlation analysis was used to show which morphometric parameters are closely related.

4.6 Results

4.6.1 Developmental biology

Developmental time in the two strains was significantly different (Figure 4.4). It took a longer day (11.05 ± 0.67, p = 0.000) for the wild REDCO strain of *Anopheles gambiae* to develop from egg to pupal stage as compared to the laboratory-adapted *Anopheles gambiae* strain which took a shorter duration of (8.19 ± 0.20, p = 0.000).

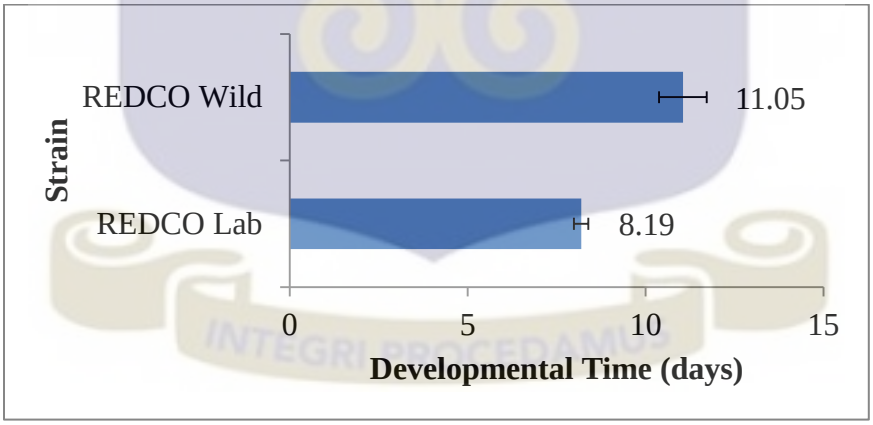


Figure 4.4 Duration of developmental time from egg stage to pupal stage for both the laboratory-adapted REDCO strain and the wild REDCO strain.

4.7.1 Reproductive capacity.

The mean eggs produced per female was significantly higher (p = 0.000) for the laboratory adapted REDCO strain (33.00 ± 1.00) than the wild REDCO strain (17.00 ± 3.00). The percentage egg hatch rates per female for both strains were the same with no significant difference between them (Table 4.2). A percentage of 73.00 ± 5.00, p = 0.000 eggs hatched in the laboratory-adapted strain which was relatively the same as the percentage hatch in the wild REDCO strain (72.00 ± 2.00, p = 0.000).

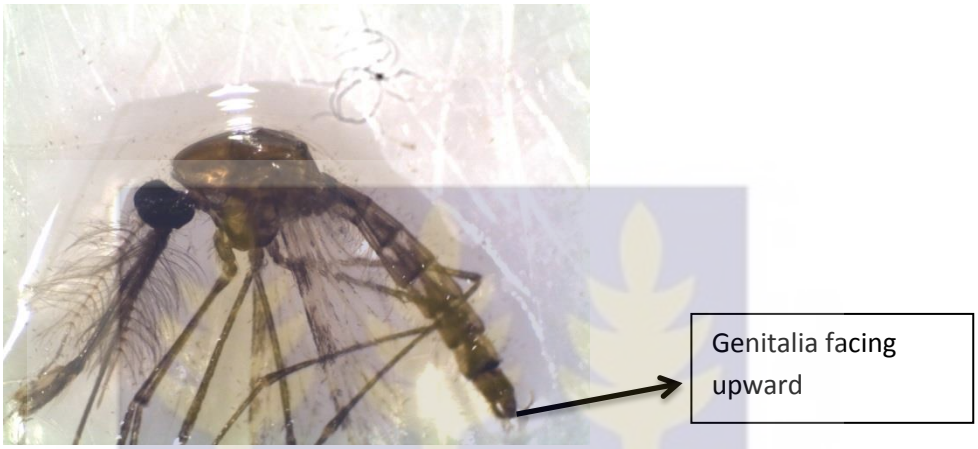
Table 4.2 Iso-female egg productions and hatchability for the two strains.

Strain	% Egg production	% Egg hatchability
Wild	17 ± 3 [a]	72 ± 2 [a]
Laboratory-adapted	33 ± 1 [b]	73 ± 5 [a]

4.7.2 Sexual maturation and reproductive capacity

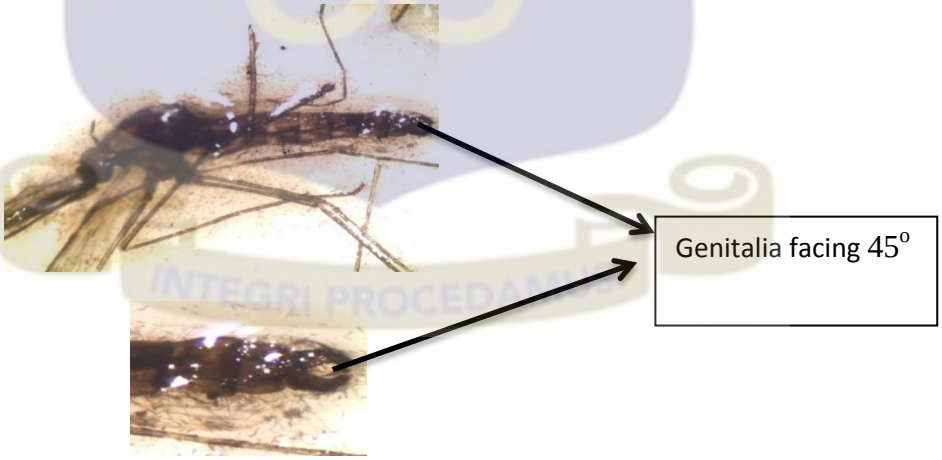
The degree of genitalia rotation with respect to time was used to determine the stage of sexual maturity in male *Anopheles gambiae* mosquitoes. The different stages of sexual maturation based on the degree of genitalia rotation are shown in Figure 4.5 shows no degree of genitalia rotation. Their claspers pointing dorsally upwards show that they are in stage 0 in their genitalia rotation. Figures 4.6 shows stage 1 since the claspers faces 45 ^{o} laterally. The claspers lie at right angle with the rest of the body in stage 2 (Figures 4.7), while in stage 3 (Figures 4.8), the claspers face 135 ^{o} to the rest of the body. Stage 4 (Figures 4.9) which shows

complete sexual maturation of the male mosquitoes has the claspers facing ventrally downwards in 180 ^{o} rotation. Genitalia are rotated in either clockwise or counter clockwise direction.



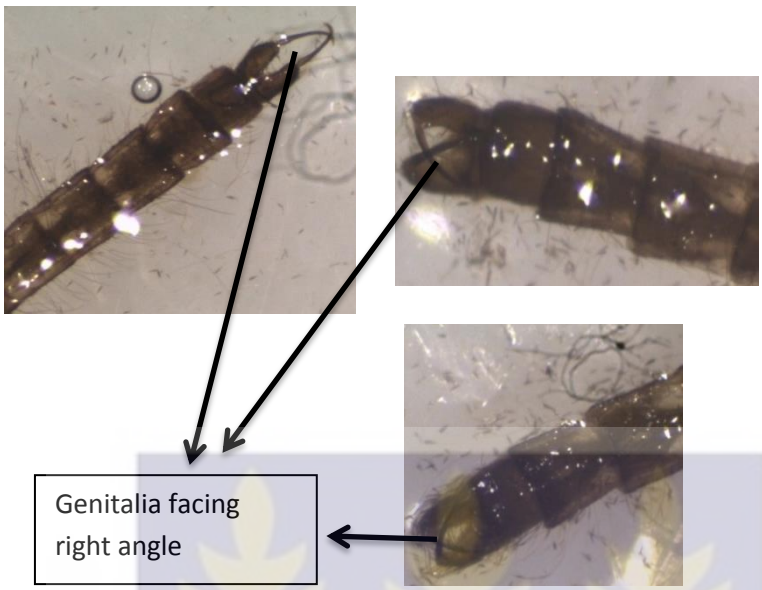
Figures 4.5 Male mosquito with no genitalia rotation (stage 0).

Claspers points dorsally upwards. Their claspers pointing dorsally upwards show that they are in stage 0 in their genitalia rotation.



Figures 4.6 Male mosquito with slight genitalia rotation (stage 1).

Claspers face the lateral direction at 45 ^{o}.

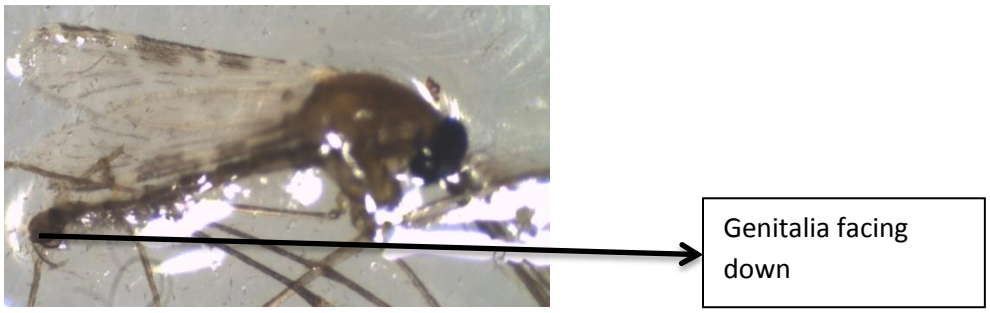


Figures 4.7 Male mosquito with perpendicular angled genitalia rotation (stage 2).

Claspers points at right angle sideways at 90 ^{o}.

Figures 4.8 Male mosquitoes with obtuse angled genitalia rotation (stage 3).

Claspers point widely at 135 ^{o}.



Figures 4.9 Male mosquitoes with full genitalia rotation (stage 4).

Claspers points completely and ventrally downwards at 180 ^{o}.

4.7.3 Morphometrics

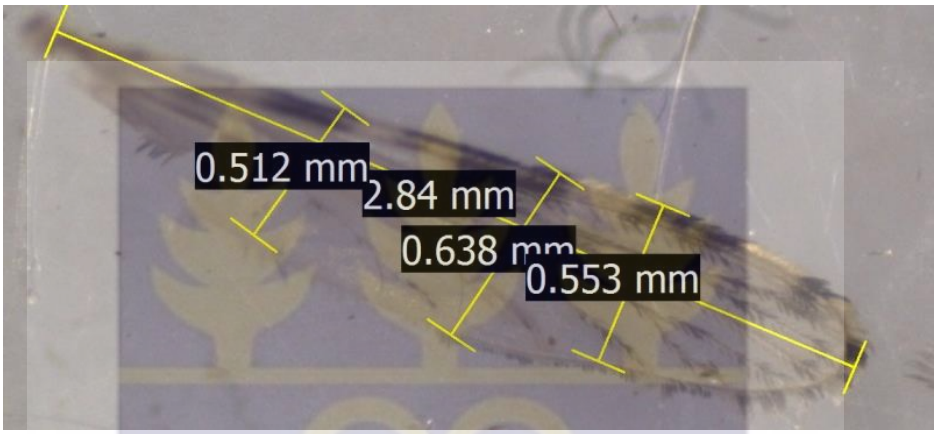


Figure 4.10 Right wing of laboratory-adapted Anopheles gambiae mosquito with morphometric dimensions.

Only a measurement of wing length was taken for each wing. For accuracy the mean of three wing width was used instead of one. Hairs along the fringes of the wing were avoided as much as possible Figure 4.10.

Table 4.3 Male morphometrics of laboratory adapted REDCO and wild REDCO strains of *Anopheles gambiae*.

Morphometric characteristics	Lab REDCO	Wild REDCO	P – value
Body length	4.24 ± 0.05[a]	4.19 ± 0.04[a]	0.462
Thoracic width	1.04 ± 0.02[a]	0.96 ± 0.02[b]	0.002

Wing length	3.07 ± 0.03[a]	2.99 ± 0.03[b]	0.005
Wing width	0.62 ± 0.02[a]	0.58 ± 0.01[a]	0.068
BSI	4.45 ± 0.10[a]	4.08 ± 0.10[b]	0.010
WSI	1.92 ± 0.07[a]	1.73 ± 0.04[b]	0.026

Values in the same column with different small case alphabets as superscripts are significantly different at the 0.05 confidence level based on Student's t-Test.

From Table 4.3 Body length of both the wild REDCO mosquito strain and the laboratory-adapted mosquito strain showed no significant difference. In terms of body length, both strains did not show any significant difference ($p = 0.46$), being 4.11 ± 0.05 and 4.24 ± 0.05 for the wild and laboratory adapted strains respectively. Similarly, wing width did not show any significant difference ($p = 0.068$), however, there was significant differences between the thoracic width ($p = 0.002$) and wing length ($p = 0.005$). Also, the indices calculated with the body measurements showed significant differences (Figure 4.3). Both body size index (BSI) and wing size index (WSI) were significantly higher ($p = 0.010$ and $p = 0.026$ respectively) for the laboratory - adapted REDCO strain than the wild REDCO strain. Correlation analysis shows that body length is the only morphometric character that correlates with all the other characters as well as with the body size index and wing size index (Figure 4.3). All the other characters were inconsistent in their correlation.

Table 4.4 Male morphometrics of directly sampled wild REDCO strain of *Anopheles gambiae.*

Morphometric characteristics	Pure wild REDCO strain
Body length	3.71 ± 0.13
Thoracic width	0.89 ± 0.07
Wing length	2.61 ± 0.13
Wing width	0.60 ± 0.03
BSI	3.32 ± 0.33
WSI	1.58 ± 0.12

The morphometric measurements of *Anopheles gambiae* sampled directly from REDCO to serve as preliminary size comparison with the wild strain reared to the F 1 generation. Non-significance was shown in the morphometric characteristics ($p = 0.000$). On the average, the measured morphometric parameters in Table 4.4 were smaller when compared to the wild REDCO strain (Table 4.3) which was reared to the F1 generation. Thus ruling out that, the small sizes of the wild REDCO strain reared to the F1 generation were due to it not adjusting to the laboratory conditions.

Table 4.5 Correlation of morphometric characteristics between laboratory-adapted and wild REDCO strains of *Anopheles gambiae*

Correlated Pair	Correlation coefficient	P-value	Significant diff.
BL X TW	0.511	0.001	significance
BL X WL	0.456	0.001	significance
BL X WW	0.172	0.015	significance
BL X BSI	0.799	0.001	significance
BL X WSI	0.281	0.001	significance
TW X WL	0.271	0.001	significance
TW X WW	0.034	0.637	NS
TW X BSI	0.921	0.001	significance
TW X WSI	0.101	0.156	NS
WL X WW	0.100	0.157	NS
WL X BSI	0.399	0.001	significance
WL X WSI	0.350	0.001	significance
WW X BSI	0.102	0.149	NS
WW X WSI	0.965	0.001	significance
BSI X WSI	0.199	0.005	significance

BL; Body length, TW; Thoracic width, WL; Wing length, WW; Wing width, BSI; Body size index and WSI; Wing size index. The analysis was done with a significance of $P < 0.05$.

4.7.4 Time for sexual maturity for both strains

According to Figure 4.11 at the age of less than 5 (< 5 hours), 13 % of the laboratory-adapted male *Anopheles gambiae* were in stage 0, 39 % were in stage 1, 42 % were in stage 2 and 7 % were in stage 3 of their genitalia rotation. Below 10 hours, 2 % were in stage 0, 3 % were in stage 1, 21 %, 41 % and 33 % were in stages 2, 3 and 4 respectively. At < 15 hours stage 4 was the highest with a value 72 % male mosquito genitalia rotation. This was followed by stage 3 (25 %) and stage 2 (3 %). Stage 4 (91 %) was the highest at < 20 hours, followed by stage 3 (9 %). At the age of < 25 hours, 97 % were in stage 4, 2 % in stage 2 and stage 3 was only 1 %. All male mosquitoes reached full rotation at the age of < 45 hours.

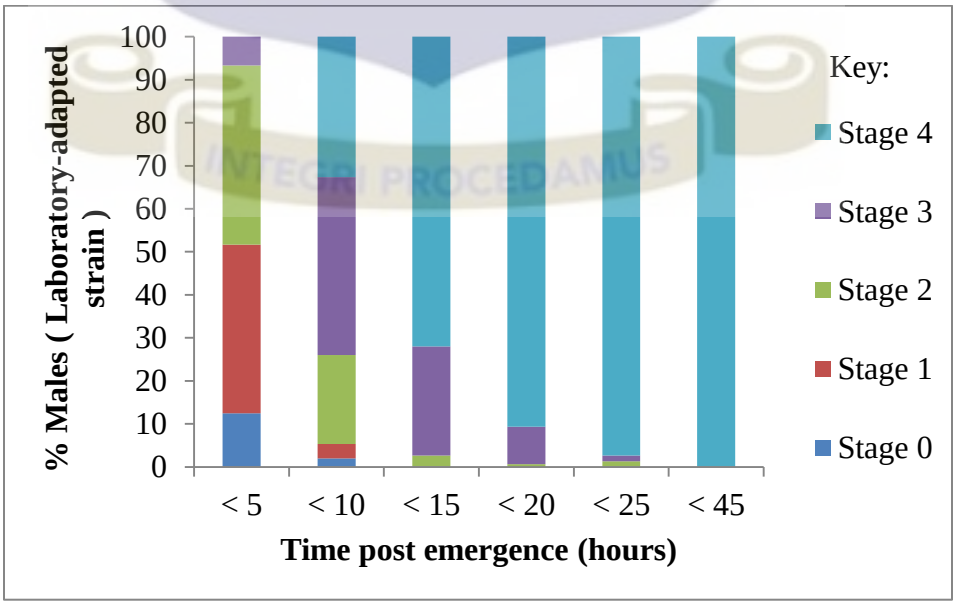


Figure 4.11 Percentage of laboratory-adapted male *Anopheles gambiae* at different stages of genitalia rotation.

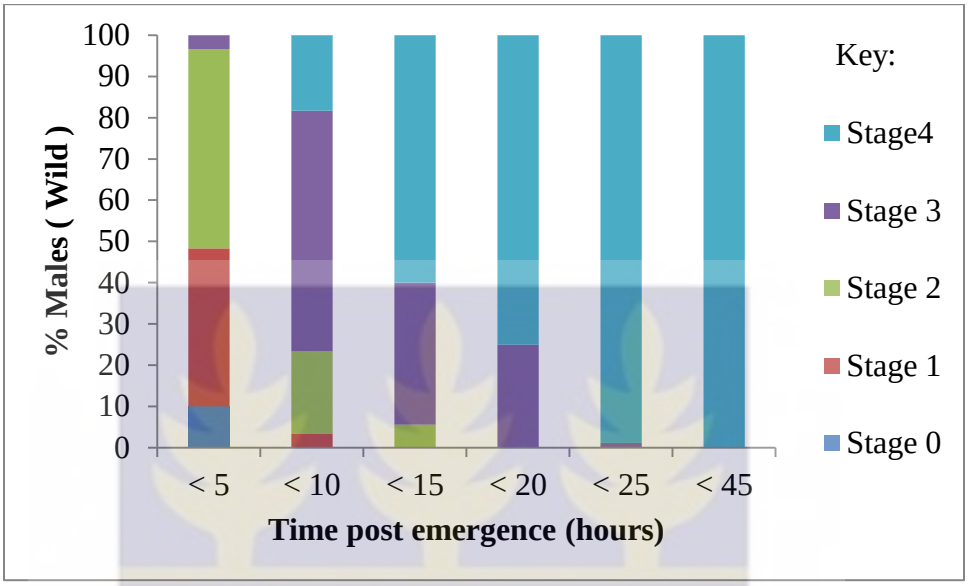


Figure 4.12 Percentage of wild male *Anopheles gambiae* at different stages of genitalia rotation.

At the age of < 5 hours, 10 % of the wild male *Anopheles gambiae* were in stage 0, 38 % were in stage 1, 48 % were in stage 2 and 3 % were in stage 3 of their genitalia rotation. Below 10 hours, stage 0 was absent, 3 % were in stage 1, 20 %, 58 % and 18 % were in stages 2, 3 and 4 respectively. At < 15 hours stage 4 was the highest with a value 60 % male mosquito genitalia rotation. This was followed by stage 3 (34 %) and stage 2 (6 %). Stage 4 (75 %) was the highest at < 20 hours, followed by stage 3 (25 %). At the age of < 25 hours, 99 % were in stage 4 and stage 3 was only 1 %. All male mosquitoes reached full rotation at the age of < 45 hours as shown in Figure 4.12.

Table 4.6 Chi squared test for both the laboratory-adapted strain and wild strain of *Anopheles gambiae* mosquitoes.

Time / hours	x^2	P – value	Significance
< 5	3.399	0.493	NS
< 10	9.351	0.053	NS
< 15	3.766	0.439	NS
< 20	8.964	0.062	NS
< 25	0.349	0.986	NS
< 45	0.000	1.000	NS

Chi square test shows that there was no significant difference ($P > 0.05$) in the extent of genitalia rotation in both the wild and laboratory adapted strains at all ages determined (Table 4.8).

4.8 Discussions

Mosquito size is very essential in mating as it boosts the chances of a male mosquito in search of a female to copulate with. Several factors can affect the size of the mosquitoes such as nutrition, favourable environmental conditions and genetic evolution. Through morphometric studies, the body size of the mosquitoes of both strains were studied by measuring wing lenghs, wing widths, wing size index, body length and thoracic width. *Anopheles gambiae* is known to have wing length ranging from 2.7 – 4.4 mm (White and Kaufman, 2014; Maiga *et al.*, 2012;

Carron, 2007). In other species like *Anopheles quadrimaculatus*, wing length was found to be about 4.5 mm (Glyshaw and Wason, 2013). Wing length of *Culex pipiens pipiens* ranges between 3.45 and 4.05 mm, which was used to represent the body size of mosquitoes (Carron, 2007 ; Ukubuiwe *et al.*, 2013). Data from this study shows that wing length of both the wild and laboratory – adapted mosquitoes fell in the normal range for *Anopheles gambiae* but was different when compared with *Anopheles quadrimaculatus* and *Culex pipiens pipiens* (Carron, 2007 ; Glyshaw and Wason, 2013 ; Ukubuiwe *et al.*, 2013). This gave the assurance that effect of long term colonization of mosquitoes does not adversely affect wing size and thus the mosquitoes used for the work were normal and fit. Positive statistical significance was seen between the wing length of the colonized male *Anopheles gambiae* mosquitoes and the wild male mosquitoes.

The colonized strain had significantly longer wings than the wild strain (Table 4.3). This difference can also mean that the laboratory – adapted mosquitoes were bigger in size than the wild mosquitoes, since wing size is directly proportional to body size (Carron, 2007 ; Ukubuiwe, 2013 ; Santos *et al.*, 2010). Bigger size in the laboratory-adapted mosquitoes gives a higher reproductive advantage than the wild mosquitoes. The reasons being that the bigger the wing size, the better formation of swarms, the faster the swarm speed during swarming, the better swarm tone produced to attract female mosquitoes and quicker flight to escape predation during swarming (Carron, 2007 ; Catora *et al.*, 2010 ; Ukubuiwe, 2013 ; Santos *et al.*, 2012). Similar results were found when Santos *et al.*, (2012) compared transgenic mosquitoes to wild mosquitoes. This could mean that the

bigger size was as a result of colonization but not necessarily genetic engineering of the mosquitoes. The body size index of the mosquitoes showed similar significant difference as the wing length. The laboratory-adapted strain recorded a bigger size than the wild strain. Bigger mosquitoes have enormous amount of food reserves. This mean that colonized mosquitoes will have more energy to mate when used for mass release programs and their survival will be assured since they will be able to live long enough to mate and pass on sterile sperms to wild females, similar to findings of Villareal-Trevino *et al.*, (2015). This is one of the reasons why the laboratory-adapted strain swarmed better and showed numerically higher insemination than the wild strain. If these are reflected in natural environmental conditions, then mass releases of sterilized or genetically modified mosquitoes will have high potential in controlling mosquitoes.

This work concludes that morphometrically, laboratory-adapted male mosquitoes have more advantages when compared to its wild strain, which contradicts findings of (Aksoy, 2008 ; Benedict *et al.*, 2009 ; Braw, 2014 ; Howell and Knols, 2009 ; Scott *et al.*,2002 ; Sharma *et al.*, 2009), who said that laboratory colonized mosquitoes are less fit and smaller in size than their wild counterpart. This results can be justified because preliminary results of wild REDCO strain which were not reared in the laboratory (Table 4.4) were smaller in size. This means that, small size of wild REDCO strain reared to F 1 under laboratory conditions was as a result of adaptation to laboratory conditions.

It has been established that through evolution, female mosquitoes have developed sexual preference to bigger and medium sized mosquitoes, which they expect to

transfer those fitness traits to their offspring (Aksoy,2008 ; Howell and Knols, 2009 ; Segoli *et al.*, 2014 ; Yong, 2008 ; Yeap *et al.*, 2013). This makes colonized male mosquitoes be of higher priority to females, thus giving them a competitive advantage to mate with the wild female mosquitoes (Villarreal-Trevino *et al.*, 2015). This size advantage can be expected in any mass rearing program if good quality control of laboratory nutrition, laboratory conditions and laboratory practices are adhered to.

According to Maiga *et al.*, (2012) bigger and intermediate sized mosquitoes (2.45 – 2.9) mm mate very well, meaning that the laboratory-adapted male mosquitoes which had wing length (3.07 mm) and body size index (4.45 mm^2) both being higher than what was recorded in Maiga *et al.*, (2012) will possibly mate better even in field releases. Greater egg production in virgin females mated by laboratory-adapted males proved this to be possible. On the other hand, wing size cannot be used to estimate body weight (Carron, 2007 ; Koella and Lyimo, 1996 ; Nasci, 1990) so therefore estimation of body size using wing length or wing area should not be confused with body weight. The results of this work show that the morphometry of laboratory-adapted mosquitoes and the wild strain although from the same origin are not the same, but the difference favours the colonized strain to the wild strain. The bigger body size of the colonized strain indicates the endowment of mating potential and competitiveness to wild strain (Villarreal-Trevino *et al.*, 2015). As it was hypothesized that inbreeding existing in the laboratory-adapted strain should reduce vigour, and possibly make weaker traits like dwarfism, stunted growth, and smaller wings dorminant in mosquito

population reared over F 90 generation, this was not the case. Instead, stronger traits such as bigger body size and wing size were exhibited, which translated into fitness and vigour in the colonized strain. The reason why the inbred colonized strain showed such advantageous traits can be due to adaptation and selection or may be due to certain polymorphic gene blocks that have high heterozygosity which have been found to exist in *Anopheles gambiae* (Turissini *et al.,* 2014). This heterozygosity has been responsible for unexpected vigour in the inbred colonized *Anopheles gambiae* strain, thus creating a micro-diversity of the gene pool of the inbred colonized strain which makes colonization of *Anopheles gambiae* mosquitoes very advantageous in mass rearing.

In order to check if there is a strong relationship between the measured attributes used in the morphometric studies, several morphometric parameters were measured in order to skew down to the most appropriate and efficient methods, since other researchers chose to use either wing length, body length, wing size index or body size index (Adeleke *et al.*, 2008 ; Carron, 2007 ; Gleiser *et al.*, 2000 ; Mwangangi *et al.*, 2004 ; Resh, and Cardé 2003; Yeap *et al.*, 2013). From the correlation in shown in Figure (4.15), wing width and thoracic width showed a non-significant correlation, which mean only these cannot be used to ascertain the size of mosquitoes. However body length, wing length, body size index and wing size index showed higher correlation, which means either of them can be used to estimate mosquito size as done by other researchers (Adeleke *et al.*, 2008 ; Carron, 2007 ; Gleiser *et al.*, 2000 ; Mwangangi *et al.*, 2004 ; Resh, and Cardé 2003; Yeap *et al.*, 2013). Body length though correlated may not be the best

choice, since other mosquito parts such as antennae and abdominal parts might be lost as a result of damage. Body size index also might be problematic, time consuming and laborious since it makes use of both body length and thoracic width. Obtaining these body lengths can be difficult, since the state in which the mosquito died and the nutritional state of the mosquitoes when they died can deform the exact width of the thorax and shorten the length of the mosquitoes which might take concave or convoluted shape. Wing length and wing size index therefore are the best choice to estimate the size of mosquitoes. The reason being that the other half pair of wing can provide an option when the other half is damaged. Also the combination of both the width and length of the mosquito wing make it a reliable morphometric method. Also, the versatility of the wing is an advantage because even in wrinkled condition of the wing, a drop of PBS or NaCl or any insect dissecting solution spreads it out uniformly for measurements to be made (Benedict, 2007).

Sexual maturation in male mosquitoes is characterized by a complete 180 ^{o} rotation of their genitalia which consist of terminal end of the male abdomen (Howell and Knols, 2009), and the rate of rotation can be accelerated or retarded based on external conditions and temperature (Oliva *et al.*, 2011). Oliva *et al.*, (2011) indicated that an average of 20 hours recorded complete genitalia rotation which was not the case for this work where there were still male mosquitoes in stage 3 and even stage two at 20 hours. This difference could be due to temperature and possibly, other environmental or rearing conditions (Oliva *et al.*, 2011). The laboratory-adapted strain showed numerically small numbers of about

9 male mosquitoes still in stage 3, than the wild strain which had about 25 % male mosquitoes in stage 3. As some experiments have shown, there was lagging in genitalia rotation in *Aedes spp.* but that was not the case in this study (Oliva *et al.*, 2011). More laboratory-adapted male mosquitoes (33) completed their genitalia rotation in 10 hours while only 18 of the wild male mosquito strain had completed their genitalia rotation in the same period. This was lower than 11 hours male sexual maturity recorded by Oliva *et al.*, (2011). One potential impedance to SIT, and other genetic control programmers such as IIT and HEG (Alphey, 2014), is the possible heterogeneity in the biology of both the long term laboratory reared strain and its wild strain, which includes sexual maturity. Some work of Mediterranean fruit flies, house flies and some insect pests have shown that prolonged colonization alters insect behaviour and shortens sexual maturity (Howell and Knols, 2009 ; Oliva *et al.*, 2011). Oliva *et al.*, (2011) concluded that the speedy genitalia rotation of the colonized strain could be linked to induced selection which is objectionable. The reason being that selection also occurred in this work too because the colonies were over F 90 generations but no such difference in genitalia rotation was found between the strains. This resulted in a synchrony in genitalia rotation of both strains. This means that in field releases, statistically speaking both strains will mature and possibly compete on the same scale, thus for genetic or SIT control to be successful, the ratio of released male mosquitoes to the wild mosquitoes should be at least 5 (mass released male mosquito) ; 1 (wild male mosquito) and the deleterious effect of radiation that might hinder the fitness of sterilized mosquitoes reduced (Helinski *et al.*, 2009 ;

Oliva *et al.*, 2012 ; Mastrangelo and Walder, 2011 ; Rodriguez *et al.*, 2013) and even 10 ; 1 ratio based on a mathematical model of Patinvoh and Susu, (2014). From the correlation test in Tables 4.3 and 4.5, although descriptively and numerically the laboratory-adapted male mosquito strain showed averagely faster genitalia rotation across all the ages, these differences were not statistically significant enough to justify that long term colonization shortens sexual maturation as noted by (Oliva *et al*., 2011).

To measure more accurately the sexual competitiveness and fitness of mosquitoes, iso-female egg production is best. After the virgin female mosquitoes were crossed separately by laboratory-adapted male mosquitoes and female mosquitoes, eggs collected from each individual female mosquito gave an estimation on how sexually fit the strains were. For the iso-female egg production, the eggs obtained from the cross involving the laboratory-adapted male strain recorded the highest of 33 eggs per female while the one involving the wild males had 17 eggs per female. This difference was highly significant Table 4.2. Thus if this conclusion should reflect in real field releases, then it is possible that the mating-favoured laboratory male mosquitoes can inseminate wild female mosquitoes, which will result in all laid eggs carrying sterility gene. Under laboratory experiments, the laboratory-adapted males still showed to exhibit better sexual competitiveness, behaviour and fitness than the wild male mosquitoes which agreed with Villarreal-Trevino *et al.*, (2015).

On the other hand when compared to iso-female egg production experiment done by other researchers, this work showed lower egg production. Other researchers

such as Oliva *et al.*, (2012) recorded an average of 50 eggs, while Hurd *et al.*, (2005) also had 51 – 76 eggs per female and Agyapong *et al.*, (2014) had 64, 140 and 236 eggs per female. This indicates that normal number of eggs per female can exceed 200 and thus the numbers recorded in this was low. This may be due to sperms from both strains being less viable, females not being able to lay more eggs since they were blood fed only once or factors unknown. Also, this wide disparity in the laboratory – adapted strain and the wild mosquito can be linked to the fact that the laboratory- adapted strain adapted to the artificial conditions better than that of the wild strain. However, similar to work done by Vikareal-Trevino *et al.,* (2015), iso-female egg production showed significant increase in the laboratory strain than the wild strain (Table 4.2), therefore it is still showing that the laboratory-adapted male mosquitoes are very competitive, fit and possibly absence of repressive changes in its biology.

Although egg production per female mosquito was significantly higher in the laboratory strain than wild strain (Table 4.2), they had the same hatch rate of 73 % for laboratory strain and 72 % for wild strain (Table 4.2). This hatch rate nonetheless was higher than 40 % hatch rate recorded in Vikareal-Trevino *et al.*, (2015) iso-female egg hatch. However it fell in the 64 – 82 % hatchability recorded by Khan *et al*., (2013) in which about a number of 64 eggs or below are classified as low hatchability. It can therefore be said that hatchability was high in both the eggs produced from the crosses which involved both strains.

Although it took a significantly shorter development time in the laboratory adapted strain (8.19 days) as compared to 11.05 days in the wild strain (Figure

4.4), both fell within the normal duration of *Anopheles* mosquitoes life cycle of 14-27 days (Vikareal-Trevino *et al.*, 2015). This shows that when immature stages of laboratory colonized mosquitoes are released into the field, it will not delay in developing into an adult. Since any delay will make it vulnerable to predation or miss the opportunity to mate with female mosquitoes which might flaw the whole control process.

4.9 Conclusions

The laboratory-adapted male mosquito strain was bigger than the wild strain of *Anopheles gambiae* since it had a significantly longer wing than the wild strain. The laboratory-adapted strain was bigger, while the wild strain was intermediate in size when compared to previous work by other researchers. After correlating the body length, wing length, body size index and wing size index of both strains, it was concluded that wing length and wing length index are the best morphometric methods to estimate the size of mosquitoes, and probably other dipterans, because measurements are simple to do, versatile and efficient. Irrespective of slight numerical variations in the genitalia rotation of both strains, there was no significant difference in the genitalia rotation of both the laboratory-adapted strain and the wild strain, therefore both strains become sexually matured at the same time. The laboratory-adapted strain had more egg production per female than the wild strain, thus can be said to be more fecund at the first gonotrophic cycle than the wild strain. Fertility was the same in both strains after

the first gonotrophic cycle. The developmental time from egg stage to pupal stage was very short in the laboratory-adapted strain than the wild strain.

CHAPTER FIVE

5.0 SEXUAL COMPATIBILITY BETWEEN THE LABORATORY-ADAPTED REDCO STRAIN OF *ANOPHELES GAMBIAE* WITH ITS WILD STRAIN FROM THE SAME ORIGIN.

5.1 Background

Reproductive behaviour is affected by factors such as population density, nutrition and environmental conditions can influence fitness of insects (Takken and Lindsay, 2005). These factors greatly affect the length of sperms of *Anopheles* reared under laboratory conditions for a very long period. On the contrary size of testes was significantly larger in colonized strain than the wild strain. Accessory glands size decreased significantly in the laboratory strain (Baeshen *et al.*, 2014). The biology of mosquitoes also focuses on survival of larvae, adult longevity, flight ability, and mating competitiveness (Lees *et al.*, 2013). Well inseminated mosquito have opaque, dark coloured spherically shaped spermathecae while uninseminated spermathecae look transparent in colour (Benedict, 2007). In sexual compatibility studies, heterosis which is a genetic characteristic of hybrid crosses that show vigor and mating competitiveness, thus showing high swarms and insemination rate (Birchler *et al.*, 2010 ; CIMMYT, 1997). According to Baeshen *et al.*, (2014), long term colonization reduces accessory gland size, shortens sperm tail, and enlarge testes size of male mosquitoes which is possible to affect the sexual compatibility of the laboratory-adapted male mosquito strain. A reduction in the size of the accessory glands can

affect the survival of stored sperms since accessory glands, which are responsible for sperm nourishment might be incapable of secreting enough nutrients to keep all the sperms viable (Pascini *et al.,* 2013). On the other hand, Fox *et al.*, (2002) suggests that large testicular size is an advantageous trait, since the larger the testes the more sex hormones produced and more matured sperms produced. It is now a known fact that 20 – hydroxy – ecdysone steroid hormone filled mating plugs make it possible for a sustained high insemination rate and also high egg production (Fox *et al.*, 2002). If true, then the laboratory-adapted male mosquitoes have an upper sexual competitive advantage over wild male mosquitoes (Akst, 2013 ; Baldini *et al.*, 2013 ; Chapman, 2009 ; Gabrieli *et al.*, 2014 ; Robinson, 2013). Lima *et al.*, (2004) concluded that laboratory-adapted male mosquitoes are more competitive, with higher rates of insemination while wild male mosquitoes had lower insemination rates. On the contrary, Richardson and Williams, (2013) gave a counter report that wild male mosquitoes rather had a higher insemination rate than the long term colonized male mosquitoes .

According to literature, pure laboratory strain crosses laid more eggs while crosses involving wild males and laboratory-adapted female mosquitoes had the least number of egg production (Lima *et al.*, 2004). Egg hatchability experiment which was done for F 40 generation mosquitoes and wild mosquitoes was around 90 % with no statistical significance (Richardson and Williams, 2013). In another experiment conducted by Maciel-de-Freitas *et al.*, (2004) and Clements, (2012) the mean hatch rate ranged from 80 % to 88.9 %.

Swarming behaviour shows fitness of male mosquitoes, since one round of swarming routine can make use of about 50% of energy reserves (Howell and Knols, 2009). Swarming can take 10 minutes, 30 minutes or 60 minutes (Howell and Knols, 2009 ; Vinogradova, 2000). This means that the most competitive males are energetic and are able to mate with more than one female. Some male mosquitoes are denied mating which can be associated to sexual immaturity and lower fitness. Shorter swarming peak period is an evolutionary adaptation in mosquitoes to reduce the rate of predation during swarming (Parrish and Hamner, 1998). Mosquito swarms are influenced by natural conditions (Irby *et al.*, 2012) and often have male mosquitoes moving in polarized pattern (Shishika *et al.*, 2014). Also, when male mosquitoes form large swarms, the probability of finding mates increases. Swarms are driven by inherent internal clock mechanism (Parrish and Hamner, 1998). Some mosquitoes complexes have the ability to form swarms over markers while some do not rely on markers to swarm (Charwood *et al.*, 2002 ; Manoukis *et al.*, 2009 ; WHO, 2007).

5.2 Materials and Methods

5.3 Mating experiment; swarm formation and insemination

About 700 pupae each of wild Recdo strain and laboratory-adapted REDCO strain were put into two cages to emerge into adults. They were sexed into different cages after about 11 hours of emergence to prevent or drastically reduce the probability of mating and ensure the virginity of both sexes.

Four (4) cages were set up and carefully labelled as Cage 1, Cage 2, Cage 3 and Cage 4. Cage 1 contained one hundred (100) wild REDCO male mosquitoes and one hundred (100) laboratory-adapted REDCO female mosquitoes. Cage 2 contained 100 wild REDCO female mosquitoes and 100 laboratory-adapted REDCO male mosquitoes. The Cage 3 and Cage 4 represented the controls. Cage 3 contained 100 females and 100 males of wild REDCO mosquitoes, while Cage 4 contained 100 females and 100 males of laboratory-adapted REDCO mosquitoes. The whole mating experiments were replicated three times. Summary of the mating crosses is given in Table 5.1.

Table 5.1 Mating Experiments

Test	Experimental cages		Control cages	
Cage	Cage 1	Cage 2	Cage 3	Cage 4
Mating crosses	100 WR ♂ and 100 LR ♀	100 WR ♀ and 100 LR ♂	100 WR ♂ and 100 WR ♀	100 LR ♀ and 100 LR ♂

WR = Wild REDCO mosquitoes LR = Laboratory-adapted REDCO mosquitoes

Cotton wool soaked into a 5 ml vial containing 20 % glucose solution was placed into each of the four cages. The glucose solution in each cage was changed after every two days. Mosquitoes in each cage were left together for seven days to ensure higher probability of copulation. Insemination of each female was checked

at the end of the seventh day, using the protocol developed by Benedict (2007). Swarming activities were visually observed with the aid of a touch light and the average number of males taking part in swarm and those resting were counted and recorded for each dark cycle, for the seven days duration. Fifty female mosquitoes were randomly sampled from both the wild and laboratory-adapted cages and the insemination status of each female was checked at the end of the seventh day, using the protocol developed by Benedict (2007). Swarming activities were visually observed with the aid of a touch light and the average number of males taking part in swarm and those resting were counted and recorded for each dark cycle, for the seven days duration as shown in Figure 5.3. Though this method was very difficult and might affect the results minimally, since male mosquitoes response to the light hampered effective counting. The effect was not very adverse since the sample size was small.

5.4 Dissection of spermathecae to determine insemination status

Protocol by Benedict, (2007) in Malaria Research 4 (MR4) for dissecting spermathecae to determine insemination status was used. In order to be sure if the laboratory-adapted mosquitoes reared in the laboratory for over several generations have the potential in effectively transferring sperms into females, it is important to know whether copulated or mated females are inseminated or not. This is done by simply dissecting the spermathecae and determining whether sperms are present or absent. The spermatheca is situated near the terminalia

inside segment VIII and sometimes be seen through the cuticle while viewing the ventral side of the abdomen. It is a spherical, fenestrated dark-brown organ which mimics a golf ball in appearance. The spermatheca surrounded by accessory tissues is gently removed by using one dissecting pin to hold the eighth segment down while using the other pin to gently pull out the terminalia and segment IX.

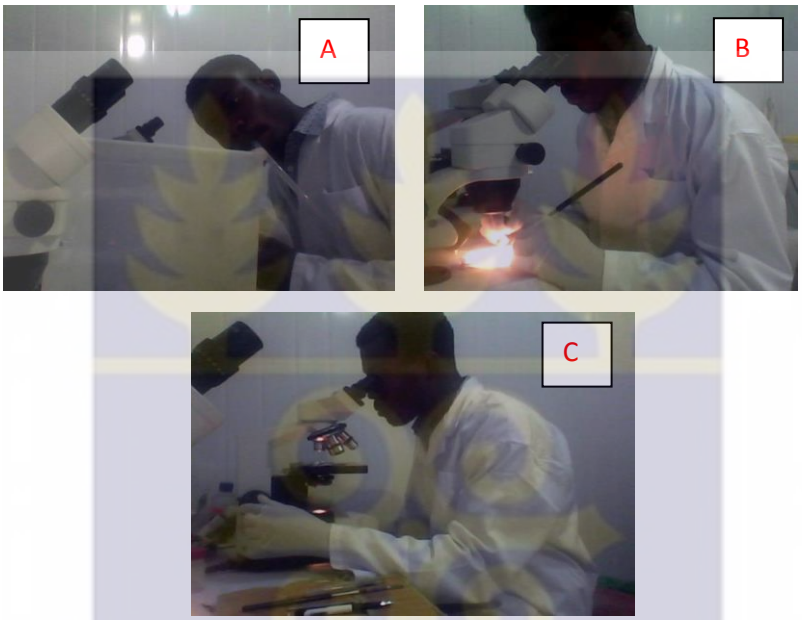


Figure 5.2 Observation of insemination status of female *Anopheles gambiae.*

A; Aspirating and mechanically knocking down of female *Anopheles gambiae* mosquitoes to check for insemination.
B; Dissecting of the spermathecae under a dissecting microscope.
C; Checking for insemination with the help of a compound light microscope.

5.5 Procedure for the dissection of spermatheca

Adult female mosquitoes were made unconscious by mechanically knocking them against cage, so as to observe sperms swim. A drop of 10 % NaCl mosquito saline solution was placed on a clean slide using a pasteur pipette. The thorax of the female mosquitoes were gently grasped with a pair of forceps and placed ventrally

with the abdomen resting in the saline solution under the stereoscope. The terminalia of each female mosquito was gently removed by grasping them and pulling away slowly using the pointed tip of the dissecting pin. The dark spherical spermatheca surrounded by accessory tissues was located within the 8th segment after removing terminalia and the ninth segment. The cadaver of the mosquito and remaining tissues were disposed. A cover slip was gently put on the spermatheca using a needle (to avoid rupturing the spermathecae). On the underside of the slide, a circle was drawn to surround the spermathecae using a permanent marker which made it simpler to locate the tiny organ under the compound light microscope. Under 100 times magnification on a compound microscope, the spermathecae were checked for insemination. Inseminated spermathecae usually have concentric threads and clusters of swimming sperms in it, while the uninseminated females had fairly transparent spermathecae.

5.6 Data Analysis

The experimental design used was the completely randomized design (CRD) and three replications were done for each experiment to ensure drastic reduction of error and bias. The data acquired was analyzed using the Statistical Package for Social Sciences (SPSS) version 16.0. One-way analysis of variance (ANOVA) was used to check for differences in means in the percentage insemination experiment. A Tukey - HSD test was done for mean separation between means that showed significant difference. A p – value of 5% was used as a standard for

statistical significance level. The analyzed data were illustrated in figures or graphs.

5.6 Results

5.6.1 Results of Swarming Experiment.

The laboratory-adapted REDCO *Anopheles gambiae* mosquitoes formed more crowded swarms than the wild REDCO *Anopheles gambiae* mosquitoes, as shown from Figures 4.1 and 4.2. More mosquitoes were captured during swarming in the laboratory-adapted strain than the wild strain.

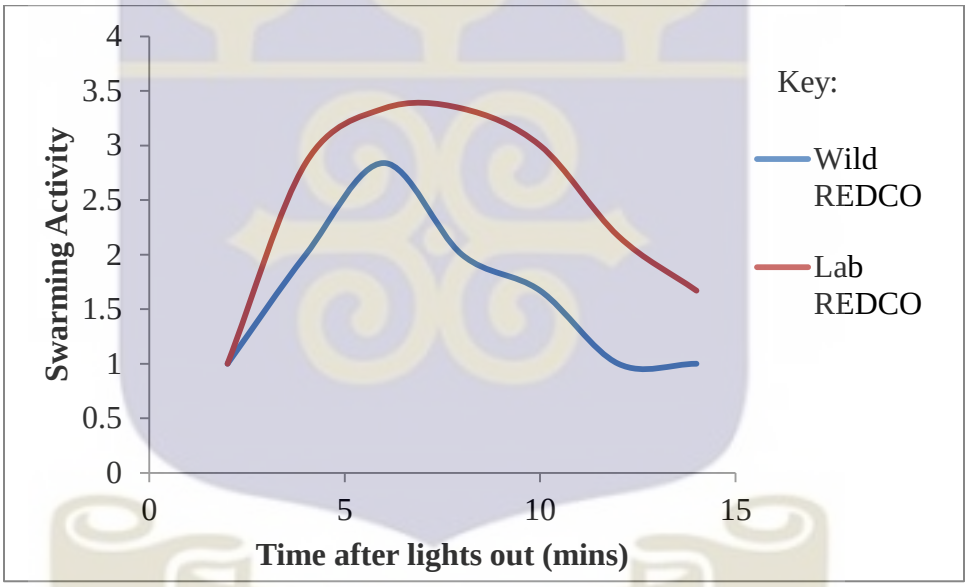


Figure 5.2 Swarming activities of both laboratory and wild strains at dusk.

The data in Figure 5.2 show two different swarm peaks between the laboratory-adapted strain and the wild strain. This difference in peaks is not statistically significant. The laboratory-adapted strain had a mean swarm number of 2.43 ± 0.30 while the wild strain had a mean swarm value of 1.71 ± 0.29, with both having a significant value of p = 0.109 and values of 3.25 and 2.75 respectively.

5.6.2 Mating Experiment and insemination rate

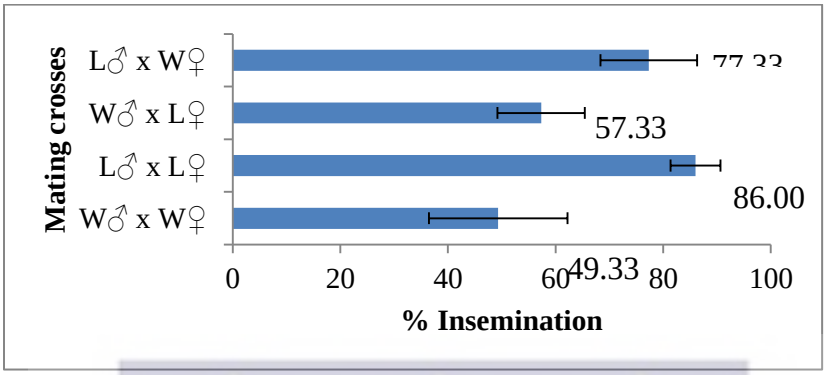


Figure 5.3 Percentage insemination in the mating crosses.

Data on the percentage insemination among the crosses between the two strains is shown in Figure 5.3. Although percentage insemination was not the same among the crosses, there were no significant differences between their values. The percentage insemination of the cross between male laboratory-adapted strain and female wild strain L♂ x W♀ was 77.33 % ± 8.97 %, p = 0.070 and the cross between the wild male strain and female laboratory-adapted strain W♂ x L♀ was 57.33 % ± 8.11 %, p = 0.070. The values of the control experiments which were the cross within the laboratory-adapted strain and wild strain were 86.00 % ± 4.62 %, p = 0.070 and 49.33 % ± 12.88 %, p = 0.070 respectively. The control experiments also had no significant difference within them.

5.7.3 Mating experiment and egg production

Data on the mean number of eggs produced among the crosses between the two strains are shown in Figure 5.5. The number of eggs produced in the cross between laboratory-adapted strain L♂ x L♀ was the highest with the value of

1098.00 ± 119.00, (p = 0.000) which had a high significant difference compared to the cross between the wild strain W♂ x W♀ (594.00 ± 20.00, p = 0.000) and the wild male strain and female laboratory-adapted strain cross, W♂ x L♀ (237.00 ± 40.00, p = 0.000) respectively. On the contrary, male laboratory-adapted strain and female wild strain L♂ x W♀ (848 ± 63.00, p = 0.000) had no significant difference between laboratory-adapted strain L♂ x L♀ and the wild strain W♂ x W♀.

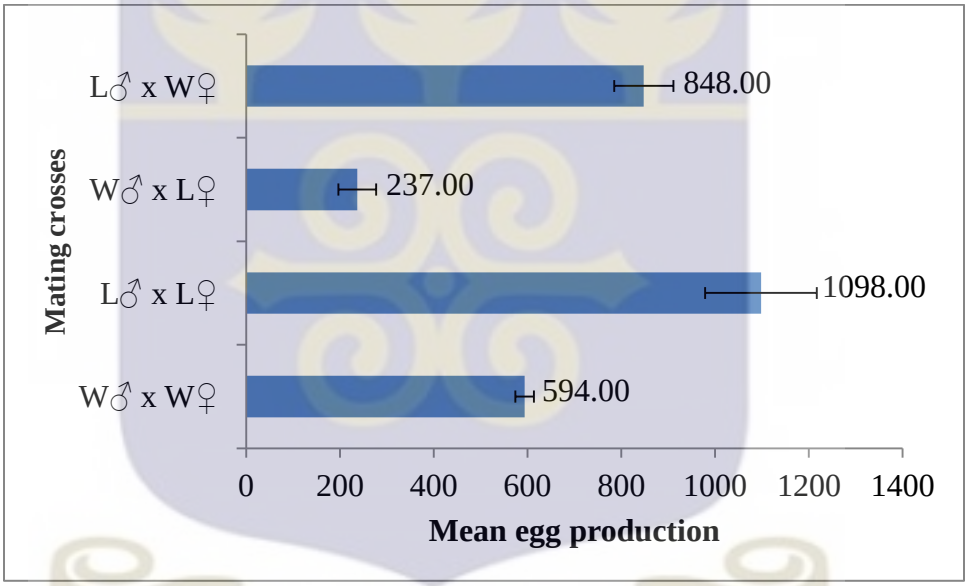


Figure 5.4 Egg production among the mating crosses.

The percentage egg production per 50 females *Anopheles gambiae* in different crosses involving the two strains.

5.6.4 Mating experiment and hatchability

The percentage egg hatch data had no significant difference among the crosses. The percentage egg hatch of the cross between male laboratory-adapted strain and

female wild strain L♂ x W♀ was 90.72 % ± 2.18 %, (p = 0.148) and the cross between the wild male strain and female laboratory-adapted strain W♂ x L♀ was 79.71 % ± 3.13 %, p = 0.148. The values of the control experiments which were the cross within the laboratory-adapted strain and wild strain were 9 0.72 % ± 2.18 %, p = 0.148 and 80.38 % ± 5.15 %, p = 0.158 respectively. The control experiments also had no significant difference within them.

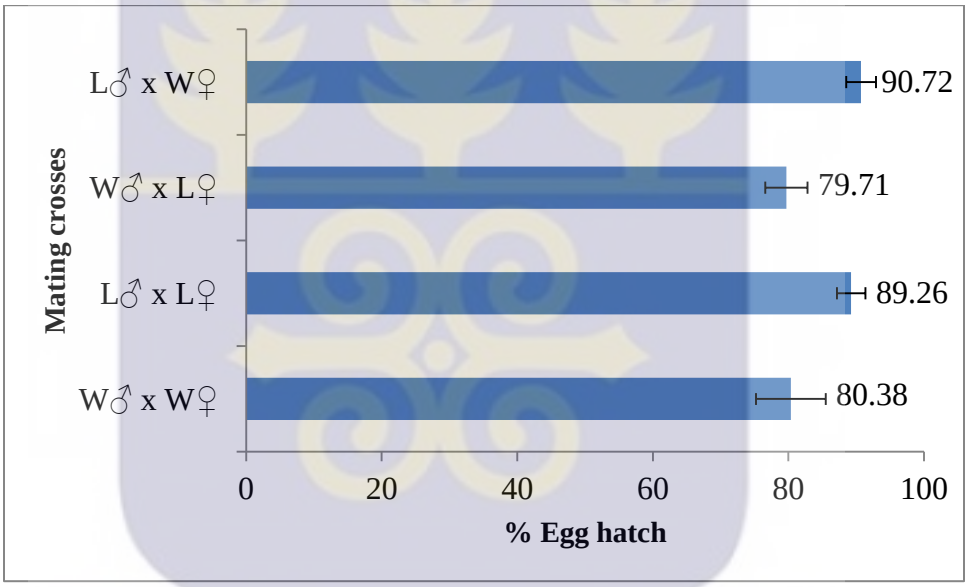


Figure 5.5 Percentage egg hatch among the eggs produced from the mating crosses.

5.7 Discussions

In this chapter, studies focused on the laboratory-adapted strain and wild strain of *Anopheles gambiae* male mosquitoes. It showed that the swarming activity of the wild strain increased from 1 to a mean swarm peak value of 3.00 ± 0.30 within 4 to 6 minutes. It sharply declined to a mean 1 ± 0.30 at 14 minutes period. This

result indicates that the first 6 minutes after dusk is when swarming by the wild male mosquito strain became intense. The peak swarming was shorter, taking about 2 minutes. The steep decline in swarming activity can be due to the probability that all wild male mosquito strain had the chance to mate with females or they had a faster depletion of energy to continue mating or probably it is an evolutionary adaptive trait to evade the risk of predation during swarming (Parrish and Hamner, 1998).

The laboratory-adapted male mosquito strain on the other hand had its swarming activity increasing from 1.00 ± 0.30 to a peak of 3.00 ± 0.30 but this was followed by a gentle decline to 2.00 ± 0.30 swarming activity after 14 minutes period. This means that the male laboratory-adapted mosquitoes sustained high swarming for a longer period than the wild strain. This phenomenon could be due to the ability of the colonized strain to feed effectively and store enough food to be used in swarming or a super swarming fitness of the laboratory-adapted male mosquito strain or probably an evolutionary adaption of the laboratory-adapted male mosquito strain, in which swarming trait has been selected over time. Since swarming activity consume about half the energy of male mosquitoes as reported by Howell and Knols, (2009) it is possible that the laboratory-adapted male mosquitoes had more energy reserves than the wild male mosquitoes. The peak swarming activity was broad, stretching from 6 – 10 minutes, thus a gap of 2 minutes more than the wild strain. However, the two strains showed no significant difference in swarming activity within the same time range. This is indicative of the fact that, the ability to form swarms, which is an essential inextricable pre-

mating characteristic, was exhibited similarly by both the laboratory – adapted strain and wild strain. In the laboratory – adapted strain, the first 10 minutes was the period of intense swarming. This period of swarming in this work agrees with the 10 – 30 minutes range recorded in the publication of Howell and Knols, (2009) and 5 minutes to 60 minutes range according to Vinogradova, (2000). This hypothesizes that there is a high probability that mass reared male mosquitoes released into the wild for the purpose of SIT or genetic control, can form swarms effectively. Effective swarms due to the reason that many male mosquitoes forming part of swarms for longer swarming periods. Thus giving equal chance to mass reared strain in forming swarms.

Although swarming between the laboratory – adapted strain and the wild strain were statistically the same, it can alter in real field conditions. The black spot myth of swarming behaviour is due to certain unknown signals employed in swarming under natural conditions. Swarming is seen as a group expression but precisely, it is being executed independently by each male mosquito. Each has a unique flight path and speed, but at the same time overlaps and interacts with each other, since they exhibit non polarized form of swarm motion (Shishika *et al.*, 2014).The interaction can be attributed to male competition since some are deprived the opportunity to mate with females. Males with the best flight speed, maneuverability and swarm tone are often the preferred choice of females. This complex-individual-interaction result in a cluster observed as swarming. One possible counter-success factor on mass releases of long term colonized mosquitoes is unstable environmental conditions. Irby *et al.*, (2012) proposed that

long term alterations in temperature and rainfall pattern present in the natural environment are capable of affecting the swarming behaviour and biology of mosquitoes. If this assertion is true in the real world, then swarming activity of laboratory-adapted male mosquitoes might be significantly lower than the wild strain. From this work it was realized that the wild male mosquito strain possessed an advantageous attribute in that it took a shorter swarm peak time than the laboratory – adapted strain.

Parrish and Hamner, (1998) and Rios and Connelly, (2013) concluded in their works that short but efficient swarming is advantageous in the natural environment. This gives mosquitoes bonus time to escape the cost of predation and possibly to inseminate females quickly and spread their genes to more females. It can therefore be postulated that under natural conditions, many mass reared male mosquitoes will be lost to predators such as dragon flies, bats and birds. This is because the duration of swarming takes a longer time in the laboratory colonized mosquitoes. Nevertheless, this can be averted by inflating the ratio of mass reared male mosquitoes to wild strain such as from 5 ; 1 to 10 ; 1 as done in SIT releases, to achieve effective eradication. (Maiga *et al.*, 2014 ; Parrish and Hamner, 1998). Although the swarm size was not significantly larger between the laboratory – adapted and wild strains, this work shows that the laboratory – adapted strain showed fairly large swarms descriptively than the wild strain. This agrees with Parrish and Hamner, (1998) who said that larger swarm size, shape, height and density increases the chance of mate pairing, thus giving them the chance to mate with female mosquitoes.

The fact that both the laboratory – adapted strain and wild strain had similar swarming peak (Figure 5.2) is evidential of the fact that the internal clock mechanism irrespective of environmental stimuli stays preserved for more than F 90 generations (Parrish and Hamner, 1998). The statistically similar swarms formed by both the laboratory – adapted male mosquito strain and wild male mosquito strain agrees with Charlwood *et al.*, (2002) who found out that swarm shape, height and size are fairly the same. One aspect of the laboratory – adapted strain which can be investigated in the future is its ability to swarm over markers. Although both laboratory – adapted and wild strains of *Anopheles gambiae s.s* inherently do not need markers, sometimes wild *Anopheles gambiae s.s* rarely do swarm over markers such as shrubs, humans, animals and water bodies (Charwood *et al.*, 2002 ; Manoukis *et al.*, 2009). This statistical non significance in swarming between both strains help them to reduce the danger of not getting a mate, avoid re-mating already copulated females and also help them attract females effectively (Manoukis *et al.*, 2009).

Fertilization of mosquitoes can only be possible if insemination of females is a success. The transparency or opacity of the spermathecae show whether a female mosquito was successfully inseminated (Benedict, 2007). In this study, insemination resulting from the cross between the laboratory-adapted male and female strain was highest (Figure 5.3). Thus this might have been possible due to adaptation to the laboratory conditions. However, the results of insemination obtained from the laboratory-adapted male mosquitoes and wild female mosquitoes, and also wild male mosquitoes and laboratory-adapted female

mosquitoes can be linked to heterosis (Birchler *et al*., 2010 ; CIMMYT, 1997). Figure 5.2 and 5.3 also showed that the reason for the non-significant rate of insemination is as a result of the laboratory-adapted male mosquito strain being able to form better swarms in crosses that involved it, such as the cross between L♂ x L♀ (86 %) and L♂ x W♀ (77.33 %) had non-significantly high insemination rate. Statistically, these changes in percentage insemination among the crosses were not significant. It can be deduced that both the laboratory-adapted male mosquito strain and the wild male mosquito strain both have equal ability to inseminate wild female mosquitoes. This makes it possible for mass reared sterile males or genetically engineered males to pass on any desired or altered gene to wild female mosquitoes in order to achieve control purpose (Alphey *et al.*, 2010 ; Benedict *et al.*, 2009 ; Howell and Knols, 2009 ; Muhenga *et al.*, 2011 ; Nolan *et al.*,, 2011 ; Singer, 2011). The same rate of insemination is a clear indication that both strains have not got deviated courtship behaviour and that isolating mosquitoes from their wild strain for several generations, as in the case of the laboratory-adapted strain still has the ability to form hormonal plug ins, which might have probably led to similar rates of insemination (Akst, 2013 ; Baldini *et al.*, 2013 ; Chapman, 2009 ; Gabrieli *et al.*, 2014 ; Ilango, 2005 ; Robinson, 2013).

This work shows that during release of mass reared laboratory-adapted strains, probability of insemination between wild male mosquitoes and laboratory-adapted male mosquitoes will be 1 ; 1 since there were no significant difference between them. This can therefore provide an equal chance for sterile sperms or any

manipulated gene of interest to be transferred from mass reared male mosquitoes into wild females. Also, since insemination rates were fairly the same for crosses involving both laboratory-adapted and wild male mosquito strains, it can be predicted that fertilized black-coloured eggs will result, because uninseminated female mosquitoes will not lay any eggs or might lay whitish eggs, which signify no occurrence of fertilization (Yamamoto *et al.*, 2013). This work also proposes that although laboratory-adapted male mosquitoes have shorter sperm tails as reported by (Baeshen *et al.*, 2014), they are still able to meet the necessary speed as compared with the wild male sperms, therefore having insemination rates which are almost at par (Figure 5.3).

Insemination follows partner pairing and mating in mosquitoes (Howell and Knols, 2009). Insemination in mosquitoes ensures that the spermatheca which is sac-like disc is well filled with sperms, to be used later for fertilization of female eggs. The results from this study show some variation exist in the insemination rate between each of the crosses. Although the differences were not statistically significant, the cross involving the pure wild mosquito strain had a lower insemination rate of 49.33 %, which means less than half of the female mosquitoes were inseminated. This can be attributed to the wild population not adapted to such artificial laboratory conditions or it is possibly a replay of what really happens in the wild. The next to have least insemination rate was the cross between wild males and laboratory-adapted females (57.33 %). Crosses involving laboratory-adapted males had the highest insemination rates of 77.33 % and 86 % respectively. The comparison of these crosses though descriptively infer that

laboratory-adapted male mosquitoes out competed their wild male counterpart in the ability to efficiently inseminate females. This suggests that laboratory-adapted male mosquitoes are not that less competitive even under laboratory conditions as reported by (Lima *et al.*, 2004 ; Richardson and Williams, 2013). It is therefore no coincidence that the rate of insemination and swarming activities reflected similar competitiveness in the laboratory-adapted male mosquito strain. The lack of significant difference in the rate of insemination in all the crossings indicate a possible synchrony of the competitiveness which is bound to happen between laboratory-reared male mosquitoes and the wild male during mass release control programmes. This synchrony will enable the mass reared male mosquitoes compete effectively or even outcompete the wild male mosquitoes if the laboratory strain are released in large numbers to unbalance the ratio in their favour. Hamady *et al.,* (2013) also recorded high rate of insemination in the colonized strain which was also not significant. Another work conducted by Lima *et al.*, (2004) had a significantly higher insemination rate in the laboratory-adapted strain (70 %) as compared to the wild strain (45 %). On the contrary, Richardson and Williams, (2013) rather had higher insemination rate in the wild strain than the laboratory-adapted strain. If this work, which is supported by Hamady *et al.,* (2013) and Lima *et al.,* (2004) are true, then this will significantly accelerate the spread of sterile sperms or any genetically altered gene of control interest (Alphey *et al.,* 2010 ; Benedict *et al.,* 2009 ; Hamady *et al.,* 2013 ; Howell and Knols, 2009 ; Singer, 2011).

Research findings by Baeshen *et al.*, (2014), that states that laboratory conditions and inbreeding affect the competitiveness of laboratory-reared male mosquitoes are not completely justified, unless there are secondary factors such as irradiation of male mosquitoes which might affect the competitiveness of the laboratory-reared male mosquitoes. This is because irradiation is able to reduce the competitiveness of male mosquitoes by half (Maiga *et al.*, 2014). Success in El Salvador mosquito control programme using SIT refutes the possibility that effective dose used in irradiation can affect the competitiveness of male mosquitoes significantly; unless probably a wrong radiation dose or over dose was used (Benedict *et al.*, 2009). For insemination to be highly successful, sperms must show qualities like energetic and healthy sperms as a result of nourishment from accessory glands, ability for sperms to swim faster, and the presence of certain peptide compounds in the male mosquito semen called mating plugin which prevents leakage of sperms from spermathecae (Akst, 2013 ; Baldini *et al.*, 2013 ; Chapman, 2009 ; Gabrieli *et al.*, 2014 ; Robinson, 2013). Since insemination from crosses that included laboratory-adapted male mosquitoes were above 50 %, it can be said to have had a successful insemination rate thus it has the qualities stated above. There is a clear indication that smaller accessory glands in laboratory-adapted male mosquitoes as recorded by (Baeshen *et al.*, 2014) do not significantly affect rate of insemination and sperm nourishment.

Contrary to the rate of insemination among the crossings, there was significant difference in egg production as illustrated in Figure 5.4. The eggs produced in cross had significantly the highest number of eggs (1098) when compared with

eggs produced in the cross between W♂ x W♀ (594) and W♂ x L♀ (237). This work shows that the two different sets of crossings that produced the greatest number of eggs both contained the laboratory-adapted male mosquitoes. It can therefore be deduced that female mosquitoes who were mated and inseminate by the laboratory males laid most eggs. It seems that a similar pattern on competitiveness from swarming activity, to rate of insemination down to egg production is favouring crossings involving laboratory-adapted male mosquitoes.

One reason which might have led to the L♂ x L♀ cross having the highest egg production is either the possibility that evolutionary selection of females with high egg laying ability or sexually virile male mosquitoes who are able to mate well have survived throughout several generations and thus might have passed on those mating abilities to their progenies over time. Also, it can be said that the laboratory-adapted mosquito strain is more habituated with the laboratory conditions, particularly; the way they feed on sugar to obtain energy, the way female mosquitoes blood feed and possibly because they are used to the temperature and humidity in the laboratory. There is likelihood that there exist inherent factors due to genetic selection causing the high egg production in the L♂ x L♀ cross than a mere micro-adaptation to laboratory conditions as this is again reflected in the cross between L♂ x W♀. It can be concluded that the laboratory adapted male mosquitoes have acquired special traits over time which might include the production of healthy, energetic and fast swimming sperms. Maybe the enlarged testes associated with mosquitoes reared in the laboratory for long time as quoted by Baeshen *et al.*, (2014) can be linked to large amount of

matured spermatozoa production. There has been a recent discovery of a peptide called 20 – hydroxy – ecdysone hormone (20 E) in male ejaculate which has the ability of totally manipulating female reproductive system. This protein forms a gel-like cork structure that is able to prevent already mated females from remating, helps in storing and sealing of sperms by preventing leakages from spermathecae. It also serves as markers to identify mating status of females (Akst, 2013 ; Baldini *et al.*, 2013 ; Chapman, 2009 ; Gabrieli *et al.*, 2014 ; Robinson, 2013).

The significantly high number of eggs produced by crosses involving laboratory-adapted males as shown in Figure 5.4 can be linked possession of advantageous genes selected by the laboratory-adapted male mosquitoes over a long time. Since individuals with desired traits are favoured in evolution, it is absolute that males unable to produce this hormone or who produced little were eliminated from the population after over F 90 generations of competition in the laboratory, thus ensuring the survival of the fittest male mosquitoes which probably caused more egg production in all the crosses that involved the laboratory-adapted male mosquito strain. The outcome of egg produced from 100 % L♂ x L♀ and W♂ x W♀ crosses can be due to almost complete gene homogeneity in each population since each was either pure wild strain or colonized strain. It can be postulated that each population has fairly homogenous gene pool characteristic of pure strains. Although inbreeding is ascribed with expression of weak, repressive traits in a population due to insufficient gene diversity and gene mixture it can be academically guessed that egg production, swarming activity and insemination

should be depressed but in this case an elevation in egg production was recorded in both the pure strains. However, the cross between the L♂ x W♀ did not show any significant difference in egg production when compared to eggs produced in both L♂ x L♀ and W♂ x W♀ crosses. This positive correlation can be due to the phenomenon known as heterosis. This is because wild-colony-hybrid vigor resurfaces after crossing two populations with uniquely different gene pools (Birchler *et al*., 2010 ; CIMMYT, 1997). That was the reason why egg production in the L♂ x W♀ cross was averagely the same as that of L♂ x L♀ and W♂ x W♀. On the contrary, eggs produced in W♂ x L♀ cross showed negative heterosis, since the advantageous trait of producing more eggs was suppressed. This counter heterotic characteristic is a vivid indication that the wild male mosquitoes exhibited lower vigor, fitness and competitiveness as compared to the laboratory-adapted male mosquito strain. According to Hamady *et al.*, (2013) the pure laboratory-adapted strain had more eggs laid than the rest of the other crosses. This finding agrees with the result in this work. Hamady *et al.*, (2013) recorded no significant difference between the pure laboratory-adapted strain (L♂ x L♀) and the next cross (L♂ x W♀) which had more eggs. The cross between the hybrid (W♂ x L♀) had the least egg production, which was the same as the results in Figure 5.4. Quite different from findings in this work, the cross between the pure wild strain (W♂ x W♀) rather was the second leader in egg production, followed by the hybrid cross (L♂ x W♀). From both experiments, it is clear that crosses involving the laboratory-adapted male mosquitoes showed high egg production, signifying sexual competitiveness of the laboratory-adapted strain.

There was no significant difference in percentage egg hatch (Figure 5.5). Hatchability in eggs produced from L♂ x L♀, W♂ x W♀, L♂ x W♀ and W♂ x L♀ crosses were fairly the same. Since the difference in the hatchability is statistically not significant, it can be concluded that rearing mosquitoes in the laboratory for as long as over F 90 generations does not affect the sexual compatibility of the mosquitoes. This proves how viable the sperms of both the wild and laboratory-adapted males can be. Thus, a high standard of quality control must be put in place to ensure that male mosquitoes targeted for mass releases are completely sterile otherwise the purpose of the mosquito control will be futile because sperms from the mass released male mosquitoes will fertilize the eggs of the wild females equally as the wild male, thereby aggravating the vector problem than solving it.

5.8 Conclusion

The results demonstrate that long term colonization of mosquitoes significantly increased egg production in the cross involving pure laboratory – adapted mosquito strain (L♂ x L♀) and hybrid cross (L♂ x W♀).

The results obtained from the study have added more knowledge to the already existing literature on the effects of long term colonization of *Anopheles gambiae* mosquitoes. They could be used to provide information in mass rearing of mosquitoes. The results could also be used to provide a baseline data for quality control of male mosquitoes, mass reared for genetic control and SIT control purposes.

The results confirm that swarming activity, rate of insemination, and egg hatchability are not significantly different in both the laboratory-adapted mosquitoes and the wild mosquito strain.

CHAPTER SIX

6.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 General conclusions

The laboratory-adapted male mosquito strain was bigger than the wild strain of *Anopheles gambiae* since it had a significantly longer wing than the wild strain. The laboratory-adapted strain was bigger, while the wild strain was intermediate in size when compared to previous work by other researchers. Wing length and wing length index are the best morphometric method to estimate the size of mosquitoes, and probably other dipterans, because measurements are simple to do, versatile and efficient. Irrespective of slight numerical variations in the genitalia rotation of both strains, there was no significant difference in the genitalia rotation of both the laboratory-adapted strain and the wild strain, therefore both strains become sexually matured at the same time.

The laboratory-adapted strain had more egg production per female than the wild strain, thus can be said to be more fecund at the first gonotrophic cycle than the wild strain. Fertility was the same in both strains after the first gonotrophic cycle.

The developmental time from egg stage to pupal stage was very short in the laboratory-adapted strain than the wild strain. This means that more *Anopheles gambiae* mosquitoes can be produced in a significantly shorter period based on the assumption that the rearing facility or laboratory maintains high quality

control standards. This can make SIT more possible since more male mosquitoes can be reared, sterilized through irradiation and released on a faster pace.

Long term colonization of mosquitoes significantly increased egg production in the cross involving pure laboratory – adapted mosquito strain (L♂ x L♀) and hybrid cross (L♂ x W♀).

The study have added more knowledge to the already existing literature on the effects of long term colonization of *Anopheles gambiae* mosquitoes. It could be used as a source of information in mass rearing of mosquitoes. It could also be used to provide a baseline data for quality control of male mosquitoes, mass reared for genetic control and SIT control purposes.

Swarming activity, rate of insemination, and egg hatchability are not significantly different in both the laboratory-adapted mosquitoes and the wild mosquito strain.

6.2 General recommendations

Though the comparison of the colonized strain and wild strain were done at the organismal level, there is a need to check for any changes in the biology and behaviour at the genetic level. This can help identify the gene(s) which might have been responsible for the higher insemination and egg production in the colonized strain. This can be isolated and artificially engineered into mass reared male mosquitoes to make them super fit.

Further mating experiments between colonized strain, wild strain, sterilized strain (irradition) and genetically modified strain should be done under complete field

environment, exposed to natural elements and if possible on an island or isolated area, to effectively measure performance of released mosquitoes.

Better swarm formation types, sizes and behaviour should be researched on using high powered infrared 3D videoing, or other higher resolution night-vision cameras.

Swarming of colonized male *Anopheles gambiae s.s* mosquitoes must be compared to both wild *Anopheles gambiae s.s* to see if mass released strains can inter-swarm with wild types.

Since swarming took a short time and occurred during the dark phase, more cutting edge detectable marking system such as gene and protein based markers must be developed. It should probably be able to make real-time tracking of mosquito swarms and dispersal of released mosquitoes possible under both laboratory and field settings.

Further research should be conducted on radiation sterilized male mosquitoes, genetically modified male mosquitoes (none irradiated) and wild male mosquitoes for competitive fittness and mating behaviour.

Further work on morphometric comparison using geometric (land mark) morphometry is recommended, since it requires more advanced features and geographic modellings and able to measure wing size (surface area), wing length and wing shape at a single scanning. Since it requires less human inputs it is more accurate.

Apart from checking for insemination, it is recommended to count sperms stored in each spermathecae.

Speed of sperms during insemination and fertilization need to be calculated. Also markers to help trace path of sperms as well as measure its velocity need to be developed.

Amount of 20 E hormone released by each strain needs to be quantified in the future, and the mystery in its link to egg production unveiled. This can help in artificially boosting the production of this hormone in colonized strains.

Egg production for more than one gonotrophic cycle is recommended to be done. As well as fecundity and fertility studies for colonized strain, irradiated strain and genetically engineered strain.

Development of 3D modeling for the genitalia rotation of male mosquitoes as well as 3D morphometric measurements.

Work on the possibility of recording swarm tone of mosquitoes and using its intensity to relate to effective swarm formation.

Lastly, a global standard for mosquito size, rate of sexual maturity, and effective behaviour of mosquitoes be developed for any mass rearing facilities before sterilized mosquitoes (irradiated) or genetically modified mosquitoes are released. This will ensure fitness, quality control and success of genetic based mosquito control.

REFERENCES

Adeleke, M. A., Mafiana, C. F., Idowu, A. B., Adekunle, M. F. and Sam-Wobo, S. O. (2008). Mosquito larval habitats and public health implications in Abeokuta, Ogun State, Nigeria, *Tanzania Journal of Health Research,* **10 (2) ;** 103 - 107.

Agyapong, J. Chabi, J., Ablorde, A., Kartey, W. D., Osei, J. H. N., De Souza, D. K., Dadzie, S., Boakye, D.A., Ohta, N., Hadi, M. P. and Suzuki, T. (2014). **Oviposition Behavior of *Anopheles gambiae* Mosquitoes. *Journal of* tropical medical health.** 42(4); **187–190. PMCID; PMC4272906** doi; **10.2149/tmh. American Mosquito Control Association. http;//www.mosquito.org/biology**

Aksoy, S. (2008). Transgenesis and the Management of Vector-Borne Disease. *Advances in Experimental Medicine and Biology* **627 (1);** 158-190 pages Springer Science & Business Media Publisher. 0387782257, 9780387782256

Akst, J. (2013). Male mosquitoes trigger egg production ; malaria-transmitting female *Anopheles gambiae* develop eggs upon mating as a result of a steroid hormone injected into them by males. *The Scientist*

http;//www.the-scientist.com/?articles.view/articleNo/38070/title/Male-Mosquitoes-Trigger-Egg-Production/ (Retrieved 12 / 08 / 2015, 8;19 pm)

Alphey, A. (2014). Genetic Control of Mosquitoes. *Annual Review of Entomology* **59 (1) ;** 205–24 doi;10.1146/annurev-ento-011613-162002 ento.annualreviews.org

Amani, H., Yaghoobi-Ershadi, M. R., and Kassiri, H. (2014). The ecology and larval habitats characteristics of anopheline mosquitoes (Diptera; Culicidae) in

Aligudarz County (Luristan province, western Iran) *Asian Pacific Journal of Tropical Biomedicine* 4 **(1);** S233–S241. Tehran, Iran doi; 10.12980/APJTB.4.2014C186 PMCID; PMC4025324

Antonio-Nkondjio, C., Fossog, B. T., Ndo, C., Djantio, B. M.**,** Togouet**, S. Z.,** Awono-Ambene, P., Costantini**, C.,** Wondji, C. S. **and** Ranson, H. **(2011).** *Anopheles gambiae* distribution and insecticide resistance in the cities of Douala and Yaoundé (Cameroon); Influence of urban agriculture and pollution, *Malaria Journal*, **10 (154) ;** 1475 - 2875 doi;10.1186/

Baeshen, R., Ekechukwu, N. E., Toure, M., Paton, D., Coulibaly, M., Traoré, S. F., et al. (2014). Differential effects of inbreeding and selection on male reproductive phenotype associated with the colonization and laboratory maintenance of *Anopheles gambiae*. *Malaria Journal*, **13(19) ;** 1-3.

Baldini, F. Gabrieli, P. South, A. Valim, C. Mancini, F. Catteruccia, F. (2013). The Interaction between a sexually transferred steroid. *PLoS Biology* **11 (10) ;** *e1001695*. doi;10.1371/journal.pbio.1001695 PMCID; PMC3812110

Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C. and Kaiser, A. (2010). Mosquitoes and their control *Springer Science and Business Media*, pp 73-74

Benedict .M.Q., (2007). Methods in *Anopheles* Research, USA. Pgs 260-280

Benedict, M. Q., Knols, B. G. J., Bossin, H. C., Howell, P. I., Mialhe, E., Caceres, C. and Robinson, A. S. (2009). Review; Colonisation and mass

rearing; learning from others *Malaria Journal*, **8 (2) ;** 4 doi;10.1186/1475-2875-8-S2-S4 http;//www.malariajournal.com/content/8/S2/S4

Benedict .M.Q., (2010). Methods in *Anopheles* Research. 2nd edition. Atlanta, USA. Pgs170-241

Benítez , H. A., Sanzana, M., Jerez, V., Parra, L. E., Hernández, C. E. and Canales-Aguirre, C. B. (2013). Sexual shape and size dimorphism in carabid beetles of the genus *Ceroglossus*; Is geometric body size similar between sexes due to sex ratio? *Zoological Science*, **30 (4) ;**289-295. Zoological Society of Japan (publisher).DOI;http;//dx.doi.org/10.2108/zsj.30.289URL; http;//www.bioone.org/doi/full/10.2108/zsj.30.289

Birchler, J. A. Yao, H. Chudalayandi, S. Vaiman, D. and Veitia, R. A. (2010). **Heterosis.** *Plant Cell.* 22 (7) ; 2105–2112 **doi; 10.1105/tpc.110.076133 http;//www.ncbi.nlm.nih.gov/pmc/articles/PMC2929104/**

Braw, E. (2014). Genetically engineered mosquitoes could be vital weapon against malaria. *Guardian sustainable business* http;//www.theguardian.com/sustainable-business/genetically-engineered-mosquitos-fight-malaria

Carron, A. (2007). Correlation between wing measurements and dry body weight in male and female *Ochlerotatus (Ochlerotatus) caspius* (Pallas, 1771) (Diptera; Culicidae). *Journal of the European Mosquito Control Association* pgs 4-8. 24 ISSN1460-6127; www.europeanmosquitobulletin.com (Retrieved 20 / 05 / 2015, 12;28 pm)

Catora, L. J., Ng'Habib, K. R., Hoyc, R.R. and Harringtona, L. C. (2010). Sizing up a mate; variation in production and response to acoustic signals

in *Anopheles gambiae. Behavioral Ecology* **21 (5) ;** 1033-1039.doi; 10.1093/beheco/arq087

Castello, L. D. (2014). Tracking mosquito swarms in the lab Rome. Pgs 3 – 4

Centers for Disease Control and Prevention. (2012). ***Anopheles* Mosquitoes** **http;//www.cdc.gov/malaria/about/biology/mosquitoes/ (Retrieved 21 / 04 / 2015, 4;00pm)**

Chapman, T. (2009). Finding the Right Plugin; Mosquitoes Have the Answer. *Plos Biology.* **7 (12) ;** e1000273. doi;10.1371/journal.pbio.1000273 School of Biological Sciences, University of East Anglia, Norwich, Norfolk, United Kingdom.

Charlesworth, B., and Charlesworth, D. (1999). The genetic basis of inbreeding depression. Genet Res, 74;329–340.

Charlesworth, D., and Charlesworth, B. (1987). Inbreeding depression and its evolutionary consequences. Annu Rev Ecol Syst, 18;237–268.

Charlwood, J. D. Pinto, J. Sousa, C. A. Madsen, H. Ferreira, C. and Rosario, V. E. (2002). The swarming and mating behaviour of *Anopheles gambiae s.s,* (Diptera; Culicidae) from São Tomé Island. *Journal of Vector Ecology.* **27 (2) ;** 178-83.

Chinery, W. A. (1995). Impact of rapid urbanization on mosquitoes and their disease transmission potential in Accra and Tema, Ghana. *African Journal of Medicine and Medical Sciences,* **24 (2);** 179 - 188.

CIMMYT, (1997). The genetics and exploitation of heterosis in crops; an international symposium, pg 11-12 Mexico City.

Clements, A. N. (2012). The Biology of Mosquitoes ; Transmission of Viruses and Interactions with Bacteria. *CABI*, **3 (1)** ; 459

Coluzzi, M. (1999). The clay feet of malaria giant and its African roots; Hypotheses and inferences about origin, spread and control of *Plasmodium. Parassitologia*, **41 (1)** ; 277 -283.

CRA-IAEA, (2013). Biology of Male Mosquitoes in Relation to Genetic Control Programmes; Coordinated Research Activities Annual Report and Statistics. 8 pp, CRPG3.40.02 http;//cra.iaea.org/crp/project/ProjectDetail?projectId=1370&lastActionName=AllClosedCRPList (Retrieved 30 / 04 / 2015, 12;20am)

Cross, C. (2004). *Welcome Trust;* Malaria in the life cycle of *Anopheles* mosquitoes. http;//malaria.wellcome.ac.uk/doc_WTD023872.html. (Retrieved 20 / 05 / 2015, 2;28 pm)

Dahan, Y. L, and Koekemoer, L. L., (2014). Analysis of the genitalia rotation in the male *Anopheles funestus* (Diptera; Culicidae). *Acta Tropica* **13(2);** 20–25

Demirci, B., Lee, Y., Lanzaro, G. C. and Alten, B. (2012). Altitudinal genetic and morphometric variation among populations of *Culex theileri* Theobald (Diptera; Culicidae) from northeastern Turkey. *Journal of Vector Ecology*, **37 (1) ;** 197–209. doi; 10.1111/j.1948-7134.2012.00217.x

Dujardin, J. (2011). Genetics and Evolution of Infectious Diseases. *Elsevier Inc.* **Chpt 16 ; 474 – 475.** France. DOI; 10.1016 / B978-0-12-384890-1, 00016-9

Dujardin, J.-P. and Slice, D. E. (2007) Contributions of Morphometrics to Medical Entomology, in Encyclopedia of Infectious Diseases; Modern Methodologies (ed M. Tibayrenc), *John Wiley and Sons, Inc*., Hoboken, NJ, USA. doi; 10.1002/9780470114209. ch25

Dyck, V. A., Hendrichs, J. and Robinson, A. S. (2005). The Sterile Insect Technique; Principles and practice in area-wide integrated pest management. *Heidelberg, Germany; Springer*. 787 pp.

European Centre for Disease Prevention and Control. (2014). Technical report; Guidelines for the surveillance of native mosquitoes in Europe. **109 pp** Stockholm; Stockholm, ISBN 978-92-9193-599-4 doi 10.2900/37227 http;//ecdc.europa.eu/en/publications/Publications/surveillance-of%20native-mosquitoes%20-guidelines.pdf

Fox, J. G. Anderson, L. C. Loew, F. M. and Fred W. Quimby, F. W. (2002). A volume in American college of laboratory animal medicine (Second Edition) *Elsevier Inc*. pg 692 ISBN; 978-0-12-263951-7

Gabrieli, .P., Kakani .E.G., Mitchell S.N., Mameli .E., Want .E.J., Anton A.M., Baldini, A. S. F., and Catteruccia .F., (2014). Sexual transfer of the steroid hormone 20E induces the post-mating switch in Anopheles gambiae. *PNAS*, 111(46) pgs 1-2 www.pnas.org/cgi/doi/10.1073/pnas.1410488111

Ghana Ministry of Health, (2008). National malaria control programme annual report. Ministry of Health, Ghana, Accra 72pp. www.ghanahealthservice.org/malaria_control.php. (Retrieved 3 / 04 / 2015, 12;30am)

Ghanahealthservice. (2014). Malaria control., Ministry of Health, Ghana, Accra www.ghanahealthservice.org/malaria_control.php. (Retrieved 09 / 09 / 2014)

Gleiser, R. M., Urrutia, J., and Gorla, D. E. (2000). Body size variation of the floodwater mosquito Aedes albifasciatus in Central Argentina. *Journal of medical and veterinary entomology.* **14 (1) ;** 38 - 43.

Glyshaw, P. and Wason, E. (2013). *Anopheles quadrimaculatus. Animal Diversity Web*. http;//animaldiversity.org/accounts/Anopheles_quadrimaculatus/ (Retrieved 30 / 06 / 2015, 3;38pm)

Guelbeogo, M. W., and Ayala, D. (2013). Advances and Perspectives in the study of the malaria mosquito *Anopheles funestus* Dia .I., *New insights into malaria vectors*. **55 (3) ;** 89 Editor Sylvie Manguin, ISBN 978-953-51-1188-7, DOI;10.5772/55389

Hamady, D., Ruslan, N. B., Ahmad, A. H., Rawi, C. H. M., Ahmad, H., Satho, T., Miake, F., Zuharah, W. F., FuKumitsu, Y., Saad, A. R., Rajasaygar, S., Vargas, R. E. M., Majid, A. H. A., Fadzly, N., Ghani, I. A. and AbuBakar, S. (2013). Colonized *Aedes albopictus* and its sexual performance in the wild; implications for SIT technology and containment.

Parasites and Vectors. **6 (1) ;** 206 PMCID; PMC3726284 doi; 10.1186/1756-3305-6-206

Harbach, R.E. (1994). Review of the classification of the genus *Anopheles* (Diptera; Culicidae) ; the foundation for comparative systematics and phylogenetic research. *Bulletin of Entomological Research,* **84 (1)**; 331-42.

Harbach, R.E. (2004). The classification of genus *Anopheles* (Diptera; Culicidae); a working hypothesis of phylogenetic relationships *Anopheles* genus, *Bulletin of Entomological Research* **94 (1)**; 513-553. Department of Entomology, The Natural History Museum, Cromwell Road, London, SW75BD, UK94, DOI; 10.1079/BER2004321

Harbach, R.E. (2013). The phylogeny and classification of *Anopheles* mosquitoes - *New insights into malaria vectors*., pgs 3 - 55. InTech. Rijeka, Croatia.http;//www.nhm.ac.uk/our-science/departments-and-staff/staff directory/ralphharbach.html#sthash.qIW9q3hd.dpufhttp;//dx.doi.org/10.5772/54695 (Retrieved 10 / 04 /2015)

Helinski, M. E. H., Parker, A. G. and Knols, B. G. J. (2009). Review; Radiation biology of mosquitoes *Malaria Journal*, **8 (2) ;** S6 doi;10.1186/1475-2875-8-S2-S6 http;//www.malariajournal.com/content/8/S2/S6 (Retrieved 22 / 06 / 2015, 03;59pm)

Howell, P. I., and Knols, B. G. J., (2009). Review; Male mating biology *Malaria Journal* **8 (2);** 8 pgs 2-4 http;//www.malariajournal.com/content/8/S2/S8

http;//www.en.citizendium.org/wiki/Plasmodium. (Retrieved 31 / 03 / 2015; 5;22am)

Hurd, H. Taylor, P.J. Adams, D. Underhill, A. and Eggleston, P. (2005). Evaluating the costs of mosquito resistance to malaria parasites Evolution. *PMC* **59 (12);** 2560–2572. PMCID; PMC1602058 EMSID; UKMS12587 NIHMSID; NIHMS12587
http;//www.ncbi.nlm.nih.gov/pmc/articles/PMC1602058/

http;//www.cdc.gov/malaria/about/biology/mosquitoes/female_diagram.html (Retrieved 11 / 03 / 2015; 3;22 am)

http;//www.pestech.com/wp-content/uploads/2013/03//ad5.gif (Retrieved 01 / 04 / 2015; 5;10 am)

http;//www.biographix.cz (Retrieved 11 / 03 / 2015; 3;30 am)

http;//www.entnemdept.ufl.edu/creatures/AQUATIC/anopheles_gambiae.htm (Retrieved 11 / 03 / 2015; 3;25 am).

Ilango .K., (2005). Structure and function of the spermathecal complex in the phlebotomine sandfly ***Phlebotomus papatasi*** Scopoli (Diptera; Psychodidae); Post-copulatory histophysiological changes during the gonotrophic cycle ***Journal of Biosciences***, **30 (5) ;** 733-747

Irby, W. Morreale, R. and Bloodworth, P. (2012). Conference Paper ; Comparative biology of disjunct populations of the pitcher plant mosquito

Wyeomyia smithii (Diptera; Culicidae) in the southeastern United States. *Conference; Entomological Society of America Annual Meeting* **(abstract).**

Khaemba, B. M., Mutani, A. and Bett, M. K. (1994). Studies on the *Anopheline* mosquitoes transmitting malaria in a newly developed highland urban area; A case study of Moi University and its environs. *East African Medicine Journal* **71 (1);** 159 - 164

Khan, I. Damiens, D. Soliban, S. M. and Gilles, J. R. L. (2013). Effects of drying eggs and egg storage on hatchability and development of *Anopheles arabiensis. Malaria Journal.* **12 (1) ;** 318. doi; 10.1186/1475-2875-12-318 PMCID; PMC3847695

Killeen, G.F., Fillinger, U., Kiche, I., Gouagna, L.C., and Knols, B.G.J., (2002). Eradication of *Anopheles gambiae* from Brazil; Lessons for malaria control in Africa. *The Lancet Infectious Diseases*, **2 (10)**; 618 – 627, Elsevier doi;10.1016/S1473-3099(02)00397-3.

Klingenberg, C. P. (2002). Morphometrics and the role of the phenotype in studies of the evolution of developmental mechanisms. *Gene* **287 (1) ;** 3–10 Elsevier.

Klinkenberg, E., McCall, **P. J., Wilson**, M. D., **Amerasinghe**, F. P. and **Donnelly**, M. J. (2008). Impact of urban agriculture on malaria vectors in Accra, Ghana *Malaria Journal*, **7 (1) ;** 151 doi;10.1186/1475-2875-7-151 http;//www.malariajournal.com/content/7/1/151

Koella, J. B.C. and Lyimo, H. O. (1996). Short communication; Variability in the relationship between weight and wing length of *Anopheles gambiae* (Diptera ; Culicidae). *Journal of Medical Entomology* **33 (2) ;** 261-264

Kumar, A., Chery. L., Biswas, C., Dubhashi, N., Dutta, P., Kumar, V.D., Kacchap, M., Kakati, S., Khandeparkar, A., Kour, D., Mahajanj, S. N, Maji, A., Majumder, P., Mohanta, J., Mohapatra, P .K., Narayanasamy, K., Roy, K., Shastri, J., Valecha, N., Vikash, R., Wani, R., White, J. and Rathod, P. K. (2012). Malaria in South Asia; Prevalence and control. *Acta Trop.*; **121 (3) ;**10 doi;10.1016/j.actatropica.2012.01.004. NIH-P

Lees .R.S, Knols .B., Bellini .R., Benedict .M.Q., Bheecarry .A., Bossin .H.C., Chadee, D. D., Charlwood .J., Dabiré .R.K, Djogbenou .L, Egyir-Yawson .A., Gato .R., Gouagna .L.C., Hassan .M.M, Khan .S.A, Koekemoer .L.L., Lemperiere .G., Manoukis .N.C., Mozuraitis .R., Pitts . R.J., Simard .F., and Gilles .J.R.L., (2014). Review; Improving our knowledge of male mosquito biology inrelation to genetic control programmes *Acta Tropica* **13 (2);** 2– 11

Lima, J. B. P. Valle, D.and **Peixoto, A. A. (2004).** Adaptation of a South American malaria vector to laboratory colonization suggests faster-male evolution for mating ability *BMC Evolutionary Biology*, **4 (1)** ; 12 doi;10.1186/1471-2148-4-12 http;//www.biomedcentral.com/1471-2148/4/12

Lorenz. C, Marques. T. C, Sallum. M. A. M, Suesdek. L, (2012). Morphometrical diagnosis of the malaria vectors *Anopheles cruzii, An. homunculus* and *An. bellator Parasites and Vectors* **5 (1) ;** 257.

doi; 10.1186/1756-3305-5-257PMC3514230 http;//www.parasitesandvectors.com/content/5/1/257 (Retrieved 05/18/2015 1;47am)

Maciel-de-Freitas, R. Gonçalves, J. M. and Lourenço-de-Oliveira, R. (2004). General biology short communication; Efficiency of rubidium marking in *Aedes albopictus* (diptera; culicidae); preliminary evaluation on persistence of egg labeling, survival, and fecundity of marked female. *Mem Inst Oswaldo Cruz,* **99(8);** 823-827 Rio de Janeiro, http;//dx.doi.org/10.1590/S0074-02762004000800007 http;//www.scielo.br/scielo.php?script=sci_arttext&pid=S007402762004000800007&lng=es&nrm=iso&tlng=es

Maïga, H., Dabiré, R. K., Lehmann,T., Tripet, F. and Diabaté, A. (2012). Variation in energy reserves and role of body size in the mating system of *Anopheles gambiae. Journal of Vector Ecology* **37 (2);** 289-297. http;//onlinelibrary.wiley.com/doi/10.1111/j.1948-7134.2012.00230.x/pdf

Maïga, H., Damiens, D., Niang, A., Sawadago, S. P., Fatherhamen, O., Lees, S. R., Roux, O., Dabiré, R. K., Ouédraogo, G. A., Tripet, F., Diabaté, A. and Gilles, J. R. L. (2014). Mating competitiveness of sterile male *Anopheles coluzzii* in large cages. *Malaria Journal*, **13 (1) ;** 460 doi ; 10.1186/1475-2875-13-460 http;//www.malariajournal.com/content/13/1/460

Malhotra, P. R, Jatav, P. C., and Chauhan, R. S., (2000). Surface morphology of the egg of anopheles stephensi stephensi sensu stricto (diptera, culicidae),

Italian Journal of Zoology, **67 (2) ;**147-151, DOI; 10.1080/11250000009356307 http;//dx.doi.org/10.1080/11250000009356307

Manoukis, N. C. Diabate, A. Abdoulaye, A. Diallo, M. Dao, A. Yaro, A. S. Ribeiro, J. M. C. and Lehmann, T. (2009). Structure and Dynamics of Male Swarms of *Anopheles gambiae. Journal of Medical Entomology*. **46 (2) ;** 227–235. PMCID; PMC2680012 NIHMSID; NIHMS111182 http;//animals.nationalgeographic.com/animals/bugs/mosquito/ (Retrieved 15 / 06 / 2015, 6;00pm)

Mastrangelo, T. and Walder, J. (2011). Use of Radiation and Isotopes in Insects, Radioisotopes. *Applications in Bio-Medical Science*, pg 67-92. Prof. Nirmal Singh (Ed.), InTech ISBN; 978-953-307-748-2, http;//www.intechopen.com/books/radioisotopes-applications-in-bio-medical-science/use-of-radiationand-isotopes-in-insects http;//cdn.intechopen.com/pdfs-wm/23690.pdf

Matthys, B., N'Goran, B. E. K., Koné, M., Koudou, B. G., Vounatsou, P., Cissé, G., Tschannen, A. B., Tanner, M. and Utzinger, J. (2006). Urban agricultural land use and characterization of mosquito larval habitats in a medium-sized town of La Côte d'Ivoire, *Journal of Vector Ecology* **31 (2);** 319 - 333.

Meyer, J. R. (2005). External anatomy; Abdomen. General entomology, ENT 425 Department of Entomology NC State University

http;//www.cals.ncsu.edu/course/ent425/tutorial/abdomen.html (Retrieved 07 / 14 / 2015, 1;00 pm)

Milius .S., (2015). Sexual conflict in mosquitoes may have worsened spread of malaria; Mating issues drove evolution of better disease carriers. *Science News*. https;//www.sciencenews.org/article/sexual-conflict-mosquitoes-may-have-worsened-spread-malaria

Minakawa, N., Githure, J. I., Beier, J. C. and Yan, G. (2001). Anopheline mosquito survival strategies during the dry period in western Kenya. *Journal Medical Entomology* **38 (1) ;** 388 - 392.

Munhenga, G., Brooke, B. D., Chirwa, T. F., Hunt, H. H., Coetzee, M., Govender, D., and **Koekemoer, L. L. (2011).** Evaluating the potential of the sterile insect technique for malaria control; relative fitness and mating compatibility between laboratory colonized and a wild population of *Anopheles arabiensis* from the Kruger National Park, South Africa' *Parasites and Vectors* , **4 (1)**; 208

doi;10.1186/1756-3305-4-208

http;//www.parasitesandvectors.com/content/4/1/208 (Retrieved 18 / 03 / 2015)

Mwangangi, J. M., Mbogo, C.M., Nzovu, J.G., Kabiru, E.W., Mwambi, H., Githure, J. I., Beier, J. C. (2004). Relationships between body size of *Anopheles* mosquitoes and *Plasmodium falciparum* sporozoite rates along the Kenya coast. *Journal of American Mosquito Control Association.* **20 (4);**390-4.

Nasci, R. S. (1990). Relationship of Wing Length to Adult Dry Weight in Several Mosquito Species (Diptera; Culicidae). *Journal of medical entomology* **27 (4) ;** 716-719 DOI; http;//dx.doi.org/10.1093/jmedent/27.4.716 PMID;2388250 http;//www.ncbi.nlm.nih.gov/pubmed/2388250

New Zealand BioSecure Entomology Laboratory, *Anopheles* Genus Anopheline mosquitoes. (2007). *A Division of Southern Monitoring Services Limited*. **2 ; 1-5** New Zealand, http;//www.smsl.co.nz/site/southernmonitoring/files/NZB/Anopheles%20species%20-%20profile%20May%2007.pdf

Nolan T., Papathanos, P., Windbichler, N., Magnusson, K., Benton, J., Catteruccia, F., and Crisanti, A., (2011). Developing transgenic *Anopheles* mosquitoes for the Sterile Insect Technique.*Genetica* **139 (1) ;** 33-39

Obala, A. A., Kutima, H. L., Nyamogoba, H. D., Mwangi, A. W., Simiyu, C. J., and Magak, G. N., (2012). Anopheles gambiae Giles (Diptera; Culicidae)and Anopheles arabiensis population densities and infectivity in Kopere village. *Western Kenya Journal Infect Dev Ctries,* **6 (8)**, 637 - 643.

Oliva .C.F., Lemperiere .G., Gilles .J., (2010). Precocious sex in laboratory reared mosquito *Anopheles arabiensis* Patton (Diptera; Culicidae). *Poster_ Eden.* IAEA/FAO www.crvoi.org

Oliva .C.F., Benedict .M.Q., Lempérière .G. and Gilles .J., (2011). Laboratory selection for an accelerated mosquito sexual development rate. *Malaria Journal*, **10 (1) ;** 135 http;//www.malariajournal.com/content/10/1/135

Oliva, C. F., Jacquet, M., Gilles, J., Lemperiere, G., Maquart, P., Quilici, S., Schooneman, F., Vreysen, M. J. B., and Boyer, S. (2012). The Sterile Insect Technique for Controlling Populations of *Aedes albopictus* (Diptera; Culicidae) on Reunion Island; Mating Vigour of Sterilized Males *PLoS One.* **7 (11) ;** e49414. Basil Brooke, Editor PMCID; PMC3504010

doi; 10.1371/journal.pone.0049414

Omlin, F. X., Carlson, J. C. Ogbunugafor, C. B. and Hassanali, A. (2007). *Anopheles gambiae* exploits the tree hole ecosystem in western Kenya; A new urban malaria risk? *The American Journal of Tropical Medicine and Hygiene.* **77 (6) ;** 264 - 269.

Onyido, A., Ezike, V., Ikpeze , O. O., and Nwankwo,, A. C. (2009). A new method of rearing and maintenance of mosquito colony in the laboratory. *African Journal of Bioscience,* **2 (1);** 17.

Parrish, J. K. and Hamner, W. M. (1998). Animal Groups in Three Dimensions; How species aggregate. Pg 100-105 *Cambridge press* USA ISBN; 9780521460248

Pascini, T. V.; Ramalho-Ortigão, Marcelo; and Martins, G. F. (2013). The fine structure of the spermatheca in Anopheles aquasalis (Diptera; Culicidae) *Journal; Annals of the Entomological Society of Americ*a, **106 (6) ;** 857 - 867 http;//hdl.handle.net/2097/17187 http;//dx.doi.org/10.1603/AN13079

Patinvoh, R. J., and Susu, A. A. (2014), Mathematical Modelling of Sterile Insect Technology for Mosquito Control *Advances in Entomology* **2 (4) ;** 13 ID;50418,13 pages 10.4236/ae.2014.24027

Ponlawat, A., and Harrington, L. C. (2009). Factors associated with Male Mating Success of the Dengue Vector Mosquito, *Aedes aegypti*. *American Journal Tropical Medicine Hygiene*, **80 (3)**; 395-400.

Rueda, L., M. (2008). Freshwater animal diversity assessment;Global diversity of mosquitoes (Insecta; Diptera; Culicidae) in freshwater. *Hydrobiologia* **595 (1);** 477–487 Springer DOI 10.1007/s10750-007-9037-x

Reinert, , J. F., Kaiser,, P. E., and Seawright,, J. A. (1997). Analysis of the Anopheles (Anopheles) quadrimaculatus complex of sibling species (Diptera;Culicidae) using morphological, cytological, molecular, genetic, biochemical and ecological techniques in an integrated approach. *Journal of the American Mosquito Control Association* , **13 (1);** 101-103.

Reinert, , J. F., Harbach R.E., and Kitching .I. (2009). Phylogeny and classification of tribe *Aedini* (Diptera; Culicidae). *Zoological Journal of the Linnean Society*. **(157)**; 100 - 794

Reisen, W.K. (2003). Lessons from the past; historical studies by the University of Maryland and the University of California, Berkeley. In Ecological aspects for application of genetically modified mosquitoes. Edited by Takken, W.; Scott, T.W. Dordrecht; Kluwer Academic; 25–32.

Resh, V. H. and Cardé, R. T. (2003). Encyclopedia of Insects *Academic Press*, 130-1266 pages ISBN; 0080546056, 9780080546056

Richardson, A. J., and Williams, C. R. (2013). Inter-population mating success in Australian dengue vector mosquitoes; effects of laboratory colonization and implications for the spread of transgenics. *Journal of Vector Ecology.* **38 (1) ;** 111 - 9. doi; 10.1111/j.1948-7134.2013.12016.x.

Rios .L.M., and Connelly ,C.R., (2007), Common malaria mosquito ***Anopheles quadrimaculatus*** Say (Insecta; Diptera; Culicidae) University of Florida. 419pp

http;//entnemdept.ufl.edu/creatures/aquatic/Anopheles_quadrimaculatus.htm#syn

Rios, L. M., and Connelly, C. R. (2013). Common malaria mosquito *Anopheles quadrimaculatus* Say (Insecta; Diptera; Culicidae) University of Florida EENY-419pp

http;//entnemdept.ufl.edu/creatures/aquatic/*Anopheles_quadrimaculatus*.htm#syn

Robinson, R. (2013). His Hormone, Her Oogenesis; How Male Malaria Mosquitoes Trigger Female Egg Development *PLoS Biol* **11 (10);** e1001694. doi; 10.1371/journal.pbio.1001694 PMCID; PMC3812113

Rodriguez, S. D., Brar, R. K., Drake, L. L., Drumm, H. E., Price, D. P., Hammond, J. I., Urquidi, J. and Hansen, I. A. (2013). The effect of the radio-protective agents ethanol, trimethylglycine, and beer on survival of X-ray-sterilized male *Aedes aegypti Parasites and Vectors.* **6 (1);** 211. doi; 10.1186/1756-3305-6-211 PMCID; PMC3723957

Romi .R., Boccolini .D., Hovanesyan .I., Grigoryan .G, Di Luca M, and Sabatinell G., (2002). *Anopheles sacharovi* (Diptera; Culicidae); A reemerging

malaria vector in the Ararat Valley of Armenia. *Journal of medical Entomology.* **39 (3) ;** 446-50.

Rutledge, R. C., Baker, R. H., Morris, C. D. and Nayar, J. K. (2005). Human malaria. *EDIS*. *http;//edis.ifas.ufl.edu/MG103* (Retrieved, 26 / 07 / 2015).

Santos, M. N., Nogueira, P. M., Dias, F. S., Valle, D. and Moreira, L. A. (2010). Fitness aspects of transgenic *Aedes fluviatilis* mosquitoes expressing a Plasmodium-blocking molecule *Transgenic Research* **19 (6) ;**1129-35. Brazil. DOI;10.1007/s11248-010-9375-8 http;//www.researchgate.net/publication/41429782_Fitness_aspects_of_transgenic_*Aedes_fluviatilis*_mosquitoes_expressing_a_*Plasmodium*-blocking_molecule

Sawadogo, S.P., Costantini. C., Pennetier, C., Diabaté, A., Gibson, G., and Dabiré, K.R., (2013). Differences in timing of mating swarms in sympatric populations of *Anopheles coluzzii* and *Anopheles gambiae s.s.* (formerly *Anopheles gambiae* M and S molecular forms) in Burkina Faso, *West Africa Parasites and Vectors*, **6 (1);** 275 http;//www.parasitesandvectors.com/content/6/1/275

Scott, T. W., Jason L. Rasgon, J. L., Black IV, W. C. and Gould, F. (2002). Fitness studies; developing a consensus methodology. Pg 171-179 DOI; 10.1007/1-4020-3799-6_16 http;//www.researchgate.net/publication/226912257_Fitness_studies_developing_a_consensus_methodology

Segoli, M., Hoffmann, A. A., Lloyd, J. Omodei, G. J. and Scott A. Ritchie, S. A. (2014). The Effect of Virus-Blocking *Wolbachia* on Male Competitiveness of the Dengue Vector Mosquito, *Aedes aegypti*. Mark Quentin Benedict, Editor *PLoS Neglected Tropical Diseases* **8 (12) ;** e3294 PMC4263406

Sehrawat, N., (2014). Research and Reviews; Scanning Electron Microscopic Observations of Eggs of *Anopheles Fluviatilis* mosquito (Diptera; Culicidae). *Journal of Zoological Sciences* mosquito (Diptera; Culicidae). Department of Genetics, M.D. University, Rohtak-124001, Haryana, India. Pgs 1-7

Sendaydiego. J. P, Torres. M. A. J, and Demayo. C.J., (2013). Describing Wing Geometry of *Aedes Aegypti* Using Landmark-Based Geometric Morphometrics. *International Journal of Bioscience, Biochemistry and Bioinformatics*, **3 (4);** 5

Sharma, A., Parasher, H., Singh, O.P., and Adak, T. (2009). Short communication; Species B of *Anopheles culicifacies* (Diptera; Culicidae) is reproductively less fit than species A and C of the complex *Acta Tropica* **112 (1);** 316-319 Publisher Elsevier

Shishikal, D. Manoukis, N. C. Butail, S. and Paley, D. A. (2014). Male motion coordination in anopheline mating swarms *Scientific reports* **4 (1);** 6318 DOI; 10.1038/srep06318

http;//www.nature.com/srep/2014/140912/srep06318/pdf/srep06318.pdf

Silva, D. S, Carbajal de la Fuente, J ., Serra-Freire, A. L., Guimarães, N. M., Érico, A., Juliana, S. S. and Jeronimo, A. (2012). Morphometric analysis of

Chagasia fajardi (Diptera; Culicidae) populations in Brazil. *Revista Colombiana de Entomología*, **38 (2) ;** 276-281. http;//www.scielo.org.co/scielo.php?script=sci_arttext&pid=S0120048820120002 00019&lng=en&tlng=en. (Retrieved 26 / 04 / 2015, 2;15pm)

Sinka, M. E. (2013). *Global distributions of the dominant vector species of malaria, Anopheles* mosquitoes - *New insights into malaria* pp 109 - 144. Manguin, S. (Ed.), *In Tech, doi*; 10.5772 / 54163 *vectors* ISBN; 978 - 953 - 951 - 1188 - 7

Singer, E. (2011). Researchers release genetically engineered mosquitoes *MIT Technology Review* http;//www.technologyreview.com/view/425989/researchers-release-genetically-engineered-mosquitoes/ (Retrieved 25 / 06 / 2015, 2;26pm)

Srikumar, K. K. and Bhat, P. S. (2012). Field survey and comparative biology of tea mosquito bug (*Helopeltis spp*.) on cashew (*Anacardium occidentale* Linn.) *Journal of Cell and Animal Biology* **6 (14) ;** 200-206, http;//www.academicjournals.org/JCAB DOI; 10.5897/JCAB11.094 Retrieved 12/6/1015 1;26 pm

Takken, W. and Lindsay, S. W. (2005). Factors affecting the vectorial competence of *Anopheles gambiae*; a question of scale *Research Gate* 75-85pp http;//www.researchgate.net/publication/40129115 (Retrieved 15 / 06 / 2015, 09;35am)

Tibayrenc, M. (2011). Modern Morphometrics of medically important insects, chapter 16 (Jean-Pierre Dujardin **ed.**) *Genetics and evolution of infectious*

diseases Elsevier Inc. France. 474-475 DOI; 10.1016 / B978-0-12-384890-1, 00016-9

Turissini, D. A., Gamez, S. and White, B. J. (2014). Genome-Wide Patterns of Polymorphism in an Inbred Line of the African Malaria Mosquito *Anopheles gambiae Genome Biology and Evolution.* **6 (11) ;** 3094-3104 doi; 10.1093/gbe/evu243

Ukubuiwe, A.C., Olayemi, I.K., Omalu, I.C.J., Jibrin A. and Oyibo-Usman, K. (2013). Molecular Bases of Reproductive and Vectorial Fitness of *Culex pipiens pipiens* (Diptera; Culicidae) Mosquito Populations, for the Transmission of Filariasis in North Central Nigeria. *Journal of Medical Sciences,* **13 (1) ;** 201-207.,**DOI;** 10.3923/jms.2013.201.207 http;//scialert.net/abstract/?doi=jms.2013.201.207

Vinogradova, E. B. (2000). *Culex Pipiens Pipiens* mosquitoes; Taxonomy, distribution, ecology, physiology, genetics, applied importance and control *Pensoft* pg 72-73

Villarreal-Treviño, C., **Vásquez**, G. M., **López-Sifuentes**, V. M., **Escobedo-Vargas**, K., **Huayanay-Repetto**, A., **Linton,** Y., **Flores-Mendoza**, C., **Lescano, A. G.** and **Stell, F. M. (**2015**).** Establishment of a free-mating, long-standing and highly productive laboratory colony of *Anopheles darlingi* from the Peruvian amazon. *Malaria Journal*, **14 (1) ;** 227 doi;10.1186/s12936-015-0733-0 http;//www.malariajournal.com/content/14/1/227

Virginio. F ., Vidal. P. O., and Suesdek. L. (2015). **Wing sexual dimorphism of pathogen-vector culicids.** ***Parasite and Vectors*****.** 8 (1) ; **159. PMC4391167 doi; 10.1186/s13071-015-0769-6**

Walter Reed Biosystematics unit (2013). *Mosquito Classification Comparison,* http;//www.wrbu.org/mq_NewClass201308.html (Retrieved 21 / 04 / 2015, 4;25pm)

White, M . T, Griffin, J . T, Churcher T . S., Ferguson, N . M., Basáñez, M. G. and Ghani, A.C. (2011). Modelling the impact of vector control interventions on Anopheles gambiae Giles (Diptera; Culicidae) population dynamics Parasites and Vectors, **4 (1);**153 http;//www.parasitesandvectors.com/content/4/1/153

Scientists against malaria (2015). *Anopheles,* The Vector. http;//scientistsagainstmalaria.net/vector/anopheles-vector (Retrieved 21 / 04 / 2015, 6;23 PM)

White, S. A. and Kaufman, P. E. (2014). African malaria mosquito scientific name; *Anopheles gambiae* Giles (Insecta; Diptera; Culicidae) Featured creatures. University of Florida EENY – 601 . http;//entnemdept.ufl.edu/creatures/aquatic/*Anopheles_gambiae*.htm

World health organization. (2006). Malaria vector control and personal protection. *World Health Organization Technical Report Series* **936;1**

World Health Organization, (2007). Anopheline species complexes in South and South-East Asia.Techniques used in the recognition of species complexes.

SEARO Technical publication **57 (1) ;** 17-79 apps.searo.who.int/pds_docs/B2406.pdf

World health organization global malaria programme (2012). Global plan for insecticide resistance management in malaria vectors. pg1-2

World health organization. (2012). Malaria Policy Advisory Committee and Secretariat. *Malaria Journal* **11(1);**424 http;//www.malariajournal.com/content/11/1/424

World Health Organization (2013). Who global malaria programme; World malaria report. 255pp

http;//www.who.int/malaria/publications/world_malaria_report_2013/wmr2013_no_profiles.pdf (Retrieved 13/ 07/ 2015, 4;48 pm)

World Health Organization Reviewed (2015). *Malaria* http;//www.who.int/mediacentre/factsheets/fs094/en/ (Retrieved 21/4/15, 4;00 pm)

Wright, L.I., Tregenza, T., Hosken, D.J. (2008). Inbreeding, inbreeding depression and extinction. Conserv Genet, **9 (1) ;** 833–843.

Yamamoto, D. S., Hatakeyama, M. and Matsuoka, H. (2013). Artificial activation of mature unfertilized eggs in the malaria vector mosquito, *Anopheles stephensi* (Diptera, Culicidae) *Journal of Experimental Biology* **216 (15);** 2960-2966 doi; 10.1242/jeb.084293

Yeap, H. L., **Axford, J. K.**, **Popovici, J.**, **Endersby, B. M.**, **Iturbe-Ormaetxe, I.**, **Ritchie,** S. A., and **Hoffmann, A. A.** (2014). Assessing quality of life-shortening ***Wolbachia***-infected ***Aedes aegypti*** mosquitoes in the field based on capture rates and morphometric assessments *Parasites and Vectors*, **7 (1)** ; 58 doi;10.1186/1756-3305-7-58 http;//www.parasitesandvectors.com/content/7/1/58 (Retrieved, 26/5/2015, 10;22am).

Yeap, H. L., Endersby, N. M., Johnson, P. H., Ritchie, S. A., and Hoffmann, A. A. (2013). Body Size and Wing Shape Measurements as Quality Indicators of *Aedes aegypti* Mosquitoes Destined for Field Release. *The American Journal of Tropical Medicine and Hygiene*, **89 (1) ;** 78–92. doi;10.4269/ajtmh.12-0719 http;//ecdc.europa.eu/en/healthtopics/vectors/mosquitoes/Pages/anopheles-sacharovi-factsheet.aspx#C1 (Retrieved 26 /03 / 2015 4;09 am)

Yong, E. (2008). Biggest is not best in mosquito mating game. *Newscientist* Magazine **19 (8) ;** 26-56. http;//www.newscientist.com/article/mg19826565.100-biggest-is-not-best-in-mosquito-mating-game.html