POPULATION GENETICS OF *ANOPHELES FUNESTUS* AND *ANOPHELES GAMBIAE* (DIPTERA: CULICIDAE) WITH REFERENCE TO INSECTICIDE RESISTANCE ON MAINLAND NCHELENGE AND THE NEIGHBOURING ISLANDS ON LAKE MWERU IN ZAMBIA

by

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(BSc., UNZA, Zambia; MSc., UZ, Zimbabwe)

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

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FEBRUARY, 2024

DECLARATION

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

Signature

Date

APPROVAL

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

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DEDICATION

I dedicate this thesis to my lovely wife, Lydia Fibwe Muleba; for supporting me throughout the long and winding journey of my studies. To our parents, thank you for providing a solid foundation and inspiration that is highly cherished.

My children, Adrian Mbanga Muleba, Fredrick Mukupa Muleba and Lydia Monde Muleba, you where the reason I would work even harder when I felt like giving up. I hope when you grow up, you will enjoy reading and get inspired by this thesis.

To my research family and to the national programme I hope this work will contribute to better understanding and engagement in the fight against malaria. Thank you all for contributing in so many ways during my studies.

ABSTRACT

Vector control strategies based on available entomological information have not impacted malaria transmission in Nchelenge. Prior studies carried out in the district determined the identity and bionomics of the main malaria vectors as well as their status of insecticide resistance. However, this information was confined to the mainland and inadequately covered malaria vector populations. An understanding of the population attributes of the major vectors in different areas of the district was needed. The overall goal of this study was to generate valuable information on malaria vector population genetics and insecticide resistance for the National Malaria Elimination Programme. The study included two major malaria vector mosquito species in the district, namely, *Anopheles* (*Cellia*) *funestus* s.s. Giles, 1900, and *Anopheles* (*Cellia*) *gambiae* s.s. Giles, 1902. The insecticide classes tested included carbamates, organochlorines, organophosphates, and pyrethroids. A total of 6068 *Anopheles* mosquitoes were collected indoors from four sites, including islands on Lake Mweru in Nchelenge district, between 2014 and 2016. A representative subsample of the collection was processed for molecular and ELISA laboratory analyses, and 394 specimens of this sub-sample had a fragment of their ITS2 DNA sequenced. Vector infectivity was assessed, and insecticide resistance tests were conducted on both wild and F1 mosquitoes. Based on the ITS2 sequences in both vectors, there was diversity within populations with no fixed genetic differences across the collection sites. *Anopheles gambiae* was more abundant on Kilwa island (71%; n = 178), and *An. funestus* was on Chisenga island (78%; n = 4275). *Anopheles funestus* was more abundant during the dry season (76%) and predominated in every collection except one from Kilwa Island. *Anopheles gambiae* was abundant during the rainy season (65%). Malaria infectivity in *Anopheles funestus* spanned all sites and seasons. Kilwa Island and Mainland both had 3.7% of *An. funestus* tested (n = 164) and (n = 82), respectively, infected with *P. falciparum sporozoites* in the dry season, and 7.1% and 5.9% of *An. gambiae tested* (n = 84) and (n = 17), respectively, in the rainy season. Chisenga Island had 3.2% of the tested *An. funestus* (n = 188) infected with *P. falciparum*. Insecticide resistance, i.e., mortality less than 98%, was recorded against pyrethroids (mortality, 35–84%) and carbamates (mortality, 38–96%). No resistance was recorded against organophosphates (mortality, 100%). *Anopheles funestus* was susceptible to organochlorines (mortality, 100%), in contrast with *An. gambiae* (mortality, 10%). Resistance intensity to deltamethrin (time to 50% mortality, 2–5 hours) and bendiocarb (time to 50% mortality, 2–7 hours) was very high in *An. funestus*. All 88 *An. gambiae* that were tested had the knockdown resistance (kdr west) allele (L1014F), and 14% of them also had the east kdr (L1014S). Insecticide resistance on islands against carbamates could be due to cross-resistance conferred through the widely used pyrethroid-treated bed nets. Vector control should be scaled up and embrace new-generation LLINs with piperonyl butoxide to manage resistance in island vector populations.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin-based Combination Therapy
ATCC	American Type Culture Collection
AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
CDC	Centres for Disease Control and Prevention
cDNA	(complementary Deoxyribonucleic acid)
COI	Cytochrome Oxidase Subunit 1
CSO	Central Statistical Office
CSP	Circumsporozoite
DDT	Dichloro-diphenyl-trichloroethane
DHA-PPQ	Dihydroartemisinin-Piperaquine
DHO	District Health Office
DnaSP	DNA Sequence Polymorphism
DNTPs	(deoxyribonucleotide triphosphates)
Da	Number of net nucleotide substitutions
DRC	Democratic Republic of Congo
Dxy	Number of Average Nucleotide substitutions
E8	Elimination 8
EDTA	(ethylene-diamine-tetra-acetic acid)
EIR	Entomological inoculation rate
ELISA	Enzyme-Linked Immunosorbent Assay
EST	Expressed Sequence Tag
ESRI	Environmental Systems Research Institute
FANG	Insecticide susceptible *Anopheles funestus* mosquito strain from Angola
F_{ST}	Fixation index
GMAP	Global Malaria Action Plan
GenAIex	Genetic Analysis in Excel
GIS	Geographical Information Systems

GPS	Global Positioning System
GST	Glutathione-S-transferase
G_{ST}	Coefficient of Differentiation
GSTe2	Glutathione S-transferase epsilon 2 gene
h	Number of haplotypes
Hd	Haplotype Diversity
HCs	Health Centres
HIV	Human Immunodeficiency Virus
HWE	Hardy-Weinberg Equilibrium
HWP	Hardy-Weinberg Principle
ICEMR	International Centres of Excellence in Malaria Research
ICT	Information Communication Technology
IGS	Intergenic Spacer gene
IPT	Intermittent Preventive Therapy
IPTp	Intermittent Preventive Treatment in pregnancy
IRR	Incidence Rate Ratio
IRS	Indoor Residual Spraying
IRTWG	Insecticide Resistance Technical Working Group
ITCZ	Intertropical Convergence Zone
ITNs	Insecticide-Treated Nets
ITS2	Internally Transcribed Spacer gene2
IVCC	Innovative Vector Control Consortium
IVM	Integrated Vector Management
K	Average number of genetic differences
Kdr	Knockdown resistance
KCM	Konkola Copper Mines
LLINs	Long-lasting Insecticidal Nets
MCMC	Markov Chain Monte Carlo tests
MoH	Ministry of Health
MR4	Malaria Research and Reference Reagent Resource Centre
Mw	Williams mean

NIH	National Institutes of Health
Nm	Number of migrants
NSTC	National Science and Technology Council
OR	Odds Ratio
PBO	Pyperonyl Butoxide
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PMI	President's Malaria Initiative
POPs	Persistent Organic Pollutants
QGIS	Quantum Geographic Information System
RAPD	Randomly Amplified Polymorphic DNA
RBM	Roll Back Malaria
RDT	Rapid Diagnostic Test
RFLP	Restriction Fragment Length Polymorphism
RHCs	Rural Health Centres
RNA	Ribonucleic Acid
S	Number of segregating nucleotide sites
SDS	Sodium Dodecyl Sulphate
SNP	Single Nucleotide Polymorphism
SUA	Suakoko Mosquito Strain
SSR	Simple Sequence Repeats
TB	Tuberculosis
Taq	*Thermus aquaticus*
TDRC	Tropical Diseases Research Centre
UPGMA	Unweighted Pair-Group Method with Arithmetic Mean
UNZA	University of Zambia
VCRU	Vector Control and Reference Unit
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme
ZNMEC	Zambia National Malaria Elimination Centre

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CHAPTER 1: INTRODUCTION

1.1 Background.

Mosquitoes are responsible for the transmission of some of the most lethal human pathogens known globally, such as malaria plasmodia, chikungunya virus, dengue fever virus, equine encephalitis virus, yellow fever virus, and Zika virus, among others. Malaria is certainly the most important and commonly widespread disease caused by mosquito-transmitted pathogens. Mosquitoes of the genus *Anopheles* are responsible for malaria disease transmission. The resulting diseases are the cause of untold human suffering. Socio-economic development is greatly hampered in mosquito-related disease-endemic countries, incurring costs in the process of seeking healthcare (Alonso et al., 2019; Kayiba et al., 2021) and due to human morbidity and mortality. Malaria remains a major public health problem in many countries around the world. In 2020 the disease was responsible for 229 million cases, leading to 409, 000 deaths in 87 endemic countries worldwide (World Malaria Report, 2020). These reported cases and deaths occurred despite the gains that have been achieved over a decade of scaled-up control efforts. Sub-Saharan Africa bears the heaviest burden of malaria, representing 94 percent and 95% of the reported cases and deaths, respectively, on a global scale (World Malaria Report, 2020). In Zambia, over 4 million malaria cases occur annually, and malaria accounts for approximately 30% of outpatient attendances and is responsible for nearly 8,000 deaths annually (ZNMEC, 2015). Sustained control of malaria with effective preventive and curative tools has led to a major reduction in the general disease burden across the country between 2003 and 2009 (Chizema-Kawesha et al., 2010). This reduction, however, has not been uniform throughout the country. Since 2010, annual reports of malaria-related in-patient cases and deaths have decreased by 52 percent and 65%, respectively (National Malaria Elimination Center, 2015). However, mixed levels of transmission are found among adjacent communities within some districts and adjacent districts within some provinces (ZNMEC, 2015). In Nchelenge, malaria cases have remained sustained with minimal or no changes observed despite the scaled-up control measures (Mukonka et al., 2014). Malaria is a vector-borne parasitic disease caused by infection with one or more of five protozoan *Plasmodium* species, namely *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knwolesi* (*Cox, 2010*). The four human malaria parasites; *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae,* are transmitted from person to person via a bite from an infected

female *Anopheles* mosquito vector. In recent years, *P. knowlesi* previously only known to infect monkeys in forested areas of Southeast Asia, has been found to infect humans as well. The transmission of *P. knowelesi* happens when the *Anopheles* female mosquito, infected from feeding on an infected monkey, bites a human. A person infected with malaria usually experiences fever, headaches, chills, and vomiting. A complicated form of the disease can lead to anaemia, respiratory distress, and cerebral malaria, usually in children. In adults, other forms of organ failure could be involved and may lead to a condition known as black water fever.

Malaria is both preventable and treatable. Preventive measures include sleeping under an insecticide-treated net (ITN), using mosquito repellents, and indoor residual spraying (IRS). In the case of an infection, prompt treatment with an effective antimalarial drug would be a life-saving measure. Drug and insecticide resistance in the parasite and the mosquito**,** respectively, are current issues threatening malaria control. The global campaign to eliminate malaria using proven control interventions in the treatment of the disease and vector control to prevent transmission is gaining ground in some regions. In southern Africa, eight countries including Zambia, have formed a partnership called, Elimination 8 (E8), to coordinate efforts for malaria elimination (ZNMEC, 2015).

1.2 Malaria burden in Nchelenge

Malaria is an enormous health problem in Nchelenge district, with a prevalence between 30 and 50% (Moss et al., 2015; Hast et al., 2019). *Anopheles funestus* Giles (1900) and *An. gambiae* Giles (1902) are the major vector species responsible for the transmission of malaria in the district. Transmission of malaria occurs throughout the year, with peaks in the dry season coinciding with peak numbers in *An. funestus,* which is the principal malaria vector for the district (Das et al., 2016; Stevenson et al., 2016). *Anopheles gambiae* is the second major vector, occurring at lower numbers when compared to *An. funestus.* Peak abundances in *An. gambiae* populations happen during the rainy season, thereby maintaining the transmission of malaria during periods of low numbers in *An. funestus*. The sustained transmission of malaria in Nchelenge district is happening despite the malaria control interventions that have been put in place by the national control programme to mitigate the disease burden. The combination of control

interventions applied includes indoor residual spraying (IRS), long-lasting insecticidal nets (ILLNs), treatment of disease with artemisinin combination therapy (ACT), and intermittent preventive treatment in pregnancy (IPTp). Why are these proven malaria control interventions not making an impact on the disease burden in Nchelenge? Elsewhere in Zambia, malaria has been reduced using these same interventions (Sharp et al., 2002; Chizema-Kawesha et al., 2010). This question was at the heart of the larger research project titled Malaria Transmission and Impact of Control Efforts in Southern Africa, run by the Southern Africa International Centres of Excellence for Malaria Research (ICEMR). This was a collaborative study involving local and international teams of scientists from a consortium of research and academic institutions. This group of research experts has been conducting studies on malaria in Nchelenge since 2012. The Southern Africa ICEMR was set up to determine factors contributing to malaria transmission in three different epidemiological settings in southern Africa. Two of these epidemiological settings are in Zambia and include an area in the country where malaria control hasn't achieved the desired impact, which is represented by Nchelenge district in northern Zambia. The other is an area where control has had an impact on malaria transmission, this is Choma District, in southern Zambia. The third epidemiological setting is an area where control was previously achieved but has been experiencing malaria resurgence, this is the Mutasa district in north-eastern Zimbabwe (Moss et al., 2015).

Important findings regarding malaria transmission in Nchelenge have been brought to the fore in the last eight to ten years that the ICEMR study has been conducted in the district. Key findings have attributed the determinants of malaria in the district to a number of factors. These include human economic activities, environmental risk factors, and aspects of malaria vectors. Studies on the vectors have brought out insights into the malaria vector's blood feeding behaviour, habitat partitioning, vector bionomics, and insecticide resistance (Choi et al., 2014; Das et al., 2015, 2016, 2017; Stevenson et al., 2016). Weighed in light of the current practices in the timing and deployment of the control interventions, the findings from these studies suggest a different approach to addressing the malaria problem in Nchelenge. These findings may have hampered the goal of achieving national malaria elimination by 2021. All these insightful studies had been restricted to the mainland of Nchelenge District. The malaria problem in the

district may not be fully appreciated by focusing studies only on one side of the district. In order to fully understand the magnitude of the malaria vector problems in the whole district, a well-represented eco-geographic and biologic sampling of the vector indices was required. Vector population biology and how this was influenced by different micro- and macro-environments characteristic of the district were needed.

1.3 Problem statement

There is failure of malaria control in Nchelenge district despite the implementation of LLINs, IRS, ACTs, and IPT malaria control interventions by the national control programme. In other parts of the country, malaria declined following the scaled-up implementation of these interventions, including the treatment of patients with the drug Coartem (Chizema-Kawesha et al., 2010). Nchelenge, on the other hand, experienced a rise in the prevalence of malaria cases, affecting 50.5% of the under-five children despite the scale-up of interventions (Masaninga et al., 2013; Mukonka et al., 2014). Although the problem of malaria control could be attributed to a number of factors within the district, the malaria vectors could be responsible for the largest part of the problem. Therefore, a clear understanding of the biological and ecological aspects of the malaria vectors in the district was required. Previous entomological surveys carried out in Nchelenge revealed that two malaria vector species, *An. gambiae* s.s. and *An. funestus* s.s., were responsible for malaria transmission and had shown resistance to commonly used insecticides in the district (Choi et al., 2014; Mukonka et al., 2014). However, critical information on the occurrence, distribution of insecticide resistance, and seasonal variations of these vector mosquito attributes across the greater district was lacking. Understanding the important aspects of vector bionomics and insecticide resistance patterns in the district would be key to the strategy and implementation of effective vector control measures that would impact malaria transmission.

The overall goal of this study was to generate information on malaria vector population genetics and insecticide resistance that would be useful to the National Malaria Elimination Programme in Zambia. The information would be useful in developing more effective and locally adaptable vector control strategies in Nchelenge district. High levels of genetic differentiation in mosquito populations due to both physical and biological barriers to gene flow, even between

neighbouring areas exist (Moreno et al., 2007; Lehmann et al., 2003). The implications of this on a vector control programme utilising the same approach and same set of control measures uniformly across the district cannot be overemphasised. Effective vector control strategies were urgently needed in Nchelenge District. Vector control for the district was and would remain the major component in the fight against malaria. Unless new evidence was made available to the malaria control programme**,** the current and planned vector control measures for the district would continue to be applied in much the same way they have been done across the district, with little or no impact. The physical environment of the study area, consisting of the mainland and islands with human habitations separated by a mix of varying areas of water bodies, wet/dry land, and vegetation cover, gave an indication of the complex variation in vector population attributes. This may call for the application of specific control measures that would be appropriate to specific local situations rather than the available standard coverage of control interventions. The Nchelenge mainland and nearby islands provided a unique opportunity for the study of malaria vector population genetics in Zambia. Further, identifying directions and barriers to gene flow among sub-populations of malaria vectors in Nchelenge would give important information for tracking and planning the implementation of effective vector control tools. There hadn't been an intensive, study on the population genetics of malaria vectors in Nchelenge. Understanding variations in population genetics of the two major vectors of the district would add an avenue that would be explored to strengthen vector control.

1.4 Study aim and objectives.

The aim or general objective of this study was to determine the population structures and variations of *Anopheles funestus* s.s. and *An. gambiae* s.s. populations, the major malaria vectors in Nchelenge district, in order to predict genetic movement and barriers to gene flow among target populations with reference to insecticide resistance and the impact of vector control interventions in the district.

1.4.1 Specific objectives:

The specific objectives of the study were to:

(i) Determine the population genetic structures of the major malaria vectors in Nchelenge District, namely, *An. funestus* s.s. and *An. gambiae* s.s.

(ii) Determine the proportions, distribution, and intensity of resistance to the insecticides DDT, pyrethroids, carbamates, and organophosphates in the malaria vector populations in Nchelenge district.

(iii) Determine the association between vector species abundance and *P. falciparum* sporozoite rates with study site and/or vector control interventions.

1.4.2 Research questions.

This study was set up to answer the following questions about malaria vector populations and insecticide resistance in Nchelenge district and the surrounding areas:

(i) Were there genetic structures in populations of *Anopheles funestus* s.s. and *An. gambiae* s.s., and barriers to gene flow across the study sites?

(ii) Was the distribution of insecticide resistance in the district related to past use of an insecticide in particular study sites affecting its future use in vector control?

(iii) Were vector species abundance and *P. falciparum* sporozoite infection rates associated with the ecology and/or vector control interventions in the study sites?

1.5 Study hypotheses.

The following hypotheses were tested in this study:

(i) There were no variations in population genetic structures of either *An. funestus* or *An. gambiae,* within and between the mainland and islands.

(ii) The current and past insecticide-based vector control interventions have had no effect on insecticide susceptibility status in malaria vector species in Nchelenge.

(iii) There were no spatial and temporal variations in vector species abundance and infection with *P. falciparum* sporozoites attributable to specific ecologies, and/or vector control activities in the district.

CHAPTER 2: LITERATURE REVIEW

2.1 History of malaria.

Anopheles mosquitoes have been transmitting diseases to humans since prehistoric times, in the absence of human knowledge. Regarding malaria, references to the symptoms of the disease can be seen in old records from China, Mesopotamia, Egypt, India, and the early Greeks spanning from 2700 to 400 BC (Cox, 2010; Garcia, 2010). During the latter part of this period, in Rome, man began to associate malaria with marshy areas (Sallares, 2002; Hempelmann and Krafts, 2013). The Italian word *mal'aria* meaning 'bad air' or 'corrupted air' is widely held as the origin of the name given to the disease malaria. The cause of malaria remained unknown for many years until 1880, when Charles Louis Alphonse Laveran discovered the parasite in human red blood cells (Arrow et al., 2004). In 1889, Ronald Ross demonstrated avian malaria parasites in mosquito salivary glands, and Italian scientists, notably Giovanni Battista Grassi, found human malaria parasites in *Anopheles* mosquitoes. These discoveries formed the basis of scientific studies that led to a wealth of information regarding malaria epidemiology and its transmission currently known by humans.

2.2 Malaria the disease

There are five species of the *Plasmodium* genus that cause human malaria. The most lethal and severe of these malaria causing pathogens is *Plasmodium falciparum,* which is found in sub-Saharan Africa and is responsible for over 90% of the global malaria cases (WHO, 2020). The other species of *Plasmodium* causing human malaria are *P. malariae*, *P. ovale,* and *P. vivax.* Recently, a fifth malaria parasite has been added to the list of those infectious to humans (White, 2008). It is the zoonotic parasite *P. knowlesi,* previously only known to infect primates. *Plasmodium knowlesi* is transmitted to humans when a monkey-malaria-infected mosquito bites a human. In terms of geographical coverage, *P. vivax* is widespread and occurs throughout the world. *Plasmodium vivax* is the main cause of malaria outside Africa (Adams and Mueller, 2017) and, unlike *P. falciparum*, has a high chance of relapsing even after treatment due to the existence of quiescent liver-stage parasites known as hypnozoites.

Malaria is transmitted to humans when an infected female *Anopheles* mosquito injects the sporozoite stage of the parasite into the human blood circulation system during biting to obtain a blood meal. Within the human body, the parasite undergoes phases of development through different stages of asexual multiplication, initially in the liver and later in the red blood cells. Parasite multiplication in the human host causes increased invasion and destruction of red blood cells, causing fevers, anaemia, and other associated complications of malaria. A portion of the malaria parasites transform into gametocytes (Henry et al., 2019) during the blood stage, and the mosquito picks them up while feeding on blood. Sexual development takes place in the mosquito midgut. The male and female parasite gametocytes undergo fertilisation within the lumen of the mosquito midgut resulting in a zygote. The moving zygote (ookinete) breaks through the mosquito gut wall and turns into an oocyst. The oocyst goes through a series of changes until the infectious stage, called sporozoites, are developed and enter the mosquito's bloodstream. The circulating sporozoites eventually accumulate in the mosquito salivary glands. The next person the mosquito bites will get the infection and the parasite cycle continues.

2.3 Scale of the malaria Burden

Malaria due to *P. falciparum* is the most deadly and accounts for more than 90% of the malaria cases reported in Zambia (Steketee et al., 2008). The most at-risk populations are children under the age of five, pregnant women, and travellers from non-malarious regions. In 2019, out of an estimated 33 million pregnancies worldwide, 35% (12 million) were exposed to malaria infections and 67% of the deaths were of children under the age of 5 (WHO, 2020).

The past decade has seen unprecedented international coordinated efforts against malaria. Global estimates for complete coverage of malaria control interventions for the most at-risk populations stand at US$ 5.6 billion per year (WHO, 2020), but this figure has never been realised as the finances received for malaria control have averaged below the global requirements. More funding is required to fight the disease on three major fronts that include chemotherapy, vector control, and prevention for those at risk. Artemisinin-based combination medicines like artemether-lumefantrine (e.g., Coartem) have replaced sulfadoxine - pyrimethamine (Fansidar) single dose and chloroquine as the first line of treatment due to *P. falciparum* resistance to these drugs (Greenwood and Mutabingwa,

2002). Recently, another drug, Dihydroartemisinin-piperaquine (DHA+PPQ), has been added to complement four other ACTs already recommended for the treatment of uncomplicated malaria (World Health Organisation, 2010). Insecticide-based vector control efforts have been scaled up in many malaria endemic countries with positive impacts (Steketee and Campbell, 2010; Oguttu et al., 2017; Musiime et al., 2019; Rocha et al., 2020; Feng et al., 2022).

2.3.1 Malaria vectors

There are over 3500 species of mosquitoes distributed worldwide in both tropical and temperate regions, only absent from a few islands and Antarctica (Service, 2008). There are about 38 genera of mosquitoes, all belonging to the family Culicidae (Service, 2008) in the order Diptera. The culicids comprise three mosquito subfamilies namely; Culicinae, Toxorhynchitinae, and Anophelinae. The Culicinae include two genera of medical importance: *Aedes* and *Culex*. The Anophelinae comprise one genus of medical importance, namely, *Anopheles*. These are the three important genera of hematophagous species of mosquitoes involved in human and animal disease transmission. These mosquito species also transmit viruses in addition to parasitic protozoa and parasitic nematodes, which are the disease-causing agents. Apart from malaria, other diseases transmitted by mosquitoes include Yellow Fever, Rift Valley Fever, Lymphatic Filariasis, Dengue, Japanese Encephalitis, chikungunya and o'nyong-nyong (Gillies and De Meillon, 1968; Brault et al., 2004; Bessaud et al., 2006; Tolle, 2009; Nasci, 2014). These diseases are the cause of significant human morbidity and mortality in the world today. Among all the vector-borne diseases, malaria causes the most harm to the wellbeing of humans.

The vectors that transmit malaria belong to the mosquito species of the genus *Anopheles*. The genus comprises about 500 species. *Anopheles* mosquitoes are known to occur worldwide, but only about 20 species have been identified to transmit malaria. Different groups of vector species notably transmit malaria in different regions of the world (Sinka et al., 2010, 2012). In the Americas, *An. darlingi* Root, *An. albimanus* Wiedemann, and *An. quadrimaculatus* Say are the major vectors. In the Europe and Middle East region, *An. atroparvus* van Thiel, *An. messeae* Falleroni, and *An. superpictus* Grassi are the dominant vectors. In Asia and the Mekong region (southeast Asia), *An. culicifacies* Giles, *An. fluviatilis* James, *An. stephensi* Liston, *An. dirus* Peyton & Harrison, and *An. minimus*

Theobald are the major transmitters of malaria. In Africa, *An. gambiae* Giles, *An. arabiensis* Patton, and *An. funestus* Giles are the major vectors responsible for malaria transmission in sub-Saharan Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). These three major malaria vector species responsible for malaria transmission in sub-Saharan Africa are found in Zambia (Gillies and De Meillon, 1968). Nchelenge District is home to *An. funestus* and *An. gambiae* (Das et al., 2016).

The female *Anopheles* mosquitoes are responsible for the transmission of malaria because, unlike their male counterparts, they need a blood meal supplement for the production of eggs. In Zambia, these three species are all present with varied distribution across the country (Gillies and De Meillon, 1968). A consolidated, up-to-date map of the distribution of these species across the country, however, is not yet available.

2.3.2 Identification of malaria vectors species.

To effectively conduct vector control, accurate knowledge of the identity, abundance, and distribution of the vectors is needed. Traditionally, mosquitoes are identified morphologically using identification keys that describe morphological features. The main features used include the length, shape, markings, hairs, and scales of their mouth parts, antennae, wings, legs, and abdomen. However, oftentimes, these malaria vectors may exist as a species group or a species complex (Collins and Paskewitz, 1996). A complex comprises morphologically very similar, difficult to distinguish, but genetically different species. In that case, additional, more invasive identification methods are used after the morphological identification. Earlier post-morphological identification methods included the use of species-specific allozymes and polytene chromosomal inversions (Collins and Paskewitz, 1996). These methods were commonly used to distinguish species. Additional alternative methods to diagnose species have been developed. These include cuticular hydrocarbon profile analyses and several techniques based on the use of DNA. Currently, molecular methods utilising the polymerase chain reaction (PCR) technique are widely used (Scott et al., 1993; Koekemoer et al., 2002). These methods are simple and robust (e.g., requiring just a leg to extract DNA and any insect developmental stage, for identification).

2.3.3 The *Anopheles funestus* complex

The *Anopheles funestus* complex comprises eleven sibling species (Gillies and Coetzee, 1987; Harbach, 2004). Members of the group can occur alone in a given area or ~~in~~ sympatrically with others within the group/complex in the area. *Anopheles funestus* group mosquitoes are much smaller and darker in colour compared to *An. gambiae* complex mosquitoes. The nine originally identified sibling species include *An. funestus* Giles 1900, *An. rivulorum* Leeson 1935, *An. parensis* Gillies 1962, *An. fluviatilis* James 1902, *An. leesoni* Evans 1931, *An. fuscivenosus* Leeson 1930, *An. aruni* Sobti 1968 , *An. brucei* Service 1960, and *An. confusus* Evans and Leeson 1935 (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The more recently recognised species in the group include *An. vaneedeni*, Gillies and Coetzee (1987), identified in South Africa (Gillies and Coetzee, 1987), while the most recently identified species in the complex is *An. rivulorum*-like sp. (Cohuet et al., 2003), identified in Cameroon. The latter ~~It~~ is morphologically very similar to *An. rivulorum*, and *An. funestus*-like from Malawi has morphological similarity to *An. funestus* but differs genetically (Spillings et al., 2009). This species, *An. funestus*-like, has not been reported to carry *P. falciparum* parasites to date. Following these recent developments, Harbach (2004) restructured the taxonomic classification of the *An. funestus* group. The group is now divided into subgroups of sibling species namely the *An. funestus* subgroup (*An. funestus*, *An. vaneedeni*, *An. parensis*, *An. aruni*, *An. confusus*, and *An. funestus*-like), the *An. aconitus* subgroup, the *An. culicifacies* subgroup, the *An. minimus* subgroup *(An. leesoni*, amongst others), and the *An. rivulorum* subgroup (*An. brucei*, *An. rivulorum*, *An. rivulorum*-like and *An. fuscivenosus*). *Anopheles funestus* s.s. is highly endophilic and anthropophilic (Gillies and De Meillon, 1968). They feed indoors on humans, preferentially to the exclusion of other alternative hosts that might be present (Gillies and De Meillon, 1968). Until now, *An. rivulorum*, which is usually zoophilic, is the only other species from the *An. funestus* group, that has been implicated in the transmission of malaria in nature in Tanzania (Cohuet et al., 1996). The other members of this group are zoophilic and do not transmit malaria to humans (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Cohuet et al., 2003). *Anopheles vaneedeni* has been shown to support malaria parasite development under laboratory conditions, but has not been identified as a malaria vector in nature

2.3.4 The *Anopheles gambiae* complex

The *An. gambiae* complex now comprises eight recognised sibling species with the addition of two new species; *An. coluzzi,* formerly the M-form of *An. gambiae* s.s., and *An. amharicus,* formerly *An. quadriannulatus* species B (Hunt et al., 1998; Coetzee et al., 2013). *An. gambiae* complex are among the most important vectors of malaria in sub-Saharan Africa (Gillies and Coetzee, 1987). This complex consists of morphologically indistinguishable species that differ in their ecology, behaviour, and feeding habits (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Hunt et al., 1998; Coetzee et al., 2013). The members of the *An. gambiae* complex include *An. gambiae* Giles, 1902; *An. arabiensis* Patton, 1905; *An. merus* Dönitz, 1902; *An. melas* Theobald, 1903; *An. bwambae* White, 1985; *An. quadriannulatus* Theobald, 1911; *An. coluzzi*, Coetzee and Wilkerson, 2013; and *An. amharicus*, Hunt, Wilkerson, and Coetzee, 2013 (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee et al., 2013). Among the members of the complex, *An. gambiae* and *An. coluzzi* are regarded as the most important vectors of malaria in sub-Saharan Africa, where the highest number of malaria cases and deaths occur annually. *Anopheles arabiensis* ranks second within this complex, extending malaria transmission into the drier seasons and geographically drier areas further north and south of sub-Saharan Africa (Collins and Hill, 2005). *Anopheles coluzzi* is distributed in the west and part of the central African region (Coetzee et al., 2013). Three of the minor vectors include *An. merus*, a saltwater breeder found on the eastern side of the African continent; *An. melas,* another saltwater breeder found on the western coast; and *An. bwambae,* found in only one locality in Uganda(Gillies and De Meillon, 1968; White, 1985; Gillies and Coetzee, 1987). *Anopheles amharicus* and *An. quadriannulatus,* the two zoophilic members of the complex, are found in Ethiopia and most of southern Africa, respectively (Gillies and Coetzee, 1987; Coetzee et al., 2013).

2.3.5 Breeding sites

Anopheles funestus has been known to prefer breeding in shallow, stagnant, or slowly moving, more permanent, and large freshwater bodies with some vegetation (Gillies and Coetzee, 1987). Examples of these water bodies would be swamps, ponds, marshlands, etc. Studies in Mali have shown *An. funestus* to also

breed in rice cultivation areas (Dolo et al., 2004; Sinka et al., 2010). *Anopheles gambiae* prefers temporal, clean, and well sunlit sites (Gillies and Coetzee, 1987). These sites occur following rains where mosquitoes may breed in footprints, temporary pools on edges of large water bodies, tyre ruts, etc. These characteristic breeding sites for *An. gambiae* generally occur in rural areas, and thus the frequency of the species in rural areas would be high compared to urban areas. In urban areas, however, *An. gambiae* has been shown to breed in uncharacteristic sites such as domestic water containers and even polluted water (Chinery, 1984; Awolola et al., 2007).

The geographic distribution of these malaria vector species spans the tropics and subtropical regions of Africa. The region provides favourable temperatures and humidity for their development. This encompasses the south of the Sahara Desert and the northern part of the Republic of South Africa (Gillies and De Meillon, 1968). Their distributions and vector statuses vary both spatially and temporally (Shililu et al., 1998; Kelly-Hope and McKenzie, 2009) across this expanse. In places where they occur together, their contribution to malaria transmission also varies at different times of the year. In general, *An. funestus* may be responsible for most of the transmission during the dry season, while *An. gambiae* contributes to transmission during the wet season and shortly after the rains (Gillies and De Meillon, 1968).

2.3.6 Lifecycle and feeding habits

The developmental life cycle of the mosquito involves four stages: egg, larva, pupa, and adult. The female adult mosquito has mouthparts that are well adapted for piercing, cutting through the skin into the capillary beds and sucking human and other vertebrate blood. Generally, both male and female mosquitoes thrive on sugars found in plant juices, fruits, and flowers, but the female must eventually take a blood meal as this is the source of protein needed for the development of her eggs. Before a mosquito becomes an adult the availability of water is essential to support the aquatic stages in its life cycle, i.e. the egg, larva and pupa.

Which mosquito vector becomes the major transmitter of malaria in a given area is determined by a given set of conditions, including the biology of the vector itself and the parasite, the availability of the host, and other environmental factors. The

two most important attributes of an efficient vector are its preference to feed on human blood and the length of time it lives after a blood meal. For most vector species, at least one week is needed after a blood meal to allow for complete development of the malaria parasite and subsequent transmission to a human host (Beier, 1998; Bennink et al., 2016). As a result, not all anophelines serve as vectors of malaria. Different vector species can be found in diverse habitats according to their species-specific ecological niches, with possible overlaps (Sinka et al., 2012). Members of a species complex, consisting of morphologically identical cryptic species but adapted to different sets of requirements, will also have different vectorial capacities and will have varied roles in malaria transmission spatially and temporally. The status of *An. funestus* and *An. gambiae* as major vectors of human malaria stems from the fact that they preferentially feed on human blood (Gillies and De Meillon**,** 1968). They are often more likely than other *Anopheles* mosquito species to inhabit places near human dwellings, enter houses, and feed at night (Gillies and De Meillon**,** 1968). Their habits of feeding indoors (endophagy) and resting indoors (endophily) make these vectors vulnerable to the vector control strategies instituted against them. However, reports of outdoor biting and outdoor resting (Reddy et al., 2011; Chaumeau et al., 2020; Keïta et al., 2021; Kinya et al., 2022) would have negative implications for these indoor-based control strategies.

2.4 Vector control.

The use of insecticide-treated bed nets or indoor residual spraying Vector control intervention are designed to reduce the contact between the human host and the disease-carrying mosquitoes entering and resting inside human dwellings. Vector control has immensely contributed to the shrinking of many vector-borne diseases (Wilson et al., 2020). Over the years, vector control strategies have undergone transformation. From control based on a complex understanding of mosquito behaviour and ecology through simplistic heavy reliance on a few insecticide classes and back to evidence-based targeted control as outlined in the following section.

2.4.1 Past control efforts.

The earlier vector control strategies were non-chemical and emphasised the prevention of mosquito breeding and mosquito bites by draining or filling swamps or other water bodies that served as mosquito breeding sites. Application of oil or Paris green to breeding places, house screening, and use of mosquito nets were practised (Rieckmann, 2006; Smith et al., 2012). The discovery of dichloro-diphenyl-trichloroethane (DDT) in the 1940's was significant in the use of insecticides to control vector-borne diseases (Martini et al., 2021). Since then, insecticides have played a major role in organised and targeted vector control programmes (Mendis et al., 2009; Nájera et al., 2011). The insecticidal properties of DDT, such as being highly effective against indoor resting adult mosquitoes, having long-lasting residual effects, and being cheap to produce, made it the best candidate for use in the malaria eradication campaign in the 1950's and 1960s (Nájera et al., 2011). The newly discovered insecticide was sprayed indoors to target resting mosquitoes. Eradication of malaria succeeded in some areas but failed in many others due to many issues, including financial, technical, and, more importantly, the vector's development of resistance to DDT, leading to the suspension of control programmes (Nájera et al., 2011). Alternative methods of control were revived and included environmental management and biological control. To a large extent, the environmental and social changes in Europe driven by economic development led to the decline and eradication of malaria (Hay et al., 2004; Piperaki and Daikos, 2016). The use of DDT in many countries around the world was banned in the early 1970s due to its detrimental effects on the environment (Aguilar and Borrell, 2005). A few other insecticide classes have been developed over the years since the banning of DDT. These other insecticides have been more expensive and not widely applied like DDT, and have not been free of insecticide resistance (Curtis and Lines, 2000). The effect of this was a significant increase in malaria transmission, including in areas where the disease had been controlled (Nájera et al., 2011). Under the Stockholm Convention on persistent organic pollutants (POPs), the use of DDT has now been restricted to public health purposes only (WHO, 2011). The World Health Organisation supports the use of DDT in indoor residual spraying in African countries, including Zambia, where malaria is still a health problem (WHO, 2011).

2.4.2 Renewed control efforts.

Beginning in 1998, commitments to fight malaria on a global scale led to the establishment of the Roll Back Malaria (RBM) partnership. Partners from both governments and the private sector committed resources, personnel, and technical expertise to fight malaria (Chanda et al., 2008). Subsequent to the RBM partnership, further international declarations for malaria control and elimination with set targets have been made. Malaria was included in the Millennium Development Goals, with the target year for most of the declarations being 2015 (Narasimhan and Attaran, 2003; WHO, 2010, 2014). The list of declarations included the following:

i) The United Nations Millennium Declaration in 2000.

ii) The Abuja declaration involving African presidents in 2010 (WHO, 2010).

iii) The World Health Assembly Resolution WHA 58.2 in 2005.

iv) The Global Malaria Action Plan (GMAP) in 2008, refined/updated GMAP objectives, targets, milestones, and priorities beyond 2011.

Vector control was emphasised, and this was to be implemented in an integrated manner. Successful malaria vector control interventions were generally based on an integrated vector management (IVM) approach. Integrated vector management is a process for rational decision making and optimal use of the available resources for vector control (Curtis, 2008). This includes the following five elements.

a) Decisions should be made based on evidence.

b) Control options should be integrated.

c) There should be a multi-sectoral collaboration coordinated at all levels by the Ministries dealing with Public health.

d) There should be advocacy, social mobilisation, and legislation, and

e) The process should include capacity building.

In 2004, the World Health Organisation recommended the IVM approach for all vector-borne diseases (Curtis, 2008) and Zambia successfully adopted and

consolidated IVM during the scale-up of malaria control interventions (Chanda et al., 2008).

Renewed interest in malaria control by many global partners has led to malaria elimination being set as a goal in areas where transmission has been reduced (Maharaj et al., 2012). In Zambia, since 2003, support and goodwill from cooperating partners have resulted in the scaling-up of vector control efforts (Chanda et al., 2013). The interventions included indoor residual spraying and the distribution of insecticide-treated nets (ITNs) and long-lasting insecticidal nets (LLINs). Indoor residual spraying campaigns increased from 5 districts in 2003 to 72 districts by 2010. Over 6 million LLINs were distributed between 2005 and 2010 through mass campaigns and other free net distribution channels (Chanda et al., 2008; Steketee et al., 2008; Masaninga et al., 2018). To a lesser extent, in Zambia, vector control at the larval stage has been initiated by the National Malaria Control Programme but has only been implemented in a few areas. Case management with effective ACTs and presumptive treatment of pregnant women were implemented alongside the vector control interventions (National Malaria Elimination Center, 2015).

These concerted efforts had a general positive impact and brought down the cases of malaria for both in-patient cases and deaths by 66% (Masaninga et al., 2013). The sustained and integrated efforts in the fight against malaria led to a general reduction in transmission and a restructuring of the malaria epidemiology in Zambia. Before the scalling-up of interventions, Zambia was generally regarded as a highly malaria endemic country. The application of effective interventions transformed the country into three (3) epidemiological zones (Chanda et al., 2013; Masaninga et al., 2013). The first stratification or zone is made up of very low transmission regions with a parasite prevalence of less than 1%. The second stratification is medium transmission, with parasite prevalence between 1% and 10%. The third stratification is high transmission with a parasite prevalence of above 10%. Clearly, the impact on the prevalence of malaria was not uniform in all regions of the country. Some areas across the country experienced a significant reduction in malaria transmission, while others did not. For instance, cases in Luapula, Northern/Muchinga, and Eastern provinces saw a rise (Masaninga et al., 2013). The resurgence in malaria cases in some parts of the country was attributed to the reduction in financial support experienced between 2008 and 2010

(Masaninga et al., 2013). This meant that fewer resources than were needed were made available, and hence the coverage of control measures was low. Little attribution was given to the role malaria vector abundance and distribution play in malaria transmission in these epidemiological zones. Chemical control of insect pests has greatly improved health care and boosted agricultural production. The development of insecticide resistance, however, has limited the effectiveness of insecticides (Mallet, 1989). Insecticide resistance in malaria vectors is the main challenge to control programmes in endemic countries (Hargreaves et al., 2000; Hemingway and Ranson, 2000). In Zambia, the chemical options for IRS have been reduced following reports of insecticide resistance. In 2003, when vector control efforts were beginning to be scaled up in the country, baseline susceptibility tests demonstrated a 100% mortality rate of malaria vectors on all the insecticides tested (Chanda et al., 2011). At the beginning of the scale-up, deltamethrin and DDT were used in IRS in all the pilot districts of Zambia. However, six years down the line, susceptibilities to deltamethrin and DDT were reduced to between 13% and 61%, respectively, in *An. gambiae* in Ndola on the Copperbelt and the central regions of Zambia (Chanda et al., 2011). Resistance has also been recorded in *An. funestus* against carbamates (Choi et al., 2014). Carbamates were introduced much later into the national vector control programme. Sadly, the problem of insecticide resistance impacts many parts of Africa and beyond. This involves the major vectors of malaria and poses a great challenge to current malaria control efforts in Africa (Ranson and Lissenden, 2016).

2.5 Insecticide resistance

Insecticide resistance is defined as 'the development of an ability in a strain of some organisms to tolerate doses of a toxicant that would prove lethal to a majority of individuals in a normal population of the same species.' (Scott, 1999). This comes about in a population as a result of change in the genetic composition of individuals within that population as a result of the selective force of a toxicant (Ferrari, 1996).

Random mutations in specific genes can alter the normal physiological, morphological, or behavioural phenotype, leading to variations in the degree of response to a toxicant (Brogdon and McAllister, 1998). Resistant individuals may be rare initially, but with continuous exposure to the same insecticidal pressure, high proportions of resistant individuals would result. At the population level, individuals possessing mutations that enable them to survive exposure to an insecticide will eventually contribute more offspring to the population with the same resistance trait (Hemingway and Ranson, 2000; Hemingway et al., 2002). This will lead to a higher frequency of a resistant gene in the population.

Regular monitoring of susceptibility to the insecticides employed in IRS and LLIN campaigns should be an integral part of the vector control programme (Brogdon and McAllister, 1998) in order to effectively manage resistance. Studies on insecticide resistance involve detecting resistance in the field with the help of WHO and/or CDC bottle bioassays, which detect the phenotypic resistance. Field collected mosquitoes are then reared within the laboratory, where the genetic control of resistance and the mechanism/s are characterised.

2.5.1 Resistance mechanisms

In a broad sense, resistance mechanisms encompass four divisions, namely; a) reduced penetration, b) behavioural; c) metabolism; and d) target site insensitivity (Brogdon and McAllister, 1998). Many insecticides are contact toxicants, meaning they have to diffuse into the insect body through the cuticle. In resistant individuals, the cuticle gets modified so that the passage of the lethal insecticides through this structure is reduced (Ferrari, 1996). With this type of resistance mechanism, cuticular protein genes become up-regulated in resistant mosquitoes (Ibrahim et al., 2016), leading to hardening of the cuticle. An increased expression of genes involved with cuticular components was found among the 136 genes associated with an increase in pyrethroid resistance in *An. coluzzi* in Burkina Faso (Toé et al., 2014).

Behavioural resistance may result in insects avoiding entering houses sprayed with insecticide or exiting treated houses upon contact with insecticide on treated

surfaces (Carnevale and Manguin, 2021). In Tanzania, behavioural adaptations following the distribution of LLINs were noted in *An. funestus* s.l. and *An. arabiensis* (Kreppel et al., 2020). Behavioural resistance may be contributing to residual malaria transmission, stalling progress towards the control of malaria since 2000 (Keïta et al., 2021; Okumu and Finda, 2021; Kinya et al., 2022).

Target-site insensitivity and metabolic (detoxification enzyme-based) are probably the major and most important biochemical resistance mechanisms. In target site resistance, the insecticide is unable to bind to its site of activity in the organism due to a change in the shape of the insecticide binding site. Target site insensitivity resistance involves acetylcholinesterases and knockdown resistance (kdr), which has been reported to occur in *An. gambiae* in Zambia (Chanda et al**,** 2011). A single point mutation in the sodium channel gene is what causes target site insensitivity (Martinez-Torres et al., 1998; Ranson et al., 2000). This causes knockdown resistance (Martinez-Torres et al., 1998; Ranson et al., 2000). The mutation is characterised by either a leucine-phenylalanine (L1014F) mutation common in West Africa or a leucine-serine (L1014S) mutation common in East Africa. Kdr is a common resistance phenomenon conferring cross-resistance against DDT, pyrethroids, and cyclodiene, rendering them ineffective. In metabolic resistance, there are elevated levels of enzyme activity. Metabolic resistance involves groups of enzymes known as mono-oxygenases e.g., P450 based, as recently reported in *An. funestus* in Nchelenge (Choi et al., 2014), esterases, oxidases, hydrolases, and glutathione-S-transferases (GST). This leads to the neutralisation of the insecticide (Brogdon and McAllister**,** 1998) before it reaches the target site. Insecticide resistance involving up-regulation of cytochrome P450s is caused mostly by the CYP6 family of genes (Nikou et al., 2003; Lycett et al., 2006; Müller et al., 2008), which have been shown to play an important role in the detoxification of various insecticides in both *An. funestus* and *An. gambiae.* A recent study (Riveron et al., 2014) has shown that a single amino acid change, L119F in overexpressed glutathione S-transferase gene in *An. funestus* confers high levels of metabolic resistance to DDT and pyrethroids.

2.6 Population genetics

The British and American biologists Ronald Fisher, John Burdon Sanderson Haldane, and Sewall Wright pioneered the theoretical basis of population genetics (Crow, 2007). Their work resulted in the integration of natural selection with Mendelian genetics. This became critical to the unification of the operations of evolutionary theory (Larson, 2004). Following these developments, population genetics principles have been applied in the study of various biological systems, including malaria vectors. They vary from low to high complexity in organisms such as microorganisms and humans, respectively, as well as the interactions between the organisms and their physical environments. Essentially, population genetics is the study of allele frequency distributions, variation, and changes (Tabachnick and Black , 1995). The study of these variations, including the factors responsible, could be conducted over time and space. Insecticide resistance could be described as a living example of insect pests' adaptability to changing conditions. The management of insecticide resistance is an opportunity to showcase the application of evolutionary biology (Mallet, 1989). The application of population genetics to insecticide resistance has essentially been focused on resistance management. The goal has been to develop control strategies designed to prevent or delay the appearance of resistance in populations. This may involve strategies on populations subjected to pesticides for the first time or strategies which cause prevailing resistance to be reduced (Croft, 1977).

Population genetics in malaria vectors has become very useful in the understanding of the role particular mosquito species play in the transmission of disease (Tabachnick and Black**,** 1995). By applying mathematical models based on Mendel's laws of inheritance and modern evolutionary theory, the occurrence of specific alleles or combinations of alleles in a population can be investigated and/or predicted (Peiris et al., 2011). These studies have been carried out to understand vector population density fluctuations, vector competence, behaviour, and the spread of insecticide resistance genes. The studies have further explored the possibilities of using innovative vector control tools such as genetically modified vectors in disease control (Crampton, 1993; Diaz et al., 2011).

The factors that influence the variations in the allele frequencies will also influence the vectorial capacity and competency traits in disease-transmitting organisms (Tabachnick and Black, 1995). The allele frequency distribution and change can be influenced mainly through the four evolutionary processes; natural selection, genetic drift, mutation, and gene flow. Application of insecticides against malaria vectors provides a selection pressure that can lead to changes in the allele frequency distribution in the subject arthropod vector population. A few studies on vector population genetics have been done in Zambia on *An. gambiae* and *An. arabiensis* (Lehmann et al., 2003; Kent et al., 2007; Lobo et al., 2015). A good number of studies on vector population genetics have been carried out on *An. gambiae* in West Africa (Mukabayire et al., 2001; Gentile et al., 2002) and East Africa (Lehmann et al., 1998, 1999; Kamau et al., 2007). Recent studies on the population genetics of *An. funestus* in southern Africa based on mitochondrial DNA (Cohuet et al., 2005; Choi et al., 2012) identified the existence of two Clades (I and II) with unknown roles in their disease transmission or insecticide resistance. Most of the studies on malaria population genetics have compared genetic variations of populations separated by very wide distances, from hundreds to thousands of kilometres apart. Furthermore, few samples have been collected, which in some cases has taken more than 8 years (Lehmann et al., 2003). This situation may render it difficult to explain the spatial and temporal variations in the local vector populations or to suggest locally adaptable and effective vector control strategies.

2.6.1 Genetic markers

To study population genetics, a choice of an appropriate marker that can be used to describe a population attribute has to be made. Cost, study objectives, and general trends of currently widely used markers will all influence the choice of a marker to use. Genetic markers are genes or DNA sequences whose location on a chromosome is known and can be used for the identification of individuals or species (Scott et al., 1993). A genetic marker could be described as an observed variation arising from a mutation or an alteration in the genomic loci. Markers are used in natural population genetic studies to define the quantity and pattern of genetic variation. They help in the understanding of mating systems and inbreeding, as well as the taxonomic and phylogenetic relationships (Taylor, 1999) among species.

The array of genetic markers available for the study of malaria vectors (Norris, 2002) includes mitochondrial DNA, allozymes (allelic variants at a single enzyme locus), restriction fragment length polymorphism (RFLP), Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single nucleotide polymorphisms (SNPs), and simple sequence repeats (SSRs), also known as microsatellites. Allozymes have been extensively used and, at some point, were the markers of choice in studies of population genetics. They were considered to be user friendly, highly polymorphic, and co-dominant. Even with those qualities, however, allozymes have limitations. Not all organisms have allozymes in their nuclear DNA (Parker et al., 1998). This has led to the development of more robust polymerase chain reaction (PCR)-based genetic markers. These PCR-based markers provide more information with the application of sophisticated statistics. PCR-based markers can be used with sensitive data, such as that obtained, e.g., from microsatellites and other single-locus markers (Sunnucks, 2000; Geffen et al., 2007). At the moment, microsatellites are the best genetic markers because, unlike other markers, they are tandemly repeated motifs found in both coding and non-coding regions of the nuclear genome of eukaryotes (Jarne and Lagoda, 1996). The high rate of mutations in microsatellites renders them potentially the most informative markers with easy and relatively lower-cost detection by PCR (Geffen et al., 2007). New sequencing technologies have further eased the laborious approach and cost of their isolation. The existence of large databases of genomic and expressed sequence tag (EST) sequences makes screening by available bioinformatics tools possible and transferable to related species (Akemi et al., 2012). Microsatellites also have the great advantage of being co-dominant making it possible to detect both homozygous and heterozygous genotypes. Statistical Software packages used to analyse population genetics data are diverse and some of these, among many commonly used, include GENEPOP (Raymond and Rousset, 1995), STRUCTURE (Pritchard et al., 2000), Arequine (Excoffier et al., 2006), etc.

2.7 Entomological studies

Entomological studies in public health are carried out to answer different aspects of arthropod-borne disease transmission. These surveys provide information on the

occurrence, distribution, and composition of disease vector species (Gnanguenon et al., 2014; Tokponnon et al., 2023). In order to plan interventions and mount defences against potential disease outbreaks, it is crucial to have established entomological surveillance programmes (Hagenlocher and Castro, 2015; Jourdain et al., 2019). Entomological information is important in the selection of available control options as well as the measurement of the impact of interventions on vector populations (Wagman et al., 2021) and assessing their effectiveness in reducing the disease burden in humans (Padonou et al., 2012; Ossè et al., 2013; Hamainza et al., 2016; Kenea et al., 2019) There is a wide array of options for mosquito collection techniques depending on the behaviour of the vector, the measured entomological parameters, and the objectives of the study (Mboera, 2005; Onyango et al., 2013).

2.7.1 Sampling techniques

The question of which mosquito sampling technique should be used for estimation of the mosquito man-biting rate in different settings arises when implementing vector control programmes. The actual estimation of man-biting rates remains a difficult undertaking (Service, 1993). There is general agreement within the medical entomology community that the most direct and accurate method of estimating the biting rate is the human landing catch (HLC) (Sikaala et al., 2013; Wong et al., 2013; Briët et al., 2015; Kenea et al., 2017). This involves a team of individuals sitting around collecting mosquitoes. This is accomplished with mouth aspirators and flashlights. The individuals would sit both inside and outside of the human dwellings, collecting mosquitoes attempting to feed on exposed body parts (usually legs). The timing of the collection is set between dawn and dusk, covering about 12 hours of mosquito nocturnal activity. This sampling technique has negative ethical implications. Those involved in collecting mosquitoes are exposed to unusually high risks of infectious mosquito bites and thus would require prophylactic treatment. In many instances, vector control programmes are faced with the problem of standardising entomological indicators and sampling methods for operational purposes (Wong et al., 2013). For instance, to determine the impact of a particular vector control intervention, one of the indicators looked at is the reduction of contact between infected mosquitoes and humans. This entomological indicator for measuring transmission intensity (Lines et al., 1991) is known as the

entomological inoculation rate (EIR). This is the product of the sporozoite rate and the man-biting rate. The EIR is the most important estimation of malaria transmission epidemiology as it gives a meaningful estimate of man-vector contact (Davis et al., 1995; Mboera, 2005). The EIR is calculated by the following formula.

$$EIR = maS$$

Where *ma* is the human-biting rate and *S* is the proportion of infected mosquitoes.

The estimated parameters are expressed as mosquito infectious bites received per person per unit of time, e.g., days, months, 6 months, or per year. Various mosquito sampling techniques have been compared in the estimation of the EIR (Mathenge et al., 2004; Mboera, 2005; Kilama et al., 2014).

Alternative methods to the HLC have been tried and Lines et al. (1991) and compared the CDC light trap hung next to individuals protected by untreated bed nets. They concluded that light traps in Tanzanian villages, when used as such, would catch a representative sample of mosquitoes that could have bitten humans sleeping in bed rooms. (Drakeley et al., 2003) collected mosquitoes by CDC light, applied an index of 1.605 to the formula, and estimated the annual EIR for Ifakara, Tanzania. Other workers in other parts of Africa have validated the applicability of the CDC light trap as a credible alternative to the HLC (Sikaala et al., 2013; Briët et al., 2015). When only light traps are considered in the estimation of EIR, leaving out the number of occupants in the house, the uncertainty (standard error) can be computed (Drakeley et al., 2003).

2.7.2 Species identification in the laboratory

Once mosquitoes are collected, they are identified morphologically, and usually the identification is confirmed molecularly. Morphological identifications using standard mosquito identification keys (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987) are carried out. Molecular identifications of vector species are done with different PCR protocols (Scott et al., 1993; Singh et al., 1999; Koekemoer et

al., 2002; Choi et al., 2013) based on their morphological identity (Singh et al., 1999; Koekemoer et al., 2002; Choi et al., 2013).

PCR is an *in vitro* method for amplifying (synthesising) DNA or RNA sequences in large amounts, starting with just a small quantity. Following its invention in 1985 (Mullis and Faloona, 1987; Rabinow, 1996), PCR became a standard laboratory procedure in a relatively short period of time (Hoy, 2013). It is a powerful tool that can be used to solve various problems in different fields of general science as well as basic and applied entomology. The list of possibilities that can be accomplished with PCR includes but is not limited to the following (Hoy**,** 2013):

Isolation of specific DNA fragments labelling of DNA endings; Cloning of genomic and complementary DNA (cDNA); Sequencing of DNA Mutation of specific DNA sequences; Alteration of DNA promoters; Quantitation of DNA and RNA amounts; Identification of molecular markers for taxonomic and ecological studies

The PCR procedure is automated and operates on a programme fed into the PCR machine. The programme runs through repeated cycles of temperatures that serve to denature template DNA, annealing and extending the primers to copy the DNA. Components of PCR include Primers, DNTPs, and template DNA (Hyndman and Mitsuhashi, 2003). These are made to react within a buffered environment under the enzymatic actions of taq polymerase (Saiki et al., 1988; McPherson, et al., 1991; Goodman and Fygenson, 1998). Prior to this, the template DNA is extracted in various ways, including the widely used salt method (Miller et al., 1988; Goldenberger et al., 1995; Steiner et al., 1995; Hammond et al., 1996; Aljanabi and Martinez, 1997). Key to the extraction process is ensuring that the DNA is free of proteases and other binding proteins that could inhibit DNA amplification.

Depending on the objectives of the study, the PCR technology has been modified to provide specific answers to specific questions, including where there is a lack of prior genetic information, when only a single DNA strand is required, or when the interest is in nucleotides up-stream or down-stream that of a specific gene.

In entomological research, the PCR technology could be applied to the detection and identification of pathogenic microorganisms in disease vectors. Pesticide resistance detection makes it possible to evaluate efficacy of control interventions.

2.7.3 *Plasmodium* sporozoite infection detection

The proportion of infected mosquitoes in a collection is another parameter that is considered an important factor in determining the transmission potential of malaria vectors. Three methods for detecting infected mosquitoes with *Plasmodium* sporozoites are used. Traditional methods involve dissection of mosquitoes and the use of light microscopes to detect and quantify malaria parasites in the salivary glands (Kumpitak et al., 2021). The choice of which detection method is used is usually a consideration of practicality, sensitivity, specificity, and through-put nature (Echeverry et al., 2017). Due to the cost of detecting sporozoites in mosquitoes (i.e., using ELISA and PCR), not all mosquitoes collected are processed. The traditional method is tedious and requires the examination of freshly collected specimens. With this method, a level of expertise and experience in mosquito dissection and microscopy are needed. However, microcopy cannot be used to distinguish *Plasmodium* species or CSP subtyping for *P. vivax* (Robert et al., 1988; Fontenille et al., 2001). The PCR methods can be used to detect and distinguish *Plasmodium* species in the upper part of the mosquito body but have limited parasite life stage specificity (Snounou et al., 1993; Singh et al., 1999; Snounou and Singh, 2002; Hendershot et al., 2021). Furthermore, PCR requires expensive equipment and well trained staff to run. The ELISA technique is a simple, high-throughput assay that can be used to examine a large number of wild mosquitoes to understand malaria transmission (Kumpitak et al., 2021). The ELISA method is prone to false positives and requires boiling of positive homogenates and re-run the assay (Durnez et al., 2011)

The detection of sporozoites involves preparing homogenates for an enzyme-linked immunosorbent assay (ELISA). Suitable ELISA readers visually read and quantify the results. The *P. falciparum* sporozoites are determined using circumsporozoite ELISA methods (Wirtz et al., 1987). The ELISAs have been developed to detect *P. falciparum*, *P. vivax* 210, and *P. vivax* 247

circumsporozoite (CS) proteins in malaria infected mosquitoes. The sensitivity and specificity of the ELISAs are based on the monoclonal antibodies used.

Mosquito heads and thoraces are crushed and dissolved in appropriate reagents before detection of sporozoites is carried out using the ELISA technique.
Some PCR and ELISA resources that include primers, genomic and cDNA, monoclonal antibodies, and positive controls are made available to the scientific community through collaborative work. The Beire Resources managed by the American type culture provides some of these reagents to registered scientists through the Malaria Research and Reference Reagents Resource (MR4) Centre.

2.7.4 Entomological research on malaria vectors in Zambia

Historically, in Zambia, initial research in malaria entomology was driven mainly by the need for immediate operational answers to vector control programmes in mining towns on the Copperbelt. The copper mines consulted with entomologists from other countries in the 1930s to aid in the management of malaria vectors. De Meillon (1937) found evidence of the malaria vectors, *An. gambiae* and *An. funestus,* resting in natives' houses and miners train shuttles in Mufulira. Previous work in the same area by Sir Malcom Watson had recommended larval control only, with an understanding that the vectors' flight range was no more than half a mile. De Meillon's finding added another dimension to vector control by targeting adult mosquitoes.

As part of a large study in many African countries, Paterson (1964) reported the sympatric existence of *An. gambiae s.l.* species A, B, and C with no hybrids in Chirundu. Back then, it was not clear whether to regard *Anopheles gambiae* as a single species or a species complex. At that time, species were separated based on taxonomic, behavioural, and cross-mating studies (Gillies and Coetzee**,** 1987). Paterson's finding was concrete evidence that these freshwater breeders were separate species. The three species, A, B, and C, would later be known, respectively, as *An. gambiae* s.s., *An. arabiensis,* and *An. quadriannulatus* (Mattingly, 1977). Only *An. gambiae* s.s. and *An. arabiensis* transmit malaria.

Anopheles quadriannulatus feeds on animals and has not been found to transmit malaria (Gillies and Coetzee, 1987).

Generally, between the 1970s and 1980s, work on malaria and its control took a dive due to a slowdown of the Zambian economy as a result of reduced copper prices (Sikamo et al., 2016). Few entomological studies in Chipata, the Eastern Province, and Chirundu, in the Southern Province, were reported (Shelley, 1973; Bransby-Williams, 1979).

At the end of the 20th century, the WHO-led global movement to control malaria rekindled interest in the disease. The rollback malaria (RBM) campaign was born. During the same period, the new owners of the Konkola Copper Mines (KCM) put up a successful malaria control programme based on indoor residual spraying (Sharp et al., 2002). With support from the global funds to fight malaria, HIV, and TB and the World Bank, as well as other partners, the national programme to scale up malaria based on the KCM approach was initiated in 2003 (Chanda et al., 2013). Sentinel sites for the collection of malaria indices were established across the country. The KCM engaged the local entomologists in the name of the TDRC to conduct studies and provide advice on the efficacy of their control programme.

By the year 2000, Johns Hopkins University had set up a site for entomological studies in Southern Zambia. Violet Siachinji, one of a few local entomologists from TDRC at the time, was engaged to carry out the first documented entomological studies in Macha. In her works in Macha, she established that *An. arabiensis* and *An. funestus* were vectors in Choma, Southern Province. Follow-up studies have noted a reduced *An. funestus* population around Macha following the 2008 drought. Further entomological studies have highlighted useful information regarding the feeding and resting behaviour of *Anopheles*, spatial and temporal genetic structure, and insecticide resistance (Kent et al., 2007; Norris and Norris Johns, 2010; Norris and Norris, 2011, 2015). Following the implementation of vector control with bed nets in 2007, studies carried out noted a positive impact on malaria transmission (Norris and Norris, 2013). Studies by Fornadel et al. (2010b) and Stevenson et al. (2014) have further shown the potential existence of

secondary vectors in the Macha area. These are very important findings that the current elimination efforts should take into consideration.

Other notable partners working at the national level include the Innovations for Vector Control Consortium (IVCC), the Liverpool School of Tropical Medicine and Hygiene, PATH's Malaria Control and Elimination Partnership in Africa (MACEPA), USAID's President's Malaria Initiative (PMI), and the International Centres of Excellence for Malaria Research (ICEMR). Chanda et al, (2012, 2013), have carried out various assessments and entomological studies in the country regarding the control programme. Their studies have documented processes, major gains, and challenges regarding the implementation of vector control tools in Zambia. Each partner mentioned above works in an area of the country, which unfortunately does not cover the whole of Zambia. It must also be mentioned that different compositions of vector species are encountered in different partner areas of operation. With so many partners coming on board to help in the fight against malaria, there has been accelerated information gathering on malaria vectors in Zambia in the last 10 years. Different workers in different parts of the country have contributed a lot of useful entomological information for use in vector control. Both local and international experts on malaria entomology have worked together to bring about a wealth of information on composition, feeding and resting behaviour, and malaria infectivity. Seyoum and others described the malaria vector's biting behaviour in *An. quadriannulatus,* a known zoophilic (bites animals) in Luangwa district (Seyoum et al., 2010, 2012).

2.7.4.1 Knowledge gap

Probably the most used entomological information provided to the national programme is data on insecticide resistance in different areas. Plans for procurement and deployment of IRS products have depended on the insecticide resistance data generated by partners (Chanda et al., 2013, 2016). Despite these efforts put in by different workers, malaria still continues to cause trouble in Zambia. The ICEMR group in Nchelenge encountered two species with complex vector bionomics (Das et al**.**, 2016**;** Stevenson et al**.**, 2016) not experienced by any other groups. Studies carried out so far have been restricted to the mainland and have not focused intently on the population genetics of the local vectors. When more molecular studies were included by one of the partners working in Luangwa

and Nyimba, a wealth of critical information on vector diversity was realised (Lobo et al., 2015). As the nation continues to grapple with malaria, more specific and effective control measures are needed. More studies on the malaria vector species in Nchelenge are very much needed.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study area

3.1.1 Introduction.

Nchelenge district was born out of a fish trading camp at Kashikishi, on the northern side of the present Nchelenge town (History of Nchelenge poster/map at Nchelenge District Commissioner's Office). The district is located at 9.3° S and 28.7° E in Luapula Province, northern Zambia. The Nchelenge district mainland largely borders Lake Mweru on the east and south, sharing an international border with the Democratic Republic of Congo (DRC) in the midsection of the lake in the west and southwest and local borders with three other Zambian districts on the north, east, and south (Figure 1). The district is situated at the mouth of the Luapula River, forming part of the Luapula-Mweru Delta. It is a rural district with an estimated human population of 147, 927 (CSO, 2011). The district is endowed with many water bodies and is home to one of the two naturally occurring Lakes in Zambia shared with another country, Lake Mweru. The lake sits between Zambia and the Democratic Republic of the Congo and plays host to a rich diversity of freshwater fish species that are consumed in the two countries. The Luapula-Mweru system interface lines-up with swamps, islands, and lagoons. Prominent amongst the islands on the Zambian side are Chisenga, Kilwa, and Isokwe Islands.

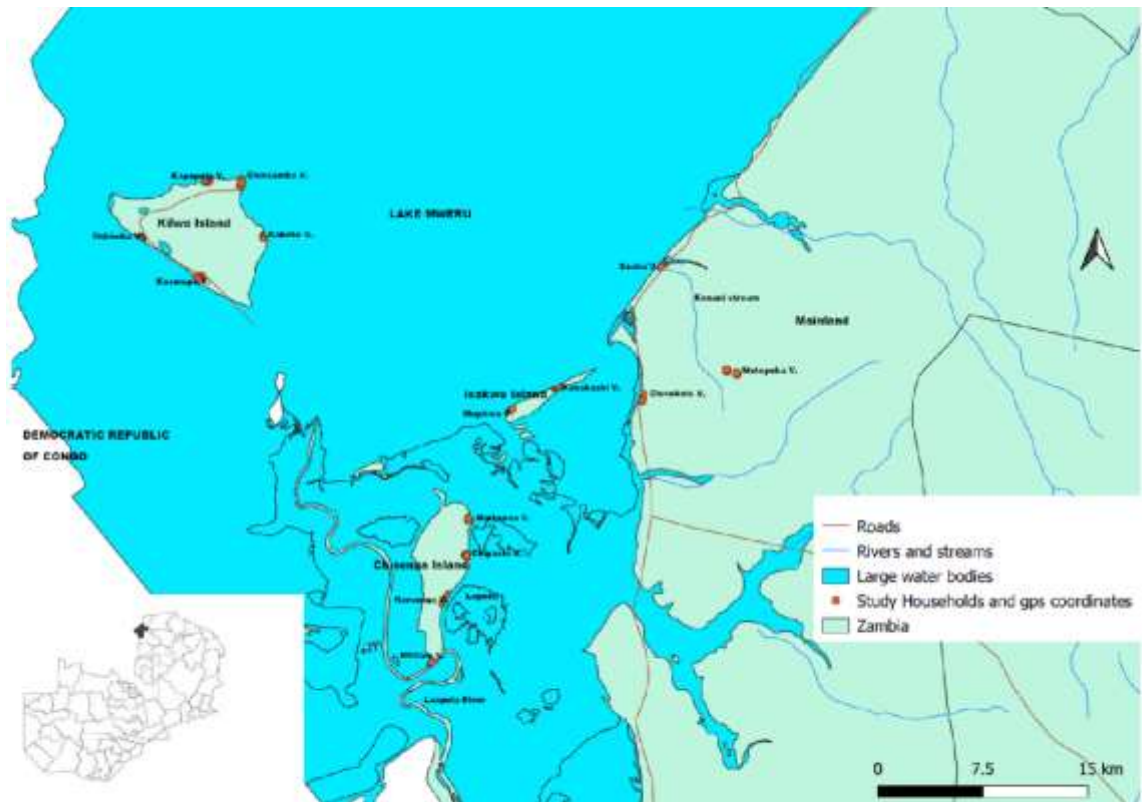


Figure 1 Map of the study area showing the location of Nchelenge in Zambia (Map composed using QGIS 3.22.7

Chisenga and Kilwa are two major islands with a combined human population accounting for 13% of the district total (CSO, 2011). The selected study sites for the present work fell within a radius of 24 km of Nchelenge town and included the mainland and the islands Kilwa, Chisenga, and Isokwe. The three islands were included in the study due to their prominence on the lake and their stable human populations. Chisenga Island (approximately 31.25 km^2), the southernmost island of the three islands studied, is part of the lower Luapula river delta. The island is situated within a few kilometres of where the river enters Lake Mweru. It is a swamp island that is south-west of Nchelenge town and surrounded by floodplains and lagoons. Chisenga Island is generally flat land with evidently good soils, as seen from cultivated crops grown without adding fertiliser. The island is located about 12 km (distance measured by Google Earth map ruler) from the southern, more rural part of the mainland and about 26 km from Nchelenge Boma, the administrative office. The swamp vegetation is largely made up of Papyrus suds, reeds, and some floating plant species. The riparian swamp vegetation system extend**s** westward within a channel of the stream from the mainland**,** widening as it goes to meet Chisenga Island. The swamp vegetation continues all the way up to

the banks of the mighty Luapula River, disrupted in places by lagoons and a series of man-made canals. The canals serve as passages for the local people to paddle their canoes between the island and the mainland. Large portions of the shores of Chisenga Island are inundated throughout the year and provide breeding sites for malaria vectors and other mosquitoes, notably *Mansonia*. The human population on Chisenga Island had been estimated at 9700 in the last census (CSO, 2011). There is a rural health centre and three primary schools on the island. The inhabitants are peasant families sustained by fishing and small-scale farming. The majority of housing structures are made of mud with thatch roofs and open eaves (Figure 2). A few houses are made of straw for both the wall and roof parts.

Figure 2 Common housing structures in Nchelenge .

Kilwa Island is the largest (approximately 45.9 km^2) of the three islands in the study. It lies about 27 kilometres to the west of the mainland, separated by Lake Mweru waters. Kilwa Island was named after Kilwa town, located about 5 km west in Pweto territory, in Katanga Province in the DRC. The island has a hilly and rocky terrain, completely surrounded by Lake Mweru's waters. The interfaces between lake waters and dry land are cliffs, valleys, and basins. The low-lying margins of the island are swampy and experience annual floods as the lake fills with water from the Luapula River. The island is under one chiefdom, namely, Chief Nshimba**,** one of the chiefs of the Lunda people of Luapula province. The estimated human population was 9**,**800 (CSO, 2011). There is a rural health centre,

four primary schools, and a secondary school. Human settlements are located in low-lying portions of Kilwa Island**,** closer to the shores of the island (Figure 3). The interior, or larger portion of the island, is unpopulated and serves as cultivation fields during the rainy season. Most of the inhabitants there too are peasant families sustained by fishing and small-scale farming, growing cassava, rice, maize, vegetables, etc. The majority of housing structures on Kilwa Island are typical village types made of mud bricks with thatch roofs and open eaves, as on Chisenga Island above. Also, a few houses are made of reed for both the wall and roof parts.

Figure 3 Lukwesa village is on the South-eastern corner of Kilwa Island. The village is located within a few metres of the shores with Lake Mweru, a common feature with all of the villages on the island.

Isokwe Island lies to the west of Nchelenge town, is the nearest to the mainland (about 3 km), and is also the smallest (about 6.8 km^2) of the three islands. It is a sandy, swampy area of land that Lake Mweru completely encircles. Due to the sandy nature of the soil, the majority of human habitations are mostly temporary and made completely of straw for both the wall and roof (Figure 4). Very few houses are made of mud walls. The inhabitants are peasant families too**,** sustained by fishing only. The island has a health post and a primary school.

Figure 4 A typical housing structure on sandy Isokwe Island. Because of the difficulty of getting clay soil, most of the houses are made of straw.

3.1.2 Climate

Nchelenge experiences high summer temperatures with an average of 30°C, according to the HOBO Micro Weather Station data logger (Onset Computer Corporation, Bourne, Maryland, USA), recorded between 2013 and 2018. In the winter season, the average temperature is around 20°C. The dry and cold seasons are quite distinct, with no rain at all falling in June, July, and August (Stevenson et al., 2016). Like the rest of Zambia, rainfall is brought by the Intertropical Convergence Zone (ITCZ), which receives about 1400mm of rain annually (2013). The rain is characterised by thunderstorms, sometimes severe, with much lightning. Flash floods occur following unusually heavy rains, causing soil erosion, destroying roads and bridges, etc., in the district. During the rainy season, substantial amounts of water collect in numerous places, providing additional mosquito breeding sites.

3.1.3 Vegetation

Nchelenge falls within the warm mesic woodlands of the Zambezian Miombo (White, 1983) or Southern Caesalpinoid Woodlands ecoregion. The vegetation is predominated by broader-leaved *Brachystegia-Julbernardia* species of wet Miombo woodlands (Timberlake et al., 2010), receiving an annual rainfall of more than 1000 mm. Based on the systems adopted for Central Africa at Yangambi, Belgian Congo (now the Democratic Republic of Congo) in the 1950s, the vegetation of Nchelenge can also be described as having the Lake Basin Chipya vegetation type along the coast (Fanshawe and Printer, 1971). This vegetation is predominantly an open forest of valley-type Miombo woodlands mixed with grass away from the coast. The vegetation type "Chipya" is an open non-miombo formation with very tall grass and a high complement of evergreen species found on richer soils (Timberlake and Chidumayo, 2011). Soil types are sandy loam with low nitrogen content (0.02–0.10 percent) and acidic (pH 4-5).The island vegetation also comprises Lake Basin Chipya mixed with grasslands that are naturally treeless, comprising dambo, floodplain, swamp, and papyrus sudd (Figure 5). The margins of many streams are covered with wet grasslands, providing breeding and resting places for mosquitoes. These margins remain flooded for months following the end of the rainy season.

Figure 5 Riparian **s**wamp vegetation between Chisenga Island and the mainland along the canal**.** Notice the papyrus, water lilies (*Nymphaea* sp.), and water lettuce (*Pistia stratiotes*) covering the water surface along the canal. These offer breeding and resting sites for *Mansonia* mosquitoes.

The natural forests occur in areas away from dense human populations on the mainland. During the rainy season, uncultivated open spaces are covered with tall *hyparrhenia* grass. The phenomenon observed above could be attributed to tree-cutting and clearing for human habitations, agriculture, and charcoal burning activities. In densely populated areas, palm trees as well as cultivated plants such as mango, pawpaw, avocado, etc. are common.

3.1.4 Drainage

The Nchelenge mainland is well endowed with a natural drainage system. There is a low gradient in the drainage system, resulting in a number of streams with slow-moving waters radiating from the district. Kenani stream is one such water body traversing the mainland study area, flowing from a south-eastern direction, with its tributaries feeding water into its course along the way before discharging its waters into Lake Mweru to the north-west. The whole margin of the western part of the mainland comprises Lake Mweru waters. Lake Mweru occupies the Mweru-Luapula graben, which is the lower western branch of the Cenozoic East African Rift Valley System (Schultz et al., 2001). The major inlet into the lake is the

Luapula River in the south, whose headwaters are located in the Bangweulu-Chambishi river swamps in Northern Zambia. The Kalungwishi River from the north-eastern part around Mweru-Wantipa, a marshy lake, is the second major inlet into the lake in the mid-section, besides numerous streams along the length of the lake. The Luvua River drains the Mweru waters into the Lualaba River and further up north into the Congo River, which discharges its waters into the Atlantic Ocean. The readily available water, coupled with warm temperatures and relatively high humidity in Nchelenge, makes it a good environment for mosquito breeding.

3.1.5 Socioeconomics of the human population

Nchelenge district has a total human population of 147,927 living in 31,724 households (CSO, 2011), giving an average of 5 people per household and an average density of 36.2 persons per square kilometre. The majority of the population, comprising 55.3%, is below the age of 18 (CSO, 2011). The larger number of houses is typical village type (Figure 3), constructed of mud bricks and grass roofs. It is a common feature, though, to find well-built formal housing units within clusters of these village houses. Improved houses are made of concrete or kiln brick, cement-plastered or without plaster, and covered with metal or asbestos roofing. Generally**,** the improved houses are more often found in urban areas as well as closer to the main road, built in close proximity to each other. Further movement away from the urban area and the main road reveals a horde of poorly constructed housing units, either sparsely built or clustered. The poorly constructed units are very porous to mosquitoes, allowing their free entry and exit through the open eaves. Nchelenge is well known for fishing, and a majority of the inhabitants are either engaged in fishing or selling fish. The human population fondly interacts with the Lake for most of their livelihoods (Figure 6). Few people are in formal employment, mainly government employees in fisheries, education, health, agriculture**,** immigration, revenue departments, etc. Fishermen spend extended periods of time on the lake and islands outside and in poorly constructed shelters, which expose them to mosquito bites. The inhabitants engaged in small-scale farming grow mostly cassava, maize, sorghum, millet, groundnuts, pumpkin, sweet potatoes, and enough vegetables for their families. On the mainland, during cultivation and harvest seasons, whole or part of the family members leave their

main houses for up to a couple of months, spending nights in poorly contracted shelters for these activities, thus exposing them to mosquito bites.

Figure 6 Lake Mweru's waters play a significant part in the lives of Nchelenge residents, from fishing to drinking to bathing and washing.

Cross-border movements into the Democratic Republic of Congo for various reasons are also common, allowing for cross-border malaria transmission.

3.2 Study design

This was a longitudinal study design employed in the communities of populations at risk of malaria. The selected households were visited at least two times during the study period. Mosquitoes were collected for 4 months in 2015 and only for 1 month in 2016, as follows: Rainy season, January and April 2015; Dry season, July and October 2015; and rainy season, again January 2016. Collection periods were also timed to ensure that insecticide resistance status was analysed before and after the vector control campaigns.

3.3 Sample size and sampling of households in study sites

3.3.1 Sample size determination

Sample size calculation of households for the human population at risk for malaria was based on the Cochran formula; $n = t^2pq/d^2$, (Cochran, 1977). The statistical parameters assumed were, 95% confidence level, a 20% disease prevalence in the rural area according to the Malaria indicator survey 2012 (NMEC, 2012) and a margin of error of 5%. The estimation gave a sample size of 245.86 of the human population at risk for malaria. Given the 2010 census of population and housing estimation of 5 persons living in one household in Nchelenge (CSO, 2011), 49.17 random samples of households across the study where therefore eligible for selection.

3.3.2 Sampling of households

Satellite images of human habitations in the study sites were overlaid with grids (Keating et al., 2003), which formed the sampling frames from which households were selected randomly. All study grids with signs of human habitation (villages) on the images were numbered, serially arranged, and systematically selected. The study grids measured 1 km^2 each, and a distance of at least two kilometres was allowed between the selected grids. From the earlier mark-release-recapture studies conducted in Kenya, the *An. gambiae* and *An. funestus* dispersal distances were less than two kilometres (Midega et al., 2007). The distance allowed between sampling points in this study was done in order to minimise the chances of sampling from the same progeny of mosquitoes. Four households were sampled from each selected village (grid). An estimation of the number of households in a selected village was made in the field, from which a sampling interval for the selection of four households was established. The selection of households started at the midpoint of the grid, identified with the help of a Global Positioning System (GPS) apparatus. The starting position for household selection was shifted for those grids whose centre was either on the lake or away from clusters of households. In two instances, the selected grids were dropped and the next grid was chosen instead. The orientation of a span bottle determined the direction of sampling within a grid (Bostoen and Chalabi, 2006). A house was selected in a specified direction, and subsequent households were selected according to the

sampling interval in that direction. The geographic coordinates of each sampled household were obtained using the Garmin OREGON® 450 Global Position System.

The inclusion criteria for a grid were that it was inhabited by humans and fell within the study area. The eligible grids in a particular study site were listed in sequence, and a 1-in-k systematic sample (Warde et al., 1987) of n grids was obtained. The first grid was picked at random from the first k = N/n by picking a corresponding number from a box containing small pieces of paper numbered from 1 to n, where k = sampling interval, N = total number of eligible grids, and n = actual number of grids possible under the selection criteria (Troyo et al., 2008) in a particular study site. Thereafter, every kth grid was selected until n grids were reached in that study area.

The ICEMR study high-resolution satellite image obtained in 2010 (Figure 7) provided an example of the images established as sampling frames (1 km2 grids) from which study households were randomly selected. The image (Figure 7) is in Natural Colour, Ortho Ready Standard, set to the appropriate Universal Transverse Mercator (UTM) WGS84 projection. The optimal image was obtained when cloud cover was minimal. Using ArcGIS software from ESRI™ (Redlands, CA), the locations of all households within the study area were identified from the satellite image. These were enumerated manually by placing a marker on the centroid of each potential residence, creating an attribute table containing unique identifiers and geographic coordinates for each grid and each household. In this study, the mainland Nchelenge study households were sampled from year two ICEMR study grids. Four study households in three, year-two ICEMR grids meeting the above-mentioned selection criteria were selected.

Mainland grids selected under the ICEMR study 2012 and 2013 shown as year1 and year2 respectively

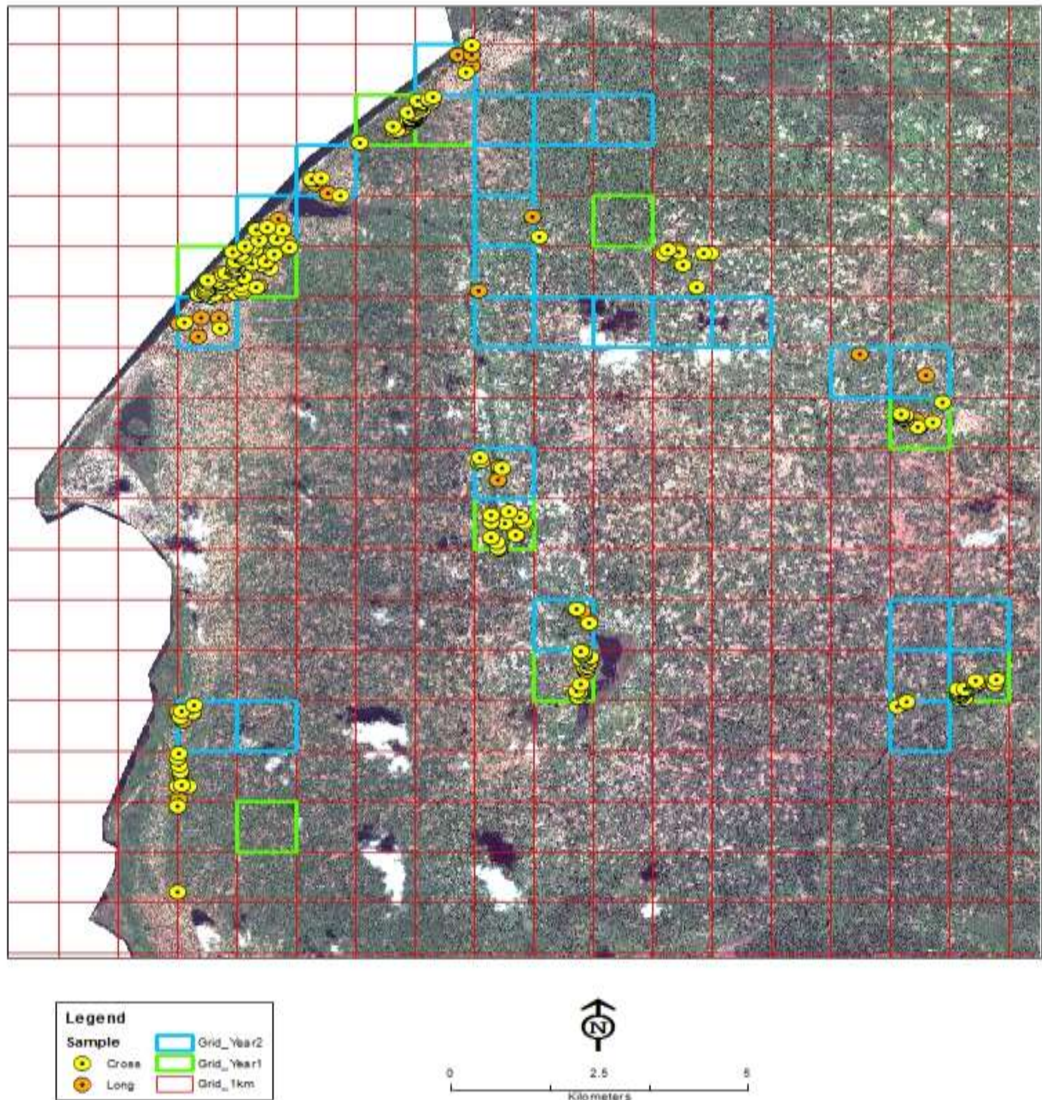


Figure 7 Mainland Nchelenge sampling frame from the ICEMR study, from which year-two grids were sampled (Map Provided by Tim Shields).

For the islands, images obtained from Google Earth in 2012 and 2014 were used. Employing ArcGIS software, sampling frames were established in the form of 1 km^2 grids as above. An attribute table containing unique identifiers and the midpoint geographic coordinates of each grid was created. The total number of grids and eventual number of households included in this study were proportional to the size of the study site. A random sample of eligible grids from each island was used to select four study households in each of the grids.

A total of 56 households from 14 villages (grids) were selected and mapped (red dots in Figure 1).

The selected households were distributed as follows:

Chisenga Island: 20 households

Isokwe Island: 8 households

Kilwa Island: 20 households

Mainland: 12 households

These households formed the sampling units that were visited at least twice and up to a maximum of five times during the study period.

3.3.3 Informed consent process

Study approval was obtained from the Tropical Diseases Research Centre (TDRC) Ethics Committee Protocol No. STC/2015/8. Recognition of traditional and political authority in the study area was deemed important for the success of the study. The district commissioner's office was made aware of the research activity. The village headmen in the study areas were very instrumental in giving consent for their villages and in sensitising their communities. Final consent was still obtained at the household level. Only consenting households were included in the study. Heads of these households or older persons (18 years and older) were asked for permission to conduct entomological studies in their houses. A questionnaire on household and community use of insecticides was administered.

3.4 Sampling of mosquitoes

This study sampled mosquitoes from an area with a high mosquito density and high parasite prevalence on the mainland and nearby islands within a radius of 24 km over a period of 2 years. The number of sampling units (households) on the islands was proportional to the size of the island. The number of households sampled per village, however, was maintained throughout all the sites. On the mainland, sampling ensured that lake-side and inland areas were represented. Analyses of vector indices were carried out at household, village, and site levels. The timing of the collections was critical to allow for the effects of seasonality and vector control interventions to be determined. Of all the sites studied, a full package of interventions (i.e., IRS and LLINs) for vector control in the District

had been carried out on the mainland. The islands received LLINs only for vector control.

Two sampling techniques, namely CDC light traps and Aspiration techniques, were used in this study. Mosquitoes were sampled from the three islands and the mainland Nchelenge District. Each of the sentinel households were sampled for a minimum of two and maximum of five times. All mosquitoes were collected indoors and included both resting and foraging vectors. All resting collections done by the backpack aspirator provided ready adult material for the study on insecticide resistance. Part of the resting collections by mouth aspirations as well as their F1s raised from eggs also provided adult material for insecticide resistance studies. Mosquitoes sampled by CDC light traps were used as material for the estimation of the following parameters: a) Foraging rates (female *Anopheles* mosquito counts per trap), b) Population genetics, c) *P. falciparum* infection rates, and d) blood feeding rates.

A total of 203 CDC light traps were set up, and 76 mouth aspirations were conducted in the study households. Five time points of collection by CDC light traps were conducted on Chisenga Island, Kilwa Island, and the mainland. Only two CDC light trap collections were conducted on Isokwe Island. Though Isokwe Island is the closest to the mainland, it was logistically difficult to sample due to the often discharged batteries following their use on Kilwa and Chisenga Islands. Sampling by CDC Light Trap was distributed as follows: 61 on Chisenga Island, 16 on Isokwe Island, 74 on Kilwa Island, and 52 on the mainland Sampling by mouth aspiration was as follows: 32 mouth aspirations were done on Chisenga Island, none on Isokwe Island, 20 on Kilwa Island, and 24 on the mainland. Additional backpack aspiration sampling was conducted in households other than the 56 selected for this study.

3.4.1 CDC light trap collection

The CDC light traps, operated by 6 Volt batteries, were set in households in January, March, July, and October of 2015 and January of 2016. Briefly, the light trap was set inside a selected household next to the sleeping space of a household occupant who was made to sleep under a mosquito net. The trap was hung about 150cm from the ground or floor on the foot side of the sleeping space.

The CDC light traps are used to collect indoor foraging mosquitoes. During CDC light trap collections, the household occupants would be sleeping under bed nets. The traps were set between 18:00 hrs. and 19:00 hrs. and removed the following morning after operating for approximately 12 hours.

3.4.2 Aspiration collection

Field collection of mosquitoes by aspiration was conducted in March 2014, March, April, and July of 2015, and January, May, and September of 2016. Mosquitoes were collected from the regular 56 households and from an additional 5–10 households within the vicinity of each of the 56 households. Collections were done by both mouth aspiration and the 12-volt battery-operated backpack aspiration methods. Briefly, the methods involved collecting indoor resting mosquitoes from the walls of selected households. The collector entered the house with a flashlight in one hand and a mouth aspirator, or the collector end of the backpack. Once inside the room, the collector carefully scanned the walls, moving the light up and down and moving in a clockwise direction from wall to wall, searching for resting mosquitoes. Once spotted, mosquitoes were gently sucked up by the mouth aspirator. When the number of mosquitoes in the tube reached between 5 and 10, they were transferred into the cage. In the case of the backpack aspirator, the trapped mosquitoes within a collection cup were held for close to 60 seconds before transferring to the cage. The cages were labelled, indicating the collection date and place of collection, and then taken to the field laboratory. Mosquitoes were held for at least 2 hours before being subjected to the standard susceptibility tests. Some of the mosquitoes collected, mainly from the mainland, made to lay eggs under laboratory conditions and the WHO standard susceptibility and intensity assays performed on their F1 progeny. All collected mosquitoes were sorted, morphologically identified by species, counted, and individually packed into desiccated 0.5-mL Eppendorf tubes for further laboratory processing and observations. The scheme of work shown (Figure 8) summarises the field and laboratory activities conducted during the study.

3.5 Subsampling of collected specimens for purposes of specific laboratory processes

Total counts of mosquito collections were highly variable from house to house and from collection site to another, as well as between seasons. Due to the cost implications of processing and running further laboratory procedures (PCR and ELISA), it was necessary to subsample a representative collection of female mosquitoes. The sub-sampling was conducted according to the following rule: *Process 10% of collected specimens from CDC light trap collections if female counts are above or equal to 50; otherwise, process all the vector mosquitoes in the collection*.

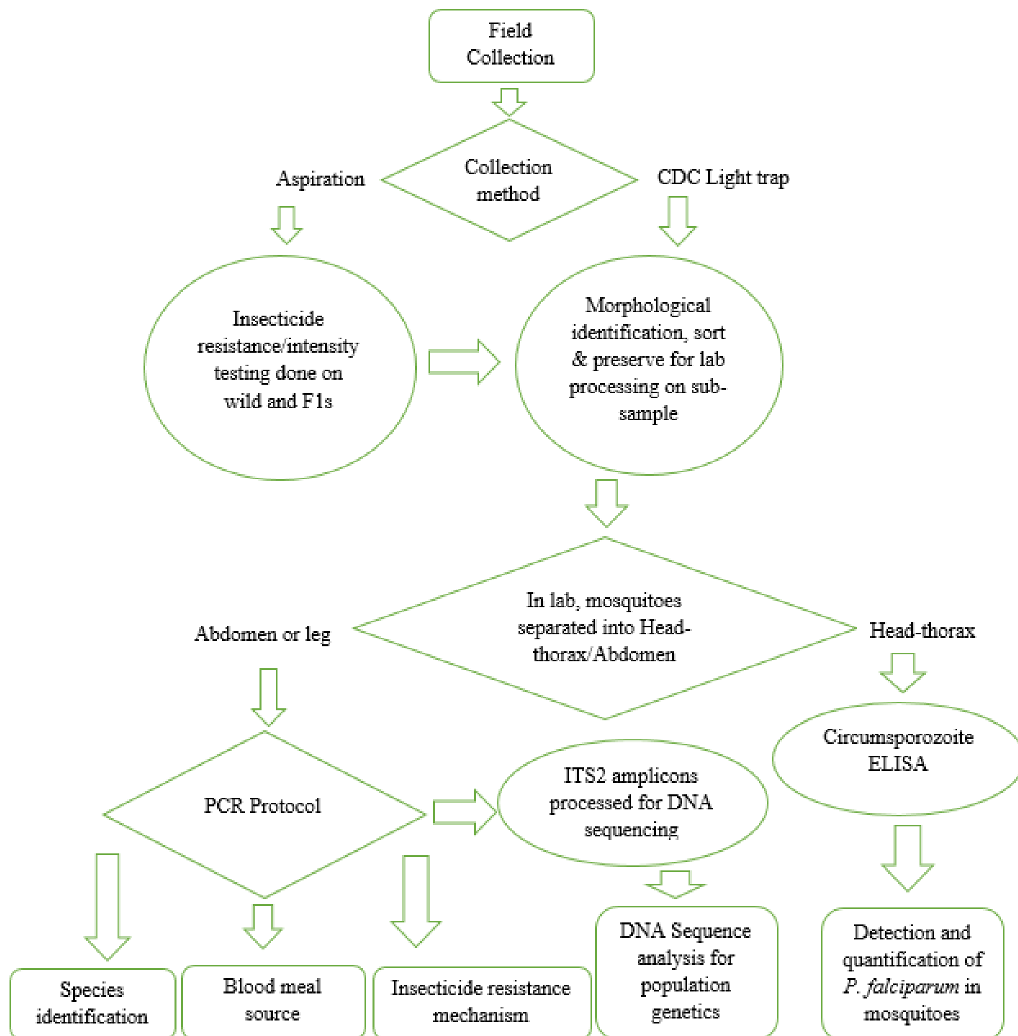


Figure 8 Summary of the workflow embarked upon during the study

3.6 Specimen Processing

The following sections provide details of the field and laboratory procedures by which the specimens were processed.

3.6.1 Species identification

a) Morphological

In the field, all the *anopheles* mosquitoes collected were morphologically identified by standard mosquito identification Keys (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). A sub-sample of the collections identified as *An. funestus* were run on the *funestus* PCR (Koekemoer et al., 2002), and all those identified as *An. gambiae* were run on the *gambiae* PCR (Scott et al., 1993).

b) PCR identification

Following morphological identifications, the mosquitoes in the selected sub-samples were prepared for further laboratory processing. Each individual mosquito was dissected into two portions by cutting its body using needle-sharp forceps through the abdominal-thoracic joint (waist). The resulting portions were Head-thorax and Abdomen. The PCR performed in this study was for mosquito DNA extracted from the mosquito abdomen portion or from the mosquito leg. The Head-thorax portions resulting from the dissections were saved for ELISA assays.
Specimens not showing a DNA band following the species PCR identification assay were re-run a second time. Following the second, if there were still no bands shown, they were denoted as no DNA band

c) DNA extraction

Extraction of DNA was done by two extraction procedures: one using an extraction kit and the other using a mixture of reagents prepared in the laboratory. The choice of DNA extraction was dependent on the laboratory where the PCR procedure was conducted. For all the PCR work done at the VCRU in South Africa, DNA was extracted using the PreGem® insect DNA extraction kit (ZyGEM Corp. Ltd.). With this method, one leg of a mosquito was added to 10 µL of the reaction mixture in a 0.2 mL PCR tube. The components of the mixture

included 8.75 µL deionized water, 1 µL 10× 10 buffer, and 0.25 µL PrepGem® insect reagent. Following sample preparation on ice, the mosquito leg was added to the tube containing the mixture. The tube was then transferred to the thermocycler and programmed as follows: step 1: Run at 75 °C for 15 minutes; step 2: Run at 95 °C for 5 minutes. According to the ZyGEM Quick-Start Guide, Step 1 activates a Proteinase enzyme by cell lysing, resulting in nuclease enzyme destruction and nucleoprotein removal. Step 2 inactivated the proteinase enzyme, and single-stranded DNA was thus extracted within 20 minutes. Extracted DNA was stored at -20 °C and only thawed when it was due for the PCR master mix. Between 1 µL and 2 µL of template DNA were added to a single reaction of the PCR master mix.The Marriott DNA extraction procedure was conducted on the mosquito abdomen in the laboratory at TDRC. Each extraction was carried out in a 1.5-mL centrifuge tube containing Bender Buffer. The constituents of Bender Buffer included 0.1M Sodium Chloride, 0.2M sucrose, 0.1M Tris-hydrochloric acid, 0.05M ethylene diamine tetra acetic acid (EDTA), and sodium dodecyl sulphate (SDS). The DNA extraction procedure involved nine steps of varying length. Step 1**:** Specimens are rehydrated for 10 minutes with High-Performance Liquid Chromatography (HPLC) water in a 1.5-mL centrifuge tube. Step 2: 100 µL Bender Buffer is added, and the specimen is completely homogenised with sterile pestles. Step 3**:** Homogenised specimens were incubated at 65 °C for 1 hour. Step 4: 15 µL cold potassium acetate is added to the specimen to precipitate the protein (there is an option to leave the process overnight at this stage). Step 5**:** Specimen is spanned in a micro centrifuge (at 14,000 revolutions per minute) for 10 minutes, followed by transfer of the supernatant to a new 1.5-mL centrifuge tube. Step 6**:** 300 µL absolute (100%) ethanol is added to the supernatant to precipitate DNA and incubated at room temperature for 5 minutes following thorough mixing. Step 7**:** Specimens are centrifuged (at 14, 000 rpm) for 15 minutes. Step 8**:** The supernatant is carefully removed and discarded, leaving a DNA pellet in the tube, and left to dry completely. Step 9**.** Pellets were resuspended in 50 µL HPLC water, stored at -20 °C, and only thawed when due for the PCR master mix. Between 1 µL and 2 µL of template DNA were added to a single reaction of the PCR master mix.

3.7 PCR procedures

3.7.1 The *Anopheles funestus* PCR (Koekemoer et al., 2002)

The *An. funestus* PCR method uses primers targeted to species-specific portions of the Internally Transcribed Space 2 region on the ITS2 gene in the five *An. funestus* complex species, namely, *An. funestus*, *An. leesoni*, *An. parensis*, *An. rivulorum,* and *An. vaneedeni* (Table 1). The assay is multiplexed such that each species detected by the assay will amplify a uniquely sized PCR fragment. The following primers were included in a single reaction mixture:

Table 1 A set of primers used to identify five common members of *An. funestus* complex

Primer	Name Sequence (5' to 3')
UV	TGT GAA CTG CAG GAC ACA T
FUN	GCA TCG ATG GGT TAA TCA TG
VAN	TGT CGA CTT GGT AGC CGA AC
RIV	CAA GCC GTT CGA CCC TGA TT
PAR	TGC GGT CCC AAG CTA GGT TC
LEES	TAC ACG GGC GCC ATG TAG TT

A single reaction mixture of 14 µL in a 0.2 mL PCR tube comprised 1.25 µ L of 10 × Buffer, 1.25 µ L of dNTPs (2.5 mM), 0.75 µL $MgCl_2$, 1.0 µL each of the primers UV, FUN, VAN, RIV, PAR and LEES, 0.1 µL Taq Polymerase (RTAQ), 3.65 µL molecular grade water and 1µL of template DNA.The PCR amplification programme for *An. funestus* was run as follows: 94 °C for 2 min, 94 °C for 30 sec, 45 °C for 30 sec, 72 °C for 40 sec, steps 2 to 3, repeated 30 times, and the final step at 72 °C for 5 min. The DNA products (amplicons) were loaded on a 2% Agarose gel (containing 1×TBE Buffer and stained with ethidium bromide. The gel was viewed and photographed under ultraviolet light.

3.7.2 The *Anopheles gambiae* PCR

The *An. gambiae* PCR method amplifies species-specific regions within the ITS2 gene. Each species that the assay identifies will amplify a distinctively sized PCR

fragment because the assay is multiplexed. The PCR amplifies four members of the *An. gambiae* complex, namely: *An. gambiae*, *An. arabiensis*, *An. quadrannulatus,* and *An. merus* (Table 2). The following primers were included in a single 12.5 µL PCR reaction mixture:

Table 2 A set of primers used to identify four members of the *An. gambiae* complex

Primer	**Name Sequence (5' to 3')**
UN	GTG TGC CCC TTC CTC GAT GT
GA	CTG GTT TGG TCG GCA CGT TT
AR	AAG TGT CCT TCT CCA TCC TA
QD	CAG ACC AAG ATG GTT AGT AT
ME	TGA CCA ACC CAC TCC CTT GA

A single reaction mixture of 13.5µL comprised 1.25 µL of 10 × Buffer, 1.25 µ L of 10× dNTPs, 0.5 µL $MgCl_2$, 0.5 µL QD primer, 1.0 µL each of the primers UN, GA, AR, and ME, 0.1µL Taq Polymerase (RTAQ), 4.9 µL molecular grade water and 1µL of template DNA.The PCR programme for *An. gambiae* was run as follows: Step 1: DNA polymerase activation at 94 °C for 10 min; Step 2: DNA denaturation at 94° C for 30 sec; Step 3: Annealing at 50° C for 30 sec; Step 4: Extension at 72° C for 30 sec (steps 2 to 4 repeated 30 times); and final extension at 72° C for 7 min. The DNA amplicons were loaded on a 2% Agarose gel (containing 1×TBE buffer) and stained with ethidium bromide. The gel was viewed and photographed under ultraviolet light.

Molecular forms (*An. gambiae* s.s.) and Clades (*An. funestus*) PCRsThe determination of the M and S molecular forms of *An. gambiae* s.s. was carried out in the Vector Control Reference Unit (VCRU) laboratory of the NICID in Johannesburg, South Africa. The M and S determinations were done by direct addition of the restriction enzyme Hha I in buffer to the PCR products of the *An. gambiae* PCR. Digestion was carried out at 37°C for 3 hours, and digested fragments were run through an ethidium bromide 2% agarose gel and photographed under ultraviolet light (Favia et al., 1997).The *An. funestus* Clades were detected as described previously (Choi et al., 2013). This technique is based

on TaqMan single nucleotide polymorphism (SNP) genotyping real-time PCR and detects clades I and II nucleotide variations in *An. funestus* s.s. In this approach, real-time PCR using TaqMan probes is used, in which either clades I or II in the mosquito vector can be detected in a single reaction. The PCR primers (Table 3) are based on the variations in cytochrome oxidase subunit I (COI). Primer probe sequences are labelled with 6-FAM and VIC at the 5' end for the detection of clades I and II, respectively. Each probe also carries a 3' nonfluorescent quencher and a minor groove binder at the 3' end.

Table 3 Clades I and II detection primer sequences used in the TaqMan PCR

Primer	**Name Sequence (5' to 3')**
Clade I	TCA GGA ATT GCT CAT GCT
Clade II	TCA GGA ATT GCC CAT GCT
Forward	GCA GGA ACA GGA TGA ACA GT
Reverse	GAA ATT CCT GCT AAA TGT AAT GAA A

A single reaction mixture of 25 μL comprised 2.5 μ L of 10 × Buffer, 2.0 μ L of dNTPs (2.5 mM), 0.3 μL ITS2A primer, 03 μL ITS2B primer, 1.0 μL Taq Polymerase (2.0U), 17.9 μL molecular grade water and 1 μL of template DNA.

The PCR programme was run as follows: **S**tep 1**:** 95°C for 10 minutes; **S**tep 2**:** 95°C for 10 seconds; and **S**tep 3**:** 63°C for 45 seconds (steps 2 and 3 repeated 45 times). The DNA amplicons were loaded on a 2% agarose gel (containing 1×TBE Buffer and stained with ethidium bromide). The gel was viewed and photographed under ultraviolet light.

3.7.3 Blood meal determination PCR

Multiplexed PCR (Kent and Norris, 2005) was used to identify the source of the blood meal in the mosquito abdomens. Mosquitoes visibly noted as blooded at the time of the collection were included in the PCR. Species-specific DNA products are amplified from the Cytochrome B gene of the mitochondrial genome using specific primers (Table 4), with the following expected fragment sizes:

Human blood meal amplifies at 334 bp, cows at 561 bp, dogs at 680 bp, goats at 132 bp, and pigs at 453 bp. The following primers were included in the master mix:

Table 4. Source of blood PCR primers used in detecting the host from which blooded mosquitoes fed

Table 4 Host blood meal PCR primers

Primer	Name Sequence
PIG573F	CCT CGC AGC CGC CGT ACA TCT C
HUMAN741F	GGC TTA CTT CTC TTC ATT CTC TCC T
GOAT894F	CCT AAT CTT AGT ACT TGT ACC CTT CCT C
DOG368F	GGA ATT GTA CTA TTA TTC GCA ACC AT
COW121F	CAT CGG CAC AAA TTT AGT CG
UNREV1025	GGT TGT CCT CCA ATT CAT GTT

A single reaction mixture of 25 µL comprised 2.5 µL of 10 × Buffer, 2.0 µL of dNTPs (2.5 mM), 0.3 µL ITS2A primer, 0.3 µL ITS2B primer, 1.0 µL Taq Polymerase (2.0U), 17.9 µL molecular grade water and 1 µL of template DNA.The PCR programme was run as follows: Step 1: 95 C for 5 min; Step 2: 95 C for 60 sec; Step 3: 56 C for 60 sec; Step 4: 72 C for 60 sec (step 2 to step 4 repeated 30 times), 72 C for 7 min. The DNA amplicons were loaded on a 2% agarose gel (containing 1×TBE Buffer and stained with ethidium bromide. The gel was viewed and photographed under ultraviolet light.

3.7.4 ITS2 PCR for subsequent sequencing of the DNA amplicons.

The PCR amplifies the internal transcribed spacer II (ITS2) region of the nuclear ribosomal DNA. Due to its robustness, this PCR can also be used to check the quality of extracted mosquito DNA. The same primers (Table 5) were used to amplify the rDNAs. The DNA amplicons produced are of varying sizes, depending on the mosquito species. In this study, *An. funestus* and *An. gambiae* mosquito DNA was included in this PCR.

Table 5 Forward and reverse primer for amplification of ITS2

Primer	Name Sequence (5' to 3')
ITS2A	TGT GAA CTG CAG GAC ACA T
ITS2B	TAT GCT TAA ATT CAG GGG GT

A single reaction mixture of 25 µL comprised 2.5 µL of 10 × Buffer, 2.0 µL of dNTPs (2.5 mM), 3.0 L $MgCl_2$, 0.5 µL ITS2A primer, 0.5 µL ITS2B primer, 0.2 µL Taq Polymerase (RTAQ), 14.3 µL molecular grade water, and 2.0 µL of template DNA.

The programme for the ITS2 PCR was run as follows: Step 1: 94 C for 2 sec; Step 2: 94 C for 30 sec; Step 3: 50 C for 30 sec; Step 4: 72 C for 40 sec (steps 2 to 4 repeated 40 times); Step 5: 72 C for 10 min. The DNA products were loaded on a 2% agarose gel (containing 1×TBE Buffer and stained with ethidium bromide. The gel was viewed and photographed under ultraviolet light.

Ten microliters of each resulting amplicon were pipetted into a labelled 1.5 mL centrifuge tube, sealed completely in Parafilm® M plastic. A few DNA amplicons (26 test amplicons) were sent for DNA sequencing to Inqaba® Biotechnical, Republic of South Africa. The majority (197 amplicons) were sent for DNA sequencing to Macrogen (Macrogen Corp., 2017) in Europe. Macrogen Inc. is a South Korean commercial public biotechnology company that provides services to the scientific community. A total of 100 *An. funestus* and 97 *An. gambiae* ITS2 DNA amplicons were sent to Macrogen. For each DNA amplicon, forward (ITS2a) and reverse (ITS2b) sequences were requested. Sequencing in both the forward and reverse directions was done in order to obtain proper and reliable sequences.

3.8 Population genetics

Population genetics analysis was based on the amplicon DNA marker as obtained from the ITS2 DNA sequences. Using this marker, genomic signatures were

compared across sites to infer spatial and evolutionary relationships and the degree of population genetic structure.

3.8.1 ITS2 Amplicon DNA sequence editing and alignment

Total of 394 *An. funestus*, *An. gambiae,* and one unknown *Anopheles sp.* DNA sequences were obtained from Macrogen, which provides sequencing services on a commercial basis. The DNA sequences were distributed as follows: 197 ITS2a forward and 197 ITS2b reverse sequences, thus representing two sequences per specimen. Each sequence was inspected for sequencing errors. If major errors were noticed in one sequence, both the forward and reverse sequences for that specimen were removed and excluded from further analyses. This was done to ensure that only good-quality sequences were included in the analyses. When the sequences were deemed okay, these were included with the rest for multiple alignment using the software Geneious Prime® 2020.1.2 (Biomatters ltd ,website: http://www.geneious.com) and MEGA-7 (Kumar et al., 2016). Following manual inspection of the sequences for quality each forward sequence was aligned to its reverse complement and a resulting single consensus sequence subjected to a BlastN programme for similarity search online to compare the particular sequence to the nucleotide sequence database stored at National Centre for Biotechnology Information (NCBI) website to confirm the species (http://www.ncbi.nih.gov/BLAST/). The percentage similarity threshold with database ITS2 nucleotide consensus sequences was set at 99% minimum. The resulting contiguous pairs of equal length for each specimen were passed on for multiple alignment within Geneious Prime, and the files were saved. Nucleotide multiple alignments were performed with MUSCLE within the default parameters. Aligned sequences were exported to other programmes: DnaSP (Rozas et al., 2017), MAGA-7 (Kumar et al., 2016), and GenAlex (Peakall and Smouse, 2006, 2012) for appropriate treatment. Nucleotide polymorphism as measured by Θ_w and diversity as measured by π were calculated using DnaSP version 6.1 (Rozas et al., 2017).

3.8.2 Amplicon DNA analysis

Analysis of molecular variance (AMOVA) was performed using GenAIex 6.503 (Peakall and Smouse, 2006, 2012) to assess genotypic variations across all the

populations. This programme partitions variation within and among populations and was used further to perform Mantel's test (Mantel, 1967; Diniz-Filho et al., 2013) to evaluate the relationship between genetic and geographic distance. The patterns in the population genetic relationship were visualised by plotting the genetic distance and geographical coordinates into principal coordinate analysis (PCoA) implemented in GenAlex 6.503 (Peakall and Smouse, 2006, 2012).

Phylogenetic and molecular evolutionary analyses were conducted using DnaSp v. 6.1 (Rozas et al., 2017) and Maximum Parsimony (MP) trees were constructed using the Near-neighbour-joining algorithm in Geneious Prime® 2020.1.2 (Biomatters ltd, website: http://www.geneious.com). A bootstrap consensus tree was inferred from 1000 replicates, and branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed.

3.9 Circumsporozoite ELISA

Plasmodium falciparum infection was determined by circumsporozoite ELISA (Wirtz et al., 1987). The circumsporozoite ELISA was conducted on the Head-thorax portion of the mosquito. A representative subsample of the total collections was subjected to the sporozoite ELISA tests to determine the infection rates in the vector mosquitoes. The sample testing took into consideration the site of collection, the number collected, and the period in which the collections were made. The ELISA reagents were obtained through a collaboration with BEIRE resources and the American Type Culture (ATCC).

The CSP-ELISA method, as described by the Malaria Research and Reference Reagent Resource Centre (MR4), was used to detect *P. falciparum* circumsporozoite protein (CSP) in the mosquito head-thorax portion. In brief terms, a 96-well U-bottom plate was incubated overnight with a *P. falciparum* CSP capture antibody. Then, each mosquito head-thorax portion was homogenised using a pestle and added to the micro-well plate (38 mosquitoes per plate) with a CSP-capture monoclonal antibody. A two-hour incubation period was allowed before the plate was washed seven times. The CSP monoclonal antibody with a conjugated peroxidase tag was added to the plate and incubated for one hour. The plate was again washed seven times, and the ABTS solution was added to visualise

the presence of CSP in the mosquito head-thorax within an hour. Qualitatively, if CSP protein was present in the mosquito head and thorax, the well containing that mosquito would turn green, showing that the mosquito was infectious. For quantitative results, the plate was placed in a spectrophotometer set at a 400-nanometer wavelength for analysis. The values associated with each mosquito that were twice the average of the negative controls on the plate were considered to be CSP or *P. falciparum*-positive (Wirtz et al., 1987). To get rid of false positives, the homogenates of all the assays that tested positive on the first run of circumsporozoite ELISA were boiled at 100 °C for ten minutes (Durnez et al., 2011), and the ELISA was done again. Only the second positive ELISA result was considered truly positive and used in subsequent processing and analyses.

3.10 Calculation of entomological inoculation rates

Entomological inoculation rate (EIR) calculations were based on CDC light traps (Kilama et al., 2014; Das et al., 2016; Stevenson et al., 2016).
Daily EIRs were determined by obtaining the product of the foraging rate and the sporozoite rate. The foraging rate (biting rate) was calculated by dividing the total female mosquito counts by the total number of humans sleeping in the household during the night of mosquito collection. The biting rate was calculated for each of the two vector species. The sporozoite rate was calculated based on the proportion of female mosquitoes found to be infected with *P. falciparum* as determined by the CSP ELISA positive result.

3.11 Insecticide Susceptibility testing

Susceptibility tests were conducted according to WHO standard susceptibility tests (WHO, 2013) on wild-caught and F1 mosquitoes. The tests were performed on two pyrethroids (0.05% deltamethrin and 0.75% permethrin), one organochlorine (4.0% DDT), two organophosphates (5% malathion and 0.25% pirimiphos-methyl), and two carbamates (0.1% bendiocarb and 0.1% propoxur). Mosquitoes were exposed for 1 hour to all the insecticides except for pirimiphos-methyl, where a 2-hour exposure period was allowed according to the guidelines. In wild collections, mosquito age was unknown, and the physiological conditions varied from unfed to gravid. For the F1s, between 2- and 5-day-old non-blooded mosquitoes were used. During the exposure period, mosquitoes were observed for knockdown at set intervals. Once the exposure period was over, the mosquitoes

were transferred back to the holding tubes, and cotton balls wetted in a 10% sugar solution were placed on the wire gauze over the tube. Mortality was scored 24 hours post-exposure. Surviving mosquitoes at this point were transferred into paper cups covered with a mesh of netting material, and cotton wool wetted with 10% sugar was placed on the net. During each test session, negative controls were run alongside. Negative controls included mosquitoes exposed to untreated paper. All the WHO test papers used were checked for efficacy by exposing the susceptible *An. gambiae* s.s. Kisumu strain and *An. funestus* FUNG. Two paper cups with surviving mosquitoes from different replicates of the same insecticide were taped together mouth-to-mouth and placed horizontally in a cooler box for transportation to the main laboratory at TDRC in Ndola, 540 km away. Once in the main laboratory, still-surviving mosquitoes were held in the same condition at room temperature for up to 12 days post-exposure.

3.12 Resistance intensity assays

Mosquitoes from wild collections and the F1 offspring of wild-collected mosquitoes raised in the laboratory were included in resistance intensities assays. The tests were performed on two pyrethroids (0.05% deltamethrin and 0.75% permethrin) and two carbamates (0.1 bendiocarb and 0.1 propoxur). For resistance intensity tests, everything was conducted according to the WHO standard susceptibility test (WHO, 2013), except the test tubes were held in a horizontal position. This ensured continuous dose exposure for a period of 8 hours, as reported elsewhere (Choi et al., 2014). During exposure time, mosquitoes were observed for knockdown at set time intervals. Once the exposure period was over, the mosquitoes were transferred back to the holding tubes, and cotton balls wetted in a 10% domestic sugar solution were placed on the wire gauze over the tube. Mortality was scored 24 hours post-exposure. In running the intensity assays, susceptible *An. gambiae* KISUMU strain and *An. funestus* FANG mortalities were compared to the control.

3.13 Insecticide resistance mechanism detection

The determination of the knockdown resistance West (kdr-w) and Knockdown resistance East (kdr-e) alleles was carried out by real-time PCR using the Taqman hydrolysis probe method on the *An. gambiae* s.s. as described previously (Bass et al., 2007). This technique is based on hydrolysis probe SNP genotyping and detects kdr-e, kdr-w, or wild-type mutations in *An. gambiae* s.s. This method is as

sensitive and specific as the gold-standard nested PCR approach. The assays were conducted at the VCRU in South Africa. The procedures and interpretation of the results were according to the protocol in appendix

4.3.14 Malaria situation and vector control activities during the study

3.14.1 Rural Health Centre malaria data

To appreciate the prevailing malaria situation during the study period, monthly data on malaria cases were obtained. Confirmed cases by Rapid Diagnostic Testing (RDT) reported from the RHCs were obtained from the District health information office. There are 12 RHCs and health posts across the district (Figure 9) that submit their data to the district health office.

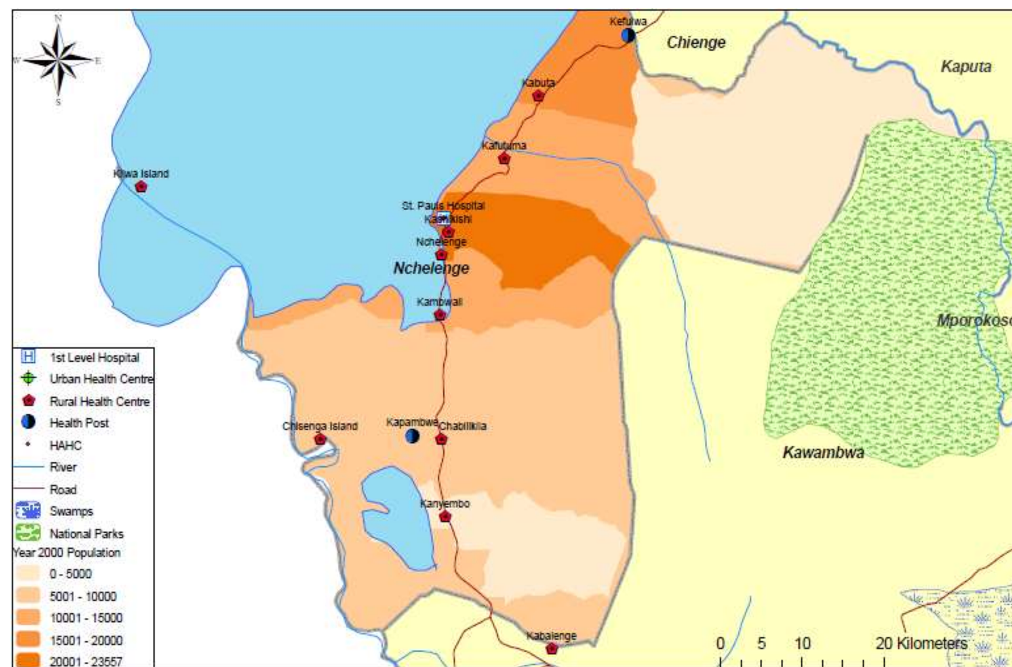


Figure 9 Nchelenge RHCs (source: Zambia National Health Facilities Atlas, 2007)

The information included age-stratified malaria cases, including malaria in children under the age of 1, children between ages 1 and 5, all ages above 5, and malaria in pregnant women. Only data from Kilwa, Chisenga, and Nchelenge urban Health centres falling within the vicinity of this study were considered. The data covered the period between January 2015 and May 2016.

3.14.2 LLIN distribution and IRS campaign

Before the study began, vector control by mass LLIN distribution had taken place in October 2014. Polyethylene bed nets, Olyset®, treated with permethrin, were distributed. These nets were encountered during household visits for mosquito collection. The mass campaigns, distributing LLINs door-to-door, were scheduled to happen every three years and were organised by the national malaria control programme.

The IRS programme has been conducted between September and December of each year since 2008 in Nchelenge district. The programme changed the insecticide product from bendiocarb to pirimiphos-methyl in 2014 due to insecticide resistance. Bendiocarb had been used in the previous two rounds of IRS, i.e., 2012 and 2013. During the study period, IRS with pirimiphos-methyl (Actellic® 300CS) was used subsequently in the 2015 and 2016 IRS seasons. At the time of this study, pirimiphos-methyl was the recommended IRS product for Nchelenge District and most parts of Zambia.

3.15 Climatological indicators

A mini-weather station, a HOBO weather data logger installed under the Southern ICEMR, was used to record the daily temperatures, humidity, and rainfall during the study period. Average monthly temperature and humidity, as well as total monthly rainfall data, were obtained and plotted.

3.16 Data analyses

3.16.1 Mosquito counts, infection rates and insecticide resistance

Due to the highly skewed nature in the data and occurrence of zeros in the mosquito counts, the female anopheline collections per trap were log-transformed [ln (n=1)] to normalize the data. The Williams mean (Mw) (Williams, 1937) for each for each species were estimated following the transformation.

The mosquito count data were described in R and analysed in STATA 15 (Release 15, College Station, TX: Stata Corp LLC) statistical programming. Variations in the total females collected from each site were evaluated by zero-inflated negative binomial regression. Vector count treatment was considered at the household level and associated with household characteristics, namely, elevation, number of people that slept in the house, number of ITNs in the house, type of roofing

material, presence of open eaves, whether fire was burned in the house, season of collection, and the site where the household was located.

The *P. falciparum* sporozoite infection rates between the malaria vectors were seasonally and spatially compared and predicted by logistic regression analysis. Insecticide resistance across the sites was compared and estimated by logistic regression analysis. The level of statistical significance in this study was p = 0.05, and any results with a p-value less than 5 were considered statistically significant.

The software, QGIS version 3.10.2 (Shekhar and Xiong, 2008) was used to create maps and plot proportions of mosquito counts, *P. falciparum* infection rates and plot graphs the level of insecticide resistance on the map.

3.16.2 rDNA amplicon data analysis

The diversity of the malaria vectors was determined using a total of 167 ITS2 consensus sequences from an initial total of 394 forward and reverse sequences of each sampled specimen. Poor-quality sequences were removed from subsequent analyses, which led to a reduction in the number of sequences included in the analyses.

3.16.3 Phylogenetic analysis

The maximum Likelihood analysis was performed on the ITS2 loci for both the *An. funestus* and *An. gambiae* sequences. A total of 84 *An. funestus* and 83 *An. gambiae* consensus sequences from across three study sites were used in the analysis. For *An. funestus,* these sequences included both those that were identified as clades I and II by the COI Taqman hydrolysis assay. Previously described *An. funestus* and *An. gambiae* sequences were obtained from NCBI BLASTn (http://www.ncbi.nih.gov/BLAST/) using Geneious Prime and included in phylogenetic tree construction. The *An. funestus* sequence was used as an out-group in the construction of the rooted *An. gambiae* phylogenetic tree. One other sequence of an unidentified *Anopheles* species was included in this *An. gambiae* tree construction.

Genetic diversity parameters were calculated for each vector. In *An. funestus,* the total nucleotide region selected in the multiple alignment of 84 consensus ITS2 sequences included 1–931 positions. In *An. gambiae,* the 83 consensus ITS2 sequences total nucleotide selection region included 1–970. Regions with missing data or alignment gaps were excluded, leaving 822 selection sites and 549 sites available for analyses for *An. funestus* and *An. gambiae,* respectively.

Phylogenetic and molecular evolutionary analyses were conducted using DnaSp v. 6.1 (Rozas et al., 2017), and Maximum Parsimony (MP) trees were constructed using the Near-neighbour joining algorithm in Geneious Prime® 2020. 1.2. A bootstrap consensus tree was inferred from 1000 replicates, and branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed.

Analysis of molecular variance (AMOVA) was performed using GenAIex 6.503 (Peakall and Smouse, 2006, 2012) to assess genotypic variations across all the populations. This programme partitions variation within and among populations and was used further to perform Mantel's test (Mantel, 1967; Diniz-Filho et al., 2013) to evaluate the relationship between genetic and geographic distance. The patterns in the population genetic relationship were visualised by plotting the genetic distance and geographical coordinates into principal coordinate analysis (PCoA) implemented in GenAlex 6.503 (Peakall and Smouse, 2006, 2012).

3.16.4 Gene flow estimation

Genetic diversity parameters were calculated for each vector. For both the malaria vectors, the analysed ITS2 sequences were drawn from three sites namely Mainland, Kilwa Is. and Chisenga Island.

In the *An. funestus*, the total nucleotide region selected in the multiple alignment consensus ITS2 sequences included 1-931 positions. A total of 85 consensus sequences were available for the analysis. Regions with missing data or with alignment gaps were excluded, and hence leaving 822 sites within the alignment data. The genetic diversity parameters calculated included the number of sequences, number of segregating sites (S) among the ITS2 sequences, number of

haplotypes (h) in a population, haplotype diversity (Hd), average number of differences (K) and nucleotide diversity (π).

In the *An. gambiae*, the consensus ITS2 sequences total nucleotide selected region included 1–9701-970. Regions with missing data or with alignment gaps were excluded, and hence leaving 549 sites available for analysis within the multiple alignment data sets

The genetic diversity parameters were calculated on the basis of number of sequences, number of segregating sites, number of haplotypes, haplotype diversity, average number of differences and nucleotide diversity.

Gene flow estimates of the coefficient of differentiation (N_{st}, $Delta_{st}$, $Gamma_{st}$ and F_{st}) and number of migrant individuals (Nm) were based on haplotype and sequence data by different algorithms (Nei and Chakraborty, 1973; Nei et al., 1983; Crease and Lynch, 1991; Hudson et al., 1992).

CHAPTER 4: RESULTS

4.1 Introduction

Mosquitoes collected comprised species in the genera *Anopheles*, *Culex* and *Aedes*. Since the mosquitoes of interest were malaria vectors in the genus *Anopheles*, mosquitoes from the other genera were only counted and discarded. Additional processing and analyses were conducted only on sampled *Anopheles* mosquitoes identified morphologically as *An. funestus* s.l. and *An. gambiae* s.l. Due to the large numbers of mosquitoes collected from some individual households, it became necessary to sub-sample at that level. This ensured that important information was obtained cost effectively. Representative samples were then processed for molecular, ELISA, and other throughput procedures. The major vectors were processed and analyzed spatially and seasonally by species. The spatial scales included households, villages, and sites, and the seasons were rainy and dry.

4.2 Sampled households

The sampled households were of varied sizes, with rooms ranging between 2 and 5. Once a household was sampled, its characteristics in terms of its physical vulnerability to mosquito entry, the human occupants, and vector control interventions were taken note of.

4.2.1 General household characteristics across different sites

The majority of the households (91% (n = 51)) had thatch roofing, and only 9% (n = 5) had metal roofs. Out of the sampled households, 96% (n = 54) had open eaves, and only 4% (n = 2) had their eaves closed. Other household characteristics examined during the study included the number of occupants, the number of ITNs in the house, the number of people who slept under ITNs the previous night, and whether the household had lit a fire the night before the trap was set up to collect mosquitoes. These characteristics varied from site to site and season to season and are summarised in Table 6.

Table 6 Household-level characteristics spatially and temporally during the study.

Site	Period	P (Q1,Q3)	No. ITNs (Q1, Q3)	No. P. in ITN (Q1, Q3)	% protected	% fire_burned
Chisenga Is.	Dry Season	6 (3,7)	2 (2, 3)	6 (3, 7)	100	12.5
	Rainy Season1	6 (4,7)	2 (2, 3)	6 (3, 7)	100	63.4
	Rainy Season2	4.5 (3,7)	2 (2, 3)	3.5 (3, 7)	77.8	none
Isokwe Is.	Dry Season	5.5 (4.5, 6.5)	2 (1, 2)	5.5 (4.5, 6.5)	100	25
	Rainy Season1	n/a	n/a	n/a	n/a	n/a
	Rainy Season2	5.5 (4, 7)	2 (1, 2)	5.5 (4, 7)	100	22.2
Kilwa Is.	Dry Season	6 (3, 7)	2 (1, 3)	3 (2, 5)	50	21.6
	Rainy Season1	5 (4, 7)	2 (2, 3)	4 (2, 6)	80	73.7
	Rainy Season2	5 (5, 7)	2 (1, 3)	4 (2, 7)	80	18.7
Mainland	Dry Season	4 (3, 5)	2 (1, 2)	2.5 (2, 4)	62.5	14.3
	Rainy Season1	4 (4, 5)	2 (2, 2)	4 (2, 5)	100	40.9
	Rainy Season2	6 (4, 7)	2 (1, 2)	3(1, 3)	50	18.2

P is the median number of people per house; No. ITNs is the median number of ITNs per house; No. P. in ITN stands for the median number of people who used ITNs the previous night, % protected for the proportion of people who used ITNs for protection, and % fire-burned for the proportion of households where fire burned inside the previous night; Q1 and Q3 is the interquartile range

Table 6 shows that the median number of people per sampled household ranged between 4 and 6. The median ITN ownership was 2 nets per sampled household and varied from nothing up to 10 nets in a single household. The use of ITNs by 2–6 people in a household indicated that between 50 and 100% of people in the

sampled households were protected from mosquito bites. The percentage of fires lit indoors increased during the rainy season. The fires were for cooking purposes.

4.3 Weather patterns during study

The weather data from May to August for both 2015 and 2016 showed that very little to no rainfall was received in the study area. Warm monthly average temperatures (20–27 °C) were experienced during the study, with lower temperatures occurring between June and July and higher temperatures between September and November. The monthly average relative humidity values ranged from 58% to 87%.

4.4 Mosquito species collected

56 households spread across 14 villages participated in this study, and CDC light traps and mouth aspirations collected a total of 10,109 mosquitoes indoors. The mosquito numbers comprised culicines and anophelines. The anophelines constituted the majority (60%) of the overall collections.

A total of 6068 *Anopheles* mosquitoes were collected from the overall collection effort during the study. Of these, 5888 were collected by CDC light traps and 180 by mouth aspirations. Morphologically identified species comprised 5809 (95.7%) *An. funestus* s.l., 252 (4.1%) *An. gambiae* s.l., 4 (0.1 %) *An. coustani,* and 3 (0.05 %) unidentified *Anopheles* mosquitoes. The total number of female *Anopheles* mosquitoes was 5705 and comprised 5447 *An. funestus* group, 251 *An. gambiae* s.l., 4 *An. coustani* s.l., and 3 unidentified *Anopheles* mosquitoes.

4.4.1 Molecular identification of *Anopheles* mosquito species

Molecular species identifications were conducted on a sub-sample of 1208 individuals, representing 21% of the total female *Anopheles* collections. The PCR molecular assay technique revealed the existence of *An. funestus* s.s. (hereinafter referred to as *An. funestus*) and *An. leesoni* as members of the *An. funestus* group (Figure 10). Further mitochondrial DNA analysis of *An. funestus* revealed the existence of Clades I and II, as reported previously by Choi and others in 2015.

Figure 10 Gel electrophoresis results for *Anopheles funestus* sl. PCR L = DNA ladder (100 bp), P = Positive Control, N = Negative Control, and S1–S28 = Samples included. S4 and S8 show faint bands for *An. leesoni* (125 BP). Samples having DNA bands the same as P are *An. funestus* s.s. (500 BP). Note that some samples did not show DNA bands (S19–S21 and S27).

The PCR results of the *Anopheles gambiae* s.l. (Scott et al., 1993) revealed only the *Anopheles gambiae* s.s. (Figure 11). Further processing by digestion of the PCR amplicons by FD Enzyme (*Hha I*) in FD Green Buffer at 37°C to determine the M- and S-forms (Figure 12) revealed the existence of the S-form, hereinafter referred to as *An. gambiae*, as the only member of this species complex found during the study.

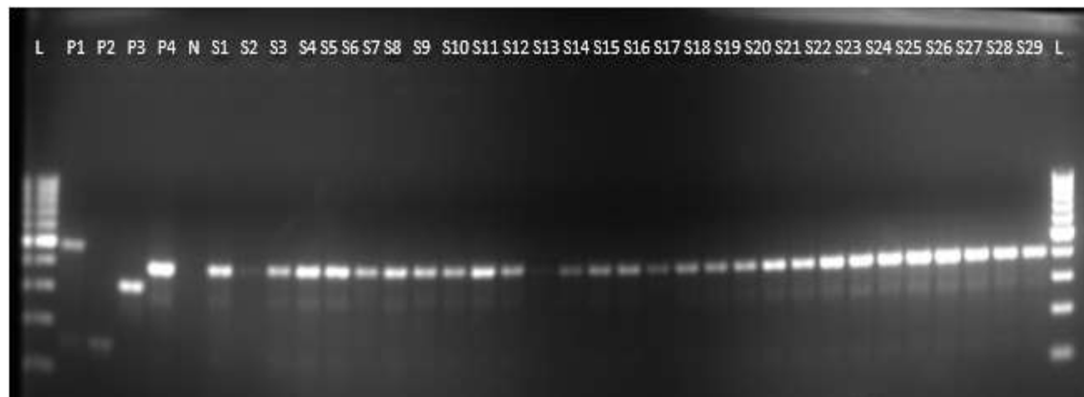


Figure 11 Elelectrophoresis results for *Anopheles gambiae* s.l. PCR L = DNA ladder (100 bp), P1 = Positive control 1 (*An.merus*,- 466bp), P2 = Positive control 2 (*An. guadrianulatus* - 153bp), P3 = Positive control 3 (*An. arabiensis* 31bp), and), P4 = Positive control 4 (*An. gambiae* – 390bp). Lane S1- Lane S29 = Samples included in PCR. Note that all samples were *An. gambiae* s.s.

The *An. gambiae* S-form digestion profile (Figure 12) was confirmed by the presence of the Hha I restriction site, which is shown by the two characteristic fragments that are 257 and 110 base pairs long. No sample tested showed the *An. gambiaM-form,*rm which lacks the Hha I restriction site and characterized by a single fragment 367bp long as shown by the M-form positive control 1(P1) lane.

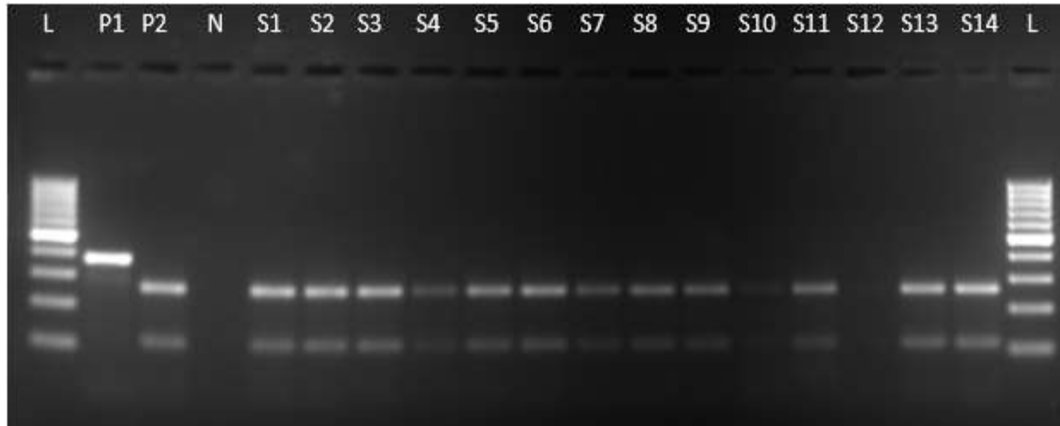


Figure 12 Electrophoresis results for the *An. gambiae* M and S molecular forms P1 = Positive control 1 (M-form), P2 = Positive control 2 (S-form), N = Negative control, S1–S14 = samples included. Note that all the samples included showed the S-form.

Table 7 provides a summary of the total species PCR assays and the success rate of the morphological identification assigned to the specimens.

Table 7 Molecular confirmation of morphologically identified vector species

Morphological ID	Total	No DNA band	Mis-identified	PCR confirmed	Success rate (%)
An. funestus s.l.	1093	119	0	974	89
An. gambiae s.l.	115	12	2	101	88
Total	1208	131	2	1075	89

Fairly high rates of success were recorded for the correct morphological identification of the two major vectors. Specimens showing No band could be a result of poor DNA extraction, mis-identifications, or other species that couldn't be identified by available PCR assays. No attempts were made to ensure that DNA was successfully extracted from the samples that did not show DNA bands.

4.5 Mosquito spatial distribution and abundance

The overall total number of collected mosquitoes from each site varied widely. Chisenga Island by far had the highest total of collected mosquitoes, representing 75% of all specimens collected. Kilwa Island contributed 13%, Mainland 11.6%, and lastly Isokwe Island at 0.4%. The household distribution of collection counts per light trap from three sites is summarised in boxplots in Figure 13.

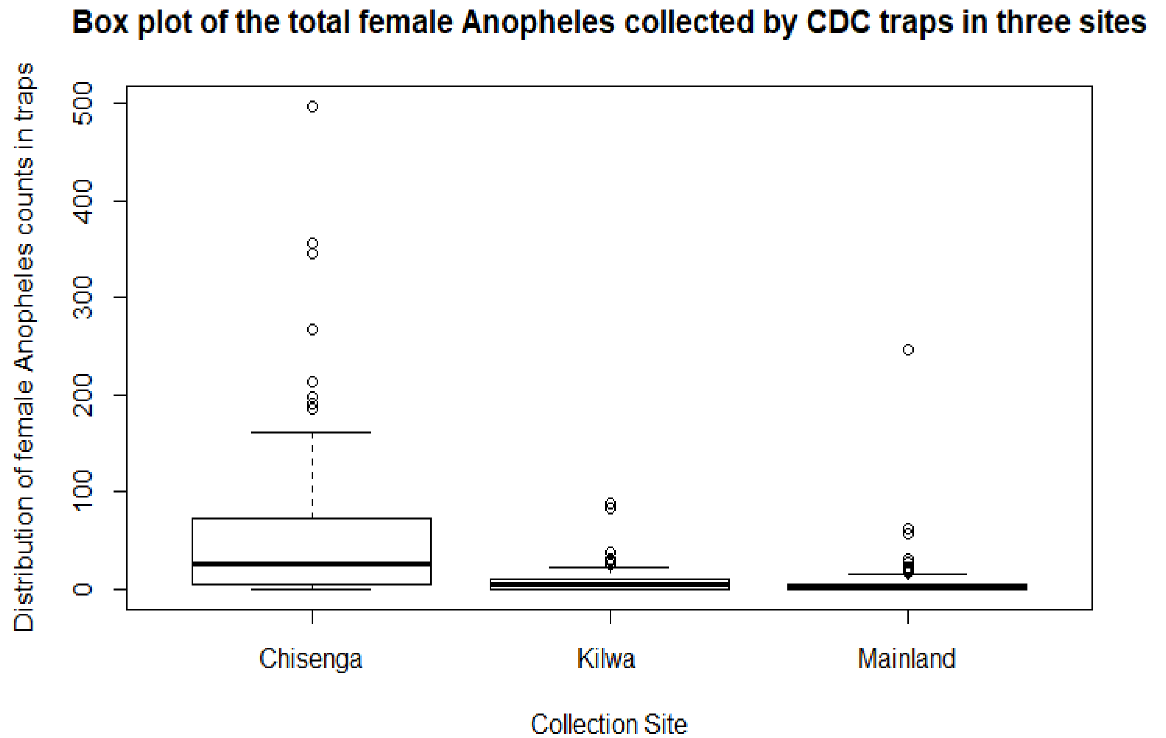


Figure 13 Distribution of female *Anopheles* counts across the three study sites with all sampling sessions

The median, interquartile range (Q1, Q3) and total collections of the two major malaria vector species from each sampled village are indicated (Table 8). Chisenga Island collections gave the highest proportion of *An. funestus* s.l., with 78% (n = 4275) of the overall total collected. Kilwa Island, on the other hand, gave the highest number of the other malaria vector, *An. gambiae* s.l., at 71% (n = 178). The highest 75th quantile of female *An. funestus* s.l. collected was from Kamonga village on Chisenga Island. For *An. gambiae*, the highest was from Chinsamba village on Kilwa island.

Table 8 Overall numbers and collections statistics of the malaria species per village

		An. funestus s.l.			*An. gambiae s.l.*		
Village	Site	Median	Q1, Q3	Total	Median	Q1, Q3	Total
Kamonga	**Chisenga**	65.5	**8, 240.5**	2277	0	0, 0.5	4
Michensa	Chisenga	36.5	6, 154	1201	0	0, 0	12
William	Chisenga	24.5	5, 54.5	482	0	0, 1	6
Chipashi	Chisenga	8.5	3, 24.5	315	0	0, 0	4
Kanakashi	Isokwe	0	0, 2.5	14	0	0, 1	9
Mupitwa	Isokwe	0	0, 1	1	0	0	0
Chinsamba	**Kilwa**	2	0, 6	151	3	**0, 8**	88
Kasompe	Kilwa	3	0, 9	187	1	0, 2	43
Kabeke	Kilwa	1	0, 1	103	1	0, 3	32
Kapopolo	Kilwa	3	0.5, 10.5	102	0	0, 1	11
Nshimba	Kilwa	0	0, 2	13	0	0, 0	4
Mutepuka	Mainland	8.5	3, 29.5	493	0	0, 1	22
Sonka	Mainland	1	0, 1	20	0	0, 0	2
Chisukulu	Mainland	1	1, 5	88	0	0, 1	14
Overall total		3	0, 23	5447	0	0, 1	251

4.5.1 Estimated Williams mean (Mw) number of female *Anopheles* species in different sites and seasons

During the dry season, Chisenga Island female *An. funestus* s.l. counts per trap were highest estimated at 56.280 (95% CI: 26.360, 118.914). The lowest estimated Mw was from Kilwa Island at 6.614 (Table 9). The highest female *An. gambiae* s.l. counts estimated per trap were from Kilwa Island at 2.466 (95% CI: 0.845, 5.510) during rainy season 2 (Table 9).

Table 9 Williams mean estimation of abundances of female *An. funestus* and *An. gambiae*

Site	Season	*Anopheles funestus*		*Anopheles gambiae*	
		Mw	95% CI	Mw	95% CI
Chisenga Island	Dry	56.28	26.360-118.914	0.166	0.055-0.439
	Rainy 1	18.421	11.077-30.230	0.361	0.162-0.594
	Rainy 2	4.925	1.730-11.860	0.105	0.115-0.380
Isokwe Island	Dry	0.891	0.832-3.977	1.091	0.112-0.339
	Rainy 1	no collections		no collections	
	Rainy 2	Nil		0.587	0.342-2.831
Mainland	Dry	8.2	3.486-17.541	0.498	0.151-1.021
	Rainy 1	2.058	0.772-4.277	0.208	0.020-0.431
	Rainy 2	0.603	0.005-1.583	0.593	0.082-1.766
Kilwa Island	Dry	6.614	3.502-11.878	0.953	0.425-1.678
	Rainy 1	0.636	0.234-1.169	0.738	0.208-1.501
	Rainy 2	0.8	0.242-1.606	2.466	0.845-5.510

Mw = Williams Mean

At the lowest level of sampling (a household), counts of female *Anopheles* mosquitoes varied between individual households within a sampled village and/or site. In some households, unusually high numbers of female anophelines would be recorded, and none would be collected from the next sampled household within a village. For example, the Kilwa island collections done in July 2015 (Figure 14)

from five villages (namely Kapopolo, Chinsamba, Kabeke, Kasompe, and Nshimba). This scenario was typical of the spatial variations in collection counts at all the sites. In the map shown, each dot represents a sampled household and the different colours of the dots correspond to the range of counts of female anopheline mosquitoes collected from that household.

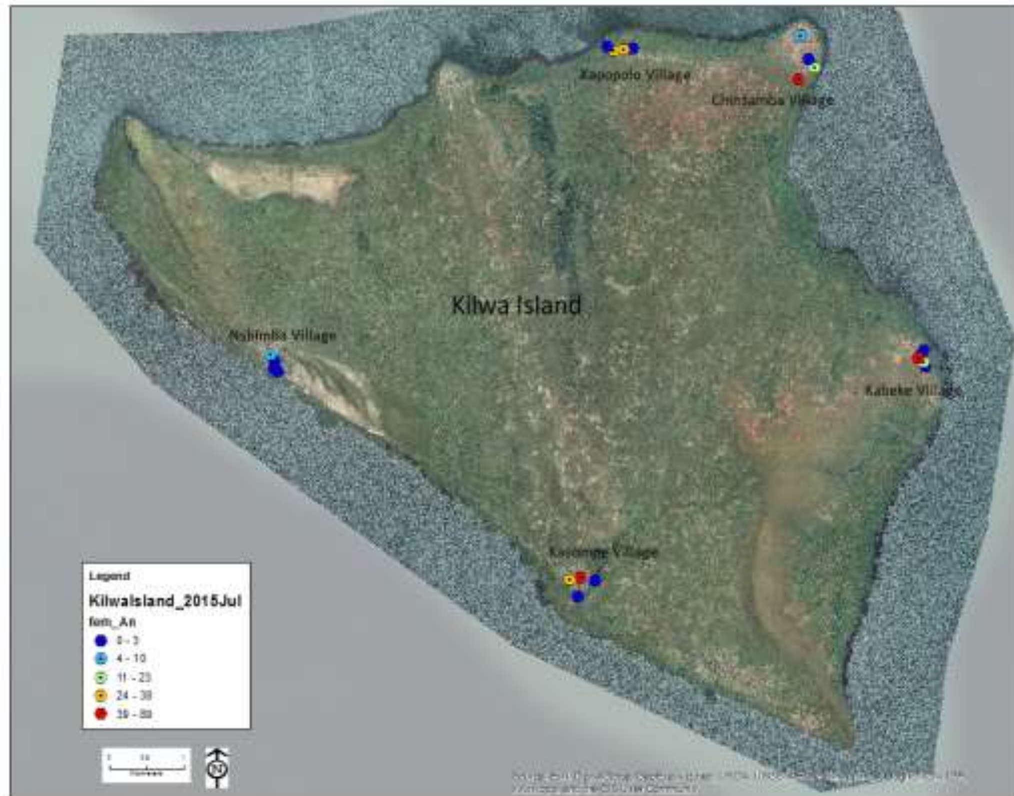


Figure 14 Variations in female *Anopheles* mosquito counts at household level, as exemplified by the July 2015 collection from Kilwa island villages (Map provided by Tim Shields, Johns Hopkins University)

4.5.2 Overall proportions of the major vectors found in study sites

The local malaria vectors occurred in different proportions across the study sites. *Anopheles funestus* overall total collections dominated in all the study sites. *Anopheles gambiae* ranked as the second major vector in overall total collections from each of the study sites. Kilwa Island had the highest proportion of *An. gambiae* compared to the other three sites, accounting for 71% of the total *An. gambiae* collected in the study. The overall mean collections for *An. funestus* and *An. gambiae* across the 14 villages in the study were plotted on a bar graph (Figure 15), and their spatial distribution was represented on the map shown (Figure 16). As can be seen from the graph and on the map, Kilwa Island villages had relatively

higher proportions of *An. gambiae* compared to villages from other sites. The lowest proportion of *An. gambiae* were noted in villages from Chisenga Island.

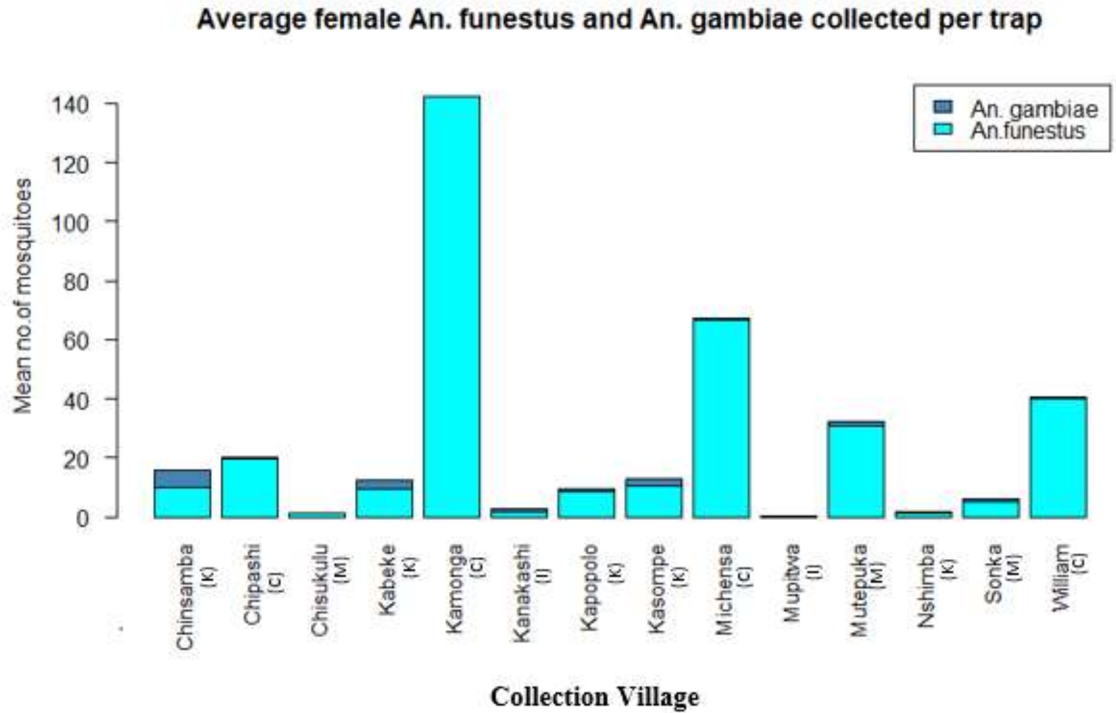


Figure 15 Overall mean proportions of *An. funestus* and *An. gambiae* across the 14 villages during the study. Letters in brackets stand for study sites where the villages are located, K, Kilwa Is., C, Chisenga Is., M, Mainland, and, Isokwe Is.

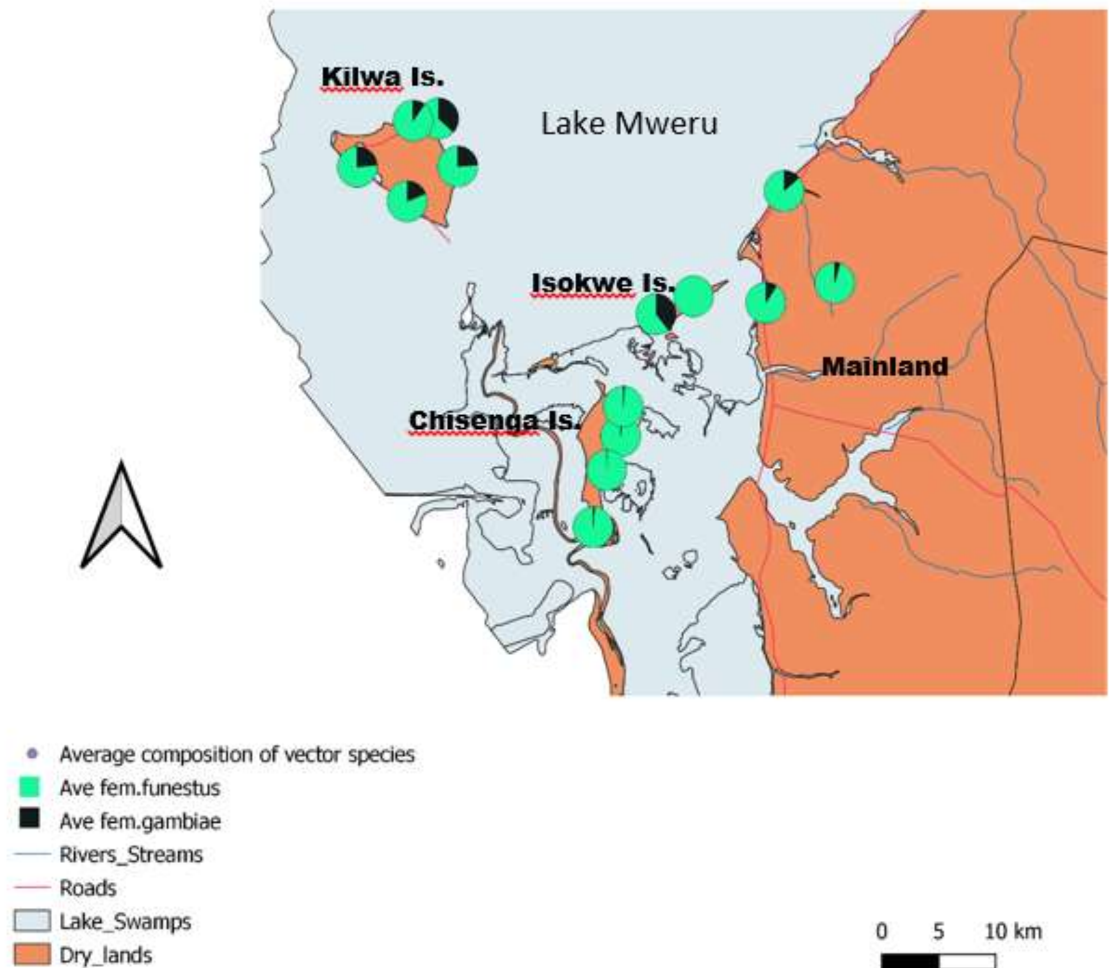


Figure 16 Spatial distribution of the mean collections of *An. funestus* and *An. gambiae* across study sites. The map was composed using QGIS 3.10.2 (Shekhar and Xiong, 2008). Shapefiles; water bodies, streams, and roads used were obtained from online sources.

4.5.3 Seasonal proportions of the two malaria vector species in site collections

In 2015, the month of July overall had the highest number of collections, representing 41% of the total collected mosquitoes. The month of October was the second-highest, with 37% of the collections. On Chisenga Island, however, the most mosquitoes were collected in the month of October. The months of January and March had lower numbers of mosquitoes, accounting for 5% and 17%, respectively. In terms of seasonal abundance, 78% of the total mosquitoes were collected during the dry season and 22% during the rainy season.

In 2016, the collections were done in January only to complete a year of the collection cycle. Total collections for January 2016 (n = 287) were within the

range of the January 2015 collections (n = 322). Within a season, collection counts of malaria vectors varied markedly from site to site. Between the seasons, the abundance of the two main vectors of malaria was in contrast with each other. *Anopheles funestus* was abundant during the dry season, but *An. gambiae* was abundant during the rainy season. A larger percentage (76%) of the major primary vector, *An. funestus,* was collected during the dry season. On the other hand, a higher percentage (65%) of the second major vector, *An. gambiae,* was collected during the rainy season.

On Chisenga Island, the rainy season 1 collections yielded a total of 3246 mosquitoes. Of these, 1063 (32.7%) were anophelines. The anophelelines were composed of 98.4% (n = 1046) *An. funestus*, 1.2% (n = 13) *Angambiae,* 0.2% (n = 2*) An. coustani, 0.2% (n = 2) An. coustani,* and 0.2% (n = 2) *An. coustani,* and 0.2% (n = 2) unidentified anophelelines *An. funestus* numbers were significantly higher compared to *An. gambiae* (Table 10, p< 0.001). The rainy season collection of mosquitoes included (35.5%) anophelines. The two vectors, *An. funestus* at 98.6% (n = 137) and *An. gambiae* at 1.4% (n = 2), were the only anophelines collected. *Anopheles funestus* was significantly higher compared to *An. gambiae* (Table 10, *p*< 001). The dry season sampling yielded a total of 4343 mosquitoes. Of these, 3323 (76.5%) were anophelines. The anopheline collection was composed of 99.6% (n = 3311) *An. funestus*, 0.3% (n = 11) *An. gambiae,* and 0.03% (n = 11) *An. coustani*. The *An. funestus* numbers were significantly higher than those of *An. gambiae* (Table 10, *p*< 0.001).

Table 10 Seasonal and temporal proportions in the two major vectors

Season	*An. funestus* s.l. (n)/*An. gambiae* s.l. (n)	Site	IRR (95% CI)	P-value
Rain 1	(1046)/(13)	Chisenga Is.	80.462 (46.85-151.62)	<0.001*

	(17)/(24)	Kilwa Is.	0.708 (0.357-1.375)	0.14
	(168)/(7)	Mainland	24 (11.395-60.611)	<0.001*
Rain 2	(137)/(2)	Chisenga Is.	68.5 (18.6-571.32)	<0.001*
	(0)/(8)	Isokwe Is.	0	
	(21)/(89)	Kilwa Is.	0.235 (0.139-0.383)	<0.001*
	(12)/(17)	Mainland	0.706 (0.308-1.568)	0.18
Dry	(3311)/(11)	Chisenga Is.	301 (168-603.56)	<0.001*
	(15)/(1)	Isokwe Is.	15 (2.308-631)	<0.001*
	(598)/(62)	Kilwa Is.	9.65 (7.42=12.73)	<0.001*
	(483)/(14)	Mainland	34.5 (20.36-63.58)	<0.001*

*indicates the results were significant at 5% significance level

On Isokwe Island, during the second rainy season 2, collections yielded a total of 92 mosquitoes. Of these, eight (8.7%) were anophelines, and all were *An. gambiae*. The dry season collections yielded a total of 105 mosquitoes. Of these 16 (15.2%) were anophelines. The anopheline collection comprised 93.7% (n = 15) *An. funestus* and 6.2% (n = 1) *An. gambiae*. The *An. funestus* numbers were significantly higher compared to *An. gambiae* (Table 10, p<0.001).

On Kilwa Island, the rainy season 1 collections yielded a total of 103 mosquitoes. Of these, 45 (43.7%) were anophelines. The anophelines comprised 37.8% (n =

17) *An. funestus*, 53.3% (n = 24) *An. gambiae,* and 2.2% (n = 1) unidentified *Anopheles* mosquitoes. The rainy season 2 collections had 164 mosquitoes, with 111 (67.7%) being anophelines. The anophelines comprised *An. funestus* at 18.9% (n = 21), *An. gambiae* at 80.2% (n = 89), and *An. coustani* at 0.9% (n = 1). *Anopheles funestus* was significantly lower compared to *An. gambiae* (Table 10, p < 0.001). The dry season collections yielded a total of 792 mosquitoes. Of these, 661 (63.9%) were anophelines. The anopheline collection was composed of 90.5% (n = 598) *An. funestus*, 9.3% (n = 62) *An. gambiae* and 0.1% (n = 1) *An. coustani*. The *An. funestus* numbers were significantly higher compared to *An. gambiae* (Table 10, p < 0.001).

On the mainland, the rainy season 1 collections yielded a total of 222 mosquitoes. Of these, 176 (79.3%) were anophelines. The anophelines comprised 95.4% (n = 168*)* *An. funestus*, and 4.0 % (n=7) *An. gambiae,* and 0.6% (n = 1) unidentified anopheline mosquito. Here the *An. funestus* numbers were significantly higher compared to *An. gambiae* (Table 10, p< 0.001). The rain season2 collections had 62 mosquitoes with 29 (46.8%) being anophelines. The anophelines comprised *An. funestus* at 41.4% (n = 12) and *An. gambiae* at 58.6% (n = 17). The dry season collections yielded a total of 588 mosquitoes. Of these 497 (54.0%) were anophelines. The anopheline collection was composed of 97.1% (n = 483) *An. funestus* and 2.8% (n = 14) *An. gambiae*. The *An. funestus* numbers were significantly higher compared to *An. gambiae* (Table 10, p < 0.001).

4.5.4 Dry and rainy seasons variations of each vector species collected per study site

Dry season and rainy season counts of each of the two vector species collected in households varied markedly from site to site (Table 11). On Chisenga Island, the *An. funestus* counts in rainy season 1 collections were significantly lower compared to the dry season collections (Table 11, IRR = 0.234, $p < 0.001$). On Isokwe Island, *An. funestus* was only collected in the dry season and was not found in the rainy season collections. No collections were conducted in Rain Season 1 from this island due to logistical issues.

Table 11 Vector seasonal count variations in collection sites

Season	Species	Site	IRR	95% CI	P-Value
Rain 1	*An. funestus*	Chisenga island	0.234	0.134-0.409	<0.001
		Isokwe island	na		
		Kilwa island	0.055	0.023-0.133	<0.001
		Mainland	0.328	0.126-0.856	0.023
	An. gambiae	Chisenga iland	1.2	0.107-15.258	0.845
		Isokwe island	na		
		Kilwa island	0.651	0.256-1.656	0.368
		Mainland	0.416	0.201-0.859	0.018
Rain 2	*An. funestus*	Chisenga iland	0.079	0.027-0.234	<0.001
		Isokwe island	na		
		Kilwa island	0.076	0.038-0.154	<0.001
		Mainland	0.051	0.017-0.149	<0.001
	An. gambiae	Chisenga iland	0.442	0.025-7.831	0.578
		Isokwe island	10.667	.770-147.666	0.077
		Kilwa island	2.873	0.980-8.427	0.054
		Mainland	1.452	0.526-4.009	0.471

An. gambiae Kilwa Island, the *An. funestus* counts in rainy season 1 collections were significantly lower compared to the dry season collections (Table 11, IRR = 0.055, p <0.001). On the mainland, *An. funestus* counts in rainy season 1 collections were significantly lower compared to the dry season collections (Table 11 IRR = 0.328, p < 0.023). The *An. gambiae* counts in rainy season 1 collections was significantly lower compared to the dry season collections (Table 11, IRR = 0.416, p = 0.018).

4.5.5 Household level factors and mosquito counts

A negative binomial regression model was used to determine the association between vector counts and household level factors. *Anopheles funestus* counts were estimated separately from *An. gambiae* counts. The results of the estimations are summarised in Table 12.

Table 12 Household level factors associated with the malaria vector counts in CDC light traps set indoors.

Species	Household Characteristic	IRR	95% Confidence interval	P - value
*An. funestus*s s.l.	Elevation	0.996	0.971-1.023	0.788
	Number of occupants sleeping in house	1.072	0.948-1.211	0.266
	Number of ITNs used in the house	1.189	0.898-1.575	0.227
	Dry season collection	reference		
	Rain season 1 collection	0.183	0.099-0.341	<0.001 *

	Rain season 2 collection	0.084	0.045-0.157	<0.001*
	Thatch roof	4.396	1.729-11.179	0.002*
	Open eaves	0.722	0.161-3.227	0.669
	Fire burned last night	1.203	0.706-2.050	0.497
	Household on Mainland	reference		
	Household on Chisenga Island	3.254	1.512-7.003	<0.001*
	Household on Isokwe Island	0.025	0.007-0.092	<0.001*
	Household on Kilwa Island	0.26	0.129-0.522	<0.001*
An. gambiae s.l.	Elevation	1	0.966-1.035	0.994
	Number of occupants slept in house	1.048	0.877=1.252	0.606
	Number of ITNs used in the house	1.165	0.785-1.728	0.448
	Dry season collection	reference		
	Rain season 1 collection	1.079	0.424=2.744	0.874
	Rain season 2 collection	2.547	1.080-6.007	0.033*

Thatch roof	0.947	0.236-3.791	0.938
Open eaves	1.011	0.130-7.877	0.992
A fire burned last night	0.587	0.257-1.339	0.206
Household on Mainland	reference		
Household on Chisenga Island	0.683	0.215-2.169	0.518
Household on Isokwe Island	0.511	0.104-2.547	0.413
Household on Kilwa Island	3.326	1.288-8.593	0.013*

For the *An. funestus* mosquito species, higher counts were significantly associated with the dry season, if the household had a thatch roof and if the household was located on Chisenga Island. Lower counts were significantly associated with rainy season and if the household was located on either Isokwe Island or Kilwa Island relative to the mainland. For the *An. gambiae* mosquito species, higher counts were significantly associated with rainy season 2 and if the household was located on Kilwa Island.

4.5.6 Proportion of blood fed *Anopheles* in light traps

The proportion of blooded *Anopheles* varied from one collection period to the next and from site to site. Overall, the highest proportion (387 of 2097, (18%) of blood-fed mosquitoes occurred in October 2015. The July 2015 collections gave the lowest proportion of 121 of 2257 (5%) even though that month had the highest overall abundance of *Anopheles*. In the other months, the proportion of blood fed *Anopheles* was as follows; January 2015 32 of 275 (11%), March 2015 16%; and January 2016 9.4%. *Anopheles funestus* overall blood feeding incidence of 18% was almost twice that of *An. gambiae* at 8.5%. In terms of sites, Isokwe Island had the highest (25%) percentage of blooded female *Anopheles*. Kilwa Island (14%), Mainland (12%), and Chisenga Island (11%) followed in that order. Net usage and proportion of blood feeding compared graphically (Figure 17) showed no apparent relationship with each other.

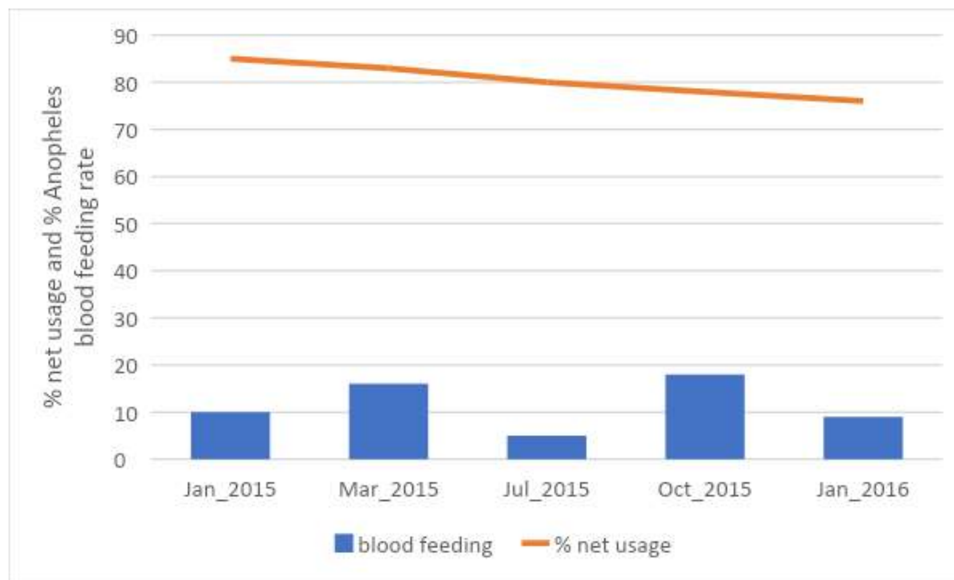


Figure 17 Net usage verses *Anopheles* mosquito blood feeding rates

Bed net usage from January 2015 to January 2016 significantly went down (reg coef. = -2.3, $p < 0.001$). This passage of time explained 99% of the variation in the usage of bed nets. The pattern of blood feeding in mosquitoes did not seem to be affected by net usage (reg. coef. 0.053 = 0.06, $p = 0.954$) and only explained 0.13% of the variation.

The source of the blood meal (blood index) was human. Of all the blood meal PCRs conducted, only human blood was detected in all results, giving a positive blood DNA. This gave a human blood index of 100% in both *An. funestus* and *An. gambiae*. However, the rate of success of the blood meal PCR was very low, at 24% of the visibly blooded mosquitoes tested.

4.6 *Plasmodium falciparum* sporozoite rates

Using the circumsporozoite ELISA assay pf-210, the number of mosquitoes with sporozoites found out of the total number of mosquitoes tested was used to estimate the *P. falciparum* sporozoite rate. This was a sub-sample of 1325 mosquitoes confirmed by PCR as *An. funestus* s.s. and *An. gambiae* s.s. The species tested included *An. funestus* s.s. Clade I and Clade II, *An. leesoni*, and *An. gambiae* s.s. Of the total mosquitoes tested on ELISA, *An. funestus* comprised 83%, *An. gambiae* comprised 16.8% and *An. leesoni* 0.2%. Table 13 gives the details of the *An. funestus* and *An. gambiae* proportions infected (those that gave a

positive ELISA test) from the four sites of study. The map (Figure 18) shows the spatial and temporal distribution of *P. falciparum* in the two major vectors.

Table 13 Spatial and temporal distribution of *P. falciparum* sporozoite infections in *An. funestus* and *An. gambiae*

Species	**Site**	**Season**	**Proportion infected (n)**	**95% Confidence interval**
An. funestus s.s	Chisenga Is.	Dry	0.005(213)	0.001- 0.033
	Isokwe Is.	Dry	0.166(12)	0.039 - 0.494
	Kilwa Is.	Dry	0.037(164)	0.017 - 0.080
	Mainland	Dry	0.037(82)	0.012 - 0.108
	Chisenga Is.	Rain1	0.032(188)	0.014 - 0.069
	Isokwe Is.	Rain1	-	-
	Kilwa Is.	Rain1	0.000(9)	-
	Mainland	Rain1	0.000(43)	-
	Chisenga Is.	Rain2	0.088(34)	0.028 - 0.244
	Isokwe Is.	Rain2	-	-
	Kilwa Is.	Rain2	0.000(16)	-
	Mainland	Rain2	0.000(7)	-
An. gambiae	Chisenga Is.	Dry	0.000(5)	-
	Isokwe Is.	Dry	0.000(1)	-

Kilwa Is.	Dry	0.000(43)	-
Mainland	Dry	0.000(8)	-
Chisenga Is.	Rain1	0.000(8)	-
Isokwe Is.	Rain1	0.000(8)	-
Kilwa Is.	Rain1	0.000(24)	-
Mainland.	Rain1	0.000(2)	-
Chisenga Is.	Rain2	0.000(40)	-
Isokwe Is.	Rain2	0.000(8)	-
Kilwa Is.	Rain2	0.071(84)	0.026 - 0.123
Mainland	Rain2	0.059(17)	0.008 - 0.334

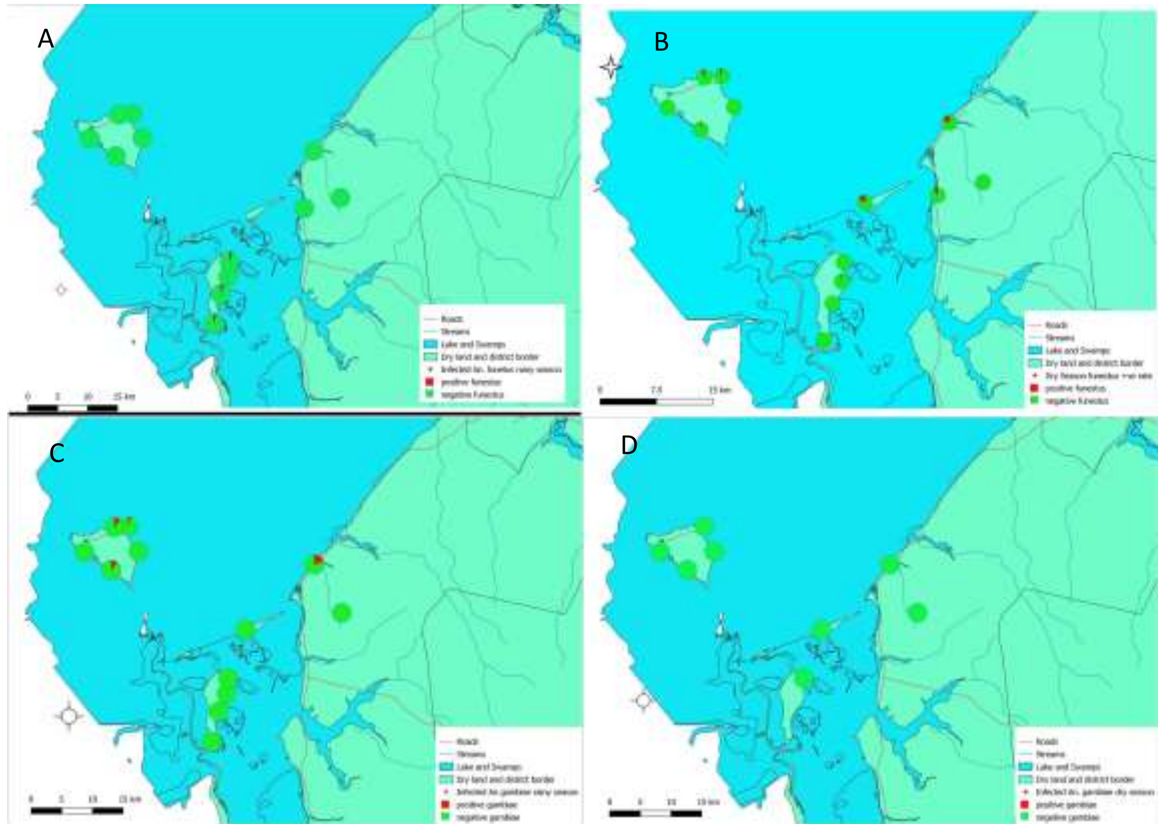


Figure 18 The seasonal and spatial distribution of *P. falciparum* rates are represented in pie charts on the study map A and B show the rainy and dry season infection rates in *An. funestus* respectively. C and D show rainy and dry season infection rates in *An. gambiae*, respectively.

More *An. funestus* were found infected with *P. falciparum* during the dry season than during the rainy season (Figures 18 A and B). During the rainy season, *P. falciparum* infected *An. funestus* were collected from Chisenga Island. The *An. gambiae* found infected with *P. falciparum* were collected during the rainy season and from Kilwa Island and the mainland (Figure 18C). This showed that *An. funestus* potential for malaria transmission was higher during the dry season compared to the rainy season. On the other hand, *Anopheles gambiae* would be transmitting more during the rainy season.

4.6.1 Analysis of the *P. falciparum* infection rates in the major vectors

The seasonal and spatial *P. falciparum* infection rates in the two major vectors were compared and predicted by logistic regression analysis. The estimated probability of finding *An. funestus* infected with *P. falciparum* sporozoites was

0.027 (95% CI: 0.016–0.039, p <0.001). On the other hand, the estimated probability of getting *P. falciparum*- infected *An. gambiae* was 0.017 (95% CI: 0.002–0.031, p = 0.025). In the dry season, the estimated probability of vector infection was 0.016 (95% CI: 0.007–0.025, p < 0.001). Rainy season 1's estimated probability for vector infection was 0.0331 (95% CI: 0.0103–0.056, p = 0.004). Rainy season 2's estimated probability for vector infection was 0.040 (95% CI: 0.013–0.068, p = 0.004).The malaria vector infection rates from each site were compared with the infection rates from the mainland. There were six times more odds of finding an infected vector on Isokwe Island compared to the Mainland (OR = 6.167, 95% CI: 0.996–38.169, p = 0.050). The predicted *P. falciparum* infection rates in malaria vectors from different sites were as follows: Mainland = 0.018 (95% CI: 0.001–0.036, p = 0.044), Chisenga island = 0.016 (95% CI: 0.007–0.025, p = 0.001), and Kilwa island = 0.042 (95% CI: 0.016–0.068, p = 0.001).

4.6.2 Entomological inoculation rates

The daily entomological inoculation rates were estimated per season and separately for each of the two major vector species in the district. The sporozoite rates shown (Table 14) were used to compute the daily EIRs in Table 14. The sporozoite rate was based on the proportion of the females found positive by the ELISA from the total of the PCR confirmed species. The biting rates (foraging rates) i.e. the number of female mosquito vector species per person per light trap night were computed from the household collections based on morphologically identified vector species and the number of household occupants. Since the focus of changes in the EIRs was dry season and rainy season, the six-month EIRs were estimated for each vector species from each site. Chisenga Island's dry season EIR was estimated to be 24.45 (95% CI: infectious bites per person per six months (ib/p/6mo), and the rainy season was 74.6 (95% CI: 12.59–110.41) ib/p/6mo. The EIR for Chisenga Island was driven by *An. funestus* for both dry and rainy seasons.

Isokwe Island's dry season EIR was also driven by *An. funestus* with an estimated 10.4 (95% CI: 2.37–30.66) ib/p/6mo. Kilwa Island's overall EIR was driven by both *An. funestus* and *An. gambiae,* with respective dry season estimation of 17.34 (95% CI: 8.03–37.59) ib/p/6mo and rainy season estimations of 12.23 (95% CI: 4.38–20.99) ib/p/6mo. The combined overall annual EIR for Kilwa Island was thus estimated to be 29.12.41–58.58) ib/p/yr. The overall EIR on the mainland was also

a function of the two vector species, both contributing in different seasons. The dry season EIR was estimated at 32 (95% CI: 10.58–95.99) ib/p/6mo and rainy season EIR was 2.92 (95% CI: 0.36–16.97) ib/p/6mo for *An. funestus* and *An. gambiae* respectively. The combined annual EIR for the mainland was thus estimated to be 34.92 (95% CI: 10.94 – 112.96) ib/p/yr. The spatio-temporal daily EIRs for the study area are summarised in Table 14. From this table, it can be seen that the EIR varied at different points of collection and from one site to another. *Anopheles funestus* posed a higher risk for malaria transmission, both spatially and temporally. On Chisenga Island, all the collection times were in both the rainy and dry seasons except for July 2015, which yielded an EIR attributed to *An. funestus*. On Kilwa Island and Mainland, *An. funestus* was involved in malaria transmission during the dry season months. *Anopheles gambiae,* on the other hand, was more seasonal in malaria transmission, with all the recorded EIRs occurring during the rainy season on Kilwa Island and the mainland. These results show that the overall situation for the District was that malaria transmission occurred during both dry and rainy seasons, with *An. funestus* responsible for malaria transmission in most parts of the district during the dry season and *An. gambiae* adding to malaria transmission on Kilwa Island and mainland during the rainy season months.

Table 14 Spatial and temporal daily Entomological Inoculation Rates for *An. funestus* and *An. gambiae* in collection sites at five collection points.

Species	Site	Season	Biting rate	Sporozoite rate	Daily EIR	95% Confidence Interval
An. funestus	Chisenga Is.	Dry	26.740	0.005	0.134	0.027 - 0.882
	Isokwe Is.	Dry	0.341	0.166	0.057	0.013 - 0.168
	Kilwa Is.	Dry	2.572	0.037	0.095	0.044 - 0.206
	Mainland	Dry	4.867	0.037	0.180	0.058 - 0.526
	Chisenga	Rain 1	5.978	0.032	0.191	0.084 - 0.412

	Is.					
	Isokwe Is.	Rain 1	n/a	0	0	-
	Kilwa Is.	Rain 1	0.18	0	0	-
	Mainland	Rain 1	2.286	0	0	-
	Chisenga Is.	Rain 2	2.479	0.088	0.218	0.069 - 0.605
	Isokwe Is.	Rain 2	0	0	0	-
	Kilwa Is.	Rain 2	0.221	0	0	-
	Mainland	Rain 2	0.197	0	0	-
An. gambiae	Chisenga Is.	Dry	0.089	0	0	-
	Isokwe Is.	Dry	0.023	0	0	-
	Kilwa Is.	Dry	0.313	0	0	-
	Mainland	Dry	0.156	0	0	-
	Chisenga Is.	Rain 1	0.096	0	0	-
	Isokwe Is.	Rain 1	n/a	0	0	-
	Kilwa Is.	Rain 1	0.260	0	0	-
	Mainland	Rain 1	0.077	0	0	-

Chisenga Is.	Rain 2	0.042	0	0	-
Isokwe Is.	Rain 2	0.190	0	0	-
Kilwa Is.	Rain 2	0.937	0.071	0.067	0.024 - 0.115
Mainland	Rain 2	0.279	0.059	0.016	0.002 - 0.093

4.7 Health Centre monthly Malaria cases confirmed by RDT obtained from the District Health Office.

Malaria data by rapid diagnostic test (RDT) were obtained from the Nchelenge District Health Office (DHO) information and communications technology (ICT) unit. Malaria cases from three Health centre (HC) facilities falling within the study sites were extracted. The data covered period from January 2015 to July 2016. The different age categories and all age groups total malaria cases by HC were as shown (Table 15).

Table 15 Malaria cases by RDT from Health centres obtained from the District office. The data covers the period from January 2015 to May 2016.

HC	1 - 5 yrs	> 5 yrs	< 1 Yr	MIP	All Age	At risk
Kilwa	3712	5893	1720	244	11569	9978
Chisenga	1739	2364	1590	172	5865	9764
Nchelenge	3975	6092	2349	244	12660	11766
Totals	9426	14349	5659	660	30094	31508

Further malaria incidence data estimates obtained for the communities serviced by the three Health centres in 2015 were as follows: Kilwa Island Health Centre, 1017 cases/thousand human population; Chisenga Island, 287 cases/thousand human population; and Nchelenge Health Centre, 476 cases/thousand human population.

These data were plotted by age category and malaria in pregnancy, as shown in figure 19.

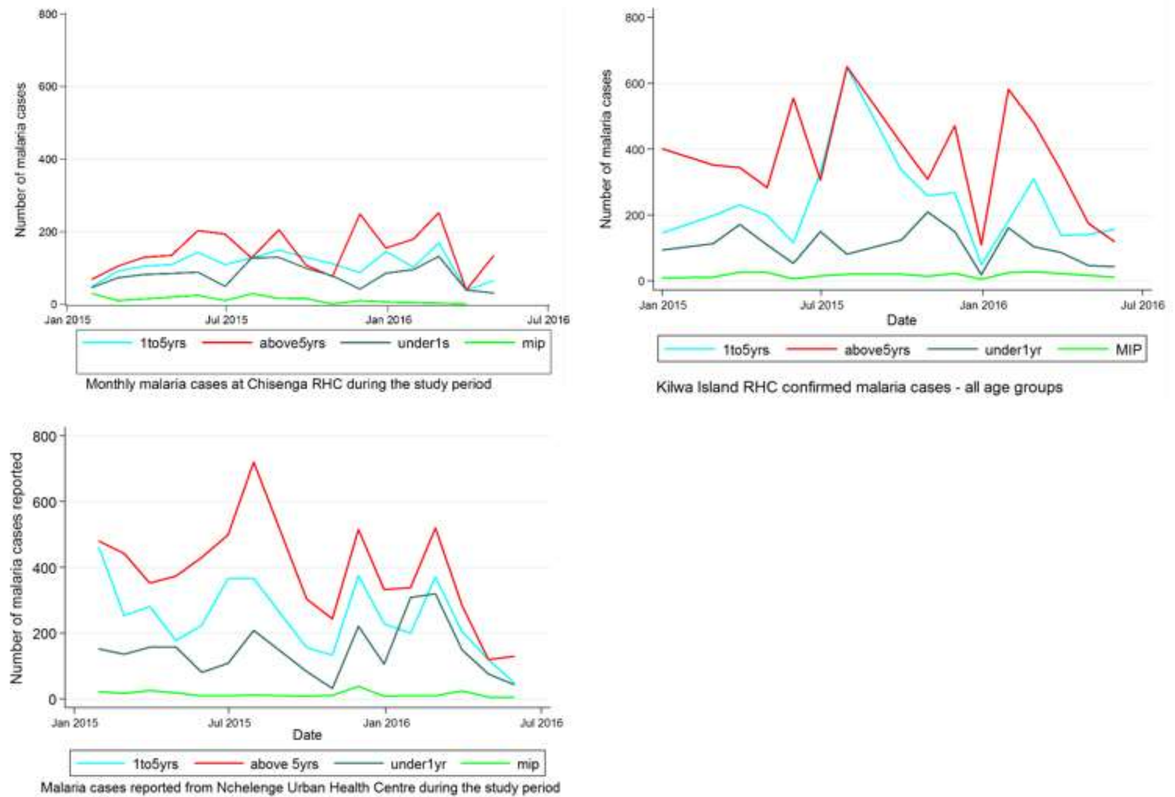


Figure 19 All age groups monthly cases of malaria obtained from the District health office Data obtained from three health centres is presented here. A was data collected from Chisenga Island Centre, B is data from Kilwa Island Health Centre, and C was data from Nchelenge urban health centre.

From the graphs (Figure 19), it was noted that malaria cases were reported at HCs throughout the study period. Slight peaks between July and November 2015 and January and April 2016 were apparent in the above five-year age group across the sites. Relatively more cases in the above five-year age group were reported at the Nchelenge urban health centre (Mainland). Negative binomial regression analysis of the malaria cases showed that the over five-year age group bore the highest burden of the reported cases across the HCs. Overall, cases in the one-to-five-year age category compared to the other age groups revealed significant differences. By age-group nearly 1.5 times more cases were reported in the five-year age group

compared to the one-to-five year age group (IRR = 1.496, 95% CI: 1.244–1.798, $p < 0.001$). About 41% fewer cases were reported in under-one-year age group compared to the one-to-five year group (IRR = 0.594, 95% CI: 0.493–0.715, p < 0.001) and a further 92% fewer cases were reported in pregnancy compared to the one-to-five year age group (IRR = 0.083, 95% CI: 0.067–0.22, $p < 0.001$). At the health centre level, the malaria case differences in age categories were significant except for Chisenga Island HC. At this HC, the difference was significant only for malaria in pregnancy compared to the one-to-five year age group. Overall, slightly lower cases were reported during the rainy seasons 10% lower (IRR = 0.90, 95% CI:0.756–1.0700, $p = 0.232$) and 20% lower (IRR = 0.800, 95% CI: 0.679–0.944, $p = 0.008$) for the 2015 and 2016 rainy seasons, respectively.

4.8 Resistance to insecticides commonly used in vector control.

Due to the low numbers of *An. gambiae* collected, most of the physiological insecticide resistance reported here was in *An. funestus*. However, insecticide resistance in the *An. gambiae* specimens was apparent. Resistance was confirmed with enough *An. gambiae* specimen that were available for genetic detection of the insecticide resistance mechanism.

4.8.1 WHO susceptibility testing

Standard WHO susceptibility testing assays were conducted in 2014, 2015, and 2016. In 2015 and 2016, *An. funestus* was more abundant and well represented across the three sites where insecticide testing was conducted. The 2015 and 2016 results were pooled and analyzed together (Table 16).

All the insecticide test papers were validated by exposing the susceptible *An. gambiae*, Kisumu, to the papers. All the WHO test papers used caused 100% mortality in this laboratory mosquitoes, showing that the test papers were efficacious (WHO., 2016). Field-collected mosquitoes recorded lower mortality rates against some WHO recommended insecticides. Of the insecticides tested, DDT, dieldrin, malathion, and pirimiphos-methyl caused a 24-hour mortality of 100%. The 24-hour mortality rates ranged from 38% to 96% on bendiocarb, propoxur, permethrin, and deltamethrin (Table 16). By the WHO criteria (WHO., 2013), this was a clear indication of resistance against these insecticides. The spatial distribution of insecticide resistance in *An. funestus* was plotted on the map

(Figure 20). The map depicts various insecticides, which are represented on the map by differently coloured bars. The height of the bars indicates the level of insecticide susceptibility, with a full bar representing complete susceptibility to that particular insecticide.

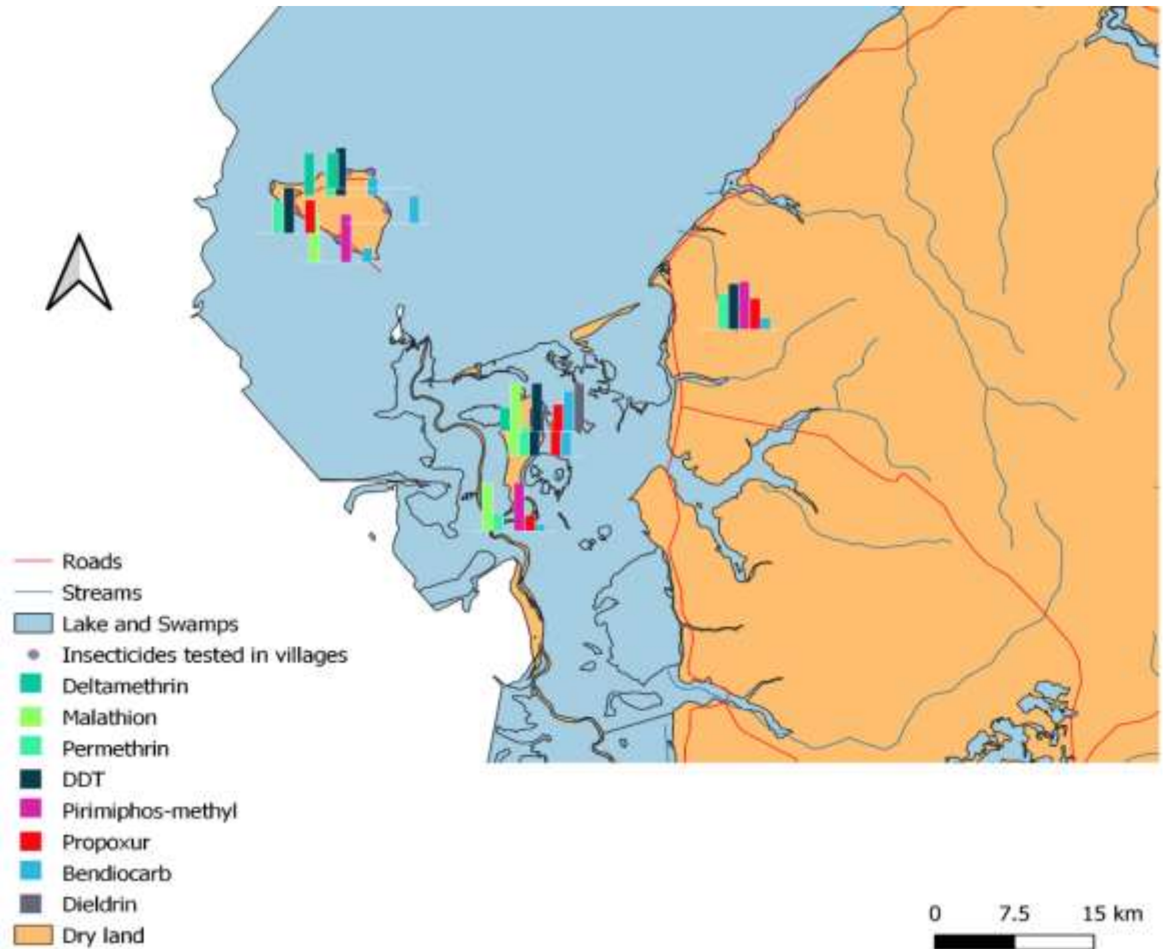


Figure 20 Distribution of insecticide resistance in *An. funestus* from villages across the study sites.

The bars on the map represent insecticides tested. The height of the bar shows the level of susceptibility to the insecticide indicated, where a full bar indicates complete susceptibility and short bars show resistance**.** Shape files of water bodies, streams, and roads used were obtained from online sources.

Table 16 2015–2016 WHO standard susceptibility tests were conducted on field-collected *An. funestus* in three of the study sites. Mortality was corrected by Abbot's formula, where control mortality was between 5% and 20% 24 hours post-exposure

site	Insecticide	Dead after 24 hours		Total tested	% Mortality (corrected)
		No	Yes		
Kilwa island	DDT	0	143	143	100
	Pirimiphos-methyl	0	85	85	100
	Bendiocarb	59	56	115	44*
	Control	130	12	142	9
	Deltamethrin	23	133	156	84*
	Malathion	0	85	85	100
	Permethrin	16	82	98	82*
	Propoxur	4	94	98	96*
Chisenga Island	DDT	0	34	34	100
	Pirimiphos-methyl	0	70	70	100
	Bendiocarb	40	67	107	58*
	Control	100	13	113	13

	Deltamethrin	16	42	58	69*
	Dieldrin	0	19	19	100
	Malathion	0	59	59	100
	Permethrin	40	29	69	35*
	Propoxur	32	48	80	55*
Mainland	DDT	0	21	21	100
	Pirimiphos-methyl	0	22	22	100
	Bendiocarb	52	32	84	38*
	Control	28	1	29	4
	Deltamethrin	9	46	55	84*
	Permethrin	7	19	26	73*
	Propoxur	7	13	20	65*

*Resistance

Logistic regression was used to estimate the effects of these insecticides compared to negative control at the three sites. Four of the insecticides (DDT, dieldrin, Pirimiphos-methyl and malathion) perfectly predicted 100% success throughout. The levels of resistance against the other four insecticides (bendiocarb, deltamethrin, permethrin and propoxur) varied across the three sites.

The overall marginal effects on *An. funestus* predicted for the insecticides that registered resistance, holding all the effects at their mean, were as follows:

1. Bendiocarb was predicted to cause 38% mortality (95% CI: 227.7%–48.5%)
2. Deltamethrin was predicted to cause 84% mortality (95% CI: 73.7% - 93.4%)
3. Permethrin was predicted to cause 73% mortality (95% CI: 56.0% - 90.1%)

Propoxur was predicted to cause 65% mortality (95% CI: 44.1% - 85.9%). The observed effects of bendiocarb and DDT on *An. gambiae* were the opposite of their effects on *An. funestus* (Table 17). Bendiocarb caused 100% mortality in *An. gambiae*. On the other hand, DDT recorded a 10% mortality rate in *An. gambiae*. Pirimiphos-methyl caused 100% mortality on *An. gambiae,* while deltamethrin recorded a mortality rate of 6%. From these results, using the WHO criteria, *An. gambiae* was resistant to DDT and deltamethrin but susceptible to pirimiphos-methyl and bendiocarb.

Table 17 2014 WHO standard susceptibility tests conducted on F1s raised from field-collected *An. gambiae* and *An. funestus* in three of the study sites. Mortality was corrected by Abbot's formula where control mortality was between 5% and 20% 24hrs post-exposure.

Species	Insecticide	Dead 24h Yes	Dead 24h No	Total tested	Replicates	% Mortality
An. gambiae	Bendiocarb	74	0	74	4	100
	DDT	1	10	11	1	10*
	Deltamethrin	2	34	36	2	6*
	Pirimiphos-methyl	42	0	42	2	100
	Control	0	31	31	2	0

An. funestus	Bendiocarb	58	30	88	5	66*
	DDT	82	0	82	3	100
	Deltamethrin	-	-	-	-	-
	Pirimiphos-methyl	84	0	84	4	100
	Control	0	45	45	2	0

*Resistance

In 2016, some *An. funestus* females from Kilwa Island that survived the 60 min WHO standard exposure time and were placed in holding cups potentially lived beyond 12 days. These could have survived longer had they not been killed by freezing! The distribution of the survivors that were frozen at 12 days following exposure was 13% (n = 70) on bendiocarb, 15% (n = 13) on propoxur and 13% (n = 32) on permethrin.

4.8.2 Resistance intensity

Resistance intensities (Figs. 21, 22, 23, 24, 25 and 26) are shown for both the wild mosquitoes and in the laboratory on F1s. Table 18 gives a summary of the mortalities at the end of 8 hours of continuous exposure and 24 hours post-exposure.

Table 18 Resistance intensity testing carried out on *An. funestus* using WHO standard tubes laid horizontally, allowing mosquito exposure to insecticide for 8 hours. Mortality was scored 24hrs post exposure period.

Year - Site	Insecticide	Total tested	Replicates	8-Hour exposure % Knockdown	24-Hour Mortality

Year - Site	Insecticide	Total tested	Replicates	8-Hour exposure % Knockdown	24-Hour Mortality
2014 - Mainland					
F1s	Bendiocarb	196	8	100	100
	Deltamethrin	102	4	75	83*
	Control			100	100
2016 - Mainland					
Wild	Bendiocarb	37	2	54	54*
	Deltamethrin	55	3	71	84*
	Control			100	100
2016 - Chisenga Island					
Wild	Bendiocarb	45	2	77	77*
	Deltamethrin	85	4	89	93*
	Permethrin	38	2	100	100
	Propoxur	85	4	99	100
	Control			100	100

Year - Site	Insecticide	Total tested	Replicates	8-Hour exposure % Knockdown	24-Hour Mortality
2016 - Kilwa Island					
	Bendiocarb	23	1	91	91*
Wild	Deltamethrin	42	2	90	93*
	Permethrin	6	1	100	100
	Propoxur	17	1	100	100
	Control			100	100

*Resistance

The resistance intensities against bendiocarb from all study sites varied inconsistently compared to the susceptible colony mosquitoes. A quick glance at Figure 22 shows that the resistance intensity was highest on the mainland, followed by Kilwa Island and then Chisenga Island. In 2016, the knockdown effect of bendiocarb on the wild mosquitoes from the mainland compared to its effect on colony mosquitoes was significant at 50% less (IRR = 0.500, 95% CI: 00.206–1.142, p=039). On the other hand, the bendiocarb knockdown rate on wild *An. funestus* (Figure 22) compared to F1s recorded in 2014 (Figure 21) was significantly lower by 44% (IRR = 0.540, 95% CI: 0.323 – 0.858, p = 002). A 100% knockdown rate within the 8-hour exposure time was recorded on F1s in 2014 (Figure 21). In 2016, both the knockdown and mortality rates recorded on wild mosquitoes against bendiocarb (Figure 22). For both 2014 and 2016 the 8-hour %knockdown was the same as the 24-hour post-exposure % mortality on bendiocarb.

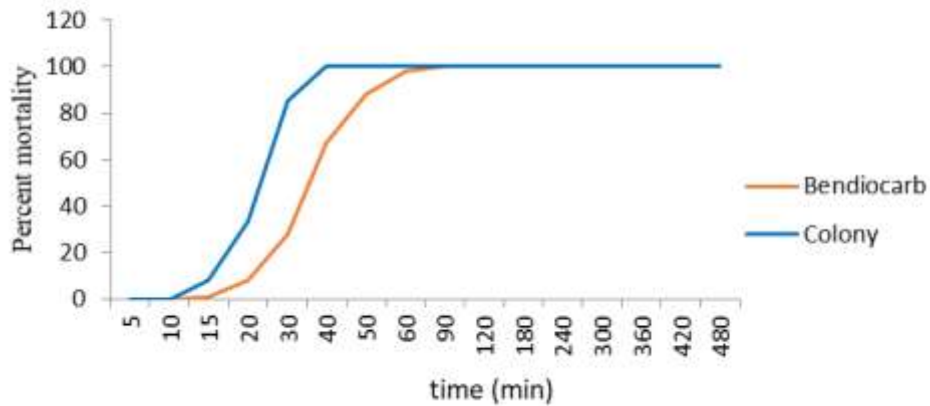


Figure 21 Resistance intensity tests of 0.1% bendiocarb on F1s of female *An. funestus* from mainland, 2014

In 2014 (Figure 21), a slight delay in achieving a 100% knockdown rate against bendiocarb on the mainland was observed. It took two and a half times that of the susceptible colony mosquitoes to achieve 100% knockdown in F1s of the mainland wild mosquitoes. In 2016, the 8-hour continuous exposure to bendiocarb on wild mosquitoes from the three sites neither resulted in 100% knockdown nor 100% mortality 24 hours later (Figure 22). For two-thirds of the resistance intensity events, the rate of knockdown translated into the same percentage mortality rate 24-hours post-exposure (Figure 22).

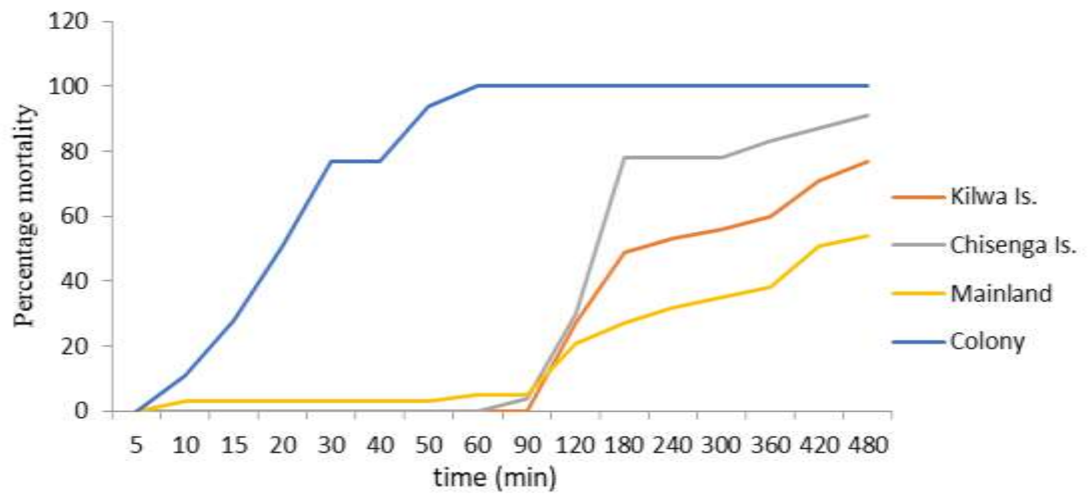


Figure 22 Resistance intensity testing of 0.1% bendiocarb on wild *An. funestus* from the study sites, 2016

Quite a different scenario was obtained with propoxur, another carbamate, in 2016 (Figure 23). There was 100% knockdown at the end of the 8-hour exposure period on both the Chisenga Island and Kilwa Island wild *An. funestus*. Just about half of the mosquitoes were knocked down by the 60 min standard time,sure time, whereas all the colony mosquitoes were knocked down by 60 min standard exposure time.

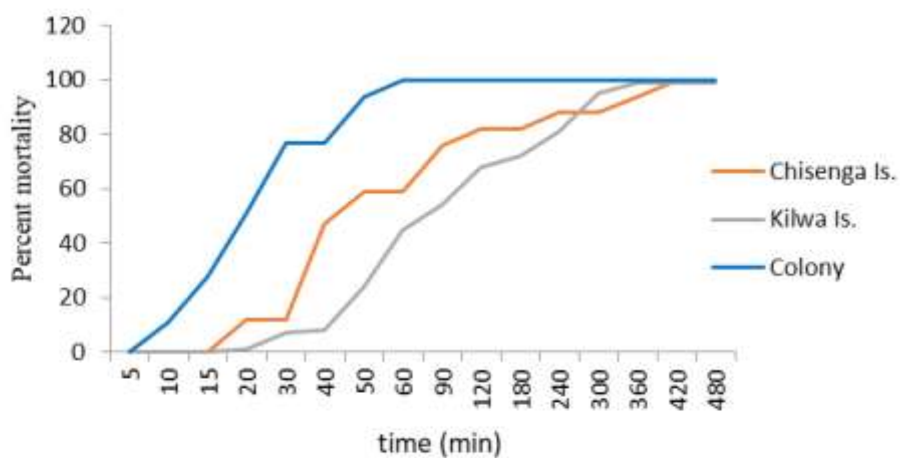


Figure 23 Resistance intensity tests of 0.1% propoxur on wild *An. funestus* from the study sites, 2016

Although 100% knockdown was achieved within the 8-hour continuous exposure against propoxur, delay times to achieving 100% knockdown were slightly different between the Kilwa and Chisenga Islands. The length of time required to achieve 100% knockdown on Kilwa Island was six times that of the susceptible control colony mosquitoes. For Chisenga Island, the delay was seven times that of the colony mosquitoes. The results recorded in 2014 (Figure 24) and 2016 (Figure 25) on deltamethrin resistance intensity for the mainland were similar for both years. In 2016, the intensity of resistance against deltamethrin (type-II pyrethroid) was higher on the mainland compared to the two islands, which recorded similar knockdown rates during the 8-hour exposure time (Figure 25).

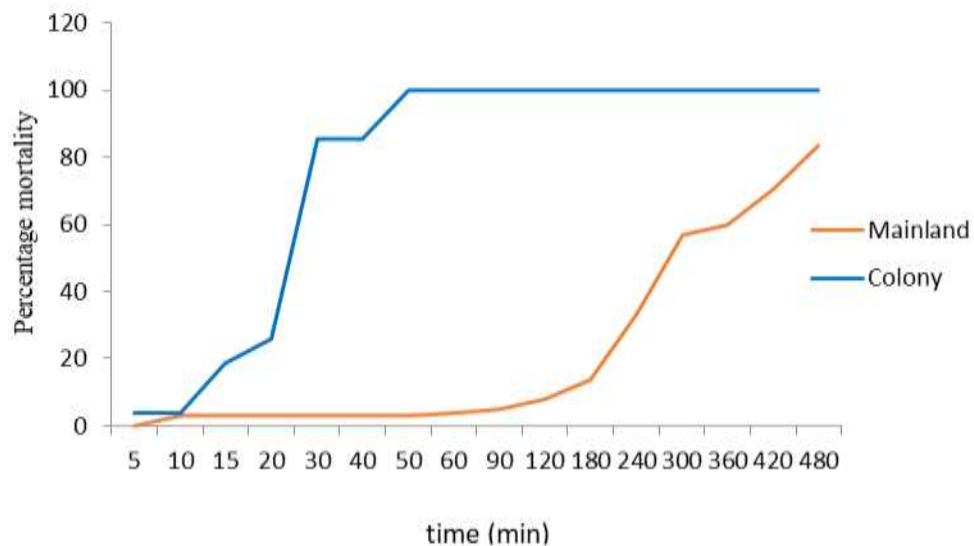


Figure 24 Resistance intensity tests of 0.05% deltamethrin on F1s of *An. funestus* from Mainland, 2014

Deltamethrin knockdown effects on colony mosquitoes compared to wild mainland *An. funestus* delayed and were nearly 30% less (IRR = 0.696, 95% CI: 0.337–1.413, p = 0.142).

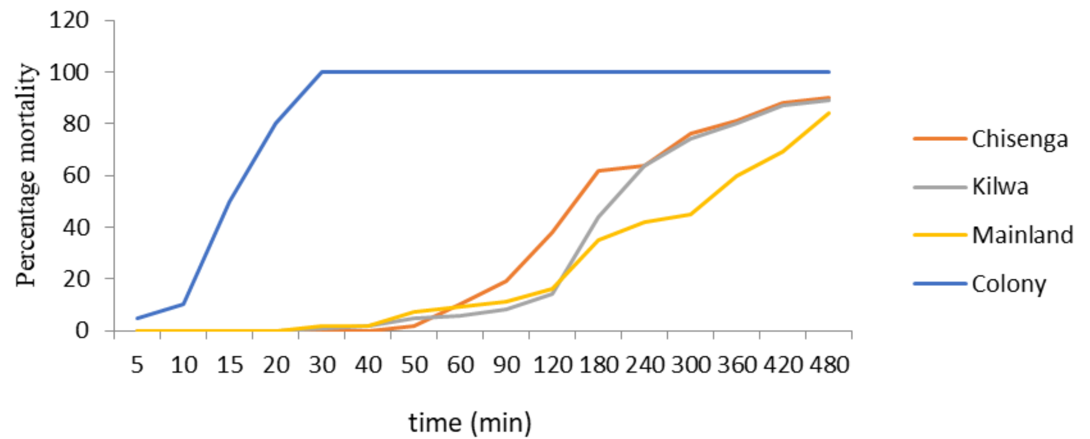


Figure 25 Resistance intensity tests of 0.05 deltamethrin on wild An. funestus in the study sites, 2016

Resistance intensity tests on permethrin resulted in 100% knockdown by the 7th hour of exposure, whereas the colony mosquitoes were all down by 30 min (Figure 26).

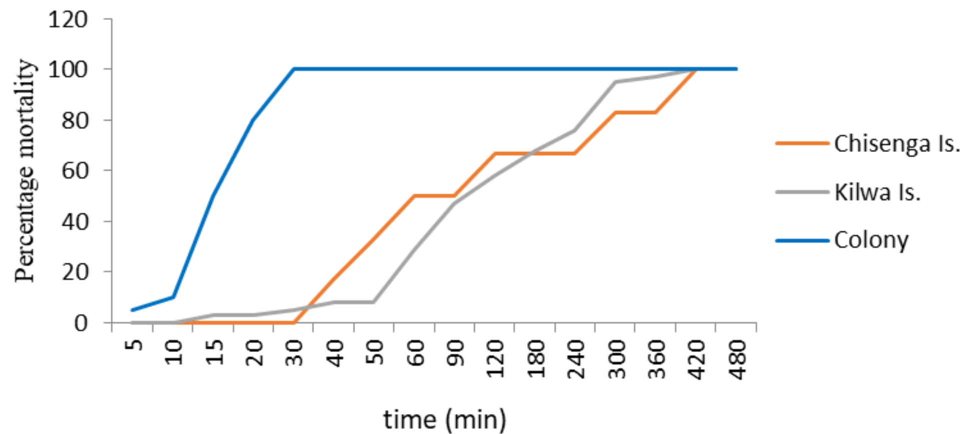


Figure 26 Resistance intensity tests of 0.75% permethrin on wild *An. funestus* in the study sites, 2016

Although 100% knockdown was achieved within the 8-hour continuous exposure for resistance intensity against permethrin, delay times were slightly different between the Kilwa and Chisenga Island mosquitoes (Figure 26). The length of time for 100% knockdown on Kilwa Island was 12 times that of the susceptible

control colony mosquitoes. For Chisenga Island, 100% knockdown was achieved at 14 times the rate of the colony mosquitoes.

4.9 Insecticide usage in the community

Fifty households with one respondent each were interviewed on the usage of insecticide in the community. The results of the survey regarding the demographics of the respondents and their responses are summarised in Figure 27.

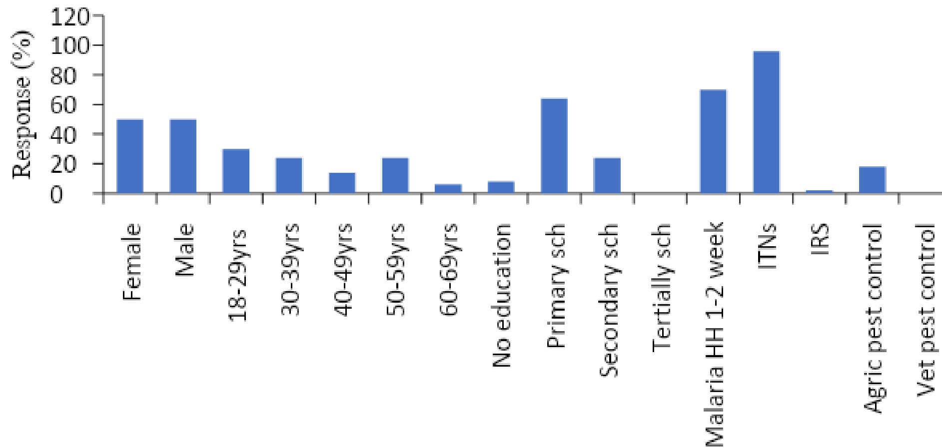


Figure 27 Demographics and responses regarding usage of insecticides against public health, crops and veterinary pests

Equal numbers of male and female participants were interviewed, with the majority having a primary school education. The youngest age interviewed was 18 years old, and the oldest was 66 years old. Ninety-six percent of the respondents indicated that they owned at least one ITN and they used the net during the night to prevent mosquito bites. Seventy percent reported that at least one member of their family had suffered malaria within one- to-two weeks from the date of the interview and had been treated with Coartem. Almost every household interviewed engaged in some agricultural activity, 18% used agricultural pesticides, and none of the respondents reported using pesticides on pets or other domestic animals they kept. The names of insecticides they reported using on agricultural pests included Boxer™ (Emamectin Benzoate), Lamdacyhalothrin and Super Actellic Chirinda Matura dust (pirimiphos-methyl).

4.9.1 Resistance mechanisms detected in *An. gambiae*

The kdr-West allele RR was detected in all 86 *An. gambiae* mosquitoes tested (Figures 28 and 29). The majority of the *An. gambiae* mosquitoes came from Kilwa Island. The kdr mutation L1014F common in west, central, and southern Africa seems to be fixed in *An. gambiae* of Nchelenge. The kdr-East type mutation L1014S (Figure 30), common in east Africa, was also detected in the same samples, with only 14% of these showing the RS allele kdr mutation.

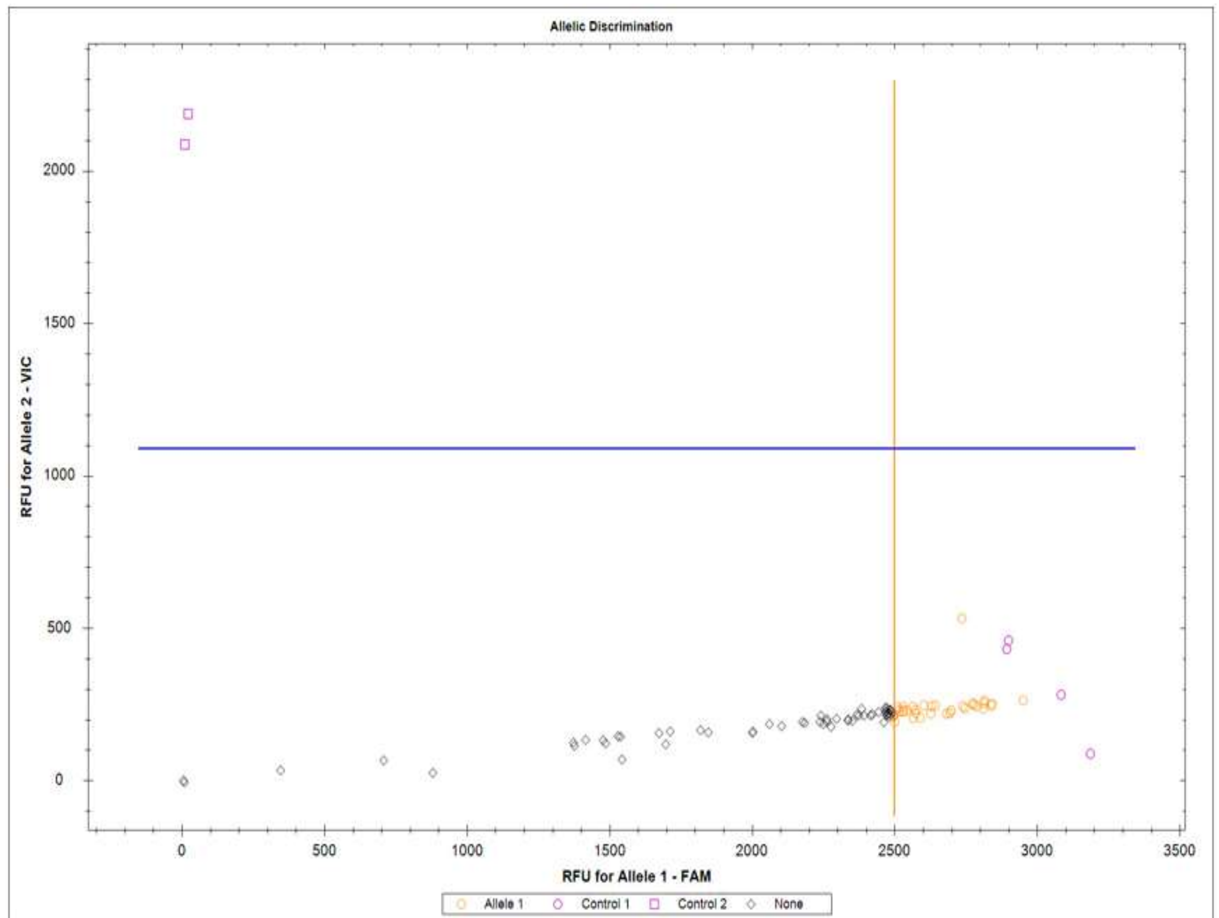


Figure 28 Genotype assignment by the total relative fluorescence units (RFU) showing the RR allele (Allele1) in all the tested samples Allele 1 represents individual samples with the L1014F mutation for knockdown resistance (KDR) against Pyrethroids and DDT.

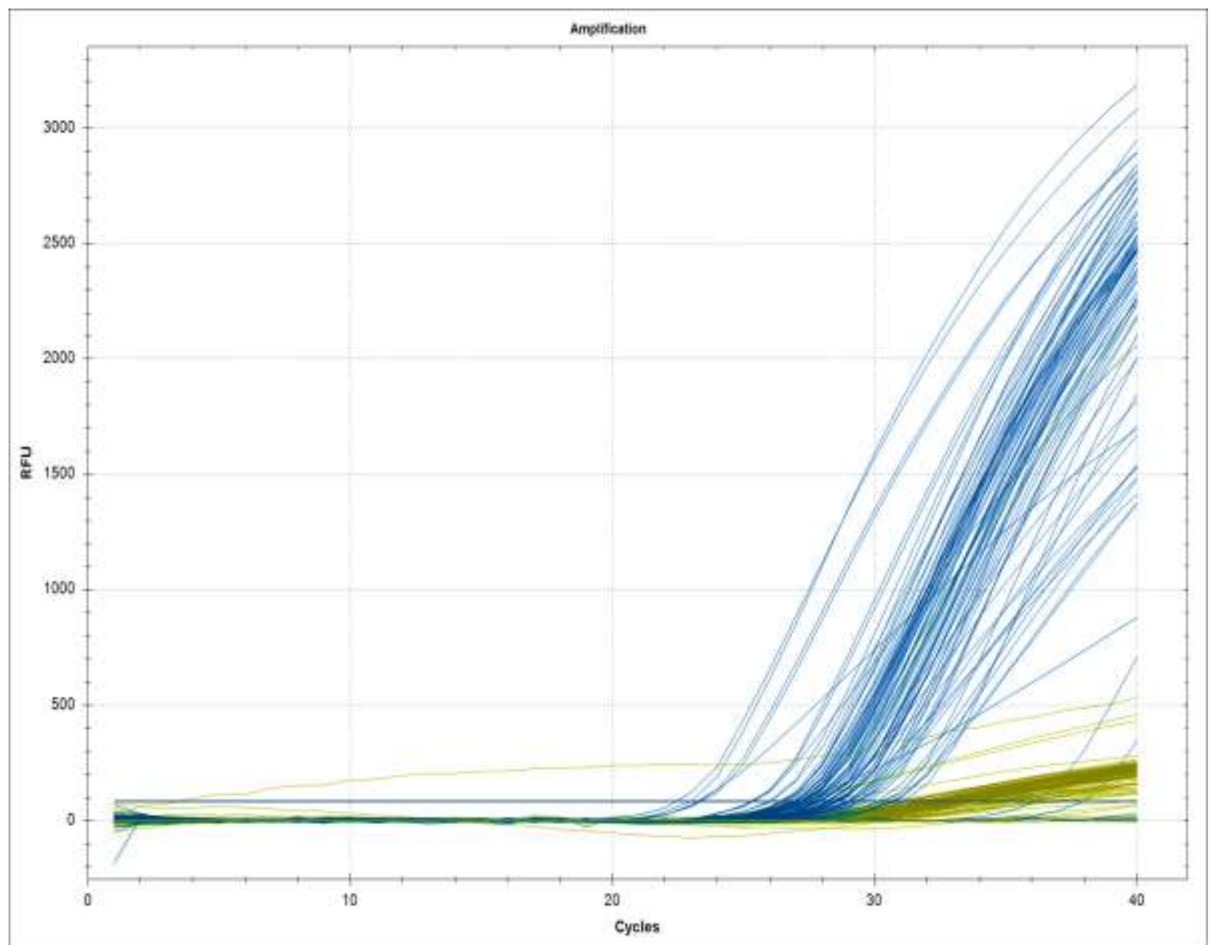


Figure 29 Detection of the Kdr-w mutation. Cycling of the FAM-labeled probe specific for the Kdr-w allele.

The KDR east mutation was also noted in the *An. gambiae* specimens (Figure 30).

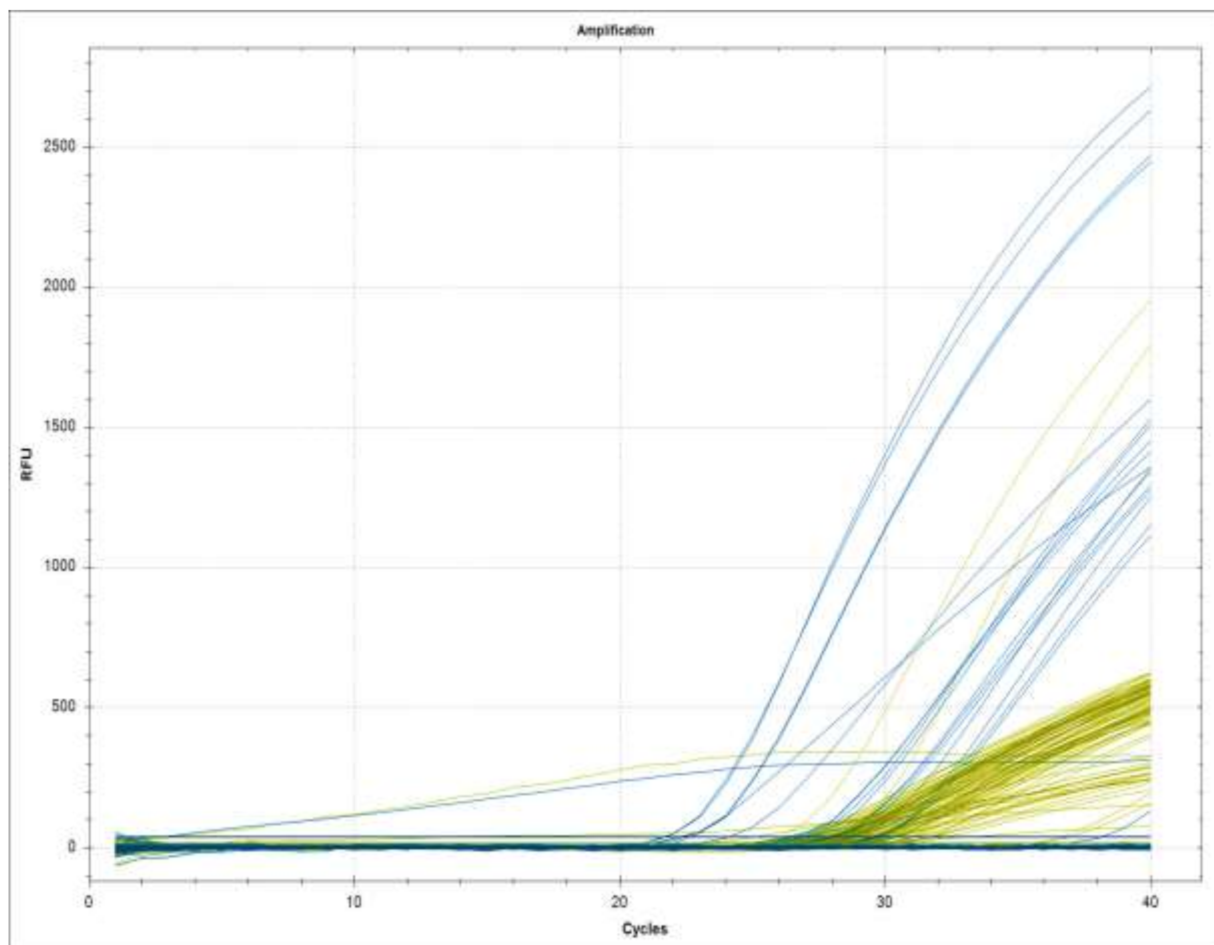


Figure 30 Detection of the Kdr-e mutation. Cycling of the FAM-labelled probe specific for the Kdr-e allele.

Resistance mechanisms in *An. funestus* from Nchelenge were determined and reported elsewhere (Choi et al., 2014). Pre-exposure of the *An. funestus* mosquitoes to the piperonyl butoxide (PBO) nullified the phenotypic resistance against deltamethrin and bendiocarb. Mono-oxygenase-based P450 resistance phenotype was suspected by the use of the synergist PBO.

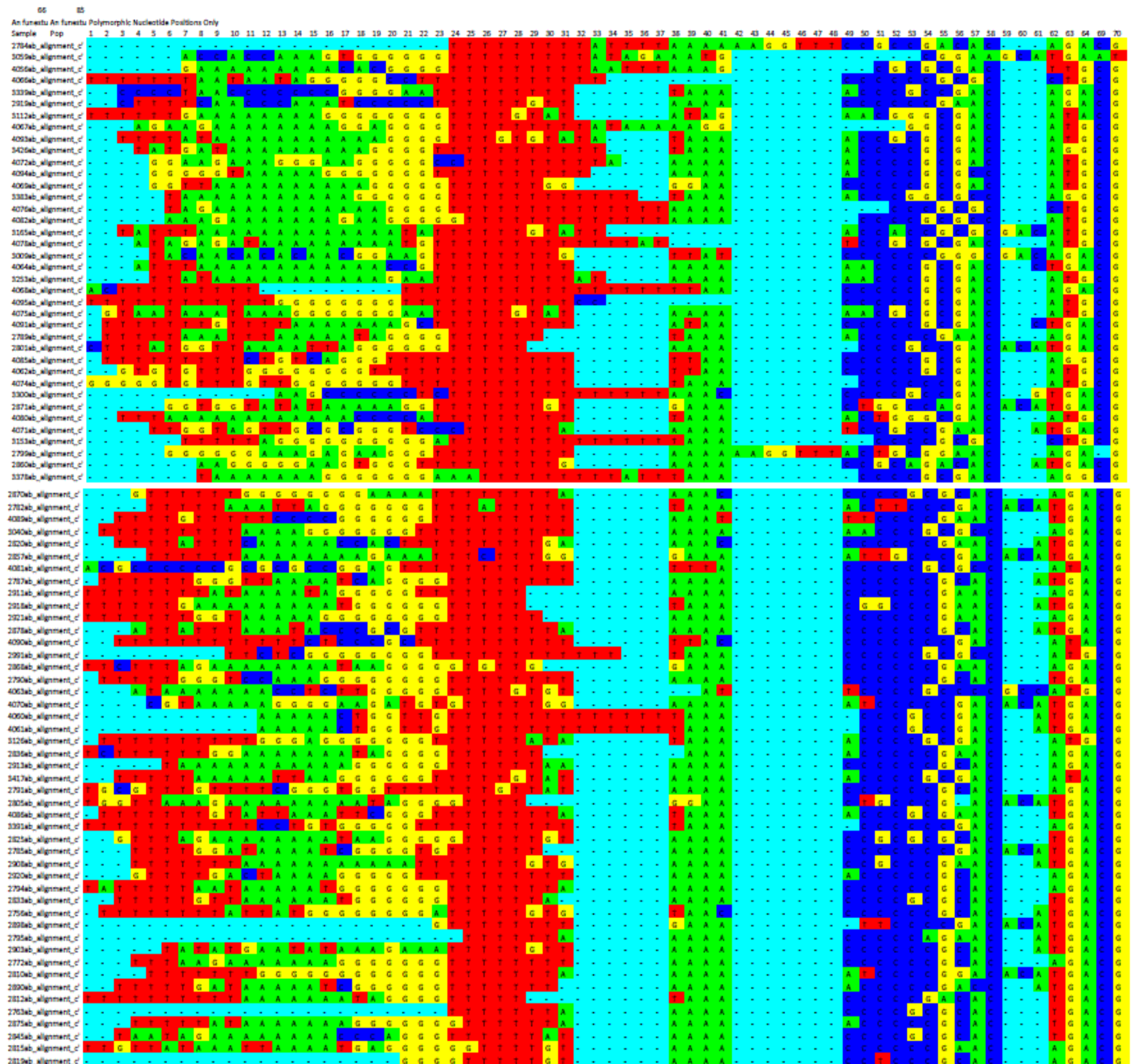
4.10 Population genetics of the malaria vectors of Nchelenge

The population genetics of *An. funestus* and *An. gambiae* were determined based on the rDNA ITS2 sequences obtained from Macrogen, a commercial service provider. Both *An. funestus* and *An. gambiae* sequence lengths varied. The *An. funestus* sequences ranged from 770 to 805 bases, while that of *An. gambiae*

ranged from 506 to 526 bases Sequence annotation for the database-matched samples indicated that the gene of our interest was found between the 5.8s and 28s of the ribosomal RNA gene. The ends of the 5.8s and 28s ribosomal RNA genes were partial sequences, and the ITS2 was a complete sequence of either *An. funestus* or *An. gambiae* isolates. Database matches are included for voucher specimens as well as a host of isolates with different accession numbers in the database.

Multiple alignments of the sequences were followed by different analyses in different population genetics analysis software, to which sequence data was exported. Figure 31 is an example of multiple alignment as generated in GenAIex 6.503 shows only the region with nucleotide differences.

Figure 31 Multiple alignments in the *An. funestus* ITS2 rDNA generated with



GenAIex show polymorphic sites only. Color-coded nucleic acid bases are shown where; red is T, dark blue is C, green is A and yellow is G

4.10.1 Genetic diversity

The diversity of the malaria vectors was determined using a total of 167 ITS2 consensus sequences from an initial total of 394 forward and reverse sequences of each sampled specimen. Poor quality sequences were removed from subsequent analyses, and this led to a reduction in the number of sequences included in the following analyses.

4.10.2 Phylogenetic Analyses

The maximum Likelihood analysis was performed for the ITS2 loci for both the *An. funestus* and *An. gambiae* s.s. sequences. A total of 84 *An. funestus* and 83 *An. gambiae* consensus sequences from across three study sites were used in the analysis. For *An. funestus* these sequences included both those that were identified as clade I and Clade II by the COI Taqman hydrolysis assay. Previously described *An. funestus* and *An. gambiae* sequences were obtained from NCBI BLASTn (http://www.ncbi.nih.gov/BLAST/) using Geneious Prime and included in phylogenetic tree construction. The *An. funestus* sequence was used as an out-group in the construction of the rooted *An. gambiae* phylogenetic tree. The phylogenetic tree constructed for *An. funestus* (Figure 32) revealed that 97% (82/84) sequences clustered together as *An. funestus* with 82% bootstrap support. Very minor sub-clusters were seen with bootstrap support ranging from 50.7 to 65.2 %. Previously described *An. funestus* sequences from NCBI BLASTn clustered together with the 82 consensus sequences. Two sequences, one each from Chisenga Island and Kilwa Island, occurred e separately from the rest. There was no evidence for clade clustering in the phylogenetic tree constructed for *An. funestus,* as both clades I and II, as determined by the mitochondrial DNA,were seen clustering together on one branch in this ITS2 genetic locus. The database ITS2 sequences of *An. funestus* clustered together with the study *An. funestus* ITS2 sequences. The *An. funestus* Clade II shown in Figure 32 which were classified as such using the COI TaqMan hydrolysis assay did not cluster together but rather mixed evenly with the Clade I *An. funestus* in this study. The phylogenetic tree constructed for *An. gambiae* (Figure 33) revealed that 100% (85/85) sequences included clustered together as *An. gambiae* with 100% bootstrap support. Very minor sub-clusters were seen with bootstrap support ranging from 54.3 to 89.7%. The consensus sequences analysed in this study clustered well with previously described *An. gambiae* sequences from NCBI BLASTn. Besides the *An. funestus* out-group sequence, one other sequence of an unidentified *Anopheles species* was included in the *An. gambiae* phylogenetic tree construction.

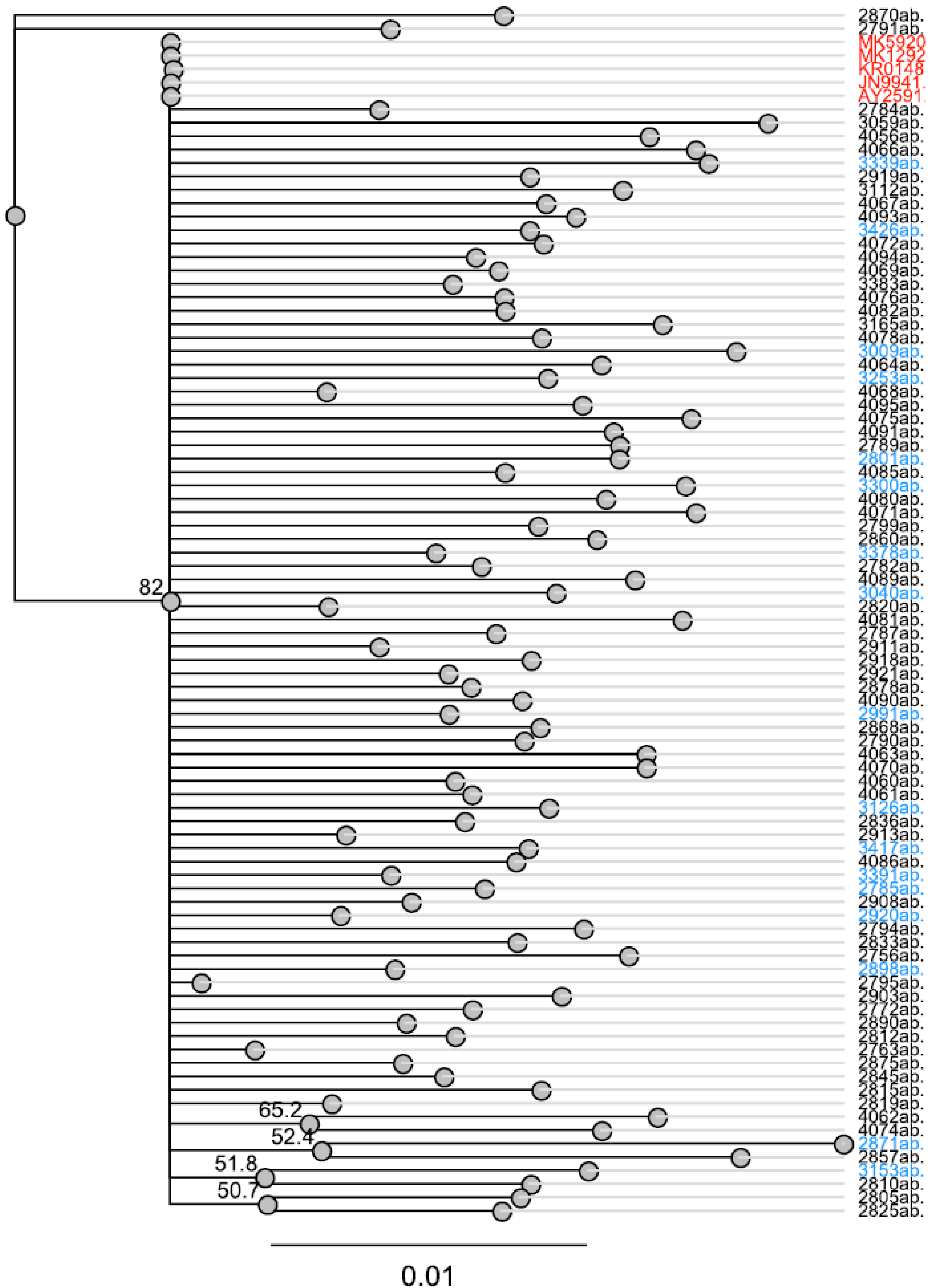


Figure 32 *An. funestus* near-neighbour joining phylogenetic tree constructed with default settings in Geneous Prime

The Blue colour coded specimen represents Clade II as determined by the COI using the TaqMan assay. The red-coloured specimen were *An. funestus* ITS2 isolates from the NCBI database and included in the constructed tree.

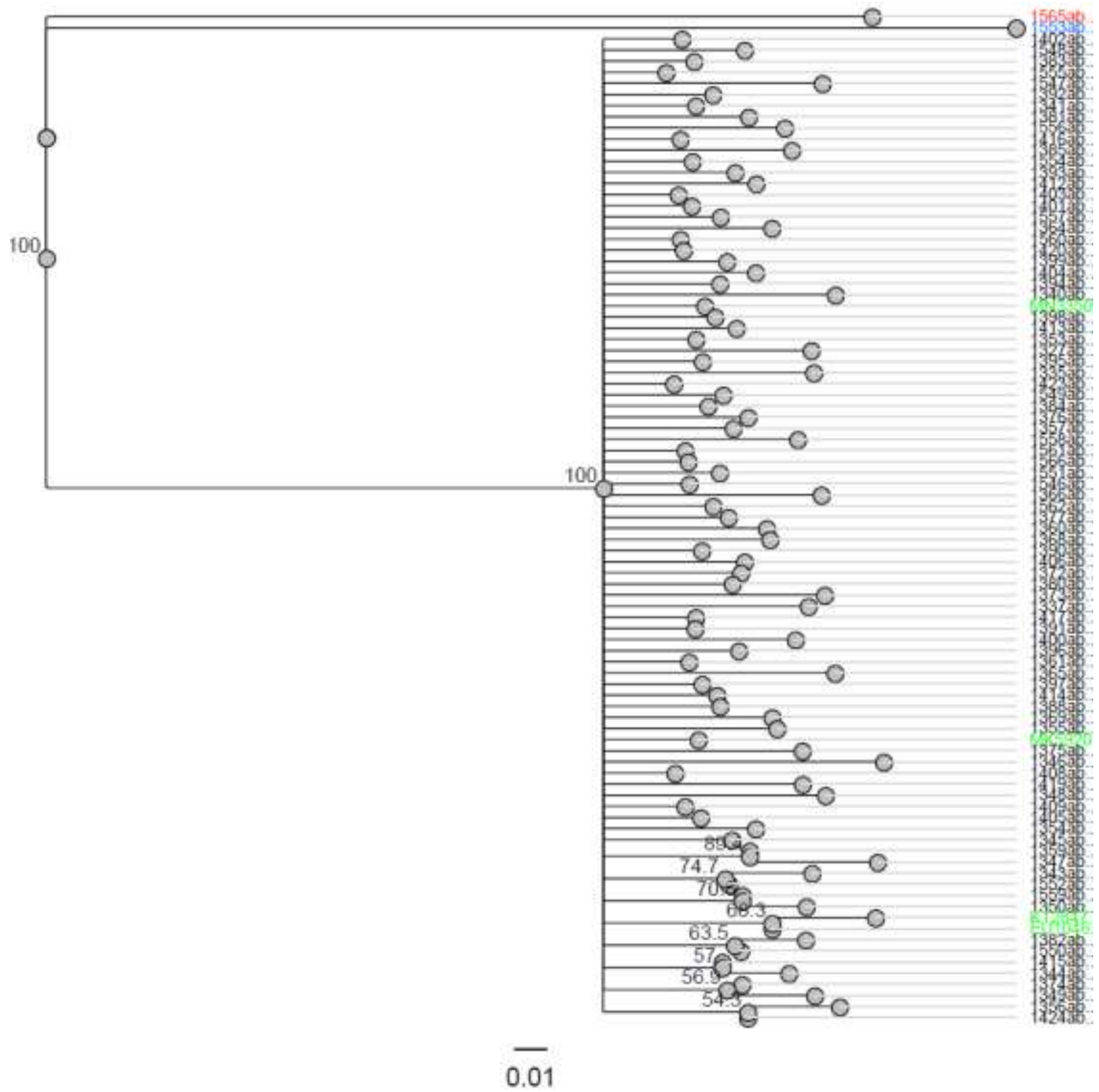


Figure 33 *An. gambiae* near-neighbour joining phylogenetic tree constructed with default settings in Geneous Prime

The red and Blue colour-coded specimens represent *An. funestus* and the unidentified *Anopheles* mosquito. The in green were accession numbers of *An. gambiae* ITS2 isolatthe from the database in NCBI and included in the constructed tree.*Anoplese gambiae* ITS2 database sequences clustered together with the study *An. gambiae* sequences very well. The unidentified *Anopheles* species ITS2 sequence appeared closer to *An. funestus* than *An. gambiae* (Figure 33).

4.10.3 Gene flow and genetic differentiation analysis

In the total data estimates, it was shown that haplotype diversity was 0.999 in *An. funestus* (Table 19) and that of *An. gambiae* was 0.97297 (Table 20). An abundance of singleton haplotypes is what drives this diversity. Nucleotide diversity in *An. funestus* was at 0.01042 and that of *An. gambiae* was 0.00619. The average number of nucleotide differences (Kt) in *An. funestus* was 8.568 and that of *An. gambiae* was 3.40053.

Table 19 *Anopheles funestus* - genetic differentiation parameters

Population	No. of sequences	No. of segregating sites (S)	Number of Haplotypes (h)	Haplotype diversity (Hd)	Ave. no. of differences (K)	Nucleotide diversity (π) (JC)
Mainland	23	28	23	1.0000	8.41107	0.01031
Kilwa Is.	20	27	20	1.0000	8.65263	0.01061
Chisenga Is.	41	30	41	1.0000	8.63780	0.01059
Total	84	37	82	0.99943	8.56799	0.01042

Table 20 *Anopheles gambiae:* genetic differentiation parameters

Population	No. of sequences	No. of segregating sites (S)	Number of Haplotypes (h)	Haplotype diversity (Hd)	Ave. no. of differences (K)	Nucleotide diversity (π)
Mainland	11	7	9	0.94545	2.41818	0.00440
Kilwa Is.	63	19	45	0.97696	3.57143	0.00651
Chisenga I	9	7	8	0.97222	3.66667	0.00668
Total	83	19	49	0.97297	3.40053	0.00619

The nucleotide diversity (π) in both *An. funestus* and *An. gambiae* was lowest in the Mainland population. Nucleotide diversity in *An. funestus* was highest in the Kilwa Island population, followed by the Chisenga Island population. Nucleotide diversity in *An. gambiae* was highest in Chisenga Island population, followed by Kilwa Island population. Genetic differentiation estimated probabilities in both *An. funestus* and *An. gambiae* populations obtained by the permutation test with 1000 replicates were all not significant. In *An. funestus*, gene flow estimates of the coefficients of differentiation and their related migrant individuals were G_{st} was 0.00062, with Nm at 400.10; $Delta_{St}$ was 0.00023, and $Gamma_{St}$, 0.2276,6 with Nm at 10.7; *N*st was -0.00278 with*N*m at -90.26.; *F*st was -0.00274 with Nm at -91.50.In *An. gambiae*, gene flow estimates of the coefficients of differentiation and their related migrant individuals were G_{st} = 0.01271 with Nm at 19.43; $Delta_{St}$ was 0.00009 and, $Gamma_{St}$, 0.01399 with Nm at 17.63; Nst = -0.02473 with Nm at -10.36; (Fst) was -0.02457 with Nm at -10.43.

4.10.4 DNA Divergence between populations

4.10.4.1. *Anopheles funestus:* – Mainland and Chisenga Island populations

A comparison of the *An. funestus* populations from Mainland and Chisenga Island was based on a total of 64 ITS2 consensus sequence samples, which included 23 from Mainland and 41 from Chisenga Island. Within the Mainland sample the number of polymorphic sites was 29 with a total number of 40 mutations detected. The average number of nucleotide differences (k) was 8.498 and nucleotide diversity (π) 0.0103±0.0007. Chisenga Island sample, the number of polymorphic sites was 31, with 48 mutations detected. The average number of nucleotide differences (k) was 8.733 and nucleotide diversity (π) was 0.0106±0.0007.Considered together as one sample with 64 consensus sequences from the mainland and Chisenga Island, there were 37 polymorphic sites with a total of 56 mutations detected. The average number of nucleotide differences (k) was 8.659 with a Nucleotide diversity (π) of 0.0105.Regarding sampled population comparisons, there were no fixed differences found between the two populations. From a total of 56 mutations detected, 32 mutations were shared between the two populations. Out of these mutations, 8 were polymorphic in Mainland populations but monomorphic in Chisenga Island populations. It was the other way around for another set of 16 mutations, which were polymorphic on Chisenga Island but monomorphic in Mainland populations. The Average number of nucleotide differences between populations was 8.638.

4.10.4.2 *Anopheles funestus:* Mainland and Kilwa Island populations

A comparison of the *An. funestus* populations from Mainland and Chisenga Island was based on a total of 43 consensus sequence samples, which included 23 from Mainland and 20 from Kilwa Island. Within the Mainland sample, the number of polymorphic sites was 43, with 66 mutations detected. The average number of nucleotide differences (k) was 15.154 and nucleotide diversity (π) o0.018±0.00098.

Within the Kilwa Island sample, the number of polymorphic sites was 40 with 62 total mutations detected. The average number of nucleotide differences (k) was 14.716 and nucleotide diversity (π) was 0.017±0.0008.Considered together as one sample with 43 consensus sequences from mainland and Kilwa Island, there were 48 polymorphic sites with a total of 79 mutations detected. The average number of nucleotide differences (k) was 14.942 with a Nucleotide diversity (π) of 0.0178 Regarding sampled population comparisons, there were no fixed differences found

between the two populations. From a total of 79 mutations detected 49 mutations were shared between the two populations. Out of these mutations 17 were polymorphic in Mainland populations but monomorphic in Kilwa Island populations. Conversely, 13 mutations were polymorphic in Kilwa Island populations but monomorphic in Mainland populations. The Average number of nucleotide differences between populations was 14.920.

4.10.4.3 *Anopheles funestus:* Chisenga Island and Kilwa Island populations

A comparison of the *An. funestus* populations from Chisenga Island and Kilwa Island was based on a total of 61 consensus sequence samples, which included 41 from Chisenga Island and 20 from Kilwa Island. Within the Island sample the number of polymorphic sites was 31 out of a total of 48 mutations detected. The average number of nucleotide differences (k) was 9.016 and nucleotide diversity (π) of 0.011±0.0005 (Jukes and Cantor).Within the Kilwa Island sample, the number of polymorphic sites was 28, with 38 mutations detected. The average number of nucleotide differences (k) was 9.068 and nucleotide diversity (π) of 0.011±0.0006. Considered together as one sample with 61 consensus sequences from Kilwa Island and Chisenga Island, there were 35 polymorphic sites with a total of 56 mutations detected. The average number of nucleotide differences (k) was 9.004 with a Nucleotide diversity (π) of 0.0109. Regarding sampled population comparisons, there were no fixed differences found between the two populations. From a total of 56 mutations detected, 30 mutations were shared between the two populations. Out of these mutations, 18 were polymorphic in Chisenga Island populations but monomorphic in Kilwa Island populations. Conversely, 8 were polymorphic in Kilwa Island populations but monomorphic in Chisenga Island populations The Average number of nucleotide differences between populations was 8.978.

4.10.4.4 *Anopheles gambiae:* Mainland and Chisenga Island populations

A comparison of the *An. gambiae* populations from Mainland and Chisenga Island was based on a total of 20 consensus sequence samples, which included 11 from Mainland and 9 from Chisenga Island. Within the Mainland sample, the number of

polymorphic sites was 11, with 19 mutations detected. The average number of nucleotide differences (k) was 5.618 and nucleotide diversity (π) was 0.0099 ± 0.00116.

Within the Chisenga Island sample, the number of polymorphic sites was 11, with 16 mutations detected. The average number of nucleotide differences (k) was 5.649 and nucleotide diversity (π) of 0.01003 ± 0.00088.

Considered together as one sample with 20 consensus sequences from the mainland and Chisenga Island, there were 17 polymorphic sites with a total of 22 mutations detected. The average number of nucleotide differences (k) was 5.637 with a Nucleotide diversity (π) of 0.00985. Regarding sampled population comparisons, there were no fixed differences found between the two populations. From a total of 22 mutations detected, 13 mutations were shared between the two populations. Out of these mutations, six were polymorphic in Mainland populations but monomorphic in Chisenga Island populations. It was the other way around for another set of three mutations, which were polymorphic on Chisenga Island but monomorphic in Mainland populations. The Average number of nucleotide differences between populations was 5.626. Average number of nucleotide substitutions per site between populations,Dxy= 0.00991±0.00168 (JC). Number of net nucleotide substitutions between populations, Da = -0.00005±0.00175

4.10.4.5 *Anopheles gambiae:* – Mainland and Kilwa Island populations

A comparison of the *An. gambiae* populations from Mainland and Chisenga Island was based on a total of 74 consensus sequence samples, which included 11 from Mainland and 63 from Kilwa Island. Within the Mainland sample, the number of polymorphic sites was 7, with 8 mutations detected. The average number of nucleotide differences (k) was 2.418 and nucleotide diversity (π) of 0.00442± 0.00066.Within the Kilwa Island sample, the number of polymorphic sites was 19, with a total of 24 mutations detected. The average number of nucleotide differences (k) was 3.571 and nucleotide diversity of 0.00053. Considered together as one sample with 74 consensus sequences from the mainland and Kilwa Island,

there were 19 polymorphic sites with a total of 25 mutations detected. The average number of nucleotide differences (3.401,s 3.401 with a Nucleotide diversity (π) of 0.00620.Regarding sampled population comparisons, there were no fixed differences found between the two populations. From a total of 25 mutations detected, 7 mutations were shared between the two populations. Out of these mutations 1 was polymorphic in Mainland populations but monomorphic in Kilwa Island populations. On the other hand, 2 mutations were polymorphic in Kilwa Island population but monomorphic in the Mainland population. The Average number of nucleotide differences between populations was 3.000. The average number of nucleotide substitutions per site between populations, Dxy = 0.00549±0.00057 (JC). Number of net nucleotide substitutions per site between population, Da = 0.00001±0.00060.

4.10.4.6 *Anopheles gambiae* – Kilwa Island and Chisenga Island populations

The comparison of the *An. gambiae* populations from Kilwa Island and Chisenga Island was based on a total of 72 consensus samples, which included 9 from Chisenga Island and 63 from Kilwa Island. Within the Kilwa samples, the number of polymorphic sites was 19, with a total of 24 mutations detected. The average number of nucleotide differences (k) was 3.571 and nucleotide diversity (π) of 0.00654±0.00053.

Within the Chisenga Island sample, the number of polymorphic sites was 7, with a total of 10 mutations detected. The average number of nucleotide differences (k) was 3.667 and nucleotide diversity (π) of 0.00671±0.00065.Considered together as one sample with 72 consensus sequences from Kilwa Island and Chisenga Island, there were 19 polymorphic sites with a total of 24 mutations detected. The average number of nucleotide differences (k) was 3.547 with a Nucleotide diversity (π) of 0.00646. Regarding sampled population comparisons, there were no fixed differences found between the two populations. From a total of 24 mutations detected, 10 mutations were shared between the two populations. Out of these mutations, 14 were polymorphic in Kilwa Island populations but monomorphic in Chisenga Island populations. The Average number of nucleotide differences (k) between populations was 3.455. The average number of nucleotide substitutions

per site between populations, Dxy = 0.00633±0.00063 (JC). Number of net substitutions per site between populations, Da = -0.00030±0.00068 (JC).

4.10.5. Analysis of Molecular variance

The analysis by GenAIex (Peakall and Smouse, 2006, 2012) revealed that *An. funestus* populations had a PhiPT (Φ_{ST} = fixation index) value of -0.001, an indication of no genetic differentiation between the *An. funestus* populations. All of the total variations obtained were within population variations (Table 21).

Table 21 Hierachical analysis of molecular variance (AMOVA) within/among *An. funestus* populations.

Source of variation	d.f.	SS	MS	Estimated Variance	Total variance (%)
Among Populations	2	4.906	2.453	0.000	0%
Within Populations	82	207.378	2.529	2.529	100%
Total	84	212.284		2.529	100%

d.f.: degrees of freedom; SS: Sum of Squares; MS: Mean Squares

The *An. gambiae* population had a PhiPT (Φ_{ST} = fixation index) value of 0.018, which was not significant (p = 0.107). The partitioned variations observed in *An. gambiae* too were to a large extent due to within populations varians than between populations (Table 22). The PhiPT value obtained here too is an indication of very minimal to no genetic differentiation in the *An. gambiae* populations.

Table 22 Hierarchical analysis of molecular variance (AMOVA) within/among *An. gambiae* populations.

Source of Variation	d.f.	SS	MS	Estimated Variance	Total variance (%)
Among Populations	2	172.872	86.436	1.225	2
Within Populations	80	5309.610	66.370	66.370	98
Total	82	5482.482		67.596	100

Principal coordinate analysis (PCoA), which was done in GenAIex 6.503 to see how the *An. funestus* populations at the different study sites are related to each other, did not show any signs of isolation by distance (Figure 34).*An. funestus*

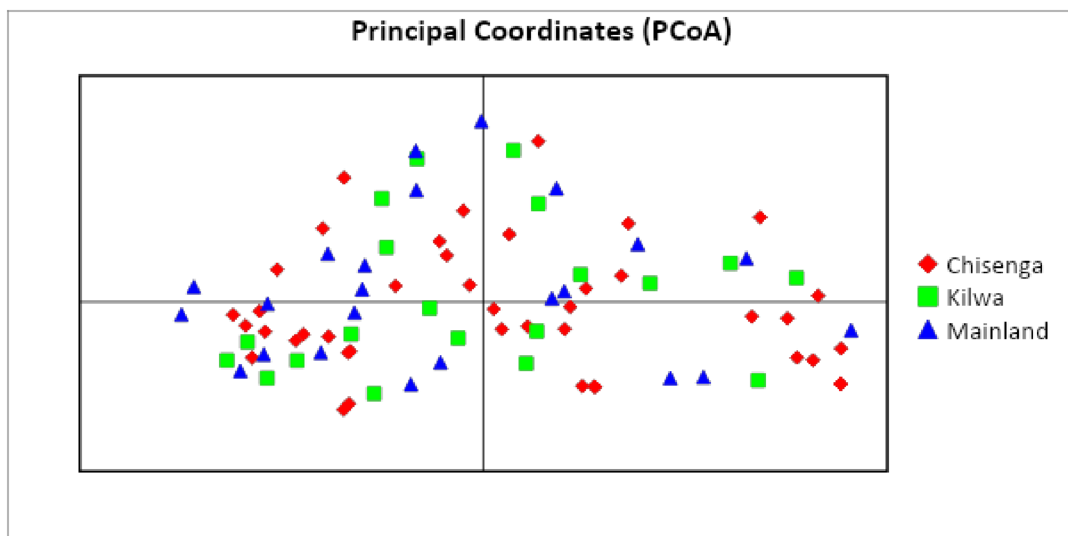


Figure 34 Distribution of *An. funestus* populations across the three study sites. The different colours represent different study sites

The first 3 axes explained 17.3 % of the total variation. All the populations from the study area were distributed fairly in all four axes. Principal coordinate analysis (PCoA) conducted in GenaIex 6.503 to visualise patterns of relationships in the *An. gambiae* populations across the study sites did not reveal any indications of isolation by distance except for small clustering between the Mainland and Kilwa populations and Chisenga and Kilwas populations in the upper two and lower two axes, respectively (Figure 35).

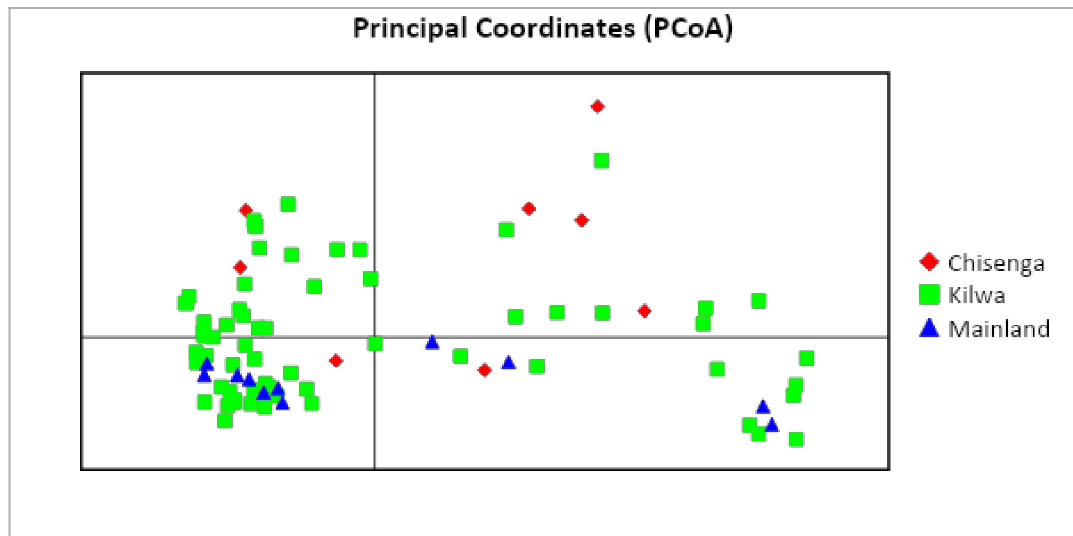


Figure 35 Distribution of *An. gambiae* populations across the three study sites. The different colours represent different study sites

The first 3 axes explained 38.0% of the total variation, much less than 80% of the total variance in relatedness between genetic and geographical distance for the *An. gambiae* ITS2 locus. The first two axes are the most important and should account for at least 80% as threshold for statistical significance. The *An. gambiae* population from the mainland clustered in the lower two axes compared to populations from Kilwa Is. and Chisenga Island.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1 Introduction.

The study determined the genetic diversity of the two major malaria vector mosquito species in Nchelenge district, including islands. While no fixed differences were observed in either malaria vector, *An. funestus* s.s. populations were genetically more diverse in comparison to *An. gambiae* s.s. Spatial and temporal abundance and *P. falciparum* sporozoite rates in these vector species were compared. There were significant differences in the abundance of *An. funestus* s.l. on Chisenga Island and of *An. gambiae* s.l. on Kilwa Island compared to the mainland. The *An. funestus* s.l. was more abundant during the dry season, and the *An. gambiae* was abundant during the rainy season. Malaria infectivity in *Anopheles funestus* spanned all sites and seasons, whereas *An. gambiae* infection with *P. falciparum* happened during the rainy season and was on Kilwa Island and the mainland. Insecticide resistance, i.e., mortality less than 98%, was recorded against pyrethroids and carbamates. No resistance was recorded against organophosphates. *Anopheles funestus* was susceptible to organochlorines, in contrast with *An. gambiae*. Resistance intensity to deltamethrin and bendiocarb was very high in *An. funestus*. The knockdown resistance (kdr) allele was detected in all *An. gambiae* specimens tested.

5.2 Genetic structuring

The first objective was determined with the help of nucleotide sequences of the `internally transcribed spacer second subunit gene (ITS2) of the rDNA. Proportional samples of each vector from study sites were represented in each bioinformatics analysis. The question I proposed to answer in this regard was whether there were genetic structures in populations of the two major malaria vectors, *An. funestus* and *An. gambiae.* The hypothesis I set for this question was that no genetic structures existed within and between populations across the study sites. This study addressed this fundamental question regarding genetic structuring in the major malaria vectors of Nchelenge. With the genetic marker used here (ITS2), there was no evidence for genetic structuring among sub-populations of either *Anopheles funestus* or *An. gambiae* at the study sites. All the metrics for population differentiation obtained in this study (i.e. *G*st, *Φ*st, *F*st, *N*st) were very

low including negative values. Although none of these metrics measuring within and among population diversity is perfect (Ma et al., 2015) the most commonly used metric in population genetic studies, F_{ST} gave a negative value for both *An. gambiae* and *An. funestus* populations. This would be considered zero, meaning no genetic differentiation, even though the value of F_{ST} is expected to fall between 0 and 1 (Ma et al., 2015). This would be due to the genetic marker used in this study. No fixed differences were found between the subpopulations from different sites within the district except for within subpopulation variations. It was quite clear in the results that subpopulations in both *An. funestus* and *An. gambiae* showed differences in levels of nucleotide diversity within each subpopulation in specific sites. Between the two malaria vector species studied, there was almost twice as much total nucleotide diversity in the *An. funestus* population compared to the total nucleotide diversity in the *An. gambiae* population. The higher genetic diversity in *An. funestus* could explain the success of this mosquito as the major primary malaria vector species in Nchelenge. The high genetic diversity observed in *An. funestus* in this study is in agreement with recent findings from studies conducted regionally and nationally on *An. funestus*. Within populations, diversity was observed in *An. funestus* collected from Zambia, including Nchelenge and Choma, Malawi, Mozambique, Uganda, and Tanzania (Jones et al., 2018; Kaddumukasa et al., 2020).

In Equatorial Guinea, scientists noted varied genetic diversity among *An. gambiae* populations from Bioko and nearby islands (Moreno et al., 2007). Larger islands exhibited higher diversity compared to the smaller islands. They also noted a decrease in diversity the further away the Island was from the mainland. It would be easier to understand that vector populations on Kilwa Island, which is furthest away from Nchelenge and completely surrounded by Lake Mweru waters, would share fewer alleles with the Nchelenge Mainland populations. However, this may not be the case with the Mainland in the DRC on the other side and more closer to this island. Besides, the islands surrounded by seawater may not present a similar scenario as the freshwater and continental islands. From the three sites considered here, the Mainland malaria vector populations recorded the lowest Nucleotide diversity in both *An. funestus* and *An. gambiae*. This finding is in contrast with the principles of biogeography, where biological (and hence genetic) diversity (being highest on the mainland) is positively correlated with the size of the island but

negatively so with distance from the mainland (Gillespie and Roderick, 2002). This phenomenon of lowest diversity on the Mainland compared to the islands in Nchelenge could probably be attributed in part to the level of vector control pressure in form of IRS campaign and LLINs distribution programmes carried out on the mainland in Nchelenge. On Kilwa and Chisenga Islands, the main vector control intervention is the distribution of LLINs. The sustained combination of LLINs and IRS for controlling malaria vectors on the mainland could have impacted negatively on the genetic diversity of malaria vectors in this site. Very little to no vector control activities are undertaken on the DRC side of the islands.

To what extent did the respective malaria vector subpopulations mix and were there any barriers to gene flow? The results showed that there was no definite barrier to the movement of genes among the vector populations of Nchelenge. Lake Mweru waters sitting between the islands and Mainland may not be a definite barrier to gene flow. The values of gene flow (Nm) obtained in this study were 400.1 and 19.43 for *An. funestus* and *An. gambiae*, respectively, may attest to this fact. The *An. funestus* Nm value of 400.1 obtained here was in agreement with those obtained by other workers ranging 17 - 483 using microsatellite loci and mitochondrial DNA as genetic markers in West, East and Southern Africa (Braginets et al., 2003; Michel et al., 2005; Ogola et al., 2019; Kaddumukasa et al., 2020). The high figure of Nm obtained in this study indicate a fairly high amount of genes flowing between populations. In this study the value of Nm obtained in *An. funestus* is a further confirmation of a lack of genetic differentiation between populations of this vector across the sites under study. Mosquito movements beyond the > 1km flight range in *An. gambiae* (Costantini et al., 1996) between the islands and mainland could be facilitated by wind as well as human activities (De Meillon, 1947; Gillies and De Meillon, 1968; Lindsay et al., 1995). Human movements between the mainland and islands happen more frequently in Nchelenge. These movements include fishing activities and family visits between and beyond Nchelenge, reaching into the DRC. In addition to humans, chunks of floating vegetation from the Luapula River into Lake Mweru capable of transporting fairly large animals like waterbucks are a regular feature. These floating mats would be capable of transporting both resting adult mosquito and larvae. The *An. gambiae* Nm value of 19.43 and the *An. funestus* value of 400.1 obtained here indicate a lower rate of gene flow in *An. gambiae* compared to that of *An. funestus*.

The depth of variation expected from the genetic markers used in this study is mostly at the higher level of sibling species, subspecies, and molecular forms within a species group, with very little variation at the lower population level of the same species. In many studies the rDNA ITS2 and the intergenetic spacer (IGS) genes have been used to diagnose sibling species (Scott et al., 1993; Koekemoer et al., 2002). Molecular forms in *An. gambiae* s.s. from Mali, Mopti, Besau, and Savanna are identified on the basis of differences in the rDNA nucleotide composition in the right arm of the second chromosome (2R) (Gentile et al., 2002). Studies that have attempted to quantify the variations within and between populations have utilised more sensitive, co-dominant, and genome-wide simple sequence repeats known as microsatellites (Pinto et al., 2003; Lanzaro et al., 2006; Kaddumukasa et al., 2020); single nucleotide polymorphisms (SNPs) (Peiris et al., 2011) and the maternally inherited mitochondrial COI DNA (Lehmann et al., 1998; Michel et al., 2005; Foley and Torres, 2006) than the more conserved ITS2 found in rDNA. However, the values of genetic diversity and differentiation obtained in this study conform to and are within the values obtained by other workers in population genetics utilising microsatellites. From these findings obtained here, it would appear that when many samples of the same species have their ITS2 sequenced and compared, their genetic diversity would be realised.

This study further confirmed what other workers observed: that the apparent Clade I and Clade II phylogeny in *An. funestus* based on the COI gene was not correlated with the ITS2 genetic marker used here (Jones et al., 2018; Kaddumukasa et al., 2020). These findings may show that, at present, segregations of *An. funestus* into Clades based on COI DNA hasn't translated into genome-wide segregation. Further research and probably utilising more robust markers on the biological importance of this segregation are needed.

Within the limitations of the chosen markers, population sample sizes, and techniques used, there was enough evidence to the effect that no major differences existed between the populations of vectors from different sites in the study area. However, within these populations, diversity was apparent, a reflection of their differences in levels of interaction with both the physical environment and human

activity. I therefore fail to reject the set hypothesis in the part where it says no variations existed between subpopulations. Further, I reject the hypothesis in part where it says there are no within population variations.

5.3 Insecticide resistance

The second objective was addressed with the help of the WHO standard susceptibility testing on both field-collected mosquitoes and their F1 generation raised in the laboratory. The insecticides used in the tests represented a range of classes recommended for indoor residual spraying and/or use on treated bed nets. The SNP TaqMan assay for determination of the KDR resistance mechanism in *An. gambiae* was utilised.

The second objective was to answer the question: What was the distribution of resistance to the following insecticides: DDT, pyrethroids, carbamates, and organophosphates in different populations in the light of historical and future use in IRS for the district? The hypothesis to this question was that exposure to insecticides with different modes of action had no varied effects on different mosquito vector populations, leading to varying levels of susceptibility and intensity of resistance to the insecticides.

This study established that insecticide resistance against pyrethroids and carbamates in *An. funestus* was widespread across the study sites. The level of resistance varied from site to site. No resistance was recorded against the organophosphates pirimiphos-methyl and malathion. Both *An. funestus* and *An. gambiae* at all sites were susceptible to the organophosphates. A mixed scenario of resistance and susceptibility was recorded against the organochlorine DDT and against carbamates bendiocarb and propoxur. The reported insecticide resistance in this study confirms reports of widespread resistance in malaria vectors across the African region (Hancock et al., 2018). In Uganda, Tanzania, Cameroon, and Equatorial Guinea, *An. gambiae* s.l. was found resistant to DDT and Pyrethroids, which was associated with increasing frequencies of the kdr L1014F/S allele (Verhaeghen et al., 2010; Mawejje et al., 2013; Protopopoff et al., 2013; Reddy et al., 2013; Matowo et al., 2014).

Insecticide resistance against DDT was clearly species-specific in this study. There was complete susceptibility to DDT in *An. funestus* but high resistance to DDT in *An. gambiae*. Varying resistance patterns to DDT in *An. gambiae* and *An. arabiensis* were also observed in eastern Uganda (Mawajje et al., 2013). In Kwazulu Natal, the difference in DDT resistance status of two vector species led to an upsurge in malaria cases when DDT was replaced with pyrethroids in the IRS programme (Hargreaves et al., 2000) due to the proliferation of pyrethroid-resistant but DDT-susceptible *An. funestus* (Maharaj et al., 2005).
Anopheles gambiae switched from susceptible to resistant to carbamate (bendiocarb) from 2014 to 2016, respectively. This signifies the importance of regular monitoring of insecticide resistance to ensure the efficacy of vector control. Consistent resistance was recorded in both *An. funestus* and *An. gambiae* against the pyrethroids deltamethrin and permethrin. The resistance determined here could have developed as a result of historical exposure and cross-resistance as a result of similar mechanisms of action across some insecticide classes (Etang et al., 2022).

Resistance intensity was determined for pyrethroids (deltamethrin and permethrin) and carbamates (bendiocarb and propoxur). Due to low numbers of *An. gambiae*, resistance intensity tests were only conducted on *An. funestus* on both the F1s and wild mosquitoes. Resistance intensity against bendiocarb and deltamethrin was consistent across all populations of *An. funestus.* A longer period of exposure (8 hours) to bendiocarb and deltamethrin did not result in 100% mortality. At the time when resistance intensity tests were conducted in this study, there were no standardised WHO guidelines on resistance intensity testing. The new guidelines provide test papers impregnated with different multiples of the diagnostic concentration of the particular insecticide. Recent studies conducted in Nchelenge (Venter et al., 2017) under the new WHO guidelines were in agreement with the levels of resistance intensities reported here. A longer period of exposure to propoxur and permethrin resulted in 100% mortality. This was consistent with the fact that *An. funestus* continues to be exposed to deltamethrin through deltamethrin-treated bed nets as well as previous exposure to bendiocarb through IRS. This phenomenon was the case even though propoxur and permethrin are in the same insecticide class as bendiocarb and deltamethrin, respectively. Spatially, the highest deltamethrin and bendiocarb resistance intensities were on the

mainland. This further confirmed the longer historical exposure on the mainland. Similarly, varying patterns of resistance to DDT and pyrethroids were observed in *An. gambiae* s.l. from Uganda (Verhaeghen et al., 2010). From the findings in this study, it was evident that different levels of resistance and resistance intensity were a product of past and longer exposure to the insecticides. Therefore, I fail to accept the hypothesis that exposure to insecticides with different modes of action had no effect on mosquito vector populations in different study sites.

Long-term exposure to pyrethroids could have selected for kdr resistance in *An. gambiae*. There could be some enzyme-based resistance mechanisms as well. In *An. funestus*, pyrethroid exposure could have led to the development of oxidase-based resistance mechanisms. These findings strongly suggest that kdr mediates cross-resistance between DDT and pyrethroids in *An. gambiae* in Nchelenge. The results also strongly suggest that an underlying cross-resistance mechanism, such as CYP6Z1, is involved in the resistance to both pyrethroids and carbamates, as was recently shown (Ibrahim et al., 2016). Synergist test results on mosquitoes from Nchelenge reported earlier (Choi et al., 2014) indicated elevated monoxygenases in the Cytochrome P450 gene.

Cross-resistance was so strong that islands with no history of IRS recorded similar rates of resistance to insecticides as IRS-exposed mosquitoes from the mainland. This was also the case regarding *An. gambiae* resistance to DDT. The District has no history of DDT use; however, due to cross-resistance mediated by kdr, *An. gambiae* was highly resistant to the insecticide. The intensity of the resistance noted against bendiocarb and propoxur was similar across all study sites, with subtle differences probably reflecting different levels of direct and indirect exposure over time. The marked difference in bendiocarb resistance intensity on the mainland between 2014 and 2016 happened following two rounds (2012 and 2013) of IRS with bendiocarb and a switch over to pirimiphos-methyl as a new IRS insecticide. At the time of the study, pirimiphos-methyl was the recommended IRS product and had been used in five rounds of IRS. Fludora® Fusion became the recommended IRS insecticide following a switch from Actellic® 300 CS.

5.3.1 The probable drivers of resistance

District-wide insecticide resistance probably developed following the widespread use of pyrethroid-treated bed nets. Mass LLIN distributions meant that mosquito populations from all the sites were exposed immensely, almost at the same time. As insecticide-treated net ownership increased, the insecticidal pressure on the target malaria vectors increased too, leading to the development of insecticide resistance. In Tanzainia, a review of studies conducted between 1997 and 2017 established that *An. funestus* s.l. was highly resistant to pythroids and, unlike what this study found, this vector species was also highly resistant to DDT (Matiya et al., 2019). Insecticide resistance developed following the upscaling of vector control activities involving the use of insecticide-treated nets and IRS.

Anopheles gambiae s.s. was very resistant to DDT and pyrethroids in this study, which made sense since the RR kdr allele was found in all populations of this vector species. The existence of two kdr mutations noted in this study in the same populations takes resistance to another level. This high level of cross-resistance to DDT in *An. gambiae* without prior exposure to the insecticide emphasises the need for determining resistance mechanisms involved in an area before the selection of an alternative insecticide. The involvement of oxidase-based cytochrome P450 gene resistance mechanisms was evident in the *An. funestus* of Nchelenge. High levels of bendiocarb resistance in *An. funestus* were recorded in the district, including on the islands where no IRS insecticides have ever been sprayed.

5.3.2. Mosquito longevity post-exposure to insecticide

One of the major objectives of the use of insecticides in IRS or bed nets is to reduce the longevity of the concerned vector of disease (WHO, 2013). When the mosquito comes into contact with a lethal dose of an insecticide and is not killed immediately, it is expected that the insect will not survive long enough to be able to support the developing parasite inside the mosquito to the stage where it actually transmits this parasite to the next person. The fact that some mosquitoes were able to survive for over 10 days after exposure to WHO papers treated with

bendiocarb, propoxur, and permethrin is a major concern for the control programme in Nchelenge. Depending on environmental temperatures, between 10 and 18 days are required for the malaria parasite to fully develop from the gametocyte stage (within the mosquito) to the sporozoite stage (Beier, 1998) which is infectious to humans. The survival period of mosquitoes following insecticide exposure under WHO standards reported here underscores the high resistance intensity and hence the failure of vector control efforts observed in Nchelenge. A study in Uganda found the expression of insecticide resistance alelle, kdr, significantly higher in *P. falciparum*-infected mosquitoes (Verhaeghen et al., 2010). This showed that resistant mosquitoes had better adult survival and were highly likely to get infected and transmit *P. falciparum*.

5.3.4. Efficacy of insecticide change for Indoor Residue Spraying

Was the change to organophosphate effective where pyrethroid/carbamate resistance in the malaria vectors was known to have occurred in the Nchelenge district? This question should be seen in light of the malaria cases seen at each site of the study. From the Health centre data obtained from the District, there was very minimal to no impact on malaria cases on the Mainland compared to the islands, as determined by Hast and others (Hast et al., 2019).

5.4 Mosquito collections

The third and last objective was addressed by statistical comparisons of vector counts, *P. falciparum* infection proportions, feeding rates, and Health Centre data on malaria incidences. Further, in addressing this objective, I took note of the different seasons and the vector control interventions implemented at each site.

The third objective set out to provide answers to the question: was there an association between vector control measures in use and the sporozoite rate and/or population density of malaria vectors in different parts of the District? The hypothesis to this question was that there were no spatial and temporal variations in malaria vector subpopulation densities and their contribution to malaria transmission (sporozoite rates) as a result of the existence or non-existence of vector control measures.

All the mainland villages had received IRS besides LLINs; net coverage was lowest, with between 42% and 80% of people protected. The Net coverage was between 1.9 and 2.5 per household, with household members ranging from 1 to 10 per household. Net usage was highest on Isokwe Island, with 100% of people protected. Chisenga Island was next on LLIN's usage list, with 94% to 98% of people protected. Kilwa Island was third in LLIN usage, with 69% to 77% of people protected. The probable reasons as to why there was such a wide discrepancy in net usage for some sites (higher for Chisenga and Isokwe Islands) compared to other sites (Kilwa Island and Mainland) could be twofold, namely the entomological and the nature of houses common to the site. The availability of abundant mosquitoes, including malaria vectors, could be the major reason for the high usage of LLINs by the inhabitants of Chisenga Island. On the other hand, very poor housing structures, highly prone to mosquito entry, could force all the residents of Isokwe Island to hang their nets to prevent mosquito bites. Despite these scenarios, on Chisenga and Isokwe Islands, incidents of net abuse (e.g., using these nets for fishing) were common.

Overall mosquito density and abundance were highest on Chisenga Island (LLINs only), followed by Kilwa Island (LLINs only), then Mainland (LLINs + IRS), and lastly Isokwe Island (LLINs only). Only two methods of mosquito collection out of the four proposed were used in this study. With so many mosquitoes collected by just one method, the CDC light trap set inside houses, there remained only one other collection method to be added in order to address the objectives of the study. The determination of insecticide resistance required live mosquitoes. The methods for collecting live mosquitoes include the use of a mouth or backpack aspirator and larval scooping. In this study, two aspiration methods (mouth and backpack) were used. The data from these two methods was used under the section on insecticide resistance. At the time of designing the study, only indoor collections were considered sufficient to achieve the study objectives. Outdoor collections are equally important, especially for evaluating interventions that may impact mosquito feeding and resting behaviour, understanding residual malaria transmission, or the role of secondary vectors (Hii et al., 2021; Mustapha et al., 2021).

The collected mosquito species composition was in good agreement with earlier findings from earlier works on the mainland and across the other side in the DRC (Das et al., 2016). In general, subsequent PCR identification confirmed the accuracy of morphological identification. The more sensitive and specific PCR assays for both *An. funestus* and *An. gambiae* confirmed 89% of the morphologically identified specimens as such species. Samples run on PCR that did not produce a DNA band for that particular species were re-run. If the specimen still didn't produce a DNA band on the second PCR assay, that specimen was regarded as unidentified. Recent studies in Nchelenge have revealed the existence of *An. gibbinsi* (Gebhardt et al., 2022), which could easily be morphologically misidentified as *An. funestus* if careful attention to wing and leg markings is not paid during morphological identification. *Anopheles gibbinsii* had not been reported in previous entomological collections in Nchelenge. This finding presents Nchelenge as a potential place for understudied mosquito species that could still be transmitting malaria, besides the widely distributed major vectors, *An. funestus* and *An. gambiae*.

5.4.1. Seasonal abundance and distribution

The seasonal abundance and distribution of the two major vector species across the district yielded interesting results. The foremost point of interest was the observed scale of difference in the density of the primary vector, *An. funestus,* on Chisenga Island compared to either Kilwa Island or the mainland. This finding brings to the fore the challenges lurking in the background for the malaria control programme, which up until now relied on treated bed nets only for vector control on the islands. This study went further to show that Chisenga Island had disproportionally higher densities of *An. funestus* s.l. compared to either Kilwa Island or the mainland. The highest counts of *An. gambiae* s.l. were recorded from Kilwa Island. These findings seem to reflect local differences in the ecologies of these different study sites, which may be important for targeting control interventions (Epopa et al., 2019; Nkya et al., 2022). The second observation was in the variations in the proportion of the major primary and minor primary vectors in different site collections. In agreement with previous works in the district (Das et al., 2016; Stevenson et al., 2016), the primary vector, *An. funestus,* dominated

generally and was more abundant during the dry season. The spatio-temporal heterogeneity of vector infectivity and abundance was reported in that study. Interestingly, on Kilwa Island, *An. gambiae* was dominant in the January 2016 collections. The highest proportion of the minor of the two major primary vectors (*An. gambiae* and *An. funestus*) was noted on Kilwa Island. These findings are very important to the vector control programme in terms of understanding the different scales of seasonal transmission across different areas of the District. Furthermore, these findings would be helpful in guiding the timing and targeting of control interventions. The current practise of spraying once during the rainy season seems to have minimal impact on the abundance of the primary malaria vector during the dry season. Two rounds of IRS, if using a shorter-lasting insecticide like pirimiphos-methyl, may be helpful to the control programme.

5.4.2. Blood feeding rates

The CDC light trap collections were used as proxies in determining blood feeding rates, not absolute blood rates. The use of CDC light traps to determine absolute blood feeding rates could be misleading, and other methods that are used to sample resting mosquitoes would be more reliable in this aspect. Conventionally, light traps should sample mosquito vectors that are seeking a blood meal and are thus not expected to be blood-fed when they find themselves in the trap. The assumption is that mosquitoes on a mission to get a blood meal would be coming from outside human sleeping spaces at night. As they make their way for a meal in response to specific cues triggering their host-searching activities, the light trap will detract them as they near the household and get attracted to it. The mosquitoes already in the household before the light trap is setup would probably be blooded, especially if the occupants of that household were not protected by bed nets. In this study, the aspect of blood feeding rate was included to get an insight into the effectiveness of vector control by bed nets at preventing blood feeding. It was assumed that the mosquitoes found bloodied had taken their meal the previous night on human beings inside the same sleeping structures they were caught in. The blood meal PCR showed that the source of blood in the mosquitoes was human. These mosquitoes were already blooded, were resting within the household, and got attracted to the light trap as they set out to embark upon their nocturnal activities. It was also assumed that the household occupants sleeping

under the treated bed nets were correctly using the bed nets. Under these conditions, the mosquitoes would only have access to the sleepers by biting through the mesh of an intact net or accessing the net through the holes of a torn net. Blood feeding wouldn't be expected, even when net integrity was compromised by a few holes, due to the excito-repellency property of the insecticide in the insecticide-treated bed net (Randriamaherijaona et al., 2015).

Three months prior to this study, the communities within Nchelenge had received long-lasting insecticidal bed nets through a mass campaign in October 2014. The nets distributed and found in the study households were made of hard polyethylene material. Olyset® was the brand name, manufactured by Sumitomo Industries, Japan. These nets are expected to remain effective against mosquitoes for about three years (Mejía et al., 2013). The trend in the percentage of net use by household occupants observed during the study was a gradual reduction over time. This trend did not show any relationship, e.g., blood feeding increasing with a reduction in net use and pushing up the proportions of blooded mosquitoes in the collections conducted over the seasons. Two probable explanations for these observations will be given. The first explanation would be that mosquitoes were resistant to the insecticide on the bed nets. The chapter on insecticide resistance shows that resistance to pyrethroids, the insecticide class with which bed nets are treated, was widespread across the district, in agreement with earlier studies in Nchelenge (Choi et al., 2014; Venter et al., 2017). Second, the large mesh size of the net material in use at the time, compromised the LLIN's ability to prevent mosquito passage through the mesh. It was not uncommon to hear the household owners complain about finding blooded mosquitoes inside the intact bed nets they were sleeping under. This finding elicits the need for conducting local-based field evaluations for quality control of interventions before they are implemented on a wider scale.

5.4.3. *Plasmodium falciparum* sporozoite rates

The parasite rates in malaria vectors revealed interesting findings. Areas with more abundant vectors per trap were not necessarily the areas with higher proportions of

infected vectors. The highest densities of the primary vector *An. funestus* collected from Chisenga came in third in terms of infection rates, and the lowest primary vector density on Isokwe Island had the highest infection rates. Similar findings were observed in cote d'Ivoire where two villages each comprising three main vector species contributing variably to *P. falciparaum* transmission (Adja et al., 2011). Furthermore, the mainland and Kilwa Island were the only sites where both major vector species were found infected with *P. falciparum*. Despite their low numbers, *An. gambiae* had similar proportions of infection compared to *An. funestus* on both Kilwa Island and the mainland. This would entail that *An. gambiae* in Nchelenge could still be regarded as a more efficient vector, which has taken a minor primary role in malaria transmission due to low numbers, being localised, and being more seasonal in occurrence.

The hypothesis that there were no spatial and temporal variations in vector population density, contributing to malaria transmission in different sites of the district is rejected here. Vector counts were significantly associated with the season and site of collection.

Using the dry season as a reference, lower counts of *An. funestus* were significantly associated with the rainy season, but higher *An. gambiae* counts. Interestingly, however, the odds for vector infection with *P. falciparum* were higher during the rainy season when vector counts were low. This was due to the fact that the spatio-temporal daily proportions of vector infections were higher during the rainy season compared to the dry season. The EIR calculations showed higher figures occurring during the dry season due to the higher abundance of vector counts during the dry season.

Using the mainland as a reference, higher counts of *An. funestus* were associated with Chisenga Island, while lower counts were significantly associated with Kilwa Island and Isokwe Island. Despite recording the least abundant vectors of malaria, Isokwe Island recorded the highest proportions of infected *An. funestus* s.s. In Kenya high sporozoite rates in vectors were attributed to higher proportion of *P. falciparum* gametocytes circulating in the human population on this island and

attracting mosquitoes (Busula et al., 2017). From these findings, it is rather difficult to see clearly if the number of vector control interventions had any effect on the abundance and *P. falciparum* sporozoite infection rates in the vectors.

5.4.4. Entomological inoculation rates

The value of the biting rate, and hence the EIR, went up with the number of vector mosquitoes collected. The sporozoite rate was highest in *An. funestus* on Isokwe Island in the dry season, while the estimated EIR came out highest on Chisenga Island in the same season. The EIR is regarded as an important indicator of the intensity of malaria transmission in an area. A reduction in EIR following interventions targeted at the vector is one of the indices used to measure the impact of vector control (Coleman et al., 1971). The EIR is a product of the human biting rate and parasite sporozoite rate in malaria vectors. Methods for measuring EIR are a subject of debate and lack standardisation across a spectrum of epidemiological settings (Mboera, 2005; Kelly-Hope and McKenzie, 2009; Fornadel et al., 2010a; Wong et al., 2013; Briët et al., 2015).

The most direct method of measuring biting rates involves trapping mosquitoes as they land on people, the human-landing catch (HLC), as this is believed to catch those mosquitoes actively searching for a human blood meal. However, putting humans at risk of malaria infection and other vector-borne diseases during HLCs is an ethical issue that many Institutional Review Boards (ERBs) or Ethics Committees (ECs) are not willing to approve. Alternatives to HLCs involve trapping host-seeking mosquitoes with CDC light traps, tent traps, or electrified traps (Mboera et al., 2005).

The study used an ELISA-based method to determine sporozoite rates and estimate the EIRs of Nchelenge District. The seven false-positive specimens could have led to an overestimation of the EIRs for Kilwa and Chisenga Islands, but they were removed. It’s always good practise to run confirmatory tests on all ELISA-positive assays.

Eighteen months of concomitant malaria incidence data were obtained from health centres servicing the three individual collection sites. With 1017 cases per 1000 people, Kilwa Island had the highest incidence of malaria, while Nchelenge HC on the mainland had the lowest incidence at 287 cases per 1000 people. In contrast, the estimated EIRs were highest on Chisenga Island, followed by the Mainland, and then Kilwa Island. This discrepancy between the high estimated EIR and malaria cases in humans may be due to the increased use of nets or other vector control measures, which could reduce case incidence. Generally, net usage by the human population on Chisenga Island was much better compared to net usage on Kilwa Island and the mainland.

From these findings, it seems local factors, including the ecology, demographics, malaria control use, and mosquito bionomics in a particular area, need to be considered in the estimation and interpretation of the EIR in Nchelenge, as well as an understanding of how the EIR was estimated. The EIRs measured in a consistent, standardised way are likely more useful as indicators of change over time in a given location than as a comparison measure across different settings. Monitoring how EIRs are affected will be important in the evaluation of the scale-up of the malaria control package that will include IRS and community case management on the islands of Nchelenge.

5.5 Limitations

Apart from the data on insecticide resistance, most of the other data collected were interpreted based on observational causal-effect relationships. The dangers of interpreting data based on a causal-effect relationship are many. There could be bias, the existence of confounding factors, or chance alone. For example, the low number of cases of malaria reported at Chisenga Island Health Centre, where the risk for malaria transmission was highest, could have been due to the fact that the health-seeking behaviour of the community members was also involved. No behavioural and social science studies at different sites were conducted to provide more insight into this observation. The long distance to the Health centre for some community members could be another reason why few cases were reported.

The study had no way of quantifying the amount of IRS coverage for each study village on the mainland. In certain instances, the particular household sampled may not have received the IRS intervention within a reasonable time before the survey happened. Even when the village was targeted for IRS intervention, not all households received it. In this regard, the broad consideration given to Mainland villages as receiving both IRS and ITNs may not hold true.

Community malaria prevalence surveys would be a useful tool to provide a real-time determination of the burden of malaria on the islands. This would also be useful in measuring the impact of malaria control interventions at the different sites of the study. Future studies on the islands should consider these aspects of malaria research.

5.6 Conclusions

This study determined that no genetic structures existed between the populations at different study sites in Nchelenge District. The genetic diversity was high within each vector population, with *An. funestus being* more diversified compared to *An. gambiae*. The combination of IRS and LLINs on the mainland might have impacted genetic diversity in Mainland malaria vector populations but had a very minimal impact on malaria transmission. No defined barrier to gene flow was apparent. The insecticide resistance gene kdr, based on point mutations in the voltage-gated sodium channel gene, has spread throughout the populations at the study sites. The occurrence of two kdr mutations (L1014F/S, now known as L995F/S), with the L995F occurring at high frequencies, is responsible for the high levels of insecticide resistance noted against pyrethroids and DDT in *An. gambiae*.

No resistance to organophosphates was recorded across vector populations at the study sites. Resistance to DDT, pyrethroids, and carbamates varied across sites and between the two vector species. Widespread and sustained use of pyrethroid-based LLINs has contributed to pyrethroid resistance in both *An. funestus* and *An. gambiae*. The different resistance mechanisms in each vector species have resulted in cross-resistance to carbamates in *An. funestus* and to DDT in *An. gambiae*. The

interplay of kdr- and cytochrome p450-based resistance mechanisms is responsible for the high rates and intensity of insecticide resistance in malaria vectors in Nchelenge District.

The nature of housing and the elevated risk of mosquito bites led to varied usage of LLINs in the different communities studied. High bed net usage by the communities on Chisenga Island seems to have reduced man-vector contacts. This seems to have resulted in lower incidences of malaria cases seen at Chisenga Health Centre, despite being an area with the highest risk for malaria transmission.

5.7 Recommendations

The findings of this study have led to the following recommendations for the National Malaria Elimination Programme in Zambia:

i. Given the high burden of malaria, the national malaria elimination programme should consider the option of deploying malaria vaccines in line with the recent WHO declaration
ii. Sustained vector control at all sites should be emphasised, as should the extension of IRS coverage to the islands, as well as adding new tools like PBO nets that take care of pyrethroid resistance.
iii. Evaluation of novel vector control tools to assess their efficacy and practical applications before deployment in the national vector control/elimination programme should consider Nchelenge.
iv. Non-chemical interventions designed to prevent or control mosquito bites, like house improvements, should be considered for Nchelenge. This may call for the involvement of multi-sectoral stakeholders, including traditional leaders, local authorities, and other technocrats.
v. Continued research on the vectors and vector control tools, the parasite biology, and the human host factors, including their beliefs and perceptions towards malaria, is required to fully understand disease epidemiology and evaluate the knowledge, attitudes, and practises of the inhabitants of Nchelenge.

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APPENDICES

Appendix 1. ICEMR/TDRC Mosquito Aspiration, Spray catch and Light Trap Consent Form

The University of Zambia

Study Title: POPULATION GENETICS OF *ANOPHELES FUNESTUS* GILES 1900 AND *ANOPHELES GAMBIAE* GILES 1902 (DIPTERA: CULICIDAE) WITH REFERENCE TO INSECTICIDE RESISTANCE ON MAINLAND NCHELENGE AND THE NEIGHBORING ISLANDS ON LAKE MWERU IN ZAMBIA.

Investigator: Mbanga Muleba

Ethics Clearance No.:

PI Version Date: February 15, 2013

What you should know about this study

. You are being asked to participate in a research study.

. This consent form explains the research study and your part in the study.

. Please read it carefully or ask someone to read it for you and take as much time as you need.

You and your family are volunteers in the study and you can choose not to take part. Even if you join, you may quit at any time. There will be no penalty of any kind if you decide to quit the study.

During the study, we will tell you if we learn any new information that might affect whether you wish to continue to be in the study.

Purpose of research project

The Tropical Diseases Research Centre is working with the Johns Hopkins Malaria Research Institute to learn about malaria so that we can help to improve malaria control. We want to know why some people get malaria and what things increase the chance of getting malaria. We want to know who carries the stage of malaria parasite that is transmitted to mosquitoes and how the body fights malaria. We also want to know more about the mosquitoes that transmit malaria and the parasite that causes malaria. The information we collect should help guide malaria control in your community.

Why you are being asked to participate

You have been chosen to participate in this study because of where you live. You live in the study area and your house was chosen by chance or because it is in an area at risk for malaria.

Procedures

If you agree to be in the study, we would like to collect mosquitoes which carry malaria in your house. This may mean asking you to have a small mosquito trap in your house at night or for us to enter your house in the morning so we can collect the mosquitoes with the backpack aspirator or by spray catch. The aspirator does not cause harm to you. We will collect resting mosquitoes from different rooms especially those rooms were people spent the nit last night. We will search for mosquitoes for about 10 min and you can either stay outside or you or one of the members of your household can observe the catching of mosquitoes in the house.

The spray catch method will kill the mosquitoes resting inside your house. With this method, you and all your household members, including pests, uncovered food and drink will be required to move out of the house for about 30minutes. In the house, we will cover white sheets on all your furniture and floors of the rooms chosen. After this an insecticide aerosol commonly sold in shops will be sprayed in the air inside the house. All mosquitoes that will fall on the sheets will be collected sand taken to the laboratory for identification and further analysis.

Risks and Discomforts

We will call on you very early in the morning which may disturb your sleep ask you to remain outside of your house during the collection time. There are no other risks or discomforts to participating in this study.

Benefits

There will be no direct benefit to participating in this study. This part of the study will help us learn about the mosquitoes that carry malaria in this area. Learning about the mosquitoes will help us to control malaria in this area.

Payment

There will be no payment for participating in the study.

Protecting data confidentiality

We will record whether we were able to collect mosquitoes or not, and will identify the types of mosquitoes. Only the study investigators will have access to the results. The information will be kept in secure computers. We will not use names in reports about the study.

Alternatives to procedures or treatments

Your participation is voluntary and you can choose not to participate in the study at any time.

Biological specimens

The mosquitoes collected from your home during this study are important to understanding malaria. You will not receive any financial benefit from any product or idea created by the investigators using the information or materials collected from you.

Cost of participation in the study

Your participation in this study will not cost you any money.

What happens if you leave the study early?

Even if you do not want to join the study, or if you leave the study, you will still get the same care available to you at your local clinic or hospital.

Sharing your health information with others

Only information linked to a study identification number, and not your name, will be shared with other researchers.

Payment of treatment costs for injury or illness from study participation

We will not provide payment of treatment costs for injury or illness related to study participation.

Who do I call or ask for if I have questions or problems?

Call the principal investigator, Mbanga Muleba, at 0977 899 583 if you have questions, complaints, or get sick or injured as a result of being in this study.

E-mail: mulebem@yahoo.com

Call or contact the TDRC Ethics Committee if you have questions about your rights as a study participant. Contact the the TDRC Ethics Committee if you feel you have not been treated fairly or if you have other concerns. The TDRC Ethics Committee's contact information is:

Local contact: Mr Shephard Khondowe

Telephone: +260212620737

Address: P.O. Box 71769, Ndola, Zambia

E-mail: khondowes@tdrc.org.zm

What does your signature or thumbprint on this consent form mean?

Your signature or thumbprint on this form means:

You have been informed about this study's purpose, procedures, possible benefits and risks.

You have been given the chance to ask questions before you sign.

You have voluntarily agreed to be in this study.

You understand that samples and information may be stored and used in ethically-approved research studies.

Please indicate if you agree to the following:

Do you agree to have a mosquito spray catch conducted in your house?

Yes . No

Do you agree to have a mosquito light trap set-up in your house overnight?

Yes . No

________________________ _____________________________ __________

Print name of Head of Household Signature of Head of Household Date

Ask the participant to mark a "left thumb impression" in this box if the participant (or participant's parent) is unable to provide a signature above.

________________________ _____________________________ __________

Print name of Person Obtaining Signature of Person Obtaining Consent Date Consent

Give one copy to the participant and keep one copy in study records

Appendix 2. INSECTICIDE USAGE QUESTIONNAIRE

Study Title: POPULATION GENETICS OF *Anopheles funestus* (DIPTERA: CULICIDAE) AND *Anopheles gambiae* (DIPTERA: CULICIDAE) WITH REFERENCE TO INSECTICIDE RESISTANCE ON MAINLAND NCHELENGE AND NEIGHBORING ISLANDS ON LAKE MWERU IN ZAMBIA

1) Date of survey ………/………/…………
2) Village/institution _________________
3) Household Number __________________
   Coordinates:___________________
4) Name of adult interviewed __________________
5) Occupation of adult interviewed ________________
   i) Fisherman/Peasant farmer____
   ii) Civil Servant ____
   iii) Business executive ______
   iv) Businessman/woman _________
   v) Housewife ______
   vi) Unemployed ______
   vii) Student _________
   viii) Other ____ Specify___________________________
6) Sex
   i) Male ___
   ii) Female _____
7) Age _____ years
8) Can you read and write?
   i) Yes ____
   ii) No ___
9) What is your educational level? _____ years of formal school
10) Have you or any other household/Institution member suffered from malaria
   i) Yes___
   ii) No___
11) When was the last time any member of household/institution fell sick with malaria
   i) Today ____
   ii) Yesterday ____
   iii) Last week ____
   iv) More than two weeks ago ____
12) What treatment was given to the patient?
   i) Coartem
   ii) Fansida
   iii) Quinine
   iv) Other, specify

13) How many methods of malaria vector prevention/control do you know______

---

14) Which of these methods you know are being used in your community______

---

15) If insecticides are used do you know which types/names of insecticides that are commonly used in your community
    i) Yes ____
    ii) No ___

16) Of the insecticide-based methods, which ones do you/your institution use _____

---

17) What are the types/names of insecticides used in this area? (Researcher/ assistant please check for labels and note the name and class of insecticide if available)

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--

18) For how long have you been using these vector control methods
    i) Less than one week___
    ii) More than one week but less than a month___
    iii) More than a month but less than 6 months
    iv) More than 6 months but less than 1 year
    v) More than 1 year
    vi) More than 2 years

19) Do you/your institution grow any crops
    i) Yes
    ii) No

20) What type of crops do you grow
    i) Vegetables___
    ii) Fruits___
    iii) Cash crops___
    iv) Cassava/maize___
    v) Other ___ Specify___

21) Do you use any agricultural pesticides to control insect infestations
    i) Yes
    ii) No

22) What type(s) or name (s) of pesticides do you use? (Researcher/ assistant please check for labels and note the name and class of insecticide if available)

---

23) For how long have you been using these pesticides
    i) Less than one week___
    ii) More than one week but less than a month___
    iii) More than a month but less than 6 months
    iv) More than 6 months but less than 1 year
    v) More than 1 year
    vi) More than 2 years
24) Do you/your institutions rear/keep animals?
    i) Yes
    ii) No
25) What type of animals?
    i) Goats
    ii) Pigs
    iii) Cattle
    iv) Wild animals
    v) Others specify
26) Do you use pesticides to treat your animals?
    i) Yes
    ii) No
27) Do you use pesticides to treat shelters where animals live e.g. kennel s/kraals /cages etc.
    i) Yes
    ii) No
28) If yes what types or names of pesticides do you use? (Researcher/ assistant please check for labels and note the name and class of insecticide if available)

    -
29) For how long have you been using these pesticides
    i) Less than one week___
    ii) More than one week but less than a month___
    iii) More than a month but less than 6 months
    iv) More than 6 months but less than 1 year
    v) More than 1 year
    vi) More than 2 years

THANK YOU

Appendix 3. Data collection forms

A. Form I Mosquito Collection Form: Month & year

Setting team to fill															Collection team to fill								Lab to fill	
Date	**HH ID**	**Hse**	**Room**	**Grid**	**UTM X**	**UTM Y**	**Coll Method**	**Trap location**	**Outdoor structure**	**Roof Type**	**House eave**	**Wall type**	**Fire burn last night**	**IRS**	**# ITN hanging**	**# pSleeping in house**	**# p under ITN in house**	**Number animals**	**Time p outside**	**Time p enter at night**	**Time p exit morning**	**Time collected**	**Total Mosquitoes**	**Comment**

A.

B. Form II Mosquito counts form.	**Month &Year:**											
to fill at lab or field house												
Date	**HH ID**	**Hse**	**Room**	**#fem An.**	**#bl An.**	**#male An.**	**Total An.**	**#fem Culicines**	**#male Culicines**	**Total Culicines**	**Total Mosq**	**Comments**

C. Form III Individual Anopheles form.				**Month&Year:**												
	To fill immediately after collection								To fill after completion of PCRs/ELISAs							
MosqID	**Collection Date**	**Collection Method**	**HH ID**	**Hse**	**Room**	**Sex**	**Morph species**	**Blooded**	**Extraction date**	**PCR species**	**PCR date**	**Blood PCR**	**PCR date**	**ELISA Pf**	**ELISA date**	**comment**

Appendix 4. The WHO Process for Standard Susceptibility testing in malaria vectors-2016 guidelines

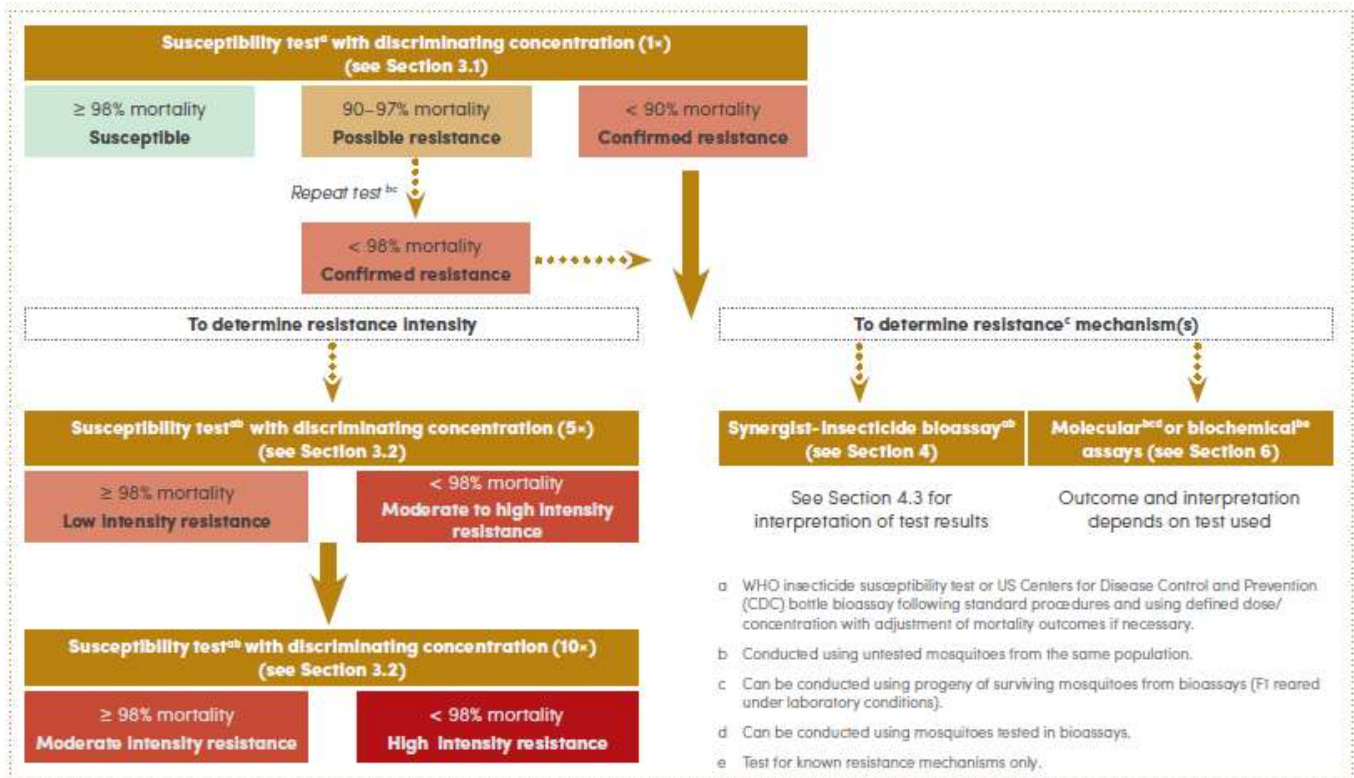

FIG. 3.1

Process for insecticide susceptibility testing of malaria vectors with a given insecticide using the established discriminating concentration (1×) and multiplier concentrations (5× and 10×) for resistance intensity (also indicated are further field and laboratory investigations for resistance mechanisms)

BOX 3.1

MEASURING SUSCEPTIBILITY TO INSECTICIDES IN ADULT MOSQUITOES: THE WHO INSECTICIDE SUSCEPTIBILITY TEST PROCEDURES FOR DISCRIMINATING, 5× AND 10× CONCENTRATIONS

1. The investigator puts on gloves. Six sheets of clean white paper (12 × 15 cm), rolled into a cylinder shape, are inserted into six holding tubes (with the green dot), one per tube, and fastened into position against the wall of the tube with a steel spring wire clip. The slide unit is attached to the tubes at the other end.

2. Ideally, 120–150 active female mosquitoes are aspirated (in batches) from a mosquito cage into the six green-dotted holding tubes through the filling hole in the slide, to give six replicate samples of 20–25 mosquitoes per tube.

3. Once the mosquitoes have been transferred, the slide unit is closed and the holding tubes set in an upright position for 1 hour. At the end of this time, any moribund mosquitoes (i.e. those unable to fly) and dead mosquitoes are removed.[a]

4. The investigator inserts one oil-treated paper (the control) into each of two yellow-dotted tubes, ensuring that the label of the paper is visible on the outside of the tube. The paper is fastened with a copper clip and the tube closed with a screw cap.

5. Four exposure tubes with red dots are prepared in much the same way as the yellow-dotted tubes. Each of the four red-dotted exposure tubes is lined with a sheet of insecticide-impregnated paper such that print label is visible on the outside. Each paper is then fastened into its position against the wall with a copper spring-wire clip and the tube is closed with a screw cap.

6. The empty exposure tubes are attached to the vacant position on the slides and, with the slide unit open, the mosquitoes are blown gently into the exposure tubes. Once all the mosquitoes are in the exposure tubes, the slide unit is closed (usually a cotton wool plug is inserted into the hole to lock the slide) and the holding tubes are detached and set aside. The investigator now removes the gloves.

7. Mosquitoes are kept in the exposure tubes, which are set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour (unless otherwise specified). The tubes are placed in an area of reduced lighting or covered with cardboard discs to reduce light intensity and to discourage test mosquitoes from resting on the mesh-screen lid.

8. At the end of the 1-hour exposure period (or longer for certain compounds, as outlined in Table 3.1), the mosquitoes are transferred back to the holding tubes by reversing the procedure outlined in Step 6. The exposure tubes are detached from the slide units. A pad of a cotton wool soaked in 10% sugar water is placed on the mesh-screen end of the holding tubes.

9. Mosquitoes are maintained in the holding tubes for 24 hours (or longer for slow-acting compounds). During this time, it is important to keep the holding tubes in a shady, sheltered place in the laboratory or in a chamber maintained at 27 °C ± 2 °C temperature and 75% ± 10% relative humidity. Temperature and humidity should be recorded during the recovery period.

10. At the end of recovery period (i.e. 24 hours post-exposure or longer for slow-acting compounds), the number of dead mosquitoes[a] is counted and recorded. An adult mosquito is considered to be alive if it is able to fly, regardless of the number of legs remaining. Any knocked down mosquitoes, whether or not they have lost legs or wings, are considered moribund and are counted as dead. A mosquito is classified as dead or knocked down if it is immobile or unable to stand or take off.

On completion of the susceptibility test, mosquitoes may be transferred to individual, clearly labelled microcentrifuge tubes with a lid for airtight locking (separating dead and live mosquitoes into separate tubes) for preservation until such time as they can be transferred to suitable facilities for species identification and supplementary testing if necessary. A schematic representation of the procedure is shown in Fig. 3.2.

[a] For the purpose of insecticide bioassays, the definition of knockdown and mortality involves not only the state of the insect but also the time at which the observation is made. A mosquito is classified as "dead" or "knocked down" if it is immobile or unable to stand or take off. The distinction between knocked down and dead is defined only by the time of observation. The assessment of knockdown is made within 1 hour of exposure. Mortality is determined at least 24 hours after exposure. The holding container may be tapped a few times before a final determination is made. In the case of slow-acting insecticides, the recovery (holding) period may be extended beyond 24 hours. Control mortality should be measured over the same recovery period. Mortality after 24 hours should be recorded; in some cases, repeated observations may be appropriate. Classification of adult mosquitoes as alive, knocked down or dead in bioassays is summarized below:

ALIVE	KNOCKED DOWN OR DEAD AFTER EXPOSURE	
	MORIBUND	DEAD
Can both stand and fly in a coordinated manner	- Cannot stand (e.g. has only one or two legs) - Cannot fly in a coordinated manner - Lies on its back, moving legs and wings but unable to take off - Can stand and take off briefly but rapidly falls down	- No sign of life - Immobile - Cannot stand

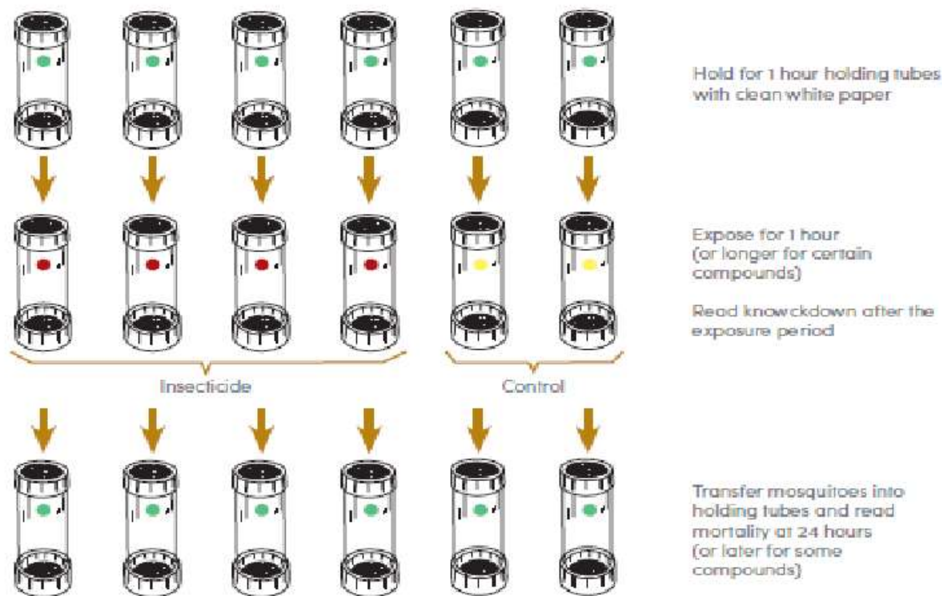

FIG. 3.2
Steps to perform the WHO insecticide susceptibility test for discriminating, 5× and 10× concentrations

BOX 3.2
DETERMINING RESISTANCE IN VECTOR POPULATIONS

Use of discriminating concentrations

To determine resistance in vector populations it is necessary to first establish baseline susceptibility data for individual insecticides in a normal or "susceptible" population of a given species. This is achieved by exposing nonresistant vectors to filter papers impregnated with serial concentrations of a given insecticide compound, and plotting the percentage mortality against exposure on logarithmic-probability paper. The graph can be used to estimate the concentrations required to produce various levels of kill; this calculation can also be done using a log-probit statistical model. Using this method, it is possible to derive the concentration corresponding to 99.9% mortality (the $LC_{99.9}$ value); at this concentration there is a high probability that all individuals in a susceptible population will be killed. Double this concentration is conventionally known as the discriminating (or diagnostic) concentration (i.e. 1×). Once discriminating concentrations for individual insecticides have been established under standardized laboratory conditions

using known susceptible strains or populations of a range of mosquito vector species, it is not necessary for routine monitoring purposes to conduct susceptibility tests at the full range of test concentrations. Instead, it is sufficient to conduct a standard bioassay resistance test using the discriminating concentration, because any survivors at this concentration may be considered to be resistant. This approach has obvious advantages in terms of the feasibility, cost and efficiency of testing. However, confirmation of resistance using discriminating concentrations may not necessarily correlate with operational failure of insecticide formulations used for IRS, or for ITNs or LLINs. Further assays designed to assess the extent of resistance intensity can be used to inform operational decisions (see below). Discriminating concentrations for various insecticide compounds either used in vector control or evaluated through research have been established for different mosquito species (Table 3.1). For new insecticide compounds, in cases where mosquito species are not routinely monitored or in specific situations where baseline data are not available, it is necessary to first establish the baseline susceptibility as described above.

Appendix 5. Standard Operating Procedure for clades of *An. funestus* Using the hydrolysis probe analysis (Taqman assay)

Document number:

Version number: 1

(Changes from previous version highlighted)

Written by: Dr Kwang Shik Choi

Checked by: ( )

Approved by: ( )

Active date: 5 March 2014

Purpose

Michel et al. (2005) and Choi et al. (2012 and 2014) reported that *An. funestus s.s*. from Mozambique, Madagascar and Zambia has two clades based on the DNA sequence analysis of mitochondrial DNA (*ND5* and *COI*). However, these are unable to identify morphologically. This method is able to accurately and rapidly identify the two clade types of *An. funestus s.s.*

Source

Choi, Kwang Shik, Maureen Coetzee and Lizette L Koekemoer (2013) Dectection of clade types (clades I and II) within *Anopheles funestus sensu stricto* by the hydrolysis probe analysis (Taqman assay). *Parasites&Vectors*, **6:**173.

Principle of the Method

- This technique is based on TaqMan SNP genotyping and detects clades I and II variation nucleotides in *An. funestus s.s.*
- This method is as sensitive and specific as the gold standard nested PCR approach. In this approach, real-time PCR using TaqMan probes are used in which either clades I or II in the mosquito vector can be detected in a single reaction.
- Nucleotide alignments of the *An. funestus s.s. COI* sequences available in the National Center for Biotechnology Information (NCBI) database were compared and a region around the *COI* variation site which was conserved in all isolates/species was selected for primer/probe design.

- Two minor groove binding (MGB) probes (Applied Biosystems) that bind over all SNPs (clade I and II) were designed using the Primer3.
- The probe clade I (5′- TCA GGA ATT GCT CAT GCT -3′) and clade II (5′- TCA GGA ATT GCC CAT GCT -3′) were labelled with 6-FAM and VIC at the 5' end for the detection of clades I and II respectively.
- Each probe also carried a 3' nonfluorescent quencher and a minor groove binder at the 3' end.
- The minor groove binder provides more accurate allelic discrimination by increasing the TM between matched and mis-matched probes. Forward (5’- GCA GGA ACA GGA TGA ACA GT -3’) and Reverse (5’- GAA ATT CCT GCT AAA TGT AAT GAA A -3’) primers that flank the probe binding site were also designed.

Potential Hazards

Taqman reagents may be considered a hazardous chemical in case of eye contact (irritant). The correct PPE should be used i.e. gloves and labcoat should be worn at all times.

Nature and Volume of Specimen Required

The genomic DNA extracted from a whole or parts of a single mosquito specimen are used.

Equipment and Reagents Required

Reagents:

IQ Supermix

10 µM Forward primer

10 µM Reverse primer

10 µM Clade I probe

10 µM Clade II probe

dH_2O

Template DNA

Thermal Cycler:

A real-time PCR cycler

Pipettes:

0.1 -10 ulm, 10-200ul and 100-1000 ul pipettes

Centrifuge:

One that can accommodate 96 well plates.

Consumables:

96 well plates for conducting qPCR, 1.5ml tubes

Preparation and Storage of Reagents

- Stock primers are stored in a stock box at -20 ºC freezer. For each aliquot primer, add 90 µL sterilized distilled H_2O and 10 µL stock primer to obtain a 10 µM solution.

Detailed Step by Step Instructions

PCR reactions:

The PCR is set-up as shown in Table 1.

Table 1. Taqman assay set-up

Reagent	1 x
IQ Supermix	10 µl
Forward primer (10 µM)	1.6 µl
Reverse primer (10 µM)	1.6 µl
Clade I probe (10 µM)	0.8 µl
Clade II probe (10 µM)	0.8 µl
dH_2O	4.2 µl
DNA	1.0 µl
Total	**20 µl**

***Add 19 µl to each tube + 1 µl DNA

Recommended Cycling Conditions:

95 °C – 10 mins

95 °C – 10 secs

63 °C – 45 secs

Plate read

VIC- yellow (530nm excitation & 555nm emission)

FAM – green (470nm excitation & 510 emission)

Necessary Calculations

Primer/Probe:

Primer stock- 100 μM

Volume needed- ?

Primer conc. needed- 10 μM

Volume to make up to-100 μl

X. 100 μM = 100 μl. 10 μM

x= 10 μl

Therefore add 10 μl of stock primer in 90 μl dH_2O to get a volume of 100 μl.

Or add 5 μl of stock primer in 45 μl dH_2O to get a volume of 50 μl.

Test Acceptance Criteria

- The samples are considered acceptable if the amplification curve is substantially higher than the baseline curve.
- All positive controls have to work and the negative control should not amplify any product.

Interpretation of Test Results

- This assay uses two probes, the first labelled with 6-FAM detects clade I and the second, labelled with VIC, detects clade II.
- Thus, a substantial increase in 6-FAM fluorescence during PCR indicates the presence of clade I whilst a substantial increase in VIC fluorescence indicates the presence of clade II as shown in Figure.

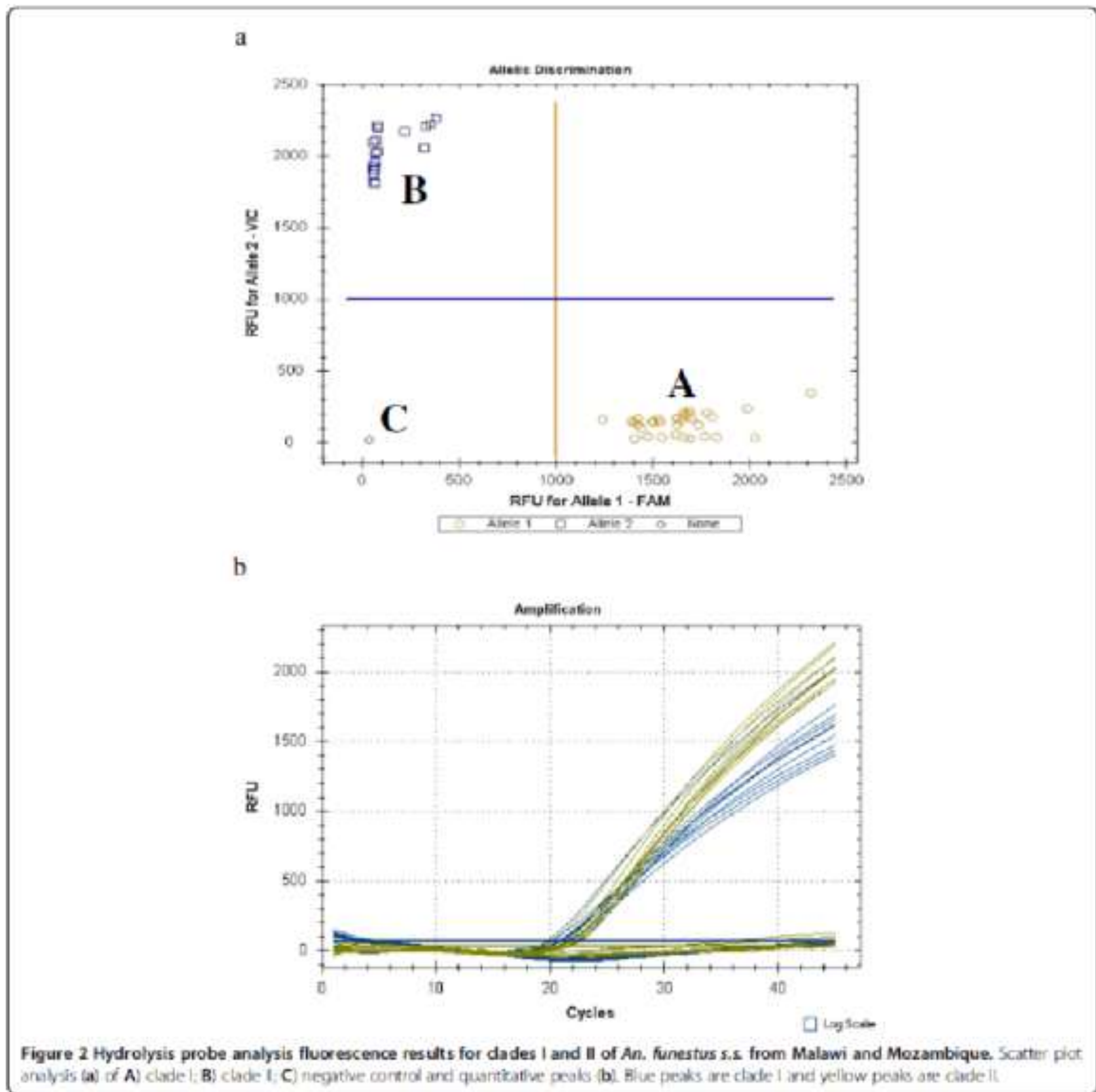

Figure 2 Hydrolysis probe analysis fluorescence results for clades I and II of *An. funestus s.s.* from Malawi and Mozambique. Scatter plot analysis **(a)** of **A)** clade I; **B)** clade I; **C)** negative control and quantitative peaks **(b)**. Blue peaks are clade I and yellow peaks are clade II.

Biological Reference Intervals and Units

None

Potential Sources of Variability

None

Internal Quality Control and External Quality Assessment Procedures

- PCR positive and negative controls are included in each run.
- The positive controls are DNA of clades I and II.
- The negative control consists of no template.

Appendix 6. Standard Operating Procedure for detection of kdr mutation types using the Taqman Assay

Document number:

Version number: 1

(Changes from previous version highlighted)

Written by: Dr Kwang Shik Choi, Vector Control Reference Laboratory

Checked by: (Dr Belinda Spillings)

Approved by: (Prof Lizette Koekemoer)

Active date: 5 October 2012

The purpose of the method

- To detect the presence of East (*kdr*-e) and West (*kdr*-w) Knockdown Resistance (*kdr*) mutation phenotypes in *Anopheles gambiae s.s.*

The source of the method and/or literature reference

Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A, Vontas J, Field LM. 2007. Detection of knockdown resistance (*kdr*) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. *Malar J* **6:**111.

The principle of the method

- This technique is based on hydrolysis probe SNP genotyping and detects *kdr*-e, *kdr*-w or wild type mutations in *An. gambiae s.s.*
- This method is as sensitive and specific as the gold standard nested PCR approach. Here, real-time PCR using hydrolysis probes is used. The reaction consists of either *kdr*-e and wild type or *kdr*-w and wild type probes such that the genotype of a single mosquito vector can be detected in a single reaction.
- Nucleotide alignments of the *An. gambiae* domain II sodium channel gene sequences available in the National Center for Biotechnology Information (NCBI) database were compared and a region around the *kdr* site which was conserved in all isolates/species was selected for primer/probe design.

- Three minor groove binding (MGB) probes (Applied Biosystems) that bind across the SNPs (*kdr*-e, *kdr*-w and wild type) were designed using the Primer Express™ Software Version 2.0.
- The *kdr*E probe (5' -ACGACTGAATTTC- 3') and *kdr*W probe (5' -ACGACAAAATTTC- 3') are labelled with 6-FAM at the 5' end for the detection of *kdr*-e and *kdr*-w respectively. The WT probe (5'-CTTACGACTAAATTTC-3') is labelled with VIC for the detection of wild type.
- Each probe carries a 3' nonfluorescent quencher and a minor groove binder at the 3' end.
- The minor groove binder provides more accurate allelic discrimination by increasing the TM between matched and mis-matched probes.

  kdr-Forward (5' -ATTTTTCTTGGCCACTGTAGTGAT- 3')

  kdr-Reverse (5' -CGATCTTGGTCCATGTTAATTTGCA -3').
- Primers that flank the probe binding site have also been designed.

Identification of potentially hazardous procedures/operations/ substances where these may occur in the method

- Taqman reagents may be considered a hazardous chemical in case of eye contact (irritant). The correct PPE should be used i.e. gloves and labcoat should be worn at all times.

The nature and volume of specimen required

- The genomic DNA extracted from a whole or parts of a single mosquito specimen is used.

Equipment and reagents required

Reagents: IQ Supermix

10 µM Forward primer

10 µM Reverse primer

10 µM WT probe

10 µM *kdr* E or W probe

PCR grade dH_2O

Template DNA

Thermal Cycler: A real-time PCR cycler

Pipettes: 0.1 -10 µl, 10-200 µl and 100-1000 µl pipettes

Centrifuge: One that can accommodate 96 well plates.

Consumables: 96 well plates for conducting qPCR, 1.5 ml tubes

The preparation and storage of reagents

- Stock primers are stored in a stock box at -20 ºC. To each 10 µl stock primer (already aliquoted), add 90 µl sterilized distilled H_2O to obtain a 10 µM solution.

Detailed step-by-step instructions on how to perform the test

Step 1:

Count how many samples you will need to do KDR identification on. Remember to include the positive controls and the negative control for the hydrolysis probe reaction. This number will be the value that you use when calculating your master mix volumes

Step 2:

Prepare a master mix. Use the table below to assist in your calculations.

Reagent	1 x (VCRL modified)***	1 x (Bass et al. 2007)
IQ Supermix	10 µl	12.5 µl
Forward primer (10 µM)	1.6 µl	2.25 µl
Reverse primer (10 µM)	1.6 µl	2.25 µl
WT probe (10 µM)	0.4 µl	0.5 µl
kdrE/kdrW probe (10 µM)	0.4 µl	0.5 µl
dH_2O	5.0 µl	6 µl
DNA	1.0 µl	1 µl
Total	**20 µl**	**25** µl

***Add 19 µl to each tube

Step 3:

Aliquot 19ul of master mix to each tube/well. Pipette in your DNA.

Step 4:

Program the real time machine. Use the recommended cycling conditions listed below.

Recommended Cycling Conditions:

95 °C – 10 mins

95 °C – 10 secs

60 °C – 45 secs

Plate read

VIC- yellow (530nm excitation & 555nm emission)

FAM – green (470nm excitation & 510 emission)

Any necessary calculations

None.

Test acceptance criteria

- The samples are considered acceptable if the amplification curve is substantially higher than the baseline curve.
- All positive controls have to work and the negative control should not amplify any product.

Interpretation of test results

- This assay uses two probes, the first labelled with 6-FAM detects *kdr-e* or *kdr-w* and the second, labelled with VIC, detects the wild type.
- Thus, a substantial increase in 6-FAM fluorescence during PCR indicates the presence of *kdr-e* or *kdr-w* whilst a substantial increase in VIC fluorescence indicates the presence of the wild type as shown in Figure1. An increase in both dyes would indicate a mixed genotype.

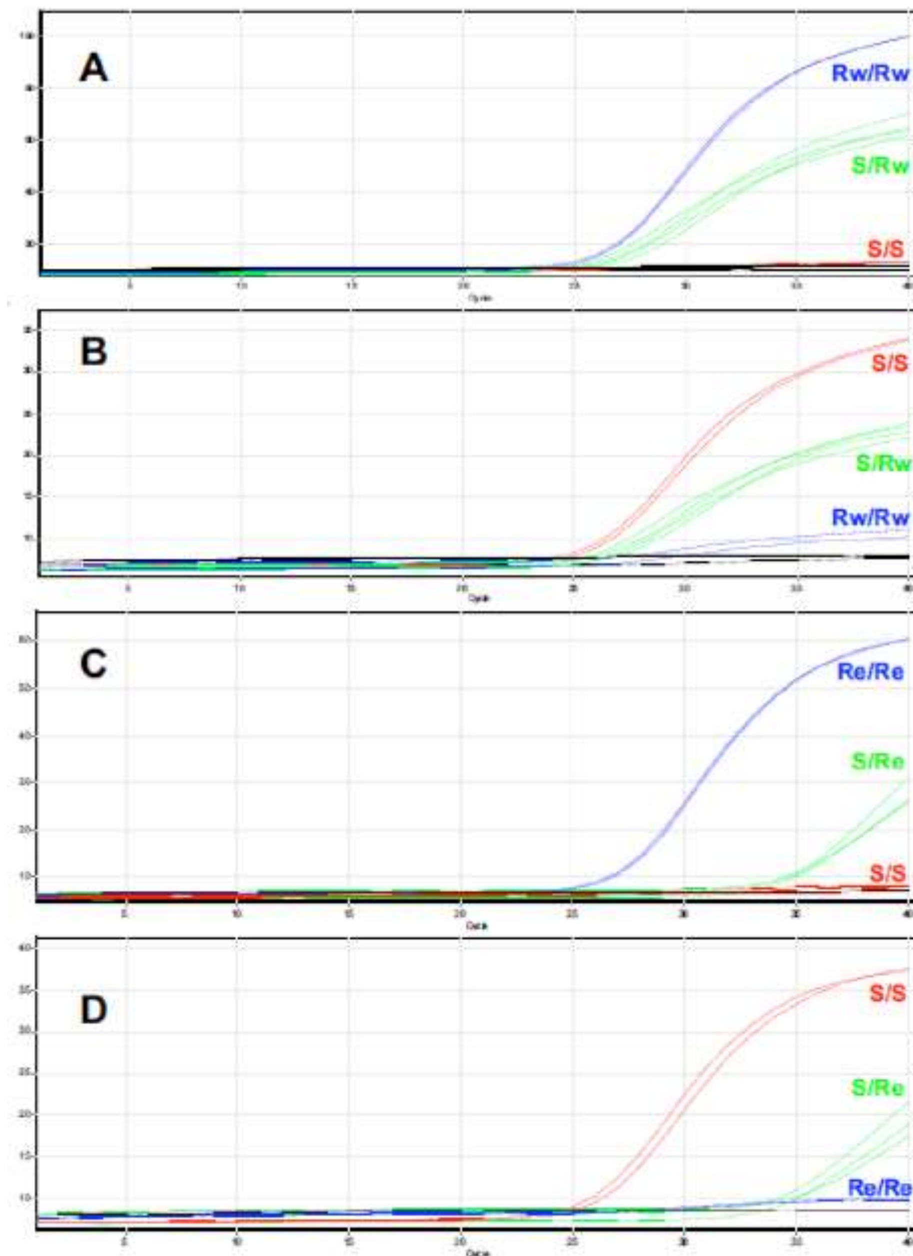

Figure 1. A) and B) Detection of the *kdr*-w mutation. C) and D) Detection of the *kdr*-e mutation. A) Cycling of FAM-labelled probe specific for the *kdr*-w allele. C) Cycling of the FAM-labelled probe specific for the *kdr*-e allele. B) and D) cycling of the VIC labelled probe specific for the wild type allele. S: Wild type allele (L1014), Rw: Resistant allele, West African mutation (L1014F), Re: Resistant allele, East African mutation (L1014S).

Where applicable, biological reference intervals and units

- None

Potential sources of variability

- None

Internal quality control and external quality assessment procedures

- PCR positive and negative controls are included in each run.
- The positive controls are DNA of east, west and wild *kdr* mutations.
- The negative control consists of no template.

Appendix 7. Abstract PA-040 - Presented at the 8th EDCTP Forum 6 -9 November, 2016 at Government Complex Conference Centre, Lusaka Zambia

PA-039

Impact of an integrated community case management of fever due to malaria and pneumonia on child mortality: a cluster randomised controlled trial in Burkina Faso

Mohamadou Siribie[1], Alfred Tiono[2], Abdel G A Babiker[3], Armande Sanou[1], Luc Serme[1], Zakaria Gansane[1], Abdoulaye Traore[1], Melba Filimina Gomes[4], Franco Pagnoni[4], Sodiomon Sirima[1]

1. GRAS, Burkina Faso; 2. CNRFP, Burkina Faso; 3. MRC CTU, United Kingdom; 4. WHO-TDR, Switzerland

Background | An integrated approach in community case management of malaria and pneumonia may increase the proportion of children receiving prompt and effective treatment, reducing the rate of child mortality.
Methods | A stepped–wedge cluster randomised controlled community trial involving children aged 2–59 months was conducted in a rural area of Burkina Faso. Community health workers in the intervention clusters were trained to recognise and to treat children with hot body due to malaria and/or pneumonia with artemether-lumefantrine alone or artemether-lumefantrine and cotrimoxazole.
Results | A total of 42,919 hot body due to malaria and 9592 pneumonia episodes were managed at community level. A 20% reduction in the all-cause mortality rate was shown in the integrated community case management of malaria and pneumonia arm (iCCMmp) compared with the control arm (IRR=0.798; 95% CI= 0.510 to 1.247; p= 0.321). However, this difference was not statistically significant. Similarly, no difference in mortality rate was detected between clusters with community case management of malaria (CCMm) and control clusters (IRR=1.048; 95% CI= 0.753 to 1.459; p=0.7794) and between clusters with iCCMmp and clusters with CCMm (IRR=0.821; 95% CI=0.597 to 1.130; p=0.2272).
Conclusions | The trial failed to show a significant impact of the intervention. It is likely due to the all-cause mortality rate used to calculate the sample size which was higher than the one measured in the study area. However, the trial showed that community case management of pneumonia can be integrated into ongoing CCMm programs in sub-Saharan Africa, thus contributing to increasing access of children to prompt and adequate treatment, and potentially leading to a reduction of child mortality in underserved populations.

PA-040

Seasonal abundance and sporozoite rates in malaria vectors in Nchelenge including islands of Lake Mweru an area with a high burden of malaria in northern Zambia

Mbanga Muleba[1], Jennifer Stevenson[2], Keith Mbata[3], Modest Mulenga[1], Maureen Coetzee[4], Douglas Norris[5]

1. TDRC, Zambia; 2. Macha Research Trust, Zambia; 3. University of Zambia, Zambia; 4. Wits University, South Africa; 5. JHSPH, United States of America

Background | Nchelenge district is a holoendemic malaria transmission zone in northern Zambia. The district occurs in a region characterised by a mix of water, marshes, islands and lagoons presenting a uniquely suitable ecology for mosquitoes. Annual indoor residual spraying (IRS) campaigns are carried out between September and December synchronised with other regions in the country with different environmental conditions. Targeted vector control interventions have been applied since 2008 without appreciable impact on disease burden. The timing and targeting of vector control measures are not guided by an informed entomological baseline. This study was aimed at providing entomological information on the seasonal abundance, spatial distribution and *Plasmodium falciparum* sporozoite infections in the local malaria vector species in order to guide implementation of vector control in the district.
Methods | Entomological studies were conducted intermittently spanning the rainy, cold-dry, and hot-dry seasons between 2015–2016. Mosquitoes were collected by CDC light traps, identified to species both morphologically and by PCR techniques. Circumsporozoite ELISA assay was used to detect *P. falciparum* in mosquito salivary glands.
Results | A total of 5437 female *Anopheles funestus* and *An. gambiae* and over 6000 culicines, mostly *Mansonia* mosquitoes were collected. The peak number of the *An. funestus* from all sites occurred in July. Overall *P. falciparum* infection rates in *An. funestus* were; Kilwa island 2.7% (4/146), Mainland 2.5% (3/122), Chisenga island 0.4% (1/220), Isokwe 5.9% (2/34) and *An. leesoni* from Kilwa 33% (1/3). The highest number of *An. gambiae* was collected from Kilwa and none was found infected with *P. falciparum* regardless of collection site.
Conclusions | The annual IRS conducted between September and December may be ineffective in controlling malaria as this misses the vector peak abundance and peak transmission season. Two rounds of IRS covering more areas would be needed to control the two vector species with different population peak seasons and malaria transmission.

Appendix 8. Abstract 867 - Presented at the sixty-sixth annual meeting of the American Society of Tropical Medicine and Hygiene November 5 – 9, 2017, Baltimore Convention Centre, Baltimore, Maryland, USA.

2030, rely for a large part on vector control interventions. Then, updated knowledge on *Anopheles* vectors is important to obtain for developing malaria surveillance plan and risk assessment. In Asia, the biodiversity of *Anopheles* mosquitoes is particularly rich compared to the other continents. The vector system is composite as each malaria vector belongs to a species complex with morphologically indistinguishable sibling species that may present different trophic behaviors and vectorial capacities. Recent advances in molecular systematics have allowed reliable species identification for their precise study. Another specific trait in Asia is that most malaria endemic areas occur in *foci* distributed along forested international borders with population movement maintaining the endemicity. The main aim of this work is to obtain information on the human-vector system, which is of primordial importance for applying appropriate vector control programs targeting the correct vector species and protecting the human population. Several approaches are being developed, in collaboration with Asian partners (Cambodia, China, Thailand, Vietnam), for answering crucial questions and improving vector control strategies. Do we know all the vectors, even secondary ones that may play a role in transmission? What is the role of the mosquito microbiota in its vectorial capacity? Can we evaluate human antibody responses to *Anopheles* saliva to evaluate the efficacy of vector control methods? Are there alternatives to synthetic insecticides capable of protecting the human population and less harmful to the environment? These joint studies are providing reliable data on *Anopheles* mosquitoes throughout Asia highlighting also the gaps to fill up. Today, there is an urgent need to extend this network at the regional scale as malaria vectors do not know boundaries and their control will be a lot more efficient if an international taskforce is identified which will increase the chance to meet the goal of malaria elimination by 2030.

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INSECTICIDE-TREATED COW-BAITED TENTS AS A TOOL TO CONTROL OUTDOOR BITING MALARIA VECTORS

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Insecticide-treated cow-baited tents were tested in Cambodia over one year as an outdoor-biting mosquito control and monitoring tool. Insecticide-treated cow-baited tents exploit the animal feeding tendencies of outdoor vectors and could potentially target and eliminate large numbers of different anopheline species that contribute to outdoor, or residual, malaria transmission. The potential success of this strategy is that anophelines with a successful bloodmeal may linger on treated tent walls, increasing their rate of contact with insecticide treated or impregnated netting. Even if the mosquitoes are not knocked down directly, this exposure could drastically reduce the number of surviving females that are able to transmit, thus reducing malaria transmission. Since many outdoor biting mosquitoes carry other diseases, this type of approach could target not only vectors of residual malaria, but also those of filariasis and other zoonoses. Large tents fitted with deltamethrin-impregnated panels with a cow in the center were compared to untreated control tents every three months during 2016 in Pursat and Preah Vihear, Cambodia, provinces with emerging artemisinin-resistant malaria parasite populations. Mosquitoes were collected hourly from 6pm to 6am and were tested for knockdown effects exactly two hours after collection. The same diverse set of more than 25 *Anopheles* species were captured in treated versus untreated tents, hundreds of anophelines were entering treated tents every night, and some of those mosquitoes were carrying malaria parasites with Kelch-13 mutations, a marker that is linked to artemisinin-resistance. These results show that insecticide-treated cow-baited tents could be a valuable tool for targeting outdoor vector species.

867

CROSS-RESISTANCE INVOLVING THREE CLASSES OF INSECTICIDES AND TWO MAJOR MALARIA VECTOR SPECIES IMPACTING MALARIA CONTROL IN NCHELENGE, A HIGH MALARIA TRANSMISSION REGION IN NORTHERN ZAMBIA

Mbanga Muleba[1], Freddie Masaninga[2], Jennifer C. Stevenson[3], Keith Mbata[4], James L. Sichivula[1], Ian Mwelwa[1], Hunter G. Chilusu[1], Nelius Venter[5], Davis Mwewa[6], Modest Mulenga[1], Maureen Coetzee[5], Douglas E. Norris for the Southern African ICEMR[7]

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For over a decade insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) have been the vector control tools in Nchelenge District, northern Zambia, although prevalence of malaria remains high. Some areas, particularly the islands, have only received ITNs. Studies carried out in the district provided insight into the problem of malaria on the mainland. We included islands in Lake Mweru with no history of IRS and determined the status of insecticide resistance and the intensity of this resistance using the World Health Organization (WHO) standard assays. We tested field-collected and F1 mosquitoes on five insecticides representing four classes between 2014 and 2016. Ninety-seven percent of the collections were *Anopheles funestus* group and 3% *An. gambiae sensu lato*. *An. funestus* from all the sites were resistant to pyrethroids and carbamates but susceptible to organophosphates and DDT. *An. gambiae* was resistant to pyrethroids and DDT but susceptible to organophosphates and bendiocarb. The intensity of resistance in *An. funestus* from all collection sites was high against bendiocarb and deltamethrin. On the mainland there was a significant increase in resistance intensity to bendiocarb between 2014 and 2016 (ANOVA $F = 93$, p-value< 0.001). We recorded 10% mortality in *An. gambiae* to DDT, an insecticide that has never been used before in the district. We recorded cross-resistance between pyrethroids and carbamates in *An. funestus* and between pyrethroids and DDT in *An. gambiae*. We detected cytochrome p450 resistance mechanism in *An. funestus* and knockdown resistance (kdr) in *An. gambiae*. Insecticide resistance may explain why control efforts have not made an appreciable impact on the transmission of malaria in Nchelenge. Well thought out integrated vector control and resistance management strategies are required, as options for vector control in Nchelenge are very limited. The use of new innovations such as ITNs incorporated with an insecticide synergist on the islands may be helpful. Alternative insecticides are needed in this area and Nchelenge could provide a good field site for evaluating efficacy of new vector control tools.

868

PYRETHROID RESISTANCE INTENSITY AND MECHANISMS OF INSECTICIDE RESISTANCE IN THE MALARIA VECTOR *ANOPHELES GAMBIAE* S.L. IN SELECTED DISTRICTS IN NORTHERN GHANA

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Appendix 9. Abstract 1265 - Presented at the annual meeting of the American Society of Tropical Medicine and Hygiene November 15 – 19, 2020, Virtual Meeting.

(ITNs) are now available that either have more than one active ingredient in addition to pyrethroids or contain a pyrethroid and the insecticide synergist piperonyl butoxide. These next-generation ITNs have been shown to be effective against pyrethroid-resistant mosquitoes and are alternatives to standard, pyrethroid-only long-lasting insecticidal nets (LLINs). We produced preliminary estimates of effectiveness, cost, and cost-effectiveness for the mass distribution of these ITNs compared to standard LLINs for varied transmission and insecticide resistance settings. A transmission model for *Plasmodium falciparum* was parameterized using experimental hut data quantifying the entomological impact of next-generation ITNs. A relationship was fit between the proportion of mosquitoes surviving the World Health Organization bioassay test and the standard LLIN induced mosquito mortality from experimental hut data. Then associations between standard LLIN mosquito mortality and the next-generation ITN mosquito mortality from trials determined their additional benefit. These relationships are combined with the feeding inhibition and deterrence measured in experimental huts to determine parameters used in the model. The efficacy can then shift as the level of pyrethroid resistance changes. The transmission model is used to project the clinical cases averted by each net type relative to standard LLINs, assuming the same net coverage. Total cost of deployment, including uncertainty intervals, were estimated using data from a systematic review and incremental cost-effectiveness ratios were calculated. Results will be updated and validated using randomized controlled trials and observational studies which are currently underway. Preliminary estimates indicate that the optimal choice of ITN is highly dependent on the baseline transmission and resistance context indicating that local decisions will be needed to make the most of scarce resources in future bednet campaigns.

1265

EFFICACY OF ENTOMOLOGICAL INOCULATION RATES IN NCHELENGE, A MALARIA HOLOENDEMIC DISTRICT IN NORTHERN ZAMBIA

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The entomological inoculation rate (EIR) is an important indicator of intensity of malaria transmission in an area. Reduction in EIR is one of the indices used to measure impact following vector control activity. The EIR is a product of the human biting rate and parasite sporozoite rate in malaria vectors. Methods for measuring EIR lack standardization across a spectrum of epidemiological settings. The most direct method of measuring biting rates is the human-landing catch (HLC). The HLC is believed to catch mosquitoes actively searching for a human blood meal. Risk of malaria infection and other vector-borne diseases during HLCs becomes an ethical issue. We used CDC light-traps, one of the alternatives to HLCs, set next to people indoors to determine biting rates. We used ELISA-based method to determine sporozoite rates and estimated the EIRs on two islands and the mainland areas of Nchelenge District, northern Zambia in January, March, July, October 2015 and January 2016. We obtained 18 months of concomitant malaria incidence data from health centres servicing the three individual collection sites. Kilwa Island had the highest incidence of malaria followed by the mainland with Chisenga Island registering the lowest incidence. In contrast the estimated EIRs were highest on Chisenga Island followed by the mainland, then Kilwa Island. In an area with high vector counts, the discrepancy between estimated EIR and malaria cases may be due to the increased use of nets or other vector control measures, which could reduce case incidence. Local factors including the ecology, demographics, malaria control use and mosquito bionomics need to be considered in the estimation and interpretation of the EIR in Nchelenge, as well as an understating of how this was estimated. Measured in a consistent standardized way, EIRs are likely more useful as indicators of change over time in a given location than as a comparison measure across different settings. Monitoring how EIRs are affected will be important in the evaluation of the scale-up of the malaria control package that will include indoor residual spraying and community case management on the islands of Nchelenge.

1266

AN OPERATIONAL REAL-TIME DASHBOARD TO TRACK VECTOR CONTROL ACTIVITIES ON BIOKO ISLAND

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The Bioko Island Malaria Elimination Project (BIMEP) has delivered 15 years of vector control, case management and social behavioral change communication. Decision-making and intervention targeting are guided by data obtained through a robust monitoring and evaluation system. In 2020, an integrated vector control strategy was adopted in an attempt to further reducing malaria prevalence that had stalled since 2015 and increased in 2019. The strategy included the expansion of indoor residual spraying to ~ 34,000 households, a top-up distribution of long-lasting insecticidal bed nets to ~20,000 households, larval source management in high prevalence areas and an integrative malaria sensibilization package. With such a huge deployment and limited resources, the use of data can improve allocation, optimize productivity and monitor vector control coverage. The BIMEP developed a comprehensive dashboard to facilitate real-time assessment of work progress, productivity and coverage. The dashboard used three main components: (1) a *coverage-map* dashboard, which displays spatial coverage at 100x100 m sectors (the units for vector control deployment); (2) a *productivity dashboard*, which displays productivity by field teams; and (3) an *overall metrics dashboard* that shows cumulative coverage and other general metrics over time. The dashboard sourced data directly from the BIMEP PostgreSQL server. Weekly meetings were held to visualize and interpret the data and guide the teams to make decisions accordingly. This facilitated adjusting deployment strategies in order to optimize production and meet targets. Although evidence is crucial for effective malaria vector control, timely and relevant data are often not integrated and readily available to inform decision-making. Online tools, such as the dashboards presented here, can help malaria control programs to track critical components of project performance and enhance decision-making. In order to achieve this, however, programs require to develop robust data assemblies and hosting systems in dedicated servers.

1267

HABITAT ADAPTATION OF *ANOPHELES COLUZZII* AND *AN. MELAS* TO NEGLECTED POLLUTED SWIMMING POOLS, ABANDONED BOATS AND CRAB HOLES ON BIOKO ISLAND

Godwin Fuseini[1], Michael von Fricken[2], Raul Ncogo Nguema[3], Jeremias Nzamio[1], Jordan Smith[1], Carlos A. Guerra[4], Guillermo A. Garcia[4]

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Insecticide resistance and changes in biting behavior of malaria vectors have renewed interest in larval source management (LSM) on Bioko Island as a useful supplement to the core vector control interventions of indoor residual spraying and insecticide-treated nets. Typically, anopheline breeding habitats have included small puddles, car tyre tracks, marshes and mangrove swamps, open drains and roadside ditches. *Anopheles* mosquitoes, however, can adapt to a diversity of breeding habitats due to changes in vector ecology. During an effort to characterize anopheline

Appendix 10. Publication in Malaria Journal

Muleba *et al. Malaria Journal*
https://doi.org/10.1186/s12936-023-04746-5
Malaria Journal

RESEARCH

Open Access

Spatial–temporal vector abundance and malaria transmission dynamics in Nchelenge and Lake Mweru islands, a region with a high burden of malaria in northern Zambia

Check for updates

Mbanga Muleba[1*], Keith J. Mbata[2], Jennifer C. Stevenson[3] and Douglas E. Norris[4]

Abstract

Background Over a decade of vector control by indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) distribution on the mainland, and only LLINs on islands had a minimal impact on disease burden in Nchelenge district, northern Zambia. *Anopheles funestus* and *Anopheles gambiae* are vectors known only from the mainland. Understanding vector bionomics in the district is necessary for planning and targeting effective vector control. This study aimed to provide information on abundance, seasonality, and *Plasmodium falciparum* sporozoite infectivity of malaria vectors in Nchelenge, including islands.

Methods Mosquitoes were collected in 192 CDC indoor light traps set in 56 households between January 2015 and January 2016. Morphological and molecular species identifications and *P. falciparum* circumsporoites by ELISA were performed. Mosquito counts and relative abundances from the islands and mainland were compared, and household factors associated with vector counts were determined.

Results A total of 5888 anophelines were collected during the study. Of these, 5,704 were female *Anopheles funestus* sensu lato (*s.l.*) and 248 female *An. gambiae s.l.* The highest proportion of *An. funestus* (n = 4090) was from Chisenga Island and *An. gambiae* (n = 174) was from Kilwa Island. The highest estimated counts per trap for *An. funestus s.l.* were from Chisenga island, (89.9, p < 0.001) and from the dry season (78.6, p < 001). For *An. gambiae* the highest counts per trap were from Kilwa island (3.1, p < 0.001) and the rainy season (2.5, p = 0.007). The highest estimated annual entomological inoculation rate was from Chisenga Island with 91.62 ib/p/y followed by Kilwa Island with 29.77 ib/p/yr, and then Mainland with 19.97 ib/p/yr.

Conclusions There was varied species abundance and malaria transmission risk across sites and seasons. The risk of malaria transmission was perennial and higher on the islands. The minimal impact of vector control efforts on the mainland was evident, but limited overall. Vector control intervention coverage with effective tools needs to be extended to the islands to effectively control malaria transmission in Nchelenge district.

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Keywords Malaria, *Anopheles funestus*, *Anopheles gambiae*, Nchelenge, Sporozoite rate, Entomological inoculation rate

Background

The use of long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), artemisinin-based combination therapy (ACT), and intermittent preventive therapy in pregnancy (IPTp) in the last decade has led to successes against malaria in sub-Saharan Africa [1–5]. Sadly, not all areas receiving control interventions face an easy transition from malaria endemicity to malaria elimina-tion [6]. For some high-transmission areas, the package of control interventions may need to be prescribed dif-ferently and specifically. In Zambia, Nchelenge District in the north is an area with high malaria transmission, with a sustained malaria prevalence between 30 and 50%, despite incremental coverage in control interven-tions comprised of ACTs, IRS, LLINs, IPTp since 2008, and more recently, integrated community case manage-ment (ICCM) [7, 8]. Studies from mainland Nchelenge have reported complex vector bionomics of two malaria vector species, *Anopheles funestus sensu stricto* (*s.s.*) and *An. gambiae s.s*. These studies have estab-lished that *An. funestus* is widely distributed and has a population peak during the dry season, while counts of *An. gambiae* remain relatively and consistently low throughout the year and are more abundant near Lake Mweru on the western border of the district [9–12]. The few genomics studies completed in Nchelenge have revealed the sympatric existence of Clades I and
II of *An. funestus s.s*. and only S-form *An. gambiae*, with populations exhibiting varied insecticide suscep-tibility to carbamates, pyrethroids, and DDT [13–15]. It is unknown, however, to what extent these findings can be extended to the island settings within the dis-trict, where control measures are much more challeng-ing to implement. The two big islands of the district, Kilwa Island and Chisenga Island, comprise nearly 13% of the district's total population and, at the time of the study, had received only LLINs as malaria control interventions.

During the study period, cases of malaria reported between January and December 2015 for all age groups from Chisenga and Kilwa Islands rural health centres were 4, 282 and 8, 439, respectively (Nchelenge DHIS). These represented cases per 1000 people of 375 and 746 for Chisenga and Kilwa islands, respectively. Nchelenge Health Centre on the mainland reported 9360 cases with an incidence rate of 476/1000 (Nchelenge DHIS). For each health facility, almost half of the cases
(2353 Chisenga Island, 4061 Kilwa Island, and 4477 Nchelenge) were in children 0–5 years old.
Given the enormity of malaria incidence in Nchelenge District, critical data is needed to better understand the underlying factors leading to the lack of success of malaria control in Nchelenge, especially in the context of malaria elimination [16]. Data on the two primary malaria vector species from the islands of Nchelenge is incredibly sparse. Published data was only from Kilwa Island mosquitoes, dating back to a 1926 report on non-malaria vectors [15]. The other known data on vectors came from a recent publication on *An. funestus s.s.* com-plete mitogenome [17], which included specimens col-lected from Kilwa Island during the initial preparatory stages of this study. This study aimed to expand ongoing malaria entomological studies in Nchelenge District to include the main islands in Lake Mweru. The focus was on vector seasonal and spatial abundance, *Plasmodium falciparum* sporozoite infection rates, and how these data may impact current and future malaria control strate-gies in the district. The study period was January 2015 to January 2016 as part of the Southern and Central Africa International Centres of Excellence for Malaria Research (ICEMR), a research collaboration set up to study malaria transmission and the impact of control interventions in Zambia, Zimbabwe, and the Democratic Republic of the Congo (DRC).

Methods

Study area

Nchelenge District is located in northern Zambia in Lua-pula Province (Fig. 1) at the mouth of the Luapula River, forming part of the Luapula-Mweru delta. Lake Mweru and the Luapula River form the border with the DRC, and the Luapula-Mweru system interface includes vast swamps and lagoons and a number of islands. Prominent among the islands on the Zambian side of Lake Mweru are Chisenga, Kilwa, and Isokwe (Fig. 1). The mainland along Lake Mweru hosts a network of shallow streams with slow-moving waters and expansive vegetation cover. Mosquitoes are hypothesised to breed along the edges of these streams, which remain inundated for extended periods of the year.
Nchelenge is largely a rural district with an estimated human population at the time of the survey of 147,927 [18]. The mainland constituted about 77% of the total district population. The island populations by head count from the district health office were estimated at 11409

Fig. 1 Map of Nchelenge and sampled households across the District (map composed using QGIS 3.22.7)

for Chisenga Island, 3501 for Isokwe Island, and 11305 for Kilwa Island (Nchelenge DHO). The majority of the island's inhabitants are involved in fishing, while also engaging in subsistence farming. The formal housing structures with metal roofing are typical of the district's urban areas. The rural areas comprise houses with mud walls and thatch roofing. Makeshift houses, which expose the inhabitants to mosquito bites, are characteristic of farming fields on the mainland and nearly all houses on Isokwe Island and in fishing camps on the islands. Seasonal human movement between the interior mainland, lakeside, and islands is common. On the mainland, the beginning of the rainy season necessitates the shift to the interior, where there are farming fields. The rainy season is usually between November and May, and the dry season comes between June and September. The collections conducted during the rainy season in 2015 were defined as rainy season 1, and those done in 2016 were designated rainy season 2.

Vector control

Indoor residual spraying and bed net distribution form the main vector control interventions conducted in Nchelenge by the national programme. The IRS pro-gramme is normally conducted between September and December of each year. During® the study period, IRS with pirimiphos-methyl (Actellic 300CS) was conducted ®in 2014 and 2015, and a mass campaign for LLIN (Olyset) distribution took place in October 2014.

Selection of study households

Satellite imagery of the three islands from Google Maps and an ARCGIS image of mainland Nchelenge District were overlaid with 1 -km^2 grids [19]. Grids with human habitation were enumerated, and study households were selected by simple random sampling. On the ground, houses within a selected grid (hereafter referred to as a village) were enumerated, and four houses were randomly selected. A total of 56 houses across 14 villages in the study area were subsequently selected and their geographic® coordinates recorded (using a Garmin OREGON 450 GPS gadget). The 14 villages were distributed as follows: Chisenga Island, 4; Isokwe Island, 2; Kilwa Island, 5; and Mainland, 3.

Mosquito collection

The collection of mosquitoes was conducted using CDC light traps hung indoors approximately 150 cm above the floor at the foot end of the sleeping space. A total of 192 traps were set indoors during the entire study period. A

total of five rounds of collection were conducted, as follows: First rainy season: 32 households (HH) in January 2015 and 36 HH in March 2015. During the dry season, 37 HH were recorded in July 2015 and 42 HH in Octo-ber 2015. Finally, the second rainy season 44 HH in Janu-ary 2016 Each of the selected households was sampled a minimum of one and a maximum of five times (Table 1) over the entire sampling period. Only data from 42 households, a total of 165 trap nights (marked with § in Table 1) sampled 3–5 times, were included in the analysis of malaria vectors' spatial and temporal abundances.

Processing of collected mosquitoes

All anopheline mosquitoes collected were morphologically identified by standard identification keys to species groups upon collection in the field [20, 21]. These were individually packed into 0.5-mL centrifuge tubes with desiccant for further laboratory processing. A representative sub- sample of 20% of specimens morphologically identified as female *An. funestus sensu lato* (*s.l.*) and *An. gambiae s.l.* were processed for polymerase chain reaction (PCR) and circumsporozoite ELISA.

Mosquitoes were dissected at the thoracic and abdomi-nal joints into the head, thorax, and abdomen. DNA was extracted from either a leg or abdomen and processed by PCR for species identification [22, 23]. The M and S molecular form determinations of *An. gambiae s.s.* were completed by PCR, as previously described [24].

TaqMan PCR, as previously described [25], identi-fied the *An. funestus* clades. The *P. falciparum* sporozo-ite infections were determined by the circumsporozoite ELISA assay pf-210 [26, 27]. To remove false positives, the homogenates of all the assays that tested positive on the first run of circumsporozoite ELISA were boiled at 100 °C for ten minutes [28], and the ELISA was repeated. The *P. falciparum* sporozoite rate was calculated as the number of mosquitoes detected with *P. falciparum* sporozoites out of the total tested by circumsporozoite ELISA [29]. The biting rate was taken as previously done by Das et al. [10], taking into account the total number of people in the households. Annual entomological inoculation rates were estimated as the product of the biting rate and sporozoite rate multiplied by 365 days.

Data analysis

The mosquito counts were entered in Excel sheets and exported to R and STATA 15 (Release 15, College Station, TX: Stata Corp LLC) for analysis. Morphological data were used for the analyses of species abundance and distribution. Only data from households sampled at least three times were included in the analyses of the seasonal and spatial abundance of vector species. The species PCR data were used to compare vector species infection rates and their spatial and seasonal occurrence.

Variations in total female anophelines collected from each site were evaluated separately for each of the two vector species by multilevel mixed-effects negative bino-mial regression with household as a random covariate. The covariates of fixed effects were season and site of collection. Marginal effects and means of vector counts were predicted for the season and site of collection. The *P. falciparum* sporozoite infection rates in *Anopheles* species were seasonally and spatially compared and predicted by logistic regression analysis. QGIS version 3.10.2 was used to create maps and plot proportions of mosquito counts as well as *P. falciparum* infection rates.

Results

Proportions of major vectors in the study area

A total of 5888 Anopheles mosquitoes were collected indoors from 56 households in 14 villages (Fig.2). A total of 858 people slept in the houses on the nights of the mosquito sample collection. Of the total collection, 5579 were female anophelines, distributed as follows: *Anopheles funestus s.l.* = 5323, *An. gambiae s.l.* = 249, *Anopheles coustani s.l.* = 4, and unidentified = 3.

The count data of the two major malaria vectors were highly skewed, with 31 traps recording zero *An. funestus*

Table 1 Distribution of households and sampling efforts across sites over the course of the study

No. of times sampled	No. of households from study site				
	Chisenga island	Isokwe island	Kilwa island	Mainland	Total sampling
5	4§	0	3§	5§	60
4	7§	0	5§	3§	60
3	4§	0	7§	4§	45
2	1	6	4	0	22
1	0	2	1	0	3
Total households	16	8	20	12	

§ Number of households included in analysis of seasonal and spatial abundance

Fig. 2 Mean composition of the primary malaria vectors *An. funestus s.l.* and *An. gambiae s.l.* across the study area (map composed using QGIS3.22.7)

s.l. collections and 97 traps recording zero *An. gambiae s.l.* collections. The maximum count of *An. funestus s.l.* in a single light trap was 497 from Chisenga Island, and the maximum for *An. gambiae s.l.* was 30 from Kilwa Island (Table 2).

Anopheles funestus s.l. count (n = 5240) were domi-nant over *An. gambiae s.l.* (n = 238) across all study sites (Table 2). Chisenga Island had the highest proportion of *An. funestus s.l.* (n = 4090), and Kilwa Island had the highest proportion of *An. gambiae s.l.* (n = 174).

Factors associated with malaria vector counts

The estimated counts per trap for the two vector spe-cies across the study sites ranged from 8.602 to 89.97 for *An. funestus s.l.* and 0.499 to 3.083 for *An. gambiae s.l.* (Table 3). On the other hand, seasonal counts per trap for

Table 2 Proportions of the total vectors collected and the maximum count in a single light trap in different sites

Site	Variable	*An. funestus s.l*	*An. gambiae s.l*
Chisenga island	Trap nights	60	60
	Total mosquitoes collected	4090	26
	Maximum	497	9
Kilwa island	Trap nights	56	56
	Total mosquitoes collected	552	174
	Maximum	88	30
Mainland	Trap nights	49	49
	Total mosquitoes collected	598	38
	Maximum	242	13

Table 3 Factors associated with the malaria vector counts in CDC light traps set indoors

Covariate	*An. funestus* s.l			*An. gambiae s.l*		
	IRR/Predicted mean count	95% CI	p-value	IRR/Predicted mean count	95%CI	P-value
Season	Ref.					
Dry						
Rainy 1	0.189	0.115–0.311	< 0.001*	0.77	0.365–1.627	0.494
Rainy 2	0.069	0.038–0.124	< 0.001*	1.868	0.858–4.065	0.404
Site						
Mainland	Ref.					
Chisenga Island	9.341	3.516–24.820	< 0.001*	0.805	0.282–2.300	0.685
Kilwa Island	0.877	0.329–2.335	0.793	4.573	1. 619–12.915	0.004*
Spatial abundance						
Chisenga Island	89.944	46.995–132.893	< 0.001*	0.499	0.175–0.823	0.003*
Kilwa Island	9.461	4.871–14.051	< 0.001*	3.083	1.546–4.620	< 0.001*
Mainland	8.602	0.578–16.626	0.036*	0.639	0.061–1.217	0.030*
Seasonal abundance						
Dry	78.606	38.997–118.215	< 0.001*	1.382	0.510–2.254	0.002*
Rainy 1	16.613	8.244–24.983	< 0.001*	0.818	0.378–1.258	< 0.001*
Rainy 2	4.869	1.815–7.923	0.002*	2.548	0.686–4.410	0.007*

IRR incidence rate ratio

*Shows results were significant at 95% confidence level

these vectors ranged from 4.869 to 78.606 for *An. funestus* and 0.818 to 2.548 for *An. gambiae* (Table 3).

The *An. funestus* counts were strongly associated with Chisenga Island (IRR = 9.341, p < 0.001), and those of *An. gambiae* were so with Kilwa Island (IRR = 4.573, p = 0.004). There was a significant reduction in the estimated *An. funestus* count during rainy seasons 1 and 2, IRR = 0.189, p < 0.001, and IRR = 0.069, p < 0.001, respectively (Table 3). The *An. gambiae* count reduced during rainy season 1 (IRR = 0.770, p = 0.494), but the count increased during rainy season 2 (IRR = 1.868, p = 0.115) (Table 3).

Molecular identifications

Molecular species identifications by PCR on a sub-sample (1108 females) successfully amplified 978 (88%) of the specimens processed. DNA from 131 specimens did not amplify.

Identification of species by PCR showed *An. funestus s.s.* (n = 773) and *Anopheles leesoni* (n = 10) as mem-bers of the *An. funestus* group. Further mitochondrial DNA analysis of *An. funestus s.s.* revealed the existence of Clade I (n = 736) and Clade II (n = 37), as previously reported [13]. The *An. gambiae s.l.* PCR analysis of specimens revealed only *An. gambiae s.s.* (n = 195). Further processing by TaqMan hydrolysis PCR to determine the M-form (*Anopheles coluzzii*) and S-forms revealed the S-form of *An. gambiae s.s.* as the only member of the complex collected.

Plasmodium falciparum infection rates in the vectors

A total of 30 specimens gave a positive *P. falciparum* sporozoite ELISA result on the first run. When the homogenates were heated to remove false positives, 25 still came out positive. The *P. falciparum* circumsporozoite ELISA showed positive results in *An. funestus s.s.* Clade I, 18 (n = 736), and *An. gambiae,* 7 (n = 195). No sporozoites were detected in *An. funestus s.s.* Clade II (n = 37) or *An. leesoni* (n = 10).

In the study sites, *P. falciparum* infections were found in both primary vectors, *An. funestus s.s.* and *An. gam-biae s.s.*, from Kilwa Island and the mainland. For Isokwe Island and Chisenga Island, only *An. funestus s.s.* was found infected with *P. falciparum*. Using the foraging rates based on the count of mosquitoes and household occupants the annual entomological inoculation rates (EIR) were estimated for each collection site. The EIR for Chisenga Island was 91.62 infectious bites per per-son per year (ib/p/yr), all of which came from *An. funes-tus s.s.* Kilwa Island's EIR was 29.77 ib/p/yr, with species contributions of 21.21 ib/p/yr and 8.57 ib/p/yr from *An. funestus s.s.* and *An. gambiae s.s.*, respectively. Mainland EIR was estimated to be 19.97 ib/p/yr, with *An. funestus s.s.* contributing 18.12 ib/p/yr and *An. gambiae s.s.* giving 1.85 ib/p/yr.

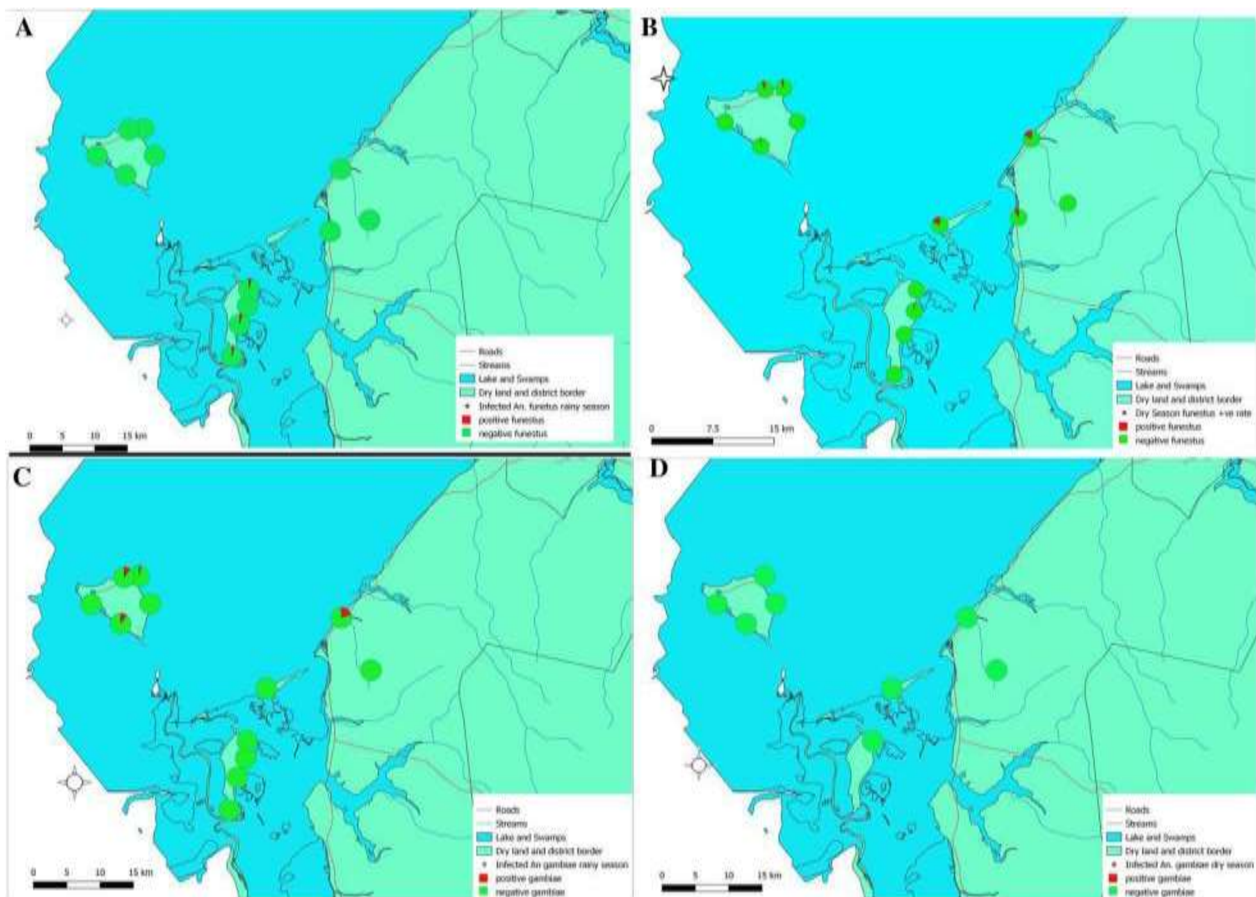

Fig. 3 Spatial and seasonal distribution of *P. falciparum* sporozoite infections in the primary vectors across study sites. **A** and **B** represent infections in *An. funestus s.l.* in the rainy and dry seasons, respectively. **C** and **D** represent infections in *An. gambiae s.l.* rainy and dry seasons, respectively (maps composed using QGIS 3.22.7)

Seasonally, *P. falciparum* sporozoites-infected vectors were detected in both dry and rainy seasons (Fig. 3). In the rainy season, *P. falciparum* sporozoites were detected among *An. funestus s.s.* samples from Chisenga Island (Fig. 3A) and in *An. gambiae s.s*. samples from Kilwa Island and the mainland (Fig. 3C). In the dry season, *P. falciparum* sporozoite infections were detected in *An. funestus* s.s. from Isokwe Island, Kilwa Island, and the mainland (Fig. 3B). No dry-season *P. falciparum* sporozoites were detected in *An. gambiae s.s.* from any site (Figure 3D).

Seasonal and spatial comparisons in *P. falciparum* infection rates by logistic regression (Table 4) in the two vector species revealed 54% lower odds of infection in *An. gambiae s.s.* compared to *An. funestus s.s.* Clade I (OR = 0.460, 95% CI: 0.184–1.150, p = 0.097). The odds of infection with *P. falciparum* in vectors were lower on Chisenga Island compared to the mainland (OR = 0.646, 95% CI: 0.198–2.109, p = 0.649). The odds of infection in vectors were higher on Isokwe Island compared to the mainland (OR = 3.564, 95% CI: 0.506–25.100, p = 0.202), and higher on Kilwa Island compared to the mainland (OR = 1.322, 95% CI: 0.388–4.506, p = 0.655). The estimated probability of *An. funestus s.s.* Clade I infection with *P. falciparum* sporozoites during the study was 0.042 (95% CI: 0.16-0.68, p = 0.001). The estimated probability of infection in *An. gambiae s.s.* was 0.020 (95% CI: 0.004–0.036, p = 0.014). Seasonal predicted probabilities of infection with *P. falciparum* in vectors were: dry season 0.021 (95% CI: 0.008–0.035, p = 0.002), rainy season 1, 0.015 (95% CI: 0.000–0.034, p = 0.092), and rainy season 2, 0.073 (95% CI: 0.026–0.119, p = 0.002).

Discussion

This study determined the spatial and seasonal abundances of the two primary vectors of malaria in Nchelenge, including islands. The infection with *P. falciparum* sporozoites in the two primary vectors of malaria, *An. funestus s.s.* and *An. gambiae s.s.,* in Nchelenge was determined. There were significant differences in the

Table 4 *Plasmodium falciparum* infection analysis by logistic regression

Covariate	OR	95% CI	p-value	Predicted SR	95% CI	p-value
Vector						
An. funestus s.s	Ref.			0.042	0.016–0.068	0.001*
An. gambiae s.s	0.46	0.184–1.50	0.097	0.02	0.004–0.036	0.014*
Season						
Dry	Ref.			0.021	0.008–0.035	0.002*
Rainy 1	0.722	0.216–2.413	0.596	0.015	0.000–0.034	0.092
Rainy 2	3.596	1.593–8.118	0.002*	0.073	0.026–0.119	0.002*
Site						
Mainland	Ref.			0.022	0.000–0.045	0.056
Chisenga Island	0.646	0.200–2.109	0.469	0.014	0.003–0.026	0.017*
Isokwe Island	3.564	0.506–25.100	0.202	0.075	0.000–0.215	0.173
Kilwa Island	1.322	0.388–4.506	0.655	0.029	0.010–0.048	0.003*

OR odds ratio, *SR* sporozoite rate

*Shows results were significant at 95% confidence level

abundance of *An. funestus s.l.* on Chisenga Island and of *An. gambiae s.l.* on Kilwa Island compared to the main-land. The *An. funestus s.l.* was more abundant during the dry season, and the *An. gambiae* was abundant dur-ing the rainy season. Predicted probabilities for *P. falci-parum* infection were significant for both *An. gambiae s.s.* and *An. funestus s.s.*, with *An. funestus s.s.* showing a slight edge over *An. gambiae s.s.* When the two vectors were considered together, the predicted probability of a malaria vector for infection with *P. falciparum* during both the dry and rainy seasons was significant. The esti-mated annual entomological inoculation rate, which is a measure of human exposure to the risk of malaria trans-mission, was highest on Chisenga Island with 91.62 ib/p/ yr, followed by Kilwa Island with 29.77 ib/p/y, and lastly Mainland with 19.97 ib/p/yr.

These results show a dynamic entomological picture driving malaria transmission in the district. The results obtained here both confirmed what was already known about Nchelenge and provided new insights about the malaria vectors on the islands. At the time of this study, the only form of vector control for the islands was net distribution. *Anopheles funestus s.l.* dominated and was more abundant during the dry season, in agreement with previous findings [10, 12]. This study went further to show that Chisenga Island had disproportionally higher densities of *An. funestus s.l.* compared to either Kilwa Island or the mainland. The highest counts of *An. gam-biae s.l.* were recorded from Kilwa Island. These findings seem to reflect local differences in the ecologies of these different study sites, which may be important for target-ing control interventions [30, 31].

The findings of this study are in support of prior stud - ies that assessed household risk factors for mosquito abundance on the mainland [9, 12]. Mosquito counts found in this study suggest that high bed net usage could be driven by high indoor densities of pyrethroid-resistant mosquitoes [9]. The findings from this study indicate that this scenario would be widespread across Nchelenge, including islands. These scenarios would raise concern as to the efficacy of the use of LLINs as the only vector control intervention for the islands in Nchelenge District. Other interventions, including the use of PBO-syner-gized LLINs designed to address the high rates of pyre-throid resistance, need to be included. Other household factors, such as thatch roofing and open eaves indica-tive of poor construction, allowed easier mosquito entry. Improved household structures should be considered a compliment to or integrated into the vector control programme for the district. Studies done in Cameroon, The Gambia, and Kenya have demonstrated that simple and low-cost improvements to house structure would be effective at preventing mosquito entry and reducing indoor densities [32–35].

The parasite sporozoite rates observed suggested varia-tions in spatial intensity but a sustained, perennial risk of vectors infected with *P. falciparum* sporozoites. *Anoph-eles funestus s.s.* contributed to transmission at all study sites during the dry season and on Chisenga Island dur-ing the rainy season. In contrast, *An. gambiae* appeared to drive transmissions during the rainy season on Kilwa Island and the mainland. The mainland and Kilwa Island were the only sites where both vector species were found infected with *P. falciparum*. Despite their relatively low numbers, *An. gambiae* had comparable rates of *P. falci-parum* sporozoite infections with the more abundant *An. funestus s.s.* on both Kilwa Island and the mainland. This demonstrated that *An. gambiae* made substantial

contribution to malaria transmission in Nchelenge, a fact that needs to be considered when designing and implementing vector control interventions. This study found no *P. falciparum*-infected Clade II *An. funestus s.s.* This was in contrast with the prior study by Choi *et al.* [13] on the mainland that could not attribute any difference in malaria transmission to either Clade, and thus our find-ings could be attributed to sample size.

Two previous studies, one intensive and another extensive, both conducted in 2012 and 2013, made comparable estimates of the EIRs for the Nchelenge mainland with different calculations of the human biting rate [10, 12]. The EIR estimated for the mainland in the current study was much lower for both *An. funestus s.s.* and *An. gambiae s.s.* The EIR is an important indicator of the intensity of malaria transmission in an area. A reduction in EIR is one of the indices used to measure the impact of vec-tor control activity [36, 37]. This study followed a mass LLIN distribution campaign and two years of IRS with pirimiphos-methyl on the mainland. Two studies on the mainland that looked at the effects of three years of IRS with pirimiphos-methyl on parasite prevalence and the number of malaria vectors found that the number of vectors in sprayed homes and targeted areas went down, but parasite prevalence did not change much [38, 39]. The present study findings on the mainland suggest reduced EIR estimates, which seem to agree with the reduced vector abundances and parasite prevalence reported during the study period.

Twelve months of concomitant malaria incidence data were obtained from health centres (HCs) servicing the three individual collection study sites. According to these data, malaria incidence was highest on Kilwa Island HC, then on the mainland Nchelenge HC, and lowest on Chisenga Island HC. In contrast, Chisenga Island had the highest estimated EIR, followed by Kilwa Island, and then the mainland. In an area with high vector counts, the discrepancy between estimated EIR and reported malaria cases may be due to the unreported cases that get treated at home within communities. No prevalence studies were conducted in the present study, nor had they been conducted before on the islands. For the islands in Nchelenge, local factors including ecology, demographics, malaria control use, and mosquito bionomics would need to be considered in the estimation and interpreta-tion of the EIR. Monitoring how EIRs would be affected would be important in the evaluation of the scale-up of the malaria control package on the islands of Nchelenge.

Conclusions

Consistent with prior studies in Nchelenge District, this study reports a high abundance of the primary vector, *An. funestus s.s.,* and a relatively low abundance of *An. gambiae*, including island sites for the first time. In sites and periods where both vector species were involved in malaria transmission, sporozoite infection rates were comparable, thereby adding to a higher risk for malaria transmission. The combination of IRS and LLINs on the mainland might have a very minimal impact on malaria transmission. Sustained vector control at all the sites should be emphasised with expanded coverage of intervention on the islands. Non-chemical mosquito bite control and prevention options should be considered for Nchelenge. Continued research on the vectors, the parasites, and the human host at different sites is required to fully understand the burden and effectively target control in different areas of Nchelenge.

Abbreviations

ACT Artemisinin-based combination therapy
ARCGIS Aeronautical Reconnaissance Coverage Geographic Information System
CDC Centres for Disease Control and prevention
DDT Dichloro-diphenyl-trichloroethane
DHIS District Health Information System
DHO District Health Office
DNA Deoxyribonucleic Acid
DRC Democratic Republic of Congo
EIR Entomological Inoculation Rate
ELISA Enzyme-linked Immunosorbent Assay
ERC Ethics Review Committee
GPS Global Position System
HC Health Centre
HH Household
ICCM Integrated Community Case Management
ICEMR International Centres of Excellence for Malaria Research
IPTp Intermittent Preventive Therapy in Pregnancy
IRS Indoor Residual Spraying
ITNs Insecticide-Treated Nets
LLINs Long-lasting Insecticidal Nets
M & S Mopti & Savanna strain
Mw Williams Mean
OR Odds ratio
PCR Polymerase chain reaction
QGIS Quantum Geographic Information System
STC Scientific and Technical Committee
TDRC Tropical Diseases Research Centre

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Author contributions

MM designed the study, collected and analysed the data, and drafted the first manuscript. KJM, JS, and DN provided guidance on study design and data interpretation. All authors reviewed and approved the manuscript.

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Availability of data and materials
Raw data on the mosquito collections and entomological indices is available upon reasonable request from the corresponding author.

Declarations

Ethics approval and consent to participate
This study did not use human specimens, and written consent for indoor collection of mosquitoes was obtained from household heads. This study was approved and cleared by the Tropical Diseases Research Centre, Ndola, Zambia (TDRC, ERC, STC/2015/8).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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