UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES
SCHOOL OF BIOLOGICAL SCIENCES

INVESTIGATING LARVAL ECOLOGY, GENETIC DIVERSITY AND INSECTICIDE RESISTANCE STATUS OF *ANOPHELES FUNESTUS* IN DIFFERENT ECOLOGICAL LANDSCAPES IN WESTERN KENYA

This thesis/dissertation is submitted to the University of Ghana, Legon

In partial fulfilment of the requirements for the award of PHD IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES DEGREE

BY

ISAIAH DEBRAH
(10704644)

WEST AFRICAN CENTRE FOR CELL BIOLOGY OF INFECTIOUS PATHOGENS (WACCBIP), DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

JANUARY 2023

DECLARATION

I, Isaiah Debrah, hereby declare that all original work presented in this thesis was performed by me, under the direct supervision of Professor Yaw Asare Afrane (Department of Medical Microbiology, University of Ghana Medical School, University of Ghana, Ghana), Professor Linda Eva Amoah (Noguchi Memorial Institute for Medical Research, University of Ghana) and Dr Andrew K. Githeko (Centre for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya). I have duly cited and acknowledged all references and materials used in the thesis.

……

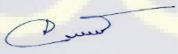
Isaiah Debrah (PhD Candidate)

……..

Professor Yaw Asare Afrane (Main Supervisor)

……

Professor Linda Eva Amoah (Co-supervisor)



……

Dr Andrew Karanja Githeko (Co-supervisor)

ACKNOWLEDGEMENTS

I am grateful to God for His sustenance, grace, and protection throughout my study.

Sincere thanks to Professor Guiyun Yan of the University of California, Irvine and WACCBIP Director, Professor Gordon Awandare, for the fellowship awards.

I also extend warm appreciation and sincere gratitude to my main supervisor, Professor Yaw Asare Afrane and the co-supervisors, Professor Linda Eva Amoah and Dr Andrew Karanja Githeko for their guidance, support and advice in completing this work successfully.

Thanks to the staff of the International Center for Excellence Malaria Research (ICEMR), Homabay, Kenya for the assistance in data collection and cooperation. Special thanks to Dr Maxwell Machani and Mr Robin Oriango for driving the car to and from the field during sample collection.

To the entire WACCBIP faculty, I sincerely appreciate your outstanding lectures and significant contributions to enhance my success in the program.

To my course mates at WACCBIP, I say thank you very much for your support in diverse ways.

My appreciation goes to the Message of the Hour Assemblies in Kisumu, Rodi and all the sister churches in the western and Rift valley provinces of Kenya for your prayers and support

Finally, thanks go to my wife and the children for their support through prayers and tolerance during my continual absence.

God bless you all

DEDICATION

To my late parents, Maku Mlehuno and Agborkey Abraham Debrah who had a vision and foresaw me attaining this high education.

To my lovely wife, Mrs Sarah Edo Debrah and my dear children, Micaiah Addo Debrah, Pendo Anne Debrah, Baraka Stefan Debrah and Seline-Norah Mawuhi Debrah

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABCs: ATP-binding cassettes

An: *Anopheles*

CI: confidence interval

COEs: carboxylesterases

CPs: cuticular proteins

CYPs: cytochrome P450s

DDT: dichlorodiphenyltrichloroethane

EIR: entomological inoculation rate

ELISA: enzyme-linked immunosorbent assay

FC: fold change

FDR: false discovery rate

GO: gene ontology

GSTs: glutathione s-transferases

GTS: global technical strategy

HBI: human blood index

ICEMR: International Centre for Excellence Malaria Research

IMPs: immunity proteins

IRS: indoor residual spraying

ITNs: insecticide-treated nets

ITS: internal transcribed spacer

kdr: knockdown resistance

LLIN: long-lasting insecticide-treated nets

LSM: larval source management

ncRNAs: non-coding RNAs

OPs: olfactory proteins

PBO: piperonyl butoxide

PCA: principal component analysis

PCR: polymerase chain reaction

PMI: President Malaria Initiative

PSC: pyrethrum spray collection

RT-PCR: real-time polymerase chain reaction

s. l: sensu lato

s.s: sensu stricto

UGTs: UDP-glycosyltransferases

WHO: World Health Organization

THESIS OUTLINE

This thesis work is composed of six chapters.

Chapter 1: General introduction which serves as the bedrock of the thesis.

Chapter 2: Contains the literature review of the study.

Chapter 3: Original results presented in a form of a manuscript already published in PLOS ONE Journal as **Debrah, I.**, Afrane, Y. A., Amoah, L. E., Ochwedo, K. O., Mukabana, W. R., Zhong, D., Zhou, G., Lee, M.-C., Onyango, S. A., Magomere, E. O., Atieli, H., Githeko, A. K., & Yan, G. (2021). **Larval ecology and bionomics of *Anopheles funestus* in highland and lowland sites in western Kenya**. *PLOS ONE*, *16*(10), e0255321. https://doi.org/10.1371/journal.pone.0255321

Chapter 4: Original results presented in the form of a manuscript already published in MDPI Insects as **Debrah, I.**; Ochwedo, K.O.; Otambo, W.O.; Machani, M.G.; Magomere, E.O.; Onyango, S.A.; Zhong, D.; Amoah, L.E.; Githeko, A.K.; Afrane, Y.A & Yan, G. (2023) Genetic Diversity and **Population Structure of *Anopheles funestus* in Western Kenya Based on Mitochondrial DNA Marker *COII***. *Insects*, *14*, 273. https://doi.org/10.3390/ insects14030273

Chapter 5: Original results presented in the form of a manuscript submitted to BMC Genomics Journal as **Metabolic Resistance to Pyrethroids with Possible Involvement of Non-coding Ribonucleic Acids in *Anopheles funestus*, the Major Malaria Vector in Western Kenya**

Preprint available online as **Debrah, I.**, Zhong, D., Machani, M. G., Nattoh, G., Ochwedo, K. O., Morang'a, C. M., Lee, M.-C., Amoah, L. E., Githeko, A. K., & Afrane, Y. A. (2024). **Non-Coding RNAs Potentially Involved in Pyrethroid Resistance of *Anopheles funestus* Population in Western Kenya**. *Research Square*.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10925441/

Chapter 6: General discussion, conclusions and recommendations

ABSTRACT

Background

Malaria is the deadliest protozoan disease transmitted by female *Anopheles* mosquitoes. Africa region continues to lead the heaviest malaria burden globally. Africa region continues to lead the heaviest malaria burden globally. *Anopheles funestus* is one of the efficient vectors of *Plasmodium. falciparum* malaria exhibits high anthropophilic, endophilic and anthropophagic behaviours. Its competence to transmit *P. falciparum* outpaced other main malaria vectors in certain countries. *An. funestus* is a dominant vector in western Kenya responsible for year-round high malaria transmission. However, its larval ecological requirements, the genetic basis of adaptations and mechanisms of resistance to public health insecticides are poorly understood. Therefore, this study investigated its larval ecology and bionomics, population structure, diversity and molecular basis of insecticide resistance in western Kenya.

Methods

A larval survey was conducted, and all potential mosquito aquatic habitats were identified, georeferenced, characterized and carefully examined for the presence of mosquito larvae and predators. Endophilic mosquitoes were collected using pyrethrum spray catches, prokopack and mouth aspirators. Mosquito species were identified using molecular and morphological methods. Molecular techniques were used to detect sporozoite infections and sources of blood meals. WHO insecticide susceptibility test was employed to assess *An. funestus* resistant status to the insecticides. Sanger sequencing was employed to sequence the *COII* gene to determine genetic diversity and population structure. Whole transcriptomic analysis using RNA-sequencing was performed to investigate the molecular basis of insecticide resistance.

Results

An. funestus larvae were found in greater numbers in the highland site, Bungoma (85%, 95%, CI, 8.722–17.15) than in the lowland site, Kombewa (15%, 95%, CI, 1.33–3.91). Approximately 59%, 35% and 5% of *An. funestus* larvae co-existed with *An. gambiae s.l*, *Culex spp* and *An. coustani* in the same habitats respectively. High *P. falciparum* sporozoite rate and human blood index were recorded. *An. funestus* populations from western Kenya had high haplotype diversity but low nucleotide diversity. No genetic differentiation or structure and a high level of gene flow were observed among the *An. funestus* populations. A high level of pyrethroid resistance was observed in various study sites (Bungoma, Teso, Siaya, Port Victoria and Kombewa) in western Kenya with Port Victoria having the highest level of resistance to both the type I (permethrin) and type II (deltamethrin) pyrethroids. The average mortality rate (MR) of pyrethroid resistance observed in all sites was 57.6%. Resistance to dichlorodiphenyltrichloroethane (DDT) was observed in Kombewa and Port Victoria but suspected resistance to DDT was observed in Siaya and Teso. However, *An. funestus* population was fully susceptible to 0.25% pirimiphos- methyl in all the sites. The preexposure to piperonyl butoxide (PBO) revealed high susceptibility to the pyrethroids in all the sites except for Port Victoria where suspected resistance for PBO + deltamethrin was observed (MR=96%). Whole transcriptomic analysis showed that most of the gene families associated with pyrethroid resistance comprised non-coding RNAs (67%), followed by immunity proteins (10%), cytochrome P450s (6%), cuticular proteins (5%), olfactory proteins (4%), glutathione S-transferases (3%), UDP-glycosyltransferases (2%), ATP-binding cassettes (2%) and carboxylesterases (1%). The top cytochrome P450 genes that were overexpressed in the *An. funestus* in western Kenya include CYP6P9A, CYP6P9b, CYP6N1, CYP9J5, CYP49A1, AFUN020895, AFUN019365, CYP9K1, CYP304B. GSTs (GSTD1,

GSTT2, GSTD7, GSTD11, GSTD3, GSTE6), peptidase S1 domain-containing protein (AFUN018482, AFUN018981, AFUN018580), cuticular protein (AFUN021427, AFUN021428, AFUN019106), UGTs (UGT310B2, UGT308D2, UGT306A3, AFUN003620) and sulfotransferase (AFUN016205, AFUN016207) were also overexpressed in *An. funestus* populations in western Kenya**.**

Conclusions

This study revealed the high adaptability of *An. funestus* to various breeding habitats and its larva co-exist with other mosquito larvae in the same aquatic habitats. *An. funestus* population is under selective pressure in western Kenya resulting in demographic expansion and the spread of variants through breeding. Population expansion and a high level of insecticide resistance suggest the high adaptability of *An. funestus* to various habitats, hence sustaining its vectorial capacity and malaria transmission. This study unveils the molecular basis of insecticide resistance in *An. funestus* in western Kenya, highlighting for the first time the potential role of non-coding RNAs in pyrethroid resistance. Targeting non-coding RNAs for intervention developments could help in insecticide resistance management.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Globally, the African region continues to bear the heaviest malaria burden accounting for more than 95% of all cases reported and a majority of reported deaths (WHO, 2021). *Plasmodium falciparum* is the main causative agent and is transmitted mainly through the bite by an infectious female *Anopheles* mosquitoes accounting for more than 78% of diagnosed episodes in Sub-Saharan Africa (WHO, 2021).

Malaria control is largely reliant on vector control strategies such as insecticide-treated nets/ long-lasting insecticide-treated nets (ITNs/LLIN), indoor residual spraying (IRS), and medication strategy using artemisinin-based combination therapies (ACTs). Among the two control options, vector control has immensely contributed to lowering malaria transmission cases. This is largely a result of their increased efficacy and effectiveness in inhibiting infection and reducing transmission (WHO, 2019), which accounted for a significant reduction in malaria as was shown previously (Bhatt *et al*., 2015).

Malaria in Sub-Saharan Africa is spread mainly by members of the *Anopheles funestus* group and *the Anopheles gambiae* species complex (Sinka *et al*., 2010). The *An. funestus* group (here referred to as *Funestus* Group) is made up of five subgroups of which three of these subgroups is made up of at least thirteen (13) species that colonize various ecological niches across the Afrotropical region (Harbach, 2013). The *Funestus* Group includes the *Funestus* Subgroup (*Anopheles funestus sensu stricto* (s.s.), *Anopheles funestus-like, Anopheles vaneedeni, Anopheles confuses, Anopheles parensis, Anopheles longipalpis type C* and *Anopheles aruni*), the *Rivulorum* Subgroup (*Anopheles rivulorum, Anopheles brucei, Anopheles rivulorum-like*,

Anopheles fuscivenosus), and the *Minimus* Subgroup (*Anopheles longipalpis type A* and *Anopheles leesoni*) (Harbach, 2013). Of all these members, *Anopheles funestus sensu stricto* (s.s.) (hereafter *An. funestus*) is the most predominant and competent vector of human malaria in Africa. Furthermore, its behaviour makes it highly competent to transmit *P. falciparum* and is thought to be emerging as the major vector outpacing *An. gambiae* and *An. arabiensis* in the eastern and southern African regions (Coetzee & Fontenille, 2004; Kaindoa *et al*., 2017; Spillings *et al*., 2009).

An. funestus exhibits high anthropophagic (biting humans), endophagic (indoor biting), anthropophilic (prefer human habitation), and endophilic (indoor resting) behaviours (Kaindoa *et al*., 2017). It has once played an important role in the malaria epidemic in southern Africa (Hargreaves *et al*., 2000). *An. funestus* is a threat to malaria elimination due to its high distribution, high resistance, and high vectorial capacity (Coetzee & Koekemoer, 2013). In endemic areas of western Kenya, *An. funestus* is one of the principal vectors mediating malaria transmission (Ogola *et al*., 2018; Zhou *et al*., 2011). Historically, western Kenya witnessed a decline in *An. funestus* population after the introduction of insecticide-based control tools (Gimnig *et al*., 2003), and this continued for several years until a re-emergence was noticed between 2010 to 2011 (McCann *et al*., 2014). Several factors were attributed to this reemergence, one is the ecology of the aquatic stages which could have contributed in the buildup to the comeback. The second hypothesis is its population structure and resistance to insecticides.

The biology of the aquatic stage of *An. funestus* is not well understood. Adult *An. funestus* is much easier to find than larvae. As a result, this species is rarely found in *Anopheles* larval surveys. Hence, researchers and field entomologists more often rely on adult collections rather

than larval sampling to get enough samples for insecticide resistance testing (Kaindoa *et al.*, 2017), an approach that requires F1 progenies with synchronized age groups to meet the guidelines stipulated by the WHO to undertake assays such as insecticide resistance test (WHO, 2018b).

Available evidence indicates that *An. funestus* is resistant to most insecticides that are recommended for vector control such as those with pyrethroids (Mulamba *et al*., 2014; Riveron *et al*., 2015), and the main challenge to its control is the insufficient data on its bionomics (Ngowo *et al.*, 2021). This is critical in several ways. First, its re-emergence poses a serious threat to the gains made thus far in malaria reduction. Second, a lack of larval ecological behaviour means that countermeasures targeting adults may not be sufficient in halting its geographical spread. Third, a shift in insecticide behaviour towards resistance suggests that current frontline strategies could be counterproductive. Therefore, deep ecological understanding, followed by enhanced targeting of *An. funestus* has been suggested as a way to increase control and minimize malaria transmission in places where the vector predominates (Kahamba *et al*., 2022; Kaindoa *et al*., 2017). To achieve this, an adequate larval survey and efficient characterization of potential breeding habitats of *An. funestus* in potential ecological settings were proposed (Kahamba *et al*., 2022; Killeen, 2003). Moreover, there have been growing concerns about the adaptation of *Anopheles* mosquito larvae to different breeding habitats (Emidi *et al*., 2017; Kabula *et al*., 2011; Kudom, 2015). Such ecological adaptions could have a long-term impact on controlling the aquatic stages of the disease vectors since finding larval habitats is crucial in larval source management (Nambunga *et al*., 2020). Unlike other major human malaria vectors, the larval ecological requirements of *An. funestus* is not well-known in many endemic regions.

External environmental stress has an impact on the vector survival rate, behaviour, ecology, vectorial capacity, and host-pathogen interactions. Temperature variations, land-use changes, host migration, and insecticide use are all factors that influence mosquito population selection (Zouache *et al.*, 2014). The evolution of different species could be due to the selection pressure of insecticide-based control tools. Advances in sequencing strategies, population genetic and genomic methods are increasingly becoming important tools for monitoring and understanding vector population structure, diversity, dynamics and dispersal ultimately aiming to inform malaria control (Main *et al*., 2015, 2016). However, only a few genetic studies have been conducted on *An. funestus* mitochondrial markers in sub-Saharan Africa (Jones *et al*., 2018; Krzywinski *et al*., 2006; Mukabayire *et al*., 1999).

Pyrethroids and dichlorodiphenyltrichloroethane (DDT) resistance have been observed in *An. funestus* with the main mechanism of resistance being metabolic resistance due to the over-expression of detoxifying enzymes (Mulamba *et al.*, 2014). The metabolic resistance was identified as the major threat to vector control (Riveron *et al*., 2014a). Multiple resistance involving cytochrome P450 detoxifying enzymes and L119F-GSTe2 resistance marker has been observed in *An. funestus*. Although up-regulation of cytochrome P450 genes plays a little role in DDT resistance, pyrethroid resistance is mainly dependent on cytochrome P450 metabolic enzymes while L119F-GSTe2 resistant mutation is dependent on DDT resistance (Djouaka *et al*., 2011, 2016). The reduction in LLIN efficacy in Southern Africa was ascribed to the expression of cytochrome P450 allele, CYP6P9a_R (Weedall *et al*., 2019). Furthermore, a study has linked the loss of efficacy of bed nets including piperonyl butoxide (PBO)-based bed nets against *An. funestus* to over-expression of cytochrome P450s particularly the CYP6P9a_R allele in Mozambique, Southern Africa (Riveron *et al*., 2019). Different alleles of the cytochrome P450

have also been implicated in metabolic resistance to pyrethroid across Africa and variations in these alleles have been observed in various geographical areas in *An. funestus* populations (Ibrahim *et al*., 2018; Sandeu *et al*., 2020). This diversity in gene expression profiles in Africa suggests the molecular basis of pyrethroid resistance in *An. funestus* may differ within the continent, national and local populations. Insecticide resistance in mosquitoes involves numerous genes and multiple complex pathways, many of which have not yet been investigated (Djouaka *et al*., 2011; Riveron *et al*., 2019). Indeed, studies have associated new gene families and mechanisms potentially involved in insecticide resistance in *Anopheles* mosquitoes (Al-Yazeedi *et al*., 2024; Logan *et al*., 2024). However, there is a limited body of evidence on the insecticide-resistance profile and molecular basis of pyrethroid resistance in *An. funestus* populations in western Kenya.

1.2 Statement of the Problem

Despite the immense investment in efforts to reduce and eliminate malaria, high transmission in endemic areas of sub-Saharan Africa still exists. *An. funestus* is a dominant vector responsible for high malaria transmission in east, south, central and west African regions (Dadzie *et al*., 2013; Kaindoa *et al*., 2017; Mapua *et al*., 2022). In western Kenya, *An. funestus* predominates in various ecological zones and mediating transmission (McCann *et al*., 2014; Zhou *et al*., 2016a), but its larval ecological requirements have not been fully elucidated. Furthermore, unravelling host genetic traits of its ecological adaptations to various settings could enrich our understanding of how the population is structured or segregated, but this is poorly understood. Considering insecticide resistance, the genetic structure and diversity of *An. funestus* population in western Kenya has not been well characterized. Quantitative information on levels of insecticide resistance in field populations of mosquitoes to the spectrum of vector control insecticides is

vital to comprehend the operational significance of resistance. Insecticide susceptibility surveys have been conducted in southern, eastern, and western Africa, with regional variations being observed (Mulamba *et al*., 2014; Riveron *et al*., 2015; Weedall *et al*., 2015a). However, there is limited evidence on the insecticide resistance profiles of *An. funestus* in western Kenya. Also, the molecular basis of *An. funestus* mosquito resistance to insecticides in western Kenya has not been fully elucidated.

1.3 Rationale

Despite achievements in reducing malaria transmission, resurgence and increased morbidity have been reported in endemic areas mainly in WHO African regions (Abiodun *et al*., 2020; Epstein *et al*., 2022). With the current efforts to achieve the elimination goal, it is imperative to fully incorporate larval source management which once played a crucial role in malaria eradication in once malaria-endemic countries. Indeed, one of the principles of the WHO Global Technical Strategy (GTS) for malaria elimination goal was that countries should expedite their efforts toward elimination through combinations of control interventions customized for the local circumstances (WHO, 2015). However, larval source management requires a deep understanding of the ecology of the larval stage of the vectors as well as a better characterization of the breeding habitats of vector species. Nonetheless, finding aquatic habitats of *An. funestus* is cumbersome as it infrequently breeds in the aquatic habitats of other mosquitoes. Furthermore, more focus on adult vector control methods and challenges in sampling aquatic habitats have limited the larval ecology studies of *Anopheles spp*, particularly *An. funestus* in Africa. Studies have reported the adaptation of other mosquito larvae to different breeding environments (Emidi *et al*., 2017; Kudom, 2015), but information on *An. funestus* is unknown. In the malaria-endemic region of western Kenya, *An. funestus* is the main vector that mediates malaria transmission, but

its larval ecology is poorly understood.

Understanding genetic diversity and genetic structure is a step towards uncovering sub-population differentiation, vector population dynamics and understanding if differences in vector genetic diversity have an impact on variations in the vector biology, behaviour, and susceptibility to malaria parasites and insecticides. These will have an impact on the management, control and surveillance of the malaria disease. Moreover, delineating the population structure of vectors is useful for investigating the genetic basis of speciation and local adaptation processes.

Understanding the pattern of insecticide resistance and characterization of mechanisms involved in the vector population is very crucial for successful resistance management. It is important to bring together conventional and more advanced tools to unveil the mechanisms of insecticide resistance in this vector population at different locations in western Kenya. This will serve as the bedrock for policymakers and stakeholders involved in vector control to aid in resistance management and rational deployment of insecticide-based vector control tools. In general, the outcome of this study will contribute significantly to the existing body of knowledge to help in vector control to achieve the elimination and eradication goal.

1.4 Hypothesis and Objectives of the Study

1.4.1 Hypothesis

It is hypothesized that *An. funestus*, the main malaria vector in western Kenya, has varied adaptations to breeding environments that shape diversity and population structure which influence the host's response to insecticides largely because of an emerging response mechanism associated with these variations.

1.4.2 Objectives of the Study

This study aimed to investigate *An. funestus* larval ecology and bionomics, population structure, diversity and the molecular basis insecticide resistance in western Kenya. Specifically, to:

1. Investigate larval ecology and bionomics of *An. funestus* in different ecological landscapes in western Kenya
2. Determine the population structure and genetic diversity of *An. funestus* in different landscapes in western Kenya
3. Assess the insecticide resistance status of *An. funestus* and the underlying molecular mechanisms of resistance in different ecological zones in western Kenya

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The global malaria burden

One of the oldest and deadliest diseases in the world is malaria (Abanyam, 2020). Since 2010, there has been a decrease in malaria mortality worldwide, mostly because of the expansion of interventions for diagnosis, treatment, and insecticide-based vector control tools (Thellier *et al*., 2020). The World Health Organization's regions of Africa, Southeast Asia, and the Western Pacific have seen a standstill in the rates of decline since 2016, while the Eastern Mediterranean and the Americas have even seen a reversal (WHO, 2020). In 2020 compared to 2019, there were reportedly 47000 more fatalities from malaria and 14 million more cases (WHO, 2021). The WHO's global malaria strategy 2020 main target was to lower the incidence of malaria cases and the death rate by at least 40% (WHO, 2015). However, this has been missed; the 2030 elimination goal may not be attained if the current efforts and investments remain the same (Patouillard *et al*., 2017).

With an expected 228 million malaria cases in 2020, the WHO African Region accounted for over 95% of cases, while the WHO Southeast Asia region accounted for only 2% of the global total. In the WHO African region, case incidence decreased between 2000 and 2019 from 368 to 222 per 1000 people at risk, then rose to 232 in 2020, primarily due to service disruptions during the COVID-19 pandemic (WHO, 2021). Globally, the malaria fatality rate (defined as deaths per 100, 000 people at risk) decreased by half, from roughly 30 in 2000 to 15 in 2015 (Bhatt *et al*., 2015), and subsequently slowed down, dropping to 13 in 2019. The death rate rose once more to 15 in 2020 (WHO, 2021). These observations indicate that malaria despite being a global

problem, the majority of cases are concentrated in sub-Saharan Africa, where emerging health challenges such as Covid-19 are likely to push back efforts made towards malaria.

Pregnant women and children under the age of five are the demographic groups most susceptible to malaria morbidity (Hajison *et al*., 2018; Kwenti *et al*., 2017). In the WHO African region, 33 moderate and high transmission countries (some including, Kenya, the Gambia, Ghana, Equatorial Guinea, Guinea-Bissau, Guinea, Liberia, and Madagascar) had anticipated 33.8 million pregnancies, of which 11.6 million (34%) were exposed to malaria infection. Prevalence of malaria exposure during pregnancy was highest in west Africa (39.8%), followed by central Africa (39.4%), and lowest in west and southern Africa (22%) (WHO, 2021). Eight hundred and nineteen thousand (819, 000) newborns with low birth weight were projected to have been born as a result of malaria infection during pregnancy, with 54.1% of these children living in the subregion of west Africa (WHO, 2021). Due to the high prevalence of asymptomatic *Plasmodium falciparum* infection (sub-clinical malaria), school-aged children and adults act as the primary reservoir of gametocytes necessary to maintain transmission (Walldorf *et al*., 2015). Malaria remains a challenge to the most vulnerable communities which bear the bluntest in terms of case and fatality rates.

2.2 Malaria burden in Kenya

Approximately 70% of Kenya's 47 million population is at risk of malaria, with the majority of cases occurring in western Kenya. (National Malaria Control Programme (NMCP), 2016). Despite attempts by the Ministry of Health to scale up different malaria control tools, such as long-lasting insecticidal nets, indoor residual spraying and artemisinin-based combination therapy (ACT), the prevalence of malaria in Kenya remains high (Alegana *et al*., 2021). This was partly attributed to high resistance to insecticides used for vector control (Zhou *et al*., 2011).

Plasmodium falciparum is the main parasite causing the highest mortality and morbidity of more than eighty-five per cent (>85%). *P. viva* and *P. ovale* also contribute 5-10% and <5% mortality and morbidity respectively (President Malaria Initiative, 2022). The susceptibility of the local vectors to parasite infections is the cause of the spread of malaria. The principal malaria vectors transmitting malaria parasites are *An. funestus, An. gambiae*, *An. arabiensis* and *An. coustani* (President Malaria Initiative, 2022).

Malaria risk is heterogeneous; temperature, rainfall patterns, and altitude all have an impact on the disease's epidemiology. Four epidemiological zones—endemic (lake and coast), epidemic (highland), seasonal (semi-arid), and low risk—are recognized in the country (National Malaria Control Programme (NMCP), 2016; President Malaria Initiative, 2022). Based on the prevalence of malaria parasites, the most susceptible group is children under five years old. Modelling of Kenya Malaria Indicator Surveys (KMISs) conducted from 2000 to 2015 revealed that all counties in the lake endemic zone have changed from high to low-to-moderate transmission (Snow *et al*., 2015). Kenya is among six countries (the United Republic of Tanzania, Malawi, Rwanda, Mozambique, and Zambia) that reduced incidence by less than 40% in 2020 compared

to 2015 even though the GTS target was not realized in these countries in the East and Southern Africa sub-region (WHO, 2021).

2.3 *Anopheles funestus* group and their role in malaria transmission in Sub-Saharan Africa

The term "*Funestus* group" was first coined by Gillies and De Meillon in 1968 (Gillies & De Meillon, 1968a). It refers to a group of vector species that have morphological relatedness to *An. funestus* (Gillies & De Meillon, 1968a). The heterogeneity within the *An. funestus* population was first suspected in the 1930s based on the larval studies and the presence of "varieties", most of them were later identified as species within the group (Evans & Leeson, 1935; Evans & Symes, 1937). This group demonstrated the absence of morphological features at the adult stage. The classification under the *Funestus* group and their identification were based on their eggs, larvae or pharyngeal armature (Gillies & De Meillon, 1968a).

Cytogenetic studies later confirmed the taxonomical observation of the *Funestus* group (Green & Hunt, 1980; Christopher & Green, 1982) but due to the laborious and time-consuming nature of the use of these techniques, simple and sensitive molecular identification tools were developed to distinguish between species of the *An. funestus* group (Cohuet *et al*., 2003; Koekemoer *et al*., 2002). With advances in research through the employment of morphological, and molecular tools as well as using the status and position of each species of the *Funestus* group, the earlier classification was reexamined. It is now generally accepted that *An. funestus* is one of the members of the five subgroups. Of these five subgroups, three groups (*funestus*, *minimus* and *rivulorum*) are found in the Afrotropical region and contain a total of 13 species (Harbach, 2013) (**Table 2.1**). These thirteen known members of the *An. funestus* group are distributed in malaria-endemic regions of Africa region **(Figure 2.1)**.

The species within the *An. funestus* group exhibit unique behaviors such as feeding on human and non-human hosts but the degree of anthropophagism and zoophagism varies from species to species. The medical importance of most species of the *An. funestus* group is not well understood although there is growing evidence that some could be acting as malaria secondary vectors (**Table 2.1**).

Table 2.1: *An. funestus* group in Africa, distribution and role in malaria transmission

Species of the *Anopheles funestus* Group in Sub-Saharan Africa					
Subgroup	**Species**	**Geographical distribution**	**Host Preference**	**Role in malaria Transmission**	**References**
Funestus	*An. funestus*	continental	anthropophagic	major	(Dadzie *et al*., 2013; Dia *et al*., 2013; Mapua *et al*., 2022; Mendis *et al*., 2000)
	An. aruni	local	unknown	unknown	(Sobti, 1968)
	An. vaneedeni	local	anthropophagic	minor	(Burke *et al*., 2017)
	An. funestus-like	local	unknown	unknown	(Mouatcho *et al*., 2018 ; Vezenegho *et al*., 2009)
	An. confusus	regional	zoophagic	unknown	(Garros *et al*., 2005; Harbach, 2013)
	An. longipalpis type C	local	zoophagic	minor	(Ogola *et al*., 2018)
	An. parensis	regional	zoophagic	minor	(Burke *et al*., 2019)
Minimus	*An. longipalpis type A*	local	zoophagic	unknown	(Koekemoer *et al*., 2009)
	An. leesoni	continental	zoophagic	minor	(Kopya *et al*., 2021; Temu *et al*., 2007)
Rivulorum	*An. rivulorum-like*	continental	potentially variable	unknown	(Dahan-Moss *et al*., 2018; Wragge *et al*., 2021)

	An. brucei	local	unknown	unknown	(Cohuet *et al*., 2003)
	An. rivulorum	continental	zoophagic/ anthropophagic	minor	(Cohuet *et al*., 2003; Hackett *et al*., 2000; Kawada *et al*., 2012a; Ogola *et al*., 2018)
	An. fuscivenosus	local	unknown	unknown	(Green, 1982)

Adapted from (Dia *et al*., 2013) with modifications

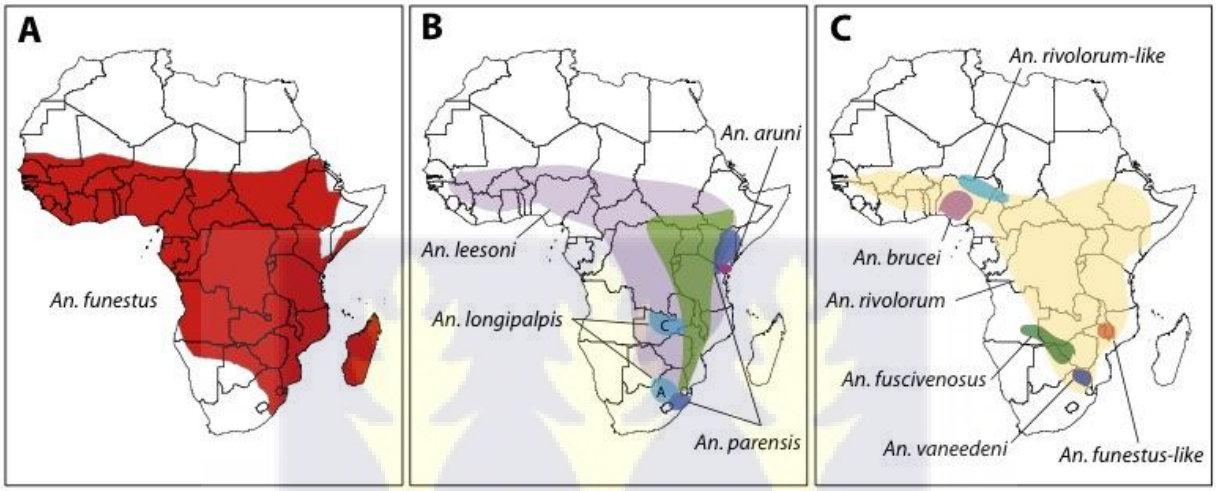


Figure 2.1: Continental distribution of the thirteen members of the *An. funestus* group (Dia *et al*., 2013). **A**: *Anopheles funestus*, **B**: *An. aruni*, *An. leesoni*, *An. longipalpis* (type A and C), and *An. parensis* and **C**: *An. funestus-like*, *An. vaneedeni*, An. *rivolorum*, *An. rivolorum-like*, *An. fuscivenosus* and *An. brucei*

The role of the species of some of the members of the *An. funestus* group are described below.

An. rivulorum is a secondary vector and hence plays a minor role in malaria transmission. It feeds early in the evening hours (Kawada *et al*., 2012b), and also has an exophilic tendency (Ogola *et al*., 2018). A study in Kenya has reported that it had a human blood index comparable to *An. funestus* (Ogola *et al*., 2018). It was reported to feed on both human and animal hosts suggestive that it has a broad-host-range feeding behavior (Ogola *et al*., 2018).

An. parensis was earlier found to be nulliparous in areas not sprayed with insecticides and hence considered not an efficient vector of human malaria (Gillies, & Furlong 1964). However, a recent study has documented that it tested positive for *P. falciparum* and could play a role as a secondary vector in residual malaria transmission (Burke *et al*., 2019). It has also been reported that it has a strong zoophilic tendency (Mouatcho *et al.*, 2007), but its endophilic (12/25) and exophilic (13/25) tendencies were comparable (Ogola *et al*., 2018).

An. longipalpis C was first identified in Kenya and was infected with *P. falciparum* highlighting its role in malaria transmission as a secondary vector (Ogola *et al*., 2018). It was also found to have a broad range of feeding behaviors on hosts such as cows and goats confirming the zoophilic nature (Kent *et al*., 2006).

An. leesoni exhibits more exophilic tendencies than endophilic. It feeds on both human hosts (Ogola *et al*., 2018), and non-human hosts including goats, sheep, and bovine (Dadzie *et al*., 2013). *An. leesoni* was found to be the second most abundant vector aside *An. funestus,* resting indoors, feeding largely on humans and infected with *P. falciparum* (Temu *et al*., 2007). This indicates that it could play a significant role as a secondary vector in residual transmission.

An. vaneedeni was found resting outdoors and infected with *P. falciparum* for the first time in South Africa, and this suggested that it could also play a role as a secondary vector of residual malaria transmission in that region (Burke *et al*., 2017).

An. rivulorum-like was found across the Afrotropical region but its role in malaria transmission, as well as resting behaviour, remains unknown (Moutatcho *et al*., 2018; Norris & Norris, 2015; Wragge *et al*., 2021). However, its feeding preferences were reported to be potentially variable (Norris & Norris, 2015).

An. funestus-like: Despite being sympatric with *An. funestus* and was first discovered in Malawi, an experiment has demonstrated that the two species are distinct (Spillings *et al*., 2009). A study has documented that it is potentially a zoophilic vector (Norris & Norris, 2015), but its role in malaria transmission has not been elucidated.

2.4 *Anopheles funestus* as a major vector of human malaria in sub-Saharan Africa

As far back as 1900, *An. funestus* is documented as one of the efficient dominant vectors of human malaria (Gillies & Coetzee, 1987). *An. funestus* has been largely ignored in comparison to its counterpart *Anopheles gambiae*, but it is an essential component of any comprehensive vector control program aimed at eliminating malaria from the African continent (Dia *et al*., 2013). The difficulty of adapting *An. funestus* to standard insectary environments, despite notable molecular and epidemiological developments over the past three decades, has slowed progress in understanding this vector (Ngowo *et al*., 2021). *An. funestus* role in malaria transmission surpasses that of *An. gambiae* and *An. arabiensis* in some endemic areas (Coetzee & Fontenille, 2004; Mapua *et al*., 2022).

The major Afrotropical vectors of human malaria exhibit anthropophilic and endopihlic behaviours. Many *Anopheles* mosquitoes have been implicated in transmitting human malaria parasites, but the most devastating efficient vectors exhibit notorious anthropophilic and synanthropic behaviours (live in association with humans) (Dadzie *et al*., 2013; Kaindoa *et al*., 2017; Sougoufara *et al*., 2014). *An. funestus* is known to possess these traits and is therefore considered a primary vector of human malaria. It has shown consistent endophilic and endophagic behaviours (Dadzie *et al*., 2013; Kaindoa *et al*., 2017; Sougoufara *et al*., 2014). Changes in feeding behaviour possibly arising from the implementation of vector control interventions from anthropophilic to much more zoophilic were previously reported in Senegal

(Lochouarn *et al*., 1998). However, feeding on both human and animal hosts was also observed in *An. funestus* indicates that it has a wide host range feeding propensity (Ogola *et al*., 2018). This biting preference was also associated with chromosomal polymorphisms between the two populations. Different chromosomal inversion combinations were associated with various biting and resting behaviours (Costantini *et al*., 1999).

In regions with high coverage of insecticide-based control tools, *An. funestus* mediates all year-round transmission, approximately 97% of malaria infections and a high entomological inoculation rate compared to other major vectors (Mapua *et al*., 2022). *An. funestus* alone now causes roughly nine out of every 10 new infections (Kaindoa *et al*., 2017; Lwetoijera *et al*., 2014), even in communities where it was outnumbered by *Anopheles arabiensis*, according to recent findings in the Ulanga and Kilombero districts in Tanzanian (Kaindoa *et al*., 2017; Lwetoijera *et al*., 2014). These districts were historically dominated by *Anopheles gambiae* in malaria transmission but *An. funestus* was responsible for transmission in dry seasons (Charlwood *et al*., 2000). However, following the deployment of ITNs, the *Anopheles gambiae* population and its role in malaria transmission have reduced drastically to a level that was not detectable (Lwetoijera *et al*., 2014). Recent studies have confirmed the absence of *Anopheles gambiae* in this setting outnumbering the sibling species, *Anopheles arabiensis* (Kaindoa *et al*., 2017; Limwagu *et al*., 2019).

A similar trend was observed in western Kenya following the introduction of insecticide-based interventions leading to the re-emergence and subsequent dominance of *An. funestus* population and responsible for all-year transmission (McCann *et al*., 2014; Zhou *et al*., 2016). In southern and east African countries, the majority of the all-year-round malaria transmission is still mediated by *An. funestus* (Burke *et al*., 2019; Kaindoa *et al*., 2017; Mapua *et al*., 2022; McCann

et al., 2014). Moreover, in central and west Africa, *An. funestus* is responsible for a higher entomological inoculation rate (Cohuet, *et al*., 2004; Konate *et al*., 2001). These demonstrate the crucial role of *An. funestus* in residual malaria transmission in areas it has colonized.

2.5 Larval habitats requirements of *Anopheles funestus*

Larval habitats or aquatic habitats also refers to as 'breeding sites' are areas where mosquitoes lay eggs, larvae hatch, change in stars, pupate, and emerge as adults. Larval habitats are the fundamental determinants of adult distribution, abundance, and fitness (Gimnig *et al*., 2001; Paaijmans *et al*., 2008). They comprise water bodies, whether they are man-made or natural, permanent or transient, large or small, freshwater or saltwater (Paaijmans *et al*., 2008).

It was documented that *Anopheles funestus* breeds in permanent and semi-permanent water bodies with floating or emergent vegetation, both natural and artificial (Gimnig *et al*., 2001; Nambunga *et al*., 2020). In places with both types of flora, however, this mosquito prefers breeding in habitats with emergent vegetation. Natural breeding occurs at the edges of swamps, in weedy and grassy areas of rivers, furrows, ditches, streams, furrows, ditches, and ponds (Minakawa *et al*., 2008; Nambunga *et al*., 2020) (**Figure 2.1**). The larval stage of *An. funestus* rarely survive in habitats directly exposed to sunlight without a vegetation cover (Minakawa *et al*., 2008). Vegetation plays a crucial role in mosquito breeding, larval survival and development (Msugupakulya *et al*., 2024).

Studies have reported that the juvenile stages of *Anopheles* mosquitoes are adapting to different ranges of aquatic habitats (Emidi *et al*., 2017; Kudom, 2015). The adaptation of the juvenile stage of malaria mosquitoes to a different range of breeding environments could have serious implications on the epidemiological outcome of malaria transmission. A recent study revealed

the co-occurrence of *An. funestus* with other mosquito species (Nambunga *et al*., 2020). Unfortunately, *An. funestus* larval ecological requirements and survival are poorly understood. Unlike *An. gambiae s.l*, few studies in western Kenya have examined the larval ecology of *An. funestus.* Moreover, most studies on *Anopheles* larval ecology never consider studying *An. funestus* separately hence limited evidence on the larval ecology of this important anthropophilic vector (Kahamba *et al*., 2022). Inadequate species-specific study on the immature stages of mosquitoes could mask peculiar host factors associated with early-stage development and abundance.

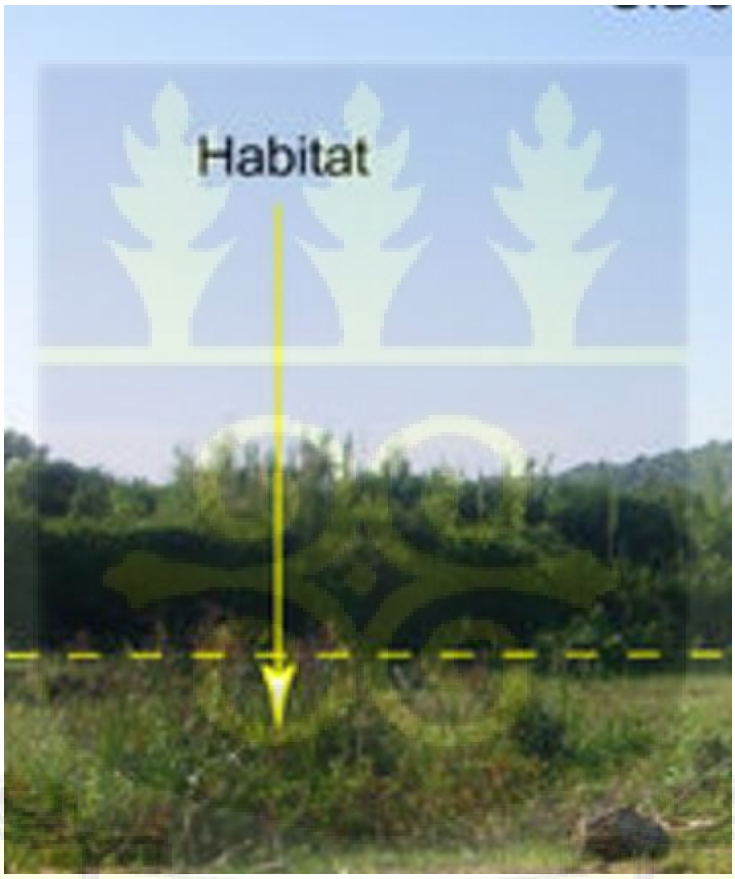


Figure 2.2: The typical breeding habitat of *An. funestus* (Adapted from (Minakawa *et al*., 2008)

2.6 Determinants of *Anopheles* larval density

Natural ecosystems are being drastically changed by human activities all around the planet. Due to population pressure, natural ecosystems are changed to support agriculture, road construction, dams, open-pit mines, irrigation projects, and uncontrolled colonization by humans (Myers & Patz, 2009; Patz *et al*., 2008). Approximately 2% to 3% of the world's tropical forests are lost annually, and land use changes for agriculture are the main factor changing land cover all over the planet (Carpenter *et al*., 2011; Patz *et al*., 2008). The increase in the number of ideal mosquito breeding habitats is facilitated by irrigation development efforts, which could increase the likelihood of high malaria transmission or potential outbreaks (Hawaria *et al*., 2020).

Mosquitos also respond to variations in environmental change, and even small changes in elements like humidity, temperature and the availability of suitable aquatic habitats for larvae have a big effect on the mosquito's density, dispersal and survival (Grillet, 2000; Wamae *et al*., 2010). Furthermore, the number and availability of productive larval habitats and their proximity to human or animal habitation to obtain blood meal determine the density of adult mosquitoes (Takken & Verhulst, 2013). Likewise, the density of the mosquito species in a particular ecological niche is largely influenced by types of breeding habitats, biotic and abiotic factors (Carlson *et al*., 2004; Minakawa *et al*., 2002).

Aquatic vegetation also known as hydrophytes or aquatic macrophytes play a crucial role in aquatic and wetland ecosystems. Since the early 1930s, aquatic vegetation was noted to have affected the oviposition of mosquitoes, anopheline mosquito larval survivorship and development (Matheson, 1930). Aquatic plants offer mosquito larvae food, shelter, and a good environment for oviposition. The air-plant-water interface, often known as the intersecting line, is particularly significant. Because the intersection line is where the larvae get food and refuge

and adults discover the water surface is broken up into countless calm cells ideal for ovipositing, the intersection line is significant to anopheline larvae (Merritt *et al*., 1992; Orr & Resh, 1992). Evidence from numerous studies has shown that the quantity of plant cover or intersecting line and larvae density are positively correlated (Hess & Hall, 1943; Orr & Resh, 1992; Walker *et al*., 1988). Emergent vegetation typically offers the most intersection lines out of the four main macrophyte categories—freely floating, emergent, submerged, and floating-leaved. Given the advantages of aquatic vegetation cover in general and areas of dense emergent vegetation, mosquitoes that choose high-density vegetation patches as larval habitats should have a significant selective advantage (i.e., greater fitness) (Orr & Resh, 1992). Although most Anopheline species are fairly generalists and not extremely selective for a particular type of vegetation, others have closer phytoecological relationships.

Predation on larval stages of *Anopheles* mosquitoes appears to be one of the several natural ecological mechanisms regulating vector populations (Mereta *et al*., 2013; Ohba *et al*., 2010). Tadpoles, aquatic insects (Heteroptera, Coleoptera and Odonata), and planktivorous fish are only a few examples of the wide variety of natural predators of mosquito larvae (Mogi, 2007). It has been demonstrated that interspecific competition for scarce resources can have significant impacts on mosquito larvae. Tadpoles compete with mosquitoes and this competition was also observed between other mosquito species and cladocerans (Blaustein & Margalit, 1994; Mokany & Shine, 2003). The role of predation on mosquito larvae may be more significant in large permanent aquatic habitats while interspecific competition among mosquito species may be more significant in small ephemeral aquatic habitats (Juliano, 2009).

The aquatic habitats' physicochemical properties have been reported to have influenced the *Anopheles* larval density (Emidi *et al*., 2017; Kudom, 2015; Lawal *et al*., 2022). Increased

density of *Anopheles* larvae was significantly correlated with high salinity and conductivity (Emidi *et al*., 2017). In *Anopheles* breeding sites in residential areas, biological oxygen demand, conductivity, Na+ and Cl- had positively influenced the larval density whereas biological oxygen demand and chemical oxygen demand negatively affected the densities in the larval habitats in petrochemical areas (Abdel-Meguid, 2022; Imam & Deeni, 2015). In the agricultural areas, a significant increase in larval density in the larval habitats was associated with biological oxygen demand, dissolved oxygen, Na^+, K^-, Cl^- and $NO3^-$ (Lawal *et al*., 2022). The density of *Anopheles gambiae* was associated with a water temperature range of 20.7-28.0°C and a pH between 5.8-7.8 (Garba & Olayemi, 2015) while *An. funestus* density was associated with a water temperature range of 25.2-28.8°C, and a pH between 6.5-6.7 (Nambunga *et al*., 2020). Water turbidity range of 26.6-54.8 NTU and total dissolved solids (TDS) range from 60.5-80.3 mg/L was associated with *An. funestus* larval density (Nambunga *et al*., 2020). In general, *Anopheles* mosquito larvae cannot withstand water temperatures beyond 40°C (Paaijmans *et al*., 2008). A study by Kinga *et al*., (2022) revealed that *Anopheles* abundance was specifically influenced by biochemical oxygen demand, total dissolved solids, and alkalinity, whereas *Culex* abundance was significantly influenced by total suspended solids, sulphate, ammonium, phosphate, and salinity. The key determinants of *Anopheles* larvae density are summarized in the diagram below **(Figure 2.3**).

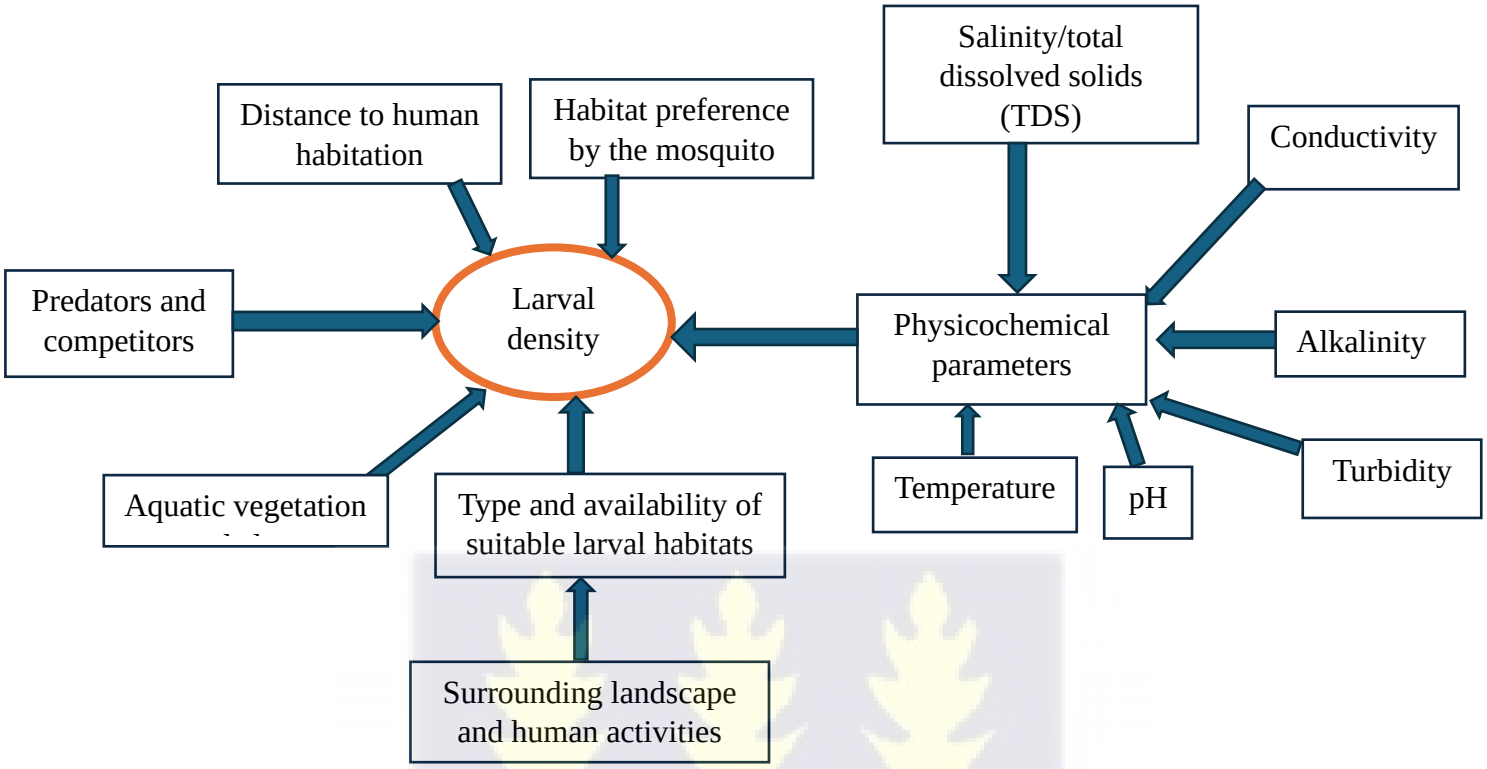


Figure 2.3: Key variables that determine *Anopheles* larval density

2.7 Genetic diversity of *Anopheles funestus* in sub–Saharan Africa

An. funestus exhibits notable genetic variation throughout its distribution. Genetic heterogeneity of *An. funestus* population was observed in the Afrotropical region (Jones *et al*., 2018; Ogola *et al*., 2019). There are numerous genetic and chromosomal polymorphisms present in this vector (Dia *et al*., 2013). *An. funestus* was found to be separated into three major blocks: west Africa, east Africa, and central Africa, these clustering are based on microsatellite markers observed throughout the African continent (Michel *et al*., 2005). *An. funestus*, which was found in southwest Uganda, shared similarities with all three divisions. This structure was confirmed by mitochondrial DNA (mtDNA), however, the resolution of the mtDNA gene trees was less (Michel *et al*., 2005). This subdivision was roughly identical to that of *An. gambiae* observed throughout Africa (Lehmann *et al*., 2003). This indicates that the same geographic and climatic factors are likely to have influenced both species' populations.

Further cryptic subdivision of *An. funestus* population was discovered by mitochondrial DNA further adding to the already available discovery from nuclear genome analysis. Two mtDNA lineages, clade I and clade II, were present in the samples from Mozambique and Madagascar. These lineages were distinguished by two fixed differences and a mean of 2% divergence, indicating that they have existed separately for about a million years (Michel *et al*., 2005).

Chromosomal inversion polymorphisms were found to be considerably different across the coastal and western samples of Kenya's *An. funestus* population, suggesting restricted gene flow between the two samples, which were 700 km apart (Kamau *et al*., 2002). Ogola *et al*., (2019), identified three genetically distinct *An. funestus* clusters (FUN1, FUN2, and FUN3) in Kenya using microsatellite markers. The largest cluster (FUN1) is made up of samples from the Rift

Valley and western regions, while the other clusters (FUN2 and FUN3) are made up of samples from the coast.

The use of mtDNA sequences for genetic diversity, molecular phylogeny and species identification is becoming increasingly important in mosquito research (Anoopkumar *et al*., 2019). This is because mtDNA sequences are genetic drift-sensitive, have high copy numbers, availability of conserved primer binding sequences, and ease of amplification hence they are widely employed in interpreting molecular taxonomy, phylogenetic relationships, population structure, and genetic diversity in malaria vectors (Norris, 2002). However, in western Kenya dominated by *An. funestus*, the genetic diversity using the mtDNA is poorly understood.

2.8 Insecticide resistance and mechanisms in malaria vectors

Vector control is the frontline strategy for malaria control and it is considered a critical component and key preventive strategy for the fight against malaria (Hemingway, 2014). Insecticide-treated mosquito nets (ITNs), primarily long-lasting insecticidal nets (LLINs) treated impregnated with a synthetic pyrethroid and indoor residual spraying (IRS) of insecticides are two key insecticide-based vector control interventions. The widespread use of these two insecticidal interventions has resulted in significant decreases in malaria morbidity and mortality since 2000 (Bhatt *et al*., 2015; WHO, 2018c). However, malaria vectors across the world have developed resistance to at least one of the four classes of public health insecticides (organophosphates, carbamates, pyrethroids, and organochlorines) frequently utilized in vector control interventions (Hemingway, 2018; Ranson & Lissenden, 2016; WHO, 2018a). Globally, 29 countries have already observed resistance to pyrethroids, carbamates, organophosphates and organochlorines across various sites, and 19 countries have confirmed resistance to all four of these insecticide classes in at least one site and at least one local vector (WHO, 2021). Of the 88

malaria-endemic countries that provided data for 2010-2020, 78 have found resistance to at least one insecticide class in at least one malaria vector and one collection site (WHO, 2021). In 68% of the sites in malarious regions for which data were available, at least one malaria vector was resistant to pyrethroids, the main insecticide class now used in ITNs. In 64% of the sites, there was evidence of organochlorine resistance. Resistance to organophosphates and carbamates though less prevalent in the locations was reported at 28% and 34% respectively (WHO, 2021).

Pyrethroid resistance is of particular concern, given this insecticide class is included in all WHO recommended LLINs and is also used for IRS in most countries (WHO, 2017). Resistance to pyrethroids has been considered a serious threat to malaria control programs (Agossa *et al*., 2015; Churcher *et al*., 2016; Hemingway, 2018). According to the WHO's Global technical plan for malaria 2016–2030 (WHO, 2015), one of the primary biological obstacles to malaria control and elimination is malaria vectors' resistance to insecticides employed in core preventative strategies. It is against this background that the WHO launched the Global Plan for Insecticide Resistance Management (GPIRM) in 2012 (WHO, 2012) for planning, executing and monitoring insecticide management at the national level, developing novel vector control tools, filling up the gaps in understanding of resistance mechanisms and the impact of current resistance management strategies as well as enabling systems for better advocacy and the development of human and financial resources (WHO, 2012, 2016).

Insecticide resistance mechanisms underlying the phenotypic resistance have been classified into four main categories; a metabolic mechanism, target-site insensitivity, behavioural avoidance and cuticle thickening to prevent or reduce the penetration of the insecticide (Hemingway, 2000; WHO, 2018a).

Metabolic detoxification mechanism: Three main metabolic detoxification gene families—cytochrome P450s (P450s), esterases, and glutathione S-transferases (GSTs)—are involved in the detoxification of insecticides in mosquitoes. One of the largest gene groups found in all living things, P450s carry out a wide range of biochemical and physiological tasks. P450s are essential for the activation and/or detoxification of endogenous and xenobiotic substances (Feyereisen, 2005). The metabolism, detoxification, and excretion of several endogenous and foreign substances are all facilitated by soluble dimeric proteins known as GSTs (Ranson *et al*., 2001; Ranson & Hemingway, 2005). An important characteristic of insect P450s and GSTs is transcriptional upregulation, which leads to higher levels of protein synthesis and enzymatic activity. As a result, insects are better able to metabolically detoxify insecticides, a phenomenon that causes them to develop resistance (Feyereisen, 2005; Rakotondranaivo *et al*., 2018; Scott, 1999).

Initial investigations to determine if metabolic detoxification is involved in the development of insecticide resistance in mosquitoes have mostly made-up synergistic studies (Brogdon *et al*., 1999; Cuamba *et al*., 2010; Enayati *et al*., 2003). The chemical synergists PBO, S,S,S,tributylphosphorotrithioate, and diethyl maleate, which are inhibitors of cytochrome P450 monooxygenases, esterases, and GSTs, respectively, have been the subject of some potential mechanisms uncovered by these research. Researchers can determine whether and which detoxification systems are involved in the emergence of resistance by comparing the toxicity levels with and without the synergists. Strong evidence for the significance of metabolic detoxification in insecticide resistance can be found in synergism research on pyrethroid resistance in various mosquito species (Djouaka *et al*., 2016). Although utilizing synergists may reveal the role of detoxification proteins in insecticide resistance, in some instances synergists

may not be effective in inhibiting some of the detoxification enzymes involved in resistance (Feyereisen, 2005).

Target-site insensitivity: It is caused by point mutations or structural alterations in the genes that code for target proteins that interact with insecticides (Casida & Durkin, 2013). Sodium channels are the target of insecticides like pyrethroids and dichlorodiphenyltrichloroethane (DDT). They bind to the sodium channels, resulting in the repeated discharge of the insect's neural system and depolarization of its nerve membranes leading to death (Narahashi, 1988). An important enzyme in the process of the neural system, acetylcholine neurotransmitters are hydrolyzed, and nerve impulses are terminated; this is the target of both carbamate and organophosphate insecticides. The diminished target-site sensitivity of sodium channels causes insects and other arthropods to become resistant to DDT and pyrethroid insecticides, a phenomenon referred to as knockdown resistance (*kdr***)** (Knipple *et al*., 1994; Li *et al*., 2012)**.** It occurs as a result of single nucleotide polymorphism (point mutations) at locus 1014 of the sodium channel genes resulting from the substitution of leucine for phenylalanine (Leu/Phe), also known as *kdr* west (L1014F) (Martinez-Torres *et al*., 1998), and substitution of leucine for serine (Leu/Ser), known as *kdr east* (L1014S) (Ranson *et al*., 2000). *Kdr* resistance confers cross-resistance to DDT and pyrethroids (Chandre *et al*., 1999). Pyrethroids and DDT target the voltage-sensitive sodium channel whereas organophosphate and carbamate inhibit the esterase resulting in the accumulation of acetylcholine in the synapses thereby impairing the nerve function and finally death of the insect (Sparks & Nauen, 2015).

Behavioural avoidance: The capacity of an insect to leave (escape) an area treated with insecticide is referred to as behavioural avoidance (deterrence), and it is frequently done without fatal consequences (Grieco *et al*., 2007). This type of reaction can also be further broken down

into non-contact spatial repellency and direct contact excitation (also known as "irritancy"). The term "contact irritancy" refers to an insect leaving an area that has been treated with an insecticide only after making direct physical contact (tarsal contact) with the chemical, whereas "spatial repellency" refers to an insect leaving an area that has been treated with an insecticide thereby avoiding direct contact with the chemical (Chareonviriyaphap *et al*., 1997; Roberts *et al*., 1997).

Reduced Penetration (Cuticular resistance): A thickening of the cuticle to alter insect cuticle chemistry to reduce the insecticide penetration of insect bodies (Karunaratne *et al*., 2018). This can reduce the metabolic processes by allowing penetration and giving extra time for detoxifying enzymes. Given that most insecticides are lipophilic by nature, cuticular resistance is thought to confer cross-resistance to several insecticides (Messenger *et al*., 2021; Nkya *et al*., 2014). Genes are overexpressed in resistant insects, increasing the number of cuticular hydrocarbon deposits in the epicuticle and increasing their resistance to insecticide intake. The quantitative alteration of cuticular hydrocarbons in populations of the highly pyrethroid-resistant *An. arabiensis*, *An. gambiae* and *Ae. aegypti* is correlated with increased expression of the cytochrome P450 enzymes, CYP4G16 and CYP4G17, which catalyze epicuticular hydrocarbon production (Balabanidou *et al*., 2016). It has been hypothesized that enhanced expression of cytochrome P450 monooxygenases is responsible for the cuticular resistance observed in these mosquitoes as seen by the lower rate of ^{14}C deltamethrin penetration (Balabanidou *et al*., 2016; Bass & Jones, 2016).

2.9 Insecticide resistance and mechanisms in *Anopheles funestus*

The first evidence of *An. funestus* insecticide resistance was suggested in the Nigerian species when three years of constant DDT, benzene hexachloride (BHC), and dieldrin spraying failed to eradicate target population in the north (Bruce-Chwatt *et al*., 1955). Subsequently, resistance to dieldrin, DDT and malathion was reported in Mali (Touré, 1982) and resistance to dieldrin was reported in four countries (Benin, Cameroon, Ghana, and Kenya) (Brown, 1986). On the insecticide susceptibility of *An. funestus* in east Africa especially in Kenya, few investigations have been conducted. The initial data from two sites in western Kenya were reported in 2007 (Kamau *et al*., 2008), and despite the lack of genetic identification for these specimens, earlier identified collections from the same two sites revealed the presence of solely *An. funestus* (Kamau *et al*., 2003). Seven *An. funestus* adults were collected in western Kenya in 2009, and susceptibility testing on their F1 offspring revealed permethrin resistance while DDT susceptibility ranged from 83.3% to 100% (Kawada *et al*., 2011). Previous research in Kisumu, western Kenya and Uganda has shown that there was widespread resistance to DDT and both types I (permethrin) and II (deltamethrin) pyrethroids but all the populations were highly susceptible to organophosphate, dieldrin and carbamate insecticides (Mulamba *et al*., 2014). The rise of multiple resistance to two or more of these insecticides has also been reported in western, southern Africa and east Africa (Djouaka *et al*., 2016; Mulamba *et al*., 2014; Riveron *et al*., 2015). It is now undeniably proven that African populations of *An. funestus* exhibit resistance to at least four of the insecticide classes advised by WHO for the control of vectors (WHO, 2021). Results from such studies imply that insecticide resistance in mosquitoes involves numerous genes and multiple complex pathways, many of which have not yet been investigated (Djouaka *et al*., 2011; Riveron *et al*., 2019).

The resistance mechanism in *An. funestus* is mainly metabolic, which is mediated by P450 monooxygenases, according to molecular and biochemical investigations (Brooke *et al*., 2001; Wondji *et al*., 2022). However, other strategies such as esterase hydrolysis, glutathione S-transferases (GSTs and carboxyl/cholinesterases (CCEs), UDP-glycosyltransferases that are involved in the detoxification of insecticides have also been reported to mediate metabolic resistance in certain scenarios (Al-Yazeedi *et al*., 2024; Hemingway & Ranson, 2000; Logan *et al*., 2024).

Pyrethroid resistance in *An. funestus* mosquitoes have also been associated with cuticular thickening, and it has been observed that resistant mosquitoes had an increased expression of cuticle protein-coding genes (Awolola *et al*., 2009; Gregory *et al*., 2011). In Africa, certain cytochrome P450 alleles have also been linked to metabolic resistance to pyrethroid, and regional differences in these alleles have been reported (Djouaka *et al*., 2016; Ibrahim *et al*., 2015, 2018). A study connected the over-expression of a single cytochrome P450s allele, CYP6P9a R to the loss of bed net efficacy against *An. funestus* in Mozambique and Southern Africa (Riveron *et al*., 2019; Weedall *et al*., 2019). This includes next-generation bed nets made of piperonyl butoxide (PBO) (Riveron *et al*., 2019). There was a resurgence of endophilic *An. funestus* in western Kenya (increased 20-fold since 2007) suggesting this species could be more resistant to pyrethroids used in ITNs impregnation (Zhou *et al*., 2016). This study also revealed that ITNs are ineffective in reducing the endophilic *An. funestus* population in western Kenya (Zhou *et al*., 2016). *An. funestus* have a superior capacity to colonize a niche once they develop resistance and demonstrate behavioural plasticity.

A previous study in east Africa using a microarray-based genome-wide transcription technique had shown up-regulation of cytochrome P450s (CYP6M7, CYP9K1, CYP4H18, CYP4C36,

CYP4H17) in pyrethroid-resistant *An. funestus* population (Matowo *et al*., 2022; Sandeu *et al*., 2020). However, the expression levels of the main genes were observed to vary across geographical zones. Genome-wide high-throughput technologies have also made it possible for identification of genes expressed across the entirety of a mosquito species' genome in response to insecticides (Bonizzoni *et al*., 2012; Messenger *et al*., 2021). Furthermore, they have made it feasible to identify and characterize additional genes/gene families, and possible pathways, involved in insecticide resistance outside of the known metabolic detoxification systems (Bonizzoni *et al*., 2012; Messenger *et al*., 2021; Weedall *et al*., 2020). To date, only a few genomic studies employ RNA-Seq technology to unveil novel genes responsible for resistance in *An. funestus* population in Africa (Matowo *et al*., 2022; Weedall *et al*., 2015a). Employment of technologies such as RNA-Seq could reveal novel genes and mechanisms mediating insecticide resistance in the field population of *An. funestus.*

2.10 Knowledge gaps

There is a paucity of information on the larval ecology of *An. funestus* in different ecological zones where the species has established a niche. Also, the population structure of *An. funestus* in western Kenya is not well understood and no comprehensive information on the resistance profile and molecular mechanisms underlying insecticide resistance in western Kenya hence the need to explore these areas.

CHAPTER THREE

3.0 LARVAL ECOLOGY AND BIONOMICS OF *ANOPHELES FUNESTUS* IN HIGHLAND AND LOWLAND SITES IN WESTERN KENYA

Published as: Debrah, I., Afrane, Y. A., Amoah, L. E., Ochwedo, K. O., Mukabana, W. R., Zhong, D., Zhou, G., Lee, M.-C., Onyango, S. A., Magomere, E. O., Atieli, H., Githeko, A. K., & Yan, G. (2021). Larval ecology and bionomics of *Anopheles funestus* in highland and lowland sites in western Kenya. *PLOS ONE*, *16*(10), e0255321. https://doi.org/10.1371/journal.pone.0255321

3.1 Abstract

Background

An. funestus is a major Afrotropical vector of human malaria. In the last few years, there have been reports of the resurgence of this vector in western Kenya after the deployment of insecticide-based interventions. However, there is a limited body of evidence on the larval ecology of this efficient primary vector. This study investigated the larval ecology, sporozoite infection rates and blood meal sources of *An. funestus* in western Kenya.

Methods

A larval survey was carried out in two study areas: Bungoma in the highland and Kombewa in the lowland of western Kenya from November 2019 to December 2020. All potential mosquito aquatic habitats were identified, georeferenced, characterized and carefully examined for the presence of mosquito larvae and predators using the standard dippers (350 ml) and 10L bucket. Endophilic mosquitoes were collected using pyrethrum spray catches. Molecular and

morphological methods were used to identify the species of the adults and larvae. Samples that failed to amplify after three attempts were sequenced. Real-time Polymerase Chain Reaction (RT-PCR) and ELISA were used to detect sporozoite infections and sources of blood meals, respectively.

Results

Of the 151 aquatic habitats assessed, 62/80 (78%) in Bungoma and 58/71(82%) in Kombewa were positive for mosquito larvae. Of the 3,193 larvae sampled, *An. funestus* larvae constitute 38% (1224/3193). Bungoma recorded a higher number of *An. funestus* larvae (85%, 95%, CI, 8.722- 17.15) than Kombewa (15%, 95%, CI, 1.33- 3.91). Molecular identification of larvae showed that 89% (n=80) were *An. funestus*. Approximately 59%, 35% and 5% of *An. funestus* larvae co-existed with *An. gambiae s.l*, *Culex spp* and *An. coustani* in the same habitats respectively. Of 1,221 *An. funestus s.l* adults sampled, molecular identifications revealed that *An. funestus* constituted 87% (n=201) and 88% (n=179) in Bungoma and Kombewa, respectively. The *Plasmodium falciparum* sporozoite rate of *An. funestus* in Bungoma and Kombewa was 2% (3/174) and 1% (2/157), respectively, and the human blood index of *An. funestus* was 84% (48/57) and 89% (39/44) for Bungoma and Kombewa, respectively.

Conclusion

Man-made ponds had the highest abundance of *An. funestus* larvae. Multiple regression and principal component analyses identified the distance to the nearest house as the key environmental factor associated with the abundance of *An. funestus* larvae in aquatic habitats. This study serves as a guide for the control of *An. funestus* and other mosquito species to complement existing vector control strategies

3.2 Introduction

Malaria continues to be the deadliest vector-borne disease in sub-Saharan Africa, accounting for approximately 215 million cases in 2019 and 94% of all cases worldwide (WHO, 2020). Anti-vectorial programs, which primarily use long-lasting insecticide-treated nets and indoor residual spraying in malaria-endemic areas of sub-Saharan Africa with high entomological inoculation rates (EIR, infective bites per person per year), have made a significant contribution to reducing malaria incidence and mortality in these regions (Bhatt *et al*., 2015; Ferguson *et al*., 2010; WHO, 2020). In terms of a reduction in malaria incidence from 2015 to 2020, 17 countries were mentioned, with Kenya being one of them (WHO, 2020). The worldwide milestone for reducing death and morbidity set by the GTS 2020 has not been met despite this exceptional accomplishment in the fight against malaria (WHO, 2020).

There is no "silver bullet" to the GTS's set goal of malaria eradication and elimination currently. Larval source management is a crucial aspect of malaria vector control that needs to be considered in the plan to eradicate the disease. According to historical records, source reduction through modification of larval habitats was a crucial part of efforts to eradicate malaria in Israel, Italy, and the United States of America (Kitron & Spielman, 1989). In malaria-endemic regions of Africa, the use of an insecticide-treated net in conjunction with larval control has been predicted to reduce adult mosquito emergence by 50% and subsequently lower the entomological inoculation rate (EIR) by up to 15 to 25-fold (Killeen *et al*., 2000). However, the implementation of larval control strategies requires a deep understanding of the ecology of the larval stage of malaria vectors as well as better characterization of the aquatic habitats of vector species in various ecological contexts (Zogo *et al*., 2019). Depending on the anopheline species, the diversity of aquatic habitats, and the closeness to human habitations, it may be cost-effective to

target the most productive aquatic habitats for larval control (Gu *et al*., 2008; Walker & Lynch, 2007).

An. funestus is a primary Afrotropical malaria vector, displaying anthropophilic and endophilic behaviours (Dia *et al*., 2013; Gillies & De Meillon, 1968b). Due to its resistance to pyrethroids used for bed net impregnation, high sporozoite rate, and persistence in indoor malaria transmission, *An. funestus* is one of the main human malaria vectors in western Kenya (Ndenga *et al*., 2014; Zhou *et al*., 2016). *An. funestus* populations historically decreased in western Kenya following the introduction of insecticide-based control methods (Gimnig *et al*., 2003), and this decrease was noted for some time before a resurgence was documented a decade ago (McCann *et al*., 2014; Zhou *et al*., 2016). Despite the possibility that this resurgence is mediating malaria transmission in western Kenya, relatively few studies have examined and described the larval habitats of *An. funestus.* In contrast to findings on *An. gambiae s.s.* and *An. arabiensis* in this region, only a small number of investigations have discovered *An. funestus* larvae in sizable permanent habitats with dense aquatic vegetation and algae (Gimnig *et al*., 2001; Minakawa *et al*., 2008).

Anopheles mosquitoes breed in a variety of aquatic habitats, thus it's important to identify those habitats to control vector immature stages. Finding *An. funestus* breeding habitats, however, are challenging because it rarely breed in the same habitats as other vectors. Therefore, before larval source management can make malaria elimination and eradication goals attainable, a greater understanding and characterization of the breeding habitats and ovipositional behavioural patterns of *An. funestus* in endemic locations is essential. Furthermore, understanding the feeding preferences of *An. funestus* and the prevalence of *Plasmodium falciparum* infection can aid in forecasting the intensity of malaria transmission. This study was designed to assess the larval

ecology, blood meal sources and *Plasmodium falciparum* sporozoite infection rates of *An. funestus* in highlands and lowlands in western Kenya. This addressed the specific objective one of this thesis.

3.3 Materials and Methods

3.3.1 Study sites

This research was carried out in two different sites, Bungoma, a highland town, and Kombewa, a lowland town, both in western Kenya, and they are roughly 55 kilometres apart (**Figure 3.1**).

Bungoma [00.54057˚N, 034.56410˚E, 1386–1,545m above sea level (asl)] is characterized by a perennial malaria outbreak. The mean annual rainfall and temperatures are 150 mm and 22.5 °C respectively (Juma & Kelonye, 2016). This area historically recorded >45% *Plasmodium falciparum* malaria outbreaks with up to 55% hospitalization rates (Okiro *et al*., 2010). The mainstay of Bungoma's rural economy is agriculture, which includes growing crops like tobacco, cereals, sugar cane, onions, and other vegetables as well as producing livestock including cattle, sheep, goats and poultry. In addition, moulding clay pots by using clayey soil also forms part of the income-generating activities for the locals. Three species—*An. funestus*, *An. gambiae*, *and An. arabiensis*—are the main human malaria vectors in Bungoma (Machani *et al*., 2020; Ochomo *et al*., 2013).

Kombewa (340 30’E, 00 07’N, 1150 -1300 m asl) is in Kisumu County, a lowland area with slow water drainage, in the Lake Victoria basin. There are two rainy seasons in this region: a long one from March to May during which malaria transmission peaks and a brief one from October to November. The average annual rainfall is 1200 mm-1300 mm, and the average annual temperature ranges from 20°C to 35°C (Kisumu County, 2020). The pattern of rainfall in the area

does, however, vary yearly. With a parasitemia rate of *P. falciparum* of 57.5%, it is a hyperendemic malaria zone (Zhou *et al*., 2016b), and an entomological inoculation rate (EIR) of 31.1 infective bites per person per year (Ndenga *et al*., 2014). *An. funestus* has reportedly been identified as the main malaria vector that predominates in this study location (Ndenga *et al*., 2014; Ototo *et al*., 2015).

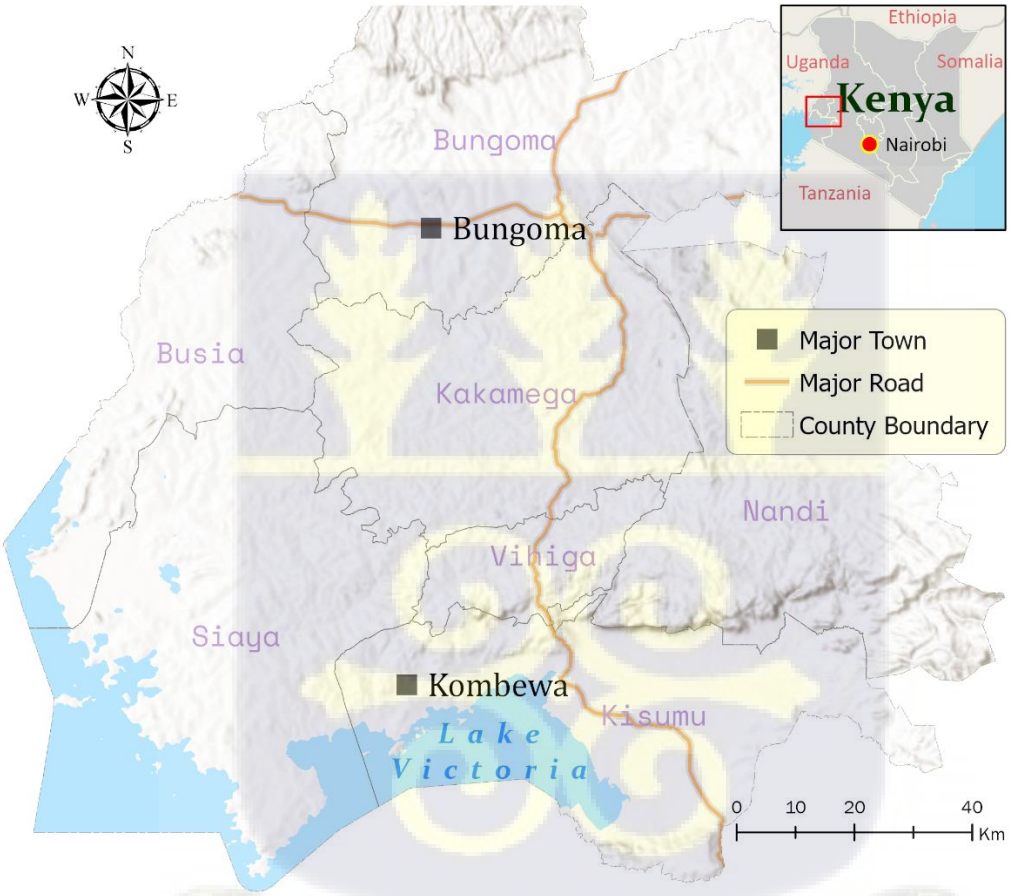


Figure 3.1: A map of the western Kenyan study sites. Software known as ArcGIS Pro 2.6 was used to create the map. source of the map: USGS, ESRI and CGIAR, (available at: www.esri.com).

3.3.2 Larval sampling

Monthly larval surveys were carried out between November 2019 and November 2020. Aquatic habitats were found within two kilometres of the study sites. Standard 350 ml dippers and a 10 L bucket were used to sample the larvae (WHO, 1992). To determine the presence or absence of larvae and aquatic predators, a maximum of 20 dips were performed at each habitat. After waiting for 3-5 minutes for any larvae to rise to the surface of the water if there were any, mosquito larvae were sampled at the margins of the habitats. To identify later using molecular analysis, *An. funestus sensu lato (s.l)* larvae were stored in 100% ethanol. Larvae were identified morphologically using referenced keys (Coetzee, 2020; Gillies & Coetzee, 1987). Using the ODK software on an Android Samsung tablet (Version SAM-T380), every potential breeding site was georeferenced using a GPS. Variables on larval habitats were described following a questionnaire that was imputed using ODK software.

3.3.3 Characterization of aquatic habitats

All potential mosquito larvae breeding habitats were identified and classified into the following categories: man-made ponds, natural ponds or rain pools, drainage ditches, swamps or marshes, and tyre tracks. In Bungoma, man-made ponds and pits were specifically created for moulding clay pots, whereas, in Kombewa, they were excavated for sand winning. If an aquatic habitat could retain water for more than three weeks, it was considered to be permanent. For each aquatic habitat, environmental factors such as the presence of vegetation, the type of vegetation, the height and coverage of the vegetation, the substrate type, the distance to the nearest house, the dimensions (length, width, and depth) of the habitat, the water physics (flow status and clarity), and the presence or absence of aquatic predators were

noted. Also recorded were the nearby land uses (cultivated land/cropland, grassland/pasture, wetland/swamp, and road).

The vegetation cover was classified as: freely floating, submerged, emergent and no vegetation. Using a meter stick, the height of the vegetation cover was measured and classified into three categories: 5, 6–10, and > 10 m. The distance to the nearest human dwellings was estimated and grouped as (A) < 100 m, (B) 100-200 m, and (C) 201-500 m. Using graphic charts, the kinds of plants and predators found in the aquatic habitats were identified. Each habitat's length, width, and depth were measured using meter sticks, and the average depth was recorded. The level of cyanobacteria (blue-green algae) in water samples taken from the aquatic environments was determined using an AquafluorTM meter (Model: 8000-010, Turner Design, San Jose, CA, USA).

The clarity of the water in each aquatic habitat was observed and classified as (A) clear (transparent like a glass), (B) opaque (not transparent and impenetrable to light), (C) cloudy (normally appeared white) and (D) muddy/brownish (brown due to disturbance stirring its deposits). The substrate type was classified as (A) mud/dirty, (B) sand and (C) stone. Land-use types for the surrounding aquatic habitats were described as (A) cultivated land/cropland (farmland used for cultivation of food crops and other crops), (B) grassland/pasture (lands with suitable grasses for grazing animals) and (C) wetland/swamp (surroundings characterized by mostly aquatic plants species covered by water/ low-lying ground not cultivated and covered with water) and (D) road (land meant for passage of vehicles and people).

3.3.4 Sampling of adult mosquitoes

Adult mosquitoes were sampled indoors using the pyrethrum spray catches (Silver, 2007) from randomly selected human dwellings near the aquatic habitats from November 2019 to August 2020 to determine whether larval abundance correlated with the adult *An. funestus* density. For each field visit, sixty (60) homes were randomly chosen near the larval habitats at each study site since there was an equal distribution of houses. The selection of homes was based on the presence of residents in the house, permission to get access to the indoor living rooms and proximity to nearby aquatic habitats.

3.3.5 Adult sampling using pyrethrum spray catches

This method collects indoor resting mosquitoes on white cotton sheets after knock-down by spraying the resting rooms with pyrethrum mix with a paraffin solution. The collection was done in the morning hours between 06:30 hours to 10:00 hours before noon (WHO, 1975). In the procedure, the inhabitants of the households have been requested to empty the living rooms. The specially made white sheets (2×2 m) were laid to completely cover all surfaces in the living rooms from wall to wall. All eave opens were completely closed using netting materials. After, a collector sprayed the pyrethrum solution inside the living rooms using spray pumps until the whole room was filled with a fine mist. He immediately leaves the room and shuts the doors for ten minutes. After ten minutes, the doors were opened and the sheets were removed carefully by lifting the four corners, starting from the door before moving into the interior of the living room. The sheets were gently removed so that the knockdown mosquitoes fall in the middle of the sheets. A flashlight was used to enhance visibility. The sheets were then transferred outside for examination in daylight. The *Anopheles* mosquitoes were collected with entomological forceps and put in Eppendorf tubes containing cotton wool and silica gel.

The *Anopheles* mosquitoes were visually categorized according to their physiological status as either blood-fed (dilated and bright red abdomen), unfed (empty abdomen/no blood meal), half-gravid (abdomen is whitish posteriorly and dark reddish anteriorly), or gravid (dilated and whitish abdomen) (Service, 2012). The tubes were labelled and transferred to the International Centre for Excellence Malaria Research (ICEMR) laboratory, Tom Mboya University for species identification. The number of mosquitoes collected in each living room in the household, the time and date of sampling, the number and age of people living in the house, the GPS of each household, roofing material, wall material, distance to the nearest breeding habitats, the weather conditions and use of any mosquito control or prevention method were recorded by completing a questionnaire on ODK collect software in the Samsung Galaxy Tablet (Version SAM-T380).

3.3.6 Morphological identification of *An. funestus* mosquito

Using morphological characters of Gilles and Coetzee (1987) under x 20 Zeiss light microscope. The identification focused on dark spots at the upper margins of the wings which is common to all *Anopheles*. The palps are elongated and segmented into three. A pale spot on the second dark area, a light spot between the two dark spots on vein 6 and an absence of fringes on vein 6 are features of *Anopheles funestus*. In addition, *An. funestus* has 4-5 pale bands on the wing margin and the legs are not speckled. Speckles on the legs, a third preapical dark area on vein 1 with a pale interruption and terse 1-4 with conspicuous pale bands are features of *Anopheles gambiae.* In general, *Anopheles* larvae have no respiratory siphon and normally lie parallel to the surface of the water. *An. funestus* larvae appear dark without spikes or any noticeable white ring-like mark on the head.

3.3.7 DNA extraction from larvae

DNA was extracted from the larvae of *An. funestus s.l.* using the ethanol precipitation method (Collins *et al*., 1988). Using this method, a sterile pestle was used to crush the larvae, and 100 uL of grind buffer was added to the Eppendorf tubes. Following the grinding of the larvae, the tubes were immediately placed in a water bath on a floating rack and heated to +650˚C for 30 minutes to destroy any nucleases released, preventing DNA degradation. 14uL of 8M potassium acetate (KAc) was added to each warm tube to reach a final concentration of 1M, and the tubes were well mixed by vortexing or tapping. The tubes were then incubated for at least 30 minutes on ice. The tubes were then centrifuged for 15 minutes at room temperature with a maximum speed of 13,200 rpm. The supernatants were placed into fresh, clean tubes that had been labelled. The precipitates were handled carefully to prevent their transmission. The supernatants were stirred well by inverting the tubes after 200uL of ice-cold 100% ethanol was added. The DNA was then precipitated by incubating them for 5 minutes at room temperature. To pellet the DNA, the tubes were centrifuged for 20 minutes at room temperature at a maximum speed of 13,200 rpm. The ethanol was quickly drained off, being careful not to disturb the pellets. The tubes were spun again for 5 minutes after the pellets came loose in some cases, and then all the ethanol was poured out. After adding 200 uL of cold 70% ethanol, the pellets were washed by spinning at maximum speed for 5 minutes. The final stage of washing was completed by adding 200uL of cold 100% ethanol and spinning at a maximum speed of 13,200 rpm for 5 minutes. The DNA pellets were washed and then dried on a bench for at least an hour. The DNA was suspended in 100uL of Tris-EDTA buffer and pellets were dissolved by gently tapping the tubes. For molecular analysis, the DNA was kept at -20˚C.

3.3.8 DNA extraction from adults

Before extraction of the DNA, adult mosquitoes were cut into two parts: the head and thorax, and the abdomen. DNA was extracted from the head and the thorax using the Chelex®-100 method (Musapa *et al*., 2013), while the abdomen was stored for blood meal analysis. 190 µl of autoclaved 1×PBS and 10 µl of 10% saponin solution were added to the sample homogenate and the submerged mosquito was ground into a uniform suspension and was incubated for 20 minutes. The samples were centrifuged at 10,000 rpm for 10 minutes and supernatant were discarded. The pellets were resuspended in 200µl of 1×PBS and centrifuged again at 10000 rpm for 10 minutes. Supernatants were discarded and allowed to dry for at least 10 minutes till they were well-dried. The pellets were resuspended each in 100µl of 20% w/r chelex-100 resin suspension in deionized water. The samples were then boiled in a water bath at 85^0C on floating racks for 5 minutes and vortex for 10 seconds. They were then centrifuged at 10,000 for 1 minute and the DNA solution was transferred into pre-labelled 1.5ml storage vials and stored at -20˚C for molecular analysis.

3.3.9 Molecular identification of species

Members of the *An. funestus* group (*An. funestus, An. rivulorum, An. vaneedeni, An. parensis, An. leesoni,* and *An. rivulorum-like*) were all molecularly identified by utilizing multiplex polymerase chain reaction (PCR) using species-specific primers to amplify the polymorphic ITS2 region of ribosomal DNA (Cohuet *et al*., 2003; Koekemoer *et al*., 2002). Species-specific primers used include *An. funestus* (5`-GCA TCG ATG GGT TAA TCA TG-3`), *An. vaneedeni* (5`-TGT CGA CTT GGT AGC CGA AC-3`), *An. rivulorum* (5`-CAA GCC GTT CGA CCC TGA TT-3`), *An. parensis* (5`-TGC GGT CCC AAG CTA GGT TC-3`), *An. leesoni* (5`-TAC ACG GGC GCC ATG TAG TT-3`) and Primers for *An. rivulorum-like* (D3A (5'-GAC CCG

TCT TGA AAC ACG GA-3') and D3B (5'-TCG GAA GGA ACC AGC TAC TA-3'). A final volume of 14.5 µl of PCR mixture containing 1µl of genomic DNA, 6.5 µl DreamTaq Green PCR Master Mix (2x), 0.5 µl of each of the primers and 4.0 µl of PCR water. Genomic DNA amplification was performed using T100 thermal cycler (Biorad).

The PCR conditions include initial denaturation at 95˚C for 3 seconds, denaturation of 94˚C for 30 seconds, annealing at 55˚C for 30 seconds for 34 cycles, extension at 72˚C for 45 seconds and final extension at 72˚C for 6 seconds. Using PCR, a sub-sample of 641 (551 adults and 90 larvae) was detected. 20% (n=110) of the adult mosquitoes and 11% (n=10) of the larvae in this population failed to amplify. 65 specimens of *An. funestus s.l* (60 adults and 5 larvae) were randomly selected from those that failed to amplify by PCR after three attempts and sent to the University of California, Irvine for Sanger sequencing. The internal transcribed spacer (ITS) 2 (ITS2) region of nuclear ribosomal DNA was amplified using the forward primer ITS2A (TGTGAACTGCAGGACACAT) and the reverse primer ITS2B (TATGCTTAAATTCAGGGGGT); amplicons were sequenced using ABI Big Dye Terminator Cycle Sequencing Kits with the same primers as described by *(*Daibin *et al*., 2020). A detail of the sequencing procedure is described in Chapter 4, section **4.3.4**

3.3.10 Agarose gel electrophoresis and visualization of the DNA

For the preparation of agarose gel, 1.2g of agarose was added into 80 ml 1 X TBE buffer in a microwave beaker and heated until uniformly dispersed agarose in a microwave on high power for bubbles to appear. The beaker was removed from the microwave oven and gently swirled with the agarose solution to resuspend any particles. The solution was heated again until all particles dissolved. The solution was allowed to cool for 1-2 minutes at room temperature. Then, 4µl smart glow stain was added and swirls gently to mix the solution. The gel was poured into

the casting unit to a depth of 3-4mm and immediately after pouring, the comb was inserted and any air bubbles that may have been trapped under or between the teeth of the gel were removed. The gel was completely allowed to solidify at room temperature for approximately 30-45 minutes. After solidification, it was flooded with TBE buffer and the comb and the casting dams were carefully removed. The gel was placed in the electrophoresis chamber and the TBE until the gel is submerged under 3-5 mm of the TBE buffer. Using a P20 pipette, 10 µl of the amplified DNA samples were loaded into individual wells taking care not to puncture the sample wells. 10 µl 100bp DNA ladder was added to two of the empty wells to serve as controls. The gel cover and power lead were attached, and current (100V) was applied to the unit. Bubbles appeared around the electrode when the current was flowing. After 30 minutes, the power supply was turned off when the samples had dissolved sufficiently. The gel was moved into the smart doc imaging system (Accuris ™ instrument) where the DNA bands were viewed in agarose gels and images were taken using a smartphone camera.

3.3.11 *Plasmodia* species genotyping using multiplex real-time polymerase chain reaction (RT-PCR)

Plasmodia species (*P. falciparum*, *P. ovale*, and *P. malarae*) were detected in DNA samples using a modified TaqMan assay (Shokoples *et al.*, 2009; Veron *et al.*, 2009). *Plasmodium falciparum* species-specific 18S ribosomal primers and probes used are presented in **Table 3.1**. A final volume of 12µl containing 2 µl of sample DNA, 6 µl of PerfeCTa® qPCR ToughMix™, Low ROX™ Master mix (2X), 0.5 µl of each probe, 0.4 µl of each of the forward primers (10 µM), 0.4 µl of each reverse primers (10 µM) and 0.1 µl of double-distilled water was loaded in QuantStudio™ 3 Real-Time PCR System. The thermal profile used was set as follows; 50˚C for 2 min, (95˚C for 2 min, 95˚C for 3 sec and 58˚C for 30 sec) for 45 cycles. The real-time PCR

was performed in the QuantStudio 3 real-PCR instrument (Applied Biosystems, Thermo Fisher Scientific). The *Plasmodium* infection was analyzed by comparing melt curves with the positive controls using QuantSudio ™ Design and Analysis Desktop software v1.5.1.

Table 3.1: Species-specific primers and probes used to genotype *Plasmodia* species

Name of malaria parasite	Forward primer	Reverse primer
P. falciparum	ATTGCTTTTGAGAGGTTTTGTTACTTT	GCTGTAGTATTCAAACACAATGAACTCAA
P. malariae	AGTTAAGGGAGTGAAGACGATCAGA	CAACCCAAAGACTTTGATTTCTCATAA
P. ovale	AACCCAAAGACTTTGATTTCTCATAA	CCGACTAGGTTTTGGATGAAAGATTTTT
Probes		
P. falciparum	FAM- CATAACAGACGGGTAGTCAT-MGB	
P. malariae	VIC-ATGAGTGTTTCTTTTAGATAGC-MGB	
P. ovale	NED-CGAAAGGAATTTTCTTATT-MGB	

3.3.12 Blood meal analysis

Direct enzyme-linked immunosorbent assay (ELISA) was used to detect the origin of blood meal in the abdomen of blood-fed *Anopheles* mosquitoes following existing protocol (Beier *et al.*, 1988). 50µl of phosphate-buffered saline (PBS) was added to the abdomen of each mosquito specimen and was incubated overnight. Following grinding, 950µl of PBS was added to each sample for washing and 50µl of each sample was loaded into each of the wells of the ELISA plate and incubated for two hours. Hosts-specific positive controls and negative control from unfed lab-raised mosquitoes were also added. Following incubation and washing, anti-host specific conjugates (antibodies) against human, goat, chicken and dog proteins were added to the wells. ABTS (2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) peroxidase substrate for each human, goat, chicken and dog blood meal source was added to

each of the wells. The samples were incubated for thirty minutes for the reaction to occur. The non-reacting samples were tested subsequently using bovine immunoglobulin G. All ELISA results were read visually (Beier & Koros, 1991).

3.3.13 Data analysis

Graph Pad Prism V.8.0.1 and SPSS software (Version 21 for Windows, SPSS Inc., Chicago, IL) were used to analyze the data. The relative abundance of each species was expressed as a percentage of the number of larvae per species divided by the total number of larvae collected for all species combined per habitat type. The relative abundance of *An. funestus* was calculated as the number of *An. funestus* larvae from a specific habitat divided by the total number of *An. funestus* larvae in all samples from a habitat type.

The types of breeding sites, the number of mosquito larvae sampled and species, and the number of aquatic predators were presented in tables and figures. The Kruskal-Wallis test was used to compare the number of larvae in various habitat types and for samples with more than two groups. The following factors were taken into consideration: habitat type (man-made pond, natural pond/rain pool, drainage ditch, swamp/marshes, tyre tracks), distance to the nearest house (100, 100-200, 201-500), water clarity (clear, opaque, brownish/muddy, cloudy), aquatic plant species in a habitat (aquatic plant species in a habitat [*Pennisetum purpureum* (elephant grass), *Schoenoplectus californicus*, others/unknown], and land-use type (cultivated land/cropland, grassland/pasture, wetland/swamp, road). The Mann–Whitney U test was used to compare samples with two variables: vegetation (present or absent), category of vegetation (emergent or non-emergent), water flow status (stagnant or flowing water), and predators (present or absent). Multiple regression and principal components analyses were used to identify environmental variables associated with the abundance of *An. funestus* in aquatic habitats. The HBI was

estimated as the percentage of *Anopheles* mosquitoes that fed on humans over the total number of blood-fed *Anopheles* for which the blood meal origins were determined. The sporozoite rate of *P. falciparum* was calculated as the proportion of *Anopheles* tested for sporozoites over the total genotyped. Spearman's correlation (r_s) was used to find the relationship between the adult *An. funestus* population and the larval density at each study site. The results were considered statistically significant at P <0.05. For the sequence data analysis of the *An. funestus s.l.* the specimen that failed to amplify by PCR, the de novo assembly of reads was performed using geneious software (Kearse *et al*., 2012). Basic Local Alignment Search Tool (BLAST) was used to identify sequence similarities against sequences in GeneBank. Sequences with high identity scores or low E-values were retrieved and used in the construction of a phylogenetic tree to identify the unknown**.** Evolutionary analyses were conducted in MEGA X after basic alignment using the ClustalW algorithm (Tamura *et al*., 2021). All sequences of ITS2 are available at GenBank under accession numbers MZ435355-MZ435414.

3.4 Results

3.4.1 *An. funestus* and other mosquitoes larval distribution in Bungoma and Kombewa

A total of 151 potential mosquito aquatic habitats were examined. Of these, 62/80 (78%; 95% CI: 0.68-0.87) and 58/71 (82%; 95% CI: 0.73-0.91) in Bungoma and Kombewa were positive for mosquito larvae, respectively. The number of aquatic habitats with *An. funestus* larvae in Bungoma were 55/80 (69%; 95% CI; 0.58-0.79), whereas 23/71 (32%; 95% CI: 0.21-0.43) were in Kombewa. In all, *An. funestus* larval habitats constituted 65% (n=78) of the mosquito-positive habitats, whereas *An. gambiae s.l, An. coustani*, and *Culex spp*-positive habitats made up 57% (n=69), 14% (n=17) and 36% (n=43), respectively.

A total of 3,193 mosquito larvae (39% *An. funestus*, 30% *An. gambiae* s.l., 28% *Culex* spp. and 3% *An. coustani*) were collected from the various habitats in Bungoma and Kombewa. Bungoma had a higher number of *An. funestus* larvae (85% 95%, CI, 8.722-17.15) than Kombewa (15% 95%, CI, 1.33-3.91). Approximately 59% of *An. funestus* larvae were collected from breeding sites with co-existing *An. gambiae s.l* larvae in the same habitats (**Figure 3.2**). Similarly, 35% and 5% of *An. funestus* larvae were found co-existing with *Culex spp*., and *An. coustani* larvae, respectively (**Figure 2**). No pupae were identified during the sampling. Molecular results on the sibling species of the *An. funestus* group larvae revealed that 89% (n=80) were *An. funestus*, 11% (n=9) were *An. rivulorum* and 1% (n=1) was *An. sp.9*.

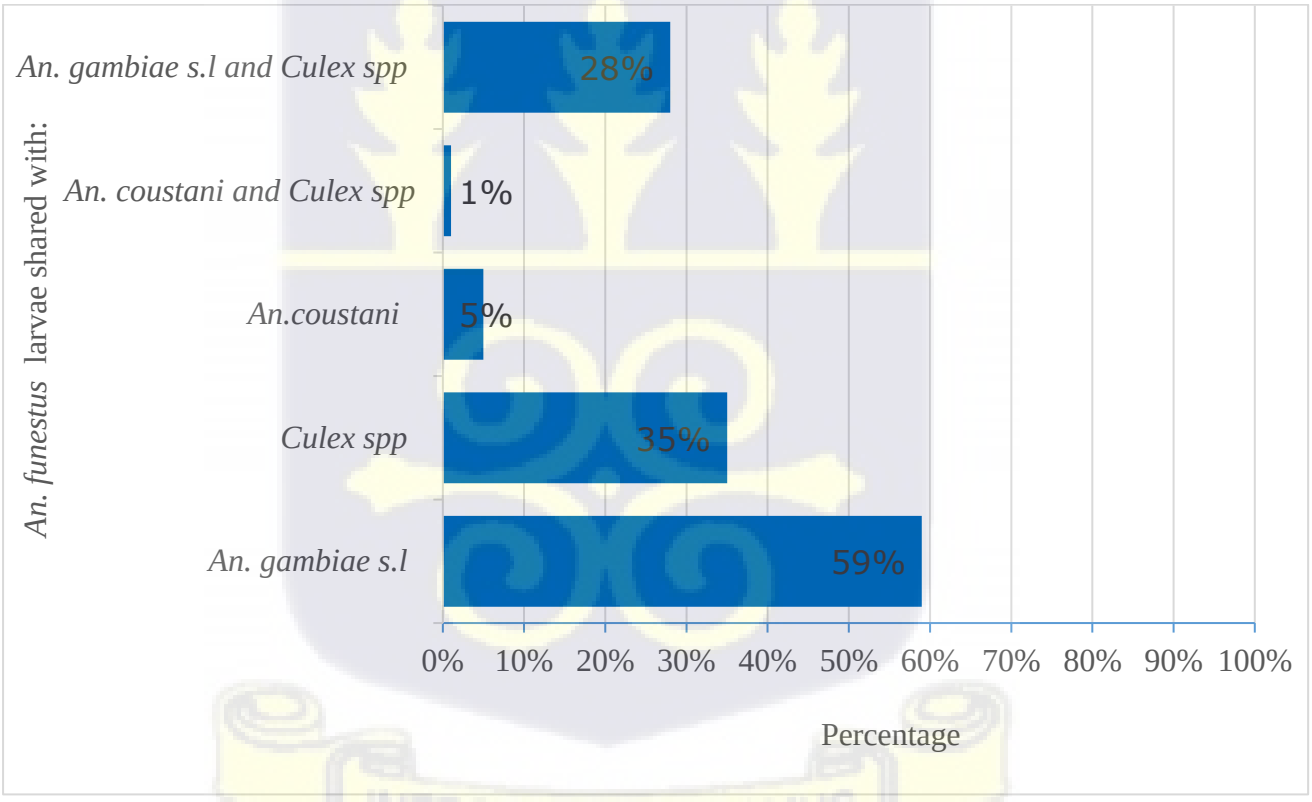


Figure 3.2: Proportion of *An. funestus* larvae shared with other mosquitoes in the aquatic habitats

3.4.2 Characteristics of mosquito aquatic habitats

An. funestus larvae were found in various habitats, with or without vegetation: man-made ponds, natural ponds/rain pools, drainage ditches, swamp/marshes, and tyre tracks. There were no significant differences in *An. funestus* larval density between the various habitat types of *An. funestus* ($\chi 2$= 8.917, df=4, *P*=0.063). However, man-made ponds comprised the highest number of *An. funestus*-positive habitats (36%, n=28) and had the highest proportion of larvae with *An. funestus* in Bungoma (53%, n=553) and in Kombewa (61%, n=115) (**Table 3.2**). The larval abundance of *An. funestus* in man-made ponds, natural ponds/rain pools and drainage ditches was significantly different between the study sites (*P*< 0.05) (**Table 3.2**). Field observations showed that man-made ponds constituted the main permanent aquatic habitat type in the study areas. This was followed by swamp/marshes, natural ponds/rain pools, drainage ditches and tyre tracks, at frequencies of 27%, 19%, 15%, and 3%, respectively. There was no significant difference in the means among the various predators found in the larval habitats (*P*=0.05). **Figure 3.3** shows the mean distribution of the various predators.

Table 3.2: Number of different mosquito species collected from aquatic habitats in Bungoma and Kombewa in Kenya (November 2019 to November 2020)

Study site	Habitat		Larval Counts per Mosquito Species (n (%))			
	Type	N	*An. funestus s.l*	*An. gambiae s.l*	*An. coustani*	*Culex spp*
Bungoma	Man-made ponds	26	553 (53)[a]	81 (37)	0 (0)	15 (47)[i]
	Natural ponds/rain pools	12	100 (10)[c]	2 (1)[g]	0 (0)	0 (0)
	Drainage ditches	19	374 (36)[e]	120 (54)	0 (0)	3 (9)[k]
	Swamps/marshes	21	8 (1)	18 (8)	0 (0)	14 (44)
	Tyre tracks	2	0 (0)	0 (0)	0 (0)	0 (0)
Kombewa	Man-made ponds	20	115 (61)[b]	67 (9)	33 (32)	162 (19)[j]
	Natural ponds/rain pools	11	25 (13)[d]	249 (34)[h]	24 (23)	188 (21)
	Drainage ditches	17	40 (21)[f]	286 (39)	47 (45)	489 (56)[l]
	Swamp/marshes	22	9 (5)	137 (18)	0 (0)	34 (4)
	Tyre tracks	1	0 (0)	0 (0)	0 (0)	0 (0)
Bungoma	**Total**	**80**	**1035**	**221**	**0**	**32**
Kombewa	**Total**	**71**	**189**	**739**	**104**	**873**

N is the number of habitats and n is the number of mosquitoes of different species in each habitat type. a and b, c and d, e and f, g and h, i and j, k and l superscripts indicate statistical significance at $P<0.05$ between the number of mosquito larvae in each habitat type between the study sites.

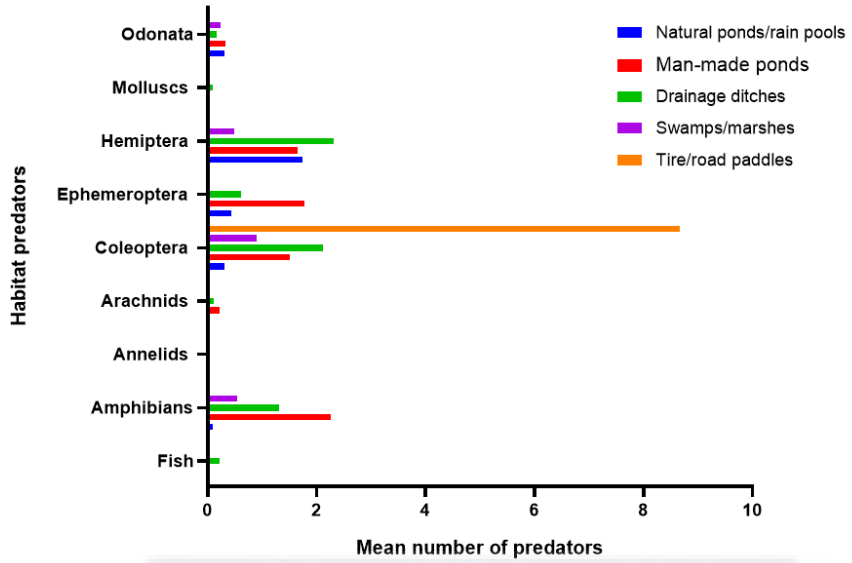


Figure 3.3: Distribution of aquatic predators in the various larval habitats.

Fish: tilapia, Amphibians: frogs/toads/tadpoles, Annelids: Segmented worms, Arachnids: spiders/ticks/mites, Coleoptera: water beetles/weevils, Ephemeroptera: mayfly, Hemiptera: backswimmer/giant water bugs/cacidas, Molluscs: snails/slugs/mussels, Odonata: damselfly/dragonfly

3.4.3 Association between environmental variables and *An. funestus* larval abundance

Table 3.3 indicates the numerous environmental variables in the habitats where larvae of *An. funestus* and other mosquito species were found to be present.

Table 3.3: Characteristics of aquatic habitats of *An. funestus* and other mosquito species at Bungoma and Kombewa western Kenya (November 2019-November 2020)

Environmental variable	All aquatic breeding sites n (%)	Potential habitats without mosquito larvae n (%)	Aquatic habitats with *An. funestus* n (%)	Aquatic habitats with *An. gambiae s.l* n (%)	Aquatic habitats with *An. coustani* n (%)	Aquatic habitats with *Culex spp* n (%)
Habitat type						
Man-made pond	46 (31)	6 (19)	28 (36)	13 (19)	6 (35)	12 (28)
Natural pond/rain pool	23 (15)	3 (10)	15 (19)	13 (19)	4 (24)	6 (14)
Drainage ditch	36 (24)	12 (39)	12 (15)	19 (27)	1 (6)	8 (19)
Swamp/marshes	43 (28)	10 (32)	21 (27)	22 (32)	6 (35)	16 (37)
Tyre tracks	3 (2)	0 (0)	2 (3)	2 (3)	0 (0)	1 (2)
Vegetation						
Present	133 (88)	28 (90)	67 (86)	63 (91)	17 (100)	41 (95)
Absent	18 (12)	3 (10)	11 (14)	6 (9)	0 (0)	2 (5)
Category of vegetation						
Emergent	101 (76)	19 (68)	48 (72)	53 (84)	14 (82)	34 (83)
Free-floating	29 (22)	9 (32)	16 (24)	8 (13)	3 (18)	5 (12)
Submerged	3 (2)	0 (0)	3 (4)	2 (3)	0 (0)	2 (5)
Water Flow Status						
Stagnant/standing water	145 (96)	31(100)	74 (95)	64 (93)	17 (100)	40 (93)
Flowing/Disturbed Water	6 (4)	0 (0)	4 (5)	5 (7)	0	3 (7)
Predators						
Present	100 (66)	12 (39)	54 (69)	56 (81)	16 (94)	35 (81)
Absent	51 (34)	19 (61)	24 (31)	13 (19)	1 (6)	8 (19)
Distance to Nearest House (m)						
<100	39 (26)	17 (55)	12 (15)	11 (16)	4 (24)	10 (23)
100-200	76 (50)	13 (42)	38 (49)	34 (49)	8 (47)	25 (58)
201-500	36 (24)	1 (3)	28 (36)	24 (35)	5 (29)	8 (19)

Water Clarity						
Clear	43 (28)	11 (35)	26 (33)	17 (25)	6 (35)	11 (25)
Opaque	69 (46)	13 (42)	39 (50)	27 (39)	9 (53)	18 (42)
Cloudy	30 (20)	4 (13)	10 (13)	21 (30)	2 (12)	12 (28)
Muddy/Brownish	9 (6)	3 (10)	3 (4)	4 (6)	0 (0)	2 (5)
Land-use type						
Cultivated land/cropland	82 (54)	18 (58)	56 (72)	36 (52)	0 (0)	13 (30)
Grassland/Pasture	61 (40)	11 (36)	18 (23)	31(45)	16 (94)	27 (63)
Wetland/swamp	4 (3)	1 (3)	2 (2.5)	2 (3)	0 (0)	0 (0)
Road	4 (3)	1 (3)	2 (2.5)	0 (0)	1 (6)	3 (7)

The strongest predictor of *An. funestus* larval density in the habitats, according to multiple regression analysis models, was the distance to the nearest dwelling (P=0.0122). (**Table 3.4**). The ANOVA table's F-ratio showed that, at the time of larval sampling, the *An. funestus* larval density was statistically significantly correlated with important habitat variables (habitat type, land use type, vegetation coverage, vegetation height, distance to house, habitat size, water depth, water clarity, algae abundance, and predator counts). Furthermore, the principal component analysis indicated that the primary environmental element most strongly associated with the abundance of *An. funestus* larvae were the distance to the nearest human dwelling. (**Figure 3.4**).

The Mann–Whitney U and Kruskal–Wallis tests showed that *An. funestus* larval density had no significant difference between present and absent vegetation (U=1192, *P*=0.978) (**Figure 4A**), emergent and non-emergent vegetation (U=1369, *P*=0.166) (**Figure 3.5A**), stagnant and flowing water (U=287, *P*=0.136) (**Figure 3.6C**), present and absent of aquatic predators (U=2215, *P*=0.162) (**Figure 5B**), among clear, opaque, cloudy and muddy/brownish water ($\chi 2$=7.316, df=3, *P*=0.062) (**Figure 4C**), or with the presence of *Pennisetum purpureum, Schoenoplectus californiscus* and other aquatic plant species in the habitats ($\chi 2$=2.671, df=2, *P*= 0.263) (**Figure**

4B) respectively. However, *An. funestus* larval density showed statistically significant differences associated with distances to the nearest house (<100, 100-200 and 201-500 m) (χ2=25.138, df=2, *P*<0.0001) (**Figure 3.6A**) and land-use types (cultivated land/cropland, grassland/pasture, wetland/swamp and road) (χ2=29.197, df=3, *P*=0.000) (**Figure 3.5C**). Field observation showed that *An. funestus is* most abundant in habitats surrounded by cultivated land compared to those grassland areas, wetlands, and roads.

Table 3.4: Multiple Regression analysis of variables associated with *An. funestus* larval density

Habitat variable	Beta	Std.Err. of Beta	B	Std.Err. of B	t(114)	*P*-level
Habitat Type	-0.0876	0.0984	-0.0613	0.0688	-0.8902	0.3752
Land use type	-0.1816	0.1047	-0.2464	0.1420	-1.7348	0.0855
Vegetation coverage	0.0957	0.0997	0.0024	0.0025	0.9598	0.3392
Vegetation Height	-0.0219	0.0890	-0.0058	0.0236	-0.2466	0.8056
Distance to house	0.2355	0.0925	0.0016	0.0006	2.5468	*0.0122
Habitat size	-0.0594	0.0929	-0.0006	0.0009	-0.6391	0.5240
Water depth	0.1088	0.0894	0.2402	0.1975	1.2163	0.2264
Water clarity	0.0171	0.1065	0.0033	0.0207	0.1605	0.8728
Algae abundance	-0.1588	0.0980	-0.0014	0.0008	-1.6193	0.1081
Predator counts	-0.0568	0.0909	-0.0045	0.0072	-0.6254	0.5329

Beta standardized coefficient, B the unstandardized coefficient value, * significant at *P*<0.05

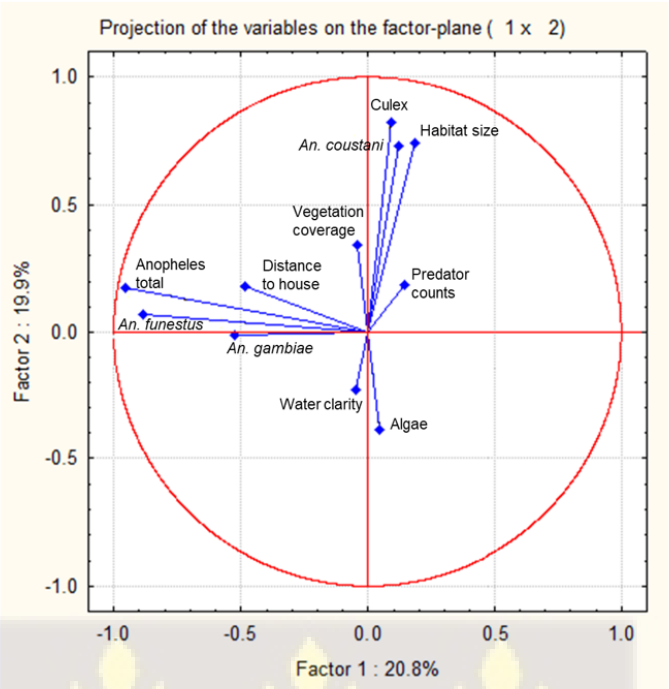


Figure 3.4: Principal component analysis of *An. funestus* larval abundance

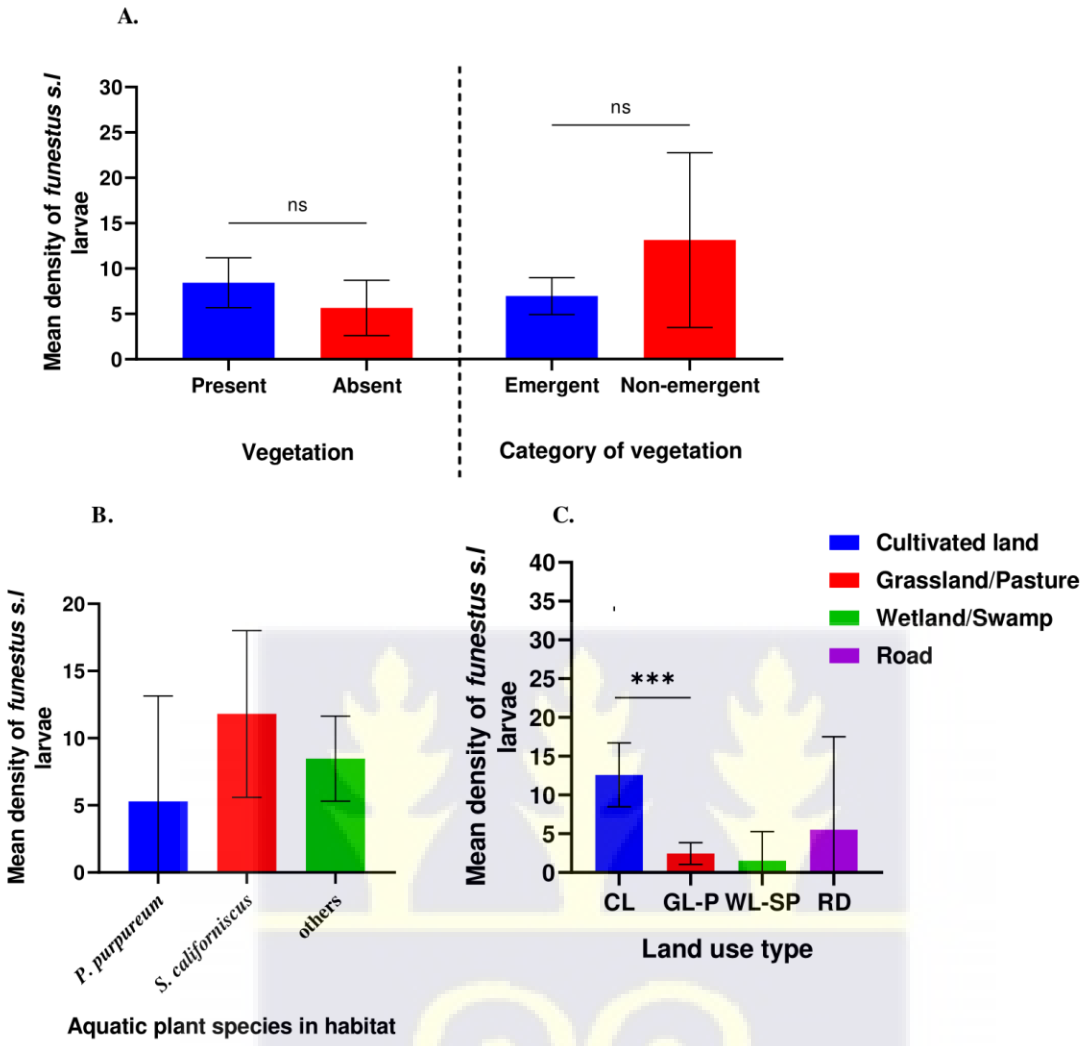


Figure 3.5: Association of habitat variables with *An. funestus* density. (A) vegetation (present and absent) and category of vegetation (emergent and non-emergent), (B) aquatic species in the habitats, (C) land use type

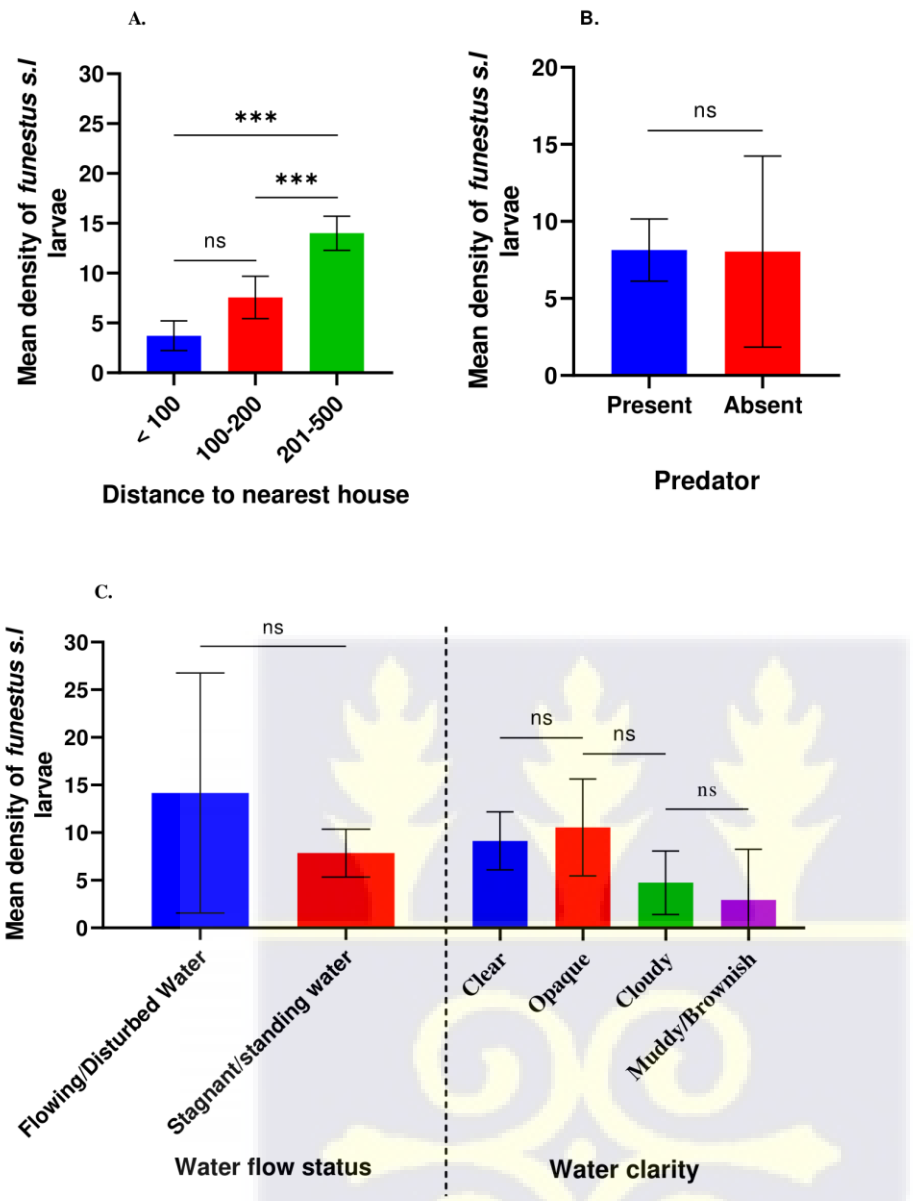


Figure 3.6: Association of habitat variables with *An. funestus* larval density. (A) distance to the nearest house, (B) predator (present and absent in habitats) and (C) water flow status and water clarity

3.4.4 Relationship between larval abundance and adult mosquitoes sampled indoors

The findings of the Pearson correlation test revealed a weakly positive linear association between the number of adult mosquitoes captured indoors in Bungoma and the presence of *An. funestus* larvae (rs=0.178, P=0.026). However, there was no correlation between the number of adult mosquitoes sampled indoors in Kombewa and the abundance of *An. funestus* larvae (rs=0.003, P=0.972).

3.4.5 Composition of indoor resting *Anopheles* mosquitoes

Morphological identification of *Anopheles* mosquitoes has shown that in Bungoma and Kombewa, indoor collections of 1,221 *An. funestus s.l* and 195 *An. gambiae s.l* were made. Of the 1,221 *An. funestus s.l.* sampled, Bungoma and Kombewa provided 46% (n=565) and 54% (n=656) of the samples, respectively (**Table 3.5**). A sub-sample of 551 *Anopheles*, including 380 *An. funestus s.l.* and 171 *An. gambiae s.l.* from the study sites were analyzed for molecular identification. Of the *An. funestus s.l.* analysed, 201 and 179 were from Bungoma and Kombewa, respectively. Per the findings, *An. funestus* predominated in the two research areas. In Bungoma and Kombewa, respectively, *An. funestus* made up 87% (n=201) and 88% (n=179) of the population, whereas *An. rivulorum* made up 13% (n=201) and 12% (n=179) of the population.

Of the 171 *An. gambiae s.l.* analysed, 76 and 95 were from Bungoma and Kombewa, respectively. The predominant species found in Bungoma (86%, n=76) and Kombewa (81%, n=95) was *An. gambiae*. In Bungoma and Kombewa, *An. arabiensis* made up 15% (n=76) and 19% (n=95), respectively. The evolutionary relationship of the unamplified sequenced data is shown in **Figure 3.7**

Table 3.5: Physiological status and distribution of *Anopheles* mosquitoes sampled indoors using pyrethrum spray catches (PSC)

Stage	Bungoma		Kombewa	
	An. funestus s.l.	*An. gambiae s.l.*	*An. funestus s.l.*	*An. gambiae s.l.*
Blood fed	306 (54)	62 (75)	346 (53)	42 (38)
Gravid	11 (2)	0 (0)	48 (7)	9 (8)
Half gravid	194 (34)	13 (15)	213 (32)	56 (50)
Empty	54 (10)	8 (10)	49 (8)	5 (4)
Total	**565 (87)**	**83 (13)**	**656 (85)**	**112 (15)**

Numbers in the brackets represent percentages

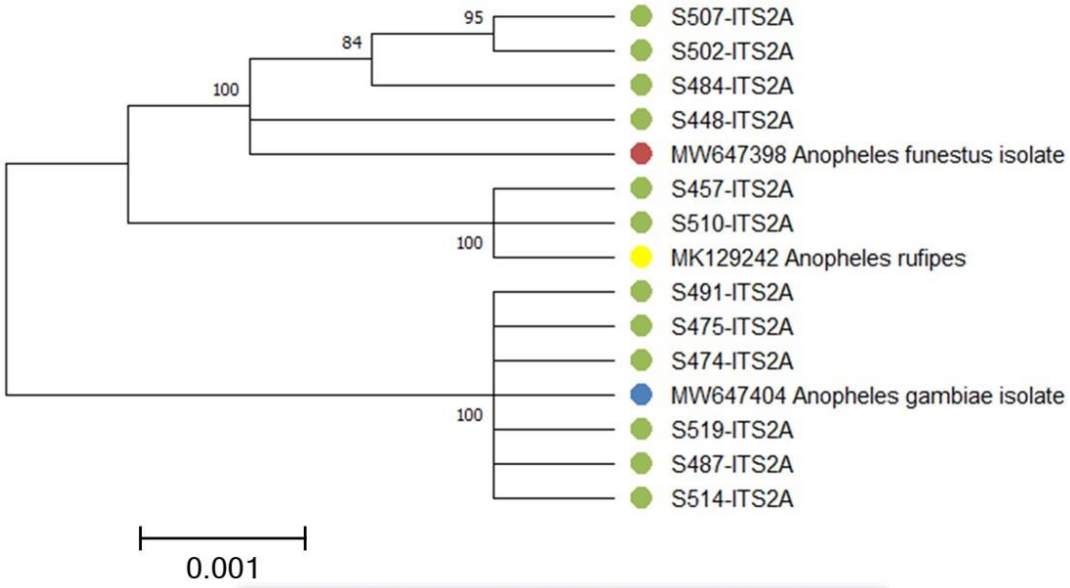


Figure 3.7: Evolutionary relationships of taxa.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987)**.** The optimal tree with the sum of branch length = 3.05389016 is shown (**Figure 3.7**). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were calculated using the Kimura 2-parameter method and are expressed in base substitutions per site. A gamma distribution with a shape parameter of 1 was used to simulate the rate variance between sites. There were 15 nucleotide sequences in this analysis. For each sequence pair, all unclear positions were eliminated (pairwise deletion option). The final dataset contained 815 locations altogether. In MEGA X, evolutionary analyses were performed (Kumar *et al*., 2018).

3.4.6 *Plasmodium falciparum (Pf)* sporozoite infection rate

All the sub-samples (n=551) subjected to molecular identification were genotyped to detect sporozoite. The *Pf* sporozoite rate of *An. funestus* in Bungoma and Kombewa was 2% (3/174) and 1% (2/157), respectively (**Table 3.6**). However, none of the *An. rivulorum* found in Bungoma and Kombewa was positive for *Pf* sporozoite (**Table 3.6**). Only one *An. gambiae* mosquito from Kombewa (1%, 1/77) tested positive for *Pf* sporozoite. However, sporozoites were not detected in *An. arabiensis* in the study sites.

Table 3.6: Sporozoite rate of *Anopheles* mosquitoes sampled from indoors in Bungoma and Kombewa, November 2019 to November 2020

Site	*An. gambiae s.l*	No. tested	*Pf* +ve (%)	*An. funestus s.l*	No. tested	*Pf* +ve (%)
Bungoma	*An. gambiae*	65	0 (0)	*An. funestus*	174	3 (2)
	An. arabiensis	11	0 (0)	*An. rivulorum*	27	0 (0)
	Total	**76**			**201**	
Kombewa	*An. gambiae*	77	1 (1)	*An. funestus*	157	2 (1)
	An. arabiensis	18	0 (0)	*An. rivulorum*	22	0 (0)
	Total	**95**			**179**	

Numbers in the bracket indicate sporozoite rate; *Pf, Plasmodium falciparum*, +ve, positive

3.4.7 *Anopheles* blood meal origins

A total of 208 samples (Bungoma, n=114 and Kombewa, n=94) were analysed for the origin of the mosquito blood meals. The HBI of *An. funestsus* was 84% (48/57) and 89% (39/44) for Bungoma and Kombewa, respectively (**Table 3.7**). **Table 3. 7** shows the HBI for all species tested**.**

Table 3.7: Origin of blood meals *Anopheles* mosquitoes in Bungoma and Kombewa (November 2019 to November 2020)

Site	**Species**	**No. tested**		**Origin of blood meals**		**HBI**
			human	Bovine	goat	
Bungoma	*An. gambiae*	43	21	15	7	49
	An.arabiensis	6	4	2	0	67
	An. rivulorum	8	6	2	0	75
	An. funestus	57	48	7	2	84
	Total	**114**	**79**	**26**	**9**	
Kombewa	*An. gambiae*	34	20	10	4	59
	An. arabiensis	3	3	0	0	100
	An. rivulorum	13	10	3	0	77
	An. funestus	44	39	3	2	89
	Total	**94**	**72**	**16**	**6**	

HBI, human blood index

3.5 Discussion

The great diversities of anopheline larval habitats in addition to their inaccessibility makes larval ecology studies of malaria mosquitoes methodologically cumbersome (Gimnig *et al*., 2001). The presence of quality larval habitats is significant in determining the abundance and distribution of adult mosquitoes. This study was designed to add to the limited amount of information on the larval ecology of *An. funestus* in western Kenya. Two study areas were selected, a highland site (Bungoma) and a lowland site (Kombewa), and their aquatic habitats were examined and characterized to determine if there have been changes in the breeding habitats of *An. funestus* in the village sites. This study revealed that *An. funestus* is a major vector influencing malaria transmission in the region, confirming a previous report that *An. funestus* has re-emerged and could be responsible for malaria transmission in western Kenya (Zhou *et al*., 2016b).

The findings revealed that *An. funestus* larvae thrive in a wide range of aquatic habitats and co-breeds with other malaria vectors in the same habitats. Although there were no significant differences observed in the various habitat types, man-made ponds had the highest proportion of *An. funestus* larvae. Man-made ponds, created mostly for making clay pots and sand winning, contributed remarkably to the proportion of *An. funestus* habitats and larvae abundance in the study areas. This corroborates previous findings in western Kenya where man-made habitats accounted for an increase in populations of *An. gambiae* (Gimnig *et al*., 2002; Imbahale *et al*., 2011). Field observations have shown that man-made ponds are permanent habitats that hold water for a longer period compared to other habitat types. This suggests that malaria transmission in the study areas is partly man-made, and thus, proper environmental management specifically through habitat manipulation could curtail malaria transmission by major vectors of human malaria. This study confirms how anthropogenic modification of ecosystems can

contribute greatly to the abundance and distribution of malaria vectors (Yasuoka & Levins, 2007).

It was found that more than 50% of *An. funestus* larvae co-existed in aquatic habitats with *An. gambiae s.l* larvae. Moreover, *An. funestus* shared the same habitats with *Culex spp* and *An. coustani*. Previous studies in neighbouring countries, Tanzania (Nambunga *et al*., 2020) and Uganda (Musiime *et al*., 2020), have reported that *An. funestus* shared habitats with other *Anopheles* and *Culex spp*., indicating that any larval control programme targeting *An. funestus* would have a profound effect in controlling other equally important vectors of human malaria and other mosquito-borne diseases.

Hitherto, it has been reported that *An. funestus* prefers breeding in aquatic habitats with thick vegetation (Gimnig *et al*., 2001; Minakawa *et al*., 2008), but this study revealed that *An. funestus* can breed in habitats with aquatic vegetation or without vegetation. The presence of vegetation has been noted to be an important environmental variable associated with *Anopheles* mosquito larvae density (Getachew *et al*., 2020). For example, aquatic macrophytes play an important role in the oviposition, larval survival and development of anophelines as they serve as a food source, protection for the larval stages and provide an enabling environment for mosquito breeding (Balling & Resh, 1984; Hall, 1972; Hess & Hall, 1943; Orr & Resh, 1992). However, this study revealed that *An. funestus* can breed in habitats with or without aquatic vegetation. These data showed that there was no significant difference in the means between habitats with aquatic vegetation and habitats without aquatic vegetation.

Multiple linear regression analysis showed that the abundance of algae in the habitat was not a predicting factor for the density of *An. funestus* in this study. However, a study noted that algae abundance was positively correlated with *An. gambiae* density in western Kenya (Gimnig *et al*.,

2001) and also an important factor in predicting the abundance of *An. pseudopunctipennis* in the Americas (Manguin *et al*., 1996) and crucial for the development of *An. pretoriensis* in the Rift Valley province of Ethiopia (Aklilu *et al*., 2020).

While algae abundance was an important factor, distance to the nearest house was not an important component associated with *An. gambiae s.l* and *An. pretoriensis* abundance (Aklilu *et al*., 2020). However, the proximity of productive larval habitats to human or animal habitation to obtain a blood meal can determine the density of adult mosquitoes (Takken & Verhulst, 2013). In western Kenya, a previous study reported that the distance to the nearest house was significantly associated with the abundance of *An. gambiae* (Minakawa *et al*., 1999). Among the environmental variables assessed in this study, principal component and multiple linear regression analyses identified the distance to the nearest house as a major predictor of *An. funestus* abundance in habitats, in agreement with a previous finding for *An gambiae* (Minakawa *et al*., 1999). These findings suggest that larval source management targeting *An. funestus* aquatic habitats located near houses could reduce the adult mosquito population.

Aquatic predators are well known to influence the abundance of mosquito larvae in breeding environments and are considered beneficial biological control agents for mosquito larvae (Koenraadt & Takken, 2003; Kweka *et al*., 2011; Munga *et al*., 2014; Shaalan & Canyon, 2009). Notwithstanding, there was no significant difference in *An. funestus* larval density between aquatic habitats with predators and habitats without predators. A similar study has noted that the presence of predators was not significantly associated with the low density of *An. gambiae s.l* larvae (Ndenga *et al*., 2011). Conversely, a previous study in Tanzania (Dida *et al*., 2015) and central Sudan (Mahgoub *et al*., 2017), reported that most predators were identified in habitats with fewer densities of mosquito larvae. However, no reduction in the density of *An. funestus*

larvae in the presence of predators in shared habitats were observed. This could be ascribed to the presence of other prey in larval habitats. A study has documented that in the presence of alternative prey, the habitats, the larval consumption-ability of predators was significantly reduced in the habitats (Kumar *et al*., 2008).

This study revealed that *An. funestus* was the predominant indoor resting vector corroborating the findings of previous investigations in Bungoma (Machani *et al*., 2020; Ochomo *et al*., 2013) and Kombewa (Ototo *et al*., 2015; Zhou *et al*., 2016b). The relative abundance, high sporozoite rate and HBI of *An. funestus* suggest that it is the main vector mediating malaria transmission in the study areas. I speculate that the adaptation of *An. funestus* to breed in warmer, open sunlit habitats may significantly reduce the developmental time of larval stages and increase the adult population of this species.

Because of a recent increase of 1° C due to climate change in Bungoma, its temperatures at 1,500 m asl are now equivalent to those at 1,347 m asl, assuming an altitudinal lapse rate of 154 m per degree Celsius at this altitude (Dhimal *et al*., 2014). Thus, ecologically, an elevation of 1,500 m asl must now be treated as equivalent to a Kombewa's low-altitude vector ecosystem in western Kenya (Githeko, 2009, 2021). Essentially, climate change has made the malaria risk for the Kenya highlands similar to lowland habitats.

The limitations of this study are: first, this study did not integrate detailed water chemistry analysis as part of the variable in assessing the larval ecology of *An. funestus*. Hence, further studies should be undertaken to assess the physicochemical characteristics of aquatic habitats that allow the co-existence of *An. funestus* with other mosquito species. Second, the productivity of *An. funestus* aquatic habitats and their ability to facilitate the development of larvae to emerged adults was not examined.

3.6 Conclusion

Several aquatic habitats were found to be breeding habitats for *An. funestus*, which coexisted there with the larvae of other mosquito species and aquatic predators. *An. funestus* was found in aquatic habitats that were both permanent and semi-permanent, with or without aquatic vegetation, slow-moving/disturbed, opaque, foggy/cloudy, or brownish water. The distance to the closest human habitation was the only significant variable that accurately predicted the abundance of *An. funestus* in aquatic habitats. To control the adult population of *An. funestus*, larval source management programs should target aquatic habitats close to human habitations. This study provides recommendations for larval source management or larviciding as a complementary tool for controlling aquatic stages of *An. funestus*.

An. funestus was found breeding in a variety of aquatic habitats and co-existing with larvae of other mosquito species and aquatic predators. *An. funestus* was found in permanent and semi-permanent aquatic habitats, with or without aquatic vegetation, and slow-moving/disturbed or stagnant water that was clear, opaque, cloudy and brownish. The only significant factor predicting the abundance *of An. funestus* in the aquatic habitats was the distance to the nearest house. Thus, larval control programmes should aim at targeting aquatic habitats near human dwellings to reduce the abundance of adult *An. funestus*. This study serves as a guide for the control of aquatic stages of *An. funestus* using larval source management or larviciding to complement existing vector control strategies. The importance of *An. funestus* in malaria transmission and the need for persistent vector surveillance before initiating vector control interventions are further confirmed by the relative abundance, high sporozoite rate, and HBI.

CHAPTER FOUR

4.0 GENETIC DIVERSITY AND POPULATION STRUCTURE OF *ANOPHELES FUNESTUS* IN WESTERN KENYA BASED ON MITOCHONDRIAL DNA MARKER, *COII*

Published as: Debrah, I.; Ochwedo, K.O.; Otambo, W.O.; Machani, M.G.; Magomere, E.O.; Onyango, S.A.; Zhong, D.; Amoah, L.E.; Githeko, A.K.; Afrane, Y.A & Yan, G. (2023) Genetic Diversity and Population Structure of *Anopheles funestus* in Western Kenya Based on Mitochondrial DNA Marker *COII*. *Insects*, *14*, 273. https://doi.org/10.3390/ insects14030273

4.1 Abstract

The mitochondrial marker, *COII* was employed to assess the genetic structure and diversity of *Anopheles funestus*, a very important malaria vector in Africa, that adapts and colonizes different ecological niches in western Kenya. Mosquitoes were collected using mechanical aspirators in four areas (Bungoma, Port Victoria, Kombewa and Migori) in western Kenya. Following morphological identification, PCR was used to confirm species. The *COII* gene was amplified, sequenced and analyzed to determine genetic diversity and population structure. A total of 126 (Port Victoria-38, Migori-38, Bungoma-22 and Kombewa-28) sequences of *COII* were used for population genetic analysis. *Anopheles funestus* had high haplotype diversity (Hd= 0.97 to 0.98) but low nucleotide diversity (π =0.004 to 0.005). The neutrality test revealed negative Tajima's *D* and Fs values indicating an excess of low-frequency variation. This could be attributed to either population expansion or negative selection pressure across all the populations. No genetic differentiation or structure (Fst = -0.01) and a high level of gene flow (Gamma St, Nm =17.99 to 35.22) were observed among the populations. Population expansion suggests the high

adaptability of this species to various ecological requirements hence sustaining its vectorial capacity and malaria transmission.

Keywords: *Anopheles funestus*, western Kenya, *COII*, genetic diversity, gene flow

4.2 Introduction

Malaria is a public health problem in Sub-Saharan Africa, spread mainly by the members of the *Anopheles funestus* group and *the Anopheles gambiae* species complex (Sinka *et al*., 2010). *An. funestus* group comprises five subgroups of which three of these subgroups contain at least thirteen (13) species, identified in various ecological niches across Africa (Harbach, 2013). *Anopheles funestus sensu stricto* (s.s.) (hereafter *An. funestus*) belongs to the *Funestus* subgroup which has seven members, namely: *An. funestus, An. aruni, An. vaneedeni, An. funestus-like, An. confuses, An. longipalpis type C* and *An. parensis* (Garros *et al*., 2005; Harbach, 2013). Of this group, *An. funestus* is a major vector responsible for high malaria transmission in sub-Saharan Africa. Three of the members of the *An. funestus* group; *An. funestus, An. rivulorum and An. leesoni* were found sympatrically in various ecological zones in Kenya, (Kamau *et al*., 2003) Sudan (Makhawi *et al*., 2015) and Nigeria (Awolola *et al*., 2005; Temu *et al*., 2007), suggesting that they have effective reproductive isolation mechanisms.

While most of the species of the *Anopheles funestus* group can be found only in certain geographical areas in Africa, *An. funestus* has a wide range of geographical distribution across various climatic types. This vector remains one of the most devastatingly efficient human malaria vectors exhibiting consistently notorious anthropophilic (preferring human habitation), anthropophagic (biting humans), endophilic (indoor resting) and endophagic (indoor biting) behaviours (Kaindoa *et al*., 2017; Sougoufara *et al*., 2014). Its capacity to transmit human

malaria far outpaced *Anopheles gambiae* and *Anopheles arabiensis* in some endemic areas in Africa (Coetzee & Fontenille, 2004; Kaindoa *et al*., 2017).

An. funestus can adapt and colonize different ecological niches owing to its high resistance to insecticides and changing breeding environments. A previous study in Kenya revealed that *An. funestus* breeds in various habitats and co-breeds with *An. gambiae sensu lato*, *Culex spp* and other vectors in the same habitats (Debrah *et al*., 2021). The vector survival rate, behaviour, ecology, vectorial capacity, and host-pathogen interactions are all affected by external environmental stress including temperature changes, land-use changes, host migration, and insecticide use (Zouache *et al*., 2014). These environmental factors have been demonstrated to influence mosquito population selection (Zouache *et al*., 2014). A study using microsatellite markers identified three genetically different *An. funestus* clusters namely: FUN1, FUN2 and FUN3 in Kenya (Ogola *et al*., 2019). The largest cluster (FUN1) was identified in samples collected from Rift Valley and Western regions, while FUN2 and FUN3 were identified in coastal region samples.

The mitochondrial DNA (mtDNA) is sensitive to genetic drift and has high copy numbers, highly conserved primer binding and ease of amplification making it a good marker for interpreting molecular taxonomy, phylogenetic relationships, population structure, and genetic diversity (Norris, 2002). Indeed, mtDNA markers have been utilized to study the genetic variances and evolutionary relationships of many mosquito species, as well as to correctly quantify gene flow and changes between populations (Bunmee *et al*., 2021; Weeraratne *et al*., 2018).

As a mitochondrial marker, *COII* has a number of properties such as maternal inheritance devoid of recombination, intraspecific polymorphism, higher level of differentiation between

populations and small effective population size makes it a good marker for studying genetic diversity, gene flow and population structure (Chang *et al*., 2016). Mitochondrial genetic diversity and molecular phylogeny are becoming increasingly important in mosquito research (Zhang *et al*., 2022).

The population structure and genetic diversity of *An. funestus* might influence its adaptation and efficiency of malaria transmission in western Kenya. Delineating the fine-scale population structure of vectors could be useful to investigate the genetic basis of speciation and local adaptation processes. Moreover, understanding gene flow among *An. funestus* populations could help to assess their movement in natural populations and therefore, how the populations are segregated. This study was designed to investigate the genetic structure, diversity and gene flow of a major vector, *An. funestus* in a malaria-endemic region of western Kenya using the mitochondrial marker, *COII*. This addressed the specific objective two of this thesis.

4.3 Materials and Methods

4.3.1 Mosquito sampling

Anopheles funestus mosquitoes were collected from four malaria transmission areas (Port Victoria, Migori, Bungoma and Kombewa,) in four counties in western Kenya from November 2020 to October 2021. Sample collection sites are shown in **Figure 4.1.** Adult mosquitoes were sampled indoors using pyrethrum spray catches and mechanical aspirators. All mosquitoes were morphologically identified using morphological keys (Coetzee, 2020) and the *An. funestus sensu lato* was stored in 1.5ml Eppendorf tubes containing cotton wool and silica gel (Coetzee, 2020). Detailed morphological identifications were described in Chapter Three, section 3.3.6. Samples were stored at -20°C for subsequent molecular analysis.

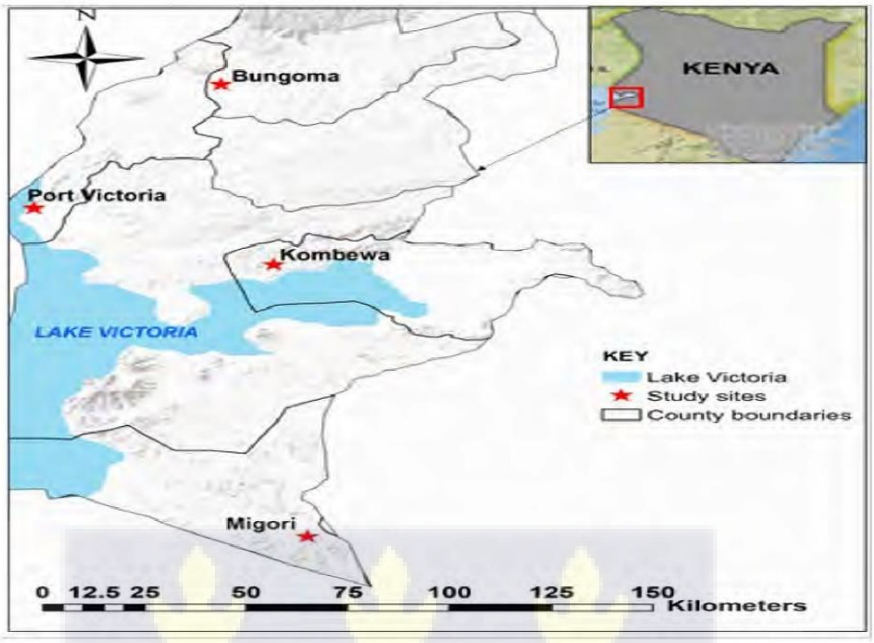


Figure 4.1: Map of study areas in western Kenya

This map was prepared with ESRI ArcGIS Pro 2.8 using field survey results and publicly available datasets. The Open Database License, which is used to make the data available, was used to compile the material on the map from OpenStreetMap and the OpenStreetMap 115 Foundation.

4.3.2 DNA Extraction from mosquito samples

Genomic DNA was extracted from the whole mosquito using the Chelex®-100 method (Musapa *et al*., 2013). The procedure was already described in chapter three, section 3.3.8.

4.3.3 PCR amplifications of DNA

An. funestus-specific PCR was conducted to confirm species using the species-specific primers (ITS2A/FUN) in the internal transcribed spacer region (ITS2) on the ribosomal DNA as described by (Koekemoer *et al*., 2002). This method was described in Chapter 3, section 3.3.9.

The *COII* gene was amplified using forward (5'-TCT AAT ATG GCA GAT TAG TGC A-3') and reverse (5'-ACT TGC TTT CAG TCA TCT AAT G-3') primers (Chang *et al.*, 2016). A 23µl PCR mixture containing 3µl genomic DNA, 0.5µl each of forward and reverse primers, 11.5µl master mix with PerfeCTa® ToughMix® (5x) and 7.5 of PCR water. The PCR conditions include initial denaturation at 95˚C for 3 minutes, denaturation of 95˚C for 15 seconds, annealing at 41˚C for 30 seconds, extension at 72˚C for 1 minute and 30 seconds for 35 cycles and final extension at 72˚C for 7 minutes. The gene was amplified using T100 thermal cycler (Biorad). The amplicons' quality was assessed using agarose gel electrophoresis in 1.5% w/v agarose gel stained with 2µl smart glow and visualized using the SmartDoc imaging system (Accuris ™ instruments).

4.3.4 Sequencing of the *COII* gene

Before sequencing, all positive PCR products were cleaned using Exonuclease 1-Shrimp Alkaline Phosphatase (ExoSAP). Before adding 8 µl of the amplicons, 1µl of ExoSAP was added to each of the 96 well plates. The conditions for ExoSAP activity for cleaning the amplicons were at a temperature of 37 ˚C for 15 seconds for 1 cycle. All the amplicons were bidirectionally

sequenced with the same primers used for the PCR using 3730 BigDye® Terminator v3.1 Sequencing Standard kit on ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). In the procedure, 3.63µl of nuclease-free water was added to 4µl of purified PCR product along with 0.3µl of BigDye Terminator v3.1, 1.75µl of 5X sequencing buffer, and 0.32µl of each primer. The thermal cycler was used to carry out the sequencing reactions. The following conditions were used: one cycle of polymerase activation at 96˚C for 60 sec, 25 cycles of denaturation at 96˚C for 30 sec, annealing at 50˚C for 15 sec, extension at 60˚C for 4 min, and a final holding temperature of 15˚C for 10 min. Following that, 96-well reaction plates with 90µl of the ethanol and sodium premix injected into each well underwent 10µl sequencing processes with ethanol and sodium acetate precipitation. The plates were then centrifuged at 3000 × g for 30 minutes at 4°C after being sealed with micro-seals and incubated at -20˚C for 30 minutes (5810R bench centrifuge, Eppendorf). Seals were taken off, absorbent paper towels were placed on top of the plates, and then the dishes were gently inverted to drain. The inverted plates were covered with fresh paper towels and spun at 50 × g for one minute at 4˚C. Next, 150 µl of ice cold (-20˚C) 70% ethanol was added to each well, and the plates were then sealed and spun at 3000 × g for ten minutes at 4°C. The plates were inverted once more over paper towels, and any surplus liquid was gently drained away before being covered with fresh paper towels, inverted, and spun for one minute at 50 × g at 4˚C. They were placed on the bench to air dry while being covered in brand-new paper towels. After adding 10µl of Hi-Di™ Formamide to each well, the samples were re-suspended in Hi-Di for denaturation (96˚C for 1 minute) reaction and electrophoresis using an ABI 3730 xl capillary sequencer (Applied Biosystems, UK).

4.3.5 Data analysis

De novo assembly of paired raw reads was performed using Geneious prime software version 2022.0.1 (Kearse *et al*., 2012), and CLC Genomics Workbench (Matvienko, 2015). Low-quality reads with low base calling accuracy below 99% (Phred 20) were excluded from the analysis. The ClustalW algorithm in MEGA X was used for multiple sequence alignment (Tamura *et al*., 2021). DnaSP Version 6.12.03 (Rozas *et al*., 2017), was used to compute genetic diversity indices [haplotype diversity (Hd), number of haplotypes (h), nucleotide diversity (π), number of segregating sites (S) and mean number of pairwise difference (k)] and Neutrality tests (Tajima's *D*, Fu and Li's D, Fu and Li's F, and Fu's Fs statistics). Gamma ST measurement, an inbuilt algorithm in DnaSP software was used to estimate gene flow and genetic differentiation (Higuera *et al*., 2020; Zhong *et al*., 2020). The analysis of molecular variance (AMOVA) was performed in Arlequin version 3.5.2 (Excoffier & Lischer, 2010) to partition genetic variations among groups (Port Victoria, Migori, Bungoma and Kombewa,) and within groups. Population Analysis with Reticulate Trees (PopART) version 1.7(Leigh & Bryant, 2015) was used to infer haplotype networks.

The best-fitting nucleotide substitution model was estimated with the Akaike Information Criterion (AIC) and the Bayesian information criterion (BIC) implemented in MEGA version 11.0.13 (Tamura *et al*., 2021). The Bayesian phylogenetic analysis was performed by MrBayes v3.2.7 (Ronquist *et al*., 2012), using Markov chain Monte Carlo (MCMC) methods. The MCMC was run for 2,000,000 generations with sampling tree topologies every 1,000 generations, after excluding the initial 25% as 'burn-in'. To root the tree, *Anopheles gambiae, Aedes albopictus,* and *Culex pipiens pallens* were the "outgroup" (accession # MG930872, MG930866, KX383916, and KT851543). The previously reported *COII* sequences of *An. funestus* from

western Kenya (GenBank accession: MT917174) and other locations in African countries, including southern Ghana (MT917179 and MT917180), northwestern Tanzania (MT917176 and MT917177), eastern Uganda (MT917175, MT917181, and MT917182), eastern Zambia (MT917178), and northern Malawi (MT917161), were also included in the phylogenetic tree. The 50% majority rule consensus tree was constructed with Bayesian posterior probabilities of the nodal supports. The output tree was visualized and edited with an online tool Interactive Tree of Life (iTOL) v5 (Letunic & Bork, 2021).

4.4 Results

4.4.1 Genetic diversity of *An. funestus* in western Kenya

A total of 126 (Port Victoria-38, Migori-38, Bungoma-22, and Kombewa-28) amplicon sequences of *COII* were used for population genetic analysis. A total of 64 haplotypes were identified (Table S1), suggesting a high haplotype diversity in the populations (Hd= 0.97 to 0.98) albeit low nucleotide diversity (Π=0.004) based on *COII* sequences (**Table 4.1**). Moreover, the statistical test of neutrality revealed significant negative Tajima's D and Fs values indicating a deviation from a standard neutral model and population expansion with an excess of low-frequency variation likely due to population expansion (or possibly negative selection pressure). (**Table 4.1**). There was no significant difference in observed nucleotide diversity across the four populations (X2=181.744, df =189, p=0.635).

Table 4.1: Nucleotide diversity indices based on *COII* of *An. funestus* from four areas in western Kenya

Populations	N	L	S	Π	h	Hd	Tajima's		Fs		Fu and Li's		Fu and Li's	
							D	p	Statistics	p	*D*	p	*F*	p
Port Vic.	38	774	32	0.005	30	0.98	-1.86	< 0.05	-31.51	0.000	-1.99	> 0.10	-2.31	> 0.05
Migori	38	774	26	0.004	25	0.97	-1.76	> 0.05	-21.85	0.000	-2.75	< 0.05	-2.86	< 0.05
Bungoma	22	774	19	0.004	18	0.98	-1.64	> 0.05	-16.42	0.000	-1.89	> 0.10	-2.12	> 0.05
Kombewa	28	774	17	0.004	23	0.98	-1.33	> 0.10	-25.04	0.000	-1.06	> 0.10	-1.34	> 0.10
All populations	126		41	0.004	64	0.97	-1.81	< 0.05	-32.91	0.000	-1.42	> 0.10	-1.89	> 0.05

N: Sampled population, L: number of sites analyzed, S: segregating sites, Π: Nucleotide diversity, h: Number of Haplotypes, Hd: Haplotype diversity, Statistical significance ($p<0.05$)

4.4.2 Population structure and gene flow

AMOVA results showed that there was no genetic differentiation across all four populations (Fst = -0.01). Specifically, this result revealed that there was no genetic differentiation between Port Victoria and Migori (Fst= -0.010), Bungoma and Kombewa (Fst= -0.016), Port Victoria and Bungoma (Fst = -0.020), Migori and Bungoma (Fst= -0.008), Port Victoria and Kombewa (Fst = -0.009), Migori and Kombewa (Fst= -0.003) (**Table 4.2)**. The lack of population structure was supported by a high level of gene flows across the four populations (Gamma St, Nm=15.40) with the highest gene flow occurring between Port Victoria and Bungoma (Gamma St, Nm= 35.22). This was followed by Port Victoria and Migori (Gamma St, Nm= 30.25). The lowest gene flow was between Migori and Kombewa (Gamma St, Nm=17.99) (**Table 4.2**).

Table 4.2: *Anopheles funestus* population structure in western Kenya region

Populations	No. of shared mutations (%)	Dxy	Hs	Ks	Kxy	Gst	Gamma St		Fst		
							Value	Nm	Value	Nm	*P*-Value
Port Vic. *vs* Migori	20/40 (50)	0.004	0.97	3.400	3.365	0.001	0.008	30.25	-0.010	-24.45	0.855
Bungoma *vs* Kombewa	12/25 (48)	0.004	0.98	2.878	2.836	-0.006	0.013	19.43	-0.016	-16.04	0.892
Port Vic *vs* Bungoma	18/34 (52.9)	0.004	0.98	3.389	3.222	-0.004	0.007	35.22	-0.020	-12.80	0.982
Migori *vs* Bungoma	14/32 (43.8)	0.004	0.97	3.048	2.994	0.001	0.013	19.02	-0.008	-31.71	0.649
Port Vic *vs* Kombewa	15/36 (41.7)	0.004	0.98	3.324	3.234	-0.003	0.011	23.43	-0.009	-28.29	0.838
Migori *vs* Kombewa	15/30 (50)	0.004	0.97	3.015	2.985	0.0003	0.014	17.99	-0.003	-82.77	0.468

Dxy: Average number of nucleotide substitution per site between populations, Hs: weighted average of estimated haplotype diversities in the subpopulations, Ks: the number of synonymous substitutions per synonymous site, Kxy: Average number of nucleotide differences between populations, Gst: a measure of population differentiation, Nm: Number of migrants

4.4.3 Phylogenetic relationships and network analyses

The best-fit model for nucleotide substitution was identified by MEGA 11 as a Tamura 3-parameter with gamma-distributed rate heterogeneity (T92 + G) according to the Bayesian information criterion for *COII* haplotypes. The phylogenetic tree was inferred by Bayesian analyses with the standard deviation of split frequency values (<0.01). The phylogenetic estimates from the MCMC analyses strongly supported the monophyletic group (posterior probability = 1) (**Figure 4.2)**. There is no clear clustering of haplotypes observed and all the 64 identified haplotypes (Prob=0.9983) in western Kenya shared a common ancestor with *An. funestus-like* (accession # MT917161) from Malawi, with the closest haplotype (Hap 40) coming from the Migori and Port Victoria populations, which border Tanzania and Uganda, respectively. *Anopheles funestus* samples from Port Victoria and Bungoma (Hap 2) shared a recent common ancestor with *An. funestus* (accession # MT917175) from Uganda.

Haplotype networks were constructed using the Median-Joining method in PopART software. Out of the 64 distinct haplotypes identified in western Kenya, 30 (46.9%) were found in the Port Victoria population (**Table 4.1**). Twenty (31.3%) of the 64 haplotypes were found in each of the study populations. The median-joining haplotype network of the *COII* gene revealed the genealogy of each of the observed haplotypes with haplotypes S1-S20 being shared across the study populations (**Figure 4.3**). In the four study areas, the most common haplotype was S1 (14/64, 21.9%), followed by S3 (7/64, 10.9%), S2 (7/64, 10.9%), S4 (6/64, 9.4%), S6 (6/64, 9.4%), and S8 (4/64, 6.3%). The distribution of the S1 haplotype among the 64 observed haplotypes was as follows: 8%, 6%, 5%, and 3% in Port Victoria, Migori, Bungoma, and Kombewa, respectively. The haplotype (S1) could be an ancestral haplotype (recent common ancestor) to *An. funestus* in western Kenya. Port Victoria and Bungoma populations had the most

shared mutations (52.9%) followed by Migori and Port Victoria as well as with Kombewa each at 50%. Port Victoria and Kombewa populations had the least shared number of mutated sites (42%). Nucleotide sequences of the 64 identified haplotypes were submitted to GenBank and assigned accession numbers ON931353-ON931416.

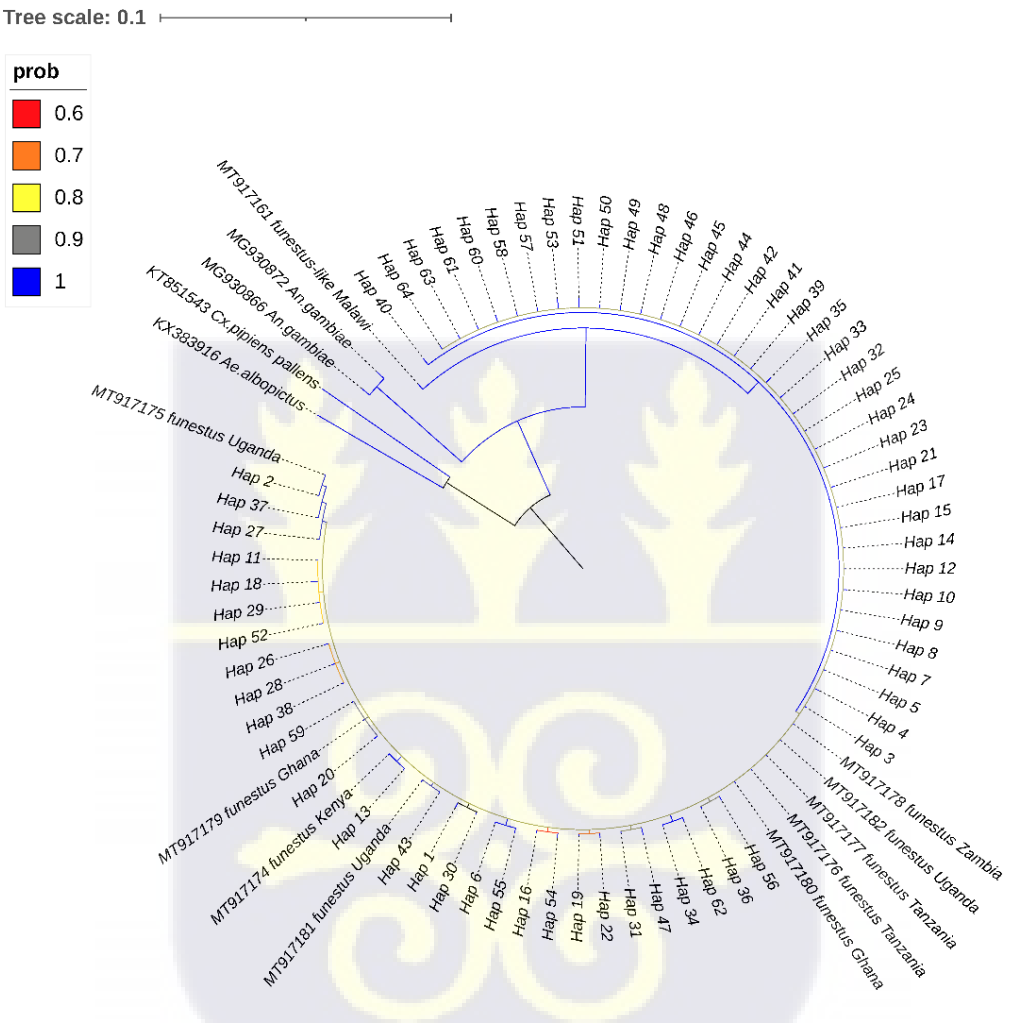


Figure 4.2: Bayesian phylogenetic tree inferred from cytochrome oxidase subunit II gene (*COII*). The 50% majority rule consensus tree was constructed using Markov chain Monte Carlo (MCMC) methods and posterior probabilities of the nodal supports are indicated by colour lines where the green corresponds to 100% support value).

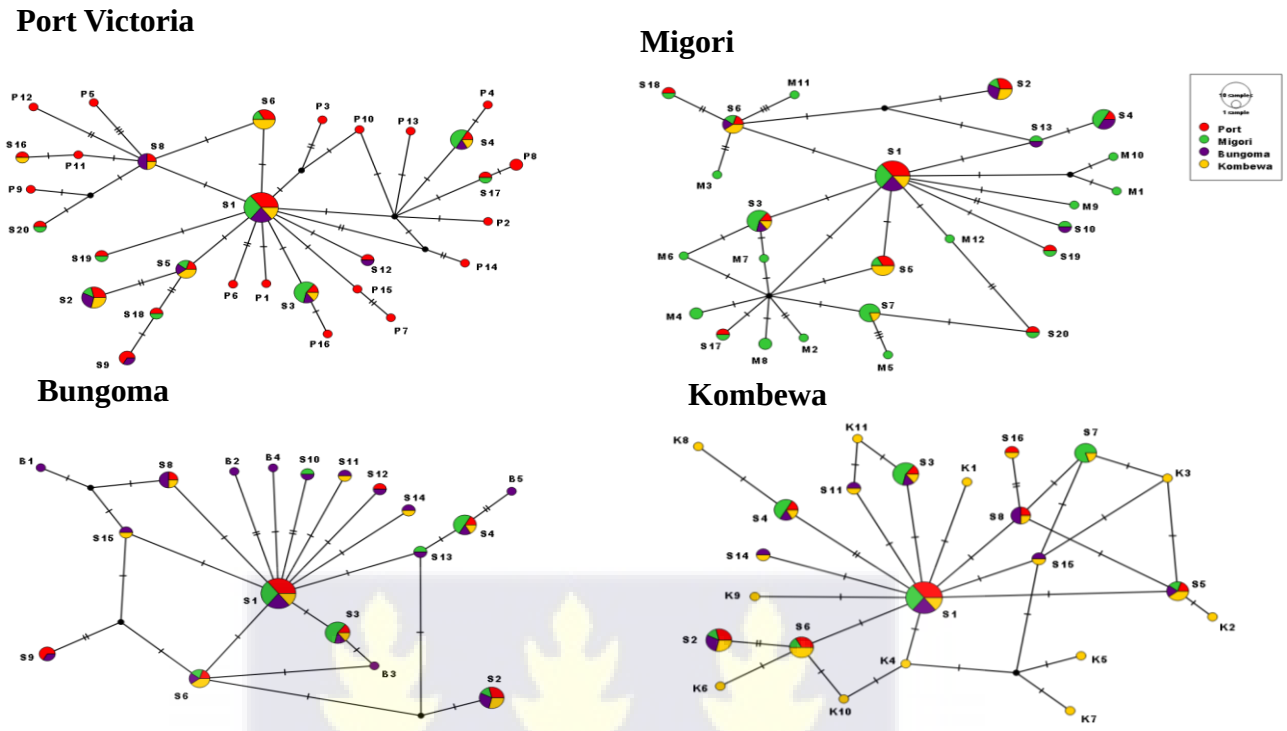


Figure 4.3. Haplotype distribution in the four study populations. S represents shared haplotypes across the four study sites, P represents haplotypes in Port Victoria only, M represents haplotypes in Migori, K represents haplotypes in Kombewa and B represents haplotypes in Bungoma. Hatch marks represent the number of mutated sites resulting in particular haplotypes whereas the size of the circle corresponds to haplotype frequency or numbers of the sample under that specific haplotype.

4.5 Discussion

This study revealed the population genetic structure of *An. funestus* population across four counties in western Kenya using a mitochondrial marker, *COII*. In the context of malaria control strategies including genetic alteration of vector species, information on genetic diversity and population structure of vectors is critical (Collins & James, 1996). In recent years, changes in the ecology of vector populations have been documented (Debrah *et al*., 2021). High tolerance to a variety of ecological niches, insecticide resistance, and vast geographic distribution make *An. funestus* a highly adaptable dominant vector species (Dia *et al*., 2013). Due to differences in the ecological zones in western Kenya, I anticipated that the barrier to gene flow could be due to variation brought about either by biotic or abiotic factors. Key biotic factors include Lake Victoria, climatic conditions, and landscape (highland versus lowland), resulting in gene flow and genetic diversity between the populations. Abiotic factors including agriculture and the use of fertilizers and pesticides could have selective pressure on the *An. funestus* population resulting in genetic diversity.

This study revealed the signature of the population expansion, with weak population structure and high levels of gene flow among the population of *An. funestus* from areas with varied *P. falciparum* transmission intensities in western Kenya. Generally, *An. funestus* exhibited low nucleotide diversity with Port Victoria exhibiting a higher number of haplotypes than other regions. In western Kenya, the high genetic diversity of *An. funestus* population compared to coastal regions based on microsatellite markers was reported a decade ago (Braginets *et al*., 2003). The observed high number of haplotypes in Kombewa, Migori, and Port Victoria among this primary vector is consistent with high levels of *Plasmodial* transmission in these study areas as compared to other malaria vectors (Ototo *et al*., 2015; Zhou *et al*., 2011). Port Victoria which

is also proximal to Lake Victoria had a significantly high number of haplotypes circulating in the area. Port Victoria is the main Lake Victoria transport corridor between Kenya, Lake Victoria Islands, and Uganda. It was earlier reported that small wooden boats played a significant role in the transportation of mosquitoes between the mainland and Lake Victoria Islands (Chen *et al*., 2004). This might have played a role in facilitating high gene flow, resulting in a high number of haplotypes and haplotype diversity reported in that population. Port Victoria has also been documented to have a high malaria prevalence with vector control interventions ongoing (Noor *et al*., 2009). The high gene flow observed in the populations could be due to migration and a lack of geographical barriers (Bunmee *et al*., 2021). Mountain ranges, rivers, forests, and other physical barriers, in combination with climatic or biological obstacles like flight range and breeding grounds, may obstruct gene flow between *Anopheles* populations. However, none of these factors served as a barrier to gene flow in the *An. funestus* population in western Kenya. The Rift Valley is known to serve as a barrier to gene flow (Kamau *et al*., 2003), nonetheless these findings showed that there are no physical barriers to hamper gene flow among mosquito populations in western Kenya. Different breeding sites, mosquito migration, environmental changes, and human activities act to shape the genetic diversity of *An. funestus* populations (Samb *et al*., 2012).

Given the low Fst values and high Gamma St, Nm values, there is a strong indication of high gene flow between populations as well as high breeding. Not only has high gene flow reduced heterozygosity between populations, but it has also resulted in most haplotypes being shared among the western Kenya region, contributing to a weak or lack of population structure. The shared haplotypes were observed between western Kenya and other African countries, suggesting that the genetic diversity of the *COII* gene might be not directly associated with

geographical divisions. However, with the excess frequency of rare alleles, a genetic signature for population expansion can persist for a long period, masking any genuine ecological population or genetic structure that may exist (Rogers & Harpending, 1992). The observed weak population structure, negative selection, and population expansion suggest that there was a free exchange of genes among *An. funestus* populations in western Kenya. The presence of a negative signature of selection on this gene is an indicator of purifying selection acting on the gene to preserve the genetic structure by eliminating deleterious mutations (Meiklejohn *et al*., 2007).

With evidence of purifying selection and population expansion (or possibly negative selection pressure), it is possible evolutionary forces shaping *An. funestus* population in western Kenya could be the usage of long-lasting insecticidal nets, indoor residual spraying and insecticide use for agricultural activities, especially those affecting larval breeding sites (Aguirre-Obando *et al*., 2015; Pasteur & Raymond, 1996). The high number of haplotypes per study site and the population expansion observed in this study suggest the high adaptability of *An. funestus* to various ecological requirements hence sustaining its vectorial capacity and malaria transmission.

4.6 Conclusion

This study has shown that the *An. funestus* population in western Kenya is under selection pressure leading to demographic expansion and the spread of variants through breeding among varied transmission sites in western Kenya. Population expansion (or possibly negative selection pressure) suggests there is high adaptability of this species to various ecological requirements hence sustaining its vectorial capacity and malaria transmission.

CHAPTER FIVE

5.0 METABOLIC RESISTANCE TO PYRETHROIDS WITH POSSIBLE INVOLVEMENT OF NON-CODING RIBONUCLEIC ACIDS IN *ANOPHELES FUNESTUS*, THE MAJOR MALARIA VECTOR IN WESTERN KENYA

Original results are presented in the form of a manuscript submitted to BMC Genomics Journal. Preprint available online as:

Debrah, I., Zhong, D., Machani, M. G., Nattoh, G., Ochwedo, K. O., Morang'a, C. M., Lee, M.-C., Amoah, L. E., Githeko, A. K., & Afrane, Y. A. (2024). Non-Coding RNAs Potentially Involved in Pyrethroid Resistance of *Anopheles funestus* Population in Western Kenya. *Research Square*. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10925441/

5.1 Abstract

Backgrounds

The resurgence of *Anopheles funestus*, a dominant vector of human malaria in western Kenya was partly attributed to insecticide resistance. However, evidence on the molecular basis of pyrethroid resistance in western Kenya is limited. This study reported metabolic resistance mechanisms and demonstrated that multiple non-coding Ribonucleic Acids **(**ncRNAs) could play a potential role in *An. funestus* resistance to pyrethroid in western Kenya. *Anopheles funestus* mosquitoes were sampled by aspiration methods in Bungoma, Teso, Siaya, Port Victoria and Kombewa in western Kenya. The F1 progenies were exposed to deltamethrin (0.05%), permethrin (0.75%), DDT (4%) and pirimiphos-methyl (0.25%) following WHO test guidelines. A synergist assay using piperonyl butoxide (PBO) (4%) was conducted to determine cytochrome P450s' role in pyrethroid resistance. RNA-seq was conducted on a combined pool of specimens

that were resistant and unexposed, and the results were compared with those of the FANG susceptible reference strain. This approach aimed to uncover the molecular mechanisms underlying the observed phenotypic pyrethroid resistance.

Results

Pyrethroid resistance was observed in all sites with an average mortality rate (MR) of 57.6%. Port Victoria had the highest level of pyrethroid resistance to permethrin (MR=53%) and deltamethrin (MR=11%). Teso had the lowest level of resistance to permethrin (MR=70%) and deltamethrin (MR=87%). Resistance to DDT was observed only in Kombewa (MR=89%) and Port Victoria (MR=85%). A full susceptibility to P-methyl (0.25%) was observed in all sites. PBO synergist assay revealed high susceptibility (>98%) to pyrethroids in all the sites except for Port Victoria (MR=96%). Whole transcriptomic analysis showed that most gene families associated with pyrethroid resistance comprised non-coding RNAs (67%), followed by immunity proteins (10%), cytochrome P450s (6%), cuticular proteins (5%), olfactory proteins (4%), glutathione S-transferases (3%), UDP-glycosyltransferases (2%), ATP-binding cassettes (2%) and carboxylesterases (1%).

Conclusions

This study unveils the molecular basis of insecticide resistance in *An. funestus* in western Kenya, highlighting for the first time the potential role of non-coding RNAs alongside metabolic detoxification in pyrethroid resistance. Targeting non-coding RNAs for intervention development could help in insecticide resistance management.

Keywords: *Anopheles funestus*, insecticide resistance, non-coding RNAs, western Kenya, RNA-seq

5.2 Introduction

Vector control particularly the use of bed nets treated with pyrethroids has had an impact on entomological parameters, such as reducing infection rate in the vector population, vector abundance, and parity rate (Ototo *et al*., 2015; Trape *et al*., 2014), leading to a decline in malaria morbidity and mortality in sub-Saharan Africa as a result of a decline in vectorial capacity (Bhatt *et al*., 2015; Eisele *et al*., 2012; O'Meara *et al*., 2010). Despite these successes, malaria resurgence and outbreaks have been reported in various transmission settings in sub-Saharan Africa where ITNs were deployed (Mwesigwa *et al*., 2015; Nabatanzi *et al*., 2022; Trape *et al*., 2011; Zhou *et al*., 2011). Hence, the effectiveness of the primary vector control methods with regard to insecticide resistance needs continuous monitoring and probing of the resistance mechanisms.

In contrast to other major vectors, *Anopheles funestus sensu stricto* (hereafter *An. funestus*) has received very scant attention owing to the difficulty in colonizing this species under laboratory conditions. *An. funestus* is distributed throughout Africa similar to the distributed union of *An. gambiae*. After developing resistance and exhibiting behavioural adaptability, *An. funestus* has a higher ability to colonize a niche (Debrah *et al*., 2021; Dia *et al*., 2013). It is one of the most ubiquitous and efficient malaria vectors in the world; highly susceptible to the *P. falciparum* parasite, highly anthropophagic and endophilic (Kaindoa *et al*., 2017; Mapua *et al*., 2022; Sougoufara *et al*., 2014). The significance of studying this mosquito is highlighted by its versatility in ecological adaptation and the emergence of resistance to recommended public health insecticides for vector control (Dia *et al*., 2013; Mulamba *et al*., 2014).

Increased resistance to pyrethroids used for bed net impregnation has led to low efficacy of conventional LLINs against *An. funestus* (Akoton *et al*., 2018). Resistance monitoring focuses on

transmission foci, hotspots of localized outbreaks, or after spikes in disease cases in pre-elimination and elimination settings (WHO, 2016). For effective insecticide resistance management, it is essential to genetically characterize insecticide resistance profiles and mechanisms in the vector populations. Metabolic resistance poses the biggest threat to the control of malaria vectors (Riveron *et al*., 2014a). Cytochrome P450s, Glutathione S-transferases (GSTs) and carboxylesterases (COEs) are well-established enzyme families in malaria vectors known to confer resistance to pyrethroids (Messenger *et al*., 2021; Wondji *et al*., 2007). These detoxification genes are pivotal in the molecular mechanism of insecticide resistance.

Non-coding RNAs (ncRNAs) form a vast class of RNAs that do not code for proteins and are ubiquitous in the insect genome. Examples of ncRNAs include transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), microRNA (miRNA), PIWI-interacting RNA (piRNA), endogenous small interfering RNA (siRNA), circular RNA (circRNA), long non-coding RNA (lncRNA), protein functional effector small ncRNA (pfeRNA), and other ncRNAs whose functions remain unknown (Cech & Steitz, 2014; Mei *et al*., 2023). They can control the expression of genes at the chromosomal, transcriptional, post-transcriptional, and translational levels and play a role in the entire developmental process. Non-coding RNAs have been demonstrated in studies on arthropods to be essential for several physiological and developmental processes, including moulting, reproduction, immunity, wing development, and insecticide resistance (Qiao *et al*., 2019). Non-coding RNAs can modify signaling pathways involved in these biological processes by targeting both DNA and RNA substrates. Sequences of regulatory ncRNAs can also help establish epigenetic alterations such as histone acetylation/deacetylation, DNA/histone methylation, etc. within the nucleus by bringing in chromatin remodeling agents that are known to change transcriptional activity (Catalanotto *et*

al., 2016; Khalil *et al*., 2009). Based on their length, ncRNAs are arbitrarily divided into two groups: small ncRNAs (scnRNAs, <200 nts) and long ncRNAs (lncRNAs, >200 nts) (Kapranov *et al*., 2007). Depending on where they are in relation to genes that code for proteins, lncRNAs can also be categorized as sense, antisense, intronic, or intergenic (Zhu *et al*., 2017). With regards to insecticide resistance in insects, lncRNAs that were found to be differentially expressed during the larval stage development of resistant *Plutella xylostella* genotypes (Etebari *et al*., 2015), and uniquely differentially expressed during the egg to adult moth stages in Bt-toxin resistant strains of the same insect (Liu *et al*., 2017). Similarly, the expression of the lncRNAs in *P. xylostella* was linked to the expression of the cytochrome P450, the ATP-binding cassette (ABC) transporter and the esterase genes involved in resistance to chlorantraniliprole insecticide (Zhu *et al*., 2017). Moreover, some long intergenic non-coding RNAs were overexpressed in deltamethrin-resistant larvae of *Plutella xylostella* exposed to deltamethrin (Etebari *et al*., 2015). ncRNAs are intriguing candidates to study when organisms are exposed to insecticides and other toxicants since they are involved in pathways linked to responses to cellular stress (Chen & Carmichael, 2010; Etebari *et al*., 2015). The genes for ribosomal proteins, such as L39 (Tan *et al*., 2007), S4 (Hu *et al*., 2007), L22 (He *et al*., 2009), and S29 (Sun *et al*., 2011), have been found to be associated with the resistance mechanism of *Culex* mosquitoes.

In the malaria-endemic region of western Kenya, there has been a resurgence of endophilic *An. funestus* and increased 20-fold over a decade ago (McCann *et al*., 2014; Zhou *et al*., 2011). The resurgence of this vector was partly attributed to resistance to pyrethroids used in ITN impregnation (Zhou *et al*., 2016a). As the country is aiming to achieve the malaria elimination goal by 2030, it is very crucial to have a comprehensive understanding of the resistant profile of this important, re-emerged vector to inform stakeholders of the right choice of control strategy to

adopt. To date, there have been few investigations on *An. funestus* susceptibility to insecticides in Kenya. The initial study on *An. funestus* susceptibility to insecticides from two study areas in western Kenya was reported in 2007 (Kamau *et al*., 2008) and even though the species were not identified using molecular techniques, previously identified *Anopheles* species from the same areas revealed that only *An. funestus* was present (Kamau *et al*., 2003). Later in western Kenya, seven adults *An. funestus* were sampled and their F1 progenies' susceptibility to insecticides revealed that they were susceptible to DDT but resistant to permethrin (Kawada *et al*., 2011). Further study in Kisumu in the lowland area of western Kenya has shown that *An. funestus* is resistant to pyrethroids (deltamethrin and permethrin) with overexpression CYP6P9a and CYP6P9b responsible for pyrethroid resistance (Mulamba *et al*., 2014). A recent study in the same Kisumu, using microarray for transcriptome analysis has revealed that overexpression of cytochrome P450s notably, CYP4H18, CYP6M7, CYP9K1, CYP4C36 and CYP4H17 in pyrethroid-resistant *An. funestus* population (Sandeu *et al*., 2020). The use of microarrays can only be used with the gene families that have been identified on the array, and they only give information on relative expression levels. The RNA-seq technology offers single nucleotide level resolution, absolute rather than relative gene expression profile, and a comprehensive view of the transcriptome in a specific state (Crawford *et al*., 2010).

This study examined the insecticide resistance profile of *An. funestus* across five sites in four counties in western Kenya and elucidated the molecular mechanisms of resistance using RNA-seq. These results provide new novel insights into insecticide resistance at the molecular level in this important malaria vector, which has received limited attention, and could help in designing effective control strategies. This addressed the specific objective three of this thesis.

5.3 Materials and Methods

5.3.1 Sampling of indoor-resting *Anopheles* mosquitoes

Anopheles mosquitoes were sampled from five sites: Bungoma [00.54057˚N, 034.56410˚E, 1386–1,545m above sea level (asl)] (highland), Teso (0°43'0" N. 34°21'0" E, 1357-1,500m asl) (highland), Siaya (0.0626° N, 34.2878° E, 1,140-1,400m asl) (lowland), Port Victoria (0° 6' 0" N / 33° 58' 0" E, 1,149 asl) (lowland) and Kombewa (0^0 07'N, 340 30'E, 1150–1300 m asl) (lowland) in western Kenya (**Figure 5.1**). These sites are malaria-endemic areas predominated by *An. funestus* mosquitoes. Adult *An. funestus* population were sampled from the indoor living room using mouth and prokopack aspiration methods after informed consent was sought and provided by the owners of the households.

Figure 5.1: Map of study sites where mosquitoes were sampled in western Kenya

The software ArcGIS Pro 2.6 was used to create the map. Map sources: USGS, ESRI, and CGIAR (www.esri.com)

5.3.2 Mosquito sampling using mouth aspiration technique

This method was used to collect mosquitoes resting indoors on walls, the underside of beds, clothing, ceilings, roofs, and furniture. The collection was done using the sucking tube or mouth aspirator and a flashlight. The mouthpiece of the aspirator was put inside the mouth and with the help of the flashlight, the vector mosquito resting on walls or bed net was carefully aspirated into the rubber or plastic tube. They were immediately transferred into the paper cups and were provided with a 10% sugar solution soaked in cotton wool. The paper cups were later put into the cool box and transported into the insectary and were reared and allowed to lay the eggs. The F1

progenies (3-5 days) were used for the insecticide susceptibility test.

5.3.3 Mosquito sampling using prokopack aspirator

This prokopack aspirator (model 140) was used to sample indoor resting mosquitoes of different age groups and physiological states. It is lightweight about 650g, highly manoeuvrable aspirator for collecting indoor resting adult mosquitoes. It has an extension pole up to 13 feet (4m) from the ground level and this facilitates collections on ceilings, upper walls and under furniture. It has a 12v amp hr gelled-electrolyte battery, an extension pole, six collection cups and lids with stainless steel mosquito mesh, and a universal charger (input 100-250 VAC, 50/60 Hz) allowing complete recharging in 2.5 hours. As it was moved along the walls to suck mosquitoes resting on surfaces into the collection cup. The cup containing mosquitoes was later released into the cage for easy sorting. The samples were put into paper cups and provided with a 10% sugar solution in cotton wool and transported to the lab for rearing.

5.3.4 Mosquito sorting and identification

Mosquitoes were sorted by separating male mosquitoes from the females and *Culex spp* from *Anopheles*. Later *Anopheles* mosquitoes were morphologically identified as *An. funestus s.l* and *An. gambiae s.l* following morphological and taxonomic keys (Coetzee, 2020; Gillies & Coetzee, 1987). *An. funestus* possess a pale spot on the second dark area, a light spot between the two dark spots on vein 6 and an absence of fringes on vein 6 are features for *An. funestus*. The legs of *An. funestus* are not speckled.

5.3.5 Raising of F1 progenies

An. funestus blood-fed, gravid and half gravid were put into cages to lay eggs on wet filter papers. The F_0 females in these physiological states were fed on 10% sugar solutions soaked in cotton wool and laying pads/Petri dishes. After laying the eggs, the eggs were allowed to hatch into larvae. The larvae were put in a pan containing spring water and were fed with the larvae feed, tetramin until they matured to a pupal stage where they were transferred into cages to emerge into adults.

5.3.6 Insecticide susceptibility tests

The F1 adult progenies aged between 3-5 days old were used for the bioassay. The WHO Insecticide Susceptibility test/ WHO bioassay was carried out following the test method developed by the WHO (WHO, 2016). Following this procedure, clean sheets of white paper the size of 12 x 15 cm impregnated with 0.05% deltamethrin, 0.75% permethrin, 4% DDT and 0.25% pirimiphos methyl were rolled into a cylinder shape tube having a slide unit at the opposite end. 120-150 female *An. funestus* mosquitoes were aspirated into the red-dotted tubes passing through a hole in the slide. This gives 6 replicates of 20-25 samples per tube. The mosquitoes were transferred into individual tubes using mouth aspirators, the slide unit was closed and the holding tubes were kept in an upright position for an hour. During this 1 hour, the number of mosquitoes that were knockdown was recorded every 10 minutes on the recording sheet until the I hour elapsed. After 1-hour exposure, the slide units are separated from the exposure tubes. On the mesh screen of the holding tubes, a pad of cotton wool soaked in 10% sugar water was put there for the mosquito to feed on and mortality was scored 24 hours post-exposure. During this period, the holding tubes were kept under standard laboratory conditions at a temperature of 27 ˚C ± 2 ˚C and a relative humidity of 75% ± 10%. The mosquito samples

were exposed to silicone oil-treated papers inserted into two yellow-dotted tubes to serve as a control.

5.3.7 PBO synergist bioassays

After establishing pyrethroid resistance in the *An. funestus* population, a synergist bioassay was conducted with PBO-impregnated papers to determine the role of P450 monooxygenases in pyrethroid resistance. The PBO inhibits these enzymes' activity in insects including mosquitoes. The mosquito samples (F1 progenies) were pre-exposed to 4% PBO for 1 hour before they were immediately exposed to the pyrethroids (0.05% deltamethrin and 0.75% permethrin). The mortality was scored 24 hours post-exposure after maintaining under standard laboratory conditions at a temperature of 27 ˚C ± 2 ˚C and a relative humidity of 75% ± 10%.

5.3.8 Preparation of samples for molecular and transcriptome analysis

Surviving resistant *An. funestus* samples after exposure to the insecticides (permethrin and deltamethrin) and unexposed (*An. funestus* F1 progenies samples that were not exposed to any insecticides) were killed immediately by keeping them in a deep freezer for about 10 minutes until they were completely knockdown. Samples were immediately stored in 0.5 ml Eppendorf tubes with RNALater and were immediately frozen at -80˚C for subsequent molecular and whole transcriptome analysis.

5.3.9 DNA extraction for species identification

DNA was extracted from the legs of each stored mosquito specimen using the Chelex®-100 method (Musapa *et al*., 2013). Following the method, 190 µl of autoclaved 1×PBS and 10 µl of 10% saponin solution were added to the sample homogenate and the submerged mosquito section was ground into a uniform suspension and was incubated for 20 minutes. The samples were centrifuged at 10,000 rpm for 10 minutes and supernatant were discarded. The pellets were resuspended in 200µl of 1×PBS and centrifuged again at 10000 rpm for 10 minutes. Supernatants were discarded and allowed to dry for at least 10 minutes till they were well-dried. The pellets were resuspended each in 100µl of 20% w/r chelex-100 resin suspension in deionized water. The samples were then boiled in a water bath at 85^0C on floating racks for 5 minutes and vortex for 10 seconds. They were then centrifuged at 10,000 for 1 minute and the DNA solution was transferred into pre-labelled 1.5ml storage vials and stored at -20˚C for molecular analysis.

5.3.10 Species identification using PCR

An. funestus-specific PCR was conducted to confirm species using the species-specific primers (ITS2A/FUN) in the internal transcribed spacer region (ITS2) on the ribosomal DNA (Cohuet *et al*., 2003; Koekemoer *et al*., 2002). Species-specific primers for *An. funestus* (5`-GCA TCG ATG GGT TAA TCA TG-3`) and universal primer (5`-TGT GAA CTG CAG GAC ACA T-3`) were used. A final volume of 12.5 µl of PCR mixture containing 1µl of genomic DNA, 6.5 µl DreamTaq Green PCR Master Mix (2x), 0.5 µl of each of the primers and 4.0 µl of PCR water. Genomic DNA amplification was performed using T100 thermal cycler (Biorad). The PCR conditions include initial denaturation at 95 ˚C for 3 seconds, denaturation of 94 ˚C for 30 seconds, annealing at 55 ˚C for 30 seconds for 34 cycles, extension at 72 ˚C for 45 seconds and final extension at 72 ˚C for 6 seconds.

5.3.11 Preparation of agarose and visualization of the DNA

For the preparation of agarose gel, 1.2g of agarose was added into 80 ml 1 X TBE buffer in a microwave beaker and heated until uniformly dispersed agarose in a microwave on high power for bubbles to appear. The beaker was removed from the microwave oven and gently swirled with the agarose solution to resuspend any particles. The solution was heated again until all particles dissolved. The solution was allowed to cool for 1-2 minutes at room temperature. Then, 4µl smart glow stain was added and swirls gently to mix the solution. The gel was poured into the casting unit to a depth of 3-4mm and immediately after pouring, the comb was inserted and any air bubbles that may have been trapped under or between the teeth of the gel were removed. The gel was completely allowed to solidify at room temperature for approximately 30-45 minutes. After solidification, it was flooded with TBE buffer and the comb and the casting dams were carefully removed. The gel was placed in the electrophoresis chamber and the TBE until the gel is submerged under 3-5 mm of the TBE buffer. Using a P20 pipette, 10 µl of the amplified DNA samples were loaded into individual wells taking care not to puncture the sample wells. 10 µl 100bp DNA ladder was added to two of the empty wells to serve as controls. The gel cover and power lead were attached, and current (100V) was applied to the unit. Bubbles appeared around the electrode when the current was flowing. After 30 minutes, the power supply was turned off when the samples had dissolved sufficiently. The gel was moved into the smart doc imaging system (Accuris ^{TM} instrument) where the DNA bands were viewed in agarose gels and images were taken using a smartphone camera.

5.3.12 RNA extraction

Total RNA was isolated and purified from individual whole mosquitoes using the ZYMO Quick-RNA miniprep kit (Vitucci & McCullough, 2022). Samples were removed from -80˚C and allowed to thaw for about 10-20 minutes or until there was a clear appearance or thawed completely. Following the grinding of the mosquito samples using pestles, 300 µL of RNA lysis buffer was added to each sample tube. Using a P1000 pipette 300 µL of 100% ethanol was added to each volume of cell homogenate in lysis buffer. The samples were vortexed thoroughly for about 4 seconds till the ethanol was in solution. At this point, the mixture was transferred to a Zymo-Spin™ IIICG Column 1 (green) in a tube. 400 µL of RNA Prep Buffer was added to the column and samples were centrifuged at room temperature at 13,000 x g for 30 seconds. After the flow-through was aspirated and 700 µL of wash buffer mix with ethanol was added to the spin cartridge. Samples were incubated at room temperature for 1 minute and were centrifuged under room temperature at 13,000 x g for 30 seconds and the flow-through was aspirated. At this point, 400 µL of wash buffer mixed with ethanol was added to the spin cartridge and incubated at room temperature for 1 minute. The samples were centrifuged at 13,000 x g for 2 minutes under room temperature conditions and the flow-through was aspirated. The collection tubes and flow-through were discarded. The spin cartridge was inserted into a labelled recovery tube. Using a P200 pipette, 100 µL RNase-free water was added to the spin cartridge and was incubated at room temperature for 1 minute. The spin cartridge was centrifuged for 30 seconds at 13,000 x g at room temperature to elute the RNA from the membrane into the recovery tubes. Samples were vortexed, columns were discarded and short-spun for five seconds to get the samples in the bottom of the tubes. The purified RNA was stored on ice was quantified using 2 µL of each sample. RNA quantity and quality were assessed using a NanoDrop 2000

spectrophotometer and a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. A260/A230 ratio in the samples was higher than 1.8, approximately 2.0 indicating a good quality for downstream applications. Equal amounts of high-quality RNA from ten mosquitoes per group (pyrethroid-resistant and unexposed to pyrethroid) were pooled for cDNA library preparation and subsequent RNA-seq analysis. Due to insufficient RNA quality in some individuals from Port Victoria, Teso, and Bungoma, I was unable to pool enough samples in certain groups. As a result, only eight pools were processed for RNA-seq analysis.

5.3.13 cDNA library preparation and RNA Sequencing

The RNA integrity number was measured using TapeStation 4200 with a High Sensitivity RNA Screen Tape (Agilent Technologies Inc., California, USA). Ribosomal RNA was depleted with Ribo-Zero Plus rRNA Removal Kit (Illumina Inc., California, USA). Samples were then heated, fragmented and randomly primed according to the manufacturer's recommendation. The first strand was synthesized with the Protoscript II Reverse Transcriptase with a longer extension period, approximately 30 minutes at 42ºC. All remaining steps for library preparation were performed according to NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (New England BioLabs Inc., Massachusetts, USA). Final libraries quantity was assessed by Qubit 3.0 and quality was assessed by TapeStation D1000 ScreenTape (Agilent Technologies Inc., California, USA). The final library size was 350bp with an insert size of 200bp. Subsequently, Illumina® 8-nt dual indices were used. Equimolar pooling of libraries was performed based on QC values and sequenced on an Illumina® NovaSeq platform (Illumina, California, USA) with a read length configuration of 150 PE for [120M PE] reads per sample (60M in each direction).

5.3.14 Bioassay data analysis

The mortality rate of the sample tested was expressed as the total number of dead *An. funestus* mosquitoes of all the replicates exposed to a particular insecticide and expressed this as the percentage of all the population exposed to that insecticide.

$$\text{observed mortality of } An.\ funestus \text{ exposed} = \frac{\text{A total number of dead } An.funestus \text{ mosquitoes}}{\text{Total exposed mosquitoes}} \times 100$$

Following the WHO criteria (WHO, 2016) for determining insecticide resistance in the malaria vector population, a population is classified as susceptible when the mortality is between 98-100%, resistant when mortality is less than 90% and suspected resistance when mortality is between 90% to 97%.

5.3.14.1 Quality control and differential gene expression analysis

Upon obtaining paired-end sequence reads from the sequencing centre (range 46,981,760 – 96,640,964 total reads), they were checked for quality using *FASTQC* (v0.11.5) (Andrews, 2017) and cleaned to remove adapters. Trimmomatic module (v.0.39) (Bolger *et al*., 2014), was used to remove the Illumina adapters (TruSeq3-PE-2) used for library preparation. A criterion to selection reads that were more than 50 bp and a Phred-Quality-Score greater than 20 for downstream analysis was implemented. The nf-core/RNAseq pipeline was used for the RNA-seq analysis (Patel *et al*., 2024). Briefly, reads were aligned to the *Anopheles funestus* FUMOZ reference genome that is available from VectorBase (AfunF3) using the STAR v 2.7.10a (Dobin & Gingeras, 2015, 2016). To reduce the size of the output SAM tools from the alignment output, they were piped to BAM files, sorted, and indexed using SAMtools v1.10 (Li *et al*., 2009). The sorted and indexed files were used as input for the Htseq-count reads, which were created using the module htseq-count (v.0.6.1) as described (Anders *et al*., 2015). A reference gene transfer

format was used to count the number of alignment mapping to each gene based on union and intersection-strict (Anders *et al*., 2015). Gene expression values were normalized using Relative Log Expression (RLE) from DESeq2. Expression abundance between different treatments (pyrethroids-resistant group vs pyrethroid susceptible FANG colony of *An. funestus* mosquitoes (Wondji *et al*., 2022), and unexposed/control vs the susceptible FANG colony) for the study sites (Teso, Port Victoria, Siaya and Kombewa) was determined using DESeq2 (v.1.18.0) (Anders & Huber, 2010). The susceptible FANG colony raw sequence data was retrieved from GenBank (accession: ERR981209- ERR981211) (Weedall *et al*., 2015b). A correlation of gene expression between biological replicates was calculated by Pearson's correlation as suggested before (Schulze *et al*., 2012), while the Benjamini-Hochberg method was applied in calibrating the p-value to decrease chances of false positives (Ferreira & Zwinderman, 2006). Therefore, differential expression between treatment groups was considered significant if the p-value <0.05 and the fold change (FC) >1.5 (Wu *et al*., 2014). To visualize the expression pattern of genes in the sample groups, principal component analysis (PCA) and heatmap were used. Hierarchical clustering analysis was applied to cluster genes exhibiting similar expression patterns/levels, while the Gene Ontology (GO) from the GO database (http://geneontology.org/) was utilized for functional analysis of the differentially expressed genes to establish their biological profiles (Dobin & Gingeras, 2016).

5.4 Results

5.4.1 Phenotypic resistance profile in western Kenya

Pyrethroid resistance was observed in all the sites with an average mortality rate (MR) of 57.6%. Port Victoria had the highest level of resistance to permethrin (MR=53%) and deltamethrin (MR=11%) pyrethroids. Teso had the lowest level of resistance to permethrin (MR=70%) and deltamethrin (MR=87%). Resistance to DDT was observed only in Kombewa (MR=89%) and Port Victoria (MR=85%). However, after samples were pre-exposed to the synergist, PBO, high susceptibility (> 98%) to the pyrethroids (deltamethrin and permethrin) was observed in all the sites except for Port Victoria where suspected resistance (96%) was observed for PBO + deltamethrin. In addition to the pyrethroid resistance, resistance to DDT was observed in Kombewa (89%) and Port Victoria (85%) (**Table 5.1**). Notwithstanding, a suspected resistance to DDT was observed in Siaya (93%) and Teso (92%). *An. funestus* was, however, fully susceptible to pirimiphos- methyl (0.25%) in all the sites.

Table 5.1: Mortality rate of *An. funestus* exposed to different insecticides and synergist (24-hr post-exposure).

Study site	Type and % of insecticide/chemical used	*An. funestus F*1 progeny		
		N	% Mortality (24hr)	status
Kombewa	Permethrin (0.75%)	280	54	Resistant
	Permethrin (0.75%) + PBO (4%)	300	99	
	Deltamethrin (0.05%)	180	59	Resistant
	Deltamethrin (0.05%) + PBO (4%)	100	100	
	DDT (4%)	100	89	Resistant
	Pirimiphos methyl (0.25%)	180	99	Susceptible
Siaya	Permethrin (0.75%)	100	78	Resistant
	Deltamethrin (0.05%)	133	52	Resistant
	0.75% permethrin + PBO (4%)	100	100	Susceptible
	Deltamethrin (0.05%) + PBO (4%)	100	100	Susceptible
	DDT (4%)	100	93	Suspected resistant
	Pirimiphos methyl (0.25%)	100	100	Susceptible
Teso	Deltamethrin (0.05%)	100	70	Resistant
	Permethrin (0.75%)	100	87	Resistant
	0.75% permethrin + 4% PBO	100	100	Susceptible
	Deltamethrin (0.05%) + PBO (4%)	100	100	Susceptible
	DDT (4%)	300	92	Suspected resistant
	Pirimiphos methyl (0.25%)	100	100	Susceptible
Port Victoria	Permethrin (0.75%)	217	53	Resistant
	Deltamethrin (0.05%)	100	11	Resistant
	Deltamethrin (0.05%) + PBO (4%)	100	96	Suspected resistance
	DDT (4%)	100	85	Resistant
Bungoma	Permethrin (0.75%)	100	69	Resistant
	Deltamethrin (0.05%)	100	43	Resistant

N: number exposed to the insecticide

5.4.2 Outcome of quality assessment of sequence reads.

The base sequence quality score is presented in **Figure 5.2.**

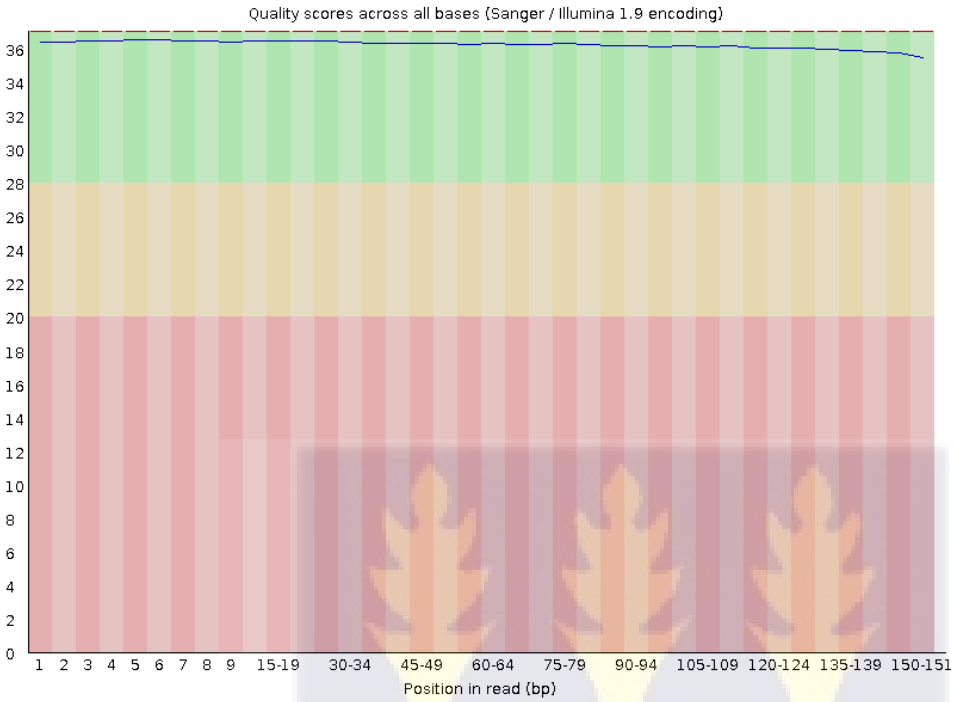


Figure 5.2: The mean quality value across each base position in the read. The base position in the read is shown on the x-axis, while quality scores are shown on the y-axis. The plot background is also colour-coded to identify good (green), acceptable (yellow), and bad (red) quality scores. For each position in the sample, the quality values do not fall below 32 indicating a high-quality score.

5.4.3 Differentially expressed genes between groups

After quality control and elimination of genes with low read counts, differential expression analysis was carried out on the transcripts. Three pairwise comparisons were performed: resistant versus susceptible, resistant versus unexposed (control) and unexposed versus susceptible. The resistant versus unexposed comparison aims to account for the induction of transcription during the pyrethroid exposure; genes were filtered by analyzing their expression profiles in the susceptible *An. funestus* population, under the assumption that constitutive resistance genes will show significant differential expression between survivors of the bioassay and the unexposed field F1 progenies when compared to the susceptible FANG colony. The volcano plots (**Figure 5.3**) showed the expression patterns of genes between resistant vs susceptible (**Figure 5.3A**), resistant vs unexposed (**Figure 5.3B**) and unexposed vs susceptible (**Figure 5.3C**). There was a clear distinction between overexpressed genes in resistant vs susceptible (**Figure 5.3A**) and unexposed vs susceptible (**Figure 5.3C**) groups. However, no difference in the gene expression between the resistant and unexposed groups was observed.

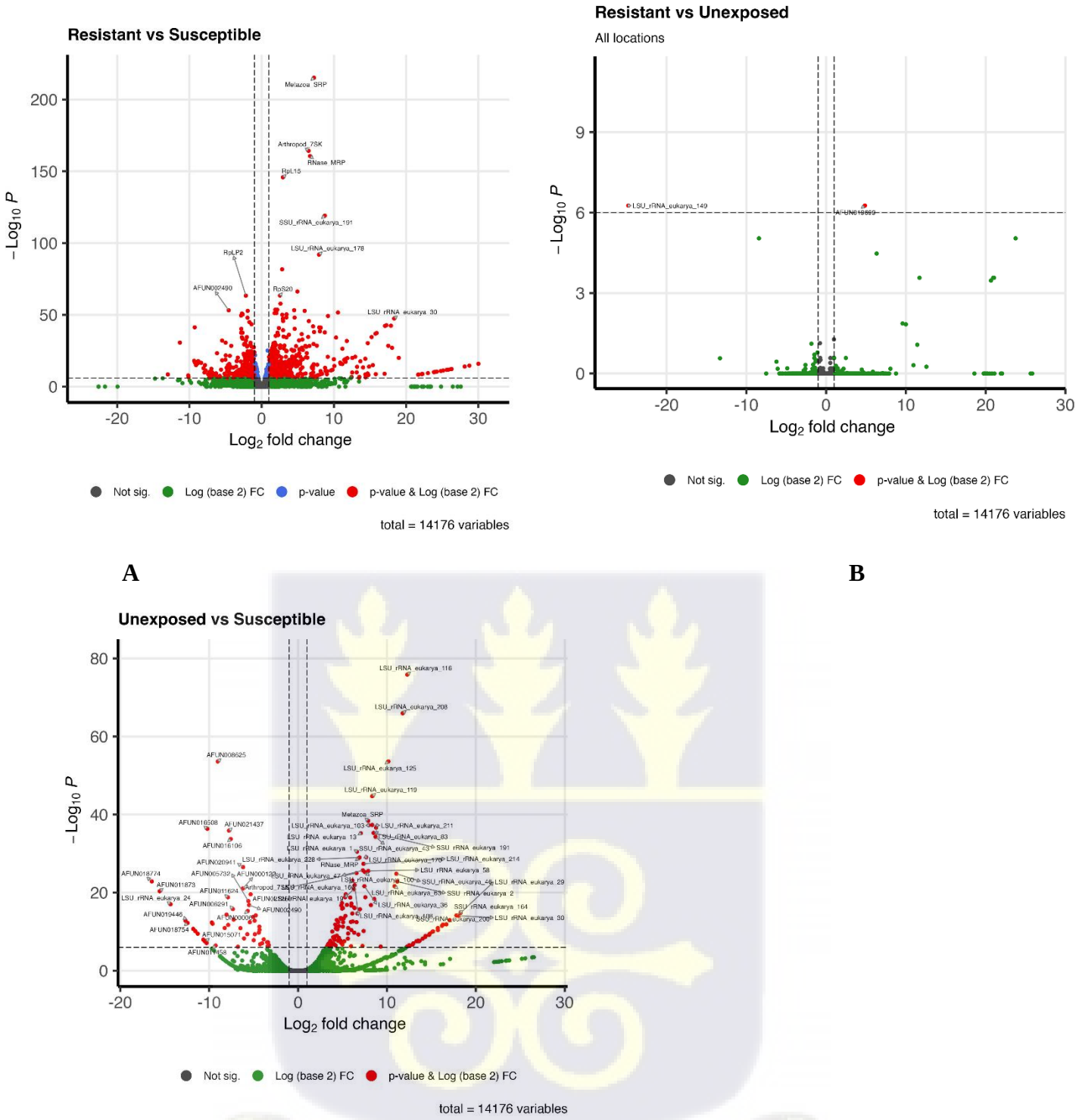


Figure 5.3. Volcano plots indicate upregulation and downregulation for resistant vs susceptible (A), resistant vs unexposed (B) and unexposed (control) vs susceptible (C). The X-axis indicates the log2 fold-change- positive and negative values are up and down-regulated respectively relative to the susceptible group in A and C. The Y-axis indicates -$\log_{10}$ of the

adjusted P-value (FDR) ($-\log_{10}$FDR values >200 for A, > 9 for B and > 80 for C). In each volcano plot, genes that are overexpressed in the population are >0 on the x-axis. P-values of < 0.05 are indicated by the horizontal line, while 2-fold expression differences are indicated by vertical dotted lines. The 14176 variables indicate the total number of genes tested

The PCA plots indicate a representation of differences in the sample groups (resistant, unexposed and susceptible). The samples in the resistant and unexposed groups clustered together to the left-hand side from the susceptible counterparts indicating similarity between them (**Figure 5.4**). The susceptible FANG group clusters towards the left side from the resistant and the unexposed groups (**Figure 5.4**).

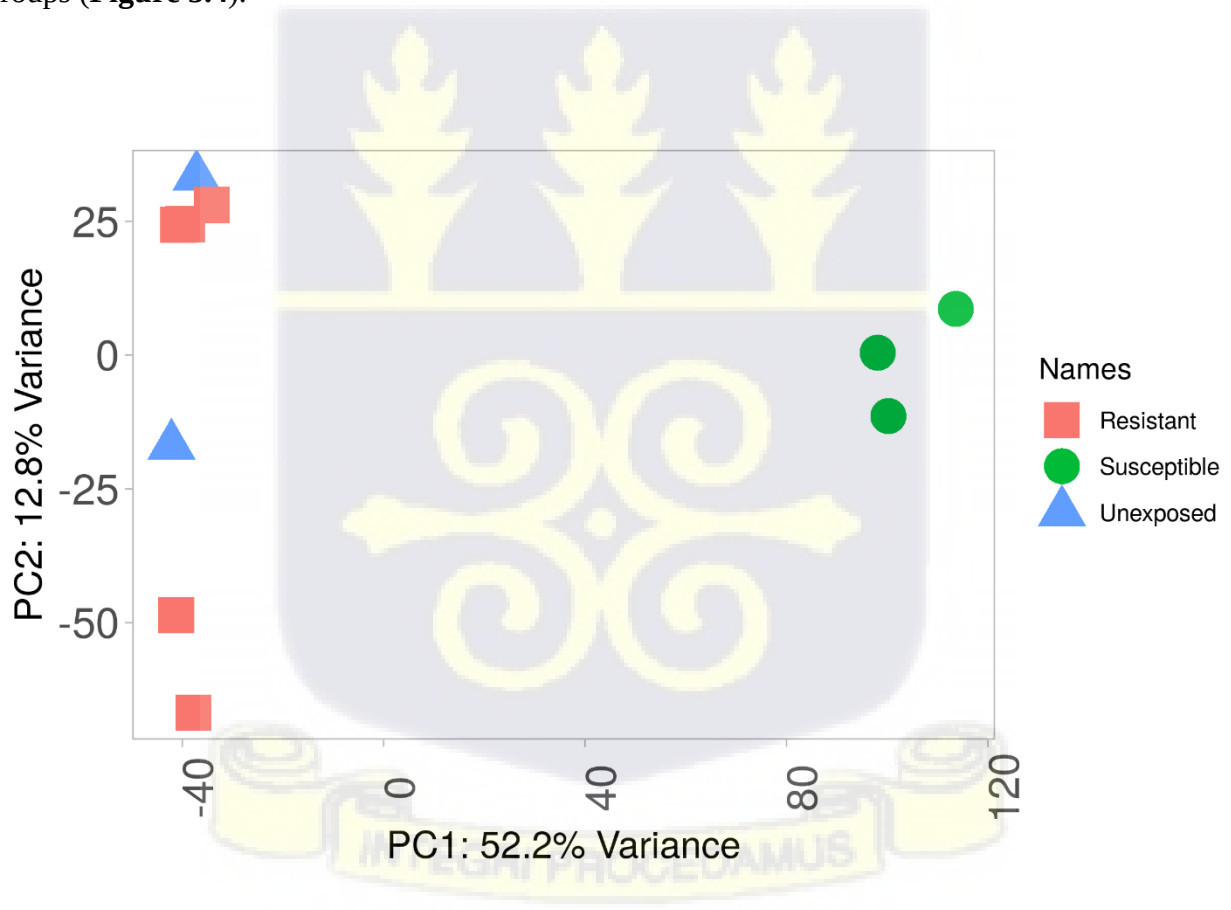


Figure 5.4: A principal component analysis showing the gene expression pattern of the sample groups relative to the susceptible group

The heatmap revealed an obvious grouping of samples into resistant, unexposed and susceptible. Dissimilarity in the gene expression levels was noticed between groups. The overall gene expression profile indicates a higher level of expression in the resistant and unexposed sample groups relative to the susceptible (**Figure 5.5**). Moreover, most of the genes were highly expressed in the Kombewa-resistant (Kr01, Kr02 and Kr03) samples. This was followed by the resistant samples from Port Victoria, Siaya and Teso. However, low levels of gene expression were observed in the susceptible samples (ERR981209, ERR981210 and ERR981211).

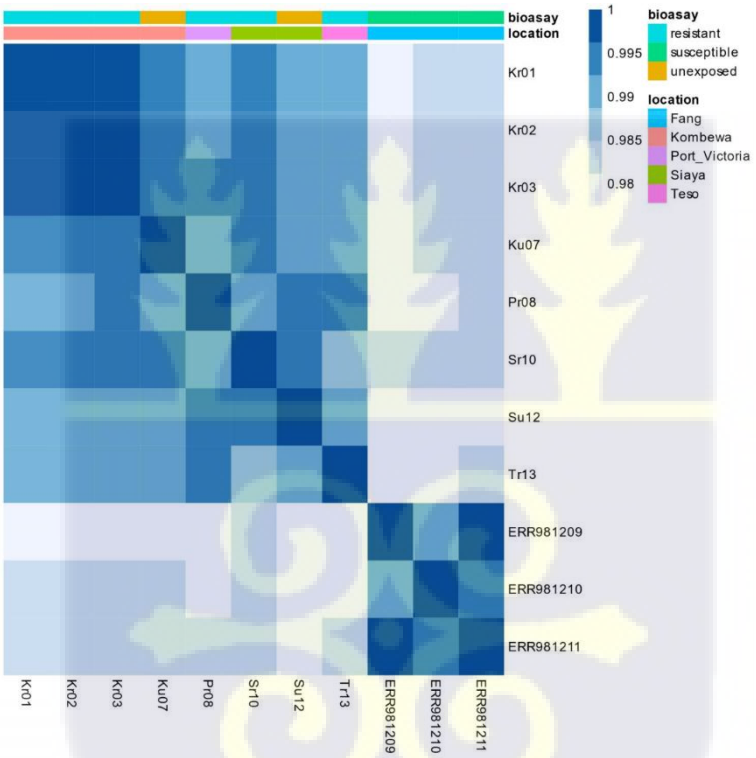
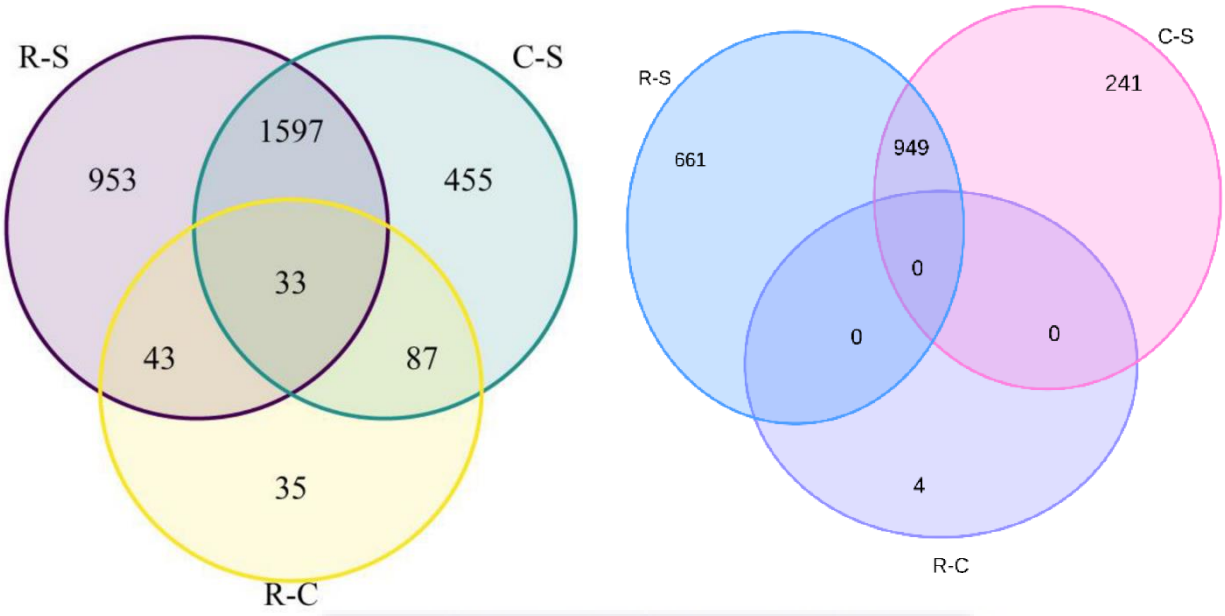


Figure 5.5: Heatmap indicating the expression of all genes in the sample groups relative to the susceptible group

Comparison using the Venn diagrams, 33 genes (n=14176) were differentially expressed in all the comparisons [resistant vs susceptible (R-S), resistant vs unexposed (R-C) and vs unexposed and susceptible (C-S)] (**Figure 5.6A**). However, 953, 35 and 455 common genes were differentially expressed in only R-S, R-C and C-S respectively. More genes (1597) were significantly differentially expressed between R-S and C-S comparisons compared to the other comparisons. This was followed by 87 differentially expressed genes observed between C-S and R-C comparisons and 43 differentially expressed genes between R-S and R-C comparisons (**Figure 5.6A**). Most of the downregulated genes were found in the R-S and this was followed by C-S (**Figure 5.6B**). Nine Hundred and forty-nine (949) genes were downregulated between R-S and C-S comparisons (**Figure 5.6B**).



A B

Figure 5.6: Venn diagram comparing upregulated and downregulated genes between-group comparisons. A indicates upregulated genes between the groups and **B** indicates downregulated genes between groups. R-S: field-resistant population that survived pyrethroid exposure vs susceptible colony, R-C: field-resistant population that survived pyrethroid exposure vs unexposed (control) field population and C-S: unexposed (control) field population vs susceptible colony.

5.4.4 Differentially expressed ncRNAs linked to pyrethroid resistance.

Differentially expressed ncRNAs between resistant vs susceptible (R-S) and unexposed vs susceptible (C-S) were determined by a fold change FC >1.5 and FDR < 0.05. The transcriptome analysis shows that ncRNAs accounted for a majority of gene families that are possibly linked to pyrethroid resistance (67%) (**Figure 5.7**). This was followed by IMPs (10%), CYPs (6%), CPs (5%), OPs (4%), GSTs (3%), UGTs (2%), ABCs (2%) and COEs (1%) (**Figure 5.7**). Besides, a

pairwise comparison of fold change between ncRNA and these gene families suggests that ncRNAs were significantly expressed (**Figure 5.8**).

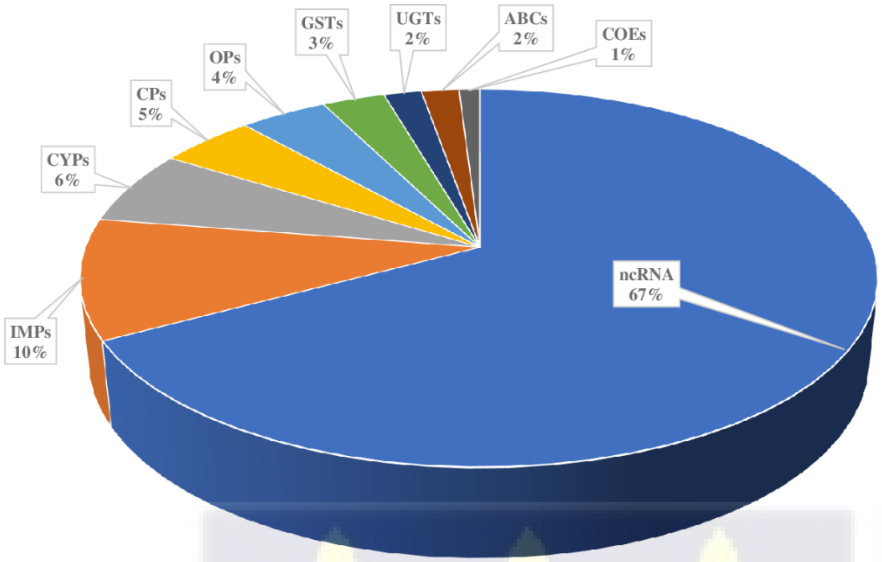


Figure 5.7: Pie chart showing the proportion of gene family associated with pyrethroid resistance

IMPs: Immunity proteins, **CYPs**: Cytochrome P450s, **CPs**: cuticular proteins, **OPs**: olfactory proteins, **GSTs**: Glutathione S-transferases, **UGTs**: UDP-glycosyltransferases, **ABCs**: ATP-binding cassettes, **COEs**: carboxylesterases and **ncRNA**: non-coding RNA

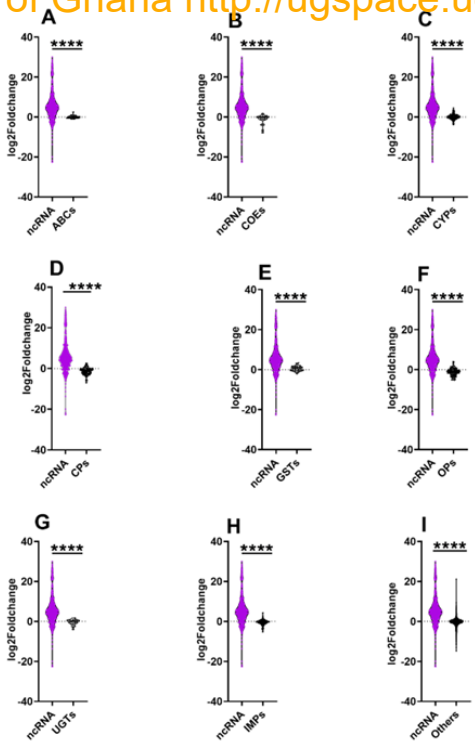


Figure 5.8: Comparative analysis of ncRNA against major gene associated with pyrethroid resistance in the resistance vs. susceptible clusters. Volcano plot representation of fold change between ncRNA and families of genes associated with pyrethroid resistance. A) ABCs: ATP-binding cassettes; B) **COEs**: carboxylesterases; C) **CYPs**: Cytochrome P450s; D) **CPs**: cuticular proteins; E) **GSTs**: Glutathione S-transferases; F) **OPs**: olfactory proteins, G) **UGTs**: UDP-glycosyltransferases; H) **IMPs**: Immunity proteins; G) Others. An unpaired t-test was used to compare means between two groups, p-value <0.05 was considered statistically significant.

The main ncRNAs that were overexpressed are in the resistant vs susceptible (R-S) and unexposed vs susceptible (C-S) are Metazoa_SR, RNaseP_nu, U3_1, Arthropod_7S, LSU_rRNA_eukarya_, SSU_rRNA_eukarya_2, LSU_rRNA_eukarya_13, SSU_rRNA_eukarya_46, LSU_rRNA_eukarya_2, LSU_rRNA_eukarya_3, SSU_rRNA_eukarya_15, LSU_rRNA_eukarya_5, LSU_rRNA_eukarya_6, SSU_rRNA_eukarya_164, SSU_rRNA_eukarya_19, SSU_rRNA_eukarya_200, LSU_rRNA_eukarya_155, LSU_rRNA_eukarya_17, LSU_rRNA_eukarya_17, LSU_rRNA_eukarya_214 and RNase_MRP (**Table 5.2)**.

Table 5.2: List of the top non-coding RNA (ncRNA) associated with pyrethroid resistance

						Resistant vs Susceptible			Unexposed vs Susceptible		
Gene ID	start	end	width	gene_ebi_biotype	Gene name	baseMean	log2FoldChange	-log10(pValue)	baseMean	log2FoldChange	-log10(pValue)
AFUN017050	90765776	90766073	298	SRP_RNA	Metazoa_SRP	101122.5	7.2	215.1	100752.7	7.9	38.4
AFUN017067	42155579	42159331	353	RNase_P_RNA	RNaseP_nuc	27310.0	4.9	66.3	2944.7	5.5	16.9
AFUN017081	74472303	74472505	203	snoRNA	U3_1	2548.6	4.6	48.1	1956.5	4.3	11.4
AFUN017253	60827945	60828260	316	ncRNA	Arthropod_7SK	8119.6	6.5	164.3	7349.6	6.6	25.0
AFUN017281	55964371	55965684	1314	rRNA	LSU_rRNA_eukarya_3	12280.4	5.9	52.8	12836.9	5.6	11.0
AFUN017331	50811594	50813597	2004	rRNA	SSU_rRNA_eukarya_20	1469.9	2.9	47.4	1330.2	3.2	5.8
AFUN017334	50799140	50800969	1830	rRNA	LSU_rRNA_eukarya_13	27686.0	7.1	42.0	33976.1	7.0	35.2
AFUN017368	50648232	50649006	775	rRNA	SSU_rRNA_eukarya_46	2694.7	10.6	51.6	3091.2	11.1	24.8
AFUN017395	50848871	50849574	704	rRNA	LSU_rRNA_eukarya_29	24076.8	17.9	42.4	28207.8	18.3	14.8
AFUN017396	50767394	50768099	706	rRNA	LSU_rRNA_eukarya_30	32757.7	18.3	47.5	23629.1	18.0	14.1
AFUN017547	48805965	48807956	1992	rRNA	SSU_rRNA_eukarya_153	161.6	4.6	40.3	181.8	6.0	6.3
AFUN017549	46029688	46031517	1830	rRNA	LSU_rRNA_eukarya_58	1910.3	6.5	45.6	1738.1	7.9	25.6
AFUN01755	499154	499173	1830	rRNA	LSU_rRNA_eukarya_6	1832.1	9.2	49.2	2672.1	10.8	21.7

4	86	15			3						
AFUN017571	46354378	46355612	1235	rRNA	SSU_rRNA_eukarya_164	15490.9	17.3	42.8	20445.0	17.8	14.2
AFUN017615	46328435	46329209	775	rRNA	SSU_rRNA_eukarya_191	10416.6	8.7	119.1	11009.1	8.5	35.3
AFUN017624	46349571	46350344	774	rRNA	SSU_rRNA_eukarya_200	13629.4	17.1	42.3	12032.4	17.0	13.0
AFUN017713	45918446	45919151	706	rRNA	LSU_rRNA_eukarya_155	790.5	4.5	53.3	632.8	3.9	7.0
AFUN017730	48236642	48237337	696	rRNA	LSU_rRNA_eukarya_170	2418.8	8.0	41.3	1872.3	7.7	29.0
AFUN017738	47110641	47111184	544	rRNA	LSU_rRNA_eukarya_178	748.8	7.9	91.9	1561.4	8.2	16.8
AFUN017782	47823339	47823797	459	rRNA	LSU_rRNA_eukarya_214	5160.7	8.2	41.0	5174.6	7.4	27.4
AFUN018392	18931658	18931991	334	RNase_MRP_RNA	RNase_MRP	72032.4	6.7	160.6	71007.8	6.8	28.8

5.4.5: Differentially expressed metabolic genes associated with pyrethroid resistance

To identify the main genes associated with high pyrethroid metabolic resistance, FDR < 0.05 and a fold change FC >1.5 were used. The main enzyme families identified include cytochrome P450s, GSTs, salivary gland proteins, Peptidase S1 domain-containing proteins, UGTs and sulfotransferases (**Table 5.3**). However, most of these genes were moderately differentially expressed.

These findings indicate that in western Kenya, different genes within these enzyme families could be responsible for resistance (**Table 5.3**). The top cytochrome P450 genes that were moderately overexpressed in the *An. funestus* in western Kenya include CYP6P9A, CYP6P9b, CYP6N1, CYP9J5, CYP49A1, AFUN020895, AFUN019365, CYP9K1, CYP304B. These genes were overexpressed in resistant vs susceptible and unexposed/control vs susceptible group comparisons. However, CYP304C1 and CYP315A1 were overexpressed only in the resistant vs susceptible comparison (**Table 5.3**). Among the GSTs, the overexpressed genes in resistant vs susceptible and unexposed/control vs susceptible groups are GSTD, GSTT, GSTE, GSTD, and GSTD3 were overexpressed only in the resistant vs susceptible comparison (**Table 5.3**). AFUN02142, AFUN021428 and AFUN019106 were the only cuticular proteins that were overexpressed in the resistant vs susceptible comparison. The differential expression analyses revealed that some of these UGTs were overexpressed in the resistant vs susceptible and unexposed/control vs susceptible groups comparisons (**Table 5.3**). These include UGT302A, UGT310B, UGT308D, UGT306A3 and AFUN003620. AFUN016205 and AFUN016207 were the sulfotransferases that were overexpressed in the *An. funestus* population from western Kenya (**Table 5.3**).

Table 5.3: List of the top genes of immunity, metabolic, cuticle and olfactory associated with pyrethroid resistance

Gene ID	Symbol	Chr.	FC (R vs S)	FC (C vs S)	Resistant (read count)	Unexposed (read count)	Susceptible (read count)	Group
AFUN008117	AFUN008117	2	2.4	2.1	120.9	145.2	178.5	Cytochrome
AFUN015889	CYP6P9b	2	6.2	4.7	946.4	1423.4	1519.4	Cytochrome
AFUN015792	CYP6P9A	2	3.5	2.5	568.3	893.9	925.1	Cytochrome
AFUN010918	CYP6N1	2	3.4	3.9	341.5	306.3	726.8	Cytochrome
AFUN001383	CYP9J5	3	2.1	2	215.7	469.7	293.4	Cytochrome
AFUN015735	CYP49A1	3	3.4	2.9	160.5	113.2	218.2	Cytochrome
AFUN005715	CYP315A1	X	2.3	NS	138.6	167.1	168.2	Cytochrome
AFUN015888	CYP6P5	2	6.3	9.5	129.3	220.2	288.1	Cytochrome
AFUN020895	AFUN020895	2	6.7	5.6	394.7	604.3	506.2	Cytochrome
AFUN019365	AFUN019365	2	10.4	10.7	267.8	333.2	412.2	Cytochrome
AFUN007549	CYP9K1	X	10.5	10.5	3310.9	5502.8	4501.7	Cytochrome
AFUN015938	CYP9M1	2	2.1	NS	384.6	452.2	414.9	Cytochrome
AFUN015956	CYP304B1	2	4.5	7	261.2	109.1	373.3	Cytochrome
AFUN015957	CYP304C1	2	2.5	NS	230.7	341.1	241.2	Cytochrome
AFUN016010	GSTD1	2	3.4	3.3	4552.7	4424.7	5215.8	Glutathione s-transferase
AFUN007291	GSTT2	X	2.5	2.2	143.4	116.3	164.3	Glutathione s-transferase
AFUN011410	GSTD7	2	2.1	NS	143	128.1	214.9	Glutathione s-transferase
AFUN015767	GSTD11	2	2.7	NS	13.3	20.1	20.3	Glutathione s-transferase
AFUN0158	GSTD3	2	2.8	NS	241.9	515.5	281.2	Glutathione s-

39								transferase
AFUN016008	GSTE6	2	9.7	6.6	99.1	122.4	106.9	Glutathione s-transferase
AFUN004194	Or42	2	2.5	NS	6	15	13.7	Odorant receptor
AFUN018482	AFUN018482	3	7.5	9.9	9.3	0	4.3	Peptidase S1 domain-containing protein
AFUN018981	AFUN018981	3	3.1	5.3	9	26.1	22	Peptidase S1 domain-containing protein
AFUN018580	AFUN018580	3	11.5	11	210.4	127.4	212.8	Peptidase S1 domain-containing protein
AFUN019220	AFUN019220	2	5.2	5.7	390.5	458.9	577.3	ABC transporter
AFUN015896	AFUN015896	2	2.1	NS	142.7	112	151.9	CLIP-domain serine protease
AFUN021427	AFUN021427	2	2.3	NS	11.6	11	8.3	Cuticular protein
AFUN021428	AFUN021428	2	2.7	NS	15.6	7	8.3	Cuticular protein
AFUN019106	AFUN019106	3	3.1	NS	8	8.7	20.4	Cuticular protein
AFUN019845	UGT302A3	3	3.2	2	421.3	681.5	634.9	UDP-glycosyltransferases
AFUN011266	UGT310B2	2	NS	3.1	16.7	16	38.6	UDP-glycosyltransferases
AFUN020198	UGT308D2	3	2	NS	22.3	46	49.1	UDP-glycosyltransferases
AFUN016302	UGT306A3	3	NS	2	279.6	318.2	391.4	UDP-glycosyltransferases
AFUN003620		2	2	2.1	320.2	325.8	437.8	UDP-glycosyltransferases
AFUN016205		3	NS	3.8	250.6	123.4	403.2	sulfotransferase
AFUN0162		3	2.1	2.4	170.8	193.5	234.9	sulfotransferase

07

R: resistant field mosquito population that survived the pyrethroid exposure, S: susceptible FANG colony, C: unexposed/control field mosquito population, FC: fold change, NS: not significant.

5.4.6: Gene ontology analysis of the differentially expressed genes

GO term annotation pathways analysis was employed to elucidate biological functions and signalling pathways that may be regulated by the differentially expressed genes in *An. funestus*. The findings revealed that these genes were engaged in a wide variety of biological functions and signalling pathways. Detailed GO enrichment for the differentially expressed genes in ontologies of cellular components, biological processes, and molecular function is represented in **Figure 5.9**. Looking at GO annotation, it is evident that differentially expressed genes in pyrethroid-resistant *An. funestus* were enriched mostly in cellular macromolecule metabolic processes, cytoplasm, cellular protein metabolic processes and gene expression (**Figure 5.9**).

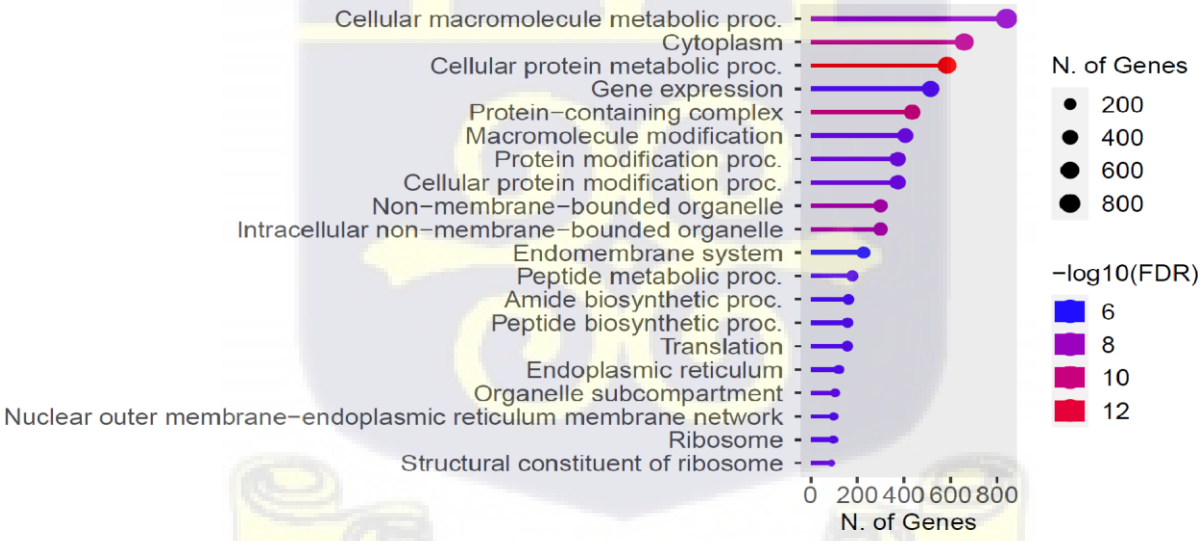


Figure 5.9: Gene Ontology (GO) enrichment analysis of the differentially expressed genes.

The x-axis indicates the gene count/number of genes while the y-axis indicates the enriched terms. Colour is used to distinguish different levels.

5.5 Discussion

The successful implementation and development of insecticide resistance management measures depends on elucidating the mechanisms underlying resistance in malaria vectors. In this study, the phenotypic resistance profile of *An. funestus* and the molecular basis of pyrethroid resistance in western Kenya have been characterized. This is one of the most comprehensive studies on the *An. funestus* susceptibility status to pyrethroids and DDT in western Kenya.

This study revealed a high level of pyrethroid resistance across western Kenya although resistance levels vary from site to site. In addition, resistance to DDT has been detected in Kombewa and Port Victoria. This confirmed a previous study in east Africa including western Kenya which reported widespread pyrethroid resistance in the *An. funestus* population (Mulamba *et al*., 2014). The rise of multiple resistance of *An. funestus* was also confirmed in a previous study in western Kenya (Mulamba *et al*., 2014), Benin, west Africa (Djouaka *et al*., 2016), and Malawi, southern Africa (Riveron *et al*., 2015). *An. funestus* was, however, fully susceptible to pirimiphos methyl, the organophosphate in all the study sites. This is congruent with a previous study in Tanzania where a full susceptibility of this vector to pirimiphos methyl was reported (Kaindoa *et al*., 2017). This is an indication that this insecticide can still be maintained for IRS programs in western Kenya. The preexposure of samples to the synergist, PBO has shown that *An. funestus* was fully susceptible to the pyrethroids in all the study sites except Port Victoria where 96% mortality was observed for the PBO + deltamethrin. This implies that the metabolic resistance mechanism (cytochrome P450 monoxygenases) was fully involved in insecticide resistance in the *An. funestus* in these sites but partially involved in Port Victoria (WHO, 2016). Other mechanism(s) might be contributing to pyrethroid resistance in Port Victoria leading to that site having the highest level of resistance compared to the other sites.

An. funestus has no *kdr* markers for resistance (Menze *et al*., 2016), hence metabolic resistance mechanism through overexpression of detoxification genes plays a crucial role in insecticide resistance (Hemingway, 2000; Hemingway & Ranson, 2000). This study has identified the top twenty ncRNAs that were differentially expressed in resistant and unexposed field populations of *An. funestus* from western Kenya. Although their mechanisms of pyrethroid resistance in *An. funestus* is unknown, they could be playing a role in regulating the expression of pyrethroid-resistant metabolic genes in *the An. funestus* resistant populations. These findings add up to a body of evidence which hypothesised that ncRNAs play roles in insecticide resistance development in insects (Etebari *et al*., 2015; Xiao *et al*., 2024). In general, the biological roles of ncRNAs in detoxification and insecticide resistance pathways are poorly understood. However, few studies have reported that some ncRNAs (notably microRNAs) interfered with the expression of insecticide-detoxifying enzymes. For instance, MiR-2b-3p has been proposed to potentially suppress the cytochrome P450 9f2 (CYP9F2) gene's transcriptional activity, which would impede the larvae of *P. xylostella* from progressing through developmental detoxification pathways (Etebari *et al*., 2018). Furthermore, it has been observed that an overabundance of miR-13664 reduced the cytochrome P450 314A1 (CpCYP314A1) gene's mRNA expression levels, increasing *Culex pipiens pallens* (Diptera: Culicidae) susceptibility to deltamethrin (Sun *et al*., 2019). Given that a few proportions of the metabolic gene families (IMP, cytochrome P450, CP, OP, GST, UGTs, ABCs and COE.) were identified in this study to be involved in pyrethroid resistance and were mostly moderately overexpressed, this large proportion of highly overexpressed ncRNAs may play a crucial role in regulating their expression.

Studies have established those genes belonging to cytochrome P450, esterase, GSTs, UGTs, cuticular proteins and ABC transporter families are implicated in insecticide resistance (Al-

Yazeedi *et al*., 2023; Messenger *et al*., 2021; Riveron *et al*., 2014b, 2018; Sandeu *et al*., 2020); as a result, ncRNAs could be considered for designing RNAi-based control systems. This will, however, require a deeper understanding of the molecular mechanisms underlying RNAi-based control systems in mosquitoes since there already existing gaps in understanding this technology in controlling other insects (Vaschetto & Beccacece, 2019). Furthermore, high-throughput sequencing techniques have recently yielded important new information about the functions of ncRNAs in insect development and the evolution of insecticide resistance (Vaschetto & Beccacece, 2019). Non-coding rRNAs constitute over 80% of the total cellular RNA in mosquitoes (Gale & Crampton, 1989). In this study, the majority of rRNA was removed using the RiboZero Plus kit. While literature indicates that rRNA comprises anywhere from 1 to 20% of the final rRNA-depleted sequencing libraries (Potemkin *et al*., 2022), it was assumed consistent rRNA levels across the samples due to standardized RNA-seq protocols. The results demonstrate that RNA-seq method can effectively detect and quantify both coding and non-coding RNA transcripts.

Two different ncRNA-based insect management approaches have been proposed following these findings: (a) using biodegradable ncRNA-insecticide solutions to control insects, (Kolliopoulou & Swevers, 2014; Powell *et al*., 2017), and (b) using metabolic engineering techniques to find and take advantage of target species' ncRNA-associated signalling pathways (Buchman *et al*., 2018). However, the molecular mechanisms behind the functioning of ncRNAs in detoxification and insecticide resistance signalling pathways are still not clear, despite the mounting body of evidence suggesting these molecules are significant regulators of insect development (Bavithra *et al*., 2023; Etebari *et al*., 2015; Vaschetto & Beccacece, 2019). This is because research in this area is still in its early stages. The straightforward CRISPR-Cas9 genome editing technique has

the potential to generate novel understandings of the roles of regulatory ncRNA sequences as well as ncRNA-based techniques targeted at managing insects including disease vectors. Moreover, using inhibitors to target specific ncRNAs might interfere with the expression levels and reduce or reverse insecticide resistance. Thus, ncRNAs could be potential targets for vector control in the future. Recently, Oberemok *et al*. (Oberemok *et al*., 2019), proposed an innovative strategy to tackle insecticide resistance and create safer compounds. Their method employs synthetic DNA oligomers to disrupt gene expression by targeting ribosomal RNA (rRNA) rather than messenger RNA (mRNA). Because rRNA makes up 80% of cellular RNA and is more plentiful and stable than mRNA, it represents a promising target for DNA antisense oligonucleotide (ASO) interventions. This strategy seeks to offer a more efficient and enduring approach to combating insecticide resistance.

An. funestus is a notorious vector of human malaria in Africa and has contributed to over 90% of all malaria transmission in some parts of eastern and southern African regions (Kaindoa *et al*., 2017; Mapua *et al*., 2022). It is noteworthy that the outcome of this study represents an advancement in the molecular basis of insecticide resistance in *An. funestus* population. Understanding the functional importance of ncRNAs in insecticide resistance pathways could enable the creation of ncRNA-based vector control techniques to control *An. funestus*. The limitation of this study is that the transcriptomic profiles for each study location were not assessed even though there were different levels of phenotypic resistance across the locations. After RNA isolation from a single mosquito, a poor-quality RNA was observed hence I resort to pooling to get enough quantity and a good quality RNA for sequencing. This has affected the number of replicates for each of the study locations. The bottom line is that all the samples are

resistant to pyrethroids and are all from western Kenya, hence I focused on the biological replicates (resistant and unexposed) rather than analysis for each of the locations.

5.6 Conclusion

An. funestus population is highly resistant to pyrethroids in western Kenya with Port Victoria recording the highest levels of resistance to the type I and type II pyrethroids. However, preexposure to PBO synergists recorded high susceptibility to the pyrethroids except in Port Victoria). The top cytochrome P450 genes that were overexpressed in the *An. funestus* in western Kenya include CYP6P9A, CYP6P9b, CYP6N1, CYP9J5, CYP49A1, AFUN020895, AFUN019365, CYP9K1, CYP304B. GSTs (GSTD1, GSTT2, GSTD7, GSTD11, GSTD3, GSTE6), peptidase S1 domain-containing protein (AFUN018482, AFUN018981, AFUN018580), cuticular protein (AFUN021427, AFUN021428, AFUN019106), UGTs (UGT310B2, UGT308D2, UGT306A3, AFUN003620) and sulfotransferase (AFUN016205, AFUN016207) were also overexpressed in *An. funestus* populations in western Kenya. We have shown for the first time that insecticide resistance in *An. funestus* is linked to the expression of ncRNAs hence a better understanding of these molecular events could help to develop resistance management strategies for future malaria control.

CHAPTER SIX

6.0 GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATION

6.1 General Discussion

This study unveiled the biology of *An. funestus* in the context of its larval ecology, population structure/genetic diversity and molecular basis of insecticide resistance in western Kenya.

These findings indicate that *An. funestus* tolerate a wide variety of aquatic habitats, co-exists with the larva of other vectors and typically breeds in permanent and semi-permanent aquatic habitats, with or without aquatic vegetation, slow-moving/disturbed or stagnant water that was clear, opaque, cloudy and brownish. It was also discovered that manmade ponds or dug out for economic activities (moulding of clay pots and sand winning) contributed immensely to the abundance of *An. funestus* larvae and the distance to the nearest house was significant factor predicting the abundance *of An. funestus* in the breeding sites. The moulding of clay pots for sale forms part of the economic activities generating income for the people in Bungoma. Digging the ground for clayey soil for this income-generating business has led to a lot of dugouts/ man-made ponds which serve as breeding sites for this important vector of human malaria. Such environmental modifications by humans including the construction of dams for irrigation purposes create additional breeding environments to enhance the breeding of mosquitoes hence spreading the diseases they transmit (Hawaria *et al*., 2020). This suggests that employing LSM strategies could help to control man-made malaria in these sites.

Vector control involving the use of any LSM strategies should be aimed at engaging all stakeholders to achieve its intended objectives. The communities' participation in LSM is key in

broadening the scope of interventions, lower operating expenses, and fostering sustainability by gaining support from the local population (Gowelo *et al*., 2020). The contribution of manmade ponds to the abundance of mosquito habitats/larvae and their proximity to human habitation observed in this study should be a wakeup to stakeholders involved in malaria elimination in Kenya. The recent outbreak of malaria in the neighbouring country, Uganda was fueled by the increased number of aquatic habitats near human habitations and the absence of indoor residual spraying (Nabatanzi *et al.*, 2022). In the 21st century, it became clear that the progress made in malaria control towards elimination and eradication has stalled (Noor & Alonso, 2022). Undoubtedly, the use of a combination of interventions is the best way to drive towards malaria elimination.

The findings indicate that *An. funestus* is highly resistant to pyrethroids but susceptible to pirimiphos- methyl used for IRS and PBO + pyrethroids. The findings also unveil that *An. funestus* population is under selective pressure in western Kenya leading to demographic expansion and the spread of variants through inbreeding. Insecticides for agricultural purposes, particularly those affecting larval breeding sites, could be evolutionary forces influencing the *An. funestus* population in western Kenya given the evidence of high resistance to pyrethroids, purifying selection and population expansion (Pasteur & Raymond, 1996; Reid & McKenzie, 2016). Field observations during larval surveys showed that farmers rely heavily on agricultural insecticides for tobacco, maize and vegetable farming. Population expansion and a high level of insecticide resistance might be the primary reason for its high adaptation to the various ecological niches in western Kenya and sustaining malaria transmission. Metabolic-based resistance mechanisms particularly cytochrome P450 monooxygenases were also detected in the *An. funestus* population. Thus, the use of LSM in combination with IRS or deployment of PBO-

based bed nets would be a good combination of intervention to effectively control this notorious vector of human malaria. With the escalating level of insecticide resistance, no single tool is deemed fit for malaria control across all settings, a right combination of vector control tools and novel tools should be developed, adopted and apply in all endemic areas (Implications of Insecticide Resistance Consortium, 2018). This should be given urgent consideration if the WHO goals of the Global Technical Strategy for malaria 2016-2030 (GTS) are to be attained (WHO, 2015). This study has shown for the first time the role of ncRNAs in pyrethroid resistance in the *An. funestus* population in western Kenya hence a better understanding of these molecular events could help to develop resistance management strategies for future malaria control.

6.2 General Conclusions

The main objective of this study was to investigate the larval ecology, population structure/genetic diversity and molecular basis of insecticide resistance of *An. funestus* in western Kenya. The conclusions from this study are presented objective by objective as indicated in chapter three, four and five.

Objective one (Chapter three): The findings revealed that *An. funestus* breeds in a variety of breeding habitats and coexist with the larva of other mosquitoes. A typical breeding habitat of *An. funestus* is permanent and semi-permanent aquatic habitats, with or without aquatic vegetation, slow-moving/disturbed or stagnant water that was clear, opaque, cloudy and brownish. The distance to the nearest house is a significant factor that predicts the abundance of *An. funestus* in the aquatic habitats. Man-made ponds made because of sand winning and moulding of clay pots had the largest percentage of *An. funestus* larvae. Furthermore, *An. funestus* had the highest HBI compared to other malaria vectors.

Objective two (Chapter four): No genetic differentiation or structure was observed but a high level of gene flow was observed among the populations across the sites in western Kenya. Population expansion suggests the high adaptability of this species to various ecological requirements hence sustaining its vectorial capacity and malaria transmission.

Objective three (Chapter five): *An. funestus* exhibited a high level of pyrethroids resistance in all the sites, resistance to DDT in Kombewa and Port Victoria but fully susceptible to pirimiphos- methyl in all the sites. The synergist bioassay revealed high susceptibility to the pyrethroids in all the sites except for Port Victoria where suspected resistance for PBO + deltamethrin was observed. The main enzyme families associated with pyrethroid resistance are ATP-binding cassettes, carboxylesterases, Cytochrome P450s, cuticular proteins, Glutathione S-transferases, olfactory proteins, UDP-glycosyltransferases, Immunity proteins. This study has shown for the first time that insecticide resistance in *An. funestus* is linked to the expression of ncRNAs. The top ten cytochrome P450 monooxygenases highly overexpressed associated pyrethroid resistance across the sites are CYP6P9A, CYP6P9b, CYP6N1, CYP9J5, CYP49A1, AFUN020895, AFUN019365, CYP9K1 and CYP304B. The top GSTs that were highly overexpressed associated with pyrethroid resistance are (GSTD1, GSTT2, GSTD7, GSTD11, GSTD3 and GSTE6.

6.3 Recommendations

1. Future studies should examine the physicochemical characteristics of aquatic habitats that favour the coexistence of *An. funestus* larvae with mosquito larvae in various habitats.

2. This study identified findable aquatic habitats that are permanent and semi-permanent for the breeding of *An. funestus* and other mosquitoes. Hence implementation of larval source management strategies in Bungoma and Kombewa and their surrounding communities will help curtail the spread of malaria in those regions leading to elimination.

3. The use of the *COII* gene for population structure studies revealed that high population expansion influences its adaptability and no genetic differentiation. Future work should examine the whole mitogenome to help fully understand variation in the lineages and genetic structure.

4. High level of insecticide resistance to pyrethroids was observed in *An. funestus* population in western Kenya. Future studies should assess the intensity of insecticide resistance in this vector population, especially in Port Victoria and Kombewa where multiple resistance was observed.

5. The use of next-generation bed nets such as PBO-based bed nets in combination with LSM strategies (habitats modifications, larviciding) could be effective vector control tools to help control *An. funestus* mosquitoes in western Kenya.

6. This study for the first time highlights the potential role of ncRNAs in pyrethroid resistance in *An. funestus*. Targeting non-coding RNAs for intervention development could help in insecticide resistance management.

REFERENCES

Abanyam, N. L. (2020). Malaria and Socio-Economic Development in Nigeria. *Lead City Journal of the Social Sciences*, *5*(1), 81.

Abdel-Meguid, A. D. (2022). Effect of physicochemical factors of breeding sites on larval density and detoxification enzymes activities of *Culex pipiens* (l.) (Diptera: Culicidae) in qalyubia governorate, Egypt. International Journal of Tropical Insect Science, 42(1), 235–244. https://doi.org/10.1007/s42690-021-00537-0

Abiodun, G. J., Adebiyi, B. O., Abiodun, R. O., Oladimeji, O., Oladimeji, K. E., Adeola, A. M., Makinde, O. S., Okosun, K. O., Djidjou-Demasse, R., & Semegni, Y. J. (2020). Investigating the resurgence of malaria prevalence in South Africa between 2015 and 2018: A scoping review. *The Open Public Health Journal*, *13*(1), 119–125.

Agossa, F. R., Gnanguenon, V., Anagonou, R., Azondekon, R., Aizoun, N., Sovi, A., Oke-Agbo, F., Sezonlin, M., & Akogbeto, M. C. (2015). Impact of insecticide resistance on the effectiveness of pyrethroid-based malaria vectors control tools in Benin: Decreased toxicity and repellent effect. *PloS One*, *10*(12), e0145207.

Aguirre-Obando, O. A., Bona, A. C. D., Duque L, J. E., & Navarro-Silva, M. A. (2015). Insecticide resistance and genetic variability in natural populations of *Aedes* (*Stegomyia*) *aegypti* (Diptera: Culicidae) from Colombia. *Zoologia (Curitiba)*, *32*, 14–22.

Aklilu, E., Kindu, M., Gebresilassie, A., Yared, S., Tekie, H., & Balkew, M. (2020). Environmental Factors Associated with Larval Habitats of Anopheline Mosquitoes (Diptera: Culicidae) in Metema District, Northwestern Ethiopia. *Journal of Arthropod-Borne Diseases*, *14*(2), 153.

Akoton, R., Tchigossou, G. M., Djègbè, I., Yessoufou, A., Atoyebi, M. S., Tossou, E., Zeukeng, F., Boko, P., Irving, H., Adéoti, R., Riveron, J., Wondji, C. S., Moutairou, K., & Djouaka, R. (2018). Experimental huts trial of the efficacy of pyrethroids/piperonyl butoxide (PBO) net treatments for controlling multi-resistant populations of *Anopheles funestus s.s.* In Kpomè, Southern Benin. *Wellcome Open Research*, *3*, 71. https://doi.org/10.12688/wellcomeopenres.14589.1

Alegana, V. A., Macharia, P. M., Muchiri, S., Mumo, E., Oyugi, E., Kamau, A., Chacky, F., Thawer, S., Molteni, F., & Rutazanna, D. (2021). *Plasmodium falciparum* parasite prevalence in east Africa: Updating data for malaria stratification. *PLOS Global Public Health*, *1*(12), e0000014.

Al-Yazeedi, T., Muhammad, A., Irving, H., Ahn, S.-J., Hearn, J., & S. Wondji, C. (2023). Overexpression and nonsynonymous mutations of UDP-glycosyltransferases potentially associated with pyrethroid resistance in *Anopheles funestus*. *bioRxiv*, 2023–08.

Al-Yazeedi, T., Muhammad, A., Irving, H., Ahn, S.-J., Hearn, J., & Wondji, C. S. (2024). Overexpression and nonsynonymous mutations of UDP-glycosyltransferases are potentially associated with pyrethroid resistance in *Anopheles funestus*. *Genomics*, *116*(2), 110798.

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Nature Precedings*, 1–1.

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics*, *31*(2), 166–169.

Andrews, S. (2017). *FastQC: A quality control tool for high throughput sequence data. 2010*. Cambridge Academic Press, pp1-3.

Anoopkumar, A. N., Puthur, S., Rebello, S., & Aneesh, E. M. (2019). Molecular characterization of *Aedes*, *Culex*, *Anopheles*, and *Armigeres* vector mosquitoes inferred by mitochondrial cytochrome oxidase I gene sequence analysis. *Biologia*, *74*(9), 1125–1138.

Awolola, T. S., Oduola, O. A., Strode, C., Koekemoer, L. L., Brooke, B., & Ranson, H. (2009). Evidence of multiple pyrethroid resistance mechanisms in the malaria vector *Anopheles gambiae sensu stricto* from Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *103*(11), 1139–1145.

Awolola, T. S., Oyewole, I. O., Koekemoer, L. L., & Coetzee, M. (2005). Identification of three members of the *Anopheles funestus* (Diptera: Culicidae) group and their role in malaria transmission in two ecological zones in Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *99*(7), 525–531.

Balabanidou, V., Kampouraki, A., MacLean, M., Blomquist, G. J., Tittiger, C., Juárez, M. P., Mijailovsky, S. J., Chalepakis, G., Anthousi, A., & Lynd, A. (2016). Cytochrome P450 associated with insecticide resistance catalyzes cuticular hydrocarbon production in Anopheles gambiae. *Proceedings of the National Academy of Sciences*, *113*(33), 9268–9273.

Balling, S. S., & Resh, V. H. (1984). Seasonal patterns of pondweed standing crop and *Anopheles occidentalis* densities in Coyote Hills Marsh. *Proceedings and Papers of the Annual Conference of the California Mosquito and Vector Control Association (USA)*.

Bass, C., & Jones, C. M. (2016). Mosquitoes boost body armor to resist insecticide attack. *Proceedings of the National Academy of Sciences*, *113*(33), 9145–9147.

Bavithra, C. M. L., Murugan, M., Pavithran, S., & Naveena, K. (2023). Enthralling genetic regulatory mechanisms meddling insecticide resistance development in insects: Role of

transcriptional and post-transcriptional events. *Frontiers in Molecular Biosciences*, *10*. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10511911/

Beier, J. C., & Koros, J. K. (1991). Visual assessment of sporozoite and bloodmeal ELISA samples in malaria field studies. *Journal of Medical Entomology*, *28*(6), 805–808.

Beier, J. C., Perkins, P. V., Wirtz, R. A., Koros, J., Diggs, D., Gargan, T. P., & Koech, D. K. (1988). Bloodmeal identification by direct enzyme-linked immunosorbent assay (ELISA), tested on Anopheles (Diptera: Culicidae) in Kenya. *Journal of Medical Entomology*, *25*(1), 9–16.

Bhatt, S., Weiss, D. J., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., Battle, K. E., Moyes, C. L., Henry, A., & Eckhoff, P. A. (2015). The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature*, *526*(7572), 207.

Blaustein, L., & Margalit, J. (1994). Mosquito larvae (Culiseta longiareolata) prey upon and compete with toad tadpoles (*Bufo viridis*). *Journal of Animal Ecology*, 841–850.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120.

Bonizzoni, M., Afrane, Y., Dunn, W. A., Atieli, F. K., Zhou, G., Zhong, D., Li, J., Githeko, A., & Yan, G. (2012). Comparative transcriptome analyses of deltamethrin-resistant and-susceptible *Anopheles gambiae* mosquitoes from Kenya by RNA-Seq. *PloS One*, *7*(9), e44607.

Braginets, O. P., Minakawa, N., Mbogo, C. M., & Yan, G. (2003). Population genetic structure of the African malaria mosquito *Anopheles funestus* in Kenya. *The American Journal of Tropical Medicine and Hygiene*, *69*(3), 303–308.

Brogdon, W. G., McAllister, J. C., Corwin, A. M., & Cordon-Rosales, C. (1999). Independent selection of multiple mechanisms for pyrethroid resistance in Guatemalan *Anopheles albimanus* (Diptera: Culicidae). *Journal of Economic Entomology*, *92*(2), 298–302.

Brooke, B. D., Kloke, G., Hunt, R. H., Koekemoer, L. L., Tem, E. A., Taylor, M. E., Small, G., Hemingway, J., & Coetzee, M. (2001). Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae). *Bulletin of Entomological Research*, *91*(4), 265–272.

Brown, A. W. (1986). Insecticide resistance in mosquitoes: A pragmatic review. *Journal of the American Mosquito Control Association*, *2*(2), 123–140.

Bruce-Chwatt, L. J., Archibald, H. M., Elliott, R., Fitz-John, R. A., & Balogun, I. A. (1955). An experimental Malaria Control Scheme in Ilaro, semi-rural holoendemic area of Southern Nigeria. Results. 1949-1953. *Inf. Bull. Gaskiya*.

Buchman, A., Marshall, J. M., Ostrovski, D., Yang, T., & Akbari, O. S. (2018). Synthetically engineered *Medea* gene drive system in the worldwide crop pest *Drosophila suzukii*. *Proceedings of the National Academy of Sciences*, *115*(18), 4725–4730. https://doi.org/10.1073/pnas.1713139115

Bunmee, K., Thaenkham, U., Saralamba, N., Ponlawat, A., Zhong, D., Cui, L., Sattabongkot, J., & Sriwichai, P. (2021). Population genetic structure of the malaria vector *Anopheles minimus* in Thailand based on mitochondrial DNA markers. *Parasites & Vectors*, *14*(1), 1–12.

Burke, A., Dahan-Moss, Y., Duncan, F., Qwabe, B., Coetzee, M., Koekemoer, L., & Brooke, B. (2019). A*nopheles parensis* contributes to residual malaria transmission in South Africa. *Malaria Journal*, *18*. https://doi.org/10.1186/s12936-019-2889-5

Burke, A., Dandalo, L., Munhenga, G., Dahan-Moss, Y., Mbokazi, F., Ngxongo, S., Coetzee, M., Koekemoer, L., & Brooke, B. (2017a). A new malaria vector mosquito in South Africa. *Scientific Reports*, *7*(1), 1–5.

Burke, A., Dandalo, L., Munhenga, G., Dahan-Moss, Y., Mbokazi, F., Ngxongo, S., Coetzee, M., Koekemoer, L., & Brooke, B. (2017b). A new malaria vector mosquito in South Africa. *Scientific Reports*, *7*, 43779.

Carlson, J. C., Byrd, B. D., & Omlin, F. X. (2004). Field assessments in western Kenya link malaria vectors to environmentally disturbed habitats during the dry season. *BMC Public Health*, *4*(1), 33.

Carpenter, S. R., Stanley, E. H., & Vander Zanden, M. J. (2011). State of the world's freshwater ecosystems: Physical, chemical, and biological changes. *Annual Review of Environment and Resources*, *36*, 75–99.

Casida, J. E., & Durkin, K. A. (2013). Neuroactive insecticides: Targets, selectivity, resistance, and secondary effects. *Annual Review of Entomology*, *58*, 99–117.

Catalanotto, C., Cogoni, C., & Zardo, G. (2016). MicroRNA in control of gene expression: An overview of nuclear functions. *International Journal of Molecular Sciences*, *17*(10), 1712.

Cech, T. R., & Steitz, J. A. (2014). The noncoding RNA revolution—Trashing old rules to forge new ones. *Cell*, *157*(1), 77–94.

Chandre, F., Darriet, F., Manguin, S., Brengues, C., Carnevale, P., & Guillet, P. (1999). Pyrethroid cross resistance spectrum among populations of *Anopheles gambiae ss* from Côte d'Ivoire. *Journal of the American Mosquito Control Association*, *15*(1), 53–59.

Chang, X., Zhong, D., Lo, E., Fang, Q., Bonizzoni, M., Wang, X., Lee, M.-C., Zhou, G., Zhu, G., & Qin, Q. (2016). Landscape genetic structure and evolutionary genetics of insecticide resistance gene mutations in *Anopheles sinensis*. *Parasites & Vectors*, *9*(1), 228.

Chareonviriyaphap, T., Roberts, D. R., Andre, R. G., Harlan, H. J., Manguin, S., & Bangs, M. J. (1997). Pesticide avoidance behavior in *Anopheles albimanus*, a malaria vector in the Americas. *Journal of the American Mosquito Control Association-Mosquito News*, *13*(2), 171–183.

Charlwood, J. D., Vij, R., & Billingsley, P. F. (2000). Dry season refugia of malaria-transmitting mosquitoes in a dry savannah zone of east Africa. *The American Journal of Tropical Medicine and Hygiene*, *62*(6), 726–732.

Chen, H., Minakawa, N., Beier, J., & Yan, G. (2004). Population genetic structure of Anopheles gambiae mosquitoes on Lake Victoria islands, west Kenya. *Malaria Journal*, *3*(1), 1–8.

Chen, L.-L., & Carmichael, G. G. (2010). Decoding the function of nuclear long non-coding RNAs. *Current Opinion in Cell Biology*, *22*(3), 357–364.

Churcher, T. S., Lissenden, N., Griffin, J. T., Worrall, E., & Ranson, H. (2016). The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *Elife*, *5*, e16090.

Coetzee, M. (2020). Key to the females of Afrotropical *Anopheles* mosquitoes (Diptera: Culicidae). *Malaria Journal*, *19*(1), 1–20.

Coetzee, M., & Fontenille, D. (2004). Advances in the study of *Anopheles funestus*, a major vector of malaria in Africa. *Insect Biochemistry and Molecular Biology*, *34*(7), 599–605.

Coetzee, M., & Koekemoer, L. L. (2013). Molecular systematics and insecticide resistance in the major African malaria vector *Anopheles funestus*. *Annual Review of Entomology*, *58*, 393–412.

Cohuet, A., Simard, F., Toto, J.-C., Kengne, P., Coetzee, M., & Fontenille, D. (2003). Species Identification Within the *Anopheles Funestus* Group of Malaria Vectors in Cameroon And Evidence for A New Species. *The American Journal of Tropical Medicine and Hygiene*, *69*(2), 200–205. https://doi.org/10.4269/ajtmh.2003.69.200

Cohuet, A., Simard, F., Wondji, C. S., Antonio-Nkondjio, C., Awono-Ambene, P., & Fontenille, D. (2004). High malaria transmission intensity due to *Anopheles funestus* (Diptera: Culicidae) in a village of savannah–forest transition area in Cameroon. *Journal of Medical Entomology*, *41*(5), 901–905.

Collins, F. H., & James, A. A. (1996). Genetic modification of mosquitoes. *Science and Medicine*, *3*, 52–61.

Collins, F. H., Mehaffey, P. C., Rasmussen, M. O., Brandling-Bennett, A. D., Odera, J. S., & Finnerty, V. (1988). Comparison of DNA-probe and isoenzyme methods for differentiating *Anopheles gambiae* and *Anopheles arabiensis* (Diptera: Culicidae). *Journal of Medical Entomology*, *25*(2), 116–120.

Costantini, C., Sagnon, N. F., Ilboudo-Sanogo, E., Coluzzi, M., & Boccolini, D. (1999). Chromosomal and bionomic heterogeneities suggest incipient speciation in *Anopheles funestus* from Burkina Faso. *Parassitologia*, *41*(4), 595–612.

Crawford, J. E., Guelbeogo, W. M., Sanou, A., Traoré, A., Vernick, K. D., Sagnon, N., & Lazzaro, B. P. (2010). De Novo Transcriptome Sequencing in *Anopheles funestus* Using

Illumina RNA-Seq Technology. *PLoS ONE*, *5*(12). https://doi.org/10.1371/journal.pone.0014202

Cuamba, N., Morgan, J. C., Irving, H., Steven, A., & Wondji, C. S. (2010). High Level of Pyrethroid Resistance in an *Anopheles funestus* Population of the Chokwe District in Mozambique. *PLoS ONE*, *5*(6). https://doi.org/10.1371/journal.pone.0011010

Dadzie, S. K., Brenyah, R., & Appawu, M. A. (2013). Role of species composition in malaria transmission by the *Anopheles funestus* group (Diptera: Culicidae) in Ghana. *Journal of Vector Ecology*, *38*(1), 105–110.

Dahan-Moss, Y., Koekemoer, L., Coetzee, M., Braack, L., Mouatcho, J., & Cornel, A. (2018). Detection of Anopheles rivulorum-like, a member of the *Anopheles funestus* group, in South Africa. *Malaria Journal*, *17*(1). https://escholarship.org/uc/item/84h625b6

Daibin, Z., Hemming-Schroeder, E., Wang, X., Solomon, K., Guofa, Z., Harrysone, A., Lee, M.-C., Afrane, Y. A., Githeko, A. K., & Guiyun, Y. (2020). Extensive new *Anopheles* cryptic species involved in human malaria transmission in western Kenya. *Scientific Reports (Nature Publisher Group)*, *10*(1).

Debrah, I., Afrane, Y. A., Amoah, L. E., Ochwedo, K. O., Mukabana, W. R., Zhong, D., Zhou, G., Lee, M.-C., Onyango, S. A., Magomere, E. O., Atieli, H., Githeko, A. K., & Yan, G. (2021). Larval ecology and bionomics of *Anopheles funestus* in highland and lowland sites in western Kenya. *PLOS ONE*, *16*(10), e0255321. https://doi.org/10.1371/journal.pone.0255321

Dhimal, M., Ahrens, B., & Kuch, U. (2014). Species composition, seasonal occurrence, habitat preference and altitudinal distribution of malaria and other disease vectors in eastern Nepal. *Parasites & Vectors*, *7*(1), 1–11.

Dia, I., Guelbeogo, M. W., & Ayala, D. (2013). Advances and Perspectives in the Study of the Malaria Mosquito *Anopheles funestus*. *Anopheles Mosquitoes-New Insights into Malaria Vectors*, *10*, 55389.

Dida, G. O., Gelder, F. B., Anyona, D. N., Abuom, P. O., Onyuka, J. O., Matano, A.-S., Adoka, S. O., Kanangire, C. K., Owuor, P. O., & Ouma, C. (2015). Presence and distribution of mosquito larvae predators and factors influencing their abundance along the Mara River, Kenya and Tanzania. *SpringerPlus*, *4*(1), 1–14.

Djouaka, R., Irving, H., Tukur, Z., & Wondji, C. S. (2011). Exploring mechanisms of multiple insecticide resistance in a population of the malaria vector *Anopheles funestus* in Benin. *PLos One*, *6*(11), e27760.

Djouaka, R., Riveron, J. M., Yessoufou, A., Tchigossou, G., Akoton, R., Irving, H., Djegbe, I., Moutairou, K., Adeoti, R., Tamò, M., Manyong, V., & Wondji, C. S. (2016). Multiple insecticide resistance in an infected population of the malaria vector *Anopheles funestus* in Benin. *Parasites & Vectors*, *9*, 453. https://doi.org/10.1186/s13071-016-1723-y

Dobin, A., & Gingeras, T. R. (2015). Mapping RNA-seq Reads with STAR. *Current Protocols in Bioinformatics*, *51*(1). https://doi.org/10.1002/0471250953.bi1114s51

Dobin, A., & Gingeras, T. R. (2016). Optimizing RNA-Seq Mapping with STAR. In O. Carugo & F. Eisenhaber (Eds.), *Data Mining Techniques for the Life Sciences* (Vol. 1415, pp. 245–262). Springer New York. https://doi.org/10.1007/978-1-4939-3572-7_13

Eisele, T. P., Larsen, D. A., Walker, N., Cibulskis, R. E., Yukich, J. O., Zikusooka, C. M., & Steketee, R. W. (2012). Estimates of child deaths prevented from malaria prevention scale-up in Africa 2001-2010. *Malaria Journal*, *11*(1), 1–11.

Emidi, B., Kisinza, W. N., Mmbando, B. P., Malima, R., & Mosha, F. W. (2017). Effect of physicochemical parameters on *Anopheles* and *Culex* mosquito larvae abundance in different breeding sites in a rural setting of Muheza, Tanzania. *Parasites & Vectors*, *10*(1), 304.

Enayati, A. A., Vatandoost, H., Ladonni, H., Townson, H., & Hemingway, J. (2003). Molecular evidence for a kdr-like pyrethroid resistance mechanism in the malaria vector mosquito *Anopheles stephensi*. *Medical and Veterinary Entomology*, *17*(2), 138–144.

Epstein, A., Maiteki-Sebuguzi, C., Namuganga, J. F., Nankabirwa, J. I., Gonahasa, S., Opigo, J., Staedke, S. G., Rutazaana, D., Arinaitwe, E., & Kamya, M. R. (2022). Resurgence of malaria in Uganda despite sustained indoor residual spraying and repeated long-lasting insecticidal net distributions. *PLOS Global Public Health*, *2*(9), e0000676.

Etebari, K., Afrad, M. H., Tang, B., Silva, R., Furlong, M. J., & Asgari, S. (2018). Involvement of microRNA miR-2b-3p in regulation of metabolic resistance to insecticides in *Plutella xylostella*. *Insect Molecular Biology*, *27*(4), 478–491. https://doi.org/10.1111/imb.12387

Etebari, K., Furlong, M. J., & Asgari, S. (2015). Genome wide discovery of long intergenic non-coding RNAs in Diamondback moth (*Plutella xylostella*) and their expression in insecticide resistant strains. *Scientific Reports*, *5*(1), 14642.

Evans, A. M., & Leeson, H. S. (1935). The *funestus* series of *Anopheles* in Southern Rhodesia, with description of a new variety. *Annals of Tropical Medicine & Parasitology*, *29*(1), 33–47.

Evans, A. M., & Symes, C. B. (1937). Anopheles funestus and its allies in Kenya. *Annals of Tropical Medicine & Parasitology*, *31*(1), 105–112.

Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, *10*(3), 564–567.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*(4), 783–791.

Ferguson, H. M., Dornhaus, A., Beeche, A., Borgemeister, C., Gottlieb, M., Mulla, M. S., Gimnig, J. E., Fish, D., & Killeen, G. F. (2010). Ecology: A prerequisite for malaria elimination and eradication. *PLoS Medicine*, *7*(8).

Ferreira, J. A., & Zwinderman, A. H. (2006). On the benjamini–hochberg method. *The Annals of Statistics*, *34*(4), 1827–1849.

Feyereisen, R. (2005). *Insect Cytochrome P450 in: Comprehensive Molecular Insect Science-Biochemistry and Molecular Biology.(Edited by Gilbert, LI, Iatrou, K. and Gill, SS)*. Elsevier Publishers, Oxford.

Gale, K., & Crampton, J. (1989). The ribosomal genes of the mosquito, *Aedes aegypti*. *European Journal of Biochemistry*, *185*(2), 311–317. https://doi.org/10.1111/j.1432-1033.1989.tb15117.x

Garba, Y., & Olayemi, I. K. (2015). Spartial variation in physicochemical characteristics of wetland rice fields mosquito larval habitats in Minna, north central Nigeria. *International Conference on Agricultural, Ecological and Medical Sciences*, 11–14.

Garros, C., Harbach, R. E., & Manguin, S. (2005). Morphological assessment and molecular phylogenetics of the Funestus and Minimus Groups of *Anopheles* (Cellia). *Journal of Medical Entomology*, *42*(4), 522–536.

Getachew, D., Balkew, M., & Tekie, H. (2020). *Anopheles* larval species composition and characterization of breeding habitats in two localities in the Ghibe River Basin, southwestern Ethiopia. *Malaria Journal*, *19*(1), 1–13.

Gillies, M. T., & Coetzee, M. (1987). A supplement to the Anophelinae of Africa South of the Sahara. *Publ S Afr Inst Med Res*, *55*, 1–143.

Gillies, M. T. & De Meillon B. (1968a). A supplement to the Anophelinae of Africa South of the Sahara, Johannesburg: The South African Institute for Medical Research. *Publ S Afr Inst Med Res*, *55*, 1–143.

Gillies, M. T., & De Meillon, B. (1968b). The Anophelinae of Africa south of the Sahara (Ethiopian zoogeographical region). The Anophelinae of Africa South of the Sahara (Ethiopian Zoogeographical Region), pp. 343

Gillies, M. T., & Furlong, M. (1964). An investigation into the behaviour of *Anopheles parensis* Gillies at Malindi on the coast of Kenya. - *Google Search*. https://www.google.com/search?rlz=1C1SQJL_enKE901KE903&ei=iQvWXtWKH6GOlwTouoj4Cg&q=+An+investigation+into+the+behaviour+of+Anopheles+parensis+Gillies+at+Malindi+on+the+coast+of+Kenya.&oq=+An+investigation+into+the+behaviour+of+Anopheles+parensis+Gillies+at+Malindi+on+the+coast+of+Kenya.&gs_lcp=CgZwc3ktYWIQDDoECAAQR1DRgQ5Y2p4OYIGrDmgBcAJ4AIAB8gGIAfIBkgEDMi0xmAEAoAEBoAECqgEHZ3dzLXdperABAA&sclient=psy-ab&ved=0ahUKEwiV86uM2eLpAhUhx4UKHWgdAq8Q4dUDCAw

Gimnig, J. E., Ombok, M., Kamau, L., & Hawley, W. A. (2001). Characteristics of larval anopheline (Diptera: Culicidae) habitats in Western Kenya. *Journal of Medical Entomology*, *38*(2), 282–288. https://doi.org/10.1603/0022-2585-38.2.282

Gimnig, J. E., Ombok, M., Otieno, S., Kaufman, M. G., Vulule, J. M., & Walker, E. D. (2002). Density-dependent development of *Anopheles gambiae* (Diptera: Culicidae) larvae in artificial habitats. *Journal of Medical Entomology*, *39*(1), 162–172.

Gimnig, J. E., Vulule, J. M., Lo, T. Q., Kamau, L., Kolczak, M. S., Phillips-Howard, P. A., Mathenge, E. M., TER KUILE, F. O., Nahlen, B. L., & Hightower, A. W. (2003). Impact of permethrin-treated bed nets on entomologic indices in an area of intense year-round malaria transmission. *The American Journal of Tropical Medicine and Hygiene*, *68*(4_suppl), 16–22.

Githeko, A. K. (2009). Malaria and climate change: Special feature. In *Commonwealth health ministers' update 2009*. Commonwealth Secretariat: Pro-Brook Publishing, GB.

Githeko, A. K. (2021). Health related vulnerabilities and enabling institutions to facilitate responses to climate change in east Africa. *East Africa Science*, *3*(1), 1–18.

Gowelo, S. A., Chirombo, J., Koenraadt, C. J., Mzilahowa, T., Berg, H., Takken, W., & McCann, R. S. (2020). Characterisation of anopheline larval habitats in southern Malawi. *Acta Tropica*, 105558.

Green, C. A. (1982). Cladistic analysis of mosquito chromosome data (*Anopheles* (*Cellia*) *Myzomyia*). *Journal of Heredity*, *73*(1), 3–11.

Green, C. A., & Hunt, R. H. (1980). Interpretation of variation in ovarian polytene chromosomes of *Anopheles funestus Giles*, *A. parensis Gillies*, and *A. aruni*? *Genetica*, *51*(3), 187–195.

Gregory, R., Darby, A. C., Irving, H., Coulibaly, M. B., Hughes, M., Koekemoer, L. L., Coetzee, M., Ranson, H., Hemingway, J., & Hall, N. (2011). A de novo expression profiling of *Anopheles funestus*, malaria vector in Africa, using 454 pyrosequencing. *PLoS One*, *6*(2), e17418.

Grieco, J. P., Achee, N. L., Chareonviriyaphap, T., Suwonkerd, W., Chauhan, K., Sardelis, M. R., & Roberts, D. R. (2007). A new classification system for the actions of IRS chemicals traditionally used for malaria control. *PLos One*, *2*(8), e716.

Grillet, M. E. (2000). Factors associated with distribution of *Anopheles aquasalis* and *Anopheles oswaldoi* (Diptera: Culicidae) in a malarious area, northeastern Venezuela. *Journal of Medical Entomology*, *37*(2), 231–238.

Gu, W., Utzinger, J., & Novak, R. J. (2008). Habitat-based larval interventions: A new perspective for malaria control. *The American Journal of Tropical Medicine and Hygiene*, *78*(1), 2–6.

Hackett, B. J., Gimnig, J., Guelbeogo, W., Costantini, C., Koekemoer, L. L., Coetzee, M., Collins, F. H., & Besansky, N. J. (2000). Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *An. Rivulorum* , and uncover a cryptic taxon. *Insect Molecular Biology*, *9*(4), 369–374. https://doi.org/10.1046/j.1365-2583.2000.00198.x

Hajison, P. L., Feresu, S. A., & Mwakikunga, B. W. (2018). Malaria in children under-five: A comparison of risk factors in lakeshore and highland areas, Zomba district, Malawi. *PLoS One*, *13*(11), e0207207.

Hall, T. F. (1972). The influence of plants on anopheline breeding. *The American Journal of Tropical Medicine and Hygiene*, *21*(5_Suppl), 787–794.

Harbach, R.E. (2013). *Mosquito Taxonomic Inventory, http://mosquito-taxonomic-inventory.info/, Accessed on June, 2020. Available from: Http:// mosquito-taxonomic-inventory.info/*. http://mosquito-taxonomic-inventory.info/

Hargreaves, K., Koekemoer, L. L., Brooke, B. D., Hunt, R. H., Mthembu, J., & Coetzee, M. (2000). *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. *Medical and Veterinary Entomology*, *14*(2), 181–189.

Hawaria, D., Demissew, A., Kibret, S., Lee, M.-C., Yewhalaw, D., & Yan, G. (2020). Effects of environmental modification on the diversity and positivity of anopheline mosquito aquatic habitats at Arjo-Dedessa irrigation development site, Southwest Ethiopia. *Infectious Diseases of Poverty*, *9*(1), 1–11.

He, J., Sun, H., Zhang, D., Sun, Y., Ma, L., Chen, L., Liu, Z., Xiong, C., Yan, G., & Zhu, C. (2009). Cloning and characterization of 60S ribosomal protein L22 (RPL22) from Culex pipiens pallens. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *153*(2), 216–222.

Hemingway, J. (2000). The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. *Insect Biochemistry and Molecular Biology*, *30*(11), 1009–1015.

Hemingway, J. (2014). The role of vector control in stopping the transmission of malaria: Threats and opportunities. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1645), 20130431.

Hemingway, J. (2018). Resistance: A problem without an easy solution. *Pesticide Biochemistry and Physiology*, *151*, 73–75.

Hemingway, J., & Ranson, H. (2000). Insecticide resistance in insect vectors of human disease. *Annual Review of Entomology*, *45*(1), 371–391.

Hess, A. D., & Hall, T. F. (1943). The intersection line as a factor in anopheline ecology. *Journal of the National Malaria Society*, *2*(2), 93–98.

Higuera, A., Muñoz, M., López, M. C., Reyes, P., Urbano, P., Villalobos, O., & Ramírez, J. D. (2020). Development of a multilocus sequence typing scheme for Giardia intestinalis. *Genes*, *11*(7), 764.

Hu, X., Wang, W., Zhang, D., Jiao, J., Tan, W., Sun, Y., Ma, L., & Zhu, C. (2007). Cloning and characterization of 40S ribosomal protein S4 gene from *Culex pipiens pallens*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *146*(2), 265–270.

Ibrahim, S. S., Amvongo-Adjia, N., Wondji, M. J., Irving, H., Riveron, J. M., & Wondji, C. S. (2018). Pyrethroid Resistance in the Major Malaria Vector *Anopheles funestus* is Exacerbated by Overexpression and Overactivity of the P450 CYP6AA1 Across Africa. *Genes*, *9*(3). https://doi.org/10.3390/genes9030140

Ibrahim, S. S., Riveron, J. M., Bibby, J., Irving, H., Yunta, C., Paine, M. J., & Wondji, C. S. (2015). Allelic variation of cytochrome P450s drives resistance to bednet insecticides in a major malaria vector. *PLoS Genetics*, *11*(10), e1005618.

Imam, A. A., & Deeni, Y. (2015). Larval productivity and detoxification enzymes profile in response to physico-chemical environmental factors of *Anopheles gambiae* breeding ecologies in Nigeria. *British Journal of Applied Science & Technology*, *5*(6), 595–612.

Imbahale, S. S., Paaijmans, K. P., Mukabana, W. R., Van Lammeren, R., Githeko, A. K., & Takken, W. (2011). A longitudinal study on *Anopheles* mosquito larval abundance in distinct geographical and environmental settings in western Kenya. *Malaria Journal*, *10*(1), 81.

Implications of Insecticide Resistance Consortium. (2018). Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: Trends in pyrethroid

resistance during a WHO-coordinated multi-country prospective study. Parasites & vectors. *Springer*, *11*(1), 550.

Jones, C. M., Lee, Y., Kitchen, A., Collier, T., Pringle, J. C., Muleba, M., Irish, S., Stevenson, J. C., Coetzee, M., & Cornel, A. J. (2018). Complete *Anopheles funestus* mitogenomes reveal an ancient history of mitochondrial lineages and their distribution in southern and central Africa. *Scientific Reports*, *8*(1), 9054.

Juliano, S. A. (2009). Species interactions among larval mosquitoes: Context dependence across habitat gradients. *Annual Review of Entomology*, *54*, 37–56.

Juma, S. G., & Kelonye, F. (2016). Projected rainfall and temperature changes over Bungoma county in western Kenya by the year 2050 based precis modeling system. *Ethiopian Journal of Environmental Studies and Management*, *9*(5), 625–640.

Kabula, B. I., Attah, P. K., Wilson, M. D., & Boakye, D. A. (2011). Characterization of *Anopheles gambiae* sl and insecticide resistance profile relative to physicochemical properties of breeding habitats within Accra Metropolis, Ghana. *Tanzania Journal of Health Research*, *13*(3), 163–187.

Kahamba, N. F., Finda, M., Ngowo, H. S., Msugupakulya, B. J., Baldini, F., Koekemoer, L. L., Ferguson, H. M., & Okumu, F. O. (2022). Using ecological observations to improve malaria control in areas where *Anopheles funestus* is the dominant vector. *Malaria Journal*, *21*(1), 1–15.

Kaindoa, E. W., Matowo, N. S., Ngowo, H. S., Mkandawile, G., Mmbando, A., Finda, M., & Okumu, F. O. (2017). Interventions that effectively target *Anopheles funestus* mosquitoes could significantly improve control of persistent malaria transmission in south-eastern Tanzania. *PloS One*, *12*(5), e0177807. https://doi.org/10.1371/journal.pone.0177807

Kamau, L., Agai, D., Matoke, D., Wachira, L., Gikandi, G., & Vulule, J. M. (2008). Status of insecticide susceptibility in *Anopheles gambiae sensu lato* and *Anopheles funestus* mosquitoes from western Kenya. *Journal of Insect Science*, *8*(1).

Kamau, L., Hunt, R., & Coetzee, M. (2002). Analysis of the population structure of *Anopheles funestus* (Diptera: Culicidae) from western and coastal Kenya using paracentric chromosomal inversion frequencies. *Journal of Medical Entomology*, *39*(1), 78–83.

Kamau, L., Munyekenye, G. O., Koekemoer, L. L., Hunt, R. H., & Coetzee, M. (2003). A survey of the *Anopheles funestus* (Diptera: Culicidae) group of mosquitoes from 10 sites in Kenya with special emphasis on population genetic structure based on chromosomal inversion karyotypes. *Journal of Medical Entomology*, *40*(5), 664–671.

Kapranov, P., Cheng, J., Dike, S., Nix, D. A., Duttagupta, R., Willingham, A. T., Stadler, P. F., Hertel, J., Hackermüller, J., Hofacker, I. L., Bell, I., Cheung, E., Drenkow, J., Dumais, E., Patel, S., Helt, G., Ganesh, M., Ghosh, S., Piccolboni, A., … Gingeras, T. R. (2007). RNA Maps Reveal New RNA Classes and a Possible Function for Pervasive Transcription. *Science*, *316*(5830), 1484–1488. https://doi.org/10.1126/science.1138341

Karunaratne, S., De Silva, W., Weeraratne, T. C., & Surendran, S. N. (2018). Insecticide resistance in mosquitoes: Development, mechanisms and monitoring. *Ceylon J Sci*, *47*(4), 299–309.

Kawada, H., Dida, G. O., Ohashi, K., Komagata, O., Kasai, S., Tomita, T., Sonye, G., Maekawa, Y., Mwatele, C., & Njenga, S. M. (2011). Multimodal pyrethroid resistance in malaria vectors, *Anopheles gambiae ss*, *Anopheles arabiensis*, and *Anopheles funestus ss* in western Kenya. *PloS One*, *6*(8), e22574.

Kawada, H., Dida, G. O., Sonye, G., Njenga, S. M., Mwandawiro, C., & Minakawa, N. (2012a). Reconsideration of *Anopheles rivulorum* as a vector of *Plasmodium falciparum* in western Kenya: Some evidence from biting time, blood preference, sporozoite positive rate, and pyrethroid resistance. *Parasites & Vectors*, *5*(1), 230.

Kawada, H., Dida, G. O., Sonye, G., Njenga, S. M., Mwandawiro, C., & Minakawa, N. (2012b). Reconsideration of *Anopheles rivulorum* as a vector of *Plasmodium falciparum* in western Kenya: Some evidence from biting time, blood preference, sporozoite positive rate, and pyrethroid resistance. *Parasites & Vectors*, *5*(1), 230.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Kent, R. J., Coetzee, M., Mharakurwa, S., & Norris, D. E. (2006). Feeding and indoor resting behaviour of the mosquito *Anopheles longipalpis* in an area of hyperendemic malaria transmission in southern Zambia. *Medical and Veterinary Entomology*, *20*(4), 459–463.

Khalil, A. M., Guttman, M., Huarte, M., Garber, M., Raj, A., Rivea Morales, D., Thomas, K., Presser, A., Bernstein, B. E., Van Oudenaarden, A., Regev, A., Lander, E. S., & Rinn, J. L. (2009). Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proceedings of the National Academy of Sciences*, *106*(28), 11667–11672. https://doi.org/10.1073/pnas.0904715106

Killeen, G. F. (2003). Following in Soper's footsteps: Northeast Brazil 63 years after eradication of *Anopheles gambiae*. *The Lancet Infectious Diseases*, *3*(10), 663–666.

Killeen, G. F., McKenzie, F. E., Foy, B. D., Schieffelin, C., Billingsley, P. F., & Beier, J. C. (2000). The potential impact of integrated malaria transmission control on entomologic inoculation rate in highly endemic areas. *The American Journal of Tropical Medicine and Hygiene*, *62*(5), 545–551.

Kinga, H., Kengne-Ouafo, J. A., King, S. A., Egyirifa, R. K., Aboagye-Antwi, F., & Akorli, J. (2022). Water Physicochemical Parameters and Microbial Composition Distinguish *Anopheles* and *Culex* Mosquito Breeding Sites: Potential as Ecological Markers for Larval Source Surveillance. *Journal of Medical Entomology*.

Kisumu County. (2020). *Kisumu County Weather Forecast 2020*. Ministry Of Environment and Forestry Kenya Meteorological Department – Kisumu County, pp. 1–4

Kitron, U., & Spielman, A. (1989). Suppression of transmission of malaria through source reduction: Antianopheline measures applied in Israel, the United States, and Italy. *Reviews of Infectious Diseases*, *11*(3), 391–406.

Knipple, D. C., Doyle, K. E., Marsella-Herrick, P. A., & Soderlund, D. M. (1994). Tight genetic linkage between the *kdr* insecticide resistance trait and a voltage-sensitive sodium channel gene in the house fly. *Proceedings of the National Academy of Sciences*, *91*(7), 2483–2487.

Koekemoer, L. L., Kamau, L., Hunt, R. H., & Coetzee, M. (2002). A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. *The American Journal of Tropical Medicine and Hygiene*, *66*(6), 804–811.

Koekemoer, L. L., Misiani, E. A., Hunt, R. H., Kent, R. J., Norris, D. E., & Coetzee, M. (2009). Cryptic species within *Anopheles longipalpis* from southern Africa and phylogenetic

comparison with members of the *An. Funestus* group. *Bulletin of Entomological Research*, *99*(1), 41–49.

Koenraadt, C. J. M., & Takken, W. (2003). Cannibalism and predation among larvae of the *Anopheles gambiae* complex. *Medical and Veterinary Entomology*, *17*(1), 61–66.

Kolliopoulou, A., & Swevers, L. (2014). Recent progress in RNAi research in Lepidoptera: Intracellular machinery, antiviral immune response and prospects for insect pest control. *Current Opinion in Insect Science*, *6*, 28–34.

Konate, L., Diop, A., Sy, N., Faye, M. N., Deng, Y., Izri, A., Faye, O., & Mouchet, J. (2001). Comeback of *Anopheles funestus* in Sahelian Senegal. *The Lancet*, *358*(9278), 336.

Kopya, E., Ndo, C., Djamouko-Djonkam, L., Nkahe, L., Awono-Ambene, P., Njiokou, F., Wondji, C. S., & Antonio-Nkondjio, C. (2021). *Anopheles leesoni Evans* 1931, a member of the *Anopheles funestus* group, is a potential malaria vector in Cameroon. *Advances in Entomology*, *10*(1), 99–109.

Krzywinski, J., Grushko, O. G., & Besansky, N. J. (2006). Analysis of the complete mitochondrial DNA from *Anopheles funestus*: An improved dipteran mitochondrial genome annotation and a temporal dimension of mosquito evolution. *Molecular Phylogenetics and Evolution*, *39*(2), 417–423.

Kudom, A. A. (2015). Larval ecology of *Anopheles coluzzii* in Cape Coast, Ghana: Water quality, nature of habitat and implication for larval control. *Malaria Journal*, *14*(1), 447.

Kumar, R., Muhid, P., Dahms, H.-U., Tseng, L.-C., & Hwang, J.-S. (2008). Potential of three aquatic predators to control mosquitoes in the presence of alternative prey: A comparative experimental assessment. *Marine and Freshwater Research*, *59*(9), 817–835.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547.

Kweka, E. J., Zhou, G., Gilbreath, T. M., Afrane, Y., Nyindo, M., Githeko, A. K., & Yan, G. (2011). Predation efficiency of *Anopheles gambiae* larvae by aquatic predators in western Kenya highlands. *Parasites & Vectors*, *4*(1), 1–7.

Kwenti, T. E., Kwenti, T. D. B., Latz, A., Njunda, L. A., & Nkuo-Akenji, T. (2017). Epidemiological and clinical profile of paediatric malaria: A cross sectional study performed on febrile children in five epidemiological strata of malaria in Cameroon. *BMC Infectious Diseases*, *17*(1), 1–13.

Lawal, N., Idoko, A. S., Abdullahi, H., Jibiya, S. A., Ibrahim, N., Osibemhe, M., & Imam, A. A. (2022). Assessment of physico-chemical characteristics of mosquito breeding sites in Northwest Nigeria. International Journal of Mosquito Research, *9*(1), 132-138

Lehmann, T., Licht, M., Elissa, N., Maega, B. T. A., Chimumbwa, J. M., Watsenga, F. T., Wondji, C. S., Simard, F., & Hawley, W. A. (2003). Population structure of *Anopheles gambiae* in Africa. *Journal of Heredity*, *94*(2), 133–147.

Leigh, J. W., & Bryant, D. (2015). POPART: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, *6*(9), 1110–1116.

Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Research*, *49*(W1), W293–W296.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., & Durbin, R. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079.

Li, T., Zhang, L., Reid, W. R., Xu, Q., Dong, K., & Liu, N. (2012). Multiple mutations and mutation combinations in the sodium channel of permethrin resistant mosquitoes, Culex quinquefasciatus. *Scientific Reports*, *2*(1), 1–9.

Limwagu, A. J., Kaindoa, E. W., Ngowo, H. S., Hape, E., Finda, M., Mkandawile, G., Kihonda, J., Kifungo, K., Njalambaha, R. M., & Matoke-Muhia, D. (2019). Using a miniaturized double-net trap (DN-Mini) to assess relationships between indoor–outdoor biting preferences and physiological ages of two malaria vectors, *Anopheles arabiensis* and *Anopheles funestus*. *Malaria Journal*, *18*(1), 1–15.

Liu, F., Guo, D., Yuan, Z., Chen, C., & Xiao, H. (2017). Genome-wide identification of long non-coding RNA genes and their association with insecticide resistance and metamorphosis in diamondback moth, *Plutella xylostella*. *Scientific Reports*, *7*(1), 15870.

Lochouarn, L., Dia, I., Boccolini, D., Coluzzi, M., & Fontenille, D. (1998). Bionomical and cytogenetic heterogeneities of *Anopheles funestus* in Senegal. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *92*(6), 607–612.

Logan, R. A. E., Mäurer, J. B., Wapler, C., & Ingham, V. A. (2024). Uridine diphosphate (UDP)-glycosyltransferases (UGTs) confer insecticide resistance in the major malaria vectors *Anopheles gambiae sl and Anopheles funestus*. https://www.researchsquare.com/article/rs-4526134/latest

Lwetoijera, D. W., Harris, C., Kiware, S. S., Dongus, S., Devine, G. J., McCall, P. J., & Majambere, S. (2014). Increasing role of *Anopheles funestus* and *Anopheles arabiensis* in malaria transmission in the Kilombero Valley, Tanzania. *Malaria Journal*, *13*(1), 331.

Machani, M. G., Ochomo, E., Amimo, F., Kosgei, J., Munga, S., Zhou, G., Githeko, A. K., Yan, G., & Afrane, Y. A. (2020). Resting behaviour of malaria vectors in highland and

lowland sites of western Kenya: Implication on malaria vector control measures. *PloS One*, *15*(2), e0224718.

Mahgoub, M. M., Kweka, E. J., & Himeidan, Y. E. (2017). Characterisation of larval habitats, species composition and factors associated with the seasonal abundance of mosquito fauna in Gezira, Sudan. *Infectious Diseases of Poverty*, *6*(1), 23. https://doi.org/10.1186/s40249-017-0242-1

Main, B. J., Lee, Y., Collier, T. C., Norris, L. C., Brisco, K., Fofana, A., Cornel, A. J., & Lanzaro, G. C. (2015). Complex genome evolution in Anopheles coluzzii associated with increased insecticide usage in Mali. *Molecular Ecology*, *24*(20), 5145–5157.

Main, B. J., Lee, Y., Ferguson, H. M., Kreppel, K. S., Kihonda, A., Govella, N. J., Collier, T. C., Cornel, A. J., Eskin, E., & Kang, E. Y. (2016). The genetic basis of host preference and resting behavior in the major African malaria vector, *Anopheles arabiensis*. *PLoS Genetics*, *12*(9), e1006303.

Makhawi, A. M., Aboud, M. A., Raba'a, F. M. E., Osman, O. F., & Elnaiem, D. E. A. (2015). Identification of *Anopheles* species of the funestus group and their role in malaria transmission in Sudan. *J Appl Indust Sci*, *3*, 58–62.

Manguin, S., Roberts, D. R., Peyton, E. L., Rejmankova, E., & Pecor, J. (1996). Characterization of *Anopheles pseudopunctipennis* larval habitats. *J*ournal of the American Mosquito Control Association, *12*(4), 619-626.

Mapua, S. A., Hape, E. E., Kihonda, J., Bwanary, H., Kifungo, K., Kilalangongono, M., Kaindoa, E. W., Ngowo, H. S., & Okumu, F. O. (2022). Persistently high proportions of plasmodium-infected *Anopheles funestus* mosquitoes in two villages in the Kilombero valley, South-Eastern Tanzania. *Parasite Epidemiology and Control*, e00264.

Martinez-Torres, D., Chandre, F., Williamson, M. S., Darriet, F., Berge, J. B., Devonshire, A. L., Guillet, P., Pasteur, N., & Pauron, D. (1998). Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae ss*. *Insect Molecular Biology*, *7*(2), 179–184.

Matheson, R. (1930). The utilization of aquatic plants as aids in mosquito control. *The American Naturalist*, *64*(690), 56–86.

Matowo, J., Weetman, D., Pignatelli, P., Wright, A., Charlwood, J. D., Kaaya, R., Shirima, B., Moshi, O., Lukole, E., & Mosha, J. (2022). Expression of pyrethroid metabolizing P450 enzymes characterizes highly resistant *Anopheles* vector species targeted by successful deployment of PBO-treated bednets in Tanzania. *PloS One*, *17*(1), e0249440.

Matvienko, M. (2015). CLC Genomics Workbench. *Plant and Animal Genome. Sr. Field Application Scientist, CLC Bio, Aarhus, DE*.

McCann, R. S., Ochomo, E., Bayoh, M. N., Vulule, J. M., Hamel, M. J., Gimnig, J. E., Hawley, W. A., & Walker, E. D. (2014). Reemergence of *Anopheles funestus* as a Vector of Plasmodium falciparum in Western Kenya after Long-Term Implementation of Insecticide-Treated Bed Nets. *The American Journal of Tropical Medicine and Hygiene*, *90*(4), 597–604. https://doi.org/10.4269/ajtmh.13-0614

Mei, Y., Khan, H., Shishikura, M., Ishiyama, S., Khan, A., Orita, H., & Brock, M. V. (2023). pfeRNAs-A Novel Class of Small Non-Coding RNAs With Real Translational Potential. *Journal of Surgical Research*, *284*, 237–244.

Meiklejohn, C. D., Montooth, K. L., & Rand, D. M. (2007). Positive and negative selection on the mitochondrial genome. *TRENDS in Genetics*, *23*(6), 259–263.

Mendis, C., Jacobsen, J. L., Gamage-Mendis, A., Bule, E., Dgedge, M., Thompson, R., Cuamba, N., Barreto, J., Begtrup, K., & Sinden, R. E. (2000). *Anopheles arabiensis* and *An.funestus* are equally important vectors of malaria in Matola coastal suburb of Maputo, southern Mozambique. *Medical and Veterinary Entomology*, *14*(2), 171–180.

Menze, B. D., Riveron, J. M., Ibrahim, S. S., Irving, H., Antonio-Nkondjio, C., Awono-Ambene, P. H., & Wondji, C. S. (2016). Multiple Insecticide Resistance in the Malaria Vector *Anopheles funestus* from Northern Cameroon Is Mediated by Metabolic Resistance Alongside Potential Target Site Insensitivity Mutations. *PloS One*, *11*(10), e0163261. https://doi.org/10.1371/journal.pone.0163261

Mereta, S. T., Yewhalaw, D., Boets, P., Ahmed, A., Duchateau, L., Speybroeck, N., Vanwambeke, S. O., Legesse, W., De Meester, L., & Goethals, P. L. (2013). Physico-chemical and biological characterization of anopheline mosquito larval habitats (Diptera: Culicidae): implications for malaria control. *Parasites & Vectors*, *6*(1), 320.

Merritt, R. W., Dadd, R. H., & Walker, E. D. (1992). Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. *Annual Review of Entomology*, *37*(1), 349–374.

Messenger, L. A., Impoinvil, L. M., Derilus, D., Yewhalaw, D., Irish, S., & Lenhart, A. (2021). A whole transcriptomic approach provides novel insights into the molecular basis of organophosphate and pyrethroid resistance in *Anopheles arabiensis* from Ethiopia. *Insect Biochemistry and Molecular Biology*, *139*, 103655.

Michel, A. P., Ingrasci, M. J., Schemerhorn, B. J., Kern, M., Le Goff, G., Coetzee, M., Elissa, N., Fontenille, D., Vulule, J., & Lehmann, T. (2005). Rangewide population genetic

structure of the African malaria vector *Anopheles funestus*. *Molecular Ecology*, *14*(14), 4235–4248.

Minakawa, N., Mutero, C. M., Githure, J. I., Beier, J. C., & Yan, G. (1999). Spatial distribution and habitat characterization of anopheline mosquito larvae in Western Kenya. *The American Journal of Tropical Medicine and Hygiene*, *61*(6), 1010–1016. https://doi.org/10.4269/ajtmh.1999.61.1010

Minakawa, N., Seda, P., & Yan, G. (2002). Influence of host and larval habitat distribution on the abundance of African malaria vectors in western Kenya. *The American Journal of Tropical Medicine and Hygiene*, *67*(1), 32–38.

Minakawa, N., Sonye, G., Dida, G. O., Futami, K., & Kaneko, S. (2008). Recent reduction in the water level of Lake Victoria has created more habitats for *Anopheles funestus*. *Malaria Journal*, *7*(1), 1–6.

Mogi, M. (2007). Insects and other invertebrate predators. *Journal of the American Mosquito Control Association*, *23*(sp2), 93–109.

Mokany, A., & Shine, R. (2003). Biological warfare in the garden pond: Tadpoles suppress the growth of mosquito larvae. *Ecological Entomology*, *28*(1), 102–108.

Mouatcho, J. C., Hargreaves, K., Koekemoer, L. L., Brooke, B. D., Oliver, S. V., Hunt, R. H., & Coetzee, M. (2007). Indoor collections of the *Anopheles funestus* group (Diptera: Culicidae) in sprayed houses in northern KwaZulu-Natal, South Africa. *Malaria Journal*, *6*(1), 30.

Mouatcho, J., Cornel, A. J., Dahan-Moss, Y., Koekemoer, L. L., Coetzee, M., & Braack, L. (2018). Detection of *Anopheles rivulorum-like*, a member of the *Anopheles funestus* group, in South Africa. *Malaria Journal*, *17*(1), 195.

Msugupakulya, B. J., Ngajuma, S. K., Ngayambwa, A. N., Kidwanga, B. E., Mpasuka, I. R., Selvaraj, P., Wilson, A. L., & Okumu, F. O. (2024). Influence of larval growth and habitat shading on retreatment frequencies of biolarvicides against malaria vectors. *Scientific Reports*, *14*(1), 1002.

Mukabayire, O., Boccolini, D., Lochouarn, L., Fontenille, D., & Besansky, N. J. (1999). Mitochondrial and ribosomal internal transcribed spacer (ITS2) diversity of the African malaria vector *Anopheles funestus*. *Molecular Ecology*, *8*(2), 289–297.

Mulamba, C., Riveron, J. M., Ibrahim, S. S., Irving, H., Barnes, K. G., Mukwaya, L. G., Birungi, J., & Wondji, C. S. (2014). Widespread pyrethroid and DDT resistance in the major malaria vector *Anopheles funestus* in east Africa is driven by metabolic resistance mechanisms. *PloS One*, *9*(10), e110058.

Munga, S., Minakawa, N., Zhou, G., Barrack, O.-O. J., Githeko, A. K., & Yan, G. (2014). Effects of larval competitors and predators on oviposition site selection of *Anopheles gambiae sensu stricto*. *Journal of Medical Entomology*, *43*(2), 221–224.

Musapa, M., Kumwenda, T., Mkulama, M., Chishimba, S., Norris, D. E., Thuma, P. E., & Mharakurwa, S. (2013). A simple Chelex protocol for DNA extraction from *Anopheles* spp. JoVE (Journal of Visualized Experiments), *71*, e3281.

Musiime, A. K., Smith, D. L., Kilama, M., Otto, G., Kyagamba, P., Rek, J., Conrad, M. D., Nankabirwa, J. I., Arinaitwe, E., & Akol, A. M. (2020). Identification and characterization of *Anopheles* larval aquatic habitats at three sites of varying malaria transmission intensities in Uganda. *Malaria Journal*, 19, 1-15

Mwesigwa, J., Okebe, J., Affara, M., Di Tanna, G. L., Nwakanma, D., Janha, O., Opondo, K., Grietens, K. P., Achan, J., & D'Alessandro, U. (2015). On-going malaria transmission in

The Gambia despite high coverage of control interventions: A nationwide cross-sectional survey. *Malaria Journal*, *14*(1), 1–9.

Myers, S. S., & Patz, J. A. (2009). Emerging threats to human health from global environmental change. *Annual Review of Environment and Resources*, *34*(1), 223–252.

Nabatanzi, M., Ntono, V., Kamulegeya, J., Kwesiga, B., Bulage, L., Lubwama, B., Ario, A., & Harris, J. (2022). Malaria outbreak facilitated by increased mosquito breeding sites near houses and cessation of indoor residual spraying, Kole district, Uganda, January-June 2019. *BMC Public Health*, *22*(1), 1–9.

Nambunga, I. H., Ngowo, H. S., Mapua, S. A., Hape, E. E., Msugupakulya, B. J., Msaky, D. S., Mhumbira, N. T., Mchwembo, K. R., Tamayamali, G. Z., & Mlembe, S. V. (2020). Aquatic habitats of the malaria vector, *Anopheles funestus* in rural south-eastern Tanzania. Malaria Journal, *19*, 1-11

Narahashi, T. (1988). Molecular and cellular approaches to neurotoxicology: Past, present and future. *Neurotox'88*, 269–288.

National Malaria Control Programme (NMCP). (2016). *Kenya malaria indicator survey 2015*. NMCP, KNBS, and ICF International Nairobi, Kenya, and Rockville, Maryland, USA. pp 1-7

Ndenga, B. A., Simbauni, J. A., Mbugi, J. P., Githeko, A. K., & Fillinger, U. (2011). Productivity of malaria vectors from different habitat types in the western Kenya highlands. *PLoS One*, *6*(4), e19473.

Ndenga, B., Githeko, A., Omukunda, E., Munyekenye, G., Atieli, H., Wamai, P., Mbogo, C., Minakawa, N., Zhou, G., & Yan, G. (2014). Population dynamics of malaria vectors in western Kenya highlands. *Journal of Medical Entomology*, *43*(2), 200–206.

Ngowo, H. S., Hape, E. E., Matthiopoulos, J., Ferguson, H. M., & Okumu, F. O. (2021). Fitness characteristics of the malaria vector *Anopheles funestus* during an attempted laboratory colonization. *Malaria Journal*, *20*(1), 148. https://doi.org/10.1186/s12936-021-03677-3

Nkya, T. E., Akhouayri, I., Poupardin, R., Batengana, B., Mosha, F., Magesa, S., Kisinza, W., & David, J.-P. (2014). Insecticide resistance mechanisms associated with different environments in the malaria vector *Anopheles gambiae*: A case study in Tanzania. *Malaria Journal*, *13*(1), 28.

Noor, A. M., & Alonso, P. L. (2022). The message on malaria is clear: Progress has stalled. *The Lancet*, *0*(0). https://doi.org/10.1016/S0140-6736(22)00732-2

Noor, A. M., Gething, P. W., Alegana, V. A., Patil, A. P., Hay, S. I., Muchiri, E., Juma, E., & Snow, R. W. (2009). The risks of malaria infection in Kenya in 2009. *BMC Infectious Diseases*, *9*(1), 1–14.

Norris, D. E. (2002). Genetic markers for study of the anopheline vectors of human malaria. *International Journal for Parasitology*, *32*(13), 1607–1615.

Norris, L. C., & Norris, D. E. (2015). Phylogeny of anopheline (Diptera: Culicidae) species in southern Africa, based on nuclear and mitochondrial genes. *Journal of Vector Ecology: Journal of the Society for Vector Ecology*, *40*(1), 16–27. https://doi.org/10.1111/jvec.12128

Oberemok, V. V., Laikova, K. V., Gal'chinsky, N. V., Useinov, R. Z., Novikov, I. A., Temirova, Z. Z., Shumskykh, M. N., Krasnodubets, A. M., Repetskaya, A. I., & Dyadichev, V. V. (2019). DNA insecticide developed from the Lymantria dispar 5.8 S ribosomal RNA gene provides a novel biotechnology for plant protection. *Scientific Reports*, *9*(1), 6197.

Ochomo, E., Bayoh, M. N., Brogdon, W. G., Gimnig, J. E., Ouma, C., Vulule, J. M., & Walker, E. D. (2013). Pyrethroid resistance in Anopheles gambiae ss and *Anopheles arabiensis* in western Kenya: Phenotypic, metabolic and target site characterizations of three populations. *Medical and Veterinary Entomology*, *27*(2), 156–164.

Ogola, E. O., Fillinger, U., Ondiba, I. M., Villinger, J., Masiga, D. K., Torto, B., & Tchouassi, D. P. (2018). Insights into malaria transmission among *Anopheles funestus* mosquitoes, Kenya. *Parasites & Vectors*, *11*(1), 577. https://doi.org/10.1186/s13071-018-3171-3

Ogola, E. O., Odero, J. O., Mwangangi, J. M., Masiga, D. K., & Tchouassi, D. P. (2019). Population genetics of *Anopheles funestus*, the African malaria vector, Kenya. *Parasites & Vectors*, *12*(1), 15. https://doi.org/10.1186/s13071-018-3252-3

Ohba, S.-Y., Kawada, H., Dida, G. O., Juma, D., Sonye, G., Minakawa, N., & Takagi, M. (2010). Predators of *Anopheles gambiae sensu lato* (Diptera: Culicidae) larvae in wetlands, western Kenya: confirmation by polymerase chain reaction method. *Journal of Medical Entomology*, *47*(5), 783–787.

Okiro, E. A., Alegana, V. A., Noor, A. M., & Snow, R. W. (2010). Changing malaria intervention coverage, transmission and hospitalization in Kenya. *Malaria Journal*, *9*(1), 285.

O'Meara, W. P., Mangeni, J. N., Steketee, R., & Greenwood, B. (2010). Changes in the burden of malaria in sub-Saharan Africa. *The Lancet Infectious Diseases*, *10*(8), 545–555.

Orr, B. K., & Resh, V. H. (1992). Influence of *Myriophyllum aquaticum* cover on *Anopheles* mosquito abundance, oviposition, and larval microhabitat. *Oecologia*, *90*(4), 474–482.

Ototo, E. N., Mbugi, J. P., Wanjala, C. L., Zhou, G., Githeko, A. K., & Yan, G. (2015). Surveillance of malaria vector population density and biting behaviour in western Kenya. *Malaria Journal*, *14*(1), 244.

Paaijmans, K. P., Jacobs, A. F. G., Takken, W., Heusinkveld, B. G., Githeko, A. K., Dicke, M., & Holtslag, A. A. M. (2008). Observations and model estimates of diurnal water temperature dynamics in mosquito breeding sites in western Kenya. *Hydrological Processes: An International Journal*, *22*(24), 4789–4801.

Pasteur, N., & Raymond, M. (1996). Insecticide resistance genes in mosquitoes: Their mutations, migration, and selection in field populations. *Journal of Heredity*, *87*(6), 444–449.

Patel, H., Ewels, P., Peltzer, A., Manning, J., Botvinnik, O., Sturm, G., Garcia, M. U., Moreno, D., & Vemuri, P. (2024). nf-core/rnaseq: Nf-core/rnaseq v3. 14.0-Hassium Honey Badger. *Zenodo Https://Doi. Org/10.5281/Zenodo*, *10471647*.

Patouillard, E., Griffin, J., Bhatt, S., Ghani, A., & Cibulskis, R. (2017). Global investment targets for malaria control and elimination between 2016 and 2030. *BMJ Global Health*, *2*(2), e000176.

Patz, J. A., Olson, S. H., Uejio, C. K., & Gibbs, H. K. (2008). Disease emergence from global climate and land use change. *Medical Clinics of North America*, *92*(6), 1473–1491.

Potemkin, N., Cawood, S. M., Treece, J., Guévremont, D., Rand, C. J., McLean, C., Stanton, J.-A. L., & Williams, J. M. (2022). A method for simultaneous detection of small and long RNA biotypes by ribodepleted RNA-Seq. *Scientific Reports*, *12*(1), 621.

Powell, M., Pyati, P., Cao, M., Bell, H., Gatehouse, J. A., & Fitches, E. (2017). Insecticidal effects of dsRNA targeting the Diap1 gene in dipteran pests. *Scientific Reports*, *7*(1), 15147.

President Malaria Initiative. (2022). U.S. President's Malaria Initiative Kenya. *Malaria Operational Plan FY 2022*. www.pmi.gov. pp1-6

Qiao, J., Du, Y., Yu, J., & Guo, J. (2019). MicroRNAs as Potential Biomarkers of Insecticide Exposure: A Review. *Chemical Research in Toxicology*, *32*(11), 2169–2181. https://doi.org/10.1021/acs.chemrestox.9b00236

Rakotondranaivo, T., Randriamanarivo, S. F., Tanjona, M. R., Vigan-Womas, I., Randrianarivelojosia, M., & Ndiath, M. O. (2018). Evidence of Insecticide Resistance to Pyrethroids and Bendiocarb in *Anopheles funestus* from Tsararano, Marovoay District, Madagascar. *BioMed Research International*, *2018*, 5806179. https://doi.org/10.1155/2018/5806179

Ranson, H., & Hemingway, J. (2005). Mosquito glutathione transferases. *Methods in Enzymology*, *401*, 226–241.

Ranson, H., Jensen, B., Vulule, J. M., Wang, X., Hemingway, J., & Collins, F. H. (2000). Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Molecular Biology*, *9*(5), 491–497.

Ranson, H., & Lissenden, N. (2016). Insecticide resistance in African *Anopheles* mosquitoes: A worsening situation that needs urgent action to maintain malaria control. *Trends in Parasitology*, *32*(3), 187–196.

Ranson, H., Rossiter, L., Li, F., Jensen, B., Wang, X., Roth, C. W., Collins, F. H., & Hemingway, J. (2001). Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochemical Journal*, *359*(2), 295–304.

Reid, M. C., & McKenzie, F. E. (2016). The contribution of agricultural insecticide use to increasing insecticide resistance in African malaria vectors. *Malaria Journal*, *15*(1), 1–8.

Riveron, J. M., Chiumia, M., Menze, B. D., Barnes, K. G., Irving, H., Ibrahim, S. S., Weedall, G. D., Mzilahowa, T., & Wondji, C. S. (2015). Rise of multiple insecticide resistance in *Anopheles funestus* in Malawi: A major concern for malaria vector control. *Malaria Journal*, *14*(1), 344.

Riveron, J. M., Huijben, S., Tchapga, W., Tchouakui, M., Wondji, M. J., Tchoupo, M., Irving, H., Cuamba, N., Maquina, M., & Paaijmans, K. (2019). Escalation of pyrethroid resistance in the malaria vector *Anopheles funestus* induces a loss of efficacy of piperonyl butoxide–based insecticide-treated nets in Mozambique. *The Journal of Infectious Diseases*, *220*(3), 467–475.

Riveron, J. M., Tchouakui, M., Mugenzi, L., Menze, B. D., Chiang, M.-C., & Wondji, C. S. (2018). Insecticide resistance in malaria vectors: An update at a global scale. In *Towards malaria elimination-a leap forward*. IntechOpen.

Riveron, J. M., Yunta, C., Ibrahim, S. S., Djouaka, R., Irving, H., Menze, B. D., Ismail, H. M., Hemingway, J., Ranson, H., & Albert, A. (2014a). A single mutation in the *GSTe2* gene allows tracking of metabolically based insecticide resistance in a major malaria vector. *Genome Biology*, *15*(2), 1–20.

Riveron, J. M., Yunta, C., Ibrahim, S. S., Djouaka, R., Irving, H., Menze, B. D., Ismail, H. M., Hemingway, J., Ranson, H., & Albert, A. (2014b). A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. *Genome Biology*, *15*(2), R27.

Roberts, D. R., Chareonviriyaphap, T., Harlan, H. H., & Hshieh, P. (1997). Methods of testing and analyzing excito-repellency responses of malaria vectors to insecticides. *Journal of the American Mosquito Control Association*, *13*(1), 13–17.

Rogers, A. R., & Harpending, H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, *9*(3), 552–569.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*(3), 539–542.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*, *34*(12), 3299–3302.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425.

Samb, B., Dia, I., Konate, L., Ayala, D., Fontenille, D., & Cohuet, A. (2012). Population genetic structure of the malaria vector *Anopheles funestus*, in a recently re-colonized area of the Senegal River basin and human-induced environmental changes. *Parasites & Vectors*, *5*(1), 1–9.

Sandeu, M. M., Mulamba, C., Weedall, G. D., & Wondji, C. S. (2020). A differential expression of pyrethroid resistance genes in the malaria vector *Anopheles funestus* across Uganda is associated with patterns of gene flow. *Plos One*, *15*(11), e0240743.

Schulze, S. K., Kanwar, R., Gölzenleuchter, M., Therneau, T. M., & Beutler, A. S. (2012). SERE: Single-parameter quality control and sample comparison for RNA-Seq. *BMC Genomics*, *13*(1), 1–9.

Scott, J. G. (1999). Cytochromes P450 and insecticide resistance. *Insect Biochemistry and Molecular Biology*, *29*(9), 757–777.

Service, M. (2012). *Medical Entomology for Students* (fifth). Cambridge University Press, New York, www.cambridge.org.

Shaalan, E. A.-S., & Canyon, D. V. (2009). Aquatic insect predators and mosquito control. *Tropical Biomedicine*, *26*, 223–261.

Shokoples, S. E., Ndao, M., Kowalewska-Grochowska, K., & Yanow, S. K. (2009). Multiplexed real-time PCR assay for discrimination of *Plasmodium* species with improved sensitivity for mixed infections. *Journal of Clinical Microbiology*, *47*(4), 975–980.

Silver, J. B. (2007). *Mosquito ecology: Field sampling methods*. springer science & business media.

Sinka, M. E., Bangs, M. J., Manguin, S., Coetzee, M., Mbogo, C. M., Hemingway, J., Patil, A. P., Temperley, W. H., Gething, P. W., & Kabaria, C. W. (2010). The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: Occurrence data, distribution maps and bionomic précis. *Parasites & Vectors*, *3*(1), 117.

Snow, R. W., Kibuchi, E., Karuri, S. W., Sang, G., Gitonga, C. W., Mwandawiro, C., Bejon, P., & Noor, A. M. (2015). Changing malaria prevalence on the Kenyan coast since 1974: Climate, drugs and vector control. *PloS One*, *10*(6), e0128792.

Sobti, S. K. (1968). A new species of the *Anopheles funestus* Complex (diptera: Culicidae) from Zanzibar, United Republic of Tanzania. *Bulletin of the World Health Organization*, *38*(3), 481.

Sougoufara, S., Diédhiou, S. M., Doucouré, S., Diagne, N., Sembène, P. M., Harry, M., Trape, J.-F., Sokhna, C., & Ndiath, M. O. (2014). Biting by *Anopheles funestus* in broad daylight after use of long-lasting insecticidal nets: A new challenge to malaria elimination. *Malaria Journal*, *13*(1), 125.

Sparks, T. C., & Nauen, R. (2015). IRAC: Mode of action classification and insecticide resistance management. *Pesticide Biochemistry and Physiology*, *121*, 122–128.

Spillings, B. L., Brooke, B. D., Koekemoer, L. L., Chiphwanya, J., Coetzee, M., & Hunt, R. H. (2009). A new species concealed by *Anopheles funestus Giles*, a major malaria vector in Africa. *The American Journal of Tropical Medicine and Hygiene*, *81*(3), 510–515.

Sun, H., Sun, L., He, J., Shen, B., Yu, J., Chen, C., Yang, M., Sun, Y., Zhang, D., Ma, L., & Zhu, C. (2011). Cloning and characterization of ribosomal protein S29, a deltamethrin resistance associated gene from *Culex pipiens pallens*. *Parasitology Research*, *109*(6), 1689–1697. https://doi.org/10.1007/s00436-011-2443-z

Sun, X. H., Xu, N., Xu, Y., Zhou, D., Sun, Y., Wang, W. J., Ma, L., Zhu, C. L., & Shen, B. (2019). A novel miRNA, miR-13664, targets CpCYP314A1 to regulate deltamethrin resistance in *Culex pipiens pallens*. *Parasitology*, *146*(2), 197–205.

Takken, W., & Verhulst, N. O. (2013). Host preferences of blood-feeding mosquitoes. *Annual Review of Entomology*, *58*, 433–453.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution*, *38*(7), 3022-3027

Tan, W., Sun, L., Zhang, D., Sun, J., Qian, J., Hu, X., Wang, W., Sun, Y., Ma, L., & Zhu, C. (2007). Cloning and overexpression of ribosomal protein L39 gene from deltamethrin-resistant Culex pipiens pallens. *Experimental Parasitology*, *115*(4), 369–378.

Temu, E. A., Minjas, J. N., Tuno, N., Kawada, H., & Takagi, M. (2007). Identification of four members of the *Anopheles funestus* (Diptera: Culicidae) group and their role in Plasmodium falciparum transmission in Bagamoyo coastal Tanzania. *Acta Tropica*, *102*(2), 119–125.

Thellier, M., Simard, F., Musset, L., Cot, M., Velut, G., Kendjo, E., & Pradines, B. (2020). Changes in malaria epidemiology in France and worldwide, 2000–2015. *Medecine et Maladies Infectieuses*, *50*(2), 99–112.

Touré, Y. T. (1982). Study of *Anopheles funestus* and *Anopheles gambiae sensu lato* susceptibility to insecticides in a rural area of Sudan savanna in Mali, chlorinated hydrocarbons, organophosphorus insecticides, carbamate insecticide. Cahiers ORSTOM Serie Entomologie Medicale et Parasitologie, *20*(2)

Trape, J.-F., Tall, A., Diagne, N., Ndiath, O., Ly, A. B., Faye, J., Dieye-Ba, F., Roucher, C., Bouganali, C., & Badiane, A. (2011). Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bednets and artemisinin-based combination therapies: A longitudinal study. *The Lancet Infectious Diseases*, *11*(12), 925–932.

Trape, J.-F., Tall, A., Sokhna, C., Ly, A. B., Diagne, N., Ndiath, O., Mazenot, C., Richard, V., Badiane, A., & Dieye-Ba, F. (2014). The rise and fall of malaria in a west African rural community, Dielmo, Senegal, from 1990 to 2012: A 22 year longitudinal study. *The Lancet Infectious Diseases*, *14*(6), 476–488.

Vaschetto, L. M., & Beccacece, H. M. (2019). The emerging importance of noncoding RNAs in the insecticide tolerance, with special emphasis on *Plutella xylostella* (Lepidoptera: Plutellidae). *WIREs RNA*, *10*(5), e1539. https://doi.org/10.1002/wrna.1539

Veron, V., Simon, S., & Carme, B. (2009). Multiplex real-time PCR detection of *P. falciparum*, *P. vivax* and *P. malariae* in human blood samples. *Experimental Parasitology*, *121*(4), 346–351.

Vezenegho, S. B., Bass, C., Puinean, M., Williamson, M. S., Field, L. M., Coetzee, M., & Koekemoer, L. L. (2009). Development of multiplex real-time PCR assays for identification of members of the *Anopheles funestus* species group. *Malaria Journal*, *8*, 282. https://doi.org/10.1186/1475-2875-8-282

Vitucci, E. C., & McCullough, S. D. (2022). *Isolation of Total RNA with the Zymo Quick-RNA Miniprep Kit*, pp 1-3

Walker, E. D., Merritt, R. W., & Wotton, R. S. (1988). Analysis of the distribution and abundance of *Anopheles quadrimaculatus* (Diptera: Culicidae) larvae in a marsh. *Environmental Entomology*, *17*(6), 992–999.

Walker, K., & Lynch, M. (2007). Contributions of Anopheles larval control to malaria suppression in tropical Africa: Review of achievements and potential. *Medical and Veterinary Entomology*, *21*(1), 2–21. https://doi.org/10.1111/j.1365-2915.2007.00674.x

Walldorf, J. A., Cohee, L. M., Coalson, J. E., Bauleni, A., Nkanaunena, K., Kapito-Tembo, A., Seydel, K. B., Ali, D., Mathanga, D., & Taylor, T. E. (2015). School-age children are a reservoir of malaria infection in Malawi. *PloS One*, *10*(7), e0134061.

Wamae, P. M., Githeko, A. K., Menya, D. M., & Takken, W. (2010). Shading by napier grass reduces malaria vector larvae in natural habitats in Western Kenya highlands. *EcoHealth*, *7*(4), 485–497.

Weedall, G. D., Irving, H., Hughes, M. A., & Wondji, C. S. (2015a). Molecular tools for studying the major malaria vector *Anopheles funestus*: Improving the utility of the genome using a comparative poly(A) and Ribo-Zero RNAseq analysis. *BMC Genomics*, *16*(1), 931. https://doi.org/10.1186/s12864-015-2114-z

Weedall, G. D., Irving, H., Hughes, M. A., & Wondji, C. S. (2015b). Molecular tools for studying the major malaria vector *Anopheles funestus*: Improving the utility of the genome using a comparative poly(A) and Ribo-Zero RNAseq analysis. *BMC Genomics*, *16*, 931. https://doi.org/10.1186/s12864-015-2114-z

Weedall, G. D., Mugenzi, L. M. J., Menze, B. D., Tchouakui, M., Ibrahim, S. S., Amvongo-Adjia, N., Irving, H., Wondji, M. J., Tchoupo, M., Djouaka, R., Riveron, J. M., & Wondji, C. S. (2019). A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy. *Science Translational Medicine*, *11*(484). https://doi.org/10.1126/scitranslmed.aat7386

Weedall, G. D., Riveron, J. M., Hearn, J., Irving, H., Kamdem, C., Fouet, C., White, B. J., & Wondji, C. S. (2020). An Africa-wide genomic evolution of insecticide resistance in the malaria vector *Anopheles funestus* involves selective sweeps, copy number variations, gene conversion and transposons. *PLoS Genetics*, *16*(6), e1008822.

Weeraratne, T. C., Surendran, S. N., Walton, C., & Karunaratne, S. H. P. (2018). Genetic diversity and population structure of malaria vector mosquitoes *Anopheles subpictus*,

Anopheles peditaeniatus, and *Anopheles vagus* in five districts of Sri Lanka. *Malaria Journal*, *17*(1), 1–8.

WHO. (1975). *Manual on practical entomology in malaria. Part 2, Methods and techniques*. Geneva : World Health Organization ; [London] : [H.M.S.O.] Vol. 9, pp 1-67

WHO. (1992). Entomological field techniques for malaria control. Part II. Tutor's guide. World Health Organization, pp 3-78

WHO. (2012). Global plan for insecticide resistance management in malaria vectors. World Health Organization, pp1-56

WHO. (2015). Global technical strategy for malaria 2016-2030. World Health Organization, pp 35

WHO. (2016). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, pp1-20

WHO. (2018a). Global report on insecticide resistance in malaria vectors: 2010–2016.pp 1--27

WHO. (2018b). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes Global Malaria Programme. Geneva: World Health Organization—Google Search. pp1-45 https://www.google.com/search?q=Test+procedures+for+insecticide+resistance+monitoring+in+malaria++vector+mosquitoes+Global+Malaria+Programme.+Geneva%3A+World+Health++Organization&sxsrf=ALiCzsbGph6ABp0D7Y73OkaVMx4hfdUHCw%3A1654093382190&source=hp&ei=RnaXYrq3B4WRgQbPrJvoBw&iflsig=AJiK0e8AAAAAYpeEVnGX8NP17BUKnSzxMZxGFeYHEObQ&ved=0ahUKEwi68t2Duoz4AhWFSMAKHU_WBn0Q4dUDCAc&uact=5&oq=Test+procedures+for+insecticide+resistance+monitoring+in+malaria++vector+mosquitoes+Global+Malaria+Programme.+Geneva%3A+

World+Health++Organization&gs_lcp=Cgdnd3Mtd2l6EANQAFgAYO8IaABwAHgAgAEAiAEAkgEAmAEAoAECoAEB&sclient=gws-wiz

WHO. (2018c). WHO, World Malaria Report 2018. pp 7-35 WHO. http://www.who.int/malaria/publications/world-malaria-report-2018/en/

WHO,. (2020). World malaria report 2020: 20 years of global progress and challenges. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO. pp 3-40

WHO. (2021). World malaria report 2021. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021. pp 4-59

Wondji, C. S., Hearn, J., Irving, H., Wondji, M. J., & Weedall, G. (2022). RNAseq-based gene expression profiling of the *Anopheles funestus* pyrethroid-resistant strain FUMOZ highlights the predominant role of the duplicated CYP6P9a/b cytochrome P450s. *G3*, *12*(1).

Wondji, C. S., Morgan, J., Coetzee, M., Hunt, R. H., Steen, K., Black, W. C., Hemingway, J., & Ranson, H. (2007). Mapping a quantitative trait locus (QTL) conferring pyrethroid resistance in the African malaria vector *Anopheles funestus*. *BMC Genomics*, *8*(1), 34.

World Health Organisation. (2015). *Global technical strategy for malaria 2016-2030. World Health Organisation*, pp 35

Wragge, S.-E., Venter, N., Touré, D., Hunt, R. H., & Coetzee, M. (2021). New distribution record of *Anopheles rivulorum-like* from Sadiola, Mali, with notes on malaria vector insecticide resistance. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *115*(5), 495–499.

Wu, Y.-J., Wu, Y.-J., Luo, X., Shen, X.-L., & Zhao, D.-G. (2014). Identification of differentially expressed genes that potentially confer pest resistance in transgenic ChIFN-γ tobacco. *Gene*, *543*(2), 181–189.

Xiao, H., Ma, C., Peng, R., & Xie, M. (2024). Insights into the role of non-coding RNAs in the development of insecticide resistance in insects. *Frontiers in Genetics*, *15*, 1429411.

Yasuoka, J., & Levins, R. (2007). Impact of deforestation and agricultural development on anopheline ecology and malaria epidemiology. *The American Journal of Tropical Medicine and Hygiene*, *76*(3), 450–460.

Zhang, Y., Zhang, C., Yang, R., Luo, C., Deng, Y., Liu, Y., Zhou, H., & Zhang, D. (2022). Molecular phylogeny of *Anopheles nivipes* based on mtDNA-COII and mosquito diversity in Cambodia-Laos border. *Malaria Journal*, *21*(1), 1–12.

Zhong, Y., Tang, Z., Huang, L., Wang, D., & Lu, Z. (2020). Genetic diversity of Procambarus clarkii populations based on mitochondrial DNA and microsatellite markers in different areas of Guangxi, China. Mitochondrial DNA Part A, 31(2), 48–56.

Zhou, G., Afrane, Y. A., Vardo-Zalik, A. M., Atieli, H., Zhong, D., Wamae, P., Himeidan, Y. E., Minakawa, N., Githeko, A. K., & Yan, G. (2011). Changing patterns of malaria epidemiology between 2002 and 2010 in Western Kenya: The fall and rise of malaria. *PloS One*, *6*(5), e20318.

Zhou, G., Lee, M.-C., Githeko, A. K., Atieli, H. E., & Yan, G. (2016a). Insecticide-treated net campaign and malaria transmission in western Kenya: 2003–2015. *Frontiers in Public Health*, *4*, 153.

Zhou, G., Lee, M.-C., Githeko, A. K., Atieli, H. E., & Yan, G. (2016b). Insecticide-treated net campaign and malaria transmission in western Kenya: 2003–2015. *Frontiers in Public Health*, *4*, 153.

Zhu, B., Xu, M., Shi, H., Gao, X., & Liang, P. (2017). Genome-wide identification of lncRNAs associated with chlorantraniliprole resistance in diamondback moth *Plutella xylostella* (L.). *BMC Genomics*, *18*(1), 380. https://doi.org/10.1186/s12864-017-3748-9

Zogo, B., Koffi, A. A., Alou, L. P. A., Fournet, F., Dahounto, A., Dabiré, R. K., Baba-Moussa, L., Moiroux, N., & Pennetier, C. (2019). Identification and characterization of *Anopheles spp*. breeding habitats in the Korhogo area in northern Côte d'Ivoire: A study prior to a Bti-based larviciding intervention. *Parasites & Vectors*, *12*(1), 146.

Zouache, K., Fontaine, A., Vega-Rua, A., Mousson, L., Thiberge, J.-M., Lourenco-De-Oliveira, R., Caro, V., Lambrechts, L., & Failloux, A.-B. (2014). Three-way interactions between mosquito population, viral strain and temperature underlying chikungunya virus transmission potential. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1792), 20141078.