EFFECTS OF FIELD AND LABORATORY LARVAL INHABITED WATER PHYSICAL CHEMICALS AND BACKGROUND COLOUR ON OVIPOSITION SITE SELECTION BY GRAVID FEMALE ANOPHELINE MOSQUITOES IN LUSAKA PROVINCE

by

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A Thesis Submitted to the University of Zambia in Fulfilment of the Requirements of the Degree of Doctor of Philosophy in Entomology of the University of Zambia.

January, 2024

DECLARATION

I, **James Simoko Phiri**, hereby declare that this thesis represents my own original work and that it has not been previously submitted for a degree, at this or any other University.

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Signature

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Date

APPROVAL

This thesis of **James Simoko Phiri** is approved as fulfilling the requirements for the award of the degree of Doctor of Philosophy in Entomology by the University of Zambia.

Signature: **Date:**

_____ _____

Principal Supervisor
Prof. Keith J. Mbata
Department of Biological Sciences
University of Zambia, Lusaka, Zambia.

_____ _____

Internal Examiner
Prof. Phillip O.Y. Nkunika
Department of Biological Sciences
University of Zambia, Lusaka, Zambia.

_____ _____

Internal Examiner
Prof. Enala Tembo-Mwase
School of Veterinary Medicine
University of Zambia, Lusaka, Zambia.

_____ _____

External Examiner
Prof. Clifford M. Mutero
International Centre for Insect Physiology and Ecology (ICIPE)
Nairobi, Kenya.

_____ _____

Chairperson of the Board of Examiners
Dr. Onesmus. M. Munyati
School of Natural Sciences
University of Zambia, Lusaka, Zambia.

DEDICATION

I dedicate this Thesis to my late father Mr. Chinyaweya Kalikali Phiri, my mother amai Tikanenji Banda, to Esther Mbewe Phiri my wife of many years, my children Cisomo Phiri, Mphatso Phiri, Simoko James Phiri and Taonga Tendai Phiri and grandchildren. When my energies faltered me, God renewed my inner strength for a purpose set for me. As the Chewa people of my late father say; "*kuona maso ya nkhono, nkudekha*" meaning that it is only with patience that you can see what many other people fail to see. I confidently claim that I saw and I share with the world the knowledge generated as a result of this study. To the memory of my late father and late grandfather Mr. Munang'ombe James Simoko Phiri who are a source of inspiration even as they are with their Maker.

To God be the glory.

ABSTRACT

Knowledge of factors that regulate oviposition site selection by gravid anopheline mosquitoes is still limited and yet it is of great importance in the quest to develop alternative malaria vector control methods. In order to contribute to data and knowledge on the subject, this study characterised and determined the effects of water physical chemicals including pH, temperature, turbidity, conductivity, larval pre-inhabited water and five different background colours on oviposition site selection behaviour by gravid anopheline mosquitoes. The study was conducted in three districts of Lusaka province, between July 2018 and February 2023.

Potential anopheline oviposition sites were sampled by scooping in water at the sites using a 350mls dipper. The anopheline oviposition sites were confirmed by the presence of anopheline larvae which were identified by their parallel water surface resting positions. Larval density was determined as mean larval counts in scoops using the dipper. Portable water testers were used to record temperature, pH, turbidity and conductivity in the field. Late instar mosquito larvae were kept in breeding containers until adults emerged from them later for mosquito identification using both morphological and molecular techniques. Field oviposition water in 1 litre capacity containers was placed in an insulated cooler box which was transported to the laboratory to determine its efficacy in influencing oviposition behaviour in gravid *Anopheles gambiae* s.s. KISUMU mosquitoes. Effects of larval pre-inhabited water on oviposition site selection was determined by presenting 30mls of the treatment water in 36mls capacity oviposition cups to groups of 30 gravid *Anopheles gambiae* s.s., KISUMU mosquitoes in 30 x 30 x 30 cm cages. The effect of water background colour on oviposition site selection, on the other hand was tested by presenting 30mls of water in five differently coloured oviposition cups to groups of 30 gravid mosquitoes in 30 x 30 x 30 cm mosquito cages. After 24 hours, the oviposition cups were inspected for eggs deposited by gravid females. The data generated were analysed statistically using IBM SPSS Statistics software, version 26 and Windows Microsoft Excel.

Out of a total of 43 sites sampled in this study, only three were confirmed as anopheline oviposition sites in the study area, two were in Nyamphande location (Site-A and Site- B) and one was in Shiala location, respectively. A total of 1,795 anopheline larvae were counted from the three sites. Two anopheline mosquito species namely, *Anopheles gambiae* s.s. (97.5%), and *Anopheles pretoriensis* (2.5%), were found to occur in Shiala location while *Anopheles coustani* (95%) and

Anopheles gambiae s.s. (5%), occurred in the Nyamphande area. Larval density was higher at Nyamphande site-A when compared to site-B, *t-test P* < 0.05(95%CI), [*t-test p* = 0.0000]. There were no significant differences P > 0.05(95%CI) in physical, and chemical properties of water between the two sites of Nyamphande. The Spearman linear regression coefficient (r_s), showed no significant correlation between larval density and water pH, temperature, turbidity, and conductivity at the three study sites. Laboratory reared gravid female *Anopheles gambiae* s.s., KISUMU laid more eggs in field oviposition water from Nyamphande site-A than in water from site-B or in distilled water, the control, P < 0.05(95%CI), one-way ANOVA (F(2,66) =11.172, *p* = 0.000 but laid fewer eggs in field oviposition water from Shiala when compared to the distilled water control *t-test P* < 0.05(95%CI), *t*-test *p* = 0.029. Gravid *Anopheles gambiae* s.s., KISUMU mosquitoes laid more eggs in water that had been pre-inhabited by the egg immature stages of conspecifics than in water of other instars or by a cohort, P < 0.05(95%CI), one-way ANOVA (F(3,88) = 3.663, *p* = 0.015. Gravid *Anopheles gambiae* s.s., KISUMU mosquitoes laid more eggs in red coloured oviposition cups than other coloured cups P < 0.05 (95%CI), one-way ANOVA (F(4,205) = 4.02, *p* = 0.004, in the order red (43%) > black (23%) > blue (16%) > green (11%) > yellow (7%).

The larval density and egg counts of anopheline mosquitoes were not related to water pH, temperature, turbidity or conductivity but to factors within the larval pre-inhabited water and to the background colour of the water. The discovery of dominance in Rufunsa district by *Anopheles coustani* mosquito, a secondary malaria vector in this study is significant for purposes of malaria control in the area and calls for an expanded study to establish whether the species plays an important role in malaria transmission in the area. This study has generated information that may be relevant for use in improving the efficacy of some existing vector control methods or for the development of alternative vector control methods. The study recommends that more research be undertaken to identify and understand factors or sources of factors that influence oviposition site selection by gravid female anopheline mosquitoes at oviposition sites of the study area.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
ATSB	Attractive toxic sugar bait
BES	British Ecological Society
Bs	*Bacillus sphaericus*
Bti	*Bacillus thuringiensis israelensis*
CDC	Centers for Disease Control and Prevention
CHIKV	Chikungunya Virus
CI	Cytoplasmic Incompatibility
DDT	Dichloro-diphenyl-trichloroethane
DENV	Dengue Virus
DMDS	Dimethyl disulfide
DMTS	Dimethyl trisulfide
DNA	Deoxyribonucleic acid
DRGS	Directorate of Research and Graduate Studies
FAO	Food and Agriculture Organisation
FD	Forestry Department
GMM	Genetically Modified Mosquito
GMO	Genetically Modified Organism
GPS	Global Positioning System
GPUA	Garden Peri-Urban Area
GRZ	Government of the Republic of Zambia
HSD	Honest Significant Difference
IIT	Incompatible Insect Technique
IPM	Integrated Pest Management
IRS	Indoor Residual Spraying
ITNs	Insecticide-Treated Nets
IVM	Integrated Vector Management
LLINs	Long Lasting Insecticide Treated Bed Nets
MoH	Ministry of Health
MWSE	Ministry of Water Development Sanitation and Environment

NMEC	National Malaria Elimination Centre
MNR	Ministry of Natural Resources
PCR	Polymerase Chain Reaction
PUAs	Peri Urban Area
RBM	Roll Back Malaria
RIDL	Release of Insects Carrying a Dominant Lethal (RIDL^{TM}) gene
SIT	Sterile Insect Technique
TDRC	Tropical Diseases Research Centre
TDS	Total Dissolved Solids
UNZA	University of Zambia
VBDs	Vector Borne Diseases
VCRU	Vector Control and Reference Unit
WB	World Bank
WHO	World health organization
YFV	Yellow Fever Virus
ZamStats	Zambia Statistics Agency
ZIKV	Zika Virus

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I acknowledge the support and guidance of my Supervisor, Professor Keith J. Mbata, PhD, Professor of Entomology, at the University of Zambia who has been a source of inspiration to me for many years. Thank you for your patience and understanding when I faced challenges but also for your constant encouraging reminder to me to make the best of the study. I also acknowledge the support and encouragement of the academic and non-academic staff in the Department of Biological Sciences, School of Natural Sciences, University of Zambia for readily and cheerfully being there to render support when I needed it. I thank the University of Zambia Management through the Dean of the School of Natural Sciences for giving me an opportunity to explore science. I enjoyed every mile of the journey. Special thanks to my wife Esther for amazingly being with me on all long drives and walking with me in the field. Special thanks also to my son Simoko James Phiri who rendered timely technical computer skills that has contributed to my improved computer competence skills. Indeed, he was God sent. I thank Dr. Elizabeth Chizema-Kawesha, Director of the Zambia National Malaria Elimination Centre (ZNMEC) and her staff and notably Mr. Willy Ngulube and many others for providing laboratory space and equipment for three years making me feel part of the Team. I also thank Dr. Gershom Chongwe, Executive Director at the Tropical Diseases Research Centre (TDRC) and his staff for his prompt response at short notice to a request to access laboratory facilities. In the same breath, I acknowledge the full support rendered by Mr. Mbanga Muleba, Head of the Entomology and Parasitology Department and his team who were readily available to render technical support. Particular thanks to. This study would have been too costly had it not been for the support I received from the Management and staff at the National Institute for Scientific and Industrial Research (NISIR) and special thanks to the Water Unit Department led by Ms. Chisha C. Mzyece and her team who readily assisted with both laboratory space and equipment.

CHAPTER 1: INTRODUCTION

1.1 Background.

Mosquitoes are vectors of many diseases such as malaria which is transmitted by *Anopheles* species, lymphatic filariasis transmitted by *Anopheles* and *Culex* species, dengue fever, Zika virus and yellow fever, all transmitted by *Aedes* mosquitoes (WHO, 2015). The global morbidity, mortality and economic loss to affected countries is immense (Lees *et al.*, 2015; Shepard *et al.*, 2014). In 2019 there were estimated 228 million global malaria cases and 409,000 deaths due to the disease (WHO, 2020). In 2019, it was estimated that Africa accounted for 93% of global malaria cases and of which 67% were children (WHO, 2019). In Zambia 2.5 million cases and 8,000 deaths were due to malaria that year (Chasaya *et al*., 2020; WHO, 2020). Earlier it was estimated that 90% and 49% of child mortalities in Gambia and Zambia, respectively were due to *P. falciparum*, a severe form of malaria (WHO, 2014).

Malaria is caused by protozoan parasites of the genus *Plasmodium* transmitted by female *Anopheles* mosquitoes (Cox, 2010). The most common and virulent malaria parasite in Zambia is *Plasmodium falciparum* which accounts for more than 95% of the cases while the other types, *P. vivax, P. malariae* and *P. ovale*, account for less than 5% of the cases (Chanda *et al.*, 2013). *Plasmodium falciparum* infection is associated with high morbidity and mortalities (MoH, 2020; MoH, 2018; Collins and Paskawitz, 1995). In the recent past, Zambia was one of the leading countries that had recorded significant gains in the fight against malaria. By the year 2008, the country had recorded a significant 66% reduction in numbers of malaria cases (WHO, 2009) and surpassing both the African Union's Abuja Declaration of 2000 and the Roll Back Malaria (RBM) targets (Chanda *et al.*, 2013). The country's successes in the fight against malaria is attributed to an integrated strategy utilizing Insecticides Treated bed nets (ITNs), Indoor Residual Spraying (IRS), chemotherapy and a clear communication strategy to ensure community and political support (Chanda *et al.*, 2013). However, due to many factors but including lack of resources, the gains made by the country have been eroded, resulting in a resurgence of malaria cases from 1 million to 4 million cases between 2010 and 2015 (Masaninga *et al.*, 2016).

Control of malaria vectors is largely based on use of chemical insecticides against which these vectors have continued to develop both physiological and behavioural insecticide resistance. Insecticide resistance has been reported against dichloro-diphenyl-trichloroethane (DDT) and the pyrethroids used in both ITNs and long-lasting insecticide treated bed-nets (LLINs). Some insecticide resistant *Anopheles* mosquitoes have been reported to be highly susceptible to *Plasmodium falciparum* (Sougoufara *et al.*, 2020). The development of drug resistance by *Plasmodium* parasites compounds the fight thereby making malaria resurgence a grim reality (Paul, 2011). In view of this, there is need for alternative vector control methods (Gari and Lindtjørn, 2018; Karunamoorthi, 2011).

One of the possible ways to overcome insecticide resistance is to develop oviposition-based vector control methods to which mosquitoes would not develop resistance (Read *et al*., 2009). Oviposition is the most important physiological behaviour to the survival of mosquito species and therefore an important stage in the transmission of mosquito borne-disease pathogens including *Plasmodium* (Chaves and Kitron, 2010). Oviposition site selection behaviour by gravid female mosquitoes is influenced by many factors including internal and external factors which are not fully understood (Okal *et al*., 2015). However, internal factors include nutrition and fertilization status of the gravid female mosquito while external factors include various environmental stimuli especially the availability and suitability of oviposition sites (Day, 2016). Although oviposition sites are heterogenous in form, they nevertheless contain common factors that influence oviposition site selection by gravid female mosquitoes. Knowledge of these factors and how they influence oviposition in gravid female anopheline mosquitoes could help in the development of alternative vector control methods. This proposition is supported by findings such as in the tsetse flies (DIPTERA: GLOSSINIDAE). The knowledge of how semiochemicals and colour of objects trigger different physiological behaviours in tsetse flies led to the discovery of novel tsetse control strategies (Kline, 2006). This led to proposals that such work in tsetse flies could be adapted for similar studies in malaria vectors for purposes of developing alternative vector control methods (Kline, 2006).

Similar knowledge of behavioural responses of crop pests to semiochemicals has led to the development of the 'Push-pull' strategy in crop protection (Pickett *et al.*, 2014; Takken, 2010) and

most recently the use of attractive toxic sugar bait (ATSB) used as a 'pull-kill' strategy in malaria vector control (Furnival *et al.*, 2020; Maia *et al*, 2018; Stewart *et al.*, 2013). Both disease vectors and crop pests control methods that have been developed and used based on knowledge of different behavioural responses of target insects have resulted in reduced use of pesticides and less environmental pollution. Unlike work on other insects of major importance, very little such similar studies have been done on malaria vectors and even much less in Zambia.

1.1.1 Malaria situation in Zambia.

Zambia has made great gains in the fight against malaria (WHO, 2009), but like other countries, sustaining the gains has been challenged by insecticide and drug resistance and also the inability to consistently undertake vector control measures (Chanda, 2016; Masaninga *et al.*, 2016; Chanda *et al.*, 2013). In Lusaka Province where the main malaria vectors are *Anopheles arabiensis, Anopheles gambiae* s.s., and *Anopheles funestus* (MoH, 2019; Kalubula *et al*., 2015) there is a contrasting situation of malaria burden where Lusaka city has very low to almost zero malaria transmission level at less than 1% (Chanda *et al.*, 2011) while the rural districts of the province such as Rufunsa and Chongwe have among the highest malaria cases in Zambia (NMEC, 2018; Kalubula *et al*., 2015). To sustain gains in areas of low transmission and to bring down malaria in areas of high endemicity, there is need for Zambia to locally develop alternative novel vector control tools and methods that are sustainable, cheaper and easier to deploy or use in the local environments.

1.1.2 Research study focus.

The focus of the study was to investigate oviposition site selection behaviour by gravid female anopheline mosquitoes in the field and in the laboratory. The aim was to identify factors that affect oviposition site selection by anopheline mosquitoes both in the field and laboratory. The study was undertaken in selected areas of three districts of Lusaka Province.

1.2 Statement of the research problem.

In spite of the many advances made in strategies and methods for control of malaria vectors, the disease still accounts for high morbidity and mortality especially in most developing countries. This is in part due to the reduced effectiveness of current vector control methods. Therefore, combating mosquito borne diseases requires new and sustainable control strategies that may be

based on utilisation of physiological conditions and behavioural needs of the mosquito such as oviposition site selection (Oliva *et al*., 2014).

1.3 Purpose of the study.

The purpose of this study was to investigate and generate data and information on the factors that may influence oviposition site selection behaviour by gravid female anopheline mosquitoes. The factors that were investigated included the following; water physical chemicals, pH, temperature, turbidity, conductivity, larval pre-inhabited water and background colour of oviposition site. Knowledge on how gravid female anopheline mosquitoes respond to these different factors may be important for control of malaria vectors.

1.4 Study objectives.

1.4.1 General objective.

The general objective of this study was to investigate the effects of water physical chemicals, immature mosquito (eggs, larvae and pupae) pre-inhabited water and oviposition site background colours on oviposition site selection behaviour by gravid female anopheline mosquitoes both in the field and in the laboratory.

1.4.2 Specific objectives.

The specific objectives of this study were to:

(i) Identify oviposition sites selected by gravid female anopheline mosquitoes in selected areas of Lusaka, Chongwe and Rufunsa districts of Lusaka Province.

(ii) Determine the physical chemical properties of the water mediums from the identified oviposition sites selected by gravid female anopheline mosquitoes.

(iii) Determine the relationships between the physical chemical properties of the water from the selected oviposition sites in the study area, with the anopheline mosquito larval densities in them.

(iv) Conduct comparisons of the efficacy of different water types from the field and laboratory on oviposition site selection by gravid female *Anopheles gambiae* s.s., KISUMU, under laboratory conditions.

(v) Determine the effect of time and pre-habitation by larval cohorts on efficacy of substrate on oviposition site selection behaviour by gravid female anopheline mosquitoes.

(vi) Determine the effect of background colour of water on oviposition site selection by gravid female anopheline mosquitoes. .

1.5 Research questions.

The following research questions were addressed by the study:

(i) Which sites in the study area had been selected for oviposition by gravid female anopheline mosquitoes?

(ii) What were the physical chemical properties of the water from the oviposition sites selected by gravid female anopheline mosquitoes in the study area?

(iii) What were the relationships between physical chemical properties of the water from the selected oviposition sites, and the anopheline mosquito egg or larval densities?

(iv) How did the different mosquito larval inhabited water in selected oviposition sites, compare in terms of efficacy on eliciting oviposition site selection by gravid female *Anopheles gambiae* s.s., KISUMU?

(v) What were the effects of time and pre-habitation of water by different larval cohorts on oviposition site selection behaviour by gravid female anopheline mosquitoes?

(vi) What were the effects of background colours provided by the oviposition cups and by the substrate of the oviposition site, on oviposition site selection by gravid female anopheline mosquitoes?

1.6 Research hypotheses.

The research hypotheses of the study were that:

(i) There were special sites selected for oviposition by gravid female anopheline mosquitoes around the study area.

(ii) There were specific physical chemical properties of water associated with oviposition site selection by gravid anopheline mosquitoes.

(iii) There were relationships between the physical chemical properties of oviposition water substrates with anopheline larval densities at oviposition sites that had been selected by gravid female anophelines.

(iv) There were differences in the efficacy of field and laboratory water in influencing oviposition site selection by gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes.

(v) There were effects by time and larval cohort pre-habitation of water on the efficacy of water in influencing oviposition site selection by gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes.

(vi) There were effects by oviposition site background colours on oviposition site selection behaviour of gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes.

1.7 Significance of the study.

The study contributed to the growing body of data and knowledge on physical chemical factors that influence oviposition site selection by gravid anopheline mosquitoes. Further the data has potential for use in the development of local, novel and sustainable alternative malaria vector control methods.

1.8 Scope of the study.

This study was a quantitative study undertaken both in the field and laboratory. The study investigated the effect or influence of field and laboratory pre-inhabited water physical chemical factors and background colour on oviposition site selection by gravid female *Anopheles* mosquitoes.

1. 9 Theoretical and conceptual frameworks.

1.9.1 Theoretical framework.

Mosquito control methods have for many years relied on the use of chemical insecticides which are increasingly failing due to increasing cases of insecticides resistance. There is need for alternative mosquito control methods to which mosquitoes would not evolve resistance. Oviposition which is controlled by internal and external factors is an obligatory physiological need in mosquitoes. It has been proposed as one possible pathway towards the development of alternative mosquito control methods because oviposition stage may be the weakest point of intervention. This is premised on the fact that a mosquito would not indefinitely delay oviposition unless it defied the normal Mendelian evolutionary process (Reiter, 2007). Knowledge of internal and external factors that affect oviposition processes could be used in the potential development of alternative sustainable non-chemical methods and resulting in reduced rate of development of insecticides resistance or environmental pollution. Ironically not much work has been done to understand factors that affect or influence oviposition site selection by gravid anopheline mosquitoes (Hoi and Roitberg, 2014).

1.9.2 Conceptual framework.

This study was undertaken to investigate the effect and influence of some factors including water physical chemicals, water background colour and factors associated with larval pre-inhabited water, on oviposition site selection behaviour by gravid *Anopheles* mosquitoes. The effect or influence was whether gravid female mosquitoes laid more eggs when presented with differently treated oviposition water in the same environment or cage in the case of laboratory experiments. The data such as on the effect or influence of colour or larval pre-inhabited water, could be used to manipulate oviposition behaviour with purpose of controlling these malaria vectors.

1.10 Ethical considerations.

This research was largely based on mosquito larvae sampling both in the field and laboratory using mosquitoes and mice bred and managed by the National Malaria Elimination Centre (NMEC) and Tropical Diseases Research Centre (TDRC). Mice were also supplied by the Department of Biological Sciences at the University of Zambia. There were no human subjects or large animals that were used at any stage of the study.

CHAPTER 2: LITERATURE REVIEW

2.1 Malaria transmission and elimination.

In malaria endemic countries, malaria transmission mostly occurs during the rainy season (Gray and Bradley, 2005). Transmission is climate or weather dependent where temperature plays a very significant role both in terms of sporogonic and gonotrophic cycles (Okuneye *et al.*, 2019). The peak period for malaria transmission season in Zambia is April-May, shortly after the rainy season (Sitali *et al.*, 2019). Some mosquitoes, are capable of surviving during the dry season and in dry savannah areas of Africa making them able to transmit malaria even under dry season (Gillies and Coetzee, 1987) or arid conditions, as eggs of some species show greater desiccation resistance (Gray and Bradley, 2005). Transmission of malaria can be eliminated through the control or elimination of the responsible vector mosquitoes using different methods (Gari and Lindtjørn, 2018; Takken and Knols, 2017; Karunamoorthi, 2011; Meisch, 1985) and the elimination of *Plasmodium*, the malaria disease causal agent, in human hosts largely by using antimalarial drugs. Knowledge of the life cycles of both *Plasmodium* and vector species are important for purposes of malaria control.

2.1.1 *Plasmodium* life cycle in mosquito and human host.

The malaria causing parasite completes its development in two different hosts; the primary definitive host which is the mosquito and the obligatory human host respectively (Fig. 1). Inside the mosquito, the parasite undergoes sexual reproduction leading to the development of human infective sporozoites which are injected into a human through a mosquito bite. The sexual development process in the mosquito host is called sporogony (Azevedo *et al*., 2019; Poonam and Dhiman, 2015; Phillips, 2001). In the human host, *Plasmodium* lives as an intracellular parasite first in liver cells where it asexually multiplies in what is called a Hepatic Cycle and later in the red blood cells (RBCs) to start its Erythrocytic Cycle (Antinori *et al*., 2012; Phillips, 2001). In the liver, sporozoites transform into schizonts which asexually multiply, rupture and release merozoites that invade erythrocytes where merozoites transform into trophozoites which asexually multiply to form schizonts in a process called schizogony (Antinori *et al*., 2012).

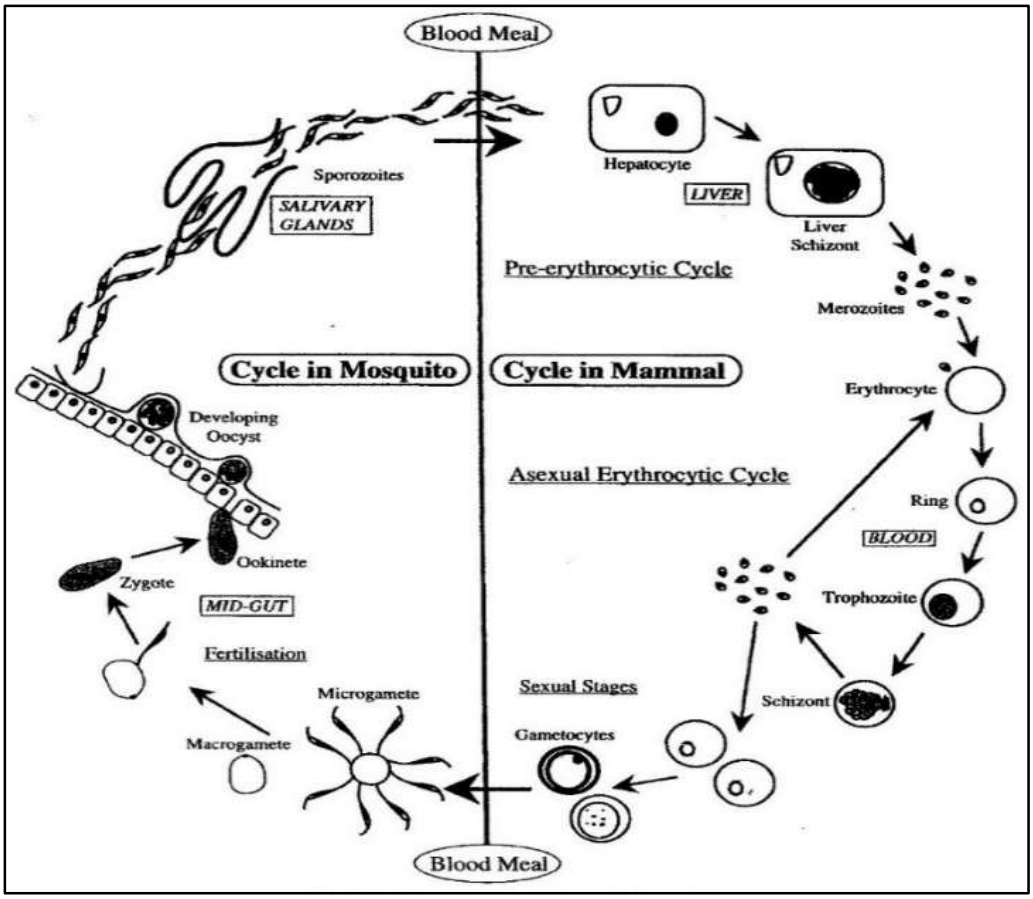

Figure 1. *Plasmodium* Life Cycle.

(Source: Phillips, 2001 p. 210).

When fully mature, schizonts rupture the infected RBCs to release merozoites, malaria pigment and toxins into the blood stream thereby causing malaria disease symptoms (Iyer *et al.*, 2007). However, some merozoites do not undergo schizogony but transform into female macrogametocytes and male microgametes. When ingested by a female *Anopheles* mosquito during a blood meal, the microgametocytes fertilize macrogametes within the mid gut region of the mosquito to continue the sporogonic cycle and malaria transmission (Antinori *et al.*, 2012).

2.1.2 Mosquito life cycle.

Mosquitoes depend on the availability of water to sustain their species. The life cycle of mosquitoes involves four distinct developmental stages; egg, larval, pupal and adult stages (Fig. 2). Apart from the adult, all other life stages of a mosquito are aquatic thereby making

water bodies as the most important environmental factor to the survival of mosquitoes. Therefore, aided by multiple environmental factors, a gravid female mosquito looks for a suitable water habitat as its oviposition site where it lays its eggs, which hatch into first instar larvae. The latter transform into second, third, and fourth instars and into pupae which in turn transform into adults which upon maturing mate and reproduce (Baranitharan *et al*, 2018). Upon mating, the anthropophilic gravid female *Anopheles* mosquitoes looks for the preferred human blood which is needed for the full development of their eggs. However, in absence of humans, gravid female *Anopheles* mosquitoes may feed on blood of some other hosts such as domestic livestock (Mbewe *et al*., 2022; Escobar *et al.*, 2020; Imbahale *et al.*, 2019). The development process of eggs to maturity within a gravid female mosquito could be completed within two days after taking a blood meal (Marques *et al*., 2018; Lu and Hagedorn, 1986).

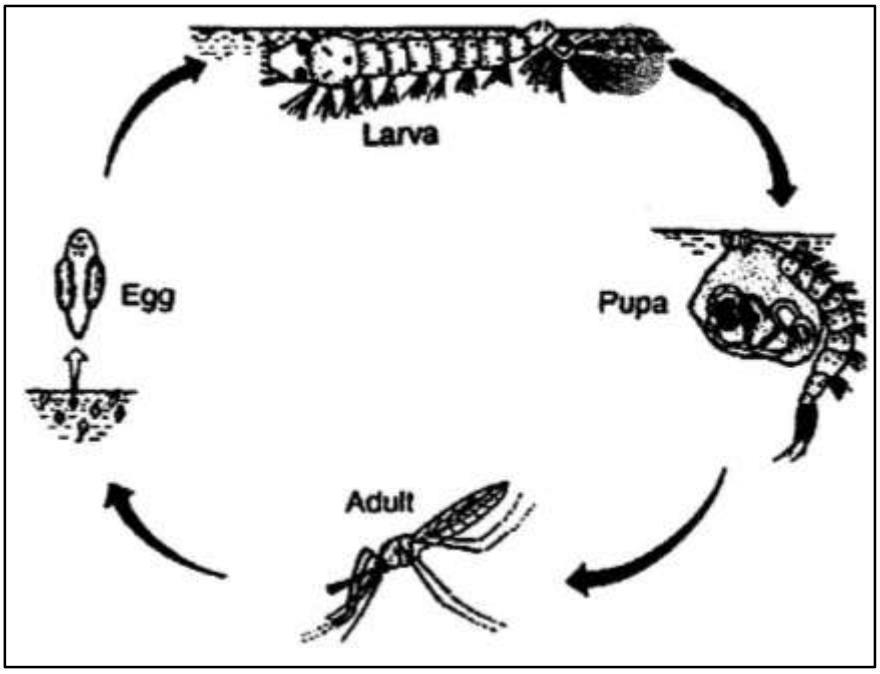

Figure 2. Life Cycle of *Anopheles* Mosquito.
(Source: Baranitharan *et al*, 2018, p. 33)

The metamorphosis process is completed within 7 to 14 days but may take longer, depending on environmental conditions (Koenraadt, 2003). The aquatic stages are the most sensitive crucial stages to survival of mosquitoes because in cases where a preferred temporal water pool dries up, desiccation of eggs and high mortalities of instars may occur (Munga *et al.*, 2006).

Therefore, the selection of an oviposition site by gravid female mosquitoes is very important to the survival of its different instar stages.

2.2 Mosquito control methods.

The control of mosquitoes has largely relied on chemical insecticides that are formulated to kill adult or immature stages of a mosquito. Another method used for the control of control mosquitoes is biological control which uses different living organisms to control mosquitoes (Aria-Castro *et al.*, 2020; Dunn and Follett, 2017; Lindh, 2007). There are several other methods used to control mosquitoes and can generally be grouped into four categories (Table 1) as chemical insecticides-based, physical environmental alteration also called larval source management (LSM), biological control including sterile and genetic technology, integrated vector control (IVC) or as part of recent concept of integrated vector management (IVM). There are other methods including odour-baited traps, semiochemicals baited toxic sugar baits and use of endectocides that kill both vectors and *Plasmodium* parasites inside hosts.

Table 1 Summary of mosquito control methods their purpose and limitations of use.

<table>
<tr><th colspan="2">METHOD</th><th>PURPOSE AND AIM OF METHOD</th><th>LIMITATIONS OF CONTROL METHOD</th></tr>
<tr><td rowspan="4">Chemical insecticides: Grouped into four according to their formulations.</td><td>FORMULATION</td><td rowspan="2"></td><td rowspan="3"></td></tr>
<tr><td>Pyrethroids: deltamethrin, permethrin, lambda-cyhalothrin</td><td rowspan="2">As adulticides and larvicides to kill adults and immature stages, respectively</td></tr>
<tr><td>Organophosphates: fenitrothion, pirimiphos-methyl, malathion, temephos.</td></tr>
<tr><td>Carbamates: propoxur and bendiocarb.</td><td rowspan="2"></td></tr>
<tr><td>Organochlorines: dichloro-diphenyl-trichloroethane (DDT).</td><td>As adulticides to kill adults.</td><td>High persistence and bio-accumulation, insecticides resistance and environmental pollution.</td></tr>
<tr><td colspan="4"></td></tr>
<tr><td>Larval source management (LSM).</td><td colspan="2">To deprive mosquitoes of breeding and oviposition sites as part of an integrated vector control programme.</td><td>Not practical to eliminate all oviposition sites, especially during the rainy season.</td></tr>
<tr><td colspan="4"></td></tr>
</table>

Biological control	Entomopathogenic Organisms.	To deliberately intervene using beneficial living organisms to enhance maximum negative pressure to control mosquitoes within a natural ecosystem interaction such as at the oviposition site.	Sustainability as most methods, require regular and repetitive application usually of an exotic biocontrol agent to attain desired outcomes
	Predators such as Larvivorous fish.		
Integrated vector management	Combination of multiple but viable vector control methods resulting in a significant drop or elimination of vector populations and disease burdens.		Sustainability as resources must consistently be pooled from different stakeholders who may not have similar commitment and resources.
Other methods of mosquito control	Trapping enhanced by attractive odours including semiochemicals and kill using endectocides treated hosts		Costs and logistical challenges in deployment.

2.2.1 Chemical insecticides.

Historically, the control of mosquitoes has largely relied on chemical formulations of insecticides that are applied into the targeted environment as adulticides to kill adult stages or as larvicides at oviposition or breeding habitat in order to kill immature stages. Although insecticide-based methods have had larger application resulting in short- and long-term positive impacts on the malaria disease burden, they have been implicated in environmental pollution (McWilliams, 2014). The effectiveness of these methods has been affected by growing development of insecticides resistance (Mathanga *et al*., 2015; Hoi and Roitberg, 2014; Takken and Verhulst, 2013). Recently, female *Anopheles* mosquitoes have exhibited a behavioural change in their resting and feeding time patterns to avoid insecticide treated houses (Carrasco *et al.*, 2019; Mohammed-Awel *et al.*, 2018; Gatton *et al.*, 2013; Moiroux *et al.*, 2012). Further, female *Anopheles* have adapted to having multiple host zoophilic feeding options (Imbahale *et al.*, 2019; Bradley *et al.*, 2016; Chareonviriyaphap *et al*., 2013; Sokhna *et al.*, 2013) while the unsustainable high cost of their use has made chemical insecticides use unaffordable in poor malaria endemic countries (Nawa *et al*., 2019; Killeen *et al*., 2017;

Oliveira *et al.*, 2017; Chanda *et al.*, 2015; Masaninga *et al*., 2013). Nevertheless, for the foreseeable future chemical insecticides will continue to be used for mosquito-malaria control along other methods.

2.2.2 Larval source management (LSM).

Larval Source Management (LSM), also referred to as environmental or physical manipulation aims to rid the environment of existing and potential mosquito breeding habitats and to eliminate larvae (McWilliam, 2014; Manjarres-Suarez *et al.*, 2013; McGraw and O'Neill, 2013; WHO, 2013; Fillinger *et al.,* 2009; Meisch, 1985; Toohey *et al.,* 1985). However, because it is unlikely that all oviposition or breeding sites can be eliminated, LSM should be used as part of an integrated vector control strategy.

2.2.3 Entomopathogenic organisms.

Entomopathogenic organisms, including bacteria, fungi and nematodes are used as biocontrol agents in mosquito vector control programs because of their ability as symbionts and parasites to kill their hosts.

2.2.4 Bacteria.

Historically, the most widely used entomopathogenic bacteria are the *Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus* (Bs), which kill mosquito larvae by hydrolyzing their gut epithelium using their endotoxins produced during sporulation (Derua *et al*., 2019; Benelli *et al.*, 2016; Kandyata *et al.*, 2012; Scholte, 2004).

The other bacteria with great global potential in mosquito control are *Wolbachia.* These are intracellular endosymbiont bacteria whose symbiotic relationship with host mosquitoes leads to cytoplasmic incompatibility (CI), resulting in death or unviable female progeny (Ding *et al*., 2020; Dodson *et al.,* 2014; Walker and Moreira, 2011). The technique of using *Wolbachia* in this manner is called the incompatible insect technique (IIT). The bacteria may confer refractoriness to disease pathogens in some mosquito hosts but may also shorten the lifespan of infected mosquitoes (Shaw and Catteruccia, 2019; Turley *et al.*, 2014; Ye *et al.*, 2013; Van den Hurk *et al.*, 2012; Moreira *et al.*, 2009). One of the likely risks of using *Wolbachia* is that

some strains may not induce CI in progeny of infected females but may enhance infectibility by some *Plasmodium* species in some *Anopheles* mosquitoes (Dodson *et al.,* 2014).

2.2.5 Fungi.

Fungi belonging to the genus *Metarhizium* and *Beauveria* are the most used in the biocontrol of some *Anopheles* mosquitoes (Litwin *et al.,* 2020; Dara, 2017; Huang *et al.,* 2017; Liao *et al.,* 2017; Mora *et al.,* 2017; Scholte *et al.,* 2004). *Metarhizium anisopliae* strain isolated in Burkina Faso, was found to be highly virulent against wild caught insecticide resistant *Anopheles coluzzii* (Bilgo *et al.,* 2018). Besides killing adults, *Metarhizium anisopliae* reduces blood feeding and fecundity in *An. gambiae* (Bilgo *et al.,* 2018). Some strains of *Microsporidia*, are known to block infection and transmission of *Plasmodium falciparum* in *Anopheles arabiensis* (Gallager, 2020; Herren *et al*., 2020; Bargielowski *et al*., 2009) which is one of the important vectors in Zambia (MoH, 2019). In Zambia, an Enterobacter that blocked infection of *Anopheles gambiae* mosquitoes by *Plasmodium* was isolated in Southern Province (Cirimotich *et al*., 2011) while microsporidia were found in Lusaka (Phiri *et al.,* 2014). In spite of their proven efficacy in control of mosquitoes the use of entomopathogenic bacteria and fungi is still limited by logistics, cost, effectiveness issues, storage and their short shelf life (Huang *et al.,* 2017; Mora *et al.,* 2017; Skinner *et al.,* 2014; Scholte *et al.,* 2004). There are also reports of bio-insecticide resistance to these biopesticides (Benelli *et al*., 2016).

2.2.6 Nematodes.

There are several nematodes that are parasites of mosquitoes and are generally larvicidal in action through cuticular infection (Kendie, 2020). They sterilize mosquitoes leading to population decline and reduced malaria transmission (Kendie, 2020). However, the use of nematodes in mosquito control is not well developed and more research is needed in this area.

2.2.7 Predators.

In nature, all life stages of a mosquito are vulnerable to predation by both vertebrates such as some fish species or by invertebrates such as members of the larger culicine mosquitoes such as *Lutzia tigripes* De Grandpré & De Charmoy, (Diptera: Culicidae) as well the *Notonecta* spp., (Hemiptera: Notonectidae) or backswimmers (Eba *et al*., 2021; Moirangthem *et al.,* 2018; Noreen *et al.,* 2017; Coetzee, 2017; Benelli *et al.,* 2016; Weterings *et al*., 2014; Mutebi, 2012;

Chandra *et al*., 2008; Skelton, 2001). Predation at an oviposition site acts together with pathogens and parasites to negatively affect growth rate resulting in small size adult mosquitoes which fail to transmit disease due to lowered vectorial capacity (Roux and Robert, 2019). To avoid negative ecosystem disruptions due to use of exotic predator species, local native predator species are preferred (Aria-Castro *et al.*, 2020; Kendie, 2020; Huang *et al*., 2017; Benelli *et al.*, 2016; Gosh *et al.*, 2005). Some predators may be species specific meaning that a predator that may be effective against culicine mosquitoes may not be effective against anophelines (Hamon, 1969).

2.2.8 Sterile insect technique and genetically modified mosquitoes.

The use and release of genetically modified organisms (GMOs) especially the non-self-refractory is part of the promising biocontrol agents in the fight against malaria and other vector borne diseases. The principles used in sterile insect techniques (SIT) as well as in gene technologies, are generally based on observations in nature where natural systems are manipulated to produce deleterious effects in a species reproductive biology with the intention of eradicating the target vector species (Dunn and Follett, 2017; Gentile *et al.*, 2015; Gilles *et al.*, 2014; Lees *et al.*, 2015; Alphey *et al.*, 2010; Vreysen *et al*., 2000). The longtime use of sterilized insects and recently the advances made in molecular biology, has seen the development of genetically modified vector mosquitoes which are refractory to pathogens such as *Plasmodium* or produce flightless female mosquitoes (Scudellari, 2019).

2.2.9 Lethal genes and gene drive technology.

Two other techniques using lethal genes have been developed. One is based on induced mutation of male gametes which produce a lethal gene in a technique referred to as release of insects carrying a dominant lethal gene-RIDL and the other is based on a synthetic lethal gene called the Gene Drive both of which rely on males as carriers and passed on to progeny where female progeny are killed or make them unviable while males survive and become carriers of the lethal genes (Nolan *et al*., 2011). Gene drives are maternally self-driving and not self-limiting (Knols *et al.*, 2007). The end result is a collapse of vector population through elimination of females while the surviving males still carry the synthetic gene unlike in the sterile insect technic (SIT) and dominant lethal gene-RIDL methods which are self-limiting because the genes die with the death of released males (Gentile *et al.*, 2015). Although the use

of genetically modified mosquito (GMM) is promising, serious concerns have been raised (Scudellari, 2019; BES, 2015; Klein *et al*., 2012; Alphey *et al.*, 2010; Robinson *et al.*, 2009) and that their use should be guided by an environmental assessment within the context of an integrated pest management (IPM) approach (BES, 2015; Gentile *et al.*, 2015; Gilles *et al.*, 2014; Alphey *et al.*, 2010; Robinson *et al.*, 2009; Knols *et al*., 2006; Budiansky, 2002).

2.2.10 Integrated vector management (IVM).

Integrated Vector Management (IVM) combines multiple but viable vector control methods resulting in a significant drop or elimination of vector populations and disease burdens (Sande *et al.*, 2019; PAN, 2013). Key to success is stakeholders' commitment and involvement. The multisector involvement and collaboration in IVM ensure optimized use of available resources and human capacity building in line with one of its principles of community involvement and sustainability (Sande *et al.*, 2019; Chanda *et al.*, 2008). The fact that other VBDs are targeted is one of the main advantages of IVM in malaria vector control. However, the requirement for sustained allocation of resources in poor countries makes IVM costly and unsustainable unless there was external support (Masaninga *et al.*, 2013).

2.2.11 Other methods.

Improvement of current and development of new mosquito-targeted malaria control methods relies on better understanding of mosquito biology including physiology and behaviour (Domingo *et al.*, 2017). Among the methods being used include mosquito traps which were previously solely used for entomological monitoring and surveillance of anopheline mosquitoes but have been improved and are also used for control purposes (Sougoufara *et al.*, 2020; Poulin *et al*., 2017; Hiscox. *et al.*, 2016; Killeen, 2016; Lima *et al.*, 2014; Kline, 2006; Kline *et al*., 1990). The efficacy of some of these technologies such as mosquito traps has been improved with the inclusion of semiochemicals or volatile odours (Mweresa *et al.*, 2020; Okumu *et al.*, 2010; Homan, 2016; Garcia-Rejon, 2011; Xue. *et al.*, 2008; Silva *et al.*, 2005; Quarles, 2003). Semiochemicals or infochemicals elicit or trigger a necessary physiological or behavioural response in organisms (Ong and Jaal, 2015; Dicke and Sabelis, 1988). Among the relevant semiochemicals at oviposition sites are infochemicals (Lindh *et al.*, 2015; Navarro-Silva *et al.*, 2009) some of which are released by organisms to send a particular message and trigger a response across or within species. Such infochemicals include pheromones and

kairomones. Other infochemicals such as apneumones are released from dead organic matter in the environment (Navarro-Silva *et al.,* 2009). Incorporation of semiochemicals in mosquito control has enhanced efficacy of some methods. However, trapping technology for mosquito control has some limitations such as cost of traps and deployment logistics needed in a major mosquito control program have been cited as some potential limitations (Gari and Lindtjørn, 2018).

The other more recent methods include the attractive targeted or toxic sugar baits (ATSBs) which is a "pull-kill" or "attract-and-kill" method (Chanda *et al*., 2023; Sougoufara *et al.,* 2020; Traore *et al*., 2020; Wooding *et al.,* 2020; Watentena and Okoye, 2019; Fiorenzano *et al*., 2017; Vanickova *et al.,* 2017; Ong and Jaal, 2015; Kline, 2007; Kline, 2006). In ATSBs both female and male mosquitoes are attracted to feed on the toxic sugars and get killed or poisoned by the substrate.

The endectocides based control method uses human or livestock mainly bovines as treated moving baits to host seeking gravid female anophelines. The common drug used is ivermectin. The method targets mosquitoes that exhibit both behavioural insecticide avoidance and those that are zoophilic but also feed on humans (Mbewe *et al*., 2022; Escobar *et al*., 2019; Imbahale *et al.*, 2019; Killeen *et al*., 2014). The method kills both *Plasmodium* and mosquitoes that feed on ivermectin treated humans and bovines resulting in reduced malaria cases (Gari and Lindtjørn, 2018). Ivermectin also reduces the fertility of some *Anopheles* mosquitoes (Sougoufara *et al.*, 2020).

2.3 Oviposition and factors influencing oviposition behaviour in gravid female mosquitoes.

Oviposition is an obligatory and critical physiological reproductive necessity and condition that should be met in order for mosquito species to reproduce, survive and be sustained or else they become extinct (Rejmánková *et al.,* 2005). Since oviposition is obligatory, it cannot be stopped although mosquitoes may skip or delay it (Oliva *et al*., 2014). However, mosquitoes cannot indefinitely skip or delay oviposition unless they evolve an alternative process to oviposition that by-passes the aquatic life stage which is very unlikely under the normal Mendelian mutation (Reiter, 2007).

Oviposition process within a gravid female anopheline mosquito is triggered by internal and external factors and causing the gravid female mosquito to take an oviposition flight in search of a suitable oviposition site (Navarro-Silva *et al*., 2009). Oviposition sites have unique and diverse ecological characteristics (Goma, 1960) and may be in form of shallow water pools or temporal stagnant water such as found in hoofprints or man's footpaths (WHO, 2019; Elyazar *et al.*, 2013; Jansen and Beebe, 2010; Koenraadt, 2003; Takken and Lindsay, 2003). The selection of an oviposition site is mediated by multiple complex factors including hydrological, colour or light reflections from a potential site, physic-chemical properties of water, availability of food, volatile odours or semiochemicals that are picked by the visual/optic, olfactory and tactile sensory organs (Li *et al.*, 2010; Navarro-Silva *et al.*, 2009). Oviposition sites may also harbour conspecific or competitor larvae of other mosquito species, pathogens or predators all of which emit volatile chemicals or semiochemicals which contribute to the multiple factors a gravid female mosquito uses in selecting an oviposition site (Asmare *et al.*, 2017; Himeidan *et al.*, 2013; Zattel *et al.*, 2013; Himeidan *et al.*, 2013; Guillermo *et al.*, 2005; Kennedy 1940). Different *Anopheles* mosquito species prefer oviposition sites that have environmental, bio-physic-chemical characteristics which may suit them and guarantee maximum survival of their progeny (Suh *et al*., 2016; Rejmánková *et al*., 2013; Lyons *et al.*, 2014; Kabula *et al.*, 2011; Gray and Bradley, 2005; Phiri, 1994). Insects may also avoid certain oviposition sites on basis of "learnt" predator avoidance (Blaustein *et al.*, 2003) as has been reported of *Anopheles gambiae* which avoids breeding sites which are inhabited by backswimmer (*Notonecta malucata*) which is a predator of immature stages of mosquitoes (Warburg *et al.*, 2011). Therefore, in selecting an oviposition site gravid female mosquitoes use different sensory systems.

Research into characterisation of oviposition sites or breeding habitats is an area of great promise but which has not yet been fully understood as to be exploited and used as an intervention point in the control of mosquitoes (Hoi and Roitberg, 2014). Oviposition is one of the weak points in the life cycle of mosquitoes that can be exploited as a basis for a strategy to control mosquito borne diseases. Oviposition process could be interfered with at two different stages either within a gravid female mosquito or externally at oviposition sites where immature stages are vulnerable. A negative intervention at oviposition stage could lead to control of mosquitoes. However, in

pursuing strategies that interfere with oviposition it is important to understand the ability of the vector to survive control measures (Budiansky, 2002) and the potential negative impact on ecosystems (Scudellari, 2019).

Characterisation of water and understanding mosquito dynamics at an oviposition site could lead to generation of new knowledge that would potentially be used for the development of potential alternative and novel mosquito control methods. Among the many factors that have been associated with *Anopheles* larval abundance and distribution are some physical chemicals temperature, pH, conductivity (µS/cm) and turbidity (NTU) which are influenced by local environmental conditions (Arcos *et al*., 2018). Many studies have reported a relationship between water physical chemicals pH, turbidity and conductivity and *Anopheles* larval abundance at oviposition sites. However, while some studies have established the relationship (Getachew *et al*., 2020; Herrera-Varela *et al*., 2014; Gopalakrishnan *et al.,* 2013; Kabula *et al*., 2011; Kenea *et al*., 2011; Chaves and Kitron, 2011; Rao *et al*., 2011; Paaijmans *et al*., 2008) other studies could not (Amini *et al*., 2020; Getachew *et al.*, 2020; Nikookar *et al*., 2017; Liu *et al*., 2012). It has been suggested that the presence of *Anopheles* larvae is related to species specific needs and not necessarily the physical chemicals Arcos *et al*., (2018). However, studies that have established a relationship between physic-chemical and larval abundance have suggested that these water variables may contribute singularly or synergistically towards eliciting a response that may influence the final selection of an oviposition site by gravid female mosquitoes (Panigrahi *et al*., 2014; Li *et al.*, 2010). Other studies on *Aedes* oviposition sites in relation to physical chemical properties of water showed a positive correlation with pH and dissolved oxygen but a significant negative correlation between conductivity and larval density of *Aedes albopictus* (Gopalakrishnan *et al.*, 2013). In other insect group the armoured ground cricket *Acanthoplus spieiseri* Brancsik (Orthoptera: Tettigoniidae, Hetrodinae), it was reported that soil pH was the single most important factor that attracted oviposition (Mbata, 2004).

Although much work has been done on effects of physic-chemical on oviposition site selection by gravid female anopheline mosquitoes, a lot more is still needed (Eneh *et al.,* 2019) hence the research by this study on the four water variables temperature, pH, conductivity (µS/cm) and turbidity (NTU).

2.3.1 Water Temperature.

Temperature regulates biochemical and physiological processes for normal development of mosquitoes but also plays an important role in regulating concentration levels of other water physical chemicals such as conductivity (Beck-Johnson *et al*., 2013; Sanford *et al*., 2013). There is a positive correlation between larval distribution with temperature (Rao *et al*., 2011). Temperature may be an enabler at an oviposition site but it may also be a serious limiting factor (Paaijmans *et al*., 2008; Gray and Bradley, 2005). Higher temperature shortens or speeds up the development cycle of mosquitoes up to a limiting level beyond which, temperature becomes lethal and kills larvae while lower temperatures slow the development of larval stages (Paaijmans *et al*., 2008).

2.3.2 Water pH.

Mosquitoes preferentially select water habitats such as ponds with different vegetation cover or had water that was pH neutral or slightly alkaline according to the species needs (Arcos *et al*., 2018; Dom *et al.*, 2016). Some studies have suggested that water pH is the most important factor that influences the selection of breeding habitats or oviposition sites by gravid female *Anopheles* mosquitoes (Kabula *et al*., 2011; Rao *et al*., 2011). Although it is agreed that pH is a very important water property in influencing the selection of an oviposition site by gravid female mosquitoes not all studies have reported the same range of pH that may be considered as most suitable in terms of relationship to *Anopheles* mosquito larval abundance and distribution. Some studies have suggested a near neutral pH range of 6.8-7.2 (Elmalih *et al*., 2018; Dom *et al.*, 2016; Gopalakrishnan *et al*., 2013; Rao *et al*., 2011) as the most suitable pH while others have suggested pH near acidic pH range as associated with *Anopheles* larval abundance and distribution (Amini *et al*., 2020; Musonda and Sichilima, 2019; Arcos *et al*., 2018; Soleimani-Ahmadi *et al*., 2014). Yet still other studies have reported alkaline pH as associated with *Anopheles* larval abundance and distribution (Amini *et al*., 2020; Arcos *et al*., 2018; Soleimani-Ahmadi *et al*., 2014; Hopkins, 1952). The variations are due to different environmental conditions in different geophysical local conditions but also shows that there is no pH range that could be considered universally suitable for different species of *Anopheles* mosquitoes in different local environmental conditions because of possible different specific species needs that may be at play (Amini *et al*., 2020). In Egypt *Anopheles gambiae* larval

abundance and distribution was found to be different in different local conditions where some *Anopheles gambiae* were found breeding in acid water of pH 4 while in other places larval abundance and distribution was associated with near alkaline conditions (Hopkins, 1952).

2.3.3 Water Conductivity (µS/cm).

Conductivity (µS/cm) of water has a positive correlation with larval densities and distribution of *Anopheles* mosquitoes in the field (Amini *et al*., 2020; Arcos *et al*., 2018; Gopalakrishnan *et al.*, 2013; Kabula *et al*., 2011; Rao *et al*., 2011). The effect of conductivity may be related to the presence of salts such as nitrates and phosphates which would also indicate possible richness in bacteria (Nkhuwa *et al*., 2015). High conductivity (µS/cm) of water may reflect presence of salts such as nitrates and phosphates which would also indicate possible richness in bacterial coliforms (Bäumle and Museteka, 2011) such that could also influence oviposition site selection by gravid female *Anopheles* mosquitoes (Mwingira *et al*., 2020; Schoelitsz *et al*., 2020; Herrera-Valera *et al*., 2014). Therefore, the relationship between conductivity (µS/cm) and larval distribution and abundance at oviposition site may also be related to anopheline mosquito species and environmental conditions (Arcos *et al*., 2018).

2.3.4 Water Turbidity (NTU).

The breeding habitats for *Anopheles gambiae* mosquitoes may be characterized by water with high turbidity (Herrera-Varela *et al.*, 2014; Kabula *et al.*, 2011; Kenea *et al.*, 2011; Paaijmans *et al*., 2008). On the other hand, others have reported that *Anopheles arabiensis* preferred water that was clear, and with low turbidity (Elmalih *et al*., 2018). Depending on species of *Anopheles* mosquitoes and cause of water turbidity, different water turbidity readings could be associated with high or low abundance of larvae because different *Anopheles* mosquitoes prefer different levels of water turbidity (Arcos *et al*., 2018). Run-off water and sedimentation during rainy season cause breeding habitats to have high content of soil particles, agrochemicals, and organic matter all which may contribute to increasing turbidity of water at an oviposition habitat (Getachew *et al*., 2020; Kenea *et al*., 2011; Chaves, and Kitron., 2011). Organic matter and agro-chemicals at an oviposition habitat not only contribute to raising turbidity but they may also provide nutrients for microbial growth that serves as food for larvae (Arcos *et al*., 2018; Chirebvu and Chimbari, 2015; Fillinger *et al*., 2009). Some studies

reported that the distribution or wide spread oviposition habitats of *Anopheles gambiae* mosquitoes was characterized by high turbid water that was near human dwellings (Kabula *et al.*, 2011; Kenea *et al.*, 2011; Paaijmans *et al*., 2008) while others have reported turbid water by gravel roadside burrow pools (Herrera-Varela *et al.*, 2014). On the other hand, others have reported that *Anopheles arabiensis* which is a sibling within the *An. gambiae* complex preferred water that was clear with low turbidity (Elmalih *et al*., 2018). Climatic weather seasons affect many physical and chemical properties of water. Run-off water and sedimentation during rainy season causes breeding habitats to have high content of soil particles, agrochemicals, and organic matter all which may contribute to increasing turbidity and conductivity of water at an oviposition habitat (Getachew *et al*., 2020; Kenea *et al*., 2011; Chaves, and Kitron., 2011). Organic matter and agro-chemicals especially fertilizers not only contribute to raising turbidity but they also provide nutrients for microbial growth that serves as food for larvae (Arcos *et al*., 2018; Chirebvu and Chimbari, 2015; Fillinger *et al*., 2009).

2.3.5 Water background colour.

Except for very few early studies on *Anopheles* mosquitoes (Bates, 1940), research on effect and application of background colour on various behavioural aspects in mosquitoes has been dominated by studies on culicine mosquitoes especially on *Aedes* species. Studies undertaken on background colour selection have shown that colour influences oviposition site selection by gravid female mosquitoes (Hellhammer *et al*., 2022; Dibal *et al.*, 2012; Bidlingmayer, 1994). It is also important to note that although colour is a physical property of water, its effect on oviposition site selection by gravid female *Anopheles* mosquitoes has been understudied when compared to other water physical properties although colour at an oviposition site may be the most important environmental stimuli when a gravid female mosquito is in an oviposition flight (Burkett, and Butler, 2005; Yanoviak, 2001). The few studies on the effect of colour on oviposition site selection by anophelines have reported preference for red colour (Omolade and Adetutu 2018). In the most recent studies, *Anopheles coluzzii* and *Aedes aegypti* mosquitoes were reported to be preferentially attracted to red colour before exposure to an olfactory stimulant which influenced *An. coluzzii* to change its preference to black (Alberto *et al*., 2022) but in a dual choice experiment *Anopheles* oviposited more on black than white surface (Pavlovich and Rockett, 2000).

Some field studies have reported that gravid female *Anopheles* mosquitoes preferred to lay eggs in water in black-coloured containers (Liu *et al.*, 2014; Badamasi *et al.*, 2008; Bates, 1940) while much recent studies both in *Anopheles* and *Aedes* mosquitoes have shown preference to red colour (Alberto *et al*., 2022; Omolade and Adetutu 2018). Colour of oviposition containers has been reported to increase egg hatchability in *Anopheles sinensis* and culicines (Liu *et al.*, 2014; Chua *et al.*, 2004; Collins and Blackwell, 2000). Field studies on colour preferences in culicine and lesser extent anopheline mosquitoes have reported that black and red colours were most preferentially selected colours over other colours (Panigrahi *et al.*, 2014; Badamasi *et al*., 2008; Muir *et al*., 1992). It has been observed that reports of different results and interpretations concerning colour stimuli-response experiments even within the same species of mosquitoes are common (Muir *et al*., 1992). Observations by Muir *et al*., (1992). Dipterans share many physiological, morphological and behavioural features. Based on these similarities, colour-odour baited traps used for control of tsetse flies, *Glossina,* (Diptera: Glossinidae), greatly influenced the concept of using traps for mosquito control (Kline, 2006; Kline *et al.,* 1990). In experiments testing colour landing preferences in tsetse flies, *Glossina morsitans* (Diptera: Glossinidae), a similar colour selection pattern was observed (Vale, 2009; Harrington *et al.,* 2008; Green, 1993; Green and Flint, 1986) and the results were used to change the design and improve the efficacy of tsetse fly traps. Similar landing experiments showed that *Musca domestica* (Diptera: Muscidae) had a preference for red colour substrate over other colours (Diclaro *et al*., 2012) in almost exact preference order as reported for *Anopheles gambiae* s.s. (Phiri and Mbata, 2023). It has been suggested the similarities in response to colour was possibly due to the similarities of the visual sensory systems of these Dipteran insects which use their compound eyes for locating objects (Alberto *et al*., 2022; Hellhammer *et al*., 2022; Muir *et al*., 1992). Adapting different aspects of technologies for control of other Diptera disease vectors has led to the development of different mosquito trap designs that are used for both entomological surveys and for control of mosquitoes (Mweresa *et al.,* 2020; Sougoufara *et al.*, 2020; Okumu *et al.,* 2013; Poulin *et al*., 2017; Hiscox. *et al.,* 2016; Homan, 2016; Quarles, 2003). The use of colour contrast in oviposition traps has led to a suggestion that these traps could be integrated as a tool in control of malaria and arbovirus (Hellhammer *et al*., 2022; Garcia-Rejon, 2011). It has also been

suggested that data and knowledge on colour preferences can be used to enhance tools used to improve management of insectaries (Liu *et al*., 2014).

Whereas background colour or light reflections coming from an oviposition site together with hydrological factors are used by gravid female mosquitoes to locate an oviposition site, the final selection of an oviposition site is dependent on other factors and especially semiochemicals (Bidlingmayer, 1994). A combination of visual and semiochemicals stimuli enhances oviposition site selection behaviour in mosquitoes (Panigrahi *et al.*, 2014; Li *et al.*, 2010; Chua *et al.,* 2004) and underlying the complex synergistic ways that different factors affect oviposition site selection.

2.3.6 Odours and semiochemicals.

Upon locating an oviposition site using the visual or optic sensory organs a gravid mosquito uses its olfactory and tactile sensory systems to navigate types of odours or semiochemicals and physical chemical properties of the water respectively in order to select an oviposition site. There are different sources of semiochemicals at an oviposition site where some of them may elicit opposite or opposing reactions in gravid female mosquitoes. Among the important semiochemicals are infochemicals which stimulate different responses or reactions in mosquitoes.

Infochemicals include pheromones and kairomones which are among the infochemicals that are found at oviposition sites (Lindh *et al.,* 2015; Navarro-Silva *et al.,* 2009). Some of these pheromones and kairomones are released by organisms to send a particular message and elicit or trigger a necessary physiological or behavioural response (Asmare *et al.,* 2017; Ponnusamy *et al.,* 2008; Dicke and Sabelis, 1988).

Other infochemicals found at an oviposition site are apneumones. Apneumones are released from dead organic matter in the environment and they also trigger a specific response in gravid anopheline mosquito (Navarro-Silva *et al.,* 2009).

Although semiochemicals play a very important role in oviposition site selection, there is limited knowledge about specifics of how mosquitoes respond to different semiochemicals (Mweresa *et al*., 2020; Sougoufara *et al.*, 2020; Wooding *et al.*, 2020; Watentena and Okoye, 2019; Vanickova *et al.*, 2017; Lindh *et al.,* 2015; Ong and Jaal, 2015).

2.3.7 Larval and predator habited sites.

The presence of mosquito larval instars or predators affects and influences the selection of an oviposition site either to lay eggs or move away and look for another site. Although there is general agreement that presence of larval instars influences oviposition site selection, different studies have reported different results, interpretation and conclusions. Some studies have reported that gravid female mosquitoes avoided water that was pre-inhabited by larval instars or predators or water that had certain bacterial-derived odours (Zattel *et al*., 2013; Warburg *et al*., 2011; Navarro-Silva *et al.,* 2009; Samanidou-Voyadjogloul *et al.,* 2007; Huang *et al*., 2006; Lowenberger and Rau, 1994). Other studies have reported that water that was inhabited by early instars was preferred for oviposition by gravid *Anopheles* mosquitoes and it was suggested that first instars emitted some semiochemicals (nonane, and 2,4 Pentane dione (2,4-PD) that were attractive to gravid females (Eneh *et al*., 2019; Maia *et al.*, 2018; Liu *et al.*, 2014; Stewart *et al.*, 2013; Takken, 2010; Sumba *et al.*, 2004; McCrae, 1984). Freshly set up artificial ponds were reported to contain twice as many larvae instars of *Anopheles arabiensis* compared to older artificial ponds leading to a suggestion that as older instars emerged and dominated the ponds the efficacy of water in those ponds to elicit gravid females to oviposit or lay eggs wained (Eneh *et al*., 2019). Recent studies have reported that emanations from first instar larvae induced egg laying in gravid female *Anopheles gambiae* (Mwingira *et al*., 2020; Schoelitsz *et al*., 2020) while late instar emanations were associated with some refractile water odours (Shmeis, 2018) or semiochemicals identified as dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) which were also known to cause egg retention in gravid female *Anopheles gambiae* mosquitoes (Schoelitsz *et al*., 2020; Maia *et al.*, 2018; Suh *et al.,* 2016; Stewart *et al.*, 2013; Takken, 2010). The refractile semiochemicals DMDS and DMTS may also be indicative of aquatic harmful bacterial activity (Flick, 2011) which would signal sub-optimal oviposition food depleted habitat to an oviposition-site searching gravid female mosquito (Arcos *et al*., 2018; Suh *et al*., 2016). The identification of such semiochemicals

associated with oviposition sites is significant in their potential use in the development of alternative methods for control of *Anopheles* mosquitoes through a possible push-pull or pull-kill system in an integrated vector control programme (Mwingira *et al*., 2020; Wooding *et al*., 2020). Based on knowledge of physiological and behavioural response to specific cues, the "Push-Pull" strategy (Zahavi, 2007), or the "Pull-Kill" strategy (Michaelakis *et al.*, 2007) for controlling crop pests have recently been adapted for use in the control of malaria vectors in Kenya (Menger *et al.*, 2015). Therefore, knowledge understanding of these factors, could significantly contribute towards development of alternative methods for combating mosquito borne diseases.

Although oviposition has recently been an active area of research, factors that influence oviposition site selection are still not well known or understood (Eneh *et al.*, 2019). This study was undertaken to understand the effect of some factors on oviposition site selection by gravid female anopheline mosquitoes in the field and under laboratory conditions.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Areas.

This study was undertaken in Zambia (Fig. 3) in three districts of Lusaka province (Fig. 4) namely, Lusaka (Fig. 5), Chongwe (Fig. 6) and Rufunsa (Fig. 7).

The study area lies between latitudes 15° and 16° S and longitudes 29° and 30° E. The distances from Lusaka city to the study area sampling sites in Chongwe and Rufunsa are 55km and 165km, respectively. The specific field sampling points in the study areas are indicated in Table. 2.

Figure 3. Map of Zambia with provinces and neighbouring countries.
(Source: mapsofindia.com).

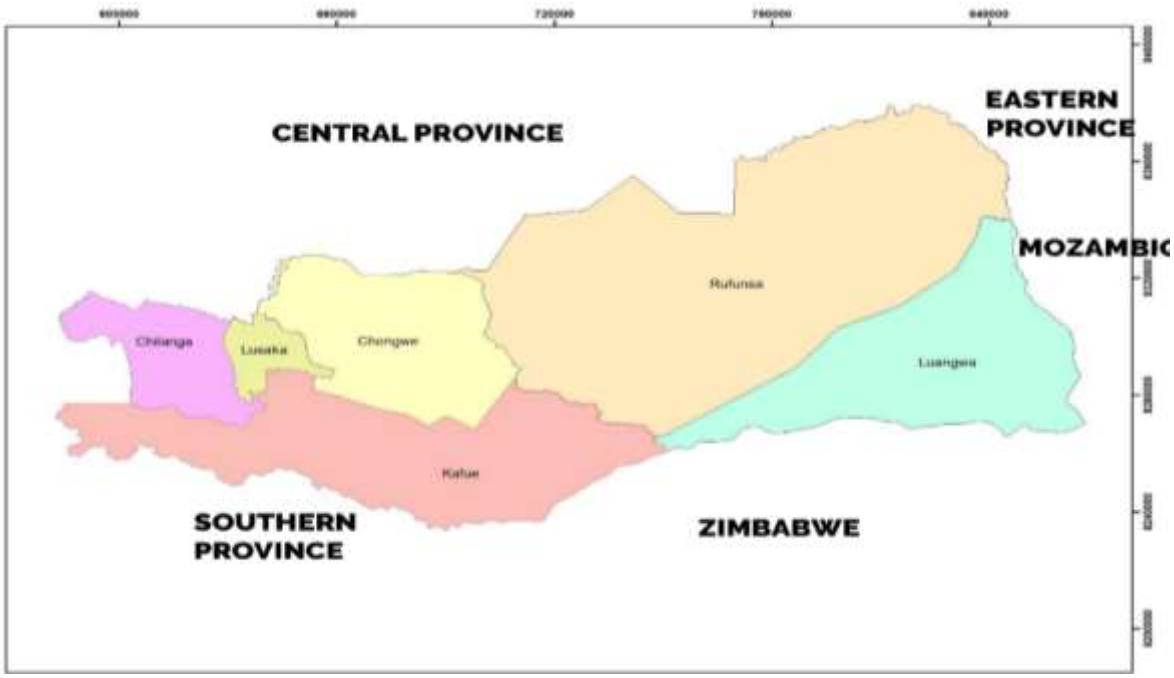

Figure 4. Map of Lusaka Province and its districts.

Figure 5. Lusaka city sampling sites at Manchinchi waste water treatment plant (MWWTP). (Source: Cartographic Office, University of Zambia.

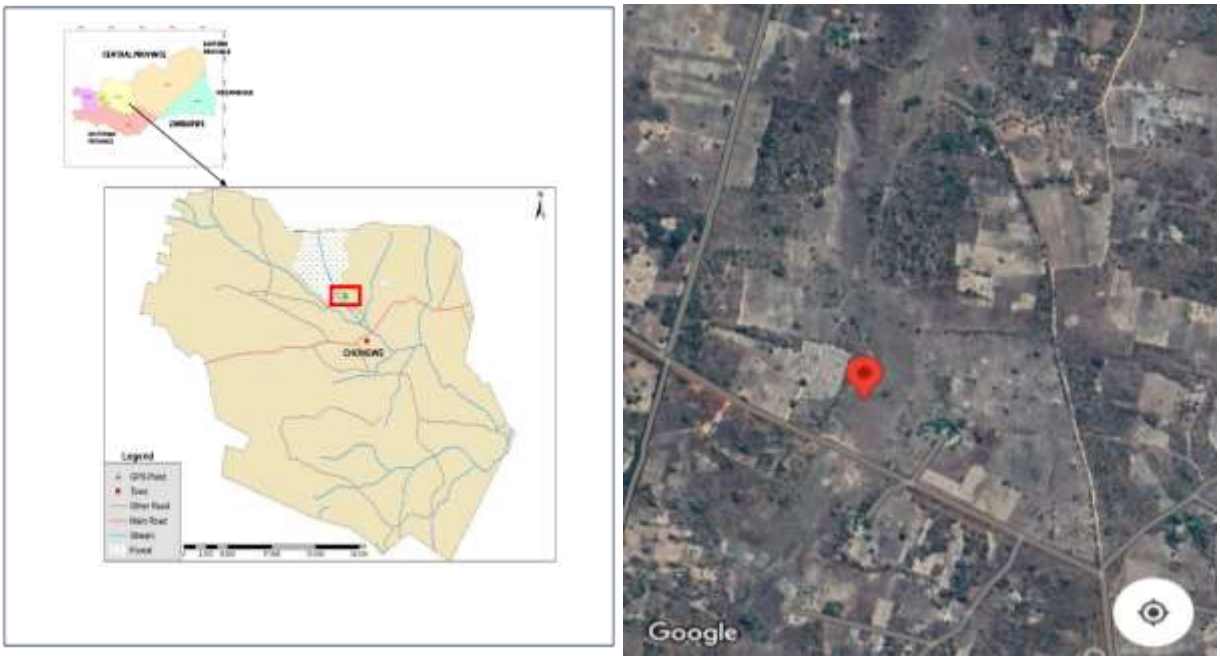

Figure 6. Chongwe district sampling site in Shiala village area.
(Source: Cartographic Office, UNZA for District cartographic maps). Satellite maps (Source: Google Maps).

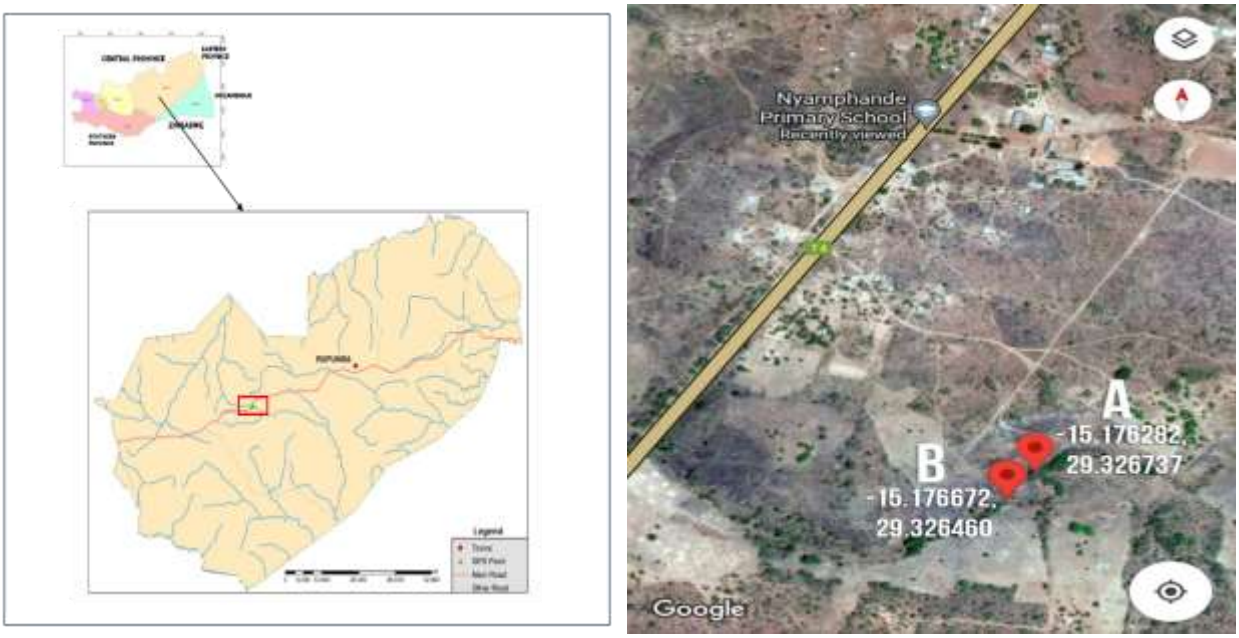

Figure 7. Rufunsa district sampling sites in Nyamphande village area.
(Source: Maps for province and district were sourced from the Cartographic Office, University of Zambia. Satellite maps sourced from Google maps.

Table 2. GPS co-ordinates for the field sampling sites.

Chongwe district		
Oviposition sampling site		GPS-co-ordinates
1	Shiala villages domestic well	Latitude, 15°27'26.9"S; Longitude, 28°65'52.1"E
Rufunsa district		
2	Nyamphande-A	Latitude, 15°17'628.2"S; Longitude, 29°32'67.4"E
3	Nyamphande-B	Latitude, 15°17'667.2"S; Longitude, 29°32'64.6"E
Lusaka city- Manchinchi waste water treatment plant (MWWTP)		
	Sampling point	Co-ordinates
4	MWWTP-1	Latitude, 15°23'33.5"S; Longitude, 28°17'49.4"E
5	MWWTP-2	Latitude, 15°23'32.4"S; Longitude, 28°17'49.3"E
6	MWWTP-3	Latitude, 15°23'30.7"S; Longitude, 28°17'47.1"E
7	MWWTP-4	Latitude, 15°23'33.8"S; Longitude, 28°17'39.9"E
8	MWWTP-5	Latitude, 15°23'33.6"S; Longitude, 28°17'40.2"E
9	MWWTP-6	Latitude, 15°23'35.3"S; Longitude, 28°17'38.0"E
10	MWWTP-7	Latitude, 15°23'38.9"S; Longitude, 28°17'37.6"E
11	MWWTP-8	Latitude, 15°23'40.9"S; Longitude, 28°17'37.8"E
12	MWWTP-9	Latitude, 15°23'42.6"S; Longitude, 28°17'39.5"E
Garden sewerage stabilisation ponds (GSSP)		
	Sampling point	Co-ordinates
13	GSSP-1	Latitude, 15°22'45.8"S; Longitude, 28°17'29.2"E
14	GSSP-2	Latitude, 15°22'46.5"S; Longitude, 28°17'29.4"E
15	GSSP-3	Latitude, 15°22'45.7"S; Longitude, 28°17'29.8"E
16	GSSP-4	Latitude, 15°22'45.5"S; Longitude, 28°17'30.8"E
17	GSSP-5	Latitude, 15°22'45.6"S; Longitude, 28°17'31.2"E
18	GSSP-6	Latitude, 15°22'46.1"S; Longitude, 28°17'31.4"E
19	GSSP-7	Latitude, 15°22'46.0"S; Longitude, 28°17'30.4"E
20	GSSP-8	Latitude, 15°22'47.0"S; Longitude, 28°17'30.1"E
21	GSSP-9	Latitude, 15°22'46.5"S; Longitude, 28°17'29.9"E
22	GSSP-10	Latitude, 15°22'47.1"S; Longitude, 28°17'29.6"E
UNZA Great East Road Campus (UNZA-GER)		
	Sampling point	Co-ordinates
23	UNZA-GER-1	Latitude, 15°23'47.5"S; Longitude, 28°19'25.3"E

24	UNZA-GER-2	Latitude, 15°23'44.9"S; Longitude, 28°19'26.9"E
25	UNZA-GER-3	Latitude, 15°23'44.5"S; Longitude, 28°19'25.9"E
26	UNZA-GER-4	Latitude, 15°23'43.2"S; Longitude, 28°19'27.6"E
27	UNZA-GER-5	Latitude, 15°23'43.6"S; Longitude, 28°19'27.8"E
28	UNZA-GER-6	Latitude, 15°23'16.7"S; Longitude, 28°19'48.4"E
29	UNZA-GER-7	Latitude, 15°23'17.5"S; Longitude, 28°19'47.7"E
30	UNZA-GER-8	Latitude, 15°23'19.5"S; Longitude, 28°19'49.3"E
31	UNZA-GER-9	Latitude, 15°23'20.7"S; Longitude, 28°19'48.3"E
32	UNZA-GER-10	Latitude, 15°23'23.1"S; Longitude, 28°20'00.6"E
Roma residential area		
	Sampling Point	Co-ordinates
33	A-Ngwezi Road	Latitude, 15°36'55.04"S; Longitude, 28°30'59. 3"E
34	B-Kiola Road	Latitude, 15°37'24.41"S; Longitude, 8°30'79.4"E

3.1.1 General climatic and socio-environmental conditions.

The study areas fall in Zambia's Agro-Ecological Region II soil and hydrological based classification with an altitude of 1,300 to 1,272m above sea level with average temperatures range of 10.1^{O}C in June to 31.6^{O}C in October and average annual rainfall of 800mm (MWSE, 2017). The study areas have two distinct environmental and social economic conditions. Whereas Lusaka city is mainly an urban built environment with a population of 2,204,059 in 2022, Chongwe and Rufunsa districts are rural districts with low populations of 312,000 and 83,000, respectively (ZamStats, 2022). The three districts sit on many wetlands and natural water streams that serve as potential mosquito breeding sites. Both Nyamphande and Shiala study areas are characterised by fertile Agro-Ecological Region II soils (WB, 2007) that support subsistence rain-fed agriculture dominated by growing of maize and soya beans. Livestock rearing is common and dominated by indigenous free-range chickens, goats and cattle. Horticultural activities dominate the dry season agriculture whose source of water are stagnant pools and shallow wells which unintendedly also serve as breeding sites for mosquitoes during the dry seasons.

3.1.2 Vegetation type.

The vegetation type found in the study areas is Miombo savannah woodland with *Brachystegia spiciformis* (Benth) and *Brachystegia boehmii* species being dominant but occasioned by other species including *Isoberlinia* (MNR, 1984; FD, 1979). The hilly stony areas are dominated by *Brachystegia spiciformis, Brachystegia boehmii, Julbernardia globiflora* (Benth.) Troupin and *Jubernadia paniculata* (Benth.) Troupin (FD, 1979). The sampling sites are characterised by wooded grassland forest type dominated by dambo and plains (FAO, 2006). In Nyamphande sites, the dominant trees were *Syzygium cordatum* while *Acacia polyacantha* species (FD, 1979) dominated the dambo grass lands.

3.2 Research Design.

Research design is a blue print of steps that are needed to undertake research study and includes experimental design (Creswell, 2009). The research design for this study is quantitative incorporating all elements from research concept, literature review, research question and quantitative experimental designs and methods. This quantitative research design was used to generate empirical data with minimised experimental errors by using replication of experimental

units to ensure that such data was reproduceable but also publishable in peer-reviewed international journals. The study was split into field and laboratory components and was implemented through randomized quantitative experiments to generate data guided by research questions of set objectives with a general Hypothesis to predict two alternative outcomes that would statistically be tested.

Therefore, the Research Design was quantitative and implemented by employing randomized field and laboratory quantitative experimental methods that increased sensitivity of each experimental treatment to reduce error associated with uncontrollable natural variables (Ross, and Morrison, 2003; Johnson, and Besselsen, 2002) for the generation of original data that were statistically used to make inferences (Creswell, 2009; Fawcet, and Downs., 1986).

The model test insect was anopheline mosquitoes both in the field and laboratory while the laboratory animal used was mice obtained from the University of Zambia but managed by laboratory authorized staff at the National Malaria Elimination Centre (NMEC) in Lusaka and at the Tropical Diseases Research Centre (TDRC) in Ndola respectively. No human subject or large animal was used throughout the research.

3.3 Sample Size.

In order to balance the needs of this study and the capacities of the two insectaries at the National Malaria Elimination Centre (NMEC) and the Tropical Diseases Research Centre (TDRC), the experimental unit sample size was decided to be 30 gravid female *Anopheles gambiae* s.s., KISUMU (Diptera: Culicidae) as recommended for studies involving animals in the laboratory (Festing, 2018) and in the field (Jachmann, 2001). The gravid female mosquitoes that were used for laboratory experiments were sourced from reared colonies at the NMEC in Lusaka and at the TDRC in Ndola.

3.4 Field and laboratory sampling and experimental methods.

The study employed longitudinal sampling techniques of mosquito oviposition sites. In the original design, field sampling for anopheline mosquito larvae were planned to be undertaken across the year to capture seasonal changes and were undertaken twice every month in December, April, September between 2018 and 2020. During the same time, water from the sampling sites in Lusaka

city was taken to both the National Malaria Elimination Centre (NMEC) and the National Institute for Scientific and Industrial Research (NISIR) in Lusaka. The water that was taken to the NMEC laboratory was used for oviposition tests. Water taken to the laboratory at NISIR was used to determine physical chemicals parameters namely pH, temperature, turbidity and conductivity. However due to failure to find anopheline oviposition sites in Lusaka city, the study was repeated for a period of nine months in areas with proven malaria transmission in Chongwe and Rufunsa districts of Lusaka Province.

The repeat experiments including field and laboratory ones were undertaken from June 2022 to February 2023. The repeated field experiments were undertaken during the dry season and a period of consecutive drought seasons in Zambia which resulted in drying up of what would normally be perennial water pools and some major water systems including those associated with dambos. This reduced the chances of finding anopheline breeding sites. Against this background, the study areas were chosen on the basis of current information indicating that the areas were located in areas of high malaria transmission (MoH, 2019; Kalubula *et al*, 2015). Further advice was sought from the National Malaria Elimination Centre (NMEC) who suggested possible areas to find anopheline oviposition sites would most likely be found in Chongwe and Rufunsa districts of Lusaka province.

Field sampling in Chongwe district was discontinued in October 2022 because the site dried up due to the consecutive droughts of the years 2020 and 2021 while the sampling in Rufunsa was terminated in the second week of November 2022 after the sampling sites were disturbed by traditional fishing poisoning methods that eliminated larvae. In total, 13 sampling events were undertaken in Chongwe district while 18 sampling events were done in Rufunsa district between June and November 2022.

Experimental mosquitoes were kept in standard WHO approved 30 x 30 x 30 cm cages. Each cage contained 30 (*n*) gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes. All mosquito cages were provided with cotton pads with a sugar (10%) solution for feeding the experimental mosquitoes. The laboratory room temperature was maintained at 28^{O}C using LG laboratory heater (Fig.5). Due to lack of an automatic humidifier, relative humidity (RH) of the room was manually maintained between RH 48 and 62 using cotton towels that were soaked in hot water basins every evening starting at 18:00 hours and repeated the following day at 10:00 hours. Temperature and humidity were monitored using a Max-Min Thermo HYGRO, INSTEN Thermo meter (Fig. 8).

Figure 8. LG Laboratory heater (Left) and INSTEN Thermo meter (Right).

3.4.1 Identification of oviposition sites selected by gravid female anopheline mosquitoes.

Identification of oviposition sites was done by sampling or scooping of any stagnant water body for presence of anopheline mosquito larvae using a standard 350mls WHO approved dipper. Anopheline oviposition sites were positively identified by presence of anopheline larvae which were identified by their parallel water surface dwelling behaviour. All ~~the~~ captured larvae at each site were counted. Larval density was determined as the number of larvae counted in a dipper (Getachew *et al*., 2020; Masaninga *et al*., 2012).

The number of sampling dips depended on the size or surface area of an oviposition site where more dips were undertaken for larger surface oviposition site than at the smaller site (Hinne *et al*., 2021). Due to the small surface area of the Shiala site in Chongwe district, 5 successful dips using a 350mls WHO approved dipper were undertaken per sampling event whereas 15 dips were undertaken at each of the larger surface area sampling sites at Nyamphande sites in Rufunsa district. All the identified anopheline oviposition sites were GPS coded using GPS Software on Samsung J6+ camera. Some unique ecological attributes of anopheline oviposition sites such as appearance of water and vegetation cover were captured on camera.

After counting larvae, third and fourth instars were separated using 3ml calibrated pipettes and put into breeders and into temperature insulated cooler boxes for transportation to the

laboratory to allow for emergence and identification of adults. Adults were identified morphologically using standard keys (Coetzee, 2020; Kent, 2006; Gillies and Coetzee, 1987). Sibling species of the *Anopheles gambiae* complex mosquitoes were identified by use of the polymerase chain reaction (PCR) technique (Scott et al., 1993).

3.4.1.1 Identification of anopheline mosquitoes using standard morphological keys.

The main morphological features used in the dichotomous keys for the identification of the species that were found in the study area were the wing venation and appearance of pale or dark spots on wing veins and hind tarsus (Gillies, and Coetzee, 1987). These dichotomous keys present a series of leading questions with two possible outcomes or choices which were followed until a species name was reached (Coetzee, 2020). In addition to the standard keys (Coetzee, 2020; Gillies and Coetzee, 1987), a simplified pictorial guide developed by Kent (2006) was also used to morphologically identify *An. coustani* and *An. pretoriensis* mosquito species.

(i) *Anopheles gambiae* complex.

The main morphological features used were wing venation, where the 3rd main dark spot of vein 1 with a pale interruption fused with preceding pale area and hindleg speckles as dark and pale spots were used to identify *An. gambiae* complex (Coetzee, 2020; Gillies and Coetzee, 1987).

(ii) *Anopheles coustani*.

The main morphological characteristics used in the identification of *An. coustani* were the hind tarsus and wing venation. The base of the hind tarsus is broadly pale (Kent, 2006) and there is no pale fringe spot opposite vein 5.2 (Gillies and Coetzee, 1987).

(iii) *Anopheles pretoriensis*.

The main morphological characteristics used in the identification of *An. pretoriensis* were the hind tarsus 1 which is broadly pale at apex and wing venation where vein 1 of wing has 2 pale spots (Gillies and Coetzee, 1987).

3.4.1.2 Identification of *Anopheles gambiae* complex mosquitoes by polymerase chain reaction (PCR) technique.

The morphologically identified adult *Anopheles gambiae sensu lato* mosquitoes were preserved individually by silica-drying them in microtubes. Later each individual mosquito was bisected into abdomen and thoracic region halves. Both halves were then crushed and used for deoxyribonucleic acid (DNA) extraction for PCR analysis. *Anopheles gambiae* sibling species were separated or identified using specific DNA primers (Table 3). The four *Anopheles gambiae* complex sibling species are *Anopheles gambiae sensu stricto, Anopheles arabiensis*, *Anopheles merus* and *Anopheles quadriannulatus* (Scott *et al*., 1993).

Table 3. Primers used to identify sibling species of *Anopheles gambiae* complex mosquitoes.

Primer	Name of sequence (5' to 3')
UN	5'GTG TGC CCC TTC CTC GAT GT3'
GA	5'CTG GTT TGG TCG GCA CGT TT3'
AR	5'AAG TGT CCT TCT CCA TCC TA3'
ME	5'TGA CCA ACC CAC TCC CTT GA3'
QD	5'CAG ACC AAG ATG GTT AGT AT3'

Reaction mixture: 25 µL.

The crushed single mosquito components were added to the tube containing 25.0 µl of PCR reaction mixture. The reaction mixture contained the following: 10 x 2.5 µL of dNTPs 2.5 mM 2.0 µL (final conc. 200 µM each), ME 3.0 µL (150 pmol), AR 3.0 µL (150 pmol), QD 3.0 µL (150 pmol), GA 0.5 µL (25 pmol), UN 1.0 µL (50 pmol), Taq 1.5 U, dH20 fill to 25µL. This mixture contained the following: 10 x 2.5 µl of double distilled water. Use 1 µL of template DNA.

PCR program.

The PCR program for *An. gambiae* was run as follows: Step 1; DNA polymerase activation at 94 °C for 10 min, Step 2; DNA denaturation at 94° C for 30sec, Step 3: Annealing at 50° C for 30sec, Step 4; Extension at 72° C for 30sec (step 2 to step 4 repeated 30 times) and final extension at 72° C for 7min. The DNA amplicons were loaded on 2% Agarose gel (containing 1 x TBE Buffer and stained with ethidium bromide.

The DNA fragments were visualized under ultra violet (UV) light and the size of the products was confirmed using the species PCR molecular ladder as follows; PCR ID DNA LADDER *An. gambiae* s.s. (390bp), *An. arabiensis* (315bp), *An. merus* (466bp), *An. quadriannulatus* (153bp) and a negative control (Scott *et al*., 1993).

3.4.2 Determination of physical chemical properties of field water on oviposition site selection.

At the oviposition site, water was randomly scooped from three points of the oviposition site area using a dipper and put into clean testing bottles and analysed for pH, temperature (^{O}C), conductivity (µS/cm) and turbidity (NTU) using portable water testing equipment. Temperature (^{O}C), conductivity (µS/cm) and pH of field water was tested using Multi-tester *PC Testr 35*, Eutech Oaklon Instruments while turbidity (nephelometric turbidity units-NTU) was analysed using *HACH* 21000 Instruments. Before re-using any testing bottle, it was rinsed with 70% alcohol. A total 54 water samples were analysed at each of the two sites in the field giving a grand total 108 samples analysed. The characterised field water with known water physical chemicals from the identified oviposition sites was put into labelled 500ml separate collection bottles and transported in a cooler box to the laboratory at the National Malaria Elimination Entre for tests on its efficacy to influence oviposition site selection by laboratory reared gravid female *Anopheles gambiae* s.s., KISUMU. The water was compared to the impact of distilled water (the control) on oviposition site selection by the gravid anopheline mosquitoes.

3.4.3 Determination of relationship between physical chemicals and larval densities.

Determination of relationship between physical chemicals and larval density was undertaken in three stages,

i. Larval densities at the two sites were compared using both descriptive and parametric statistics to determine any statistical difference.
ii. Data on physical chemicals collected at sampling stage was analysed by both descriptive and parametric statistics to determine differences.
iii. Spearman linear regression correlation (r_s) (Millar, 2001) was used on respective variables, i.e., larval densities and the four physical chemicals pH, conductivity, turbidity and temperature of water from each oviposition site to determine the correlation between each physic-chemical variable and larval counts or densities.

3.4.4 Comparison of efficacy of field and laboratory water on oviposition site selection.

Field water from Nyamphande sites A and B and distilled water was placed separately in equal amounts of 30mls in clearly marked 36mls capacity oviposition cups of the same colour and placed in three different 30 x 30 x 30 cm standard mosquito cages each containing a group of 30 gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes, to test their water preferences for oviposition. After 24hrs, the oviposition cups were inspected for presence of eggs which were counted according to the type of water in which they were deposited. The egg counts were used to determine efficacy of the different water types to influence oviposition site selection by gravid female *An. gambiae* s.s., KISUMU. Egg counts were done for three consecutive days after which mosquitoes were given a blood meal and the egg count resumed for another three days after which most of the mosquitoes had died and the few surviving ones had stopped laying eggs.

Water from Shiala site was separately but similarly compared with distilled water as was done above for Nyamphande sites but treated as an unpaired type of experiment since there was only one site found in the Chongwe area.

The experiments were repeated 23 times between August 2022 and January 2023 with the bulk of the experiments during the period November 2022 to January 2023.

3.4.5 Determination of the effect of time and larval pre-habitation of water on efficacy on oviposition site selection.

Time in this experiment was related to duration of development for specific immature stages namely the egg, first larval instar (L1), second larval instar (L2), third larval instar (L3), fourth

larval instar (L4). Time was determined as follows, egg pre-inhabited water (0 - 1 day), L1-L2 (2 - 4 days), L3 to pupae (5 - 10) and cohort representing water that had been used continuously from egg-adult (0 - 14 days). The duration time was based on local laboratory observations at NMEC, TDRC and as similarly reported in other studies (Batume *et al*., 2022).

(i) Egg water was obtained from oviposition cups and freshly laid within the past 12 hours
(ii) L1-L2, and L3-pupae water was separately processed by first cleansing the instars in distilled water and later transferring them according to their developmental groups in clean distilled water
(iii) Cohort water was a mixture of egg, L1-L2 and L3-pupae pre-inhabited water that was mixed by shaking.
(iv) All the different water was kept for 24 hours before presenting to gravid female mosquitoes.

Thirty (30mls) of the processed water pre-inhabited according to the four different immature groups was put in oviposition cups and clearly marked according to type of water in them and then presented to 30 blood-fed gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes in each of the three mosquito cages in order for the gravid female mosquitoes to select the type of water they prefer for oviposition. After 24 hours all oviposition cups were inspected for the presence of eggs and for egg counts. The experiment was repeated 23 times starting in September 2022 and completed on 13th January 2023.

3.4.6 Determination of effect of water background colour on oviposition site selection.

Thirty (30) gravid blood-fed female *Anopheles gambiae* s.s., KISUMU mosquitoes were put into each of five different mosquito cages set in a 5 x 5 Latin Square Design layout. The mosquitoes were presented a choice of five randomly laid oviposition cups of five different colours each containing 30mls of distilled water. After 24 hours all the oviposition cups in the cages were inspected for presence of eggs which were counted according to colour of oviposition cup. The experiment was repeated 42 times starting on 18th June 2018 and ending on 19th July 2019.

3.5 Data analysis.

All Data collected in each experiment were entered on spreadsheets and analysed using IBM SPSS Statistics software Version 26 and Microsoft Excel to test for the statistical differences among Means of different variables as well to test Hypotheses of the study objectives. Descriptive statistics, Mean, Standard Deviation (SD), Standard Error, and graphs were used to show differences in results of the experiments (Krzywinski and Altman, 2013; Cumming, *et al*., 2007). Significance of differences of the Means of variables in the experiments and Hypotheses were tested using inferential or non-descriptive statistics one-way analysis of variance (ANOVA) and Tukey HSD post hoc tests for multivariate analysis. The *t-test P* and Spearman regression analysis for relationships was performed using Microsoft Excel in Windows 10. Significance was at 95% Confidence Interval

CHAPTER 4: RESULTS

4.1 Identification of anopheline oviposition sites selected by gravid female anopheline mosquitoes.

4.1.1 Oviposition sites.

Three oviposition sites of *Anopheles* mosquitoes were found in Chongwe and Rufunsa districts in May 2022. No oviposition sites of anopheline mosquitoes were found in Lusaka city in this study. In Chongwe district the oviposition site was located in Shiala village area. It was a shallow domestic well whose water had some algae in it (Fig. 9). The diameter of the water well was approximately 50cm and it had a depth of 1.5m.

Figure 9 Oviposition site in Shiala village area.

In Rufunsa district, two oviposition sites were found in Nyamphande village area (Fig. 10).

Figure 10, Nyamphande site-A (Left) with *Syzygium cordatum* trees and Nyamphande site-B (Right) Oviposition Sites in Rufunsa District. Note water lilies and algae at site-B.

All the three sites were in dambo wetland areas with characteristic turbid water. The Chongwe sampling site was characterised by short grass while the Rufunsa sites were surrounded by long grass, reeds and *Syzygium cordatum* trees. The pools of water also had a lot of dead leaves under which some anopheline larvae were found. Nyamphande site-B had more aquatic biodiversity in terms of plant and animal life compared to Nyamphande site-A (Figure 7). Among the plants that were found at Nyamphande site-B include grass, water lilies, algae and reeds. Nyamphande site-A did not have water lilies or algae. The animal life found at both sites included fish, frogs and some invertebrates such as larvae of dragonflies, anopheline and culicine mosquitoes and some water mites.

4.1.2 Anopheles species found in the study areas.

4.1.2.1 Chongwe District.

A total of 875 anopheline larvae were collected at Shiala sampling site of which 153 were third and fourth instars out of which 49 emerged as adults. The adults were identified by both morphological and molecular techniques as 32 female and 16 male *Anopheles gambiae* s.s. and only 1 female *Anopheles pretoriensis* representing a 98% (*Anopheles gambiae* s.s.) and 2% (*Anopheles pretoriensis*).

4.1.2.2 Rufunsa District.

In Rufunsa district, the sites were in the form of stagnant water pools of the drying Nyamphande river in Nyamphande village areas and were named as Nyamphande-A and Nyamphande-B sampling sites. The sites were approximately 50m apart and each with surface area of approximately $35m^2$ and a depth of 1.2m. A total of 920 anopheline larvae were collected from the sites, of which 195 were third and fourth instars and from which 40 adults emerged. There were 38 *Anopheles coustani* mosquitoes segregated as 26 females and 12 males or represented as *Anopheles coustani* (95%) and 2 *Anopheles gambiae* (5%). PCR characterisation (Fig.11), confirmed the identification of *Anopheles gambiae* samples from Shiala and Nyamphande as *Anopheles gambiae* s.s. (Table.4).

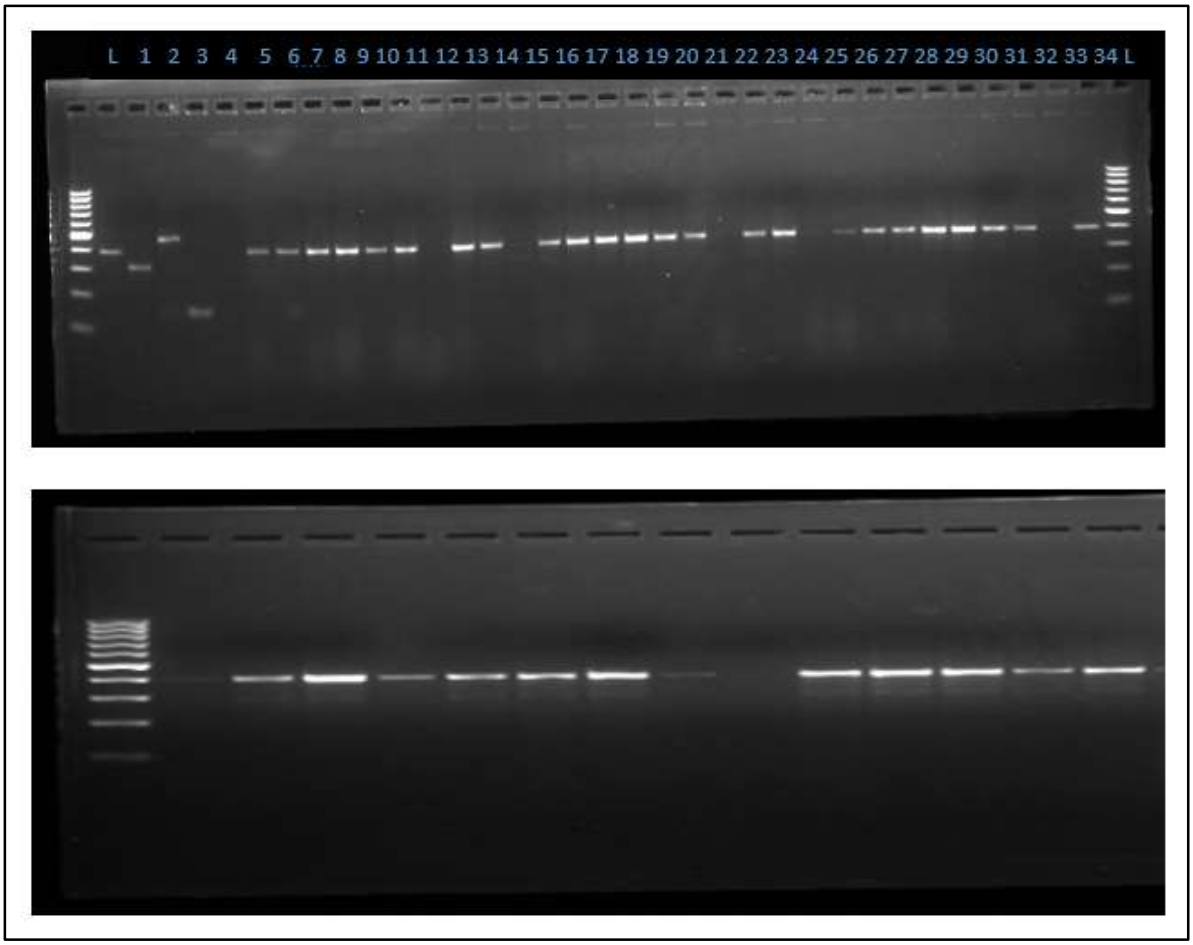

Figure 11. Polymerase chain reaction (PCR) amplified fragments for identification of *Anopheles gambiae* complex species (Scott *et al*., 1993).

Table 4. Polymerase chain reaction (PCR) primers and sizes of the amplified products for molecular forms within the *Anopheles gambiae sensu stricto* (Scott et al., 1993).

LANE #	Lab ID	PCR ID	Location	Sex
L	Ladder	DNA LADDER	n/a	
1	Control	An. gambiae s.s. (390bp)	n/a	
2	Control	An. arabiensis (315bp)	n/a	
3	Control	An. merus (466bp)	n/a	
4	Control	An. quadriannulatus (153bp)	n/a	
5	Control	Negative control	n/a	

6	JP001	An. gambiae s.s.	Shiala, Chongwe	Female
7	JP002	An. gambiae s.s.	Shiala, Chongwe	Female
8	JP003	An. gambiae s.s.	Shiala, Chongwe	Female
9	JP004	An. gambiae s.s.	Shiala, Chongwe	Female
10	JP005	An. gambiae s.s.	Shiala, Chongwe	Female
11	JP006	An. gambiae s.s.	Shiala, Chongwe	Female
12	JP007	No Band	Shiala, Chongwe	Female
13	JP008	An. gambiae s.s.	Shiala, Chongwe	Female
14	JP009	An. gambiae s.s.	Shiala, Chongwe	Female
15	JP010	No Band	Shiala, Chongwe	Female
16	JP011	An. gambiae s.s.	Shiala, Chongwe	Female
17	JP012	An. gambiae s.s.	Shiala, Chongwe	Female
18	JP013	An. gambiae s.s.	Shiala, Chongwe	Female
19	JP014	An. gambiae s.s.	Shiala, Chongwe	Female
20	JP015	An. gambiae s.s.	Shiala, Chongwe	Female
21	JP016	An. gambiae s.s.	Shiala, Chongwe	Female
22	JP017	No Band	Shiala, Chongwe	Female
23	JP018	An. gambiae s.s.	Shiala, Chongwe	Female
24	JP019	An. gambiae s.s.	Shiala, Chongwe	Female
25	JP020	No Band	Shiala, Chongwe	Female
26	JP021	An. gambiae s.s.	Shiala, Chongwe	Female
27	JP022	An. gambiae s.s.	Shiala, Chongwe	Female
28	JP023	An. gambiae s.s.	Shiala, Chongwe	Female
29	JP024	An. gambiae s.s.	Shiala, Chongwe	Female
30	JP025	An. gambiae s.s.	Shiala, Chongwe	Female
31	JP026	An. gambiae s.s.	Shiala, Chongwe	Female
32	JP027	An. gambiae s.s.	Shiala, Chongwe	Female
33	JP028	No Band	Nyamphande, Rufunsa	Female

34	JP029	An. gambiae s.s.	Nyamphande, Rufunsa	male
L	100 BP Ladder			
Repeat PCR run for samples whose DNA band were very faint				
LANE #	Lab ID	PCR ID	Location	Sex
L	Ladder		n/a	
1	Control	Negative control	n/a	
2	Control	An. gambiae s.s.(390bp)	n/a	
3	JP030	An. gambiae s.s.	Shiala, Chongwe	male
4	JP031	An. gambiae s.s.	Shiala, Chongwe	male
5	JP032	An. gambiae s.s.	Shiala, Chongwe	male
6	JP001*	An. gambiae s.s.	Shiala, Chongwe	Female
7	JP002*	An. gambiae s.s.	Shiala, Chongwe	Female
8	JP005*	An. gambiae s.s.	Shiala, Chongwe	Female
9	JP034	No Band	Shiala, Chongwe	male
10	JP016*	An. gambiae s.s.	Shiala, Chongwe	Female
11	JP021*	An. gambiae s.s.	Shiala, Chongwe	Female
12	JP0265*	An. gambiae s.s.	Shiala, Chongwe	Female
13	JP027*	An. gambiae s.s.	Nyamphande, Rufunsa	Female
14	JP029*	An. gambiae s.s.	Nyamphande, Rufunsa	male
L	100 BP Ladder		n/a	
Stared* samples were repeated as their DNA bands came out faint in the first run.				

4.2 Determination of physical chemical properties of field water on oviposition site selection.

The data on physical chemicals namely temperature, pH, conductivity, turbidity of the water from Nyamphande sites are shown in Appendix 3. There were no statistically significant differences (Table 5) between physic-chemical parameters of the water from Nyamphande sites A and B (Fig. 12).

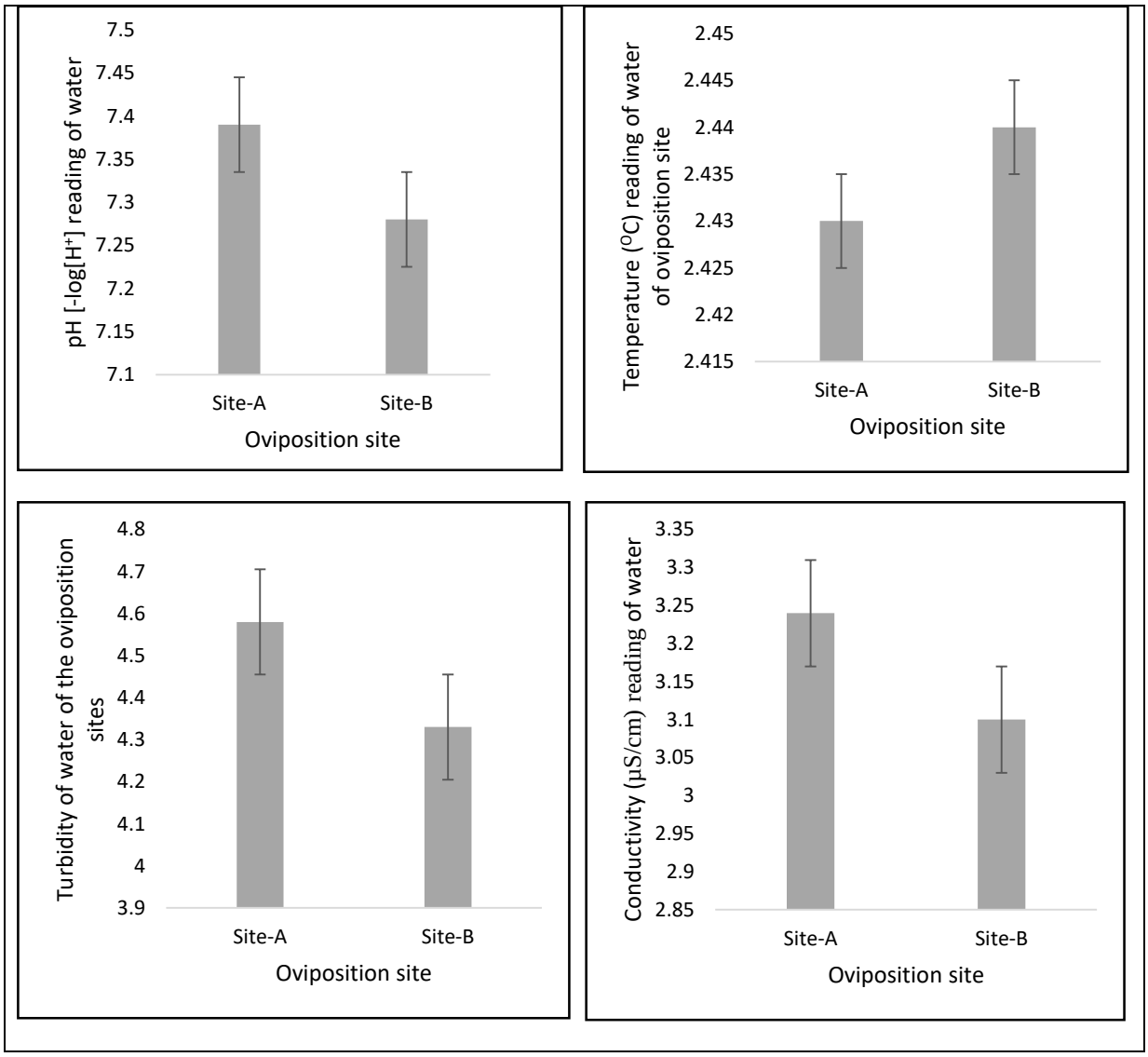

Figure 12. Characterisation of water pH, temperature, turbidity and conductivity at the Nyamphande sites.

Unpaired *t*-test $P > 0.05$ (95%CI), temperature (*t*-test $p = 0.232$), pH (*t*-test $p = 0.691$), conductivity (µS/cm) (*t*-test $p = 0.314$), turbidity (*t*-test $p = 0.622$).

Table 5. Parametric statistics test (t-test P) for significant differences in physical chemicals variables at the Nyamphande sites.

	Conductivity	Turbidity	Temperature	pH
t-test P	0.314	0.622	0.232	0.691

4.3 Determination of relationship between physical chemicals of field water and larval densities.

4.3.1 Larval density at Nyamphande sites A and B.

A total of 920 *Anopheles* larvae were counted from Nyamphande village area of which 877 (96.5%) were from Nyamphande site-A while 4.8% were from Nyamphande site-B. Both descriptive (Fig. 13) and parametric statistics showed a difference in larval densities between the two sites. There were statistically significant differences in larval densities between Nyamphande site-A and site-B at 95%CI *t*-test $P < 0.05$ (*t*-test $p = 0.000$ (95%CI).

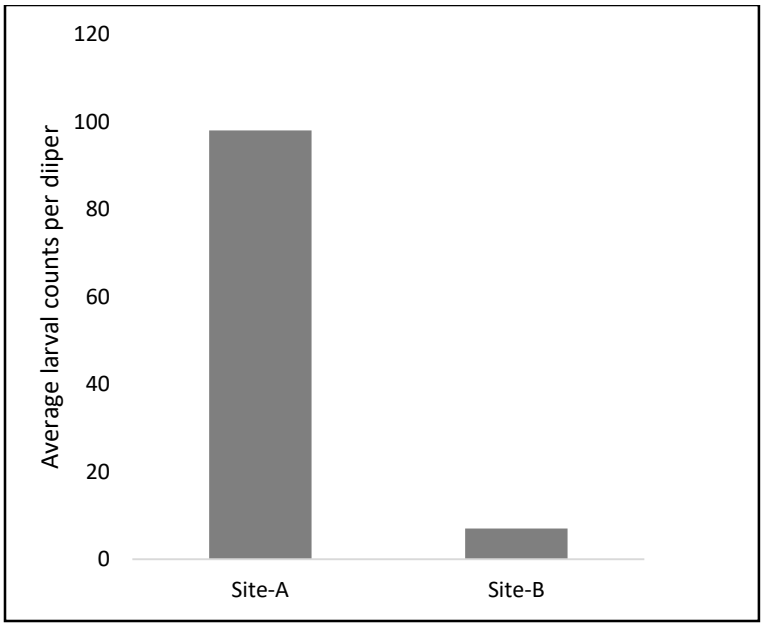

Figure 13 Larval density at Nyamphande sites-A and site-B in Rufunsa District.

4.3.2 Determination of relationship between physical chemicals and larval densities at oviposition sites.

4.3.2.1 Determination of relationship between physical chemicals and larval densities at Nyamphande site-A.

The Spearman rank correlation regression coefficient (r_s) computations (Table. 6) for water from Nyamphande site-A did not establish a significant relationship between larval density and physical chemicals namely, pH (r_s = -0.083), temperature (r_s = 0.085), turbidity (r_s = 0.264), conductivity (r_s = -0.219).

Table 6 Relationship between physical chemicals and larval density at Nyamphande site-A

Larval catch at site-A	Ranking	pH	Temperature (x10)	Ranking	Turbidity (x10)	Conductivity ($x10^2$)	Ranking
32	13	6.8	2.5	13	6.1	2.98	13
16	18	7.3	2.42	18	6.26	3	18
17	17	7.7	2.39	17	7.77	5.32	17
29	14	7.7	2.45	14	1.37	1.52	14
25	15	7	2.41	15	1.77	3.3	15
29	14	8.6	2.43	14	1.3	2.44	14
41	9	6.7	2.44	9	8.1	3.21	9
38	10	6.9	2.44	10	5.2	3.76	10
34	12	6.7	2.39	12	5.5	2.28	12
36	11	6.8	2.42	11	7.18	3.24	11
44	8	7.4	2.41	8	1.4	3.75	8
109	1	8.4	2.42	1	3.17	3.37	1
53	7	7.5	2.43	7	6	5.34	7
65	5	7.1	2.4	5	1.47	2.65	5
58	6	6.9	2.43	6	4	2.79	6
68	4	9.4	2.4	4	4.86	3.76	4
77	3	6.7	2.42	3	3.21	6.5	3
106	2	6.7	2.43	2	2.55	2.77	2
877	Spearman coefficient	-0.083	0.085		0.264	-0.219	

4.3.2.2 Determination of relationship between physical chemicals and larval densities at Nyamphande site-B.

The Spearman rank correlation regression coefficient (r_s) computations (Table. 7) for water from Nyamphande site-B established weak to no relationship between larval density and physical chemicals namely, pH (r_s = 0.235), temperature (r_s = -0.368), turbidity (r_s = -0.001), conductivity (r_s = 0.390).

Table 7 Relationship between physical chemicals and larval density at Nyamphande site-B

Larval catch at site-B	Rank	pH	Rank	Temperature (x10)	Rank	Turbidity (x10)	Rank	Conductivity ($x10^2$)
3	5	7.2	5	2.42	5	2.57	5	3.3
5	3	7.1	3	2.41	3	7.8	3	2.7
2	6	7	6	2.42	6	1.4	6	2.78
0	8	7.3	8	2.42	8	6.7	8	2.45
4	4	7.3	4	2.42	4	1.4	4	2.94
0	8	7.2	8	2.43	8	5.59	8	3.86
1	7	7.2	7	2.41	7	2.03	7	2.66
1	7	7.4	7	2.42	7	1.36	7	3.96
0	8	7.2	8	2.43	8	2	8	2.95
1	7	7.4	7	2.43	7	2.3	7	4.45
1	7	7.1	7	2.42	7	5.62	7	2.88
0	8	7.5	8	2.42	8	1.2	8	4.81
1	7	7.4	7	2.43	7	1.9	7	3.36
2	6	7.2	6	2.47	6	1.7	6	2.76
3	5	7.2	5	2.46	5	1.4	5	2.94
2	6	7.2	6	2.44	6	7.6	6	3.06
7	2	7.3	2	2.45	2	2.4	2	3.08
8	1	7.2	1	2.46	1	2.99	1	2.66
2	6	7.5	6	2.45	6	8.43	6	3.2
43	0.235	0.235		-0.368		-0.001		0.390

4.3.2.3 Determination of relationship between physical chemicals and larval densities at Shiala site.

The Spearman rank correlation regression coefficient (r_s) computations (Table. 8) for water from Shiala oviposition site did not establish relationships between larval density and physical chemicals namely, pH (r_s = 0.164), temperature (r_s = -0.188), conductivity (r_s = -0.117). Turbidity was over range.

Table 8 Relationship between physical chemicals and larval density at Shiala oviposition site.

Larval catch at Shiala	Shiala rank larvae	pH Shiala	Shiala rank larvae	Conductivity (x10)	Shiala rank larvae	Temperature (x10)	Turbidity
5	15	7.1	15	8.1	15	2.8	Over range
15	13	6.6	13	8.0	13	2.84	Over range
36	11	6.6	11	8.2	11	2.8	Over range
39	10	8	10	8.2	10	2.7	Over range
30	12	7.1	12	8.1	12	2.8	Over range
50	7	8	7	8.1	7	2.8	Over range
108	2	6.9	2	7.9	2	2.81	Over range
103	3	6.8	3	8.0	3	2.81	Over range
110	1	6.8	1	8.3	1	2.8	Over range
64	6	6.6	6	8.1	6	2.77	Over range
40	9	6.9	9	8.3	9	2.79	Over range
47	8	6.7	8	8.4	8	2.8	Over range
90	4	6.7	4	8.4	4	2.85	Over range
50	7	6.9	7	8.7	7	2.84	Over range
88	5	6.8	5	8.4	5	2.84	Over range
875	Spearman	0.164		-0.117		-0.188	

Table 9. Spearman regression coefficient on relationship analysis.

	Physical chemicals			
	pH	Conductivity (µS/cm)	Turbidity	Temperature
Nyamphande-A Regression correlation coefficient (r_s).	-0.0.083	-0.219	0.264	0.085
Nyamphande-B Regression correlation coefficient (r_s).	-0.235	0.390	-0.001	- 0.368
Shiala Spearman rank regression coefficient (r_s).	0.164	-0.117	Over range	-0.188

The Spearman rank correlation coefficient (r_s) did not establish relationship between larval density and the physical chemicals at all the three oviposition sites (Table. 9).

4.4 Comparison of efficacy of field oviposition water and laboratory water on oviposition site selection.

4.4.1 Efficacy of water from Nyamphande Sites A, B and distilled water on oviposition site selection.

A total of 2,070 gravid *An. gambiae* s.s., KISUMU mosquitoes were used. There were differences in efficacy of the three water types that were tested (Table. 10).

Table 10. Efficacy of Nyamphande and laboratory water on oviposition site selection.

Experiment	Nyamphande-A	Nyamphande-B	Laboratory Water	No. of Gravid females
1	63	0	0	3x30
2	142	31	17	3x30
3	69	0	0	3x30
4	151	25	31	3x30
5	75	0	0	3x30
6	178	43	31	3x30
7	110	11	0	3x30
8	77	29	0	3x30
9	5	0	2	3x30
10	74	62	61	3x30
11	59	2	0	3x30
12	10	0	0	3x30
13	3	0	0	3x30
14	0	8	5	3x30
15	28	55	6	3x30
16	23	44	7	3x30
17	313	122	13	3x30
18	101	88	105	3x30
19	165	1	61	3x30
20	73	10	0	3x30
21	0	3	5	3x30
22	28	53	2	3x30
23	67	23	13	3x30
				2,070
SUM	1814	610	359	

There were differences in the non-parametric statistics (Table. 11). Gravid female *Anopheles gambiae* s.s., KISUMU laid more eggs in field water from Nyamphande site-A which had higher larval density. Nyamphande site-A was previously predominantly pre-inhabited by *An. coustani* larvae than in both Nyamphande site-B which had lower larval density and laboratory water (Fig.14).

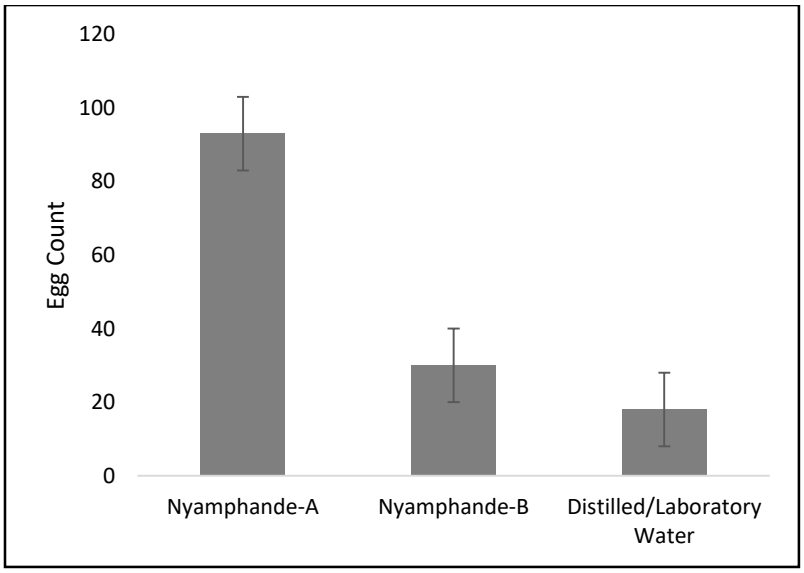

Figure 14 Efficacy of field water types on oviposition site selection.

Table 11 Comparative descriptive statistics for differences in egg counts at Nyamphande sites.

Descriptives							
Number of Eggs					95% Confidence Interval for Mean		
	N	Mean	SE	Lower Bound	Upper Bound	Minimum	Maximum
NyamphandeA	23	79.304	15.3318	47.508	111.101	.0	313.0
NyamphandeB	23	26.522	6.7665	12.489	40.555	.0	122.0
Distilledwater	23	15.609	5.5446	4.110	27.107	.0	105.0
Total	69	40.478	6.7067	27.095	53.861	.0	313.0

Parametric Statistics.

There were statistically significant differences in between groups (Table. 12) on efficacy of the different types of water to influence oviposition site selection $P < 0.05$(95%CI). One-way ANOVA ($F(2,66) = 11.172$, $p = 0.000$). Tukey HSD post hoc tests (Table. 13) for multiple means analysis $P < .05$ (95%CI). Nyamphande site-A and Nyamphande site-B ($p = 0.001$); Nyamphande site-A and laboratory water ($p = 0.000$); Nyamphande site-B and laboratory water ($p = 0.730$).

Table 12 ANOVA Comparison of efficacy of Nyamphande field water and laboratory water on oviposition site selection by gravid Anopheles gambiae s.s., KISUMU.

ANOVA

Number of Eggs_in_Water	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	53377.130	2	26688.565	11.172	.000
Within Groups	157666.087	66	2388.880		
Total	211043.217	68			

Table 13. Tukey HSD Test for comparison of efficacy of Nyamphande field water and laboratory water on oviposition site selection by gravid Anopheles gambiae s.s., KISUMU.

Post Hoc Test

Multiple Comparisons

Dependent Variable: Number of Eggs

Tukey HSD					95% Confidence Interval	
(I) Watertype	(J) Watertype	Mean Difference (I-J)	SE	Sig.	Lower Bound	Upper Bound
NyamphandeA	NyamphandeB	52.7826*	14.4128	.001	18.225	87.340
	Distilledwater	63.6957*	14.4128	.000	29.138	98.253
NyamphandeB	NyamphandeA	-52.7826*	14.4128	.001	-87.340	-18.225
	Distilledwater	10.9130	14.4128	.730	-23.645	45.471
Distilledwater	NyamphandeA	-63.6957*	14.4128	.000	-98.253	-29.138
	NyamphandeB	-10.9130	14.4128	.730	-45.471	23.645

* The mean difference is significant at the 0.05 level.

4.4.2 Efficacy of water from Shiala oviposition site and distilled water on oviposition site selection.

A total of 1,380 gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes were used for their selection of field water from Shiala and distilled water (Table. 14).

Table 14 Efficacy of Shiala and laboratory water on oviposition site selection

	Water Type		
Experiment	Shiala water	Lab water	No. of gravid females
	Number of eggs		
1	304	372	2x30
2	123	224	2x30
3	106	251	2x30
4	235	570	2x30
5	35	240	2x30
6	107	391	2x30
7	66	111	2x30
8	35	71	2x30
9	43	115	2x30
10	73	205	2x30
11	31	125	2x30
12	17	75	2x30
13	5	77	2x30
14	0	45	2x30
15	0	33	2x30
16	66	111	2x30
17	35	71	2x30
18	193	97	2x30
19	73	205	2x30
20	40	300	2x30
21	390	270	2x30
22	10	0	2x30
23	3	5	2x30
Sum	1,990	3,964	1,380
Mean	166	330	

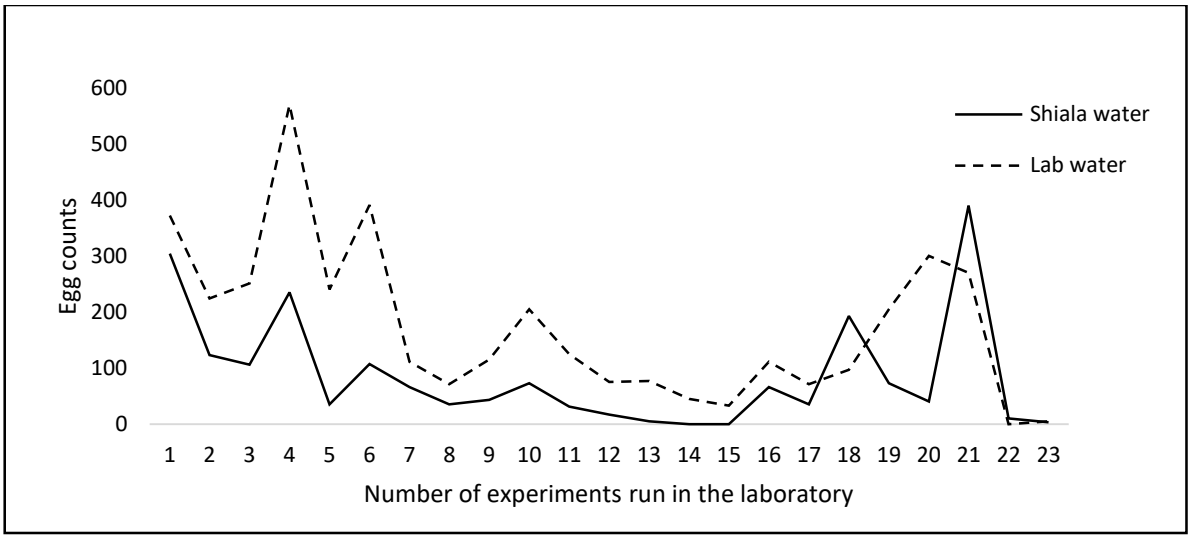

Figure 15. Efficacy of Shiala and laboratory water on oviposition site selection.

Gravid *Anopheles* gambiae s.s. mosquitoes laid more eggs in distilled water than in the Shiala field water that was previously predominantly pre-inhabited by *An. gambiae* larvae (Fig.15). The differences in egg counts between laboratory water and Shiala field water were statistically significant (*t-test P* = 0.035).

4.5 Determination of the effect of time and larval pre-habitation of water on efficacy on oviposition site selection.

A total of 2,760 gravid female *An. gambiae* s.s., KISUMU mosquitoes were used in this experiment (Table. 15). These mosquitoes laid more eggs in egg-pre-inhabited water more than other larval pre-habited water (Figs. 16), egg-water (46%) > L1-L2-water (28%) > L3-pupal-water (20.2%) > full cohort (7.2%). L1, L2, L3 stand for first, second and third larval instar development stages.

Table 15. Efficacy of egg and larval pre-inhabited water on oviposition site selection

Experiment	Egg	L1-L2	L3-L4-Pupae	Cohort	Number of mosquitoes
1	61	0	5	0	4x30
2	3	0	2	1	4x30
3	16	25	4	0	4x30
4	11	13	1	3	4x30
5	7	1	0	0	4x30
6	6	15	40	21	4x30
7	2	6	0	0	4x30
8	58	33	41	0	4x30
9	42	66	47	2	4x30
10	380	138	217	59	4x30
11	17	87	6	3	4x30
12	80	52	17	7	4x30
13	67	58	9	1	4x30
14	155	52	70	5	4x30
15	36	168	72	6	4x30
16	356	130	32	7	4x30
17	253	129	17	13	4x30
18	239	128	273	105	4x30
19	154	123	25	80	4x30
20	5	2	0	0	4x30
21	3	2	4	3	4x30
22	0	0	0	0	4x30
23	1	0	1	0	4x30
Sum	1952	1228	883	316	2,760
Mean	85	53	38	14	

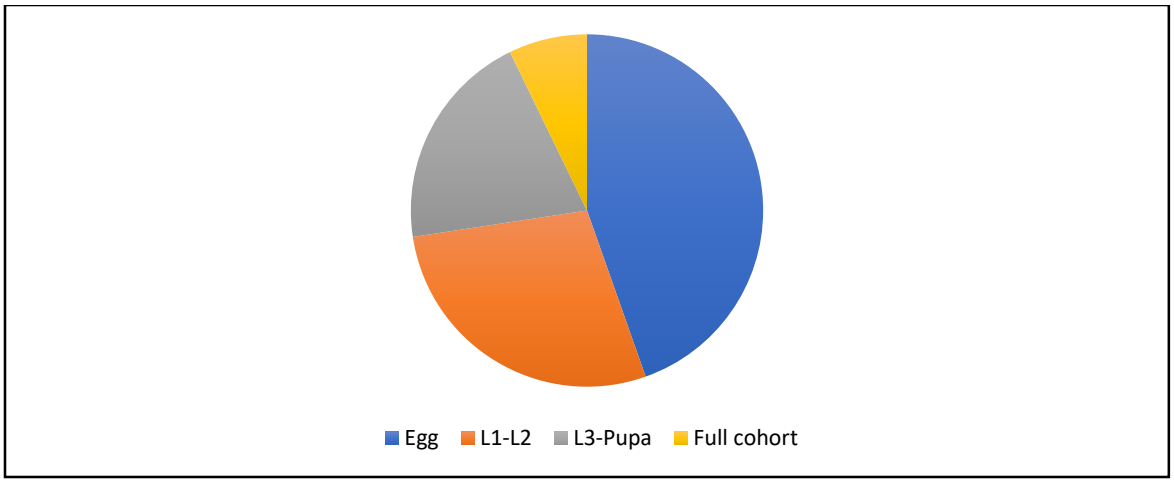

Figure 16. Efficacy of egg and different larval pre-inhabited water on oviposition site selection.

There were differences in all the means (Table. 16) and a declining trend in efficacy of oviposition water due to increased time and the developmental stage of immature instars (Fig. 17). Gravid female *An. gambiae* s.s. mosquitoes laid fewer eggs in water with increasing developmental stage which is related to time.

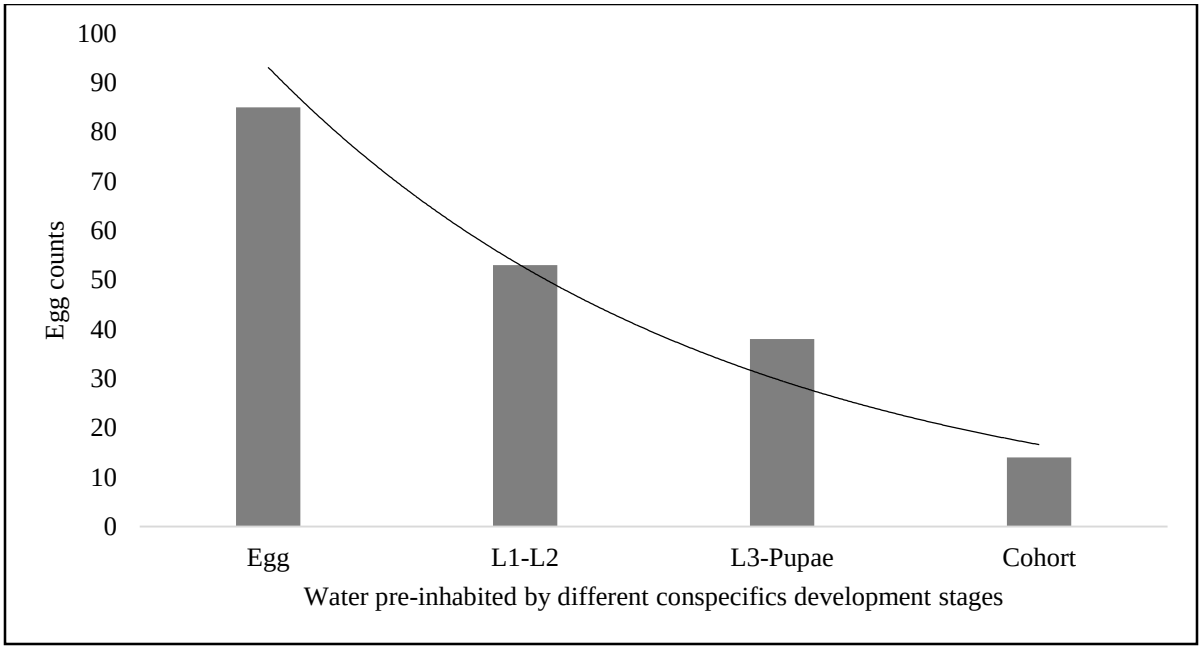

Figure 17 Effect of larval pre-habited water on oviposition selection.

Table 16 Summary Comparative Descriptive Statistics

Descriptives

Number of Eggs				95% Confidence Interval for Mean			
	N	Mean	SE	Lower Bound	Upper Bound	Minimum	Maximum
Egg-Water	23	84.87	24.193	34.70	135.04	.0	380
First-Second	23	53.39	11.729	29.07	77.72	.0	168
Third-Pupal	23	15.60	14.453	8.42	68.37	.0	273
Full Cohort	23	13.74	5.858	1.59	25.89	0	105
Total	92	47.60	8.104	31.50	63.70	.0	380

Parametric Statistics.

There were statistically significant differences in efficacy of water between the four treatment groups P < 0.05(95%CI). One-way ANOVA (F (3,88) = 3.663, p = 0.015). Tukey HSD post hoc tests, for multiple means analysis, P < 0.05 (95%CI). Egg water and L1-L2 water (p = 0.483); Egg water and L3-Pupa water (p = 0.156); Egg water and Cohort water (p = 0.009); L1-L2 water and L3-Pupa water (p = 0 .904). L1-L2 water and Cohort water (p = 0.278), L3- Pupa water and Cohort water (p = 0.677).

Table 17. ANOVA for Effect of Time and Water Pre-Inhabitation by Immatures on Oviposition Site Selection by Gravid Anopheles Gambiae s.s.

ANOVA

Number of Eggs_in_Water	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	61040.120	3	20346.707	3.663	.015
Within Groups	488848.000	88	5555.091		
Total	549888.120	91			

Table 18. Tukey HSD Post Hoc Test for Means.

Post Hoc Test

Multiple Comparisons
Dependent Variable: Egg count

Tukey HSD					95% Confidence Interval	
(I) Watertype	(J) Watertype	Mean Difference (I-J)	SE	Sig.	Lower Bound	Upper Bound
Eggwater	FirstSecondInstars	31.478	21.978	.483	-26.08	89.04
	ThirdFourthPupalInstars	46.478	21.978	.156	-11.08	104.04
	FullCohort	71.130*	21.978	.009	13.57	128.69
FirstSecondInstars	Eggwater	-31.478	21.978	.483	-89.04	26.08
	ThirdFourthPupalInstars	15.000	21.978	.904	-42.56	72.56
	FullCohort	39.652	21.978	.278	-17.91	97.21
ThirdFourthPupalInstars	Eggwater	-46.478	21.978	.156	-104.04	11.08
	FirstSecondInstars	-15.000	21.978	.904	-72.56	42.56
	FullCohort	24.652	21.978	.677	-32.91	82.21
FullCohort	Eggwater	-71.130*	21.978	.009	-128.69	-13.57
	FirstSecondInstars	-39.652	21.978	.278	-97.21	17.91
	ThirdFourthPupalInstars	-24.652	21.978	.677	-82.21	32.91

* The mean difference is significant at the 0.05 level.

As shown by one-way ANOVA (Table 17.) and Tukey HSD Post hoc test (Table 18.), egg pre-inhabited water with the shortest time was the most selected while the water that had been used to raise a cohort was the least selected for oviposition by gravid female *Anopheles gambiae* s.s. mosquitoes.

4.6 Determination of effect of water background colour on oviposition site selection.

A total of six thousand, four hundred and fifty-four (6,454) eggs of *Anopheles gambiae*, were counted over a period of 24 months starting in July 2018 until July 2020.

Gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes laid more eggs in oviposition water that was in red oviposition cups than any other colour and laid the least number of eggs in oviposition water in yellow-coloured oviposition cups (Fig.18), as follows, red (43%) > black (23%) > blue (16%) > green (11%) > yellow (7%).

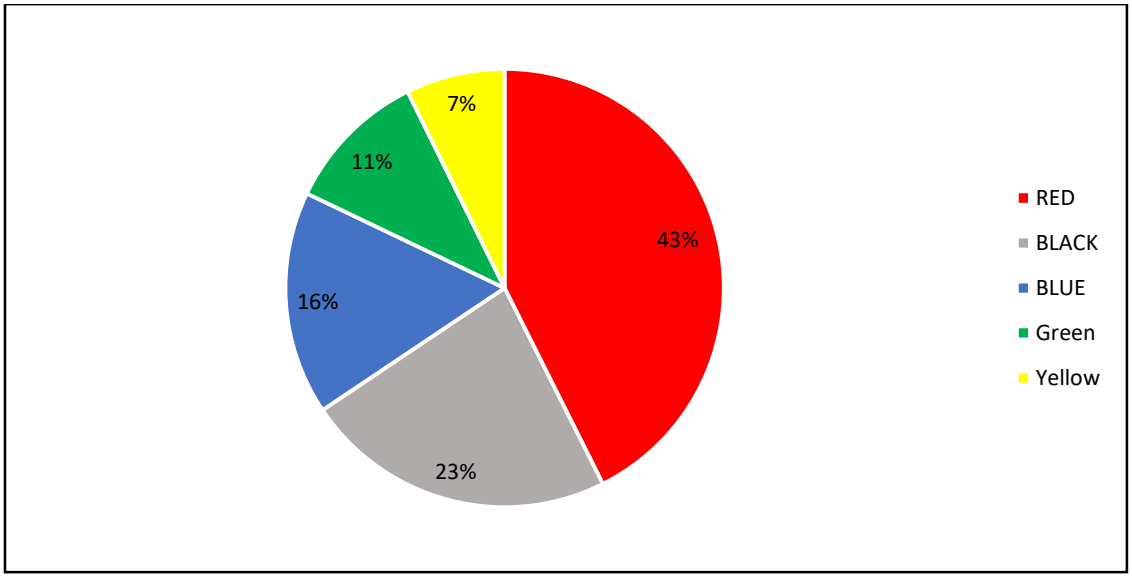

Figure 18 Effect of water background colour on oviposition by percentage of eggs.

There were clear notable differences in the mean values for egg counts (Table 19.) for different habitat background colours of oviposition containers (Fig. 19.).

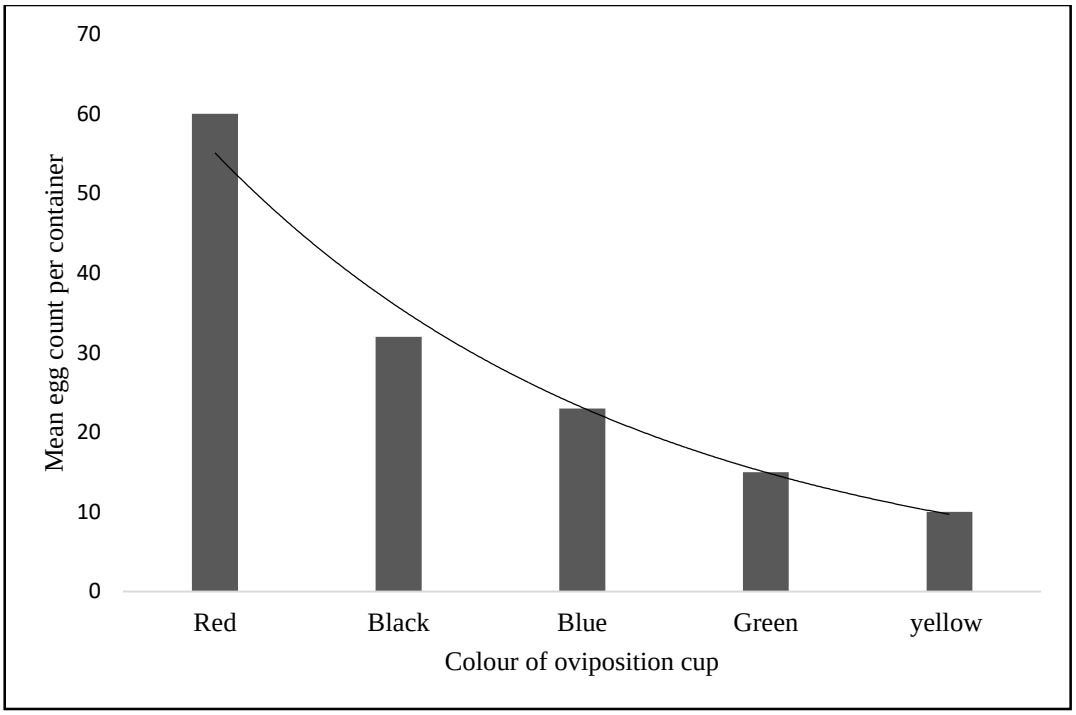

Figure 19. Effect of water background colour on oviposition site selection by *An. gambiae* s.s., KISUMU.

Table 19. Descriptive Statistics s for container colour selection for oviposition by *Anopheles gambiae.*

Descriptives

	Number of Eggs_in_Water			**95% Confidence Interval for Mean**				
	N	Mean	SD	SE	LowerBound	UpperBound	Min	Max
Blackwater	42	34.31	51.356	7.924	18.31	50.31	0	210
Bluewater	42	25.43	57.546	8.880	7.50	43.36	0	301
Redwater	42	62.62	131.497	20.290	21.64	103.60	0	700
Greenwater	42	12.95	40.998	6.326	0.18	25.73	0	215
Yellowwater	42	6.36	20.081	3.099	0.10	12.61	0	106
Total	210	28.33	73.196	5.051	18.38	38.29	0	700

Parametric Statistics.

There were statistically significant differences between the different colour treatment groups. One-way ANOVA (F(4,205) = 4.020, p = 0.004). Tukey HSD post hoc tests, for multiple means analysis, P < .05. Red coloured habitat background versus: black (*p* = 0.363); blue (*p* = 0.121); green (*p* = 0.014); yellow (*p* = .003). Black coloured habitat background versus: green (*p* = 0.644), yellow (*p* = 0.376), blue (*p* = 0.979). Blue coloured habitat background versus green (*p* = 0.929), yellow (*p* = 0.735). Green coloured habitat background versus yellow coloured habitat background (*p* = 0.993).

Table 20. ANOVA Statistic for Colour of Oviposition Cups.

ANOVA

NumberEggs_in_Water	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	81445.952	4	20361.488	4.020	.004
Within Groups	1038306.714	205	5064.911		
Total	1119752.667	209			

Table 21. Tukey HSD Post Hoc Test for Oviposition Cup Colour Selection by *Anopheles gambiae* s.s.

Post Hoc Tests

Multiple Comparisons
Dependent Variable: Number of Eggs_in_Water
Tukey HSD Test

(I) Colour	(J) Colour	Mean Difference (I-J)	SE	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Blackwater	Bluewater	8.881	15.530	.979	-33.86	51.62
	Redwater	-28.310	15.530	.363	-71.05	14.43
	Greenwater	21.357	15.530	.644	-21.39	64.10
	Yellowwater	27.952	15.530	.376	-14.79	70.69
Bluewater	Blackwater	-8.881	15.530	.979	-51.62	33.86
	Redwater	-37.190	15.530	.121	-79.93	5.55
	Greenwater	12.476	15.530	.929	-30.27	55.22
	Yellowwater	19.071	15.530	.735	-23.67	61.81
Redwater	Blackwater	28.310	15.530	.363	-14.43	71.05
	Bluewater	37.190	15.530	.121	-5.55	79.93
	Greenwater	49.667*	15.530	.014	6.92	92.41
	Yellowater	56.262*	15.530	.003	13.52	99.00
Greenwater	Blackwater	-21.357	15.530	.644	-64.10	21.39
	Bluewater	-12.476	15.530	.929	-55.22	30.27
	Redwater	-49.667*	15.530	.014	-92.41	-6.92
	Yellowwater	6.595	15.530	.993	-36.15	49.34
Yellowwater	Blackwater	-27.952	15.530	.376	-70.69	14.79
	Bluewater	-19.071	15.530	.735	-61.81	23.67
	Redwater	-56.262*	15.530	.003	-99.00	-13.52
	Greenwater	-6.595	15.530	.993	-49.34	36.15

* The mean difference is significant at the 0.05 level.

Summary of Statistics Results.

As shown by one-way ANOVA (Table 20), Tukey HSD post hoc test (Table 21), habitat background colour, statistically significantly affected oviposition habitat selection by gravid female *Anopheles gambiae* mosquitoes. Red coloured habitat background was most preferentially statistically significantly selected for oviposition over green and yellow oviposition containers but

there were no statistically significant differences in mean egg counts between red and black and also between red and blue coloured oviposition containers. Black coloured habitat background was not statistically significantly selected over, blue, green, and yellow. Blue coloured habitat background was not statistically significantly selected over green, and yellow containers. There was no statistically significant difference in selection for oviposition between green, and yellow-coloured containers. Red coloured containers, were the most selected for oviposition while green, and yellow-coloured containers were least selected in that order by gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes.

CHAPTER 5: DISCUSSION

5.1 Identification of oviposition sites selected by gravid female anopheline mosquitoes.

The finding by this study that *An. coustani* was the most dominant species while *An. gambiae* s.s. was almost absent in Nyamphande village area of Rufunsa District is significant because this area like other areas in this District is a very high malaria transmission area and yet *An. coustani* is considered a secondary vector of malaria. Although this finding was based on data collected during the dry season, other studies covering all seasons in Western Province of Zambia have reported similar findings (Cross *et al*., 2021). It is possible that the under-studied *An. coustani* could be among the major drivers of malaria in Nyamphande area of Rufunsa District. In order to establish whether *An. coustani* plays any role in malaria transmission in Rufunsa district, further and preferably long-term studies could be undertaken in the area. Such studies would involve adult female catches and blood meal analyses in addition to the entomological profile through species identification of the adults that would be caught. This proposition is supported by other studies that showed that *An. coustani* was a major malaria vector in some areas of Kenya and Madagascar (Jones *et al*., 2021; Goupeyou-Youmsi *et al*., 2020; Mwangangi, *et al*., 2013). Nevertheless, these findings by this study may have implications for malaria control in this area of Lusaka Province. On the other hand, the finding of *An. gambiae* as the dominant vector in Chongwe is consistent with the malaria entomological profile of the district (NMEC, 2018; Kalubula *et al*., 2015). However, this study is not aware of any other study that has reported presence of *An. pretoriensis* in Chongwe nor that of *An. coustani* in Rufunsa districts of Lusaka Province respectively. The none finding of oviposition sites in the selected area of Lusaka city is significant and consistent with earlier studies that reported a downward trend in availability of suitable habitats for anophelines in Lusaka city and also a declining trend in both malaria cases and adult anopheline mosquitoes (Nawa *et al.*, 2019; NMEC, 2019; Chanda *et al.*, 2013; Kandyata *et al.*, 2012; Masaninga *et al.*, 2013; Beier *et al.*, 2008; Walker and Lynch, 2007).

5.2 Determination of physical chemical properties of field water on oviposition site selection.

The finding by this study that there were no significant differences between all the four physical chemicals parameters namely, temperature, pH, turbidity and conductivity from the two sampling sites at Nyamphande-A and Nyamphande-B in Rufunsa District is expected considering the proximity of the sites to each other and the fact that they carry similar environmental load as part of the Nyamphande river system even though some variations may occur. Further it is known that water physical chemicals in an area reflect local environmental conditions including geo-physical properties and pollution (Nkhuwa *et al*., 2015).

5.3 Determination of relationships between physical chemicals and larval densities.

These results suggest that there were no relationships between larval density at oviposition sites and the selected physical chemicals parameters namely pH, temperature, turbidity and conductivity based on the Spearman rank regression correlation coefficient. Therefore, it is suggested that the differences in larval densities between the two sites may not have been caused by pH, temperature, turbidity and conductivity but by other water related factors at the two sites. This is supported by the results of other research questions by this study that showed that gravid female *An. gambiae* s.s., KISUMU mosquitoes statistically significantly laid more eggs in water from Nyamphande site-A than in water from Nyamphande site-B. These findings by this study are consistent with other recent studies that did not establish such relationship or association between larval densities and physical chemicals namely pH, temperature, turbidity and conductivity but attributed larval density and distribution to other factors (Amini *et al*., 2020; Getachew *et al.*, 2020; Nikookar *et al*., 2017; Herrera-Varela *et al*., 2014; Zattel *et al*., 2013; Liu *et al*., 2012; Warburg *et al*., 2011; Navarro-Silva *et al.*, 2009; Huang *et al*., 2006).

However, these results are not consistent with other studies that have demonstrated a relationship between *Anopheles* larval distribution and abundance to specific physical chemicals (Musonda and Sichilima, 2019; Elmalih *et al*., 2018; Dom *et al.*, 2016; Gopalakrishnan *et al*., 2013; Kabula *et al*., 2011; Kenea *et al*., 2011; Paaijmans *et al*., 2008). The differences in findings by different studies may be explained by conclusions by other studies that concluded that the density and distribution of *Anopheles* larvae was related to species specific needs and other factors such as

presence of semiochemicals including those associated with some predators and not necessarily the pH, temperature, turbidity and conductivity (Arcos *et al*., 2018; Warburg *et al*., 2011). The different conclusions by different studies demonstrate the fact that the field is still understudied and thereby justifying calls for further studies on the subject. These findings have potential application because the future identification of factors or sources of factors that influence larval density and distribution could contribute to vector control strategies based on the oviposition selection behaviour. Such strategies could include possible manipulation of an oviposition site or creation of artificial oviposition sites in strategic places for purposes of control of anopheline mosquitoes.

The Hypothesis that larval density of anopheline mosquitoes in the field was related to water physical chemicals namely pH, temperature, turbidity and conductivity could not be proved and the null hypothesis is upheld.

5.4 Comparison of efficacy of field and laboratory water on oviposition site selection.

The finding that *An. gambiae* s.s., KISUMU laid more eggs in water from Nyamphande-A than in either Nyamphande-B or laboratory water suggests the presence of some factors that influenced the laying of eggs in that manner. On the other hand, the results may also suggest that water from Nyamphande-B may have contained some factors that caused gravid female *An. gambiae* s.s., KISUMU mosquitoes to lay fewer eggs in it. These unknown factors may have influenced oviposition selection by *An. gambiae* s.s., KISUMU mosquitoes. These results corroborate the field results that showed differences in larval densities between the two sites. This suggests that field water from the two Nyamphande sites may have contained different properties that differently influenced oviposition in gravid *An. gambiae* s.s., KISUMU mosquitoes. These results further suggest that pH, temperature, turbidity and conductivity may not have influenced oviposition in gravid *An. gambiae* s.s., KISUMU. These findings by this study are consistent with other findings that attributed differences in larval densities to presence of semiochemicals emitted by some bacterial or predators (Getachew *et al*., 2020; Weterings *et al*., 2014).

The results concerning the response of gravid female *An. gambiae* s.s., KISUMU mosquitoes to Shiala field water may suggest that the water properties may have been refractory to these

mosquitoes. Further, the Shiala water results are corroborated by results of this study that showed that laboratory water that had been used to raise a cohort of conspecifics was least selected for oviposition by gravid female *An. gambiae* s.s., KISUMU mosquitoes. This may suggest that the water may have contained some conspecifics larval associated refractory semiochemicals as has been concluded by others (Mwingira *et al*., 2020; Suh *et al*., 2016; Stewart *et al*., 2013; Taken, 2010; Reiter, 2007).

These findings are significant and have implications for oviposition site-based *Anopheles* mosquito control methods currently in use or in the development of potential alternative sustainable methods based on knowledge of the oviposition site selection behaviour by gravid female anopheline mosquitoes. Identification and isolation of factors that have opposite effects on oviposition site selection behaviour could lead to development of strategies and methods for control of anopheline mosquitoes at their breeding habitats.

The Hypothesis that field larval pre-inhabited water influences oviposition site selection behaviour by gravid female anopheline mosquitoes was proved and is accepted.

5.5 Determination of the effect of time and larval pre-habitation of water on efficacy on oviposition site selection.

These findings showed a time and developmental stage related diminishing efficacy of conspecifics larval pre-inhabited water to stimulate oviposition in gravid *An. gambiae* s.s., KISUMU mosquitoes. The efficacy of larval pre-habited water to stimulate oviposition reduced with time. The higher efficacy of egg pre-inhabited water may suggest existence of some attractive substance or factor that may be associated with the eggs. Water with the least efficacy may have had little of such substance or had refractory substances that could have emanated from the late larval instars and pupae. These results are corroborated by earlier results from both the field and laboratory experiments undertaken by this study. The findings by this study may suggest the possible presence of water related factors that influenced oviposition site selection both in the field and laboratory. The identification of the possible sources of these factors in the field or laboratory could be an initial and critical step to their eventual identification and isolation and for their use in malaria vector control. The findings by this study are consistent with other studies that showed

that gravid *Anopheles gambiae* mosquitoes oviposited more in water that was pre-habited by early larval instars (Schoelitsz *et al*., 2020; Wooding *et al*., 2020; Eneh, 2019; Maia *et al.*, 2018; Suh *et al*., 2016; Herrera-Valera *et al*., 2014; Flick, 2011; Mwangangi *et al*., 2007; Munga *et al*., 2006; McCrae, 1984).

The Hypothesis that larval pre-inhabited water influences oviposition site selection behaviour by gravid female anopheline mosquitoes was proved and is accepted.

5.6 Determination of effect of water background colour on oviposition site selection.

The findings by this study showed that water background colour influenced oviposition site selection by gravid female *Anopheles gambiae* s.s., KISUMU mosquitoes. The findings are consistent with other studies which found that both anopheline and culicine mosquitoes use red colour in the process of locating target objects (Phiri and Mbata, 2023; Alberto *et al*., 2022; Hellhammer *et al*., 2022; Dibal *et al.*, 2012). These results also corroborate earlier results by this study that showed that water physical chemicals parameters namely pH, temperature, turbidity and conductivity did not influence oviposition site selection behaviour by gravid female anopheline mosquitoes both under field or laboratory conditions. These findings also contribute to the knowledge required to improve the management of insectaries to increase egg productivity by gravid *Anopheles* mosquitoes (Liu *et al*., 2014; Collins and Blackwell, 2000). Different colour combination permutations or in synergy with some semiochemicals have potential for use to improve performance of some existing vector control methods (Butail *et al*., 2013) in a similar manner to the “pull and kill” methods within integrated vector control programmes.

The Hypothesis that background colour influences oviposition site selection behaviour by gravid female anopheline mosquitoes was proved and is accepted.

5.8 Importance and possible application of findings.

The study has generated knowledge that has immediate and long-term implications for the control of malaria. The finding that oviposition site selection behaviour may not have been influenced by physical chemicals (pH, temperature, turbidity and conductivity) but by other factors is significant. The identification of these factors or their sources could contribute to possible development of modern vector control strategies. Such strategies could include possible manipulation of an oviposition site or creation of artificial oviposition sites in strategic places. Further, the discovery

on effects of red colour is significant in terms of contributing to closing knowledge gap on external factors that regulate oviposition in anopheline mosquitoes. The discovery of *An. coustani* as the most dominant vector has immediate implications for malaria transmission and control in Nyamphande area of Rufunsa district which is one of the districts in Zambia with the highest malaria transmission.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion.

Oviposition site selection behaviour by gravid anopheline mosquitoes and larval densities at oviposition sites were not influenced by or related to water physical chemicals namely pH, temperature, turbidity and conductivity but may have been related to other factors present in the water from the two sites in Nyamphande area. Further, anopheline larval associated factors and colour of oviposition cups influenced oviposition site selection behaviour by gravid female *An. gambiae* s.s., KISUMU mosquitoes. The discovery of the dominance by *Anopheles coustani*, a secondary malaria vector and the near absence of the primary vector in Rufunsa district is significant for purposes of malaria control in the area. The data and information generated by this study may be relevant for potential use in improving efficacy of some existing vector control methods or in the potential development of alternative methods.

6.2 Recommendations.

Arising from the findings by this study, the recommendations are as follows:

(i) Undertake field and laboratory research studies to characterise factors other than the known physical chemicals that may influence oviposition habitat/site selection behaviour by gravid female anopheline mosquitoes.

(ii) Undertake all-seasons research studies involving larval and adult stages in order to better understand the possible role that *An. coustani*, a secondary vector could be playing in malaria transmission in Nyamphande village areas of Rufunsa district.

(iii) Undertake field studies on the effect of habitat background colour on anopheline mosquitoes in order to better understand effects of colour as an environmental factor in the selection of oviposition site by gravid anopheline mosquitoes.

(iv) Undertake laboratory research on the possible role that colour could play in the management of mosquito insectaries.

(v) Undertake research on synergistic effect of colour and semiochemicals on performance of some odour-baited malaria vector control methods.

6.3 Challenges.

There were two main challenges that were faced:

(i) Lack of equipment needed to control internal environmental conditions such as temperature and humidity negatively impacted performance in terms of egg productivity of and survival of mosquitoes in the laboratory.

(ii) There was limited time (June to November 2022) for field observations in Chongwe and Rufunsa where the anopheline oviposition sites were found.

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APPENDICES

APPENDIX-1

Publication- https://doi.org/10.1007/s42690-023-00957-0

Phiri, J. S. and K. J. Mbata. 2023. Influence of oviposition water background colours on egg laying in gravid anopheles gambiae s.s. (diptera:culicidae). *International Journal of Tropical Insect Science*, Published on line (22 February 2023). https://doi.org/10.1007/s42690-023-00957-0

APPENDIX- 2

AUTHORISATION LETTER TO USE NMEC LABORATORY SPACE AND FACILITIES

All Correspondence should be addressed to the Permanent Secretary
Telephone: +260 211 253000/1
Fax: +260 211 251344

In reply please quote:

MOH/101/9/19

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH

NDEKE HOUSE
P. O. BOX 30205
LUSAKA

23rd January, 2018

The Professor of Entomology,
The University of Zambia,
Department of Biological Sciences,
P.O. Box 32379
LUSAKA.

Dear Professor Keith J. Mbata PhD

RE: REQUEST TO USE THE NMEC INSECTARY AND PURE LINE ANOPHELINE MOSQUITOES FOR PhD RESEARCH

Reference is made to the above subject matter.

Following your letter dated 4 January 2018 in which you requested the National Malaria Elimination Centre (NMEC) to support Mr. James Simoko Phiri for fifteen months to conduct the PhD research on behavior patterns of gravid female *anopheline* mosquitoes in relation to breeding site selection for oviposition, the NMEC has no objection to the request.

For coordination and logistical modalities, kindly liaise with the NMEC Epidemiologist, Dr. Busiku Hamainza and the Principal Malaria Control Officer, Mr. Willy Ngulube on +260977941761 and +26097936181B respectively.

We look forward to your undertaking and sharing of your research findings.

Yours sincerely,

Dr. Elizabeth Chizema Kawesha
Director NMEC/for Permanent Secretary
MINISTRY OF HEALTH

All Correspondence should be addressed to the
Permanent Secretary
Telephone: +260 211 253040/5

Fax: +260 211 253344

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH

NDEKE HOUSE
P.O. BOX 30205
LUSAKA

In reply please quote:

No. .....

MOH/101/9/19

13th June, 2022.

Professor Keith J. Mbata, PhD
The University of Zambia
Department of Biological Sciences
P.O. Box 32379
LUSAKA

RE: REQUEST TO RESUME USE OF THE NMEC INSECTARY AND LABORATORY PURE LINE ANOPHELINE MOSQUITOES FOR PHD RESEARCH

Reference is made to the subject matter above.

In response to your letter dated 26th May 2022, requesting for use of the National Malaria Elimination Centre (NMEC) facilities for academic (PhD) research project purposes, the NMEC has no objection and permits Mr. James S. Phiri to resume utilization of the facilities for the completion of his research project.

Mr. Phiri can thus report to the Vector Control Specialist, Dr. Emmanuel Kooma, for placement to commence his activities. You are also expected to share the research report with the NMEC upon completion of the project.

Yours faithfully,

for/ Dr. Busiku Hamainza
Acting Director NMEC
For/Permanent Secretary – Administration
MINSTRY OF HEALTH

APPENDIX- 3

CHARACTERISATION OF WATER PHYSICAL CHEMICALS AT NYAMPHANDE SITES

Sampling	Conductivity (µS/cm)		Turbidity (NTU)		Temperature (^{O}C)		pH (-Log[H_3O^+])	
	Site-A	Site-B	Site-A	Site-B	Site-A	Site-B	Site-A	Site-B
1	298	333	61	25.7	25	24.2	6.8	7.2
1	300	270	62.6	78	24.2	24.1	7.3	7.1
1	228	278	55	140	23.9	24.2	6.7	7
2	265	245	14.7	67	24	24.2	7.1	7.3
2	279	294	40	14	24.3	24.2	6.9	7.3
2	375	386	14	55.9	24.1	24.3	7.4	7.2
3	534	266	60	20.3	24.3	24.1	7.5	7.2
3	152	396	13.7	13.6	24.5	24.2	7.7	7.4
3	244	295	13	20	24.3	24.3	8.6	7.2
4	324	445	71.8	23	24.2	24.3	6.8	7.4
4	330	288	17.7	56.2	24.1	24.2	7	7.1
4	376	481	52	12	24.4	24.2	6.9	7.5
5	532	336	77.7	19	23.9	24.3	7.7	7.4
5	650	276	32.1	17	24.2	24.7	6.7	7.2
5	249	294	10.9	14	24.6	24.6	7.4	7.2
6	376	306	48.6	76	24	24.4	9.4	7.2
6	337	308	31.7	24	24.2	24.5	8.4	7.3
6	277	266	25.5	29.9	24.3	24.6	6.7	7.2
7	321	320	81	84.3	24.4	24.8	6.7	7.5
7	258	289	13.1	13	24.5	24.7	7.8	7.3
7	269	270	95.1	17	24.4	24.5	9.9	7.3
8	344	269	98.5	40.6	24.5	24.5	8.2	7.2
8	389	271	54	29.1	24.4	24.5	7.1	7.3
8	200	291	71	95.2	24.7	24.6	7.2	7.3
9	303	273	56.3	93.1	24.4	24.6	7.1	7.2
9	290	267	63	105	24.6	24.5	7.3	7.1
9	210	263	13.1	27.5	24.3	24.4	6.8	7.3
10	297	269	6.59	23.1	24.5	24.5	6.9	7.2
10	260	278	32	14.6	24.4	24.5	7.6	8
10	225	290	13.1	19	24.4	24.5	6.8	7.3
11	339	266	48.6	84	24	24.2	7.1	7.5
11	364	443	31.7	17	24.6	24.2	7.2	7.4
11	349	270	25.5	27.8	24.4	24.2	7.5	7.1
12	534	396	81	13	24	24.2	7.3	7.2

12	371	333	63	29	24	24.2	6.7	7.3
12	250	292	95	105	24.5	24.2	7.3	7.3
13	357	309	81	24	24.1	24.7	7.8	7.2
13	269	295	54	30.7	24.4	24.5	7	7.3
13	234	267	41	92	24.6	24.5	6.8	7.3
14	412	395	16	39	24.6	24.5	6.7	7.2
14	250	281	15	15.5	24.1	24.3	7.4	7.2
14	261	262	95	17	24.3	24.1	7.5	7.2
15	336	261	98	41	24.5	24.2	7.7	7.4
15	381	263	55	30	24.3	24.3	8.6	7.2
15	208	299	73	96	24.2	24.3	6.8	7.4
16	285	274	277	266	24.1	24.2	7	7.1
16	313	312	321	320	24.4	24.2	6.9	7.5
16	250	281	208	239	24.4	24.5	7.1	7.3
17	261	262	170	171	24.7	24.6	7.2	7.3
17	352	277	244	168	24.4	24.6	7.1	7.2
17	381	279	289	171	24.6	24.5	7.3	7.1
18	218	309	103	194	24.3	24.4	6.8	7.3
18	311	281	203	173	24	24.2	7.1	7.5
18	282	259	190	167	24.4	24.5	7.6	8
Mean	315.93	301.46	77.34	70.32	24.33	24.38	7.33	7.29
SD	90.63	51.25	74.54	71.64	0.23	0.18	0.65	0.18
95%CI	24.73	13.97	20.33	19.54	0.06	0.05	0.18	0.05
T.test P		0.314		0.622		0.232		0.691

APPENDIX- 4.

INFLUENCE OF FIVE DIFFERENT WATER BACKGROUND COLOURS ON OVIPOSITION.

Experiment	Red	Black	Blue	Green	Yellow
1	60	18	0	71	103
2	32	11	0	41	0
3	21	6	0	24	99
4	7	1	0	6	4
5	70	33	23	14	13
6	0	32	12	0	0
7	0	0	3	0	0
8	33	12	0	0	0
9	49	36	0	0	0
10	7	0	0	0	0
11	7	0	71	0	7
12	7	7	0	0	1
13	4	0	7	0	0
14	12	0	0	7	0
15	3	32	90	0	0
16	13	5	9	0	0
17	5	5	2	0	2
18	7	4	0	0	1
19	0	0	7	0	0
20	29	0	0	0	106
21	61	46	27	0	9
22	58	134	13	0	0
23	38	65	13	0	0
24	61	170	19	0	0
25	0	134	0	0	0
26	0	2	6	0	0
27	155	98	105	14	0
28	0	23	0	0	0
29	0	12	0	0	0
30	0	67	0	0	0
31	8	10	0	44	0
32	0	0	0	8	0
33	0	0	0	0	0
34	75	8	0	0	1
35	27	2	6	0	3

36	0	0	3	0	0
37	400	4	200	6	0
38	300	100	30	68	30
39	0	0	0	0	0
40	250	0	60	3	0
41	99	85	17	10	11
42	700	210	301	215	75
43	50	43	8	5	5