The Effect of Insecticide Resistance on Malaria Vector Control in Chikwawa, Southern Malawi

Thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

Justin Kumala

Reg. No. 1598631

School of Pathology, University of the Witwatersrand

Johannesburg, 2024

This work was supervised by:

Professor Maureen Coetzee,

Wits Research Institute for Malaria, School of Pathology, Faculty of Health Sciences,

University of the Witwatersrand, Johannesburg

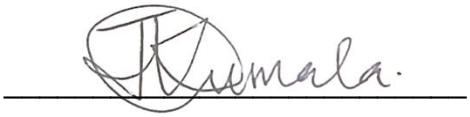
and

Dr Themba Mzilahowa

Malaria Alert Centre, Kamuzu University of Health Sciences, Blantyre, Malawi

Candidate Declaration

I, **Justin Kumala**, declare that this thesis hereby submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg, is my own independent work. It has not been submitted for any other degree or at any other university. All materials adopted from previously published work have been appropriately cited in this thesis.



Justin Kumala

31st October, 2024

Date

Abstract

Insecticide resistance poses a significant threat to malaria vector control measures, compromising the efficacy of interventions such as indoor residual spraying and insecticide-treated nets. This cross-sectional study investigated the impact of insecticide resistance on vector control tools and explored the association between resistance and mosquito plasmodium infectivity rates.

CDC bottle and WHO tube assays were used to detect phenotypic resistance in wild-collected non-blood-fed female *An. funestus*, a key vector in the region. Circumsporozoite ELISA tests were used to detect sporozoite positivity following bioassays. The Chi-square test of independence was used to investigate the relationship between bioassay mortality and sporozoite infection rates to estimate the entomological impact of resistance. Furthermore, selected long-lasting insecticidal net (LLIN) brands, Royal Sentry and Yorkool nets, were tested for their effect on a series of wild-collected and laboratory mosquito strains with variable levels of insecticide resistance. Wash resistance tests of the selected LLINs followed a WHO protocol.

The study revealed an escalation of resistance among wild *An. funestus* to pyrethroids. There was high resistance intensity against alpha-cypermethrin and moderate resistance to deltamethrin and permethrin. However, there was complete susceptibility to DDT and pirimiphos methyl, an organophosphate. The overall sporozoite positivity rate for *An. funestus* was 11.5% (n=400), highlighting high transmission in the population. The Chi-square test of independence found a significant relationship between bioassay mortality and sporozoite infection (X^2 (1, N=157) = 6.889, p= 0.009). Comparatively, Royal Sentry nets exhibited higher

mortality rates (100%) for susceptible *An. gambiae* s.s Kisumu than Yorkool nets (69.4%). Additionally, Royal Sentry nets had better mortality rates on wild pyrethroid-resistant *An. funestus* (74.04%) than Yorkool nets (7.80%). Both LLINs demonstrated better efficacy in wash resistance tests on susceptible strains, especially at baseline. However, a notable decrease in efficacy was observed against the resistance mosquito strains, suggesting the potential impact of resistance on net efficacy.

While these findings showed no evidence that insecticide resistance might compromise our efforts to control transmission, they highlighted the potential entomological impact of resistance. The findings also highlighted the importance of evidence-based decision-making in prioritising interventions that are effective against resistant populations. Thus, they emphasised the importance of tailoring intervention choices based on local insecticide resistance profiles.

Keywords:

Insecticide resistance, Malaria transmission, Long-lasting insecticide-treated net, *Anopheles funestus,* Wash resistance

Acknowledgements

I express deep gratitude to Professor Maureen Coetzee for her exceptional guidance and supervision of this PhD, even during her retirement. Sincere thanks to Dr Themba Mzilahowa for co-supervising and contributing valuable field expertise. Professor Lizette Koekemoer's mentorship during diagnostics was invaluable.

Acknowledgements to colleagues at the Vector Control Reference Laboratory, Wits Research Institute for Malaria, and Malaria Alert Centre. Special thanks to Dr Shune Oliver and contributors Gogo Christina and Nkulu Zilindile for providing mosquito colonies and space for experiments. Alison Gilbert's training on the net washing protocol and Elke Gevers' career development support are also deeply appreciated.

To colleagues including Martin Chiumia, Eunice Jamesboy, Zandile Langa, Witness Ramashia, Paul Khokhololo, Emmanuel Ngwarati, Dr Steve Gowelo, Dr Peter Ntenda, Dr Emmanuel Kaindoa, and others, your support in the field, during bioassays, diagnostics, and data analysis made my task more manageable.

Special gratitude to Mr and Mrs Kumala (parents), Pastors Franklin Nkomba and Hannes van Wyk, and role models Dr Hannah Mayamiko Dunga, Prof. Steven Dunga, Prof. Chimwemwe Chipeta, Dr Eleanor Chipeta, Prof. Costa Hofisi, Dr Mirriam Hofisi, Marion Makumbe, Dr Hattie Makumbe, Susan Dunga, and Daniel Dunga.

A heartfelt thank you to my wife, Justina, and children Jeriah, Joann and Jeriel. Your unwavering support during the most challenging times provided the strength needed. Your belief in my capabilities has been instrumental in our collective success.

This research was supported by the Consortium for Advanced Research Training in Africa (CARTA). CARTA is jointly led by the African Population and Health Research Center and the University of the Witwatersrand and funded by the Carnegie Corporation of New York (Grant No--B 8606.R02), SIDA (Grant No:54100029), the DELTAS Africa Initiative (Grant No: 107768/Z/15/Z). The statements made and views expressed are solely the responsibility of the researchers.

Original Papers and Conference Proceedings

1. **Kumala, J.**, Koekemoer, L.L., Coetzee, M., and Mzilahowa, T. Intensity of insecticide resistance in the major malaria vector *Anopheles funestus* from Chikwawa, rural Southern Malawi. *Parasites Vectors* **15**, 220 (2022). https://doi.org/10.1186/s13071-022-05299-3

2. **Kumala, J.,** Koekemoer, L., Coetzee, M., and Mzilahowa, T. 2022. Intensity of insecticide resistance in the major malaria vector *Anopheles funestus* from Chikwawa, rural Southern Malawi. 16th CARTA Annual Joint Advanced Seminar (JAS 4) conference at the School of Public Health (SPH), Makerere University, Kampala, Uganda (19th March 2019)

   Title of Presentation: The Intensity of Insecticide Resistance in the Major Malaria Vector *Anopheles funestus* from Chikwawa, Southern Malawi

   Award: Overall, best oral presentation

Table of Contents

List of Tables

List of Figures

List of Acronyms and Abbreviations

AChE	Acetylcholinesterase
ACTs	Artemisinin-based combination therapies
ANOVA	Analysis of variance
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
DDT	Dichloro diphenyl trichloroethane
DF	Degrees of freedom
ELISA	Enzyme-linked immunosorbent assay
GPIRM	Global Plan for Insecticide Resistance Management
ITN	Insecticide-treated net
IRMP	Insecticide Resistance Management Plan
IRS	Indoor Residual Spraying
IVCS	Integrated Vector Control Strategy
Kdr	Knock-down resistance
LLINs	Long-lasting insecticidal nets
MAC	Malaria Alert Centre
NMCP	National Malaria Control Program
PBO	Piperonyl butoxide
PCR	Polymerase Chain Reaction
Pf-RDT	*Plasmodium falciparum* Rapid Diagnostic Test
rpm	Rounds per minute
s.l	*Sensu lato*
spz rate	Sporozoite rate
s.s	*Sensu stricto*
WHO	World Health Organization

Chapter 1: Literature Review

1.1 Introduction

In the ongoing battle against malaria, a pervasive and devastating disease claiming hundreds of thousands of lives annually, the effectiveness of vector control strategies stands as a critical cornerstone. Vector control prevents contact between mosquitoes and the human population, disrupting the transmission cycle of malaria parasites. Typically, the success of insecticide-based interventions relies on the vectors' susceptibility of vectors to the insecticides being used during interventions.

The World Health Organization (WHO) recommends using insecticide-treated bed nets and indoor residual spraying as primary mosquito control tools in all malaria-endemic settings (Fig. 1.1) (WHO, 2019a; WHO, 2019b; WHO, 2021b). In addition to these measures and case management, the WHO has recently recommended the deployment of the RTS, S/AS01, and R21/Matrix-M malaria vaccines to complement ongoing efforts in the fight against malaria (WHO, 2023a). Larval source management (LSM) is also recommended as part of an integrated vector management (IVM) strategy with the core interventions, ITNs and IRS (Fig. 1.1) (WHO, 2023b).

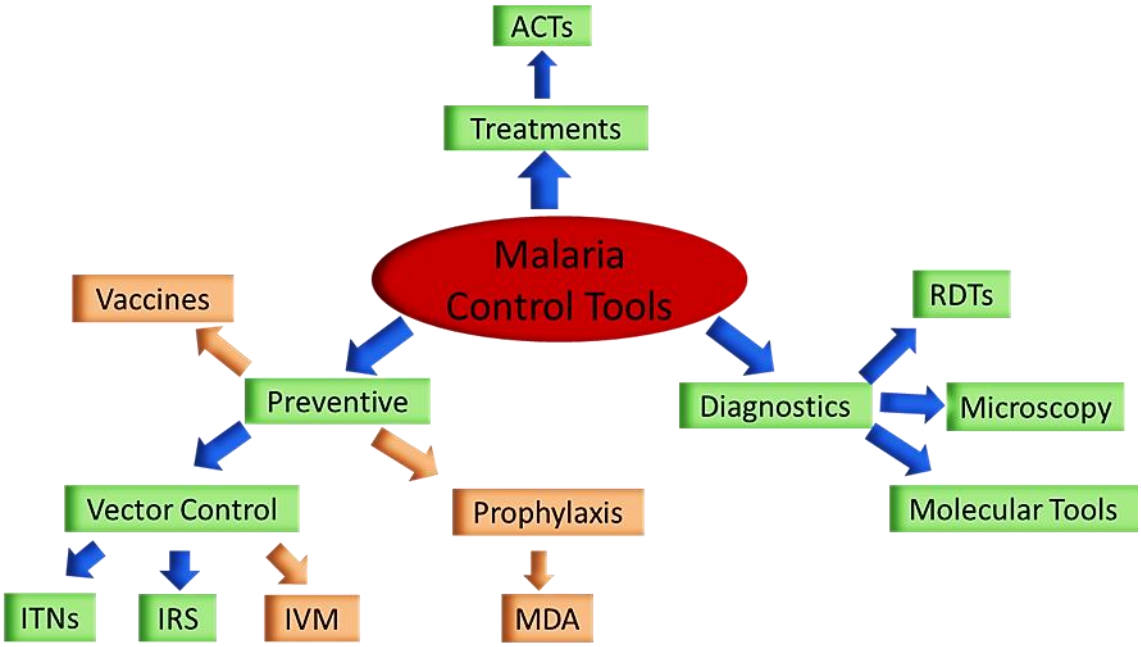


Fig. 1.1 A graphical illustration of key malaria control interventions. Highlighted in green are core interventions, while those in orange indicate complementary strategies. Interventions such as intermittent preventive therapy in pregnant women (IPTp) or in children (IPTc) are categorised as population-wide interventions and fall under mass drug administration (MDA) in this classification. ACTs= artemisinin-based combination therapies, ITNs= insecticide-treated nets, IRS= indoor residual spraying, IVM= integrated vector management (combination of ITNs and IRS, with LSM), MDA= mass drug administration and RDTs = rapid diagnostic tests. This figure, compiled by the author, collates information on malaria control from the CDC and WHO websites.

The escalating vector resistance to almost all classes of insecticides poses a significant threat to the gains made in malaria control. According to the Insecticide Resistance Action Committee (IRAC), insecticide resistance is defined as a heritable change in the sensitivity of a pest population to an insecticide product, leading to repeated failures in achieving the expected level of control with a specific product (IRAC, 2020). In practical terms, this means that the standard dose of insecticide which was once effective, no longer provides control. In disease vectors like mosquitoes, this resistance manifests as their increased ability to survive exposure

to a standard dose of insecticide that would typically be lethal. This adaptive capability may result from physiological or behavioural changes (WHO, 2018). Therefore, understanding the intricate interplay between insecticide resistance and vector control is crucial and urgent, especially as the global community grapples with the multifaceted complexities of malaria eradication (WHO, 2023b).

The confluence of insecticide resistance and malaria prevalence presents a pressing concern, potentially jeopardising the effectiveness of existing vector control measures. This thesis delves into the complex dynamics of insecticide resistance and its potential effect on malaria vector control strategies such as insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS). It focuses on Chikwawa district, southern Malawi, an area characterised by elevated levels of insecticide resistance. As we reflect on the progress made in global malaria control since 2000, it is crucial to acknowledge the persistent challenges in vector control in the fight against malaria. Despite tremendous achievements, challenges such as insecticide resistance persist and necessitate careful consideration.

Since 2000, there has been tremendous progress in reducing the burden of malaria globally and in the African region in particular. Globally, 2.1 billion malaria cases were averted, while 11.7 million lives were saved between 2000 and 2022 (WHO, 2023b). In Africa alone, malaria deaths declined by 64% (from 897,000 in 2000 to 576,000 in 2019), while the mortality rate per 100,000 population at risk dropped from 30 in 2000 to 14 in 2019 (WHO, 2022; WHO, 2023b). This tremendous success followed renewed commitments by governments and increased donor funding to end malaria globally through the scale-up of three primary interventions, namely: effective treatment with

artemisinin-based combination therapies (ACTs), and vector control through ITNs and IRS (WHO, 2020; WHO, 2021b; WHO, 2022). Arguably, vector control remains a cornerstone for malaria control, and is attributed to most of the success in reducing the global malaria burden (Bhatt *et al.,* 2015; Gari & Lindtjørn, 2018; WHO, 2021a; WHO, 2021b; WHO, 2022; WHO, 2023b).

Recent years have witnessed a concerning stagnation in the progress of malaria control, marked by a worrisome increase in reported cases and deaths annually since 2020 (Lindsay *et al.,* 2021; WHO, 2021b; WHO, 2022; WHO, 2023b). This decline in progress is attributed, in part, to stalled funding for malaria control efforts, and the WHO warns country programs and their development partners to avoid taking a "business as usual" approach as this will lead them off course (WHO, 2023b, p. 2). Furthermore, service disruptions caused by the COVID-19 pandemic have played a role in impeding the momentum gained in previous years (WHO, 2023b). Amidst these challenges, there is also a growing apprehension regarding the potential negative impact of insecticide resistance on the effectiveness of vector control interventions, thereby undermining malaria control efforts. The global community is alarmed by the fear that if not adequately managed, insecticide resistance could derail the progress made since 2000 and potentially reverse the gains achieved (Ranson & Lissenden, 2016; Hemingway *et al., 2016;* Kleinschmidt *et al., 2018;* WHO, 2023b*;* Suh *et al.,* 2023).

As mentioned earlier, the sustained control of malaria hinges on the use of effective drugs for treatment, accurate diagnostics, and the control of mosquitoes that transmit the parasites (vector control) (Birkholtz *et al., 2012;* Bhatt *et al., 2015;* Lindsay *et al.,*

2021; WHO, 2021b). However, despite the considerable progress outlined above, vector control still heavily relies on a limited range of available insecticides (Ranson & Lissenden, 2016; van den Berg *et al.,* 2021). Currently, there are six WHO-recommended chemical classes of insecticides for public health use, including organophosphates, carbamates, organochlorines, pyrethroids, and more recently, neonicotinoids (clothianidin) and pyrroles (chlorfenapyr) (WHO, 2018; Tangena *et al.,* 2020; Jobe *et al.,* 2023; WHO, 2023b).

According to the “Guidelines for Malaria Vector Control” published by the WHO in 2019, IRS deployed using a WHO-prequalified product is one of the recommended core interventions in all malaria-endemic settings (WHO, 2019a; WHO, 2019b). The organochlorine dichloro-diphenyl-trichloroethane (DDT), listed as a WHO-recommended insecticide, is not WHO-prequalified. Moreover, countries are advised only to use it when there are no equally effective and efficient alternatives, provided they adhere to the Stockholm Convention on Persistent Organic Pollutants (WHO, 2019b). Pyrethroid-PBO nets that are prequalified by the WHO are, however, conditionally recommended for settings where there are confirmed and intermediate levels of resistance to pyrethroids (WHO, 2019b). In 2023, the WHO recommended the deployment of pyrethroid-chlorfenapyr (dual active ingredient) ITNs in areas where mosquitoes have become resistant to pyrethroids (WHO, 2023b). It also recommended that dual-active ingredient ITNs be preferred over pyrethroid-PBO nets, while pyrethroid-PBO nets must be prioritised over pyrethroid-only ITNs in areas of pyrethroid resistance (WHO, 2023b).

For a long time, most malaria control programs in resource-limited countries used pyrethroids for IRS due to their cheap cost, safety and residual longevity. The WHO's World Malaria Report 2023 revealed that pyrethroid resistance was detected in 87% of all malaria-endemic countries that provided data for 2010-2020 (WHO, 2023b). The percentage of the population at risk protected by IRS in the African region declined between 2010 and 2020 (Coetzee *et al.,* 2013; Abeku *et al.,* 2019; WHO, 2021b). Consequently, the rise of pyrethroid resistance has, over the years, prompted country programs to switch from pyrethroid-based IRS to other classes of insecticides as a resistance management strategy (WHO, 2012; WHO, 2019a; WHO, 2019b; Lindsay *et al.,* 2021). The success of vector control in many disease settings has depended mainly on the success of pyrethroids, but this overdependence seems to have had a negative effect on transmission control amidst growing resistance (Ranson *et al.,* 2011; Wondji *et al.,* 2015).

The spread of insecticide resistance is likely to undermine the efficacy of these vital chemicals, resulting in compromised transmission control and a reversal of previous progress (Ranson & Lissenden, 2016; Hemingway *et al.,* 2016; Ochomo *et al.,* 2017; Selvaraj *et al.,* 2020). Losing these gains would be disastrous, as was demonstrated in South Africa in 1999/2000 when the country experienced the worst malaria epidemic since the pre-World War II period. In that epidemic, reported malaria cases increased almost fourfold due to resistance against pyrethroids in the malaria vector, *Anopheles funestus* (Coetzee *et al.,* 2013; Hargreaves *et al.,* 2000). Similar situations are now imminent across a much broader region if the current scenario of insecticide resistance is not managed urgently. Therefore, it is critical to determine the impact of vector

resistance on the effectiveness of vector control tools and its subsequent effect on malaria transmission control (Strode *et al.,* 2014; Agossa *et al.,* 2015).

In the southern African country of Malawi, where malaria is heavily endemic, pyrethroid resistance in *An. funestus* is well-known and has been reported nationwide since 2010 (Hunt *et al.,* 2010; Wondji *et al.,* 2012; Riveron *et al.,* 2015). The primary role of *An. funestus* in malaria transmission in the country cannot be overemphasised, as it consistently exhibits high sporozoite rates in the field. One study reported *An. funestus* sporozoite (spz) rates of 4% in 2015 compared to 2% for *An. arabiensis* in the same year (Lindblade *et. al*, 2015). Kabaghe *et al.,* 2018 reported up to 19% spz rates in *An. funestus* against *An. arabiensis* of 5.4% in Chikwawa district. The PMI VectorLink Malawi Annual Entomological Monitoring Report of 2022 indicated that *An. funestus* was responsible for up to 70% of malaria transmission nationwide, while *An. arabiensis* remained a secondary vector (The PMI VectorLink, 2022).

Like in most other resource-limited countries, long-lasting insecticidal nets (LLINs) remain the cornerstone of malaria control in Malawi alongside limited-scale IRS, which, until 2023, was implemented in a few malaria hotspot districts (Chanda *et al*., 2015; U.S. President's Malaria Initiative Malawi Malaria Operational Plan FY 2024). The Malawi NMCP adopted a universal coverage policy of LLINs, distributed every three years until 2022, when it decided to distribute LLINs every two years (Mangani *et al.,* 2022). The switch was prompted by field evidence, which showed that sleeping under an LLIN that was older than two years was associated with 50% increased odds of malaria infection compared to those using newer LLINs (Cohee *et al.,* 2022). It is worth noting that from 2024, Malawi has also phased out IRS from its vector control

strategy owing to significant funding constraints (U.S. President's Malaria Initiative Malawi Malaria Operational Plan FY 2024). However, it is worth noting that even the few districts that received a scale-up of IRS initially did not yield the anticipated results due to pyrethroid resistance (Wondji *et al.,* 2012) until newer formulations were introduced, which effectively increased program costs and has led to the recent phase-out of IRS (U.S. President's Malaria Initiative Malawi Malaria Operational Plan FY 2024). Thus, the need to monitor the impact of vector resistance on the effectiveness of vector control efforts cannot be over-emphasised, as this helps to guide vector control policy to prevent an impending catastrophe.

Chikwawa district is situated along the Lower Shire Valley in southern Malawi and stands out as one of the regions with consistently high malaria transmission rates. Notably, this district has grappled with elevated levels of insecticide resistance in the local *An. funestus* mosquito population over the years (Wondji *et al.,* 2012; Riveron *et al.,* 2014; Mzilahowa *et al.,* 2016; The PMI VectorLink, 2022). The rise of multiple insecticide resistance in the local mosquito population has also caused concerns, with unclear implications for malaria transmission control (Riveron *et al., 2015; Mzilahowa et al.,* 2016). Additionally, specific populations of *An. quadriannulatus* and *An. arabiensis* in the district have shown reduced susceptibility to DDT, particularly the latter which is a potent malaria vector in this area (Mzilahowa *et al.,* 2008; The PMI VectorLink, 2021; 2022). It becomes imperative to thoroughly investigate the potential impact of insecticide resistance on the current initiatives aimed at reducing malaria transmission in the district.

This study aimed to investigate the impact of insecticide resistance on malaria vector control in Chikwawa district. We hypothesised that insecticide resistance could reduce the efficacy of insecticides, consequently reducing the effectiveness of insecticide-treated bed nets and compromising transmission control. Like the rest of the country, universal coverage of ITNs, defined as two household members per ITN, serves as the district's primary strategy for vector control. A 2016 study reported that universal ITN coverage was not associated with a reduced malaria burden in Malawian children (Zamawe *et al.,* 2016). The study highlighted that this lack of association could be attributed to insecticide resistance and other factors, such as inconsistent use and low uptake of preventive malaria control measures (Zamawe *et al.,* 2016). However, no studies have been conducted to evaluate the operational significance of insecticide resistance in the country. The following sections will address some of the major concerns in malaria control. The primary focus will be on vector control while highlighting the issue of insecticide resistance. We will also draw examples from various disease control programs to highlight their successes and challenges in controlling the malaria vectors.

1.2 Research Gap

There is a lack of compelling evidence on the relationship between insecticide resistance in the malaria vector population implicated in transmission, and the effectiveness of LLINs on mosquito mortality, human biting rates, and the prevalence of malaria cases in Chikwawa. Despite debates on how to accurately measure the operational impact of insecticide resistance on malaria control, there is a consensus that insecticide resistance must be strategically managed and that not doing so would prove to be detrimental (Takken, 2002; WHO, 2012; Kabula *et al.,* 2016; Ranson & Lissenden, 2016). Researchers and policymakers further agree that resistance may ultimately impede vector control efforts and reverse the gains already achieved in controlling the disease (Ranson *et al.,* 2011; Hemingway, 2014).

Despite the well-documented occurrence of insecticide resistance in Chikwawa, there have not been any published reports associating insecticide resistance with mosquito infectivity (sporozoite) rates (Moss *et al.,* 2015). A study in neighbouring Tanzania which is north of Malawi reported a significant association between *P. falciparum* infection rates and resistance to pyrethroids by local vectors (Kabula *et al.,* 2016). Collecting such evidence would be crucial to informing the vector control policy, as it would associate resistance with compromised efforts in controlling transmission.

1.3 Fighting with a Limited Arsenal

For a long time, the success of malaria vector control relied on the long-term effective use of a limited number of insecticides belonging to four classes: organophosphates, carbamates, organochlorines, and pyrethroids (Fig. 1.2) (WHO, 2019a; WHO, 2023b). The recent recommendation of two more insecticides (a pyrrole and a neonicotinoid) is expected to increase the array of molecules that can be part of insecticide resistance management strategies, enhancing prospects in malaria control (Fig. 1.2). All these classes, except for DDT, have been prequalified by the WHO (WHO, 2023a). Pyrethroids, until recently, have been the only class of insecticides approved for treating bed nets. However, the WHO has recently prequalified a net containing a pyrethroid and pyrrole insecticide, widening options for insecticide resistance management. The other four classes are also available for indoor residual spraying. Pyrethroids, being of lower cost, are often the most accessible insecticides for malaria vector control in resource-limited settings (Curtis *et al.,* 2003). Due to their widespread use, mosquito populations worldwide have selected against this class, and therefore pyrethroid-resistant populations are geographically widely distributed (Ranson *et al.,* 2011; WHO, 2012).

In sub-Saharan Africa, most national malaria control programs have scaled up efforts in malaria vector control by using insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS). This has resulted in a steady increase in selection pressure on vector populations against the insecticides being used. Resistance against pyrethroids is the most commonly spread form of resistance across sub-Saharan Africa due to the wide use of pyrethroids during IRS and on all ITNs (Ranson *et al.,*

2011). The figure below illustrates the various classes of insecticides used in malaria vector control with some specific examples.

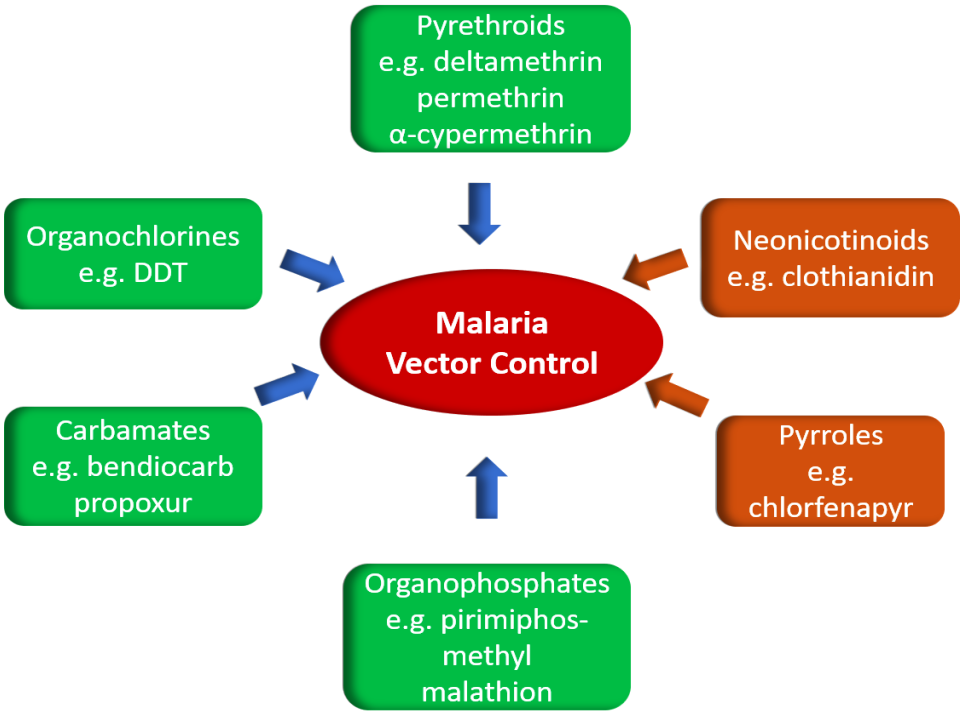


Fig 1.2. Insecticide classes that are used for vector control. Repurposed from the agricultural industry, neonicotinoids and pyrroles (highlighted in orange) are the newest classes introduced to public health to help counter the threat of resistance against the four previous classes, highlighted in green. Clothianidin was first listed by the WHO in 2017 while chlorfenapyr was first listed in 2018. The author compiled this image using information from the WHO's Malaria Vector Control Guidelines (WHO, 2019b).

1.4 Conceptual Framework

1.4.1 Factors That Influence Effectiveness of Interventions

Fig. 1.3 below highlights some of the main factors that affect the effectiveness of ITNs. The following sections then discuss how this is brought about with particular reference to vector behaviours (outdoor resting/biting) and insecticide resistance.

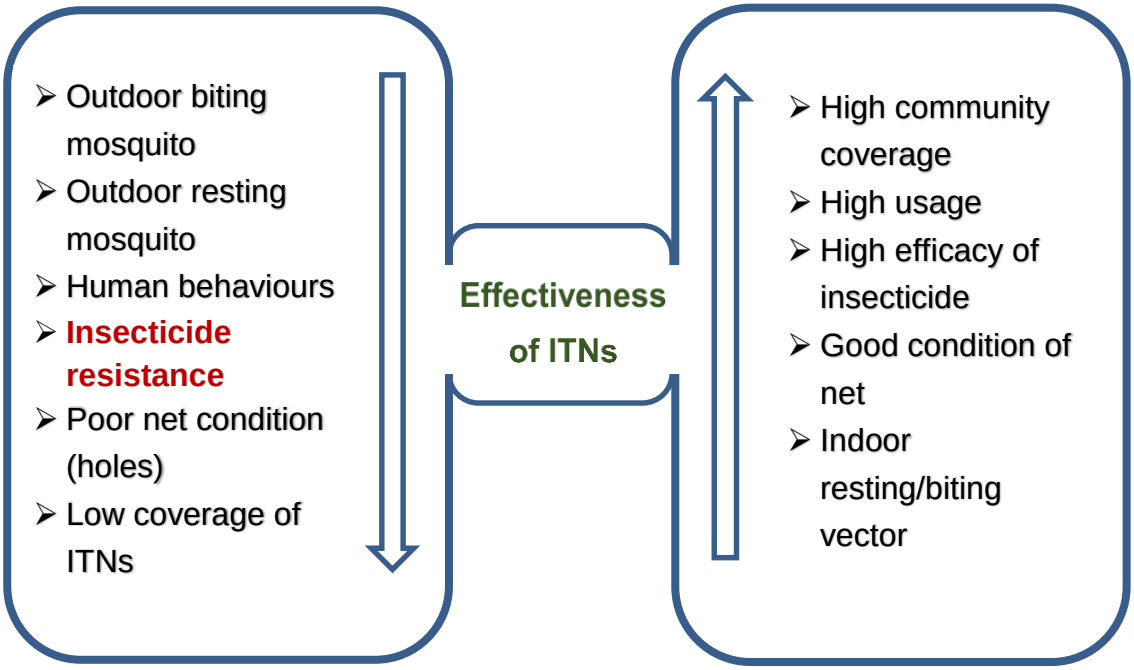


Fig.1.3 Various factors that affect the effectiveness of insecticide-treated nets (ITNs), as indicated by the direction of arrows. The up arrow signifies increasing effectiveness, while the down arrow represents decreasing effectiveness. The author compiled this diagram by combining information from different texts discussed below and from (WHO, 2012).

1.4.2 Changes in Mosquito Behaviours

Vectors that predominantly feed or rest indoors are readily controlled indoor interventions such as ITNs and IRS. However, outdoor biting and outdoor resting behaviours of vector mosquitoes compromise the protective efficacy of such interventions (Pates & Curtis, 2005). The species composition in a particular

population, coupled with vector behaviours such as the propensity to feed or rest indoors versus outdoors, what time to take a blood-meal, and what hosts the vectors prefer, may all influence the impact that interventions have in controlling the risk of biting and disease transmission (Fig. 1.4) (Okumu & Moore, 2011).

Multiple studies have shown that vectors also tend to adapt their behaviours in response to the scale-up of interventions (Reddy *et al.,* 2011; Gatton *et al.,* 2013; Sokhna *et al*., 2013). Vectors may increasingly start biting outdoors to evade indoor control measures as was reported by Moiroux *et al*., (2012). In turn, interventions may not efficiently control the risk of malaria transmission in such cases where the primary vector behaviours are not well exploited (Briet & Chitnis, 2013).

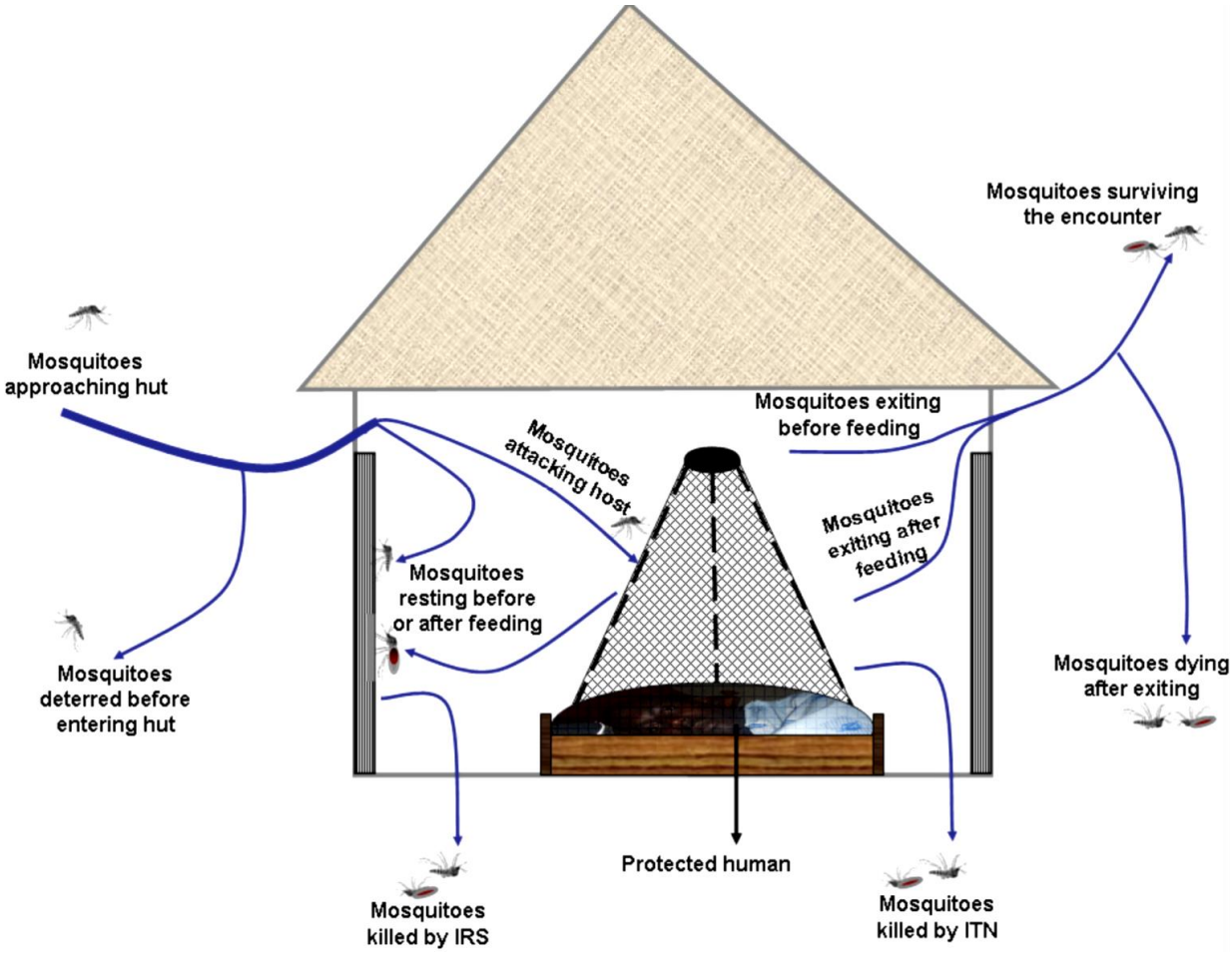


Fig. 1.4. The effect of indoor residual spraying and insecticide-treated nets in preventing human-mosquito contact (Okumu & Moore, 2011).

Mosquito that feed indoors may land on the net, picking up a lethal dose of insecticide which knocks them down, or are repelled by the excito-repellency effect of the insecticide on the net. Those that rest on the walls are knocked down by insecticides that are sprayed on the walls. Mosquitoes that survive the exposure and escape outdoors may have reduced survivability or delayed mortality effects (Kobylinski, 2011). However, those resistant to the insecticides used on the net or sprayed on the walls may still survive and continue to bite people, propelling transmission.

1.4.3 Human Behaviours Influencing Mosquito Biting Patterns

As much as experts agree on how vector behaviours influence the effectiveness of interventions, there is also a growing consensus of the role that human behaviours play in malaria transmission. Human behaviours such as misuse of interventions (e.g bed-nets used as fencing around gardens) or simply staying outdoors long and late may increase exposure to potentially infectious bites and increase malaria transmission (Moiroux *et al.,* 2012; Govella *et al.,* 2013; Killeen, 2014). In most rural communities, cultures such as story-telling are normally done outdoors. Sleeping outdoors due to the lack of sufficient space indoors, or high ambient temperatures also encourage people to spend significant time outdoors, putting them at rick of mosquito bites and malaria transmission (personal observation). Therefore, programs should consider adding community engagement activities when rolling out interventions in the communities.

1.4.4 Insecticide Resistance

Insecticide resistance is defined as the development of an ability in a strain of insect to tolerate doses of toxicants which would prove lethal to most individuals in an average population of the same species (WHO, 1957). Resistance decreases the effectiveness of ITNs by conferring better survival on the mosquito against the insecticides, thereby decreasing mosquito mortality, repellence, house entry deterrence and blood-feeding inhibition (see Fig. 1.4). Currently, insecticide resistance is widespread across sub-Saharan Africa (Fig. 1.5). The World Malaria Report 2023 indicated that resistance has been detected in over 78 of 88 countries that reported data between 2010-2020 (WHO, 2023b). The spread of insecticide resistance across Africa causes widespread concerns in this highly endemic region.

1.4.5 Some Cases of the Impact of Insecticide Resistance on Malaria Control

Insecticide resistance can result in program failure if not appropriately managed. A classic case occurred in KwaZulu-Natal in South Africa when DDT was phased out from malaria vector control in 1995 and was replaced by the pyrethroid deltamethrin in 1996 (Maharaj *et al.,* 2008; Coetzee *et al.,* 2013). This was due to DDT safety issues in agriculture and its residual effects on the environment. Following the introduction of the new insecticide for indoor residual spraying between 1996 and 1999, malaria cases increased sharply. Vector surveillance data revealed the presence of *An. funestus,* a major malaria vector species, in pyrethroid-sprayed houses (Hargreaves *et al.,* 2000). The species was resistant to the pyrethroid insecticide being sprayed, so it re-emerged where it had formerly been wiped out during the DDT-spraying era.

The malaria epidemic in KwaZulu-Natal peaked during the transmission season of 1999/2000 when registered malaria cases increased fourfold (Maharaj *et al.,* 2008; WHO, 2012). This prompted urgent discussions and negotiations by authorities to re-introduce DDT in malaria vector control in KwaZulu-Natal. When DDT was re-introduced, there was a corresponding sharp decline in registered malaria cases by 91% over the following two years and malaria case numbers returned to those seen before the epidemic (Maharaj *et al.,* 2008). This demonstrated the usefulness of continuous vector surveillance and monitoring malaria cases in the human population to avoid control failure. Furthermore, this showed that continued testing of the susceptibility of the local mosquito population to insecticides being used for vector control should be an integral part of every malaria control program.

Another classic example of the impact of insecticide resistance on malaria control occurred on the tropical island of Bioko in Equatorial Guinea (Sharp *et al.,* 2007). In 2004, the Bioko Island Malaria Control Project (BIMCP) launched an extensive intervention program to reduce malaria using case management with effective drugs and vector control (Kleinschmidt *et al.,* 2006; Sharp *et al.,* 2007). Vector control was to be done in three rounds of indoor residual spraying of homes between 2004 and 2005. The first spraying round was done using deltamethrin (a pyrethroid insecticide), after which populations of *An. melas* and *An. funestus* dropped substantially from the pre-spraying period. *Anopheles melas* dropped from an average of 5.3 to 0.8 individuals caught per trap per 100 nights, whereas *An. funestus* numbers decreased from 23.5 to 3.1 individuals per trap per 100 nights. However, *An. gambiae s.s.* numbers increased from 23.9 to 25.5 individuals per trap per 100 nights between the pre-spray period and after the first spraying round (Sharp *et al.,* 2007). This was attributed to the presence of the knock-down resistance (kdr) gene in this species, which conferred pyrethroid resistance in *An. gambiae s.s.* (Sharp *et al.,* 2007). This was mitigated by switching to bendiocarb, a carbamate insecticide with a different mode of action from pyrethroids.

Here, essential lessons can be drawn that sustained vector control can only be achieved when relevant insecticides are used and that these should be reviewed and changed from time to time with the emergence of resistance. Hence, control programs must be flexible enough to allow those switches. Also, it is noted that continuous monitoring and surveillance are critical components of every successful control program (Thomsen *et al.,* 2014). This helps to avoid program failure and ensures the achievement of programmatic goals.

1.5 The Distribution of Insecticide Resistance in Africa

In the sub-Saharan African region, insecticide resistance has been detected in all the major malaria vector species: *An. arabiensis, An. funestus,* and *An. gambiae s.s.* Many studies have been done in West, East, Central and Southern Africa, and resistance has been reported across all these regions in at least one of the insecticides and in some instances to multiple insecticide classes (Fig. 1.5) (Brooke *et al.,* 2001; Ranson *et al.,* 2009; Riveron *et al.,* 2013; IR Mapper 2024). A multi-country study done in multiple sentinel sites in three countries reported very high frequencies of DDT resistance in urban locations in Burkina Faso and Sudan, and in a cotton-growing district in Chad (Ranson *et al.,* 2009). The study also found that in locations where *An. gambiae s.s.* and *An. arabiensis* co-existed, the former tended to exhibit higher levels of resistance to pyrethroids than *An. arabiensis*. *Anopheles gambiae s.s* remained highly susceptible to organophosphates and carbamates with the exception of certain populations in a cotton-growing site in Burkina Faso, which proved to be resistant to all the then four classes of insecticides used in public health (Ranson *et al.,* 2009).

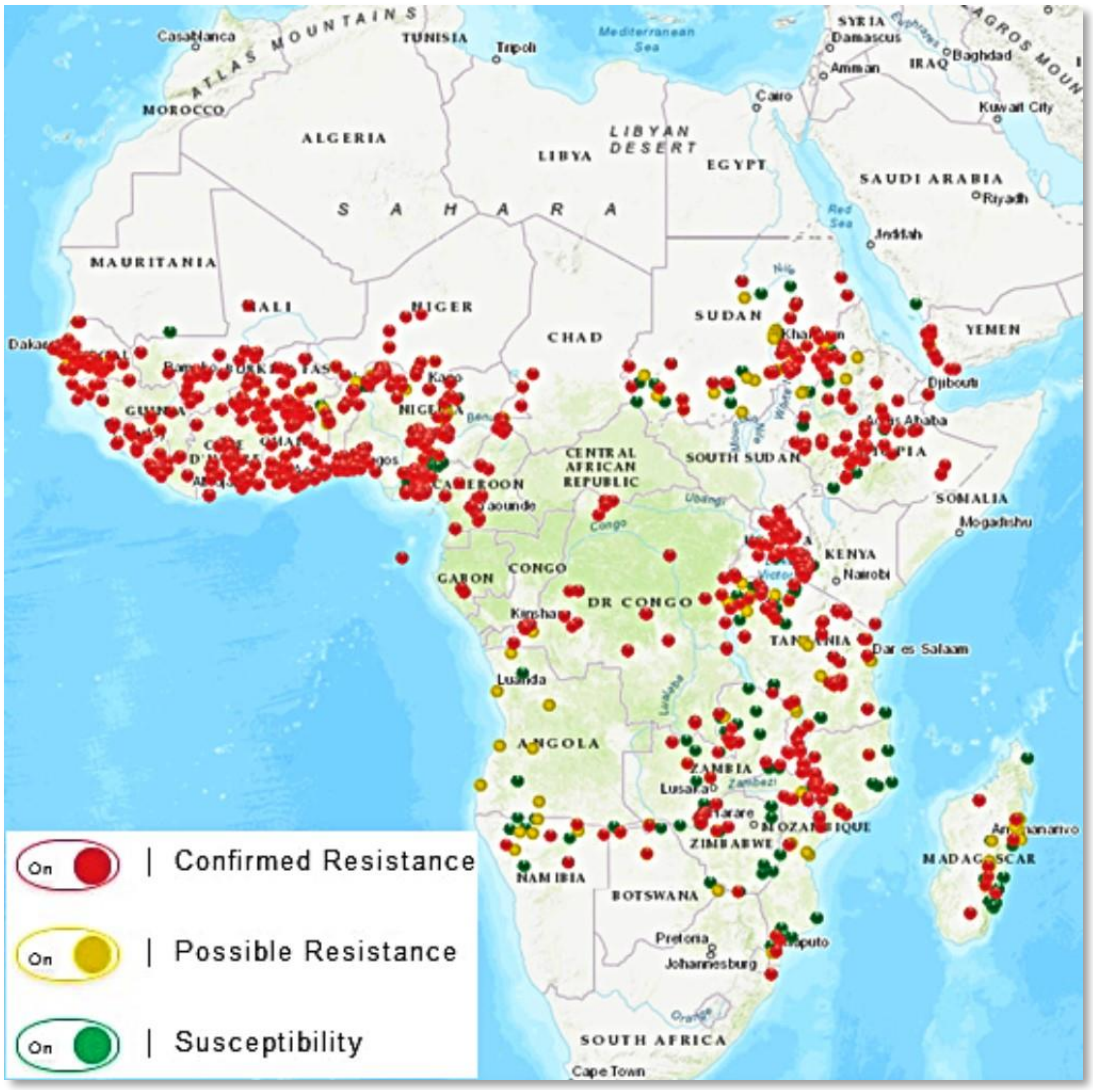


Fig. 1.5 Distribution of insecticide resistance across Africa (2015-2024). The data reported were for all malaria vectors, all insecticides, and all modes of action reported between 2015-2024. This map was downloaded from the IR Mapper V.2.0 online tool on 2 February 2024. (Link: https://anopheles.irmapper.com/)

1.5.1 The Distribution of Insecticide Resistance in Southern and Eastern Africa

Pyrethroid resistance in *An. funestus* is very widespread in southern Africa. Brooke *et al.* (2001) reported elevated levels of biochemical resistance in *An. funestus* from northern KwaZulu-Natal in South Africa and the Beluluane region in southern Mozambique. Their findings indicated that multi-function oxidase enzymes were responsible for the metabolism of pyrethroids by the mosquito species. They also found that metabolic resistance was likely conferring resistance to the carbamate insecticide propoxur, a phenomenon known as cross-resistance (Brooke *et al.,* 2001; Hemingway *et al.,* 2004; Ranson *et al.,* 2011). Casimiro *et al.* (2006) also detected high levels of pyrethroid resistance in *An. funestus* from southern Mozambique, but there was full susceptibility to DDT suggesting that a knock-down resistance (kdr) mutation type had not been selected for. This situation implies that DDT can control the species in the region through DDT-based indoor residual spraying.

In Malawi and neighbouring Mozambique, pyrethroid resistance was shown to be largely driven by the increased expression of two directionally selected cytochrome P450s in *An. funestus,* namely *CYP6P9a* and *CYP6P9b* (Riveron *et al.,* 2013). *Anopheles funestus* from Chikwawa in Malawi was resistant to both 0.75% permethrin (type I pyrethroid) and 0.05% deltamethrin (type II pyrethroid) with mortalities of 47.2% and 42.3%, respectively. However, when the mosquitoes were pre-exposed to the chemical synergist piperonyl butoxide (PBO), there was recovery of near-full susceptibility in that population with a mortality as high as 95% after one hour of exposure of females. This suggests an opportunity to adopt third-generation bed nets that incorporate PBO and a pyrethroid insecticide to improve net efficacy in areas of

high pyrethroid resistance. Recently, a study conducted in Tanzania found that a net coated with the synergist PBO reduced malaria prevalence in children by 44% over one year among users of PBO-coated nets than their non-synergist counterparts (Protopopoff *et al.,* 2018).

1.6 Major Mechanisms of Insecticide Resistance

Detecting resistance is the primary basis of implementing resistance management strategies to overcome it and maintain the effectiveness of insecticide-based mosquito control tools (WHO, 2013). Insecticide resistance can be mediated by four main mechanisms, namely target-site, metabolic, behavioural and cuticular resistance (Hemingway *et al.,* 2004; Ranson *et al.,* 2011). Target-site and metabolic resistance seem to be the main resistance mechanisms in wild mosquito populations and have been extensively studied. In contrast, the role of behavioural and cuticular resistance has not been so extensively studied (Wood *et al.,* 2010).

Behavioural resistance involves the alteration of mosquito behaviours that helps them avoid contact with insecticides or their lethal effects (Ranson *et al.,* 2011). Mosquitoes alter behaviours such as house entry, host-biting preferences and resting preferences. It has been shown that insecticides such as DDT and permethrin induce behavioural changes (Lines *et al.,* 1987; Mbogo *et al.,* 1996; Mathenge *et al.,* 2001). The importance of monitoring mosquito behaviours cannot be overemphasised as the primary vector control tools (ITNs and IRS) tend to exploit mosquito behaviours and are indoor-based interventions. There are growing concerns of higher levels of outdoor biting in some malaria vectors such as *An. arabiensis*, a behaviour that allows them to

evade contact with insecticides indoors and maintain malaria transmission (Govella *et al.,* 2013; Killeen, 2014).

Cuticular resistance involves modifications (such as hardening) in the insect's cuticle or digestive tract to prevent penetration or slow down the uptake of insecticides (Stone and Brown, 1969; Apperson and Georghiou, 1975). Hardening of the cuticle has also been associated with pyrethroid resistance in *An. funestus* (Wood *et al.,* 2010). However, it is unlikely that cuticle hardening alone may act as the primary mechanism driving resistance in any given population. It seems likely that this resistance mechanism works with other mechanisms; metabolic resistance has been demonstrated to be the primary mechanism driving resistance in *An. funestus* (Brooke *et al.,* 2001; Casimiro *et al.,* 2006; Wondji *et al.,* 2009; Riveron *et al.,* 2015).

Target-site resistance occurs when there is reduced or no affinity between the insecticide and its target site. Metabolic resistance is mediated by detoxification enzymes, which metabolise the insecticide molecule before it reaches its target (Djouaka *et al.,* 2008). Members of the cytochrome P450-dependent monooxygenases have been implicated in conferring resistance to pyrethroid insecticides in various populations of the major African malaria vector, *An. funestus*. Wondji *et al.* (2009) identified two duplicated P450 genes, namely CYP6P9 and CYP6P4, expressed at high levels in a pyrethroid-resistant strain of *An. funestus* than in a susceptible one. Djouaka *et al.* (2008) also reported elevated expression of the two cytochrome P450s, CYP6P3 and CYP6M2, in multiple populations of *An. gambiae s.s.* from southern Benin and Nigeria. The differential expression of enzymes between resistant and susceptible populations indicates biochemical metabolism as an

underlying resistance mechanism. Metabolic resistance is mainly mediated by three enzyme families: cytochrome P450s, Glutathione-S-Transferases (GSTs) and esterases (Hemingway *et al.,* 2004).

Cross-resistance is when resistance to one class of chemicals confers resistance to another. Usually, this happens when two insecticide/chemical classes share the same target site in the mosquito such that if the mosquito is exposed to one chemical class, it tends to develop resistance against the other chemical class too (Ranson *et al.,* 2011; WHO, 2012). An example of cross-resistance happens between pyrethroids and organochlorines, mainly DDT. Most vectors that have developed resistance against pyrethroids due to increased selection pressure also tend to be resistant against DDT as pyrethroids and DDT both share their target on the sodium ion channels of the mosquito (Hemingway and Ranson, 2000).

1.7 The Molecular Basis of Insecticide Resistance

Pyrethroid resistance is mediated by knock-down resistance (kdr, target-site) and metabolically by mono-oxygenases and somewhat by esterases (Nauen, 2007). Pyrethroid resistance in *An. funestus* is commonly associated with mono-oxygenases and in *An. gambiae s.l* is commonly kdr-based, although it can also be metabolic (Bergé *et al.,* 1998; Djouaka *et al.,* 2008). Pyrethroids and DDT target the voltage-gated sodium ion channels on the neurons to bind to them and prevent the gates from closing when they fire an electric signal (Hemingway *et al.,* 2004; Ranson *et al.,* 2011). This results in a sudden influx of sodium ions (Na+) moving into the cell and building up a large positive charge on the neuron, which results in the repetitive firing of electric signals, raising the fitness cost of the insect and knockdown (Ranson *et al.,* 2011).

A mutation in the voltage-gated sodium ion channels (known as kdr or *vgsc* mutation) prevents the binding of pyrethroid and DDT insecticides to their target site. This prevents the insect from being knocked down upon exposure, hence the term “knockdown resistance” (Diabaté *et al.,* 2004; Ranson *et al.,* 2011). Two forms of the kdr mutation exist, one where there is a leucine-phenylalanine substitution, L1014F (kdr-west, (Martinez-Torres *et al.,* 1998; Kulkarni *et al.,* 2006)) and another with a leucine-serine substitution, L1014S (kdr-east, (Ranson *et al.,* 2000)). Each of these arises from substituting a leucine amino acid residue on the 1014 locus of the genome (Hemingway *et al.,* 2004; Reimer *et al.,* 2008; Ranson *et al.,* 2011). Some studies have reported that kdr-west conferred higher resistance to pyrethroids than kdr-east (Reimer *et al.,* 2008). Therefore, it is critical to conduct advanced molecular testing and monitoring of insecticide resistance alleles so that resistant alleles can be detected early enough before the resistance phenotype appears. This would allow timely, informed decisions on what resistance management strategies should be deployed and what insecticides to use (WHO, 2012).

Initially, it was thought that kdr alleles were each restricted to the West or East Africa (hence the naming kdr-west and kdr-east), but evidence shows that they are no longer geographically restricted (Pinto *et al.,* 2006). In multiple studies, kdr-east and kdr-west have been detected concurrently in Gabon and Cameroon, whereas kdr-west has also been detected in Tanzania and Uganda (Kulkarni *et al.,* 2006; Verhaeghen *et al.,* 2006; Chen *et al.,* 2008; Reimer *et al.,* 2008). In their study, Reimer *et al.* (2008) found that kdr-east and kdr-west were more closely associated with DDT resistance than they were with type II pyrethroids. This shows the marked heterogeneity of resistance

across space as kdr has been more closely associated with pyrethroid resistance in *An. gambiae s.l* populations from southern Africa.

Organophosphates and carbamates target acetylcholinesterase on the nerve synapse to prevent polarisation of the next neuron, and hence an impulse is not transmitted (Apperson & Georghiou, 1975; Bisset *et al.,* 1990). A mutation occurring at the target site of organophosphates and carbamates results in alterations of the binding site, and hence, acetylcholinesterase (AChE) becomes insensitive to insecticide binding (Apperson and Georghiou, 1975; Nauen, 2007). This confers resistance in the vector against the insecticide.

1.8 Strategies for Managing Insecticide Resistance

The World Health Organization developed the Global Plan for Insecticide Resistance Management (GPIRM) in malaria vectors to provide technical recommendations to malaria-endemic countries and trigger coordinated action from stakeholders to form a framework of strategies and practices for managing insecticide resistance (WHO, 2012). The strategies are meant to sustain the effectiveness of vector control interventions despite the threat of insecticide resistance. The GPIRM has outlined four main available strategies for managing resistance, namely: rotation of insecticides, combinations of interventions, mosaic spraying and using mixtures of two or more chemical compounds (WHO, 2012)

In rotation, a different class of insecticide is used every year. Each insecticide used in a year must have a different mode of action from the one used during the previous year (WHO, 2012). In the combination strategy, one insecticide is sprayed on the walls while another with a different mode of action is used on another intervention like a bed

net. The mosaic strategy is when one chemical compound is used within a geographical area while a neighbouring area receives a different chemical compound. Lastly, mixtures mean that two or more compounds are mixed in the same intervention such that the mosquito is exposed to all the compounds simultaneously (Penilla *et al.,* 1998; WHO, 2012). It is believed that these strategies help to reduce selection pressure on the mosquito population, and hence, resistance is slowed down, or its emergence is avoided (Curtis *et al.,* 1998). However, these would work as interim solutions while new products are developed for longer-term use.

1.9 Conclusion

Most countries have made tremendous gains in controlling malaria since 2000. Insecticide-treated bed nets have played an enormous role in achieving these gains. With the evolution of insecticide resistance, malaria control programs must act immediately to implement strategies that will help to sustain the effectiveness of insecticide-based tools so that the gains in malaria control are maintained, and countries can accelerate towards elimination. To avoid failure, continuous vector surveillance and insecticide resistance monitoring must be integral to every control program (Temu *et al.,* 2012). However, the success of control must ultimately be measured in terms of epidemiological outcomes of disease (WHO, 2012).

Chapter 2. Study Overview, Objectives and Procedures

2.1 Study Population and Area

This study was conducted in 2018 in Chikwawa district, rural southern Malawi. Chikwawa is one of Malawi's highest malaria burden districts, with persistent transmission throughout the year (Mzilahowa *et al.,* 2012; Bennett *et al.,* 2013; Okiro *et al.,* 2014). The district has a tropical savannah climate, with temperatures ranging from 20°C in the coldest month of July to over 30°C in the warmest month of November. There are three main seasons: a hot-rainy season, which runs from December to April, followed by a cool and dry season from May to July, and a hot-dry season between August and November. The estimated mean annual rainfall for 2020 was 792.4 mm, with a mean annual temperature of 25.8°C. The study area was along the Lower Shire Valley (the largest river valley in Malawi). People in the study areas practice a lot of subsistence farming and small-scale activities. Farming of rice, sugarcane and maize is also widespread. In addition, extensive livestock keeping, such as cattle, goats, and sheep, is also common.

The study was conducted from the end of the rainy season into the dry season of 2018 in two sentinel sites/villages, namely Chakanira (15°58'54.1"S, 34°48'09.1"E) and Sisewo (16°04'39.9"S, 34°49'39.9"E). In terms of malaria transmission, Kabaghe *et al.* (2019) reported that 57.1% of children who were followed up for one year experienced at least one clinical episode of malaria. They also reported sporozoite rates as high as 19.2% for *An. funestus* and 5.4% for *An. arabiensis*. These two species are the district's major malaria vectors, with the former being the most prominent primary vector (Kazembe, 2006; Mzilahowa *et al.,* 2012; Okiro *et al.,* 2014). The study area is shown in Figures 2.1a and 2.1b below.

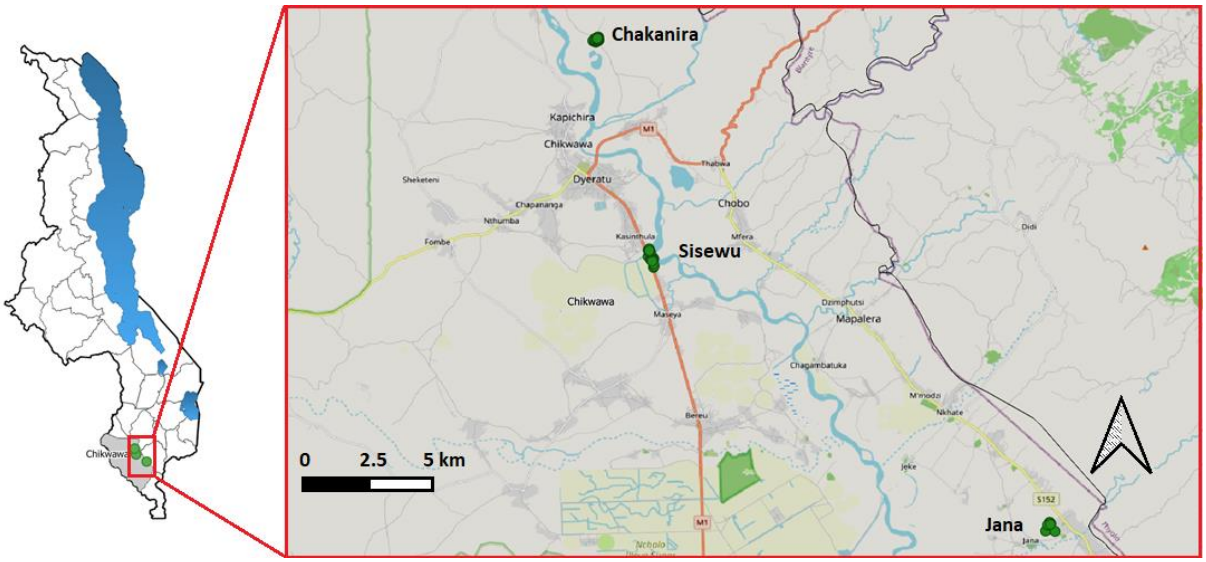


Figure 2.1a: Map of Malawi, showing the location of Chikwawa district and study sites.

Figure 2.1b: Location of study sites, showing their aerial views. These satellite images also show the proximity of the villages to the Shire River.

2.2 Study Objectives

i. To test the intensity of resistance of wild *Anopheles funestus* to deltamethrin, permethrin and alpha-cypermethrin.
ii. To determine the effect of Yorkool and Royal Sentry nets on mortality of wild-caught *Anopheles funestus* in comparison with lab susceptible mosquitoes.
iii. To determine the effect of repetitive washing of Yorkool and Royal Sentry nets on net bio-efficacy.
iv. To assess the relationship between insecticide resistance and sporozoite rates in field-caught mosquitoes.

2.3 Study design and data collection

The research adopted an experimental and quantitative approach, conducting various laboratory tests. These assessments included evaluating the effectiveness of insecticides applied to bed nets and indoor residual spraying and appraising insecticide-treated nets' bio-efficacy and wash resistance (ITNs). Live female Anopheles mosquitoes, both field-collected and from insectary-reared colonies with varying levels of susceptibility to insecticides, were used in the tests.

Field data collection involved using backpack aspirators to sample indoor-resting live female mosquitoes from households within the study area. Laboratory procedures comprised the WHO insecticide susceptibility tests, CDC bottle bioassays for assessing resistance intensity, molecular species identification using polymerase chain reaction (PCR), sporozoite detection through circumsporozoite enzyme-linked immunosorbent assay (csp-ELISA), and wash resistance testing of selected ITN brands using net cone bioassays. These laboratory methods followed WHO guidelines for wash-resistance testing (WHO, 2013; WHO, 2016).

Live female adult Anopheles were collected from randomly selected households in two villages. Entomological collections were carried out over four months, from April to July 2018, sampling thirty households in each village.

2.4 Study procedures

Procedures in this study involved field mosquito collection and laboratory-based work. For the initial part, mosquitoes were sampled from community households in the study areas and brought to the laboratory for processing. Laboratory work involved experiments with live specimens (bioassays) to test their response to insecticide exposure and diagnostic assays. We also tested the bio-efficacy of two second-generation LLINs, Yorkool and Royal Sentry, against wild *An. funestus* adult females and a susceptible lab colony of *An. gambiae* Kisumu. We further tested the wash resistance of Yorkool and Royal Sentry LLINs using a susceptible *An. arabiensis* (KGB) colony, a moderately pyrethroid-resistant colony of *An. funestus* (FUMOZ Base) and a pyrethroid/DDT-resistant colony of *An. arabiensis* (SENN DDT). The PCR technique was used for species identification confirmation, whereas the ELISA technique was used for detecting *Plasmodium spp*. in field-collected specimens.

2.5 Data analysis

Insecticide susceptibility of vector populations was reported in terms of recorded mortality rates after exposure to given insecticide concentrations per time. Mortality was calculated as the sum of dead mosquitoes across test replicates divided by the total number of samples exposed to the insecticide, expressed as a percentage. Comparisons between mean mortalities of mosquitoes exposed to various insecticides, doses and ITNs were done using analysis of variance (ANOVA). Tukey's post hoc test was also used for pairwise comparisons of mortalities at a confidence level of 95%. Resistance intensity as measured by bioassay mortality at insecticide doses of 1X the recommended diagnostic concentration (DC), 2.5X DC, 5X DC and 10X DC. The results were then summarised as tables and charts.

The Chi-square statistic was used to test for an association between insecticide resistance (as measured by bioassay mortality) and sporozoite rates. The null hypothesis assumed that the two variables were not associated, and the significance level (alpha) was set at 0.05. The efficacy of insecticides and bed nets was assessed by examining mosquito mortality rates as a response variable to exposure to given insecticides or ITNs.

2.6 Ethical approval

Ethical clearance for the study was obtained from the Human Research Ethics Committee at the University of the Witwatersrand in South Africa (clearance number M170662) and the then College of Medicine Research Ethics Committee (CoMREC) in Malawi (clearance number P.12/17/2324). Study participants were recruited as volunteers from the study area.

Before the study, all participants were tested for malaria using an antigen Pf-RDT. A recommended dosage of artemether-lumefantrine was administered if positive, while those with negative results took daily prophylactic tablets of 100 mg of Doxycycline. Prophylaxis was taken from recruitment until three weeks after completion of participation. A community health worker administered the test/treatments and followed participants for infection throughout the study up to three weeks after the study. For the household mosquito collection exercise, verbal consent was obtained from all study participants, and they could opt out of the study at any time if they wished to do so.

Chapter 3: Intensity of Insecticide Resistance in the Major Malaria Vector *Anopheles funestus* from Chikwawa, Rural Southern Malawi

3.1 Introduction

In Malawi, resistance to at least one insecticide has been reported in many areas of the country, including multiple resistance in Chikwawa district. Chikwawa is an area along the Lower Shire Valley with persistent and one of the highest transmission risk areas in Malawi with *P. falciparum* prevalence rates as high as 15% (n=9646) among participants in a recent study (The PMI VectorLink, 2022). *Anopheles funestus* s.s, the primary malaria vector in this district, has shown reduced susceptibility to pyrethroids and carbamates, and this appears to be enzyme-mediated resistance (Mzilahowa *et al.,* 2016; Abeku *et al.,* 2019; The PMI VectorLink Project, 2022). *Anopheles arabiensis*, a secondary vector in the district, has also shown reduced levels of susceptibility to pyrethroids (Abeku *et al.,* 2019; Abeku *et al.,* 2020; The PMI VectorLink Project, 2022). However, the extent to which the rise of insecticide resistance might hamper malaria control efforts in the district remains under-explored.

The level of pyrethroid resistance in Malawian *An. funestus* populations has slowly risen since it was first reported in 2008 (Hunt *et al.,* 2010). In 2015, reports from Chikwawa district indicated the rise of multiple resistance mechanisms in the local *An. funestus* population, namely over-expression of pyrethroid resistance genes (C*YP6Pa/b* and *CYP7M7*) and the dieldrin resistance mutation (A296S-RDL) (Riveron *et al.,* 2014; Riveron *et al.,* 2015; Mzilahowa *et al.,* 2016). Such reports suggest that insecticide-based vector control tools (such as ITNs and IRS) may slowly lose their efficacy, especially in the wake of multiple resistance as recently

reported in neighbouring Mozambique where the same resistance mechanisms in the local *An. funestus* population have induced a loss of efficacy of piperonyl-butoxide (PBO)-based ITNs (Riveron *et al.,* 2019), indicating other uncharacterised mechanisms at play. In Cote d'Ivoire, PBO pre-exposure of resistant *An. gambiae s.l.* did not yield a complete restoration of susceptibility to pyrethroids, and neither was there full susceptibility to chlorfenapyr, a novel insecticide for public health used in combination with pyrethroids in the newer generation ITNs (Kouassi *et al.,* 2020).

Malawi lacks compelling evidence as to what relationship exists between observed resistance and its effect on the effectiveness of ITNs on mosquito mortality in the field. It is unknown if there is compromised transmission control for people using ITNs and if there is any relationship between mosquito resistance and epidemiological profiles of clinical malaria in the district. These factors are essential in establishing the real impact of insecticide resistance on operational control (Wondji *et al.,* 2012; Kleinschmidt *et al.,* 2015; Kleinschmidt *et al.,* 2018). The current objective was to determine the intensity of resistance to deltamethrin, permethrin, alpha-cypermethrin (pyrethroids), and alternative non-pyrethroid insecticides. This was done so that an operationally important level of resistance could be determined, as it may give an early warning signal of potential loss of efficacy of the currently used insecticides. This may guide the local vector control intervention policy to minimise or entirely prevent control failure.

3.2 Methods

3.2.2 Mosquito Collections

Live adult mosquitoes were collected inside houses using a battery-powered Prokopak Aspirator. At least 30 households were randomly sampled from each of the two study villages (Chakanira and Sisewu). For a period of three months (May-July 2018), mosquitoes were collected from these households on multiple visits until the number of mosquitoes required to complete all experiments was fulfilled. Upon collection, mosquitoes were placed in paper cups in cooler boxes and provided with a sugar solution before being transported to the laboratory in Blantyre. The local vectors are *An. funestus* (most abundant) and *An. arabiensis* (Mzilahowa *et al.,* 2012).

3.2.3 CDC Bottle Assays

Tests were performed on three replicates of 20-25 wild-caught adult females of unknown age, which were exposed for one hour in 250 mL glass bottles coated with insecticide concentrations at 1X diagnostic concentration (DC), 2.5X DC, 5X DC and 10X DC of pyrethroids (permethrin, deltamethrin and alpha-cypermethrin). Negative controls were bottles coated with technical-grade acetone, a solvent used to dilute the test insecticides. A susceptible mosquito strain (*An. gambiae* s.s Kisumu) was used to evaluate the efficacy of the coated bottles as a positive control replicate. Knockdown was recorded at 10-minute intervals from exposure until one hour after which mosquitoes were transferred to holding cages for 24-hour post-exposure scoring. A small piece of cotton wool moistened with 10% sugar solution was provided to insecticide exposure survivors (WHO, 2018).

The insecticides were procured from the Centers for Disease Prevention and Control (CDC) in Atlanta, USA. Stock solutions came at 10X DC and were diluted to desired concentrations using technical-grade acetone, as shown in Table 3.1 below.

Table 3.1 Dilution of technical-grade stock insecticide solutions to reach recommended diagnostic concentrations

Stock Concentration	Target Concentration	Amount of Stock (µL)	Acetone 95% (µL)	Final concentration[1]
10X	1X	100	900	DC
10X	2.5X	250	750	2.5X DC
10X	5X	500	500	5X DC
10X	10X	1000	0	10X DC

[1]The actual concentration (in µg/ µL) of each insecticide varied according to the recommended diagnostic concentration for the given insecticide. Final concentrations were as follows: deltamethrin (DC=12.5 µg/mL, 2.5X DC= 31.3 µg/mL, 5X DC= 62.5 µg/mL, 10X DC= 125 µg/mL), permethrin (DC=21.5 µg/mL, 2.5X DC= 53.8 µg/mL, 5X DC= 107.5 µg/mL, 10X DC= 215 µg/mL), and alpha-cypermethrin (DC=12.5 µg/mL, 2.5X DC= 31.3 µg/m, 5X DC= 62.5 µg/mL, 10X DC= 125 µg/mL) (WHO, 2018).

Glass bottles were coated per the CDC bottle assay standard operating procedure (SOP) for resistance testing in anopheline mosquitoes (Brogdon and Chan, 2010). Each experiment consisted of four test bottles (DC, 2.5X DC, 5X DC, and 10X DC) and a fifth one as a negative control, with 20-25 wild-caught (F0) female *An. funestus s.l.* aspirated into each glass bottle. In addition, we had three replicates for each test dose plus a positive control. Mosquitoes that were alive at 24 hours were removed by aspiration, killed by freezing, labelled, and stored separately from the dead ones.

Where control mortality was between 5-10%, the test mortality scores were adjusted using Abbott's formula (WHO, 2018). If mortality in the control was less than 5%, results were not adjusted, and if it exceeded 10%, the whole experiment was discarded and repeated (WHO, 2018). All specimens were preserved on silica gel for molecular species identification and sporozoite detection in Chapter 6.

3.2.4 WHO Susceptibility tests

Wild-caught live female *An. funestus* mosquitoes were tested for phenotypic resistance to DDT (4%), bendiocarb (0.1%) and pirimiphos-methyl (0.25%) using standard WHO insecticide susceptibility assays (WHO, 2018). The wild-caught mosquitoes were exposed to treated papers for one hour, transferred to holding tubes, and provided with 10% sugar solution after exposure. The WHO test papers were validated by a susceptible *An. gambiae* Kisumu colony which was used as positive control during the experiments. Knockdown was recorded every 10 minutes from exposure to 60 minutes after exposure, and mortality was recorded at 24 hours.

3.2.5 Species Identification by Polymerase Chain Reaction

Following bioassay exposures, a proportion of 46% (n=869) of the morphologically – identified *An. funestus* specimens was subjected to molecular species identification using polymerase chain reaction (PCR). This sample proportion was based on the sample that would be required for further sporozoite analysis for another objective covered in Chapter 6 of this document. Samples were analysed using standard operating procedures for identifying members of the *An. funestus* group described by Koekemoer *et al.* (2002) *and* Cohuet *et al.* (2003). The PCR conditions were as follows: 1 cycle at 94^{o}C for 2 minutes, 30 cycles at 94^{o}C for 30 seconds, 45^{o}C for 30 seconds, 72^{o}C for 40 seconds and finally one cycle at 72^{o}C for 5 minutes. Detailed

steps for sample preparation, DNA extraction, PCR reactions and primers used, gel electrophoresis, visualisation and scoring of bands have been covered under section “6.2.2 Molecular Species Identification” of Chapter 6 of this document.

3.2.6 Data Analysis

3.2.6.1 CDC Bottle Assays

For the CDC bottle assays, knockdown and mortality data were analysed to assess insecticide efficacy across different concentrations of permethrin, deltamethrin, and alpha-cypermethrin on *Anopheles funestus* mosquitoes. Knockdown rates, recorded every 10 minutes during the one-hour exposure period, were expressed as cumulative proportions for each insecticide concentration and visualized over time to compare knockdown dynamics at diagnostic concentration (DC) levels: 1X DC, 2.5X DC, 5X DC, and 10X DC.

Post-exposure mortality was calculated as a percentage of mosquitoes that died within 24 hours of exposure for each insecticide concentration. Adjustments for mortality were made using Abbott's formula when control mortality ranged between 5% and 10% to account for any natural death not attributed to insecticide exposure. When control mortality exceeded 10%, the assay was considered invalid, and the experiment was repeated (WHO, 2018). Results were summarised to show the effectiveness of each insecticide at varying concentrations and analysed to establish the concentration required to achieve the highest mortality.

3.2.6.2 WHO Susceptibility Tests

The WHO susceptibility tests evaluated *An. funestus* resistance to DDT (4%), bendiocarb (0.1%), and pirimiphos-methyl (0.25%). Knockdown and mortality data from these tests were analysed following the same methodology as the CDC assays, where knockdown was recorded every 10 minutes up to 60 minutes of exposure and mortality was recorded after 24 hours. Knockdown and mortality rates were calculated for each insecticide and compared with those for a susceptible *An.*

gambiae Kisumu control colony to validate the effectiveness of the test papers and ensure quality control in the assay procedures.

Mortality data were analysed to classify *An. funestus* populations as susceptible, possibly resistant, or resistant based on WHO criteria (mortality ≥98% indicating susceptibility; 90-97% indicating possible resistance; <90% confirming resistance) (WHO, 2018). Mean mortality rates with standard error of the means (SEMs) were plotted against insecticide concentrations, to estimate resistance intensity.

3.3 Results

A total of 1,332 live wild *An. funestus s.l.* females were collected, of which 1,153 came from Chakanira village while 179 were from Sisewu village. These were used for insecticide susceptibility tests (CDC bottle and WHO tube assays). A subsample of 400 (75 from Sisewu and 325 from Chakanira) was then selected for molecular identification. A small number of *An. gambiae s.l* was also collected (1 specimen from Chakanira and 12 from Sisewu) but was not processed further.

3.3.1 CDC Bottle Assays

Table 3.2 summarises the results of pyrethroid intensity tests for the two villages, giving the numbers of mosquitoes tested per insecticide concentration, observed mortality rates, and adjusted mortality rates using Abbott's formula (WHO, 2018).

Table 3.2. CDC bottle bioassays of *An. funestus* s.l. for Chakanira and Sisewu villages.

Village	Insecticide	Dose	No. exposed[#]	Unadjusted % Mortality	Adjusted % Mortality*
Chakanira	Control	0X	133	9.0	0.0
	Deltamethrin	1X	38	84.2	82.6
		2.5X	90	82.2	80.5
		5X	75	94.7	94.1
		10X	71	100.0	100.0
	Permethrin	1X	73	93.2	92.5
		2.5X	62	93.5	92.9
		5X	68	97.1	96.8
		10X	77	100.0	100.0
	Alpha-cypermethrin	1X	52	84.6	83.1
		2.5X	53	86.8	85.5
		5X	74	95.9	95.5
		10X	65	95.4	94.9
	Control	0X	25	8.0	0.0
	Deltamethrin	1X	21	90.5	89.6
		2.5X	25	92.0	91.3

Sisewu[§]	5X	38	94.7	94.3
	10X	17	100.0	100.0

*****Adjusted mortality using Abbott's formula as described by (WHO, 2018)

[#]Low sample numbers could have affected these tests

[§]Due to limited mosquito numbers collected from Sisewu village, susceptibility tests were only carried out on one insecticide, deltamethrin.

Figures 3.1 – 3.3 show the percentage knockdown of *An. funestus s.l.* from Chakanira village to the four concentrations of the three pyrethroids. Mortalities were adjusted using Abbot's correction formula (WHO, 2016).

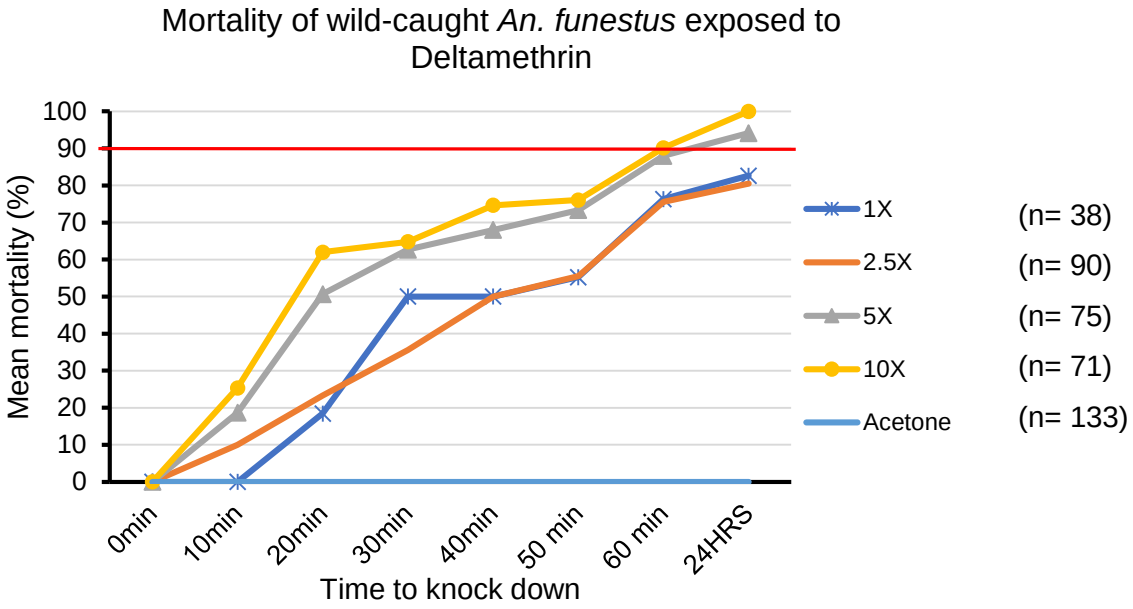


Figure 3.1 Knockdown time and 24-hour mortality rates of wild-caught *An. funestus* s.l., after exposure to various concentrations of deltamethrin. The horizontal red line represents the 90% mortality threshold for resistance confirmation.

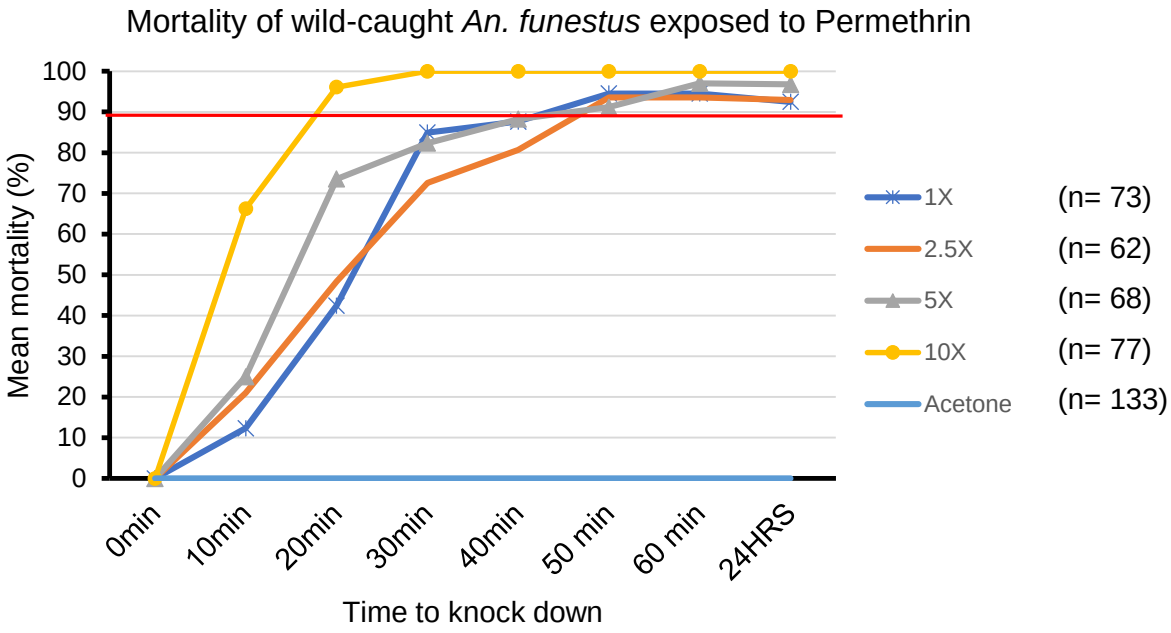


Figure 3.2 Knockdown time and 24-hour mortality rates of wild-caught *An. funestus s.l.*, after exposure to various concentrations of permethrin. The horizontal red line represents the 90% mortality threshold for resistance confirmation.

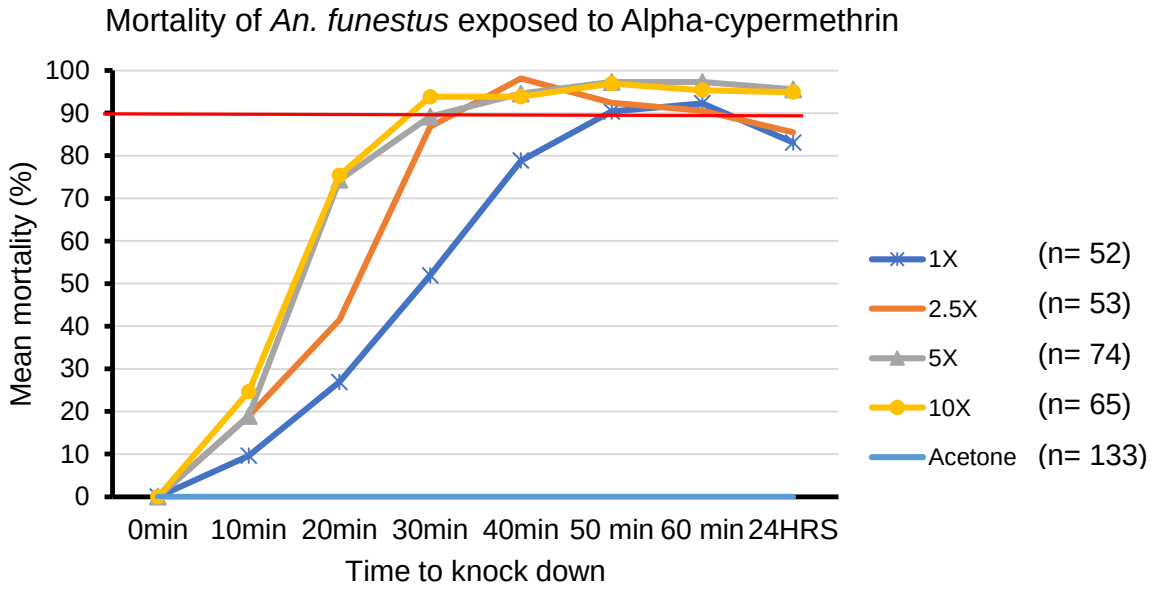


Figure 3.3 Knockdown time and 24-hour mortality rates of wild-caught *An. funestus s.l.*, after exposure to various concentrations of alpha-cypermethrin. The horizontal red line represents the 90% mortality threshold for resistance confirmation (WHO, 2018).

3.3.2 Resistance Intensity

The strength of resistance, also called intensity, was characterised for the three pyrethroids by comparing the mortality at 24 hours for each insecticide at various multiples of the diagnostic dose (Table 3.2). Resistance intensity is herein classified as low, moderate or high using a threshold of 90% for determining a resistant population at diagnostic dose (1X DC) (Bagi *et al.,* 2015; Venter *et al.,* 2017). Observed mortalities at the diagnostic dose (1X DC) were 82.6% (n=38) for deltamethrin, 92.5% (n=73) for permethrin and 83.1% (n=52) for alpha-cypermethrin indicating confirmed resistance to both deltamethrin and alpha-cypermethrin and suspected resistance to permethrin (Table 3.2, Fig. 3.4). For deltamethrin and alpha-cypermethrin, mortalities were still under the 90% threshold at 2.5X DC while they were in the range 90-98% at 5X DC indicating the presence of moderate resistance intensity in the Chakanira population (Venter *et al.,* 2017).

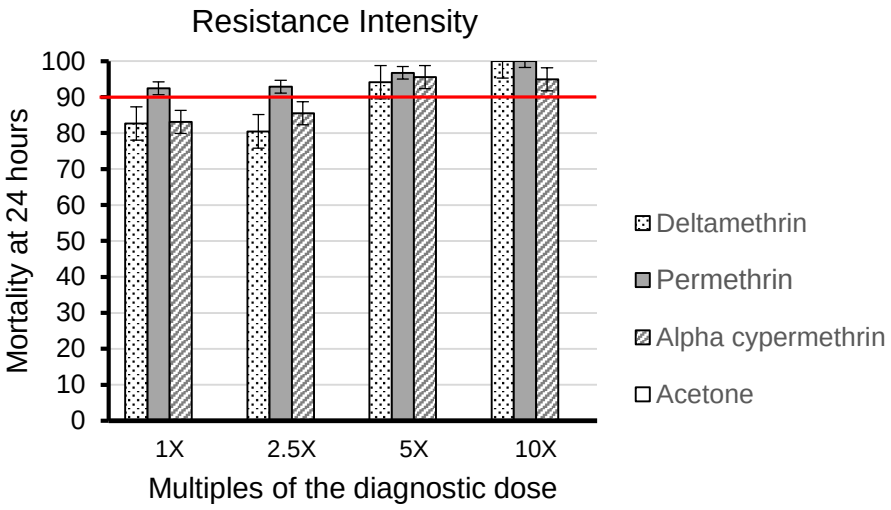


Figure 3.4 The response of wild *An. funestus s.l* to different doses of the three pyrethroids is shown. The horizontal redline at 90% represents the WHO threshold for a resistant population. Error bars represent the standard error of mean mortality rates at 24 hours.

An analysis of variance (ANOVA) was done to check for differences or similarities between mean mortalities across the insecticides at the various doses (1X DC, 2.5X DC, etc). Tukey's post hoc test was used for pairwise comparisons of group means at a confidence level of 95% (Fig 3.5).

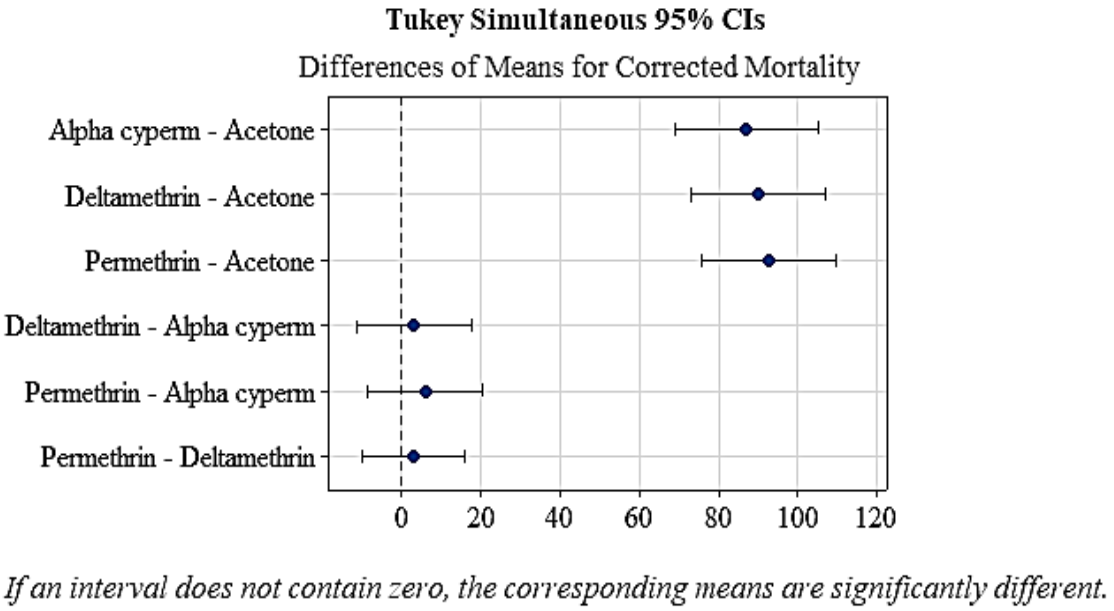


Figure 3.5 Pairwise comparisons of mean mortalities for deltamethrin, permethrin, alpha-cypermethrin and acetone negative control.

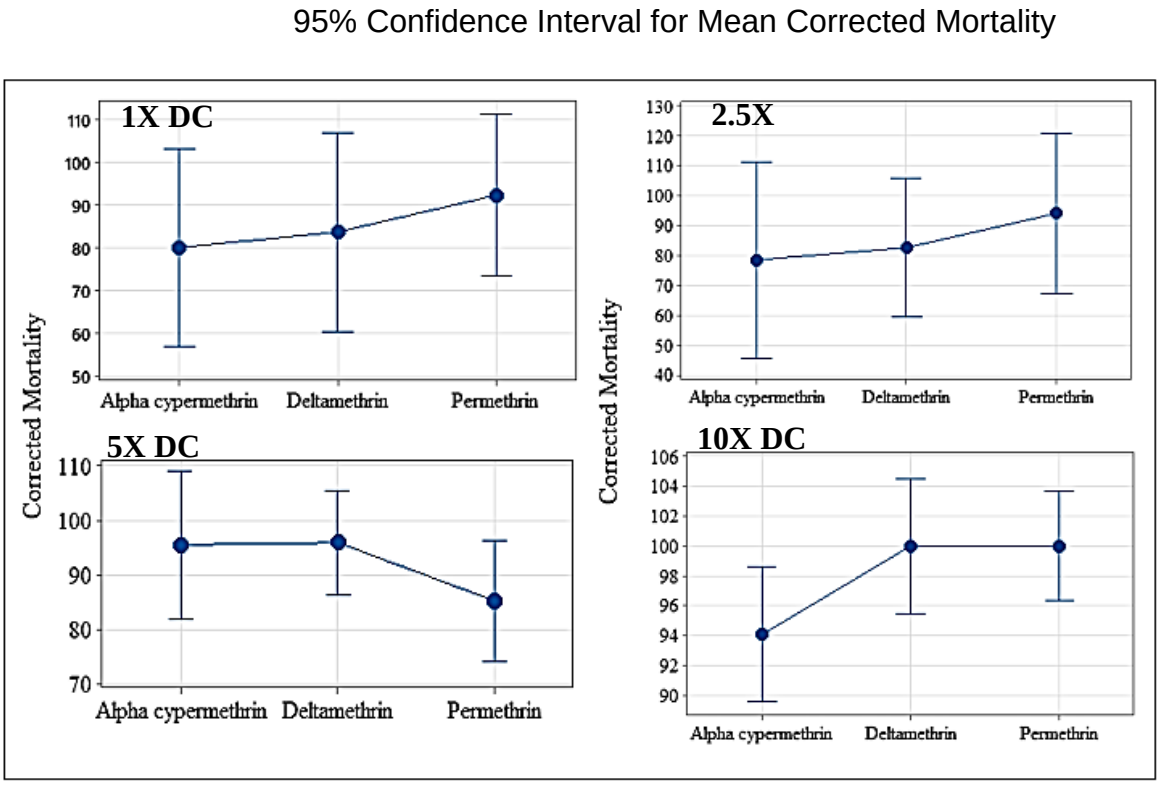


Figure 3.6 Interval plots for adjusted (corrected) mortality versus dose for the three pyrethroid insecticides.

For the given doses, ANOVA test results were as follows: 1X DC, df = 2, F= 0.71, p= 0.543; 2.5X DC, df=2, F= 0.49, p= 0.636; 5X DC, df= 2, F= 1.83, p= 0.240; 10X DC, df=2, F= 4.77, p= 0.087. The ANOVA test assumed equal variances for the analysis, with a null hypothesis of “all means were equal” against the alternative “not all means were equal” with a significance level set at 5%. The resulting ANOVA test probabilities exceeded the set significance level (alpha = 0.05). This indicated that the test could not find an instance where the group mean for each insecticide at a given dose was statistically significantly different from the others. The F-test failed to determine that the variability in the mean mortalities of the groups was sufficiently larger than the variability observed within the groups, as indicated by the small ratios and large probabilities (p-value greater than 0.05). In other words, the mean mortalities between the insecticides at each given dose belonged to the same

population (were similar). Moreover, there was no evidence that any one of the insecticides performed better than the other at a given dose. This is also indicated in the interval plots by the overlapping 95% confidence intervals of the means (Fig. 3.6).

3.3.3 WHO Susceptibility Tests

From the total live collections, 408 specimens were used for WHO susceptibility tests against DDT, bendiocarb, pirimiphos methyl and permethrin. Table 3.3 summarises *An. funestus s.l.* numbers tested for Chakanira and Sisewu villages. Only a single WHO susceptibility test was done for Sisewu village on permethrin due to the low number of mosquitoes collected.

Table 3.3 Mosquito susceptibility to non-pyrethroids in Chakanira village

Village	Insecticide	Dose	No. exposed	% Mortality
Chakanira	Control	0X	29	0
	DDT	1X	103	98.9
	Control	0X	27	0
	Bendiocarb	1X	93	33.8
	Pirimiphos-methyl	1X	103	100
Sisewu	Control	0X	28	0.0
	Permethrin	1X	25	35.4

3.3.4 Molecular Species Identification

The results for species identification of 400 wild females revealed that *An. funestus s.s.* was the most abundant member of the *An. funestus* group (n = 392) followed by *An. parensis* (n = 6) and *An. rivulorum* (n = 2). While most of these specimens were from Chakanira village, 75 were from Sisewu village and identified as 72 *An. funestus s.s*., 2 *An. rivulorum* and 1 *An. parensis*.

3.4 Discussion

Our findings reveal a high prevalence of pyrethroid resistance in *Anopheles funestus* populations from Chikwawa, with marked resistance to alpha-cypermethrin. This high-intensity resistance pattern aligns with prior reports indicating widespread pyrethroid resistance in Southern Africa (Hunt et al., 2011; Riveron et al., 2015). However, the elevated resistance level to alpha-cypermethrin observed here suggests intensified local selection pressures that could stem from historical and extensive IRS campaigns. The predominance of *An. funestus*, even amidst ongoing control measures, indicates that local environmental adaptability and possible shifts in mosquito behaviour or ecology could be contributing to the persistence of malaria transmission in Chikwawa.

The moderate resistance levels observed for deltamethrin and permethrin may indicate varying exposure levels across insecticide types, supporting previous findings of multi-pesticide exposures driving resistance (Mzilahowa *et al*., 2016; Nkya *et al*., 2014). Given that Chikwawa is an agricultural region, agricultural pyrethroid use may also contribute to the observed resistance pattern, particularly where agricultural and public health insecticides overlap (Chouaibou *et al*., 2016). This dual exposure complicates resistance management, as resistance is likely reinforced by both household IRS/LLIN use and agricultural pesticide exposure.

Additionally, our results showing the persistence of *An. funestus* populations suggest behavioural adaptations in response to vector control measures, such as shifts in resting or feeding behaviours to reduce contact with treated surfaces, a phenomenon observed in other malaria-endemic regions (Coetzee and Koekemoer, 2013; Sanou *et al*., 2021). This adaptability is further supported by studies suggesting *An. funestus* resilience in the face of environmental pressures and control measures

(Moiroux *et al*., 2012). Abundant local habitats, including water sources and vegetation, provide optimal breeding conditions, sustaining *An. funestus* populations and maintaining the transmission cycle despite IRS and LLIN interventions (Mzilahowa et al., 2016; Ondeto 2023; Namango 2024).

The findings highlight the need for locally tailored control strategies that consider the unique ecological and behavioural characteristics of *An. funestus* in Chikwawa. Approaches incorporating non-pyrethroid insecticides, such as PBO nets or new IRS formulations, may enhance control efficacy in such high-resistance areas (Oxborough *et al*., 2015; Protopopoff *et al*., 2018). Moreover, longitudinal monitoring of resistance trends and integrating molecular diagnostics to understand resistance mechanisms could inform more targeted and sustainable control interventions

3.5 Conclusion

In conclusion, the level of resistance in Chikwawa has grown over the years, and the current intensity warrants an urgent solution. It highlights the importance of a resistance management strategy to cushion against the potential negative impact of resistance. More research needs to be done to further validate the resistance intensity in the secondary vector *An. arabiensis* in this geographical region and to explore the intensity of resistance to the carbamate bendiocarb. The study's findings provide valuable insights into the resistance landscape of *An. funestus* in Chikwawa, offering essential considerations for malaria control strategies in Malawi.

Chapter 4: The Effect of Yorkool and Royal Sentry LLINs on Mortality of Wild *Anopheles funestus* and a Susceptible Laboratory Strain, *Anopheles gambiae s.s* KISUMU

4.1 Introduction

Long-lasting insecticidal nets (LLINs) constitute a category of insecticide-treated nets (ITNs) that are pre-treated in factories and designed to maintain their effectiveness for a specified number of standard washes and a defined period of use in real-world conditions (WHO, 2013). In contrast to regular ITNs necessitating re-treatment every 3-6 months, LLINs eliminate the need for such re-treatment and typically endure up to 3 years of recommended use in the field, withstanding at least 20 standard washes under controlled laboratory conditions (WHO, 2013). Their resilience, enduring insecticidal qualities, and relatively lower cost have positioned LLINs as the preferred choice for ITNs in numerous malaria-endemic nations, including Malawi (Yang *et al.,* 2018; Abeku *et al.,* 2020).

In 2015, the Malawi National Malaria Control Program (NMCP) adopted a policy advocating universal coverage of all at-risk populations through LLINs, ensuring that each household possesses at least one net for every two individuals (Mangani *et al.,* 2022). This initiative aimed for a nationwide LLIN usage rate of at least 80% (Abeku *et al.,* 2020). The Malawi NMCP distributed Royal Sentry and Yorkool LLINs to the communities in Chikwawa district in 2018 after initially testing them using a susceptible strain of *An. gambiae* s.s Kisumu in the laboratory (The PMI VectorLink, 2022). However, Chikwawa district is dominated by *An. funestus* which is also resistant to pyrethroids (Wondji *et al* 2015; Kumala *et al*., 2022) and hence the effectiveness of the nets in the field environment would likely different from the

results seen in the laboratory. The current study sought to assess the bio-efficacy of Yorkool and Royal Sentry LLINs on a natural population of *An. funestus* from Chikwawa district, southern Malawi. This evaluation was conducted in comparison with a susceptible laboratory colony of *An. gambiae* s.s. KISUMU strain. Implemented in 2018, this study foreran the widespread distribution of these nets by the Malawi NMCP during campaigns preceding the promotion of next-generation nets for managing insecticide resistance (Abeku *et al.,* 2019).

4.2 Materials and Methods

4.2.1 Yorkool LLIN

Yorkool LLIN is a type of deltamethrin-treated net produced by Tianjin Yorkool International Trading Co., Ltd. The net is treated at a 54 mg/m² concentration for its 75 denier (D) variant and 56 mg/m² for the 100 D and 150 D versions. For this study, the 100 D variant was used. WHO prequalified the net in February 2018 (source 1).

4.2.2 Royal Sentry LLIN

Royal Sentry LLIN, manufactured by Disease Control Technologies, USA, is coated with the pyrethroid alpha-cypermethrin at a concentration of 261 mg/m², which is 6.5 times the standard ITN dose for alpha-cypermethrin. The net is 100% high-density polyethylene constructed of 150 denier monofilament yarn. It was WHO-prequalified in 2019 (source 2).

4.2.3 Testing Method

The standard World Health Organization (WHO) cone bioassay technique was employed to assess the efficacy of Yorkool and Royal Sentry nets. The test involved both wild-collected *An. funestus* s.l. and a susceptible laboratory strain, *An. gambiae* s.s. KISUMU (WHO, 2013).

4.2.4 Laboratory Strain and Wild Mosquito Collection

The susceptible colony *An. gambiae* KISUMU, originally from Kisumu, Kenya, has been maintained at the Malaria Alert Centre (MAC) in Blantyre, Malawi, since 2007. Molecular (PCR) identification is conducted every six months to ensure colony integrity. Wild *An. funestus* s.l. used in the study was collected from Chikwawa, where the species has confirmed resistance to pyrethroids and carbamates (Riveron *et al.,* 2015; Mzilahowa *et al.,* 2016).

4.2.5 Experimental Setup

Six Yorkool and six Royal Sentry brand nets were selected from a 2018 batch ahead of a Long-Lasting Insecticidal Net (LLIN) distribution campaign by the National Malaria Control Program (NMCP). Following the World Health Organization (WHO) protocol, five pieces of 25 cm x 25 cm were cut from each net, labelled as S1 to S4 (sides) and T (top side) (Fig 4.1). Mosquito exposure involved attaching four WHO cones to each piece, with 20 mosquitoes of each species (*An. funestus* s.l. and *An. gambiae* s.s. KISUMU) exposed for three minutes, one species at a time. This process was repeated until at least 50 mosquitoes of each species were exposed to a net piece. Knockdown, sustained effects at 60 minutes, and 24-hour mortality were recorded. Control groups included a negative control (untreated net exposure) and a positive control (susceptible *An. gambiae* s.s. KISUMU colony exposed to a treated net). After exposure, mosquitoes were given a 10% sugar solution until the 24-hour observation concluded. This experimental design aimed to assess the bio-efficacy of Yorkool and Royal Sentry LLINs.

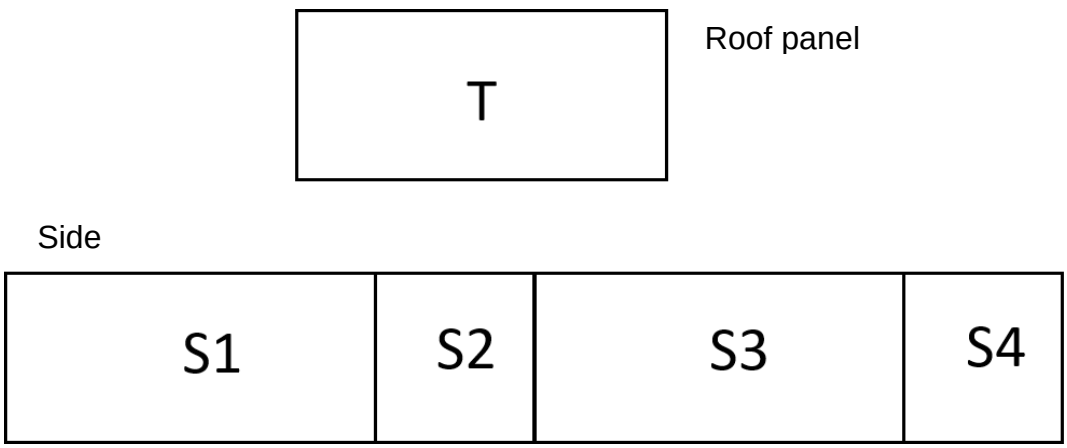


Figure 4.1 Sketch of a rectangular net showing sides where pieces were cut from

4.2.6 Data Analysis

Data analysis aimed to evaluate the bio-efficacy of Yorkool and Royal Sentry LLINs by assessing knockdown at 60 minutes, 24-hour mortality, and sustained efficacy. Mortality and knockdown rates were calculated for each net piece and corrected for control mortality using Abbott's formula (WHO, 2018). Descriptive statistics, including mean rates and standard errors, were calculated across net sections, mosquito strains (*An. funestus* and *An. gambiae* KISUMU), and LLIN brands to summarize efficacy patterns.

A one-way ANOVA was performed to compare mortality and knockdown rates between LLIN brands and across net sections, analysing each factor separately to evaluate their impact on efficacy outcomes. Tukey's post hoc tests were applied to identify specific differences where significant results were observed. To explore insecticide resistance impacts, mortality rates for the resistant *An. funestus* strain and the susceptible *An. gambiae* KISUMU strain were compared, helping to identify any variations in LLIN performance due to resistance.

4.3 Results

There were 12 nets tested, six Yorkool and six Royal Sentry nets all of which were exposed to both *An. gambiae* s.s Kisumu and *An. funestus*. There were marked differences in mortality scores between wild *An. funestus* and the susceptible colony, *An. gambiae s.s.* KISUMU, and between the two LLIN brands. As indicated earlier in Chapter 3, 98% of the total wild *An. funestus s.l*. collection was made up of *An. funestus s.s*. Therefore, the wild *An. funestus s.l*. collections are hereinafter referred to simply as *An. funestus*.

4.3.1 The Response of *An. gambiae s.s* Kisumu After Exposure to Yorkool Nets

The mortality of the susceptible *An. gambiae s.s* Kisumu strain exposed to various pieces of Yorkool nets ranged from 61.7% to 81.2%. When exposed to an untreated control, the mortality was 1.9%, so the test mortalities were not adjusted (WHO, 2016). A test for uniformity of impregnation of the nets done via a one-way ANOVA comparing the differences in mean mortalities for the various net pieces, including untreated control, showed a significant difference ($p=0.030$). A further Tukey post hoc test showed the difference between the untreated (control) net and the test nets (Table 4.1) which showed no significant differences in mortality between themselves (Table 4.2).

Table 4.1 Showing mean mortalities of *An. gambiae s.s* exposed to pieces of Yorkool Nets and an untreated control net

REPLICATE	N	Mean	St. Dev	95% Confidence Interval	Grouping[#]	
Untreated net	3	1.92	3.33	(-31.70, 35.54)	A	
S1	4	67.4	32.1	(38.3, 96.5)	A	B
S2	4	68.00	19.00	(38.88, 97.11)	A	B
S3	4	64.0	39.3	(34.9, 93.1)	A	B
S4	4	81.19	9.55	(52.07, 110.31)	A	B
T	4	61.7	35.8	(32.6, 90.9)		B

Pooled St. Dev = 27.6011

One-Way ANOVA p-value = 0.030

[#]*Grouping information using the Tukey method and 95% confidence interval. Means that do not share a letter are significantly different.*

Pairwise comparisons between the various pieces of Yorkool Nets cut from different sides of the net did not show any statistically significant differences (column five, table 4.2) among themselves. However, when the same pieces were compared with the untreated net (negative control) via Tukey's test pairwise comparisons, three pairs were only marginally significant (p ranging from 0.057-0.099). In contrast, one pair was statistically significant (column five, table 4.2). There were no individual differences between the various test replicates when compared among themselves (table 4.2).

Table 4.2 Tukey simultaneous tests for differences of mean mortalities of *An. gambiae s.s* exposed to pieces of Yorkool nets and an untreated control

Difference of Levels	Difference of Means	SE of Difference	95% Confidence Interval	Adjusted P-Value
S1 - untreated net	65.5	21.1	(-1.9, 132.9)	0.060
S2 - untreated net	66.1	21.1	(-1.3, 133.5)	0.057
S3 - untreated net	62.1	21.1	(-5.3, 129.5)	0.081
S4 - untreated net	79.3	21.1	(11.9, 146.6)	0.016
T - untreated net	59.8	21.1	(-7.6, 127.2)	0.099
S2 - S1	0.6	19.5	(-61.8, 63.0)	1.000
S3 - S1	-3.4	19.5	(-65.7, 59.0)	1.000
S4 - S1	13.8	19.5	(-48.6, 76.2)	0.979
T - S1	-5.7	19.5	(-68.0, 56.7)	1.000
S3 - S2	-4.0	19.5	(-66.3, 58.4)	1.000
S4 - S2	13.2	19.5	(-49.2, 75.6)	0.982
T - S2	-6.3	19.5	(-68.6, 56.1)	0.999
S4 - S3	17.2	19.5	(-45.2, 79.5)	0.946
T - S3	-2.3	19.5	(-64.7, 60.1)	1.000
T - S4	-19.4	19.5	(-81.8, 42.9)	0.913

Individual confidence level = 99.47%

When the scores for individual pieces of nets were put together to calculate each net's overall mean score, the overall knockdown scores (recorded at 1 hour from exposure) ranged from 86% to 100%. The overall mean mortality scores (recorded at 24 hours) for all the nets ranged from 45.6% to 88.9%. For the untreated control, the mean knockdown was zero at one hour, while the mean mortality was 1.9% at 24 hours. Table 4.3 summarises the overall scores for the nets.

Table 4.3 Mean knockdown and mortality of *An. gambiae* Kisumu exposed to individual Yorkool Nets

NETCODE	N	Knockdown[§]			Mortality[β]		
		Mean	SE* Mean	St. Dev	Mean	SE* Mean	St. Dev
20	5	93.6	3.12	7.0	84.5	4.8	10.8
26	5	100.0	0	0	88.9	2.1	4.8
3	5	91.0	2.87	6.4	55.0	12.8	28.5
8	5	86.1	6.69	15.0	45.6	12.5	28.0
Untreated net	3	0	0	0	1.9	1.9	3.3

**SE =standard error of the mean*
[§]*Knockdown was assessed at 1 hour from exposure*
[β]*Mortality was assessed at 24 hours from exposure*

4.3.2 The Response of *An. funestus* After Exposure to Yorkool Nets

Anopheles funestus exposed to various pieces of Yorkool nets did not show any significant mortality differences between the nets and to the untreated control. The results of a one-way ANOVA test for differences among mean mortalities of the various net sides for Yorkool LLINs are given in Table 4.4. Pairwise comparisons tested the differences between each net side (S1, S2, S3, S4 and T) in relation to the others. The one-way ANOVA revealed that there was no difference (p-value = 0.680) in mean *An. funestus* mortalities between the test group means (Yorkool net pieces) and untreated control net (negative control), as indicated in Table 4.4.

Table 4.4 Mean mortality of *An. funestus* exposed to Yorkool LLINs and control

REPLICATE	N	Mean	St. Dev	95% Confidence Interval	Grouping[#]
Untreated net	3	1.92	3.33	(-8.61, 12.46)	A
S1	3	11.80	2.22	(1.27, 22.33)	A
S2	3	7.50	6.61	(-3.03, 18.03)	A
S3	3	3.03	5.25	(-7.50, 13.56)	A
S4	3	10.0	17.3	(-0.5, 20.5)	A
T	3	6.67	5.77	(-3.87, 17.20)	A

Pooled St. Dev = 8.373
One-Way ANOVA p-value = 0.680
[#]Grouping information using the Tukey method of 95% confidence. Means sharing a letter are not significantly different.

The scores for individual pieces of nets were combined to calculate the overall mean score for each net exposed to *An. funestus*. The overall knockdown scores (recorded at 1 hour from exposure) for the three nets tested ranged from 0 to 4.2%. The overall mean mortality scores (recorded at 24 hours) for all the nets ranged from 4.0 % to 13.2%. For the untreated control, the mean knockdown was zero at one hour, while the mean mortality was 1.9% at 24 hours. Table 4.5 summarises the overall scores for Yorkool nets exposed to *An. funestus*.

Table 4.5 Mean knockdown and mortality of wild *An. funestus* exposed to Yorkool LLINs

NETCODE	**N**	**Knockdown[§]**			**Mortality[β]**		
		Mean	**SE* Mean**	**St. Dev**	**Mean**	**SE* Mean**	**St. Dev**
13	5	2.86	2.86	6.39	13.18	4.87	10.90
13W1	5	4.22	2.59	5.79	6.22	2.55	5.70
29	5	0.0	0.0	0.0	4.00	2.45	5.48
Untreated net	3	0.0	0.0	0.0	1.92	1.92	3.33

**SE =standard error of the mean*
[§]Knockdown was assessed at 1 hour from exposure
[β]Mortality was assessed at 24 hours from exposure

A Tukey post hoc test for pairwise differences of *An. funestus* mortality exposed to Yorkool net pieces and the negative control net did not show significant differences in pair comparisons (Table 4.6).

Table 4.6 Tukey simultaneous tests for differences of mean mortalities of *An. funestus* exposed to pieces of Yorkool nets and an untreated control

Difference of Levels	Difference of Means*	95% Confidence Interval	Adjusted P-Value#
S1 - untreated net	9.88	(-13.09, 32.84)	0.702
S2 - untreated net	5.58	(-17.39, 28.54)	0.959
S3 - untreated net	1.11	(-21.86, 24.07)	1.000
S4 - untreated net	8.08	(-14.89, 31.04)	0.837
T - untreated net	4.74	(-18.22, 27.71)	0.979
S2 - S1	-4.30	(-27.26, 18.66)	0.986
S3 - S1	-8.77	(-31.73, 14.19)	0.789
S4 - S1	-1.80	(-24.76, 21.16)	1.000
T - S1	-5.13	(-28.10, 17.83)	0.971
S3 - S2	-4.47	(-27.43, 18.49)	0.984
S4 - S2	2.50	(-20.46, 25.46)	0.999
T - S2	-0.83	(-23.80, 22.13)	1.000
S4 - S3	6.97	(-15.99, 29.93)	0.903
T - S3	3.64	(-19.33, 26.60)	0.994
T - S4	-3.33	(-26.30, 19.63)	0.996

Individual confidence level = 99.43%
**SE of difference = 6.84*
#The adjusted p-values of differences between mean mortalities were all not significant

4.3.3 The Response of *An. gambiae* Kisumu After Exposure to Royal Sentry nets

Mortality of *An. gambiae* Kisumu exposed to Royal Sentry nets and the untreated control net indicated significant differences as determined by a one-way ANOVA test (p-value = 0.000). The mean mortality scores of the susceptible species exposed to Royal Sentry test replicates were 100% against 1.92% for the untreated control net. A further Tukey post hoc test revealed that the significant difference was between the untreated control and the test replicates themselves (indicated in column six in table 4.7).

Table 4.7 Mean mortality scores of *An. gambiae* Kisumu exposed to pieces of Royal Sentry nets and an untreated control net

REPLICATE	N	Mean	St. Dev	95% CI	Grouping[#]
Untreated net	3	1.92	3.33	(0.53, 3.31)	B
S1	4	100.0	0.0	(98.8, 101.2)	A
S2	4	100.0	0.0	(98.8, 101.2)	A
S3	4	100.0	0.0	(98.8, 101.2)	A
S4	4	100.0	0.0	(98.8, 101.2)	A
T	4	100.0	0.0	(98.8, 101.2)	A

Pooled St. Dev = 1.14248
One-Way ANOVA p-value = 0.000
[#]Grouping information using the Tukey method of 95% confidence. Means that share a letter are not significantly different.

Further to the one-way ANOVA test, a Tukey post hoc analysis was done to investigate pairwise comparisons and determine where the scores' differences lay. The test revealed a strong significant difference between the untreated control net scores and the test net pieces (Table 4.8). Further, the test found no statistically significant differences between any pair of the Royal Sentry pieces being tested. Table 4.7 summarises the differences between the mean scores of all the pairwise comparisons between the various net pieces, including the untreated control net.

Table 4.8 Tukey simultaneous tests for differences of mean mortalities of *An. gambiae* Kisumu exposed to pieces of Royal Sentry nets and an untreated control

Difference of Levels	Difference of Means	SE of Difference	95% Confidence Interval	Adjusted P-Value
S1 - untreated net	98.077	0.873	(95.288, 100.866)	0.000
S2 - untreated net	98.077	0.873	(95.288, 100.866)	0.000
S3 - untreated net	98.077	0.873	(95.288, 100.866)	0.000
S4 - untreated net	98.077	0.873	(95.288, 100.866)	0.000
T - untreated net	98.077	0.873	(95.288, 100.866)	0.000
S2 - S1	0.000	0.808	(-2.582, 2.582)	1.000
S3 - S1	0.000	0.808	(-2.582, 2.582)	1.000
S4 - S1	0.000	0.808	(-2.582, 2.582)	1.000
T - S1	0.000	0.808	(-2.582, 2.582)	1.000
S3 - S2	0.000	0.808	(-2.582, 2.582)	1.000
S4 - S2	0.000	0.808	(-2.582, 2.582)	1.000
T - S2	0.000	0.808	(-2.582, 2.582)	1.000
S4 - S3	0.000	0.808	(-2.582, 2.582)	1.000
T - S3	0.000	0.808	(-2.582, 2.582)	1.000
T - S4	0.000	0.808	(-2.582, 2.582)	1.000

Individual confidence level = 99.47%

In addition to the mortality scores for the net pieces shown in Table 4.8 above, overall mean scores were calculated for each whole net from where the pieces were cut. Table 4.9 summarises the knockdown rates and mortality scores for each net tested. All Royal Sentry nets had an overall mortality score of 100% when exposed to *An. gambiae* Kisumu, whereas the mean knockdown rate rates ranged from 97.5% to 100% (Table 4.9).

Table 4.9 Mean knockdown and mortality of wild *An. gambiae* Kisumu exposed to individual Royal Sentry Nets

NETCODE	N	Knockdown[§]			Mortality[β]		
		Mean	SE* Mean	St. Dev	Mean	SE* Mean	St. Dev
32	5	100.0	0.0	0.0	100.0	0.0	0.0
36	5	100.0	0.0	0.0	100.0	0.0	0.0
40	5	98.2	1.82	4.07	100.0	0.0	0.0
46	5	97.5	2.50	5.59	100.0	0.0	0.0
Untreated net	3	0.0	0.0	0.0	1.9	1.92	3.33

**SE =standard error of the mean*
[§]*Knockdown was assessed at 1 hour from exposure*
[β]*Mortality was assessed at 24 hours from exposure*

4.3.4 The Response of *An. funestus* After Exposure to Royal Sentry nets

There was a significant difference in the mortality of wild-caught *An. funestus* exposed to Royal Sentry nets and the untreated control net (p-value = 0.000). Mean mortality scores for Royal Sentry test replicates ranged from 52.96% to 84.92% against 1.92% for the untreated control net. Tukey's post hoc test indicated the difference between the untreated control and Royal Sentry replicates (column six, table 4.10).

Table 4.10 Mortality of *An. funestus* exposed to Royal Sentry LLINs and a control net

REPLICATE	N	Mean	St. Dev	95% Confidence Interval	Grouping[#]
Untreated net	3	1.92	3.33	(-14.69, 18.54)	A
S1	3	84.92	1.38	(68.30, 101.54)	B
S2	3	84.9	19.8	(68.3, 101.6)	B
S3	3	52.96	14.75	(36.35, 69.58)	B
S4	3	74.26	3.94	(57.64, 90.88)	B
T	3	73.1	20.2	(56.5, 89.7)	B

Pooled St. Dev = 13.210
One-Way ANOVA p-value = 0.000
[#]*Grouping information using the Tukey method of 95% confidence. Means that do not share a letter are significantly different.*

The Tukey post hoc test on pairwise differences did not find significant mortality score differences among the Royal Sentry net replicates, as shown in column four of Table 4.11. The first five rows of Table 4.11 show a strongly significant difference between the Royal Sentry replicates and the negative control. The table also shows that the Royal Sentry replicates were not significantly different from each other in pairwise comparisons.

Table 4.11 Tukey simultaneous tests for differences of mean mortalities of *An. funestus* exposed to pieces of Royal Sentry nets and an untreated control

Difference of Levels	**Difference of Means**	**95% Confidence Interval**	**Adjusted P-Value[#]**
S1 - untreated net	83.0	(46.8, 119.2)	0.000
S2 - untreated net	83.0	(46.8, 119.2)	0.000
S3 - untreated net	51.0	(14.8, 87.3)	0.005
S4 - untreated net	72.3	(36.1, 108.6)	0.000
T - untreated net	71.2	(35.0, 107.4)	0.000
S2 - S1	0.0	(-36.2, 36.2)	1.000
S3 - S1	-32.0	(-68.2, 4.3)	0.096
S4 - S1	-10.7	(-46.9, 25.6)	0.913
T - S1	-11.8	(-48.0, 24.4)	0.874
S3 - S2	-32.0	(-68.2, 4.3)	0.096
S4 - S2	-10.7	(-46.9, 25.6)	0.913
T - S2	-11.8	(-48.1, 24.4)	0.874
S4 - S3	21.3	(-14.9, 57.5)	0.408
T - S3	20.1	(-16.1, 56.4)	0.463
T - S4	-1.2	(-37.4, 35.1)	1.000

Individual confidence level = 99.43%

**SE of difference = 10.80*

[#]*The adjusted p-values of mean mortality differences were all significant between the negative control and all pieces of Royal Sentry nets*

When the scores of net pieces were aggregated by individual net, the mean knockdown rates for the three Royal Sentry nets tested against *An. funestus* were 44.7%, 47.3%, and 52.4%. The mortality rates on the hand were 68.9%, 71.1%, and 82.1%.

4.3.5 The performance of Yorkool and Royal Sentry Nets on susceptible *An. gambiae s.s* Kisumu

The knockdown response of *An. gambiae* Kisumu, after exposure to the two ITN brands, was assessed in conjunction with an untreated control. Overall, the mean knockdown rate was 99% for Royal Sentry net, 89.1% for Yorkool net, and 0% for the untreated control (Table 4.12). Knockdown was assessed at one hour from the time of exposure. A one-way ANOVA found a significant difference in the knockdown rates (p= 0.000). Tukey's post-hoc test was done to determine where the significance differences laid (Table 4.13.)

Table 4.12 Mean knockdown rates of *An. gambiae* Kisumu in response to exposure to Royal Sentry, Yorkool and an untreated net

NET TYPE	N	Mean	St. Dev	95% Confidence Interval	Grouping[#]
Royal Sentry	22	99.02	3.220	(92.48, 105.56)	A
Untreated	3	0.00	0.00	(-17.70, 17.70)	B
Yorkool Net	21	89.09	22.05	(82.40, 95.78)	A

Pooled St. Dev = 15.2042
One-way ANOVA p= 0.000
[#]*Grouping information using the Tukey method of 95% confidence. Means that do not share a letter are significantly different.*

Pairwise comparisons of the nets indicated that the main difference in mean knockdown rates was between the untreated control and the two ITN brands. After one hour from exposure, there was no statistically significant difference between the knockdown rates of Yorkool and Royal Sentry nets. Results of the Tukey simultaneous tests for differences of means are summarised in Table 4.13.

Table 4.13 Tukey Simultaneous Tests for differences of mean knockdown rates *An. gambiae* Kisumu exposed to Royal Sentry, Yorkool and an untreated net

Difference of Levels	Difference of Means	SE of Difference	95% Confidence Interval	Adjusted P-Value
Untreated - Royal Sentry	-99.02	9.36	(-121.71, -76.32)	0.000
Yorkool Net - Royal Sentry	-9.93	4.64	(-21.18, 1.32)	0.094
Yorkool Net - Untreated	89.09	9.38	(66.33, 111.85)	0.000

Individual confidence level = 98.04%

Mortality rates assessed at 24 hours from exposure showed that the mean mortality rate of *An. gambiae* Kisumu exposed to Royal Sentry net was 100%. In addition, the mean mortality rates were 69.4% for Yorkool Net and 1.92% for the untreated control. The results were not adjusted as the control mortality was less than 5% (WHO, 2016). A one-way ANOVA indicated that the mean mortality results differed significantly (p= 0.000).

Table 4.14 Mean mortality of *An. gambiae* Kisumu exposed to Royal Sentry, Yorkool and an untreated net

NET TYPE	N	Mean	St. Dev	95% Confidence Interval	Grouping[#]
Royal Sentry	22	100.0	0.0	(92.2, 107.8)	A
Untreated net	3	1.92	3.33	(-19.26, 23.10)	B
Yorkool Net	21	69.40	26.65	(61.40, 77.41)	C

Pooled St. Dev = 18.1895
[#]*Means that do not share a letter are significantly different*

A post hoc test was done using Tukey's method for simultaneous pairwise comparisons of differences of means. Results indicated that all the mean mortality scores were significantly different. The difference in mean mortality for the untreated net versus the Royal Sentry net was significant (p= 0.000). Also, the mean mortality for Yorkool net versus Royal Sentry net was significantly different (p= 0.000). Lastly,

comparing the mean mortality of the Yorkool net versus that of the untreated net indicated that the two were significantly different (p= 0.000). The results are outlined in Table 4.15 below.

Table 4.15 Tukey Simultaneous Tests for differences of mean mortality of *An. gambiae* Kisumu exposed to Royal Sentry, Yorkool, and an untreated net

Difference of Levels	**Difference of Means**	**SE of Difference**	**95% Confidence Interval**	**Adjusted P-Value**
Untreated - Royal Sentry	-98.1	11.2	(-125.2, -70.9)	0.000
Yorkool Net - Royal Sentry	-30.60	5.55	(-44.06, -17.14)	0.000
Yorkool Net - Untreated	67.5	11.2	(40.2, 94.7)	0.000

Individual confidence level = 98.04%

4.3.6 The performance of Yorkool and Royal Sentry nets on wild-caught *An. funestus*

In the analysis of the performance of Yorkool and Royal Sentry nets on wild-collected *An. funestus*, knockdown and mortality rates were calculated at one hour and 24 hours post-exposure, respectively. Table 4.16 shows the mean knockdown rates calculated after one-hour post-exposure. The mean knockdown rates were 48.2% for Royal Sentry Net, 2.4% for Yorkool Net, and 0% for the untreated control. A one-way ANOVA showed a significant difference in the mean knockdown rates (p= 0.000).

Table 4.16 Showing mean knockdown of *An. funestus* exposed to Royal Sentry, Yorkool Net, and an untreated control net

NET TYPE	N	Mean	St. Dev	95% Confidence Interval	Grouping[#]
Royal Sentry	15	48.14	19.52	(40.88, 55.39)	A
Untreated net	3	0.00	0.00	(-16.23, 16.23)	B
Yorkool Net	15	2.36	4.96	(-4.90, 9.62)	B

Pooled St. Dev = 13.7607
One-way ANOVA p-value = 0.000
[#]*Grouping information using the Tukey method of 95% confidence. Means that do not share a letter are significantly different.*

A post hoc test was done to check where the mean differences in knockdown rates lay. Tukey's post hoc test revealed that the mean knockdown rates for the untreated control versus Royal Sentry nets were significantly different (p= 0.000). The mean knockdown rates for Yorkool Net and Royal Sentry Net were also statistically different (p=0.000). Lastly, Tukey's test did not find a statistically significant difference in knockdown rates of wild-caught *An. funestus* when exposed to Yorkool Net versus the untreated control net (Table 4.17).

Table 4.17 Tukey simultaneous tests for differences of mean knockdown of *An. funestus* exposed to Royal Sentry, Yorkool Net, and an untreated control

Difference of Levels	Difference of Means	SE of Difference	95% Confidence Interval	Adjusted P-Value
Untreated - Royal Sentry	-48.14	8.70	(-69.61, -26.66)	0.000
Yorkool Net - Royal Sentry	-45.78	5.02	(-58.18, -33.38)	0.000
Yorkool Net - Untreated	2.36	8.70	(-19.12, 23.84)	0.960

Individual confidence level = 98.05%

In addition to comparing knockdown rates, a one-way ANOVA analysis was done to compare the mean mortality scores of *An. funestus* exposed to Yorkool Net, Royal Sentry net and an untreated control net. Mean mortality scores were 74.04% for Royal Sentry, 7.80% for Yorkool Net, and 1.92% for the untreated control (Table 4.18). The difference between group mean mortality rates was strongly significant (one-way ANOVA, p-value= 0.000). The Tukey post hoc test showed a grouping of the mean scores for the untreated and Yorkool Nets, while the Royal Sentry net was separated into its own group (column six, table 4.18).

Table 4.18 Showing mean mortalities of *An. funestus* exposed to Royal Sentry, Yorkool Net, and an untreated control net

NET TYPE	N	Mean	St. Dev	95% Confidence Interval	Grouping[#]
Royal Sentry	15	74.04	17.15	(67.17, 80.91)	A
Untreated net	3	1.92	3.33	(-13.44, 17.29)	B
Yorkool Net	15	7.80	8.25	(0.93, 14.67)	B

Pooled St. Dev = 13.0319

One-Way ANOVA p-value = 0.000

[#]Grouping information using the Tukey method of 95% confidence. Means that do not share a letter are significantly different.

Tukey pairwise comparisons between pairs of the three types of nets revealed some differences and similarities in the group means. There was a significant difference between the untreated net and the Royal Sentry net (p=0.000). There was also a significant difference between Yorkool Net and Royal Sentry net mean scores (p=0.000). Nonetheless, the test did not find any statistically significant difference between the mean mortality rates of wild *An. funestus* exposed to Yorkool Net versus the untreated control net (p= 0.758). These results are indicated in Table 4.19 below.

Table 4.19 Tukey simultaneous tests for differences of mean mortalities of *An. funestus* exposed to Royal Sentry, Yorkool Net, and an untreated control

Difference of Levels	**Difference of Means**	**SE of Difference**	**95% Confidence Interval**	**Adjusted P-Value**
Untreated - Royal Sentry	-72.11	8.24	(-92.45, -51.77)	0.000
Yorkool Net - Royal Sentry	-66.24	4.76	(-77.98, -54.49)	0.000
Yorkool Net - Untreated	5.88	8.24	(-14.46, 26.22)	0.758

Individual confidence level = 98.05%

4.4 Discussion

The results of the study revealed substantial variations in the efficacy of Yorkool and Royal Sentry nets against *An. gambiae* s.s. Kisumu and wild *An. funestus*. We observed significant differences in mortality scores between the two mosquito species when exposed to the two LLIN brands.

4.4.1 Response of *An. gambiae* s.s Kisumu to Yorkool Nets

The susceptibility of *An. gambiae* s.s Kisumu to Yorkool nets varied across the different pieces of the net. The mortality ranged from 45.6% to 88.9%, with two nets out of four exceeding the WHO efficacy threshold of 80% mortality. This is similar to findings of a 2019 report on Yorkool net durability study in two Malawian districts which reported reduced efficacy (mortality of 67.2%) of Yorkool nets at 36 months (PMI VectorLink Project, 2019). The nets used in the current study were brand-new nets but from a 2016 batch, and two years old at the time the analyses were done. This could have also influenced the results of this study due to potential loss of efficacy during long-term storage. However, care was taken to store the net pieces in aluminium foil and at 4 ^{o}C over the course of the experiments (Musa *et al*., 2020; Mechan *et al*., 2022). The one-way ANOVA and subsequent Tukey post hoc test revealed significant differences between the untreated control and the test nets. However, pairwise comparisons between individual pieces of Yorkool nets did not show statistically significant differences.

Interestingly, the overall knockdown and mortality rates for *An. gambiae* s.s Kisumu were quite high, ranging from 86% to 100% for knockdown and 45.6% to 88.9% for

mortality. This suggests an overall effectiveness of Yorkool nets against this mosquito species.

4.4.2 Response of wild *An. funestus* to Yorkool Nets

In contrast to *An. gambiae* s.s Kisumu, wild-collected *An. funestus* exhibited lower mortality rates when exposed to Yorkool nets. The one-way ANOVA did not show a significant difference in mean mortality among different net sides and the untreated control. The Tukey post hoc test for pairwise comparisons also indicated no significant differences. This showed that the efficacy of Yorkool nets on the wild-collected *An. funestus* was no different from the untreated control net, statistically. The net may have limited efficacy *An. funestus* populations with moderate to high pyrethroid resistance profiles such as the one in Chikwawa (Kumala *et al*., 2022).

The overall knockdown and mortality rates for *An. funestus* were modest, ranging from 0% to 4.2% for knockdown and 4.0% to 13.2% for mortality. These results suggest that Yorkool nets may be less effective against wild *An. funestus* compared to the susceptible *An. gambiae* s.s Kisumu. However, evidence from other studies shows that insecticide-treated nets remain crucial in offering personal protection and continued usage is advised despite the growing threat of insecticide resistance (Strode *et al*., 2014; Lindsay *et al.,* 2021; Shah *et al.,* 2020)

4.4.3 Response of *An. gambiae* Kisumu to Royal Sentry Nets

Royal Sentry nets exhibited remarkable efficacy against *An. gambiae* s.s Kisumu, with all test replicates resulting in 100% mortality. This high mortality significantly

differed from the untreated control, as the one-way ANOVA and Tukey post hoc test confirmed.

Additionally, when analysing individual nets, all Royal Sentry nets demonstrated 100% mortality against *An. gambiae* s.s Kisumu, indicating consistent effectiveness. These results are consistent with a study conducted in Benin that showed remarkable efficacy of Royal Sentry against *An. gambiae* s.s Kisumu even up to 25 standardised washes (Ngufor *et al*., 2020).

4.4.4 Response of *An. funestus* to Royal Sentry Nets

Similar to *An. gambiae* s.s Kisumu, *An. funestus* showed high mortality rates when exposed to Royal Sentry nets. The one-way ANOVA revealed a significant difference in mean mortality between Royal Sentry replicates and the untreated control. The Tukey post hoc test further confirmed the significant differences.

When assessing individual nets, all Royal Sentry nets displayed 100% mortality against *An. funestus*, highlighting the uniform efficacy of this net brand. The net demonstrated efficacy against a wild-strain of moderate pyrethroid-resistant *An. funestus* (Kumala *et al*., 2022). This indicates that it may be a good choice of ITN in the absence of newer generation nets.

4.4.5 Comparative Analysis Between Yorkool and Royal Sentry Nets

Comparing the response of *An. gambiae* s.s Kisumu to both net brands, Royal Sentry nets exhibited a higher mean knockdown rate (99.02%) than Yorkool nets (89.09%). This means that Royal Sentry exceeded the 95% knockdown threshold for determining net efficacy by WHO guidelines, while Yorkool fell short of reaching this threshold (WHO, 2013). The mortality rates at 24 hours also favoured Royal Sentry

nets, with 100% mortality compared to 69.4% for Yorkool nets, again Royal Sentry exceeding the WHO's 80% mortality threshold while Yorkool fell short. It was expected that both nets would cause 100% mortality against this susceptible mosquito strain.

As described earlier, the *An. gambiae* s.s Kisumu colony is an insecticide-susceptible strain that is full susceptibility to the insecticides used on these nets. In addition, Yorkool and Royal Sentry nets are WHO-prequalified and the product information from the manufacturers (presented in sections 4.2.1 and 4.2.2, respectively) indicate that the nets should have had full efficacy on the susceptible strain. One limitation of this study was that chemical analysis of the nets was not carried out. This would have allowed the study to determine the amount of chemical residue present on the nets (WHO, 2013). WHO recommends that the nets used should come from at least two different batches (WHO, 2013). However, each brand of the nets used in this study came from a single batch. This could potentially affect the results if the particular batch had quality control issues. This could potentially explain why Yorkool net showed limited efficacy.

Similarly, in the case of *An. funestus*, Royal Sentry nets outperformed Yorkool nets in terms of both knockdown and mortality rates. The mean knockdown rate for Royal Sentry was 48.14%, significantly higher than Yorkool (2.36%). The mean mortality rates were 74.04% for Royal Sentry and 7.80% for Yorkool.

For Royal Sentry nets exposed to *An. gambiae* Kisumu, the mean mortality score was consistently 100%, surpassing the recommended bio-efficacy thresholds (WHO,

2013). Knockdown rates ranged from 97.5% to 100%. However, against wild *An. funestus,* only one out of three Royal Sentry nets met the 80% mortality cut-off, and none achieved the 95% knockdown requirement. Similar to Yorkool nets, Royal Sentry nets did not perform well against pyrethroid-resistant *An. funestus*.

Comparatively, Royal Sentry nets outperformed Yorkool nets when used against susceptible *An. gambiae* Kisumu, despite both being pyrethroid-impregnated. The higher concentration of alpha-cypermethrin in Royal Sentry nets (261 mg/m^2) may explain their superior performance compared to Yorkool nets (56 mg/m^2 of deltamethrin) (source 1) (source 2). This higher concentration is likely intended to act as a chemical reservoir for wash retention, contributing to prolonged bio-efficacy, which could be a factor in the observed performance differences.

After procurement, the nets were stored under controlled conditions, with relative humidity ranging from 40-70%, and precautions were taken to minimise potential loss of bio-efficacy (WHO, 2013). The net pieces were wrapped in aluminium foil during the testing period and stored in individual plastic zip-lock bags under a wooden drawer. Although some loss of efficacy during storage cannot be completely ruled out, a study in Tanzania reported that LLINs could retain their bio-efficacy even after five years of storage (Musa *et al.,* 2020).

4.4.6 Interpretation

The study demonstrates notable differences in the effectiveness of Yorkool and Royal Sentry nets against *An. gambiae* s.s Kisumu and wild *An. funestus*. Royal Sentry nets consistently exhibited high mortality rates, reaching 100% for both mosquito species, indicating robust efficacy. The wild-collected *An. funestus* was a

moderately pyrethroid resistant population (Riveron *et al.,* 2015; Kumala *et al*., 2022). The findings show that despite resistance, the nets could remain effective in the interim while complementary strategies are being sought (WHO, 2016).

On the other hand, while Yorkool nets showed significant efficacy against the susceptible *An. gambiae* s.s Kisumu, their performance against wild *An. funestus* was comparatively lower. This suggests potential variations in the response of different mosquito species to the two LLIN brands.

The observed differences in efficacy could be attributed to variations in the physical and chemical properties of the nets or variations in the behaviour and physiology of the mosquito species (Osse *et al*., 2013). Further studies are warranted to investigate the underlying factors influencing the observed differences and to optimise the design and formulation of bed nets for enhanced efficacy against a broader range of mosquito species (Okia *et al*., 2013; Obala *et al*., 2015).

These findings underscore the importance of considering mosquito species diversity in evaluating the efficacy of bed nets. The study contributes valuable insights to the ongoing efforts to combat malaria and highlights the need for continued research and innovation in vector control strategies.

4.5 Conclusion

In summary, our study reveals that both Royal Sentry and Yorkool nets demonstrate superior performance against susceptible *An. gambiae* Kisumu compared to wild-collected *An. funestus*. Despite the suboptimal performance on pyrethroid-resistant *An. funestus*, Royal Sentry nets, with their higher insecticide concentration, outperformed Yorkool nets. Although both nets may serve as a physical barrier (Okumu, 2020), it is essential to acknowledge the limitations in their efficacy against resistant mosquito populations. Considering the prevalence of pyrethroid-resistant *An. funestus* in the wild, our findings suggest that Royal Sentry nets may offer a more effective choice in such environments. Nevertheless, the use of both nets can still contribute to some level of protection. Further research and continuous monitoring are recommended to explore additional strategies for malaria control in regions with resistant mosquito populations.

Chapter 5: The Wash Resistance of Yorkool and Royal Sentry Nets, and its Effect on Susceptible and Resistant Mosquitoes

5.1 Background

The effectiveness of major malaria vector control interventions, such as long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS), has been impacted by insecticide resistance in mosquitoes (Kleinshmidt *et al*., 2018; Juache-Villagrana *et al.*, 2022; *Suh et al*., 2023). Various studies conducted elsewhere have also documented behavioural resistance in mosquitoes (Govella *et al*., 2013; Sokhna *et al*., 2013; Killeen *et al.,* 2016; Sanou *et al.,* 2021), where outdoor and early biting behaviour allows mosquitoes to mediate malaria transmission, thereby reducing the effectiveness of indoor-based vector control interventions. Different mosquito species exhibit varying levels of resistance to the chemicals primarily used for control. Additionally, various vector species contribute differently to malaria transmission; for example, in some settings, such as Malawi, Tanzania, and Kenya, *An. funestus* is responsible for higher transmission rates (The PMI VectorLink 2022; Mapua *et al.,* 2022; Ogola *et al*., 2018). Conversely, *Anopheles coluzzii* contributes to higher transmission intensity in West African regions (Fadel *et a*l., 2019). In response to these challenges, new vector control interventions are needed to complement current efforts.

The World Health Organization defines a long-lasting insecticidal net (LLIN) as a factory-treated net that can retain biological activity for at least 20 standardised washes under laboratory conditions and three years of recommended use under field conditions (WHO, 2013). LLINs have the insecticide impregnated into the fibre matrix

and come pre-treated by the manufacturer without requiring re-treatment in its lifetime (WHO, 2013). Whereas the effectiveness of conventional ITNs has been well documented since they were deployed, several limitations have led to the development of LLINs with better technologies (Yang *et al.,* 2018). Thus, LLINs emerged as the preferred means of vector control, replacing conventional ITNs, due to their longer-term effectiveness, relatively low cost and higher durability (Yang *et al.,* 2018).

To determine if a net qualifies as an LLIN, a set of experiments called wash resistance tests are conducted following standardised WHO guidelines (WHO, 2013). This involves a series of exposures of susceptible female anopheline mosquitoes (2-5 day olds) to a given net, observing mortality at 24 hours, washing the net, re-exposing mosquitoes, re-washing, and so on until a specified number of washes is reached or efficacy drops below a given threshold. While wash resistance assays typically use susceptible strains (WHO, 2013), this study used mosquito strains of different resistance profiles, including a susceptible colony.

The study investigated the wash resistance of Yorkool and Royal Sentry long-lasting nets, which were the nets of choice by the Malawi NMCP at the time the study was implemented in 2018. The durability of these nets was previously studied in another field project conducted in two Malawian districts, but wash resistance was not evaluated (PMI VectorLink Project, 2019). The study showed a subsequent decline in net efficacy (overall mortality of 67.2% for Yorkool, and 84% for Royal Sentry) at 36 months from deployment, determined by WHO cone assays using the susceptible *An. gambiae* Kisumu strain (PMI VectorLink Project, 2019). In addition, the

evaluations only used susceptible mosquitoes and did not study the potential impact of the nets on field populations. The wash resistance study was therefore done to generate additional evidence on how insecticide resistance may or may not subsequently affect the wash resistance and effectiveness of LLINs. This information might be helpful for program leaders as, in most scenarios, they are dealing with vector populations which already exhibit some level of insecticide resistance (Wondji *et al.,* 2012; WHO, 2018). In addition, unlike the laboratory strains of susceptible mosquitoes, field populations are the epidemiologically relevant cohort of malaria vectors (WHO, 2018).

5.2 Methods

5.2.1 Mosquito Strains

Three mosquito colonies with different insecticide resistance profiles were used. Firstly, the KGB colony was *An. arabiensis,* which originated from Kanyemba in the Zambezi Valley in northern Zimbabwe (Lyons *et al*., 2012). It has been maintained at the Botha de Meillon Insectary at the National Institute for Communicable Diseases (NICD) since 1975, when it was first colonised. It is still maintained as a fully insecticide susceptible strain and is a valuable control in laboratory and insectary experiments. The SENN DDT colony was *An. arabiensis* originally from the Sennar region of Sudan and was colonised at the NICD in 1990. It is resistant to pyrethroids and DDT, and periodic exposures to non-lethal doses of these insecticides maintain this resistance (Nardini *et al*., 2012). Lastly, FUMOZ Base was an *An. funestus s.s*. colony with moderate resistance to pyrethroids. It was colonised in 2000 at the NICD as a susceptible strain from Mozambique but has since developed moderate resistance to pyrethroids (Hunt *et al*., 2005; Zengenene *et al*., 2021). More

information on the KGB, SENN DDT and FUMOZ Base colonies used in this study has been previously described (Hunt *et al.,* 2005; Venter *et al.,* 2017; Glunt *et al.,* 2018).

5.2.2 Wash Resistance Experiments

This study used the standardised 'WHO guidelines for laboratory and field-testing of long-lasting insecticidal nets' published in 2013 (WHO, 2013). Initially, five pieces of 30 cm by 30 cm were cut from each net, a piece from each of the five panels. At the baseline, six nets of each brand were included. Following baseline exposure, four net pieces were randomly selected from each brand and used throughout the wash experiments. After the first exposure, the net pieces were washed and dried under a shade.

The washing process is outlined below (Table 5.1) and in Figures 5.1 and 5.2. Mosquitoes were exposed again on the following day, and mortality was recorded at 24 hours from exposure. Cycles of wash and expose were repeated until 20 washes had been completed. The net's regeneration time, defined as the period required to recover its biological efficacy when the surface insecticide has been depleted by washing, was determined prior to net wash experiments (WHO, 2013). A waiting period equivalent to the regeneration time was observed between washes to allow for the restoration of the insecticide from the netting fibre (WHO, 2013).

Table 5.1 Net washing schedule for wash resistance tests for Yorkool and Royal Sentry LLINs

Days	Day 1					Day 3					Day 5			Day 7				Day 9		Day 11				Day 13			Day 15
Total Washes	0	1		2	3		4	5		6	7	8	9	10		11	12	13	14	15		16	17	18	19	20	
	Baseline Test	Wash 1	Test	Wash 2	Wash 3	Test	Wash 4	Wash 5	Test	Wash 6	Wash 7	Wash 8	Wash 9	Wash 10	Test	Wash 11	Wash 12	Wash 13	Wash 14	Wash 15	Test	Wash 16	Wash 17	Wash 18	Wash 19	Wash 20	Test

5.2.2.1 Net Washing Process

While the WHO protocol recommends Savlon de Marseille as the standard soap for efficacy studies in phase I, this study used a common household soap (Sunlight tablet soap, Fig. 5.2A) to get locally relevant results. Sunlight Laundry Green Bar Soap is a mild bar soap used for common household needs including washing fabric and is manufactured by Unilever South Africa. Firstly, the soap was grated into small, thin pieces using a standard kitchen grater. The soap pieces were then dried at room temperature and ground to powder in a bowl using a spatula. One gram of soap was dissolved in 0.5 L of 30 ^{o}C distilled water in a beaker. A piece of net was then introduced into the beaker and washed by shaking at 155 rounds per minute (rpm). Rinsing was done following the same procedure as the washing process for 10 minutes. Figures 5.1 and 5.2 summarise this process.

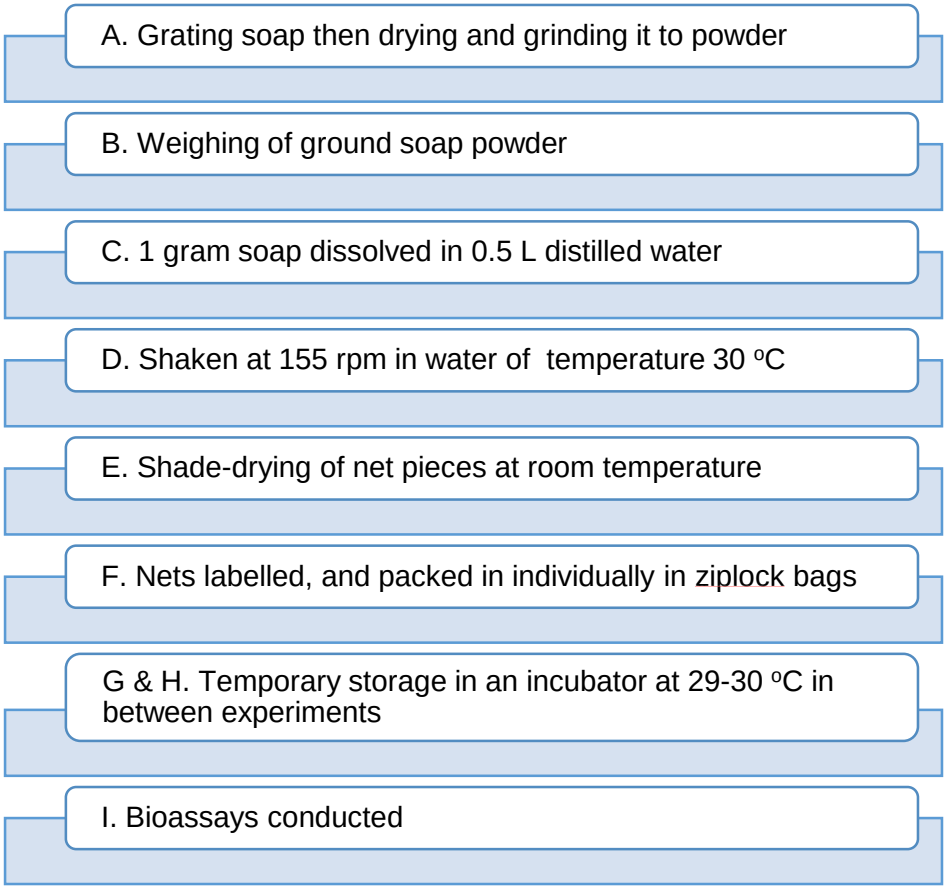


Figure 5.1 Showing steps in the process of washing nets for wash resistance tests.

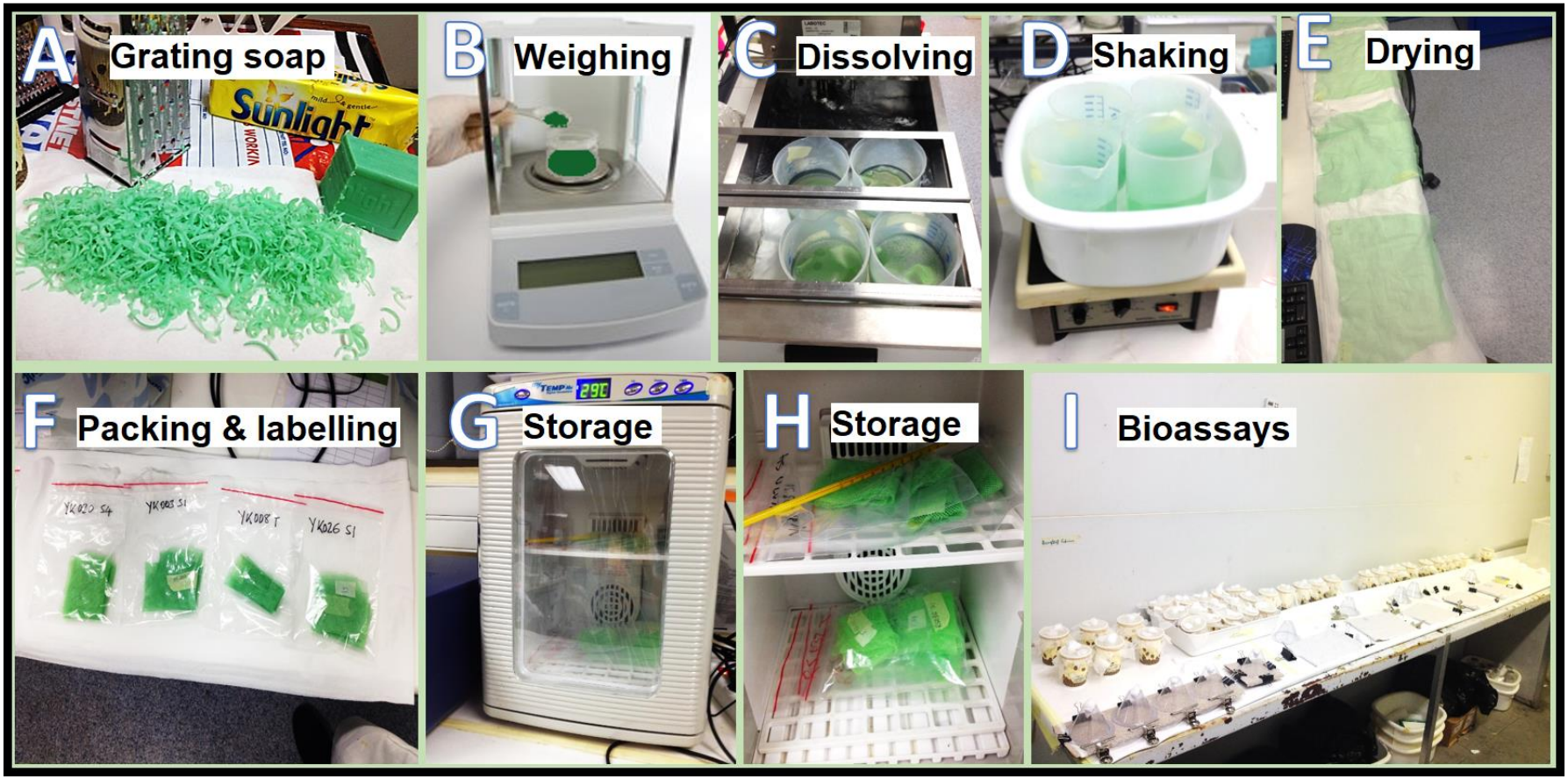


Figure 5.2 A-I, as in Fig. 5.1 above, shows the steps in net washing experiments from preparing soap (A) to conducting bioassays (I).

5.3 Data Analysis

Data analysis aimed to assess the wash resistance and regeneration efficacy of Royal Sentry and Yorkool long-lasting insecticidal nets (LLINs) against mosquito strains with distinct insecticide resistance profiles (KGB, SENN DDT, and FUMOZ Base). Mortality rates were calculated 24 hours post-exposure for each mosquito strain and tracked over the wash cycles. Mortality rates were adjusted for control mortality using Abbott's formula, ensuring an accurate comparison of LLIN effectiveness (WHO, 2018).

Descriptive statistics, including mean mortality and standard errors, were calculated across wash cycles to illustrate the progressive loss of efficacy and potential regeneration of insecticide on net surfaces. A one-way ANOVA was used to evaluate differences in mortality across mosquito strains after each wash cycle, while repeated-measures ANOVA analysed mortality trends over successive wash cycles, assessing the impact of washing frequency on LLIN performance. Where significant differences were identified, Tukey's post hoc tests were conducted to specify differences in mortality across strains.

5.3 Results

Wash resistance results have been presented by mosquito colony and brand of net. Royal Sentry nets were labelled RS01, RS02, RS03 and RS04, while Yorkool nets were labelled YK01, YK02, YK03 and YK04. All three mosquito strains (*An. funestus* FuMoz Base, *An. arabiensis* SENN DDT and *An. arabiensis* KGB) were exposed to each net piece.

All Royal Sentry pieces at baseline showed 100% efficacy on the susceptible KGB colony and efficacy ranging from 98% to 100% on SENN DDT. However, the same pieces showed reduced efficacy on the FuMoz Base colony, with mortalities ranging from 20 to 50% (Fig. 5.3.1). The effect of Yorkool nets on the same colonies was limited, ranging from 65% to 100% for KGB, 8.4% to 36.5% for SENN DDT, and 0 to 9.7% for FuMoz Base at baseline (Fig. 5.3.1).

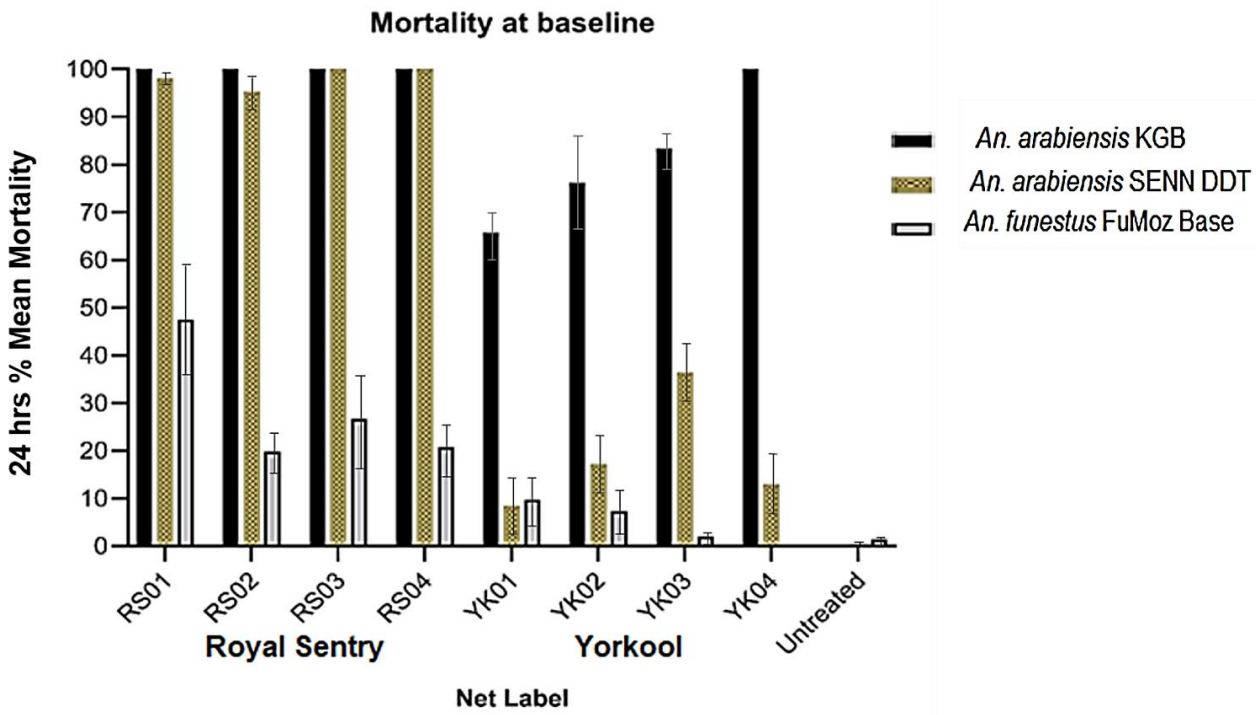


Figure 5.3.1 Mortality of KGB, SENN DDT and FuMoz Base colonies exposed to Royal Sentry and Yorkool nets at baseline (zero washes). Error bars represent standard error of the mean.

5.3.1 Wash Resistance of Royal Sentry Nets

The first piece of Royal Sentry net (RS01) had 100% efficacy on KGB for the first five consecutive washes before declining to 75% after ten washes. It then declined significantly to less than 10% after the 15th wash before picking up slightly to 27% (Fig. 5.3.2). The mortality of SENN DDT exposed to the same net went down successively from 67% after the first wash to 39% after five washes, before dropping further to 8.5% after ten washes and 1.6% by the 15th wash (Fig. 5.3.2). The final mortality of SENN DDT exposed after 20 washes was 6.7%. For FuMoz Base, the mortality went from 12.5% at the first wash, dropping to zero by the 10th wash before slowly picking up to 16.7% after 20 washes.

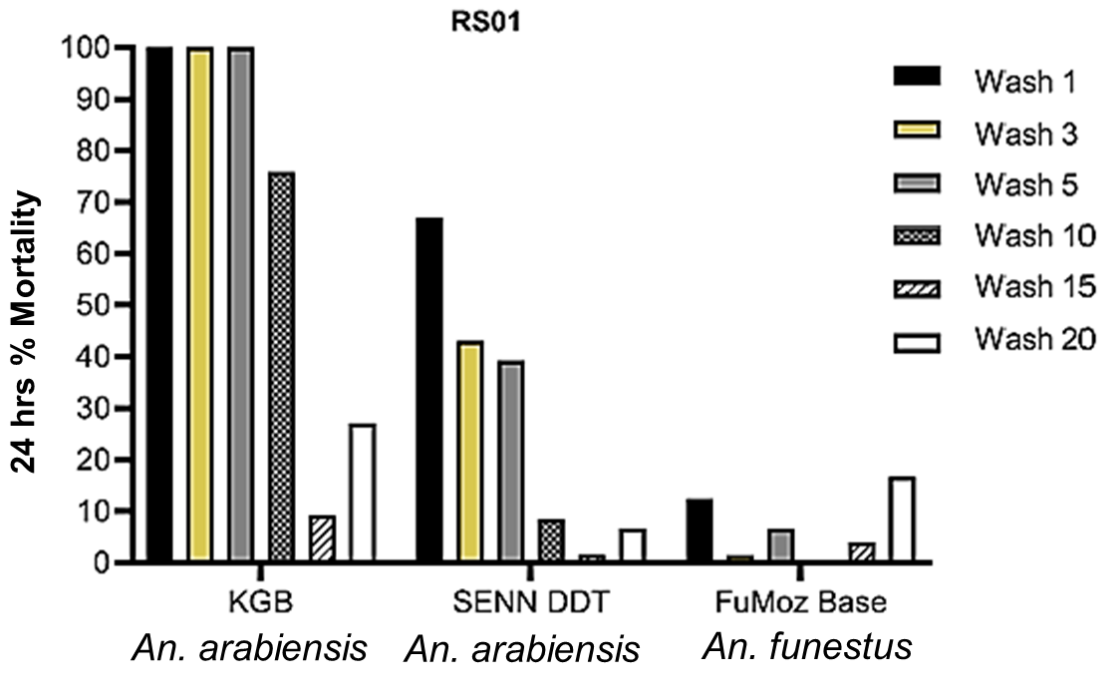


Figure 5.3.2 Mean mortality of KGB, SENN DDT and FuMoz Base colonies after exposure to Royal Sentry Net sample 1 (RS01) that was washed successively up to 20 times

Results for the second Royal Sentry piece (RS02) were similar to those of RS01. KGB's mortality was 100% for the first five washes and 95% even after ten washes. It only dropped to 9.7% after the 15th wash and then rose to 58% after 20 washes

(Fig. 5.3.3). Mortality of SENN DDT for RS02 started at 83.6% at the first wash, to 72.8% after three washes before rising to 92% after five washes. It then dropped drastically to 4.3% at the 10th wash and slowly rose through 9.3% at the 15th wash to 12.5% after 20 washes (Fig. 5.3.3). The net performed poorly on FuMoz Base with mortality at the 1st wash of 22% which went all the way down to zero by the 10th wash before rising again to 12% after the 20th wash (Fig. 5.3.3).

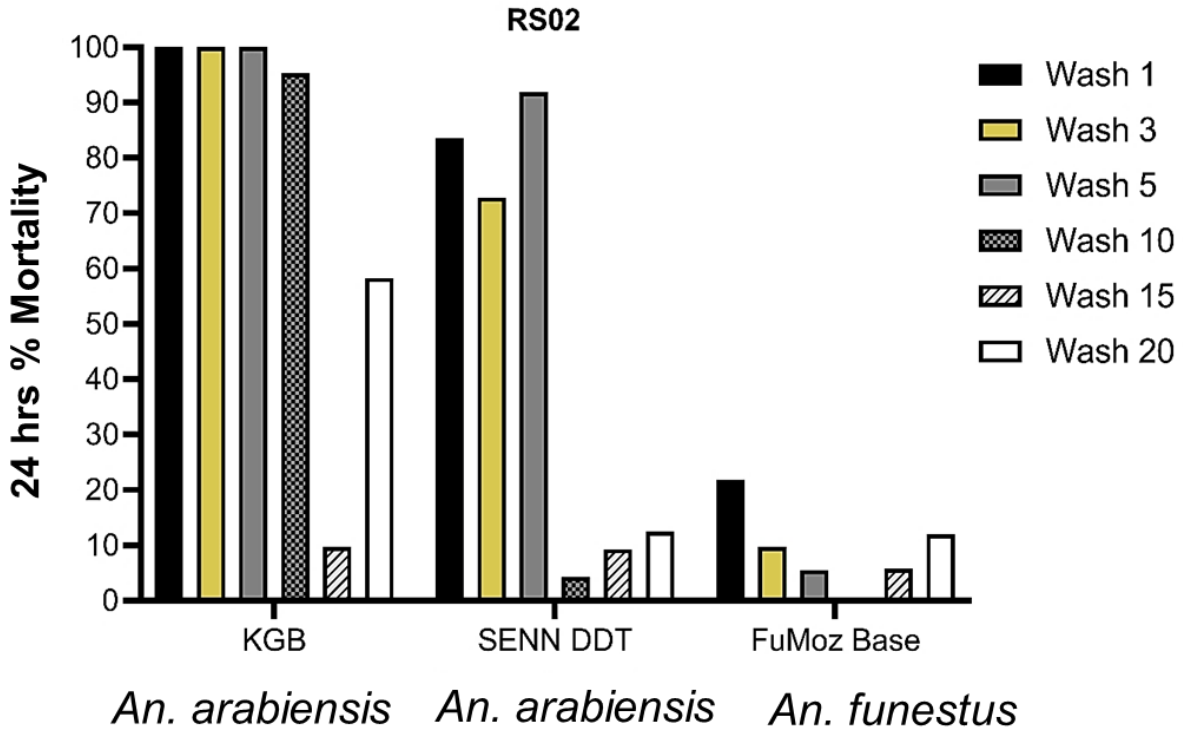


Figure 5.3.3 Mean mortality of KGB, SENN DDT and FuMoz Base colonies after exposure to Royal Sentry Net sample 2 (RS02) that was washed successively up to 20 times

Results for RS03 were also similar to those of RS02 when exposed to the three mosquito colonies. A similar trend was observed with better mortality rates for KGB, followed by SENN DDT, and lastly, poor results for the FuMoz Base colony at all washes (Fig. 5.3.4).

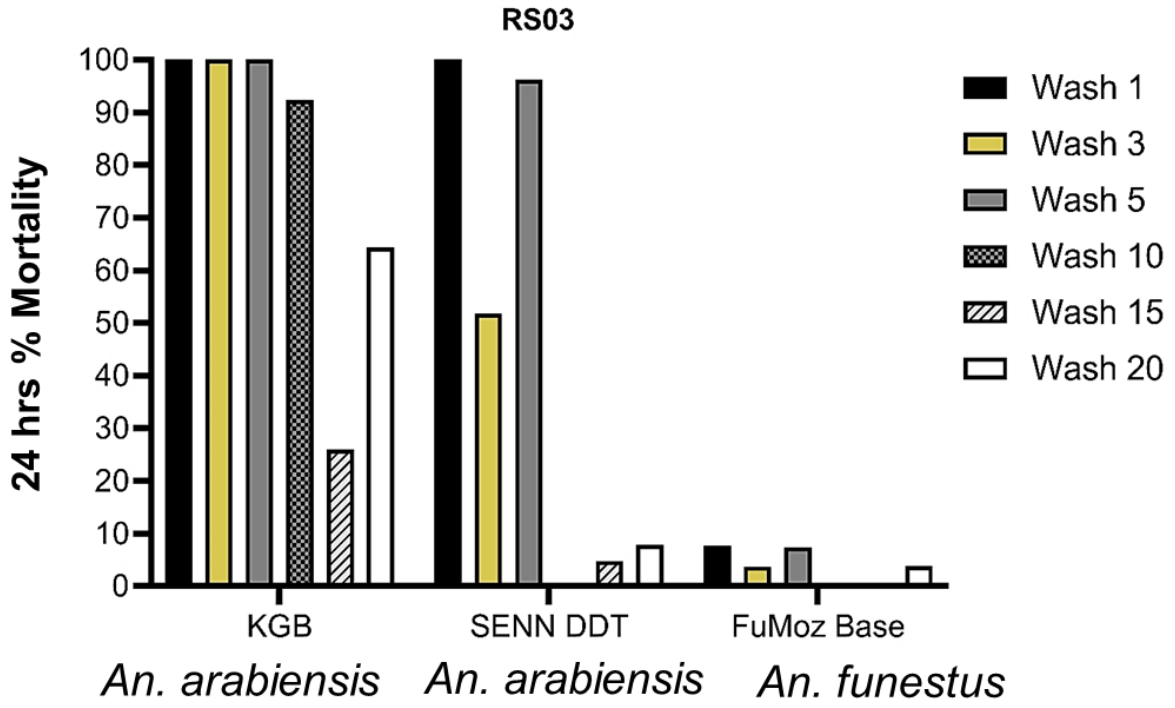


Figure 5.3.4 Mean mortality of KGB, SENN DDT and FuMoz Base colonies after exposure to Royal Sentry Net sample 3 (RS03) that was washed successively up to 20 times

For the last Royal Sentry net, RS04, mortality of susceptible KGB was 100% after five washes and slightly above 90% after the 10th wash. Mortality declined to 12.5% by the 15th wash before rebounding to 44% after the 20th wash (Fig. 5.3.5). Mortality of the pyrethroid/DDT resistant SENN DDT colony went from over 90% at the first wash, then 70% after three washes, before considerably declining to just under 10% by the fifth, 10th and 20th wash. FuMoz Base mortality was consistently under 10% from the first to the last wash (Fig. 5.3.5).

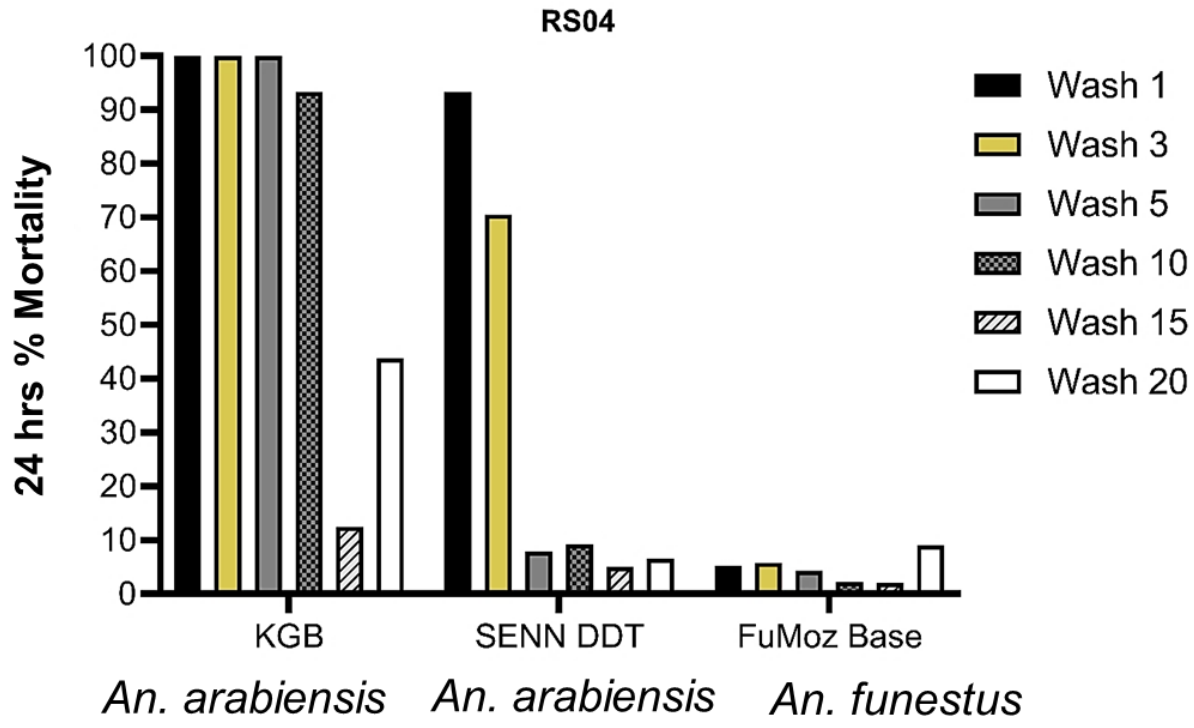


Figure 5.3.5 Mean mortality of KGB, SENN DDT and FuMoz Base colonies after exposure to Royal Sentry Net sample 4 (RS04) that was washed successively up to 20 times

5.3.2 Wash Resistance of Yorkool Nets

Yorkool nets had generally lower mortality rates for all three mosquito colonies. The first piece, YK01, started with a KGB mortality score of 80% after the first wash. After the third wash, mortality increased to 88% before declining to 44% after the fifth wash. KGB mortality was significantly reduced to 10% after ten washes, 17% after 15, and 19% after 20 washes (Fig 5.3.6). SENN DDT exposed to YK01 had 62% after the first wash (Fig. 5.3.6). Mortality rates then declined significantly to 10% or less for the rest of the washes up to the 20th wash. FuMoz Base mosquitoes exposed to YK01 consistently showed very low mortality rates from the first to the final wash, with the highest mortality of 6% at the first wash.

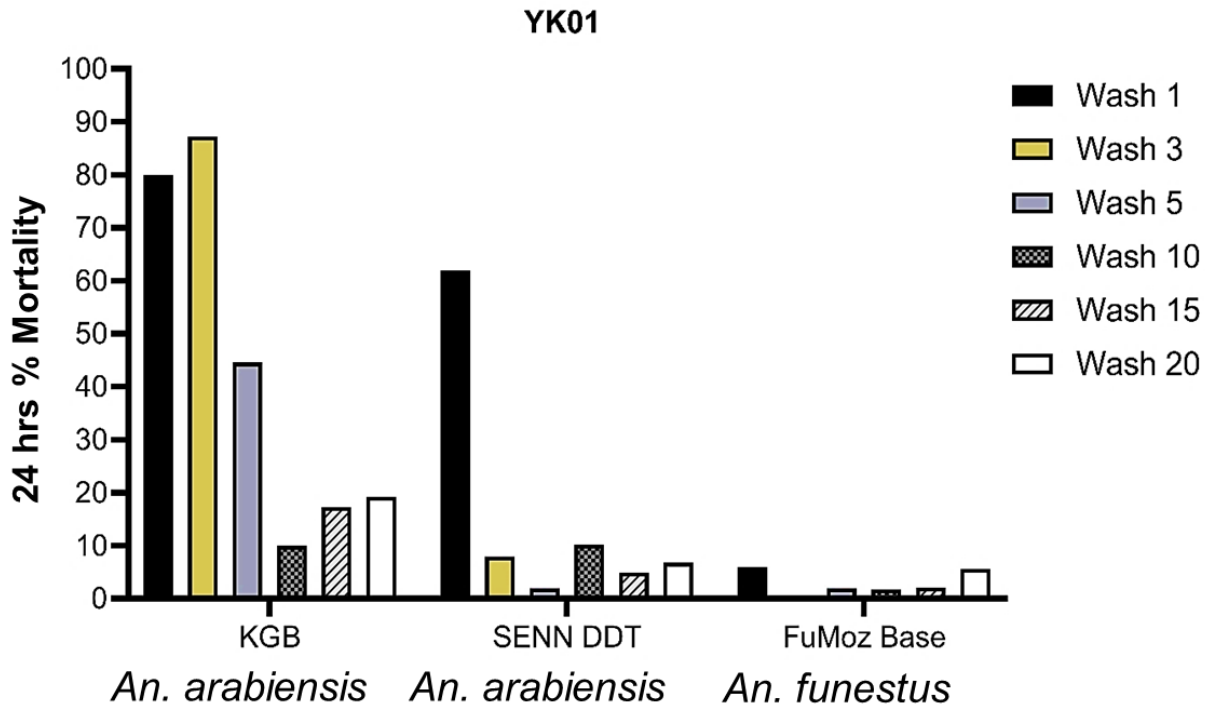


Figure 5.3.6 Mean mortality of KGB, SENN DDT and FuMoz Base colonies following exposure to Yorkool net 1 (YK01) washed successively up to 20 times

The initial mortality for KGB was 76% after the first wash, before going down to 36% after the third wash and rebounding to 58% after five washes. KGB mortality then declined to 33% (10 washes), 14% (15 washes) and 28% after the final 20th wash (Fig 5.3.7). SENN DDT and FuMoz Base mortalities were all significantly low for all washes of YK02, with the highest rate being 12% for SENN DDT at ten washes, while the rest of the mortality scores were lower.

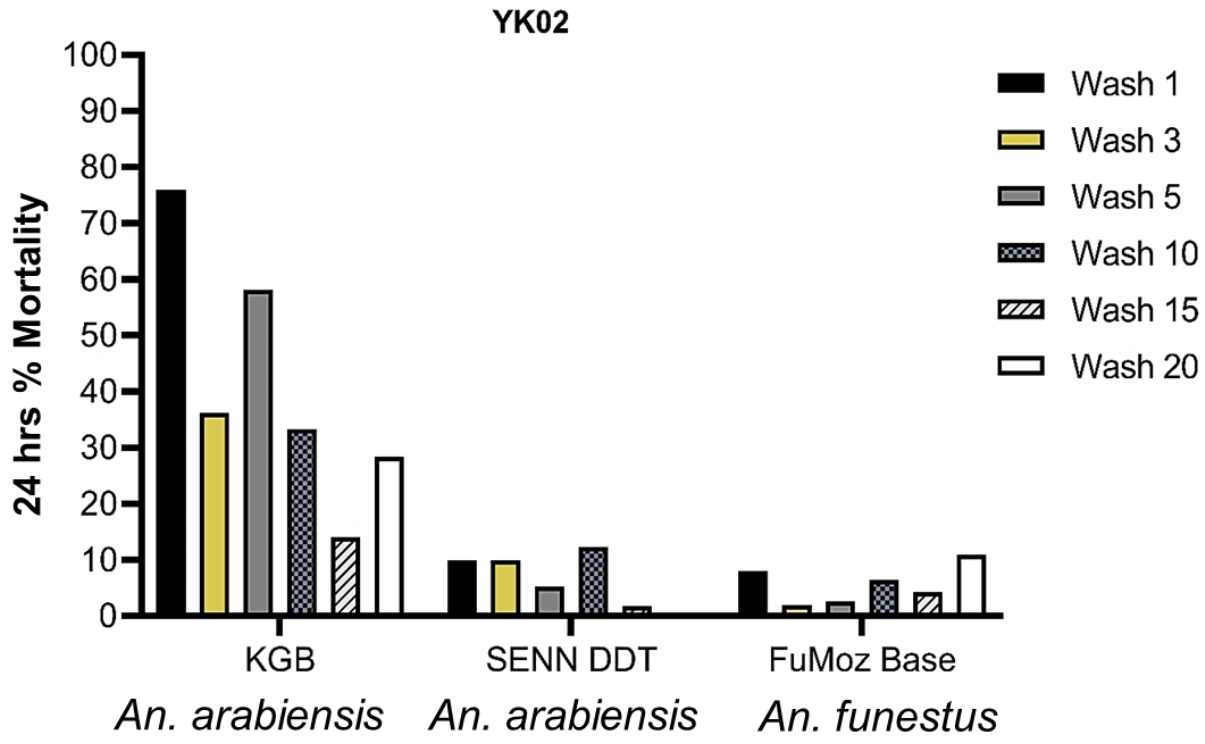


Figure 5.3.7 Mean mortality of KGB, SENN DDT and FuMoz Base colonies after exposure to Yorkool Net sample 2 (YK02) that was washed successively up to 20 times

YK03 showed a similar trend of higher mortality rates for KGB than SENN DDT and FuMoz Base, which were relatively low and similar (Fig 5.3.8). Mortality of KGB mosquitoes when exposed to YK03 net that was washed once was 86%. By the third

wash, mortality had declined to 33%, which rose to 56% after five washes. The corresponding mortality scores for KGB after the 10th, 15th and 20th washes were 23%, 30% and 38%, respectively. The highest mortality score for SENN DDT exposed to YK03 was 24% after the first wash, whereas it was 13% at the final wash. The rest of the scores for the washes in between were lower than 10% (Fig 5.3.8). FuMoz Base mortality was 10% both at the first and final washes. The scores for the washes in between were all 5% or less (Fig 5.3.8).

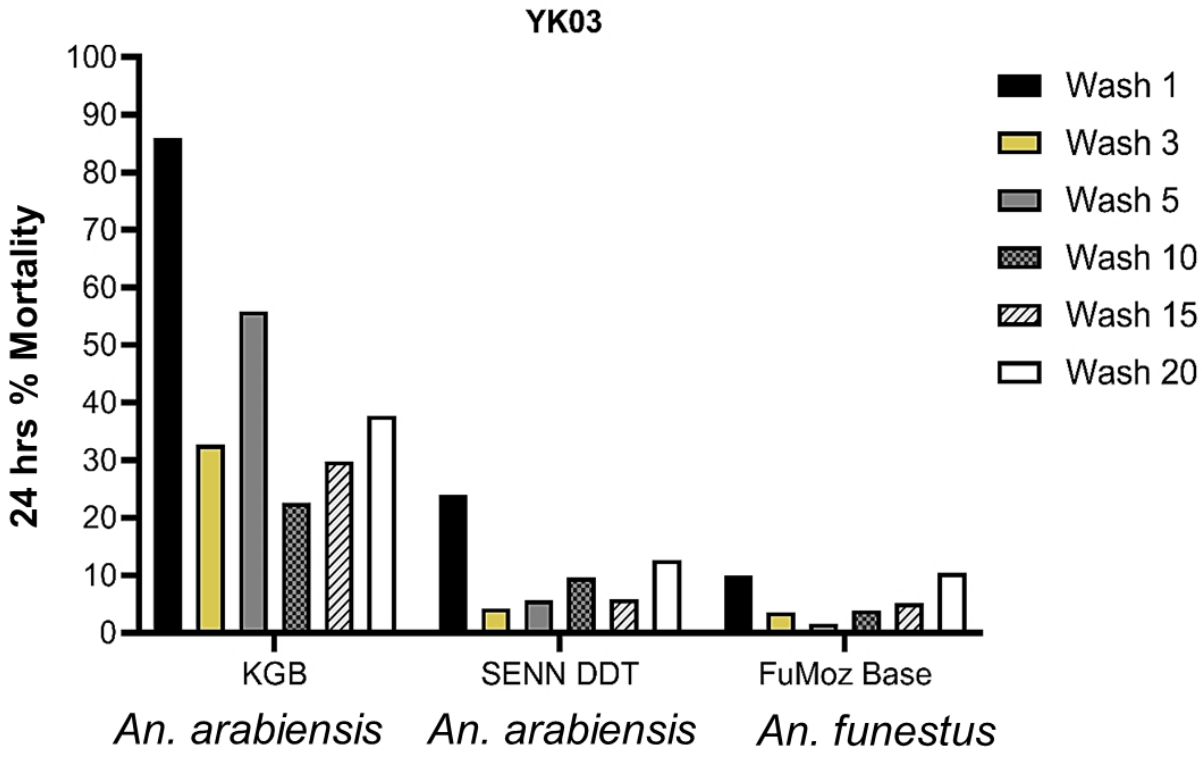


Figure 5.3.8 Mean mortality of KGB, SENN DDT and FuMoz Base colonies after exposure to Yorkool Net sample 3 (YK03) that was washed successively up to 20 times

Mortality scores for KGB exposed to YK04 were 82% after the first wash, followed by 33% after three washes. By the fifth wash, the mortality of KGB had risen to 52% before declining further to 9%, 11% and 30% by the 10th, 15th and 20th washes, respectively (Fig 5.3.9). For SENN DDT exposed to YK04, the highest observed

mortality was 18% after the fifth wash, whereas the rest of the scores were either 12% or less for all washes. Mortality scores of FuMoz Base exposed to YK04 ranged from 0-6% at all washing steps (Fig 5.3.9.).

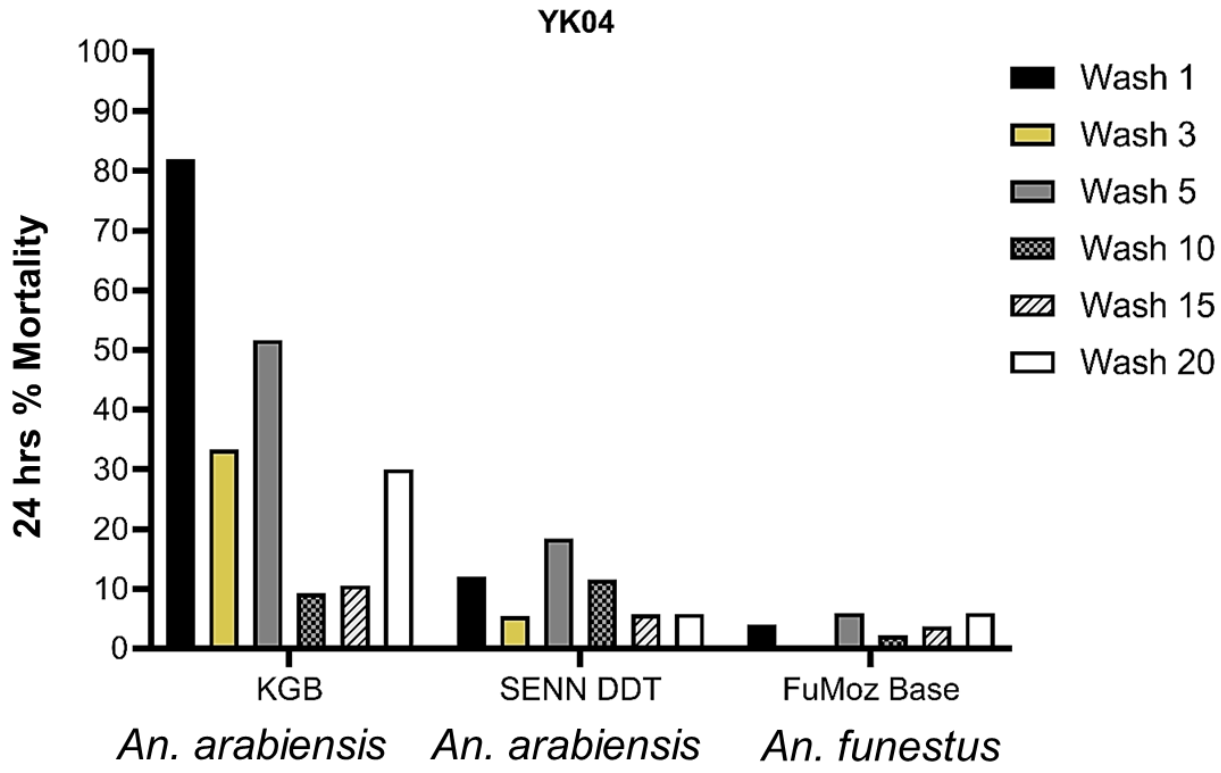


Figure 5.3.9 Mean mortality of KGB, SENN DDT and FuMoz Base colonies after exposure to Yorkool Net sample 4 (YK04) that was washed successively up to 20 times

5.4 Discussion

5.4.1 Introduction and Overview

This study assessed the wash resistance of Royal Sentry and Yorkool LLINs, focusing on their initial and post-wash efficacy against both susceptible and pyrethroid-resistant mosquito colonies. These nets have been used for vector control in various malaria-endemic countries such as Benin and Kenya (Ahogni *et al*., 2020; Achulu *et al*., 2024). Furthermore, the study evaluated the association between resistance and sporozoite infection in *An. funestus* in southern Malawi. The findings of this study reveal how resistance may compromise net performance over time, offering crucial insights for malaria control programs in regions with resistant vectors. These results highlight the need to align LLIN choices with local resistance profiles to ensure sustained protection.

5.4.2 Baseline Wash Resistance

The baseline wash resistance assessment provided valuable insights into the initial efficacy of Royal Sentry and Yorkool nets across different mosquito colonies. Royal Sentry demonstrated high efficacy against the susceptible KGB colony, consistently achieving 100% mortality at baseline (Fig. 5.3.1.). However, a notable decrease in efficacy was observed against the pyrethroid-resistant FuMoz Base colony (Fig. 5.3.1), emphasising the potential impact of insecticide resistance on net performance (Yewhalaw *et al.,* 2012; Kleinschimdt *et al.,* 2018).

These findings align with other studies conducted in Africa, highlighting the growing challenge of insecticide resistance in malaria vectors. For instance, Strode *et al.,* (2014) documented similar patterns of reduced efficacy in pyrethroid-treated nets against resistant *Anopheles* populations in East Africa. Similarly, a study by Atoyebi

(2019) in Nigeria found that pyrethroid resistance significantly affected the mortality rates of *Anopheles gambiae* exposed to treated nets, suggesting a need for novel interventions.

Conversely, some research presents differing results. A study by Musa *et al*., (2020) found that certain nets maintained efficacy against resistant strains under similar wash conditions in Tanzania. This discrepancy could stem from variations in methodology, including the types of insecticide used and the specific resistant alleles present in local mosquito populations. Such findings highlight the complexity of resistance mechanisms and the influence of local ecological factors, as observed by Antoine *et al*., (2021), who reported regional variations in resistance patterns across Burkina Faso.

Moreover, a review by Gleave *et al*., (2021) emphasizes the importance of incorporating alternative insecticides or synergists to enhance efficacy against resistant vectors. This is particularly relevant in the context of the declining effectiveness of pyrethroid-treated nets.

In addition to insecticide resistance, various factors contribute to wash resistance dynamics. These include the physical durability of the net materials, the frequency and intensity of washing, and the environmental conditions to which the nets are exposed. For instance, studies by Diane *et al.,* (2024) highlight that nets subjected to harsher washing conditions such as higher temperatures and aggressive detergents exhibit more significant degradation in insecticidal properties. These findings are corroborated by research from Mutuku *et al*., (2013), which found that repeated washing significantly reduced the insecticidal activity of long-lasting insecticidal nets (LLINs) in Kwale County, coastal Kenya (Mutuku *et al.*, 2013). While the results of

this research are consistent with findings from various studies, they also reveal critical areas where further investigation is warranted.

5.4.3 Wash Resistance Patterns

5.4.3.1 Royal Sentry Nets

The wash resistance experiments revealed distinctive patterns in the performance of Royal Sentry nets. Across the different net samples (RS01 to RS04), the initial high efficacy against KGB declined progressively with successive washes. Notably, the decline was more pronounced against the SENN DDT colony, indicating reduced effectiveness against pyrethroid-resistant mosquitoes. FuMoz Base showed consistently lower mortality rates, underscoring the challenges of wash resistance in the face of pre-existing resistance. The relationship between the number of washes and mortality rates is critical in understanding net longevity. Studies have indicated that nets treated with higher insecticide concentrations, such as the Royal Sentry nets at 261 mg/m², initially exhibit better performance, but the efficacy diminishes after multiple washes (Mutuku *et al*., 2013). The previous evidence demonstrate that treated nets with a high initial insecticide dose maintained effective mortality rates after several washes but showed a significant drop after that, suggesting a potential cut-off point around this threshold (Bubun *et al.*, 2024). It is imperative to understand these dynamics so as to determine when nets fall below protective efficacy levels for policy decisions regarding net distribution and replacement campaigns.

Furthermore, comparative studies on different net brands have reported similar trends. A study conducted in Papua New Guinea found that long-lasting insecticidal nets (LLINs) with varying treatments also exhibited significant mortality declines

post-wash (Vinit *et al.*, 2020). This work highlights that while some nets may start strong, the durability of the insecticidal effect is significantly influenced by both the type of insecticide and the wash cycles endured.

5.4.3.2 Yorkool Nets

Yorkool nets exhibited lower mortality rates across all mosquito colonies, indicating a compromised wash resistance. This diminished efficacy, particularly against the KGB strain and the limited impact on SENN DDT and FuMoz Base colonies, underscores the challenges in sustaining insecticidal activity following repeated washes. Notably, at baseline levels, some nets did not achieve 100% mortality. This could be attributed to several factors, including inherent variability in the insecticidal formulation, potential resistance mechanisms present in the mosquito populations, and the possibility of suboptimal contact with the insecticide due to net degradation or improper usage.

Furthermore, the observed increase in mortality rates after the 20th wash may suggest a potential regeneration of insecticidal properties due to factors such as the leaching of residual insecticide or changes in the physical structure of the net that enhance its insecticidal action. While this study did not directly assess regeneration mechanisms, other research has indicated that repeated exposure to washing can sometimes lead to a temporary increase in mortality, potentially due to the resuspension of insecticide particles or changes in the net's surface that affect mosquito behaviour (Syme *et al.*, 2024). Additionally, it is important to the dynamics of insecticide retention and efficacy over time, as studies suggest that continuous

washing may disrupt the insecticide matrix, leading to unexpected outcomes (Dame *et al*. 2015).

These findings underscore the complexity of wash resistance and the multifaceted interactions between insecticides and net materials. Moreover, factors such as washing technique, water quality, and the physical integrity of the nets significantly influence the outcomes of wash resistance assessments (Castellanos *et al.*, 2021). Further research is necessary to explore these regeneration effects and their implications for the long-term efficacy of treated nets in controlling mosquito populations.

5.4.4 Implications for Malaria Control Programs

The observed variations in wash resistance underscore the critical importance of considering local mosquito resistance profiles when selecting long-lasting insecticide nets (LLINs) for malaria control programs. This study revealed that while Royal Sentry exhibited initial efficacy, its effectiveness declined significantly against resistant mosquito populations over time (Ngongang-Yipmo *et al.,* 2022). Specifically, the rate of decline in efficacy was noteworthy, suggesting that even initially effective LLINs may lose their protective capabilities more rapidly than previously understood.

In contrast, the performance of Yorkool, which was below expectations, emphasises the need for continuous monitoring and evaluation of LLINs to ensure their sustained effectiveness in real-world conditions (Rafinejad *et al.*, 2008; Raghavendra *et al*., 2017). This study not only confirms existing knowledge about insecticide resistance but also contributes new insights into the dynamics of wash resistance. For instance,

quantifying the rate of decline in LLIN efficacy allows us national malaria control programs to propose timelines for the replacement or retreatment of nets, which is important for optimizing malaria control strategies.

Moreover, the complexities of wash resistance dynamics reveal that factors such as environmental conditions, the frequency of washing, and the specific mosquito resistance mechanisms involved can influence net performance (Syme *et al.*, 2024). This suggests that LLIN choices should be tailored not just to local vector resistance patterns but also to local washing practices and climatic conditions. For example, regions with high rainfall or humidity may experience different degradation rates of LLINs compared to drier climates, affecting their longevity and effectiveness (Pinchoff *et al.*, 2015).

In terms of practical implications, this study findings advocate for the establishment of localized monitoring programs that assess both insecticide resistance and the physical integrity of LLINs over time. This proactive approach could guide decisions on when to replace nets or switch to alternative insecticide formulations, ultimately improving the overall effectiveness of malaria control initiatives.

In line with the existing literatures, this study highlights the urgent need for adaptive strategies in malaria control programs (Nkahe *et al.*, 2024). Knowledge on the nature of wash resistance deepens comprehension of LLIN performance and informs more effective public health interventions. Future studies should focus on establishing standardized protocols for evaluating the rate of decline in LLIN effectiveness, thereby providing essential data that can be used to enhance malaria prevention strategies across diverse settings.

5.4.5 Limitations and Future Directions

This study investigated the efficacy of long-lasting insecticide-treated bed nets (LLINs) in a local context, with a focus on the washing protocols commonly used in households. While the research presents some limitations, such as the reliance on WHOPES published data for the regeneration time of Royal Sentry and Yorkool LLINs, and the use of a standard household soap instead of the recommended Savlon de Marseille, the interpretation and significance of these findings remain robust.

Regarding the lack of direct measurement of the regeneration time of the LLINs following initial washing, this study relied on data sourced from the WHO Pesticide Evaluation Scheme (WHOPES), which may not accurately reflect local conditions or practices (WHO, 2010). Previous studies have indicated significant variability in regeneration time based on factors such as water temperature, detergent type, and washing frequency (Atieli *et al.*, 2010; Syme *et al.*, 2024) This reliance on external data may lead to discrepancies in understanding the performance of ITNs in the specific context of our study, potentially skewing the results and their applicability to local settings.

Lastly, the study used a common household soap (Sunlight tablet soap) rather than the recommended Savlon de Marseille, which is the standard soap suggested by WHO for efficacy studies in phase I (WHO, 2013). While this choice aimed at providing locally relevant results, it may introduce variability that could impact the study's findings. Previous research indicates that the type of detergent used can significantly affect the residual insecticide levels on ITNs, thereby influencing their efficacy against mosquito populations (Ouattara *et al.*, 2013). This could have a potential impact on the generalisability of the results. Moreover, household soaps

may contain additional surfactants that could interact differently with the insecticide, potentially leading to over or underestimation of the net's effectiveness.

While models have been developed for predicting the impact of decay in bed-net efficacy on malaria transmission (Ngonghala *et al.,*2014), there is a lack of models for exploring the relationship between washes and efficacy, and the rate of this decline. We propose a future study which can develop a model that can predict how long a net performs optimally before its efficacy falls below a critical point.

5.5 Conclusion

In conclusion, this study sheds light on the complex dynamics of wash resistance in Royal Sentry and Yorkool nets. The findings emphasise the importance of a detailed approach to LLIN selection, considering both initial efficacy and sustained wash resistance against resistant mosquito populations. As malaria control programs evolve, ongoing research and surveillance will be crucial to adapt strategies and optimise the effectiveness of LLIN interventions.

Chapter 6: Assessing the Relationship Between Insecticide Resistance and Sporozoite Rates

6.1 Introduction

Insecticides have been a cornerstone for malaria vector control through the scale up on long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS). However, widespread insecticide resistance across malaria vector populations threaten to compromise the efficacy on insecticide based tools such as ITNs and IRS (reference). Despite its epidemiological importance, few studies have investigated the relationship between insecticide resistance and sporozoite rates in malaria vectors (Minetti *et al*., 2020). Conflicting findings remain among the few studies that have looked at the potential relationship between insecticide resistance and parasite infection rates; with some studies reporting higher infection rates among resistant mosquitoes than susceptible ones (Alout *et al*., 2013; Ndiath *et al*., 2014; Kabula *et al.,* 2016; Nkemngo *et al*., 2020). However, some studies report lower infection rates among resistant mosquitoes unlike among resistant ones (Lo and Coetzee, 2013). In line with the Malawi National Malaria Control Program (NMCP), which aims to eliminate malaria by 2030 (The PMI VectorLink Project, 2022), it is essential to understand the intensity of resistance in major malaria vectors and the relationship between sporozoite infection and resistance intensity to develop tailored interventions that support the elimination target outlined in the NMCP 2020–2030 policy.

To investigate the effect of resistance on malaria transmission, resistance data were associated with sporozoite infection data, using the Chi-square test of association and logistic regression analyses (WHO, 2018). The Chi-square test of independence

was performed on samples that were exposed to the diagnostic concentration (1X DC) of recommended insecticides to examine the relationship between phenotypic resistance, as indicated by bioassay results after 24 hours from exposure, and sporozoite infection rates as determined by ELISA. The null hypothesis stated no association between bioassay mortality (a proxy indicator for resistance) and sporozoite positivity rate. The alternative hypothesis was that there was an association between bioassay mortality and sporozoite positivity rates.

6.2 Methods

In chapter three, the intensity of resistance in the local population of *An. funestus* was determined. The current chapter determines the sporozoite rates in the same field-collected specimens. The two variables are then checked for association using Chi-square analysis to establish if observed resistance levels are correlated with mosquito infectivity rates.

6.2.1 Detection of Insecticide Resistance

Resistance to insecticides was investigated phenotypically in wild-collected non-blood-fed female adults of *An. funestus*. WHO susceptibility tests and CDC bottle assays were used to detect phenotypic resistance, as discussed in Chapter 3.

6.2.2 Molecular Species Identification

Samples were initially identified morphologically as belonging to the *An. funestus* group or the *An. gambiae* complex (Gillies and Coetzee, 1987). Further analysis by polymerase chain reaction (PCR) was conducted to determine their species identity. Members of the *An. funestus* group of species were identified using procedures outlined by (Koekemoer *et al.,* 2002) and (Cohuet *et al.,* 2003) as indicated in Table 6.1. No molecular or diagnostic tests were carried out on species morphologically identified as *An. gambiae* complex because of the minimal numbers collected.

DNA was extracted from a single leg or wing of each *An. funestus* s.l. specimen using an Insect DNA extraction procedure adapted from ZyGEM *prep*GEM™ Insect Kit (Cat No.: PIN00200; ZyGEM, Vienna, Austria) (www.zygem.com). Each leg or wing was placed in a 20 µL DNA-free PCR tube, to which were added 8.75 µL of

PCR grade water, 1 µL of 10X buffer and 0.25 µL of *prep*GEM™ enzyme (Table 6.1). For this leg/wing DNA extraction, grinding of the specimen was not needed. Using a thermal cycler, the tubes were incubated at 75oC for 15 minutes and 95oC for 5 minutes. After incubation, the sample solution (DNA) was ready for PCR and stored at -20ºC.

The following reagents and volumes were used for the preparation of the reaction.

Table 6.1 Reagents and sequences of primers used.

Reagent	1 reaction vol. (µL)	Primer sequence (5'→3')	Identified Species	Product size(bp)[1]
10X Buffer	1.25			
dNTP	1.00			
$MgCl_2$	0.75			
Primer UN	1.00	TGT GAA CTG CAG GAC ACA T	--	
FUN	1.00	GCA TCG ATG GGT TAA TCA TG	*An. funestus*	505
VAN	1.00	TGT CGA CTT GGT AGC CGA AC	*An. vaneedeni*	587
RIV	1.00	CAA GCC GTT CGA CCC TGA TT	*An. rivulorum*	411
PAR	1.00	TGC GGT CCC AAG CTA GGT TC	*An. parensis*	252
LEES	1.00	TAC ACG GGC GCC ATG TAG TT	*An. leesoni*	146
RIVLIKE	1.00	CCG CCT CCC GTG GAG TGG GGG	*An. rivulorum-like*	313
Taq (5U)	0.10			
dH_2O	1.90			
DNA Template	0.50			
Total	12.50			

[1]bp= base pairs. Adapted from Cohuet *et. al.* (2003).

The following thermo-cycler conditions were used:

Step 1: 1 cycle at 94^{o}C for 2 minutes

Step2: 30 cycles of: 94^{o}C for 30 seconds, 45^{o}C for 30 seconds, 72^{o}C for 40 seconds

Step 3: 1 cycle at 72^{o}C for 5 minutes

Step 4: Hold at 8^{o}C.

After completion of the PCR cycles, samples were electrophoresed on 10% Agarose gel mixed with 2 drops of Ethidium Bromide for viewing of bands under ultraviolet

light. Electrophoresis was run at 100 volts until separation of loading dye (about 60 minutes) and then bands were visualised under UV light in a Gel Doc system. Each gel had a molecular weight marker, two negative controls (one for DNA extraction and another for the PCR reaction), and positive controls for *An. funestus s.s*. and at least one other member of the *An. funestus* group. The resulting amplicons were sized against the molecular weight marker and scored against the corresponding base pair sizes of members of the *An. funestus* group (Cohuet *et al.,* 2003).

6.2.3 Sporozoite Detection

An enzyme-linked immunosorbent assay (ELISA) technique, which detects circumsporozoite proteins in malaria-infected mosquitoes, was used to detect malaria infectivity in mosquito specimens (Benedict, 2007). Abdomens were removed before the assay to avoid detecting *Plasmodium* in the midgut or blood meal, as the assay can detect these parasite stages (Benedict, 2007). This ensured that the only positive detections would be from the salivary glands and the thorax, where the parasite's infective stage migrates to.

After dissections, the specimens were homogenised in phosphate-buffered saline (PBS, pH 7.2) buffer. An ELISA plate was coated with monoclonal antibody for circumsporozoite protein, incubated overnight, and then washed. The homogenate was then added to the wells and incubated for 1-2 hours to allow for binding after which step, the plate was washed again to remove unbound material. A secondary antibody was added, and the plate incubated, and washed again. Antibodies were obtained from the National Institute of Allergy and Infectious Diseases: Pf-sporozoite ELISA Reagent Kit, MRA-890 contributed by Robert A. Wirtz (Mwamba *et al.,* 2024). Lastly, a substrate was added to produce a detectable colour change the optical

density (OD) of which was then read under a spectrophotometer. The samples were categorised as positive or negative using critical value thresholds calculated by comparing the OD values of the positive and negative controls.

However, to avoid detecting non-target antigens that cause false positivity, all csp-ELISA positive results were heated in a heat block at 100 ^{o}C for 10 minutes. This step eliminated false CSP-ELISA positives while the true positives remained positive (Durnez *et al.,* 2011).

6.2.4 Data Analysis

To investigate the relationship between mortality status and sporozoite positivity in mosquitoes, a Chi-square test of association was conducted. This test assessed whether there was a significant association between mortality (categorized as dead or alive) and sporozoite positivity outcome (positive or negative). The observed frequencies of sporozoite-positive and sporozoite-negative cases were compared across the two mortality categories. The Chi-square statistic and corresponding p-value were calculated to evaluate whether the differences in sporozoite positivity across the mortality groups were statistically significant, with a p-value less than 0.05 indicating a significant association.

In addition to the Chi-square analysis, a binomial logistic regression was performed to identify risk factors associated with sporozoite positivity. The outcome variable was sporozoite positivity (1 = positive, 0 = negative), while the independent variables included mortality status (1 = dead, 0 = alive), insecticide type (categorized as alpha-cypermethrin, bendiocarb, deltamethrin, or permethrin), and collection location (village: Chakanira or Sisewu).

The model employed was a binomial logistic regression, as the dependent variable was binary. Each independent variable was treated as categorical, and interaction terms between insecticide type and mortality status were tested to assess their combined effects on sporozoite positivity.

The model's accuracy was evaluated using the Hosmer-Lemeshow test to confirm the adequacy of the model fit; odds ratios (OR) with 95% confidence intervals were computed for each independent variable to quantify their associations with sporozoite positivity.

6.3 Results

The circumsporozoite (csp) ELISA assay detected a sporozoite positivity rate of 11.5% (n=400) after a confirmation step. Table 6.2 below summarises the number of samples that survived or did not survive exposure to given doses of pyrethroid insecticides, including their corresponding sporozoite test outcomes via ELISA. The table also indicates the percentage of positives from each category of dead or alive samples following bioassays at various insecticide doses.

Table 6.2 Summary of numbers of *An. funestus* tested for sporozoites and insecticide susceptibility at various doses

Insecticide Dose#	**Bioassay result**	**csp-ELISA result**		
		Negative	Positive n (%)*	**Total**
1X				
	Alive	66	2 (3%)	68
	Dead	75	14 (15.7%)	89
2.5X				
	Alive	13	1 (7%)	14
	Dead	69	7 (9.2%)	76
5X				
	Alive	8	0 (0)	8
	Dead	67	15 (18.3%)	82
10X				
	Alive	0	0 (0)	0

	Dead	56	7 (11.1%)	63
Total		354	46 (11.5%)	400

**row percentage for sporozoite positives*
[#]Results for pyrethroid insecticides pooled together (deltamethrin, permethrin and alpha-cypermethrin

The Chi-square test of association was used to investigate the association between bioassay results (alive or dead) and sporozoite test outcomes (positive or negative) for samples exposed to the diagnostic concentration (1X DC). The test found a statistically significant association between bioassay results and sporozoite outcomes ($X^2_{(1, N=157)} = 6.889$, p= 0.00,9), as indicated in Tables 6.3 and 6.4 below.

Table 6.3 Chi-square test of association between bioassay mortality and sporozoite test outcome

Bioassay result	**csp-ELISA result**		**Total**
	Negative	Positive	
Alive	66	2	68
	61.070	6.930	
	0.3980	3.5071	
Dead	75	14	89
	79.930	9.070	
	0.3041	2.6796	
Total	141	16	157

Cell Contents
Observed count
Expected count
Contribution to Chi-square

Table 6.4 The Chi-square test

Chi-Square Test	**Chi-Square**	**DF**	**P-Value**
Pearson	6.889	1	0.009
Likelihood Ratio	7.882	1	0.005

Using logistic regression analysis, the association between the independent variables, mortality, insecticide used and village versus the outcome variable, sporozoite positivity rate, was also investigated (Table 6.5). This was done for all the samples exposed to all insecticide concentrations, as presented in Table 6.5.

Table 6.5 below summarises the logistic regression analysis, reporting adjusted odds ratio, 95% confidence intervals and p-values for each category tested.

Table 6.5. Logistic regression analysis of risk factors for sporozoite positivity

Parameter	aOR*	95% Confidence Interval		*P*-value
Mortality				
Dead	**4.803**	**1.251**	**18.442**	**0.0223**
Alive	1.00			
Insecticides				
Alpha-cypermethrin	2.283	0.856	6.086	0.2561
Bendiocarb	1.483	0.422	5.213	0.8591
Deltamethrin	1. 932	0.830	4.498	0.4792
Permethrin	1.00			
Village				
Chakanira	**4.612**	**1.319**	**16.124**	**0.0167**
Sisewu	1.00			

*aOR=adjusted Odds Ratio

Similar to the Chi-square test reported in Table 6.2 above, table 6.3 above also shows an association between bioassay mortality and sporozoite positivity rate, with the adjusted odds ratio being 4.8 times higher among dead specimens, as compared to survivors (alive) (aOR = 4.803, p-value= 0.022). As such, the null hypothesis was rejected, and the alternative hypothesis was adopted. There was no statistically significant association between the insecticide used and the sporozoite outcome (using a significance threshold of alpha= 0.05). Lastly, sporozoite positivity was also

significantly associated with the village of sample collection, with an adjusted odds ratio of 4.61 (p-value = 0.017). Chakanira village had significantly higher sporozoite rates than Sisewu (aOR= 4.6). The final sporozoite positivity rate after confirmation (heating step followed by a re-run) was 11.5% (N=400).

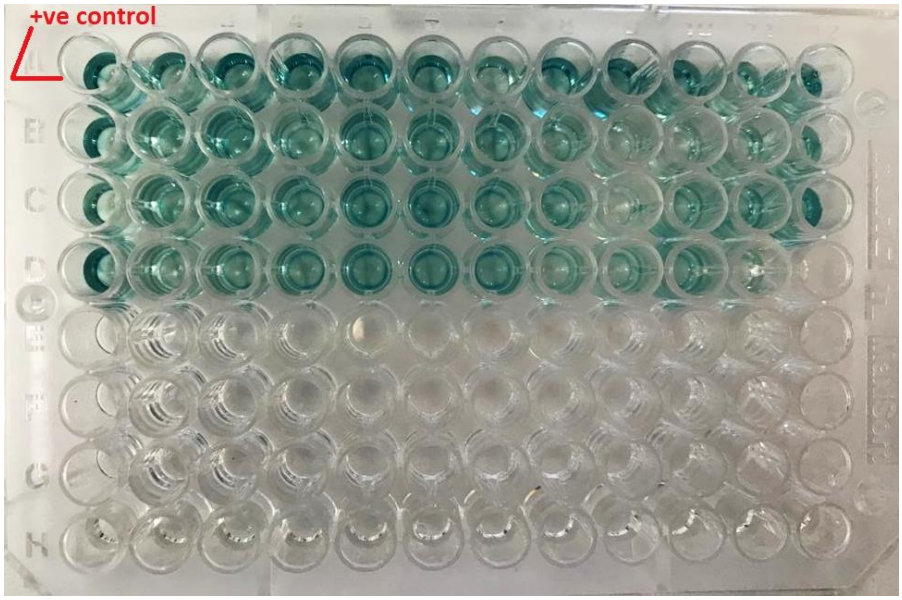


Figure 6.1 Showing an ELISA plate with results of a re-run of all positive samples after a heating step to eliminate false positives. There were 46 positive specimens out of 400 tested samples, representing a positivity rate of 11.5%.

6.4 Discussion

The Chi-square and logistic regression analyses both revealed an association between phenotypic resistance, measured by bioassay mortality, and sporozoite infection (Tables 6.3 and 6.4). This corroborates with findings from a study done in Tanzania by Pinda *et al.,* (2022) which found that older mosquitoes were more susceptible than younger mosquitoes. The study further found that the older mosquitoes had significantly higher sporozoite rates than younger mosquitoes (Pinda *et al*., 2022). However, the direction of this association was unexpected as it was initially hypothesised that resistant mosquitoes would have better survival rates, and hence higher sporozoite carriage. Contrary to this expectation, our study found higher sporozoite rates among the dead specimens (non-resistant mosquitoes) compared to bioassay survivors (resistant mosquitoes), as indicated in Tables 6.3 and 6.5.

While this finding initially seemed counterintuitive, a similar observation was reported in another study, which found that delayed mortality effects due to resistance led to a lower potential for malaria transmission (Viana *et al.,* 2016). Another study done in South Africa reported low numbers of oocysts and sporozoites *(Plasmodium berghei infection*) in pyrethroids-resistant laboratory strains of *An. funestus*, than in susceptible strains (Lo and Coetzee, 2013). Such findings may partly explain the paradoxical observation of the continued effectiveness of long-lasting insecticidal nets (LLINs) even when used against resistant vector populations. Our findings may suggest the presence of a potential fitness cost among resistant vectors, as they tended to exhibit lower sporozoite rates, as shown in Table 6.3.

The acquisition of new traits by insects may negatively affect their development or reproduction. Studies by Belinato and Martins (2016) and Kliot and Ghanim (2012) suggested that overexpression of detoxification enzymes and target-site mutations can be costly to insects, affecting their reproductive potential, dispersal ability, and general fitness or development. A potential limitation of this study is that, despite observing lower sporozoite rates among bioassay survivors, we did not further determine development fitness nor lifespan longevity. As has been noted earlier, older mosquitoes tend to be more susceptible to insecticides than younger mosquitoes, which intrinsically have lower parity rates and hence bear lower transmission potential compared to older mosquitoes (Pinda *et al,* 2022).

Our study did not find evidence to suggest that resistance is related to higher transmission rates. This may indicate that although resistance is observed, it might not yet have an observable impact on transmission rates, and thus, interventions could remain effective. There was insufficient evidence of compromised transmission control directly associated with resistance, supporting the continued efficacy of interventions. Indeed, Okumu (2020) noted that even an untreated net, if properly used, may yield protection levels as high as 60%, emphasising the encouragement for the continued use of LLINs even in areas with resistant vector populations (Clarke *et al.,* 2001; Mwangi *et al.,* 2003).

Despite the significance of our findings, caution is warranted due to several factors. Firstly, the relatively lower numbers of specimens collected in the field may have influenced the results of the statistical tests, potentially failing to detect a plausible association between resistance and sporozoite rates. Sporozoite rates in the field

generally tend to be low (<1%), requiring large sample sizes (e.g., several thousands) to establish meaningful differences or achieve sufficient statistical power (Mayagaya *et al.,* 2015). However, despite the lower number of specimens collected in our study, a sporozoite rate of 11.5% (n=400) was notably high.

To put this into perspective, historical data from a pyrethrum house spraying program in Natal (South Africa) in 1935 reported sporozoite infection rates ranging from 2.86 to 7.89% in *An. gambiae* s.l. The overall sporozoite infection rate over a six-month period was 3.62% (out of 276 mosquitoes collected). According to the PMI VectorLink Annual Entomological Monitoring Report (2018-2019) for Malawi, sporozoite infections across three sentinel districts ranged from 2.4 to 10% in the same period the study was conducted. The overall sporozoite rates were 1.3% for *An. gambiae* s.l and 3.6% for *An. funestus* s.l. Therefore, our finding of an 11.5% (n=400) sporozoite positivity rate for *An. funestus* in Chikwawa demonstrates a considerably high sporozoite positivity rate despite the fewer mosquitoes collected.

While our study presents unexpected findings regarding the association between insecticide resistance and sporozoite infection, the observed paradoxical results align with similar patterns noted in the literature discussed above. The potential fitness cost among resistant vectors and the continued effectiveness of interventions warrant further investigation, emphasising the complex dynamics of vector control in the context of evolving resistance.

6.5 Conclusion

In conclusion, while the current study did not detect evidence of compromised transmission control associated with insecticide resistance in the local *An. funestus* population, it offers valuable insights into the entomological impact of resistance in Chikwawa district. Future investigations should prioritise examining the development and fitness costs of resistance compared to susceptible mosquitoes. Moreover, it is crucial to determine the epidemiological impact by correlating changes in the actual number of clinical cases within the human population with the observed resistance.

Notwithstanding its limitations, this study provides a foundation for further research on the dynamics of insecticide resistance and its impact. Future studies should examine the specific insights gained from this investigation and explore the subtle aspects of resistance in *An. funestus* populations. This will contribute to a more comprehensive understanding of the implications of insecticide resistance for malaria transmission control in the region.

Chapter 7. Key Findings and Potential Implications

7.1 Summary of Key Findings

The study revealed a concerning escalation in resistance among *An. funestus* mosquitoes to pyrethroids. High resistance intensity to alpha-cypermethrin and moderate resistance to deltamethrin and permethrin were observed. Complete restoration of susceptibility required a 10X dose of deltamethrin and permethrin, categorising the resistance as moderate (Venter *et al.,* 2017; Kumala *et al.,* 2022). Additionally, low mortality to bendiocarb, a carbamate, suggested potential resistance. However, the population showed complete susceptibility to DDT and organophosphates. The study emphasises the urgent need for a robust resistance management strategy, prompting further research on additional vectors and alternative insecticides to safeguard malaria control efforts in Malawi (Kumala *et al.,* 2022).

Regarding LLINs, the comparative analysis between Yorkool and Royal Sentry nets indicated the superior performance of Royal Sentry against both susceptible *An. gambiae* Kisumu and wild *An. funestus*. However, wash resistance assessments revealed a decline in Royal Sentry's efficacy, especially against pyrethroid-resistant mosquitoes, emphasising the complexity of LLIN selection. Yorkool nets displayed compromised wash resistance, urging continuous monitoring for sustained effectiveness. Newer generation LLINs incorporating dual active ingredients or a synergist like PBO would offer an interim reasonable solution for resistance management. The study underscores the importance of considering local mosquito resistance profiles and tailoring LLIN choices accordingly.

Unexpected findings emerged in assessing the relationship between insecticide resistance and sporozoite rates. Despite the initial hypothesis of better survival rates correlating with higher sporozoite carriage in resistant mosquitoes, the study reports higher sporozoite rates among non-resistant specimens. While no evidence suggests compromised transmission control due to resistance, the study offers valuable insights into the entomological impact of resistance. Future research should focus on resistance development and fitness costs, correlating these with changes in clinical cases to comprehensively understand the implications for malaria transmission control in the region.

7.2 Potential Implications of This Study

The potential implications of the key findings are multi-faceted and can significantly impact malaria control strategies and public health interventions:

1. **Resistance Management Strategies:** The identification of high resistance intensity to certain insecticides underscores the urgency of implementing effective resistance management strategies. This may include rotating different classes of insecticides, combining multiple interventions, and exploring alternative vector control methods to mitigate the spread of resistance.
2. **LLIN Selection:** The comparative analysis of LLINs highlights the importance of selecting LLINs based on local vector resistance profiles. Understanding how different LLIN brands perform against both susceptible and resistant mosquito populations can inform more targeted and effective vector control interventions.

3. **Wash Resistance Considerations:** The findings on LLIN wash resistance emphasise the need for continuous monitoring of LLIN efficacy, particularly in areas with high levels of insecticide resistance. This underscores the importance of selecting LLINs with sustained wash resistance to ensure long-term effectiveness in malaria control programs.
4. **Impact on Transmission Dynamics:** The unexpected relationship between insecticide resistance and sporozoite rates raises questions about the potential impact of resistance on malaria transmission dynamics. Further research is needed to determine the epidemiological implications of resistance and its effects on malaria transmission, particularly in terms of clinical case numbers and disease burden.
5. **Public Health Policy and Interventions:** These findings have implications for public health policy and intervention strategies. They highlight the need for adaptive management approaches that consider local vector resistance profiles, incorporate evidence-based decision-making, and prioritise interventions that are effective against resistant mosquito populations.

7.3 Conclusion

Overall, these key findings provide valuable insights into the challenges posed by insecticide resistance in malaria vector control and underscore the importance of tailored and evidence-based strategies for combating malaria transmission.

References

Abeku, T., Mzilahowa, T., Beard, J., Yihdego, Y., Dandalo, L., Mokuena, M. and Burnett, J. (2019) 'Insecticide Resistance Management Plan, Malawi, 2019 – 2022', *Malaria Consortium*.

Abeku, T., Mzilahowa, T., Yihdego, Y., Dandalo, L., Burnett, J. and Mokuena, M. (2020) 'Integrated Vector Control Strategy for Malaria Control, Malawi, 2020–2024', *Malaria Consortium*.

Achulu, M., Ndungu, P., Kamau, L. and Gichuki, P.M. (2024) 'Field evaluation of Yorkool®, a long-lasting insecticidal net after 12 months of use in Seme, Kisumu County, Kenya', *International Journal of Advanced Research*, 7(1), pp.333-346.

Ahogni, I.B., Salako, A.S., Akinro, B., Sovi, A., Gnanguenon, V., Azondekon, R., Dagnon, J.F., Akogbeto, P., Tokponon, F. and Akogbeto, M.C. (2020) 'Physical integrity and survivorship of long-lasting insecticidal nets distributed to households of the same socio-cultural community in Benin, West Africa', *Malaria Journal*, 19, pp.1-13.

Alout, H., Ndam, N.T., Sandeu, M.M., Djegbe, I., Chandre, F., Dabiré, R.K., Djogbenou, L.S., Corbel, V. and Cohuet, A. (2013) 'Insecticide resistance alleles affect vector competence of *Anopheles gambiae* ss for *Plasmodium falciparum* field isolates', *PLoS One*, 8(5), p.e63849.

Atieli, F.K. *et al.* (2010) 'Wash durability and optimal drying regimen of four brands of long-lasting insecticide-treated nets after repeated washing under tropical

conditions', *Malaria Journal*, 9(1), pp. 1–10. Available at: https://doi.org/10.1186/1475-2875-9-248.

Atoyebi S.M. (2019) 'Molecular Mechanisms of Multiple Insecticide Resistance in *Anopheles funestus* sensu stricto at Akaka-Remo Southwestern Nigeria', *(Doctoral dissertation).*

Apperson, C. S. and Georghiou, G. P. (1975) 'Mechanisms of resistance to organophosphorus insecticides in Culex tarsalis', *Journal of Economic Entomology,* 68(2), pp. 153-157. doi: 10.1093/jee/68.2.153.

Bagi, J., Grisales, N., Corkill, R., Morgan, J. C., N'Falé, S., Brogdon, W. G. and Ranson, H. (2015) 'When a discriminating dose assay is not enough: measuring the intensity of insecticide resistance in malaria vectors', *Malaria Journal,* 14(1), pp. 1-9.

Benedict, M. (2007) 'Methods in Anopheles research', *Atlanta, USA: Malaria Research and Reference Reagent Resource Center*.

Bennett, A., Kazembe, L., Mathanga, D. P., Kinyoki, D., Ali, D., Snow, R. W. and Noor, A. M. (2013) 'Mapping Malaria Transmission Intensity in Malawi, 2000–2010', *The American Journal of Tropical Medicine and Hygiene,* 89(5), pp. 840-849.

Bergé, J., Feyereisen, R. and Amichot, M. (1998) 'Cytochrome P450 monooxygenases and insecticide resistance in insects', *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 353(1376), pp. 1701-1705.

Bhatt, S., Weiss, D., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., Battle, K., Moyes, C., Henry, A. and Eckhoff, P. (2015) 'The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015', *Nature,* 526(7572), pp. 207-211.

Birkholtz, L.-M., Bornman, R., Focke, W., Mutero, C. and de Jager, C. (2012) 'Sustainable malaria control: transdisciplinary approaches for translational applications', *Malaria Journal,* 11(1), pp. 431.

Bisset, J., Rodríguez, M., Díaz, C., Ortiz, E., Marquetti, M. and Hemingway, J. (1990) 'The mechanisms of organophosphate and carbamate resistance in Culex quinquefasciatus (Diptera: Culicidae) from Cuba', *Bulletin of Entomological Research,* 80(03), pp. 245-250.

Briët, O. J. and Chitnis, N. (2013) 'Effects of changing mosquito host searching behaviour on the cost effectiveness of a mass distribution of long-lasting, insecticidal nets: a modelling study', *Malaria Journal,* 12(1), pp. 1.

Brogdon, W. and Chan, A. (2010) 'Guideline for evaluating insecticide resistance in vectors using the CDC bottle bioassay', *USA: CDC Atlanta*.

Brooke, B., Kloke, G., Hunt, R., Koekemoer, L., Tem, E., Taylor, M., Small, G., Hemingway, J. and Coetzee, M. (2001) 'Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae)', *Bulletin of Entomological Research,* 91(04), pp. 265-272.

Burke, A., Dahan-Moss, Y., Duncan, F., Qwabe, B., Coetzee, M., Koekemoer, L. and Brooke, B. (2019) '*Anopheles parensis* contributes to residual malaria transmission in South Africa', *Malaria Journal,* 18(1).

Bubun, N. *et al.* (2024) ‘Insufficient duration of insecticidal efficacy of Yahe® insecticide-treated nets in Papua New Guinea’, *Malaria Journal*, 23(1), pp. 1–10. Available at: https://doi.org/10.1186/s12936-024-05005-x.

Casimiro, S., Coleman, M., Mohloai, P., Hemingway, J. and Sharp, B. (2006) 'Insecticide resistance in *Anopheles funestus* (Diptera: culicidae) from Mozambique', *Journal of Medical Entomology,* 43(2), pp. 267-275.

Castellanos, M.E., Rodas, S., Juárez, J.G., Lol, J.C., Chanquin, S., Morales, Z., Vizcaino, L., Smith, S.C., Vanden Eng, J., Woldu, H.G. and Lenhart, A., (2021) ‘Evaluation of the durability of long-lasting insecticidal nets in Guatemala’, *Malaria Journal*, 20(1), pp.1-14.

Chanda, E., Mzilahowa, T., Chipwanya, J., Mulenga, S., Ali, D., Troell, P., Dodoli, W., Govere, J. M. and Gimnig, J. (2015) 'Preventing malaria transmission by indoor residual spraying in Malawi: grappling with the challenge of uncertain sustainability', *Malaria Journal,* 14(1).

Chen, A. and Rogan, W. J. (2003) 'Nonmalarial Infant Deaths and DDT Use for Malaria Control', *Emerging Infectious Diseases,* 9(8), pp. 960-964.

Chen, H., Githeko, A. K., Githure, J. I., Mutunga, J., Zhou, G. and Yan, G. (2008) 'Monooxygenase levels and knockdown resistance (kdr) allele frequencies in *Anopheles gambiae* and *Anopheles arabiensis* in Kenya', *Journal of Medical Entomology,* 45(2), pp. 242-250.

Chouaïbou, M.S., Fodjo, B.K., Fokou, G., Allassane, O.F., Koudou, B.G., David, J.P., Antonio-Nkondjio, C., Ranson, H. and Bonfoh, B. (2016) ‘Influence of the agrochemicals used for rice and vegetable cultivation on insecticide

resistance in malaria vectors in southern Côte d'Ivoire', *Malaria Journal*, 15, pp.1-12.

Clarke, S.E., Bøgh, C., Brown, R.C., Pinder, M., Walraven, G.E. and Lindsay, S.W. (2001) 'Do untreated bednets protect against malaria?', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *95*(5), pp.457-462.

Coetzee, M. and Koekemoer, L. L. (2013) 'Molecular Systematics and Insecticide Resistance in the Major African Malaria Vector *Anopheles funestus*', *Annual Review of Entomology,* 58(1), pp. 393-412.

Coetzee, M., Kruger, P., Hunt, R., Durrheim, D., Urbach, J. and Hansford, C. (2013) 'Malaria in South Africa: 110 years of learning to control the disease', *SAMJ: South African Medical Journal,* 103(10), pp. 770-778.

Cohee, L.M., Goupeyou-Youmsi, J., Seydel, K.B., Mangani, C., Ntenda, P., Sixpence, A., Mbewe, R.B., Matengeni, A., Takala-Harrison, S., Walker, E.D. and Wilson, M.L. (2022) 'Understanding the intransigence of malaria in Malawi', *The American Journal of Tropical Medicine and Hygiene*, *107*(4 Suppl), p.40.

Cohuet, A., Simard, F., Toto, J. C., Kengne, P., Coetzee, M. and Fontenille, D. (2003) 'Species identification within the *Anopheles funestus* group of malaria vectors in Cameroon and evidence for a new species', *American Journal of Tropical Medicine and Hygiene,* 69.

Curtis, C., Miller, J., Hodjati, M. H., Kolaczinski, J. and Kasumba, I. (1998) 'Can anything be done to maintain the effectiveness of pyrethroid–impregnated

bednets against malaria vectors?', *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 353(1376), pp. 1769-1775.

Curtis, C. F., Jana-Kara, B. and Maxwell, C. A. (2003) 'Insecticide treated nets: impact on vector populations and relevance of initial intensity of transmission and pyrethroid resistance', *Journal of Vector Borne Diseases,* 40(1-2), pp. 1-8.

Diabaté, A., Baldet, T., Chandre, F., Dabire, K., Simard, F., Ouedraogo, J., Guillet, P. and Hougard, J. (2004) 'First report of a kdr mutation in *Anopheles arabiensis* from Burkina Faso, West Africa', *Journal of the American Mosquito Control Association,* 20(2), pp. 195-196.

Djouaka, R. F., Bakare, A. A., Coulibaly, O. N., Akogbeto, M. C., Ranson, H., Hemingway, J. and Strode, C. (2008) 'Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae* ss from Southern Benin and Nigeria', *BMC genomics,* 9(1), pp. 538.

Durnez, L., Van Bortel, W., Denis, L., Roelants, P., Veracx, A., Trung, H. D., Sochantha, T. and Coosemans, M. (2011) 'False positive circumsporozoite protein ELISA: a challenge for the estimation of the entomological inoculation rate of malaria and for vector incrimination', *Malaria Journal,* 10(1), pp. 195.

Fadel, A.N., Ibrahim, S.S., Tchouakui, M., Terence, E., Wondji, M.J., Tchoupo, M., Wanji, S. and Wondji, C.S. (2019) ‘A combination of metabolic resistance and high frequency of the 1014F kdr mutation is driving pyrethroid resistance in *Anopheles coluzzii* population from Guinea savanna of Cameroon’, *Parasites & Vectors,* 12, pp.1-13.

Gari, T. and Lindtjørn, B. (2018) 'Reshaping the vector control strategy for malaria elimination in Ethiopia in the context of current evidence and new tools: opportunities and challenges', *Malaria Journal,* 17(1), pp. 454.

Gatton, M. L., Chitnis, N., Churcher, T., Donnelly, M. J., Ghani, A. C., Godfray, H. C. J., Gould, F., Hastings, I., Marshall, J. and Ranson, H. (2013) 'The importance of mosquito behavioural adaptations to malaria control in Africa', *Evolution,* 67(4), pp. 1218-1230.

Gillies, M. and Coetzee, M. (1987) 'A supplement to the Anophelinae of Africa south of the Sahara (Afrotropical region)', *South African Institute for Medical Research*.

Gleave, K., Lissenden, N., Chaplin, M., Choi, L. and Ranson, H. (2021) 'Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa', *Cochrane Database of Systematic Reviews,* (5), pp. Cd012776.

Glunt, K. D., Oliver, S. V., Hunt, R. H. and Paaijmans, K. P. (2018) 'The impact of temperature on insecticide toxicity against the malaria vectors *Anopheles arabiensis* and *Anopheles funestus*', *Malaria Journal,* 17(1), pp. 1-8.

Govella, N. J., Chaki, P. P. and Killeen, G. F. (2013) 'Entomological surveillance of behavioural resilience and resistance in residual malaria vector populations', *Malaria Journal,* 12(1), pp. 1-9.

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

Hemingway, J., Ranson, H., Magill, A., Kolaczinski, J., Fornadel, C., Gimnig, J., Coetzee, M., et al. (2016) ‘Averting a malaria disaster: will insecticide resistance derail malaria control?.’ *The Lancet* 387, no. 10029 (2016): pp. 1785-1788.

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

Hemingway, J., Hawkes, N. J., McCarroll, L. and Ranson, H. (2004) 'The molecular basis of insecticide resistance in mosquitoes', *Insect Biochemistry and Molecular Biology,* 34(7), pp. 653-665.

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

Hunt, R., Edwardes, M. and Coetzee, M. (2010) 'Pyrethroid resistance in southern African *Anopheles funestus* extends to Likoma Island in Lake Malawi', *Parasites & Vectors,* 3(1), pp. 1.

Hunt, R. H., Brooke, B. D., Pillay, C., Koekemoer, L. L. and Coetzee, M. (2005) 'Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*', *Medical and Veterinary Entomology,* 19(3), pp. 271-5.

IRAC (2020) ‘Insecticide Resistance Training Basic Module: Crop Protection’, *Insecticide Resistance Action Committee,* https://irac-online.org/documents/insecticide-resistance-training-basic-module/?ext=pdf (Accessed 31 January 2024).

Juache-Villagrana, A.E., Pando-Robles, V., Garcia-Luna, S.M., Ponce-Garcia, G., Fernandez-Salas, I., Lopez-Monroy, B., Rodriguez-Sanchez, I.P. and Flores, A.E. (2022) ‘Assessing the impact of insecticide resistance on vector competence: a review’, *Insects*, 13(4), p.377.

Jobe, N. B., Huijben, S., & Paaijmans, K. P. (2023) ‘Non-target effects of chemical malaria vector control on other biological and mechanical infectious disease vectors’, *The Lancet Planetary Health*, *7*(8), e706-e717.

Kabange, S. C. (2011) ‘African Countries Debate Using DDT in Anti-Malaria Efforts’, Zambia: *VOA News*, https://www.voanews.com/africa/african-countries-debate-using-ddt-anti-malaria-efforts (Accessed 31 July 2021).

Kabula, B., Tungu, P., Rippon, E. J., Steen, K., Kisinza, W., Magesa, S., Mosha, F. and Donnelly, M. J. (2016) 'A significant association between deltamethrin resistance, *Plasmodium falciparum* infection and the Vgsc-1014S resistance mutation in *Anopheles gambiae* highlights the epidemiological importance of resistance markers', *Malaria Journal,* 15(1), pp. 1.

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

Killeen, G. F. (2014) 'Characterizing, controlling and eliminating residual malaria transmission', *Malaria Journal,* 13, pp. 330.

Killeen, G.F., Govella, N.J., Lwetoijera, D.W. and Okumu, F.O. (2016) ‘Most outdoor malaria transmission by behaviourally-resistant *Anopheles arabiensis* is mediated by mosquitoes that have previously been inside houses’, *Malaria Journal*, 15, pp.1-10.

Kleinschmidt, I., Bradley, J., Knox, T. B., Mnzava, A. P., Kafy, H. T., Mbogo, C., Ismail, B. A., Bigoga, J. D., Adechoubou, A., Raghavendra, K., Cook, J., Malik, E. M., Nkuni, Z. J., Macdonald, M., Bayoh, N., Ochomo, E., Fondjo, E., Awono-Ambene, H. P., Etang, J., Akogbeto, M., Bhatt, R. M., Chourasia, M. K., Swain, D. K., Kinyari, T., Subramaniam, K., Massougbodji, A., Okê-Sopoh, M., Ogouyemi-Hounto, A., Kouambeng, C., Abdin, M. S., West, P., Elmardi, K., Cornelie, S., Corbel, V., Valecha, N., Mathenge, E., Kamau, L., Lines, J. and Donnelly, M. J. (2018) 'Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: a WHO-coordinated, prospective, international, observational cohort study', *The Lancet Infectious Diseases,* 18(6), pp. 640-649.

Kleinschmidt, I., Mnzava, A. P., Kafy, H. T., Mbogo, C., Bashir, A. I., Bigoga, J., Adechoubou, A., Raghavendra, K., Knox, T. B., Malik, E. M., Nkuni, Z. J., Bayoh, N., Ochomo, E., Fondjo, E., Kouambeng, C., Awono-Ambene, H. P., Etang, J., Akogbeto, M., Bhatt, R., Swain, D. K., Kinyari, T., Njagi, K., Muthami, L., Subramaniam, K., Bradley, J., West, P., Massougbodji, A., Oke-Sopoh, M., Hounto, A., Elmardi, K., Valecha, N., Kamau, L., Mathenge, E. and Donnelly, M. J. (2015) 'Design of a study to determine the impact of insecticide resistance on malaria vector control: a multi-country investigation', *Malaria Journal,* 14, pp. 282.

Kleinschmidt, I., Sharp, B., Benavente, L. E., Schwabe, C., Torrez, M., Kuklinski, J., Morris, N., Raman, J. and Carter, J. (2006) 'Reduction in infection with Plasmodium falciparum one year after the introduction of malaria control interventions on Bioko Island, Equatorial Guinea', *The American Journal of Tropical Medicine and Hygiene,* 74(6), pp. 972-978.

Kobylinski, K. (2011) 'Ivermectin mass drug administration to humans for malaria parasite transmission control*', Colorado State University*.

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

Kouassi, B. L., Edi, C., Tia, E., Konan, L. Y., Akré, M. A., Koffi, A. A., Ouattara, A. F., Tanoh, A. M., Zinzindohoue, P., Kouadio, B., Andre, M., Irish, S. R., Armistead, J., Dengela, D., Cissé, N. G., Flatley, C. and Chabi, J. (2020) 'Susceptibility of *Anopheles gambiae* from Côte d'Ivoire to insecticides used on insecticide-treated nets: evaluating the additional entomological impact of piperonyl butoxide and chlorfenapyr', *Malaria Journal,* 19(1).

Kumala, J., Koekemoer, L.L., Coetzee, M. and Mzilahowa, T. (2022) 'Intensity of insecticide resistance in the major malaria vector Anopheles funestus from Chikwawa, rural Southern Malawi', *Parasites & Vectors*, 15(1), p. 220.

Kulkarni, M. A., Rowland, M., Alifrangis, M., Mosha, F. W., Matowo, J., Malima, R., Peter, J., Kweka, E., Lyimo, I. and Magesa, S. (2006) 'Occurrence of the leucine-to-phenylalanine knockdown resistance (kdr) mutation in *Anopheles*

arabiensis populations in Tanzania, detected by a simplified high-throughput SSOP-ELISA method', *Malaria Journal,* 5(1), pp. 56.

Lindsay, S.W., Thomas, M.B. and Kleinschmidt, I. (2021) 'Threats to the effectiveness of insecticide-treated bednets for malaria control: thinking beyond insecticide resistance', *The Lancet Global Health*, *9*(9), pp.e1325-e1331.

Lines, J., Myamba, J. and Curtis, C. (1987) 'Experimental hut trials of permethrin-impregnated mosquito nets and eave curtains against malaria vectors in Tanzania', *Medical and Veterinary Entomology,* 1(1), pp. 37-51.

Lo, T.M. and Coetzee, M. (2013) 'Marked biological differences between insecticide resistant and susceptible strains of *Anopheles funestus* infected with the murine parasite *Plasmodium berghei,' Parasites & Vectors*, 6, pp.1-7.

Lyons, C.L., Coetzee, M., Terblanche, J.S. and Chown, S.L. (2012) 'Thermal limits of wild and laboratory strains of two African malaria vector species, *Anopheles arabiensis* and *Anopheles funestus'*, *Malaria Journal,* 11(1), pp.1-14.

Maharaj, R., Mthembu, D. and Sharp, B. (2008) 'Impact of DDT re-introduction on malaria transmission in KwaZulu-Natal', *South African Medical Journal,* 95(11), pp. 871.

Mangani, C., Mzilahowa, T., Cohee, L., Kayange, M., Ntenda, P., Sixpence, A., Gumbo, A., Lankhulani, S., Goupeyou-Youmsi, J., Walker, E. and Laufer, M. (2022) 'Malawi ICEMR malaria research: interactions and results influencing health policies and practices', *The American Journal of Tropical Medicine and Hygiene*, 107(4 Suppl), p.49.

Mapua, S.A., Hape, E.E., Kihonda, J., Bwanary, H., Kifungo, K., Kilalangongono, M., Kaindoa, E.W., Ngowo, H.S. and Okumu, F.O. (2022) 'Persistently high proportions of plasmodium-infected *Anopheles funestus* mosquitoes in two villages in the Kilombero valley, South-Eastern Tanzania', *Parasite Epidemiology and Control,* 18, p.e00264.

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

Mathenge, E. M., Gimnig, J. E., Kolczak, M., Ombok, M., Irungu, L. W. and Hawley, W. A. (2001) 'Effect of permethrin-impregnated nets on exiting behavior, blood feeding success, and time of feeding of malaria mosquitoes (Diptera: Culicidae) in western Kenya', *Journal of Medical Entomology,* 38(4), pp. 531-536.

Mayagaya, V.S., Nkwengulila, G., Lyimo, I.N., Kihonda, J., Mtambala, H., Ngonyani, H., Russell, T.L. and Ferguson, H.M. (2015) 'The impact of livestock on the abundance, resting behaviour and sporozoite rate of malaria vectors in southern Tanzania', *Malaria Journal*, 14, pp.1-14.

Mechan, F., Katureebe, A., Tuhaise, V., Mugote, M., Oruni, A., Onyige, I., Bumali, K., Thornton, J., Maxwell, K., Kyohere, M. and Kamya, M.R. (2022) 'LLIN evaluation in Uganda project (LLINEUP): The fabric integrity, chemical content and bioefficacy of long-lasting insecticidal nets treated with and without piperonyl butoxide across two years of operational use in Uganda', *Current Research in Parasitology & Vector-borne Diseases*, 2(1), p.100092.

Mbogo, C., Baya, N., Ofulla, A., Githure, J. and Snow, R. (1996) 'The impact of permethrin-impregnated bednets on malaria vectors of the Kenyan coast', *Medical and Veterinary Entomology,* 10(3), pp. 251-259.

Moiroux, N., Gomez, M. B., Pennetier, C., Elanga, E., Djènontin, A., Chandre, F., Djègbé, I., Guis, H. and Corbel, V. (2012) 'Changes in *Anopheles funestus* biting behavior following universal coverage of long-lasting insecticidal nets in Benin', *Journal of Infectious Diseases,* 206(10), pp. 1622-1629.

Moss, W. J., Dorsey, G., Mueller, I., Laufer, M. K., Krogstad, D. J., Vinetz, J. M., Guzman, M., Rosas-Aguirre, A. M., Herrera, S., Arevalo-Herrera, M., Chery, L., Kumar, A., Mohapatra, P. K., Ramanathapuram, L., Srivastava, H. C., Cui, L., Zhou, G., Parker, D. M., Nankabirwa, J. and Kazura, J. W. (2015) 'Malaria Epidemiology and Control Within the International Centers of Excellence for Malaria Research', *American Journal of Tropical Medicine and Hygiene,* 93(3 Suppl), pp. 5-15.

Mutuku, F.M., Khambira, M., Bisanzio, D., Mungai, P., Mwanzo, I., Muchiri, E.M., King, C.H. and Kitron, U., (2013) 'Physical condition and maintenance of mosquito bed nets in Kwale County, coastal Kenya', *Malaria Journal*, 12, pp.1-14.

Musa, J.J., Moore, S., Moore, J., Mbuba, E., Mbeyela, E., Kobe, D., Swai, J.K. and Odufuwa, O.G., (2020) 'Long-lasting insecticidal nets retain bio-efficacy after five years of storage: implications for malaria control programmes', *Malaria Journal,* 19(110), https://doi.org/10.1186/s12936-020-03183-y.

Mwamba, T.M., Dahan-Moss, Y., Munhenga, G., Maposa, I. and Koekemoer, L.L. (2024) ‘Host Preferences and Impact of Climate on Blood Feeding in *Anopheles funestus* Group from South Africa’, *Tropical Medicine and Infectious Disease*, *9*(10), p.251.

Mwangi, T. W., Ross, A., Marsh, K. and Snow, R. W. (2003) 'The effects of untreated bednets on malaria infection and morbidity on the Kenyan coast', *Transactions of the Royal Society of Tropical Medicine Hygiene,* 97(4), pp. 369-372.

Mzilahowa, T., Ball, A., Bass, C., Morgan, J., Nyoni, B., Steen, K., Donnelly, M. and Wilding, C. (2008) 'Reduced susceptibility to DDT in field populations of *Anopheles quadriannulatus* and *Anopheles arabiensis* in Malawi: evidence for larval selection', *Medical and Veterinary Entomology,* 22(3), pp. 258-263.

Mzilahowa, T., Chiumia, M., Mbewe, R. B., Uzalili, V. T., Luka-Banda, M., Kutengule, A., Mathanga, D. P., Ali, D., Chiphwanya, J., Zoya, J., Mulenga, S., Dodoli, W., Bergeson-Lockwood, J., Troell, P., Oyugi, J., Lindblade, K. and Gimnig, J. E. (2016) 'Increasing insecticide resistance in *Anopheles funestus* and *Anopheles arabiensis* in Malawi, 2011–2015', *Malaria Journal,* 15(1).

Mzilahowa, T., Hastings, I. M., Molyneux, M. E. and McCall, P. J. (2012) 'Entomological indices of malaria transmission in Chikhwawa district, Southern Malawi', *Malaria Journal,* 11(1), pp. 1-9.

Namango, I.H. (2024) ‘Monitoring and control of *Anopheles* vectors in the context of residual malaria transmission in Ulanga’, *Tanzania (Doctoral dissertation, University of Basel Associated Institution*).

Nardini, L., Christian, R.N., Coetzer, N., Ranson, H., Coetzee, M. and Koekemoer, L.L. (2012) 'Detoxification enzymes associated with insecticide resistance in laboratory strains of *Anopheles arabiensis* of different geographic origin', *Parasites & Vectors*, 5(1), pp.1-12.

Nauen, R. (2007) 'Insecticide resistance in disease vectors of public health importance', *Pest Management Science,* 63(7), pp. 628-633.

Ndiath, M.O., Cailleau, A., Diedhiou, S.M., Gaye, A., Boudin, C., Richard, V. and Trape, J.F. (2014) 'Effects of the kdr resistance mutation on the susceptibility of wild *Anopheles gambiae* populations *to Plasmodium falciparum*: a hindrance for vector control', *Malaria Journal*, 13, pp.1-8.

News24 (2010) 'Malawian MPs in bizarre debate over the use of DDT to fight malaria', *News24*. Johannesburg, South Africa, https://www.news24.com/news24/Archives/City-Press/Malawian-MPs-in-bizarre-debate-over-use-of-DDT-to-fight-malaria-20150430 (Accessed 7 February 2024).

Ngongang-Yipmo, E.S., Tchouakui, M., Menze, B.D., Mugenzi, L.M., Njiokou, F. and Wondji, C.S., (2022) 'Reduced performance of community bednets against pyrethroid-resistant *Anopheles funestus* and *Anopheles gambiae*, major malaria vectors in Cameroon', *Parasites & Vectors*, 15(1), p.230.

Ngonghala, C.N., Del Valle, S.Y., Zhao, R. and Mohammed-Awel, J., (2014) 'Quantifying the impact of decay in bed-net efficacy on malaria transmission', *Journal of Theoretical Biology*, 363(1), pp.247-261.

Ngufor, C., Agbevo, A., Fagbohoun, J., Fongnikin, A. and Rowland, M. (2020) ‘Efficacy of Royal Guard, a new alpha-cypermethrin and pyriproxyfen treated mosquito net, against pyrethroid-resistant malaria vectors’, *Scientific Reports*, 10(1), p.12227.

Nkahe, D.L., Kopya, E., Siewe, N.I.N., Mbiakop, P.N., Chouakeu, N.A.K., Mimpfoundi, R., Kekeunou, S., Awono-Ambene, P. and Antonio-Nkondjio, C. (2024) ‘Durability of PBO nets (Olyset Plus®), 12 months after their distribution in Bertoua, Cameroon’, *Parasite Epidemiology and Control*, 26, p.e00373.

Nkemngo, F., Mugenzi, L.M., Terence, E., Niang, A., Wondji, M., Tchoupo, M., Nguete, N.D., Tchapga, W., Irving, H., Ntabi, J.D. and Agonhossou, R. (2020) ‘Elevated Plasmodium sporozoite infection and multiple insecticide resistance in the principal malaria vectors *Anopheles funestus* and *Anopheles gambiae* in a forested locality close to the Yaoundé airport, Cameroon’, *Wellcome Open Research*, 5, p.146.

Obala, A.A., Mangeni, J.N., Platt, A., Aswa, D., Abel, L., Namae, J. and Prudhomme O'Meara, W. (2015) ‘What is threatening the effectiveness of insecticide-treated bednets? A case-control study of environmental, behavioral, and physical factors associated with prevention failure’, *PloS One*, 10(7), p.e0132778.

Ochomo, E., Chahilu, M., Cook, J., Kinyari, T., Bayoh, N.M., West, P., Kamau, L., Osangale, A., Ombok, M., Njagi, K. and Mathenge, E. (2017) ‘Insecticide-treated nets and protection against insecticide-resistant malaria vectors in Western Kenya’, *Emerging Infectious Diseases*, 23(5), p.758.

Ogola, E.O., Fillinger, U., Ondiba, I.M., Villinger, J., Masiga, D.K., Torto, B. and Tchouassi, D.P. (2018) ‘Insights into malaria transmission among *Anopheles funestus* mosquitoes, Kenya’, *Parasites & Vectors*, 11, pp.1-10.

Okia, M., Ndyomugyenyi, R., Kirunda, J., Byaruhanga, A., Adibaku, S., Lwamafa, D.K. and Kironde, F. (2013) ‘Bioefficacy of long-lasting insecticidal nets against pyrethroid-resistant populations of Anopheles gambiae ss from different malaria transmission zones in Uganda’, *Parasites & Vectors,* 6(1), pp.1-10.

Okiro, E., Noor, A., Malinga, J., Mitto, B., Mundia, C., Mathanga, D., Mzilahowa, T., and Snow, R. J., (2014) 'An epidemiological profile of malaria and its control in Malawi', *the Roll Back Malaria Partnership and the Department for International Development*

Okumu, F. (2020) 'The fabric of life: what if mosquito nets were durable and widely available but insecticide-free?', *Malaria Journal,* 19(1), pp. 1-29.

Okumu, F. O. and Moore, S. J. (2011) 'Combining indoor residual spraying and insecticide-treated nets for malaria control in Africa: a review of possible outcomes and an outline of suggestions for the future', *Malaria Journal,* 10(208), pp. 13.

Ondeto, B.M. (2023) ‘Malaria Transmission Dynamics in Sentinel Sites and Mixed Crop Irrigation Scheme in Western Kenya’, Doctoral dissertation, *University of Nairobi)*.

Ossè, R.A., Aïkpon, R., Sovi, A., Padonou, G.G., Oké-Agbo, F., Gnanguenon, V. and Akogbéto, M. (2013) ‘Long lasting insecticidal nets use, efficacy and physical

integrity in a vector resistance area after a nationwide campaign in southern Benin, West Africa', Journal of Public Health and Epidemiology, 5(1), pp.325-35.

Ouattara, J.P.N. *et al.* (2013) 'Comparison of the Laboratory Standard Washing Using CIPAC Washing Agent and the Domestic Washing on Three Recommended Types of Long-Lasting Insecticidal Mosquito Nets', *PLoS ONE*, 8(10). Available at: https://doi.org/10.1371/journal.pone.0074824.

Oxborough, R.M. (2015) 'Laboratory and experimental hut evaluation of mosquito net and indoor residual spray (IRS) insecticides for improved malaria control', *(Doctoral dissertation, London School of Hygiene & Tropical Medicine).*

Pates, H. and Curtis, C., (2005) 'Mosquito behavior and vector control', *Annual Review of Entomology*, 50, pp. 53-70.

Penilla, P. R., Rodriguez, A. D., Hemingway, J., Torres, J. L., ARREDONDO-JIMÉNEZ, J. I. and Rodriguez, M. H. (1998) 'Resistance management strategies in malaria vector mosquito control. Baseline data for a large-scale field trial against *Anopheles albimanus* in Mexico', *Medical and Veterinary Entomology,* 12(3), pp. 217-233.

Pinchoff, J. *et al.* (2015) 'Factors associated with sustained use of long-lasting insecticide-treated nets following a reduction in malaria transmission in southern Zambia', *American Journal of Tropical Medicine and Hygiene*, 93(5), pp. 954–960. Available at: https://doi.org/10.4269/ajtmh.15-0093.

Pinto, J., Lynd, A., Elissa, N., Donnelly, M., Costa, C., Gentile, G., Caccone, A. and Rosário, V. (2006) 'Co-occurrence of East and West African kdr mutations

suggests high levels of resistance to pyrethroid insecticides in *Anopheles gambiae* from Libreville, Gabon', *Medical and Veterinary Entomology,* 20(1), pp. 27-32.

PMI VectorLink Project (2019) ‘Monitoring Durability of Yorkool and Royal Sentry Nets Distributed Nationwide in 2016 in Malawi’, Rockville, MD, U.S. *Abt Associates*.

Protopopoff, N., Mosha, J. F., Lukole, E., Charlwood, J. D., Wright, A., Mwalimu, C. D., Manjurano, A., Mosha, F. W., Kisinza, W. and Kleinschmidt, I. (2018) 'Effectiveness of a long-lasting piperonyl butoxide-treated insecticidal net and indoor residual spray interventions, separately and together, against malaria transmitted by pyrethroid-resistant mosquitoes: a cluster, randomised controlled, two-by-two factorial design trial', *The Lancet,* 391(10130), pp. 1577-1588.

Rafinejad, J., Vatandoost, H., Nikpoor, F., Abai, M.R., Shaeghi, M., Duchen, S. and Rafi, F., (2008) ‘Effect of washing on the bio-efficacy of insecticide-treated nets (ITNs) and long-lasting insecticidal nets (LLINs) against main malaria vector *Anopheles stephensi* by three bioassay methods’, *Journal of Vector Borne Diseases*, 45(2), p.143.

Raghavendra, K., Chourasia, M.K., Swain, D.K., Bhatt, R.M., Uragayala, S., Dutta, G.D.P. and Kleinschmidt, I., (2017) ‘Monitoring of long-lasting insecticidal nets (LLINs) coverage versus utilization: a community-based survey in malaria endemic villages of Central India’, *Malaria Journal*, 16(1), pp.1-8.

Ramanantsoa, A., Wilson-Barthes, M., Rahenintsoa, R., Hoibak, S., Ranaivoharimina, H., Rahelimalala, M.D., Rakotomanga, A., Finlay, A., Muela Ribera, J. and Peeters Grietens, K., (2017) ‘Can the collection of expired long-lasting insecticidal nets reduce their coverage and use? Sociocultural aspects related to LLIN life cycle management and use in four districts in Madagascar’, *Malaria Journal*, 16(1), pp.1-9.

Ranson, H., Abdallah, H., Badolo, A., Guelbeogo, W. M., Kerah-Hinzoumbé, C., Yangalbé-Kalnoné, E., Sagnon, N. f., Simard, F. and Coetzee, M. (2009) 'Insecticide resistance in *Anopheles gambiae*: data from the first year of a multi-country study highlight the extent of the problem', *Malaria Journal,* 8(1), pp. 299.

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

Ranson, H. and Lissenden, N. (2016) 'Insecticide resistance in African anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control', *Trends in Parasitology,* 32(3), pp. 187-196.

Ranson, H., N'Guessan, R., Lines, J., Moiroux, N., Nkuni, Z. and Corbel, V. (2011) 'Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control?', *Trends in Parasitology,* 27(2), pp. 91-8.

Reddy, M. R., Overgaard, H. J., Abaga, S., Reddy, V. P., Caccone, A., Kiszewski, A. E. and Slotman, M. A. (2011) 'Outdoor host seeking behaviour of *Anopheles*

gambiae mosquitoes following initiation of malaria vector control on Bioko Island, Equatorial Guinea', *Malaria Journal,* 10(1), pp. 1.

Rehwagen, C. (2006) 'WHO recommends DDT to control malaria', *BMJ (Clinical Research ed.),* 333(7569), pp. 622-622.

Reimer, L., Fondjo, E., Patchoké, S., Diallo, B., Lee, Y., Ng, A., Ndjemai, H. M., Atangana, J., Traore, S. F. and Lanzaro, G. (2008) 'Relationship between kdr mutation and resistance to pyrethroid and DDT insecticides in natural populations of *Anopheles gambiae'*, *Journal of Medical Entomology,* 45(2), pp. 260-266.

Riveron, J. M., Chiumia, M., Menze, B. D., Barnes, K. G., Irving, H., Ibrahim, S. S., Weedall, G. D., Mzilahowa, T. and Wondji, C. S. (2015) 'Rise of multiple insecticide resistance in *Anopheles funestus* in Malawi: a major concern for malaria vector control', *Malaria Journal,* 14(1), pp. 1-9.

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

Riveron, J. M., Ibrahim, S. S., Chanda, E., Mzilahowa, T., Cuamba, N., Irving, H., Barnes, K. G., Ndula, M. and Wondji, C. S. (2014) 'The highly polymorphic CYP6M7 cytochrome P450 gene partners with the directionally selected CYP6P9a and CYP6P9b genes to expand the pyrethroid resistance front in the malaria vector *Anopheles funestus* in Africa', *BMC Genomics,* 15(1), pp. 1.

Riveron, J. M., Irving, H., Ndula, M., Barnes, K. G., Ibrahim, S. S., Paine, M. J. and Wondji, C. S. (2013) 'Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector *Anopheles funestus*', *Proceedings of the National Academy of Sciences,* 110(1), pp. 252-257.

Sanou, A., Nelli, L., Guelbéogo, W.M., Cissé, F., Tapsoba, M., Ouédraogo, P., Sagnon, N.F., Ranson, H., Matthiopoulos, J. and Ferguson, H.M. (2021) 'Insecticide resistance and behavioural adaptation as a response to long-lasting insecticidal net deployment in malaria vectors in the Cascades region of Burkina Faso', *Scientific Reports*, 11(1), p.17569.

Selvaraj P, Wenger EA, Bridenbecker D, Windbichler N, Russell JR, *et al.,* (2020) 'Vector genetics, insecticide resistance and gene drives: An agent-based modeling approach to evaluate malaria transmission and elimination', *PLOS Computational Biology,* 16(8): e1008121, https://doi.org/10.1371/journal.pcbi.1008121

Shah, M.P., Steinhardt, L.C., Mwandama, D., Mzilahowa, T., Gimnig, J.E., Bauleni, A., Wong, J., Wiegand, R., Mathanga, D.P. and Lindblade, K.A. (2020) 'The effectiveness of older insecticide-treated bed nets (ITNs) to prevent malaria infection in an area of moderate pyrethroid resistance: results from a cohort study in Malawi', *Malaria Journal*, 19(1), pp.1-12.

Sharp, B. L., Ridl, F. C., Govender, D., Kuklinski, J. and Kleinschmidt, I. (2007) 'Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea', *Malaria Journal,* 6(1), pp. 52.

Sokhna, C., Ndiath, M. and Rogier, C. (2013) 'The changes in mosquito vector behaviour and the emerging resistance to insecticides will challenge the decline of malaria', *Clinical Microbiology and Infection,* 19(10), pp. 902-907.

Source 1 URL: https://extranet.who.int/pqweb/vector-control-product/yorkool-ln

Source 2 URL: https://extranet.who.int/pqweb/vector-control-product/royal-sentry-20

Stone, B. and Brown, A. (1969) 'Mechanisms of resistance to fenthion in Culex pipiens fatigans Wied', *Bulletin of the World Health Organization,* 40(3), pp. 401.

Strode C, Donegan S, Garner P, Enayati AA, Hemingway J. (2014) 'The Impact of Pyrethroid Resistance on the Efficacy of Insecticide-Treated Bed Nets against African Anopheline Mosquitoes: Systematic Review and Meta-Analysis', *PLOS Medicine*, 11(3): e1001619, https://doi.org/10.1371/journal.pmed.1001619

Suh, P.F., Elanga-Ndille, E., Tchouakui, M. *et al.,* (2023) 'Impact of insecticide resistance on malaria vector competence: a literature review', *Malaria Journal,* 22(1), p. 19. https://doi.org/10.1186/s12936-023-04444-2

Syme, T. *et al.* (2024) 'Laboratory evaluation of the regeneration time, efficacy and wash-resistance of PermaNet Dual (a deltamethrin-chlorfenapyr net) against susceptible and pyrethroid-resistant strains of *Anopheles gambiae* sensu lato', *PLoS ONE*, 19(8), pp. 1–21. Available at: https://doi.org/10.1371/journal.pone.0298513.

Takken, W. (2002) 'Do insecticide-treated bednets have an effect on malaria vectors?', *Tropical Medicine & International Health,* 7(12), pp. 1022-30.

Tangena, J. A., Hendriks, C. M. J., Devine, M., Tammaro, M., Trett, A. E., Williams, I., DePina, A. J., Sisay, A., Herizo, R., Kafy, H. T., Chizema, E., Were, A., Rozier, J., Coleman, M. and Moyes, C. L. (2020) 'Indoor residual spraying for malaria control in sub-Saharan Africa 1997 to 2017: an adjusted retrospective analysis', *Malaria Journal,* 19(1), pp. 150.

Temu, E. A., Maxwell, C., Munyekenye, G., Howard, A. F., Munga, S., Avicor, S. W., Poupardin, R., Jones, J. J., Allan, R. and Kleinschmidt, I. (2012) 'Pyrethroid resistance in *Anopheles gambiae*, in Bomi County, Liberia, compromises malaria vector control', *PloS One,* 7(9), pp. e44986.

Lancet, The (2007) 'DDT for malaria control: the issue of trade', *The Lancet,* 369(9558), pp. 248.

The PMI VectorLink Project, 2019. *Malawi Entomological Monitoring Annual Report, July 1, 2018–June 30, 2019*, https://d1u4sg1s9ptc4z.cloudfront.net/uploads/2021/03/malawi-2019-entomological-monitoring-final-report.pdf (Accessed 2 February 2024)

The PMI VectorLink Project (2022) 'Malawi Entomological Monitoring Annual Report, July 1, 2021–June 30, 2022, https://d1u4sg1s9ptc4z.cloudfront.net/uploads/2023/01/Entomological-Monitoring-Report-Malawi-2022.pdf (Accessed 2 February 2024)

Thomsen, E. K., Strode, C., Hemmings, K., Hughes, A. J., Chanda, E., Musapa, M., Kamuliwo, M., Phiri, F. N., Muzia, L. and Chanda, J. (2014) 'Underpinning

sustainable vector control through informed insecticide resistance management', *PLoS One,* 9(6), pp. e99822.

U.S. President's Malaria Initiative Malawi Malaria Operational Plan FY 2024, https://d1u4sg1s9ptc4z.cloudfront.net/uploads/2023/01/FY-2023-Malawi-MOP.pdf (Accessed 3 February 2024).

van den Berg, H. (2009) 'Global status of DDT and its alternatives for use in vector control to prevent disease', *Environmental Health Perspectives,* 117(11), pp. 1656-1663.

van den Berg, H., da Silva Bezerra, H. S., Al-Eryani, S., Chanda, E., Nagpal, B. N., Knox, T. B., Velayudhan, R. and Yadav, R. S. (2021) 'Recent trends in global insecticide use for disease vector control and potential implications for resistance management', *Scientific Reports,* 11(1), pp. 23867.

Venter, N., Oliver, S. V., Muleba, M., Davies, C., Hunt, R. H., Koekemoer, L. L., Coetzee, M. and Brooke, B. D. (2017) 'Benchmarking insecticide resistance intensity bioassays for Anopheles malaria vector species against resistance phenotypes of known epidemiological significance', *Parasites & Vectors,* 10(1).

Verhaeghen, K., Van Bortel, W., Roelants, P., Backeljau, T. and Coosemans, M. (2006) 'Detection of the East and West African kdr mutation in *Anopheles gambiae* and *Anopheles arabiensis* from Uganda using a new assay based on FRET/Melt Curve analysis', *Malaria Journal,* 5(1), pp. 1.

Viana, M., Hughes, A., Matthiopoulos, J., Ranson, H. and Ferguson, H. M. (2016) 'Delayed mortality effects cut the malaria transmission potential of insecticide-

resistant mosquitoes', *Proceedings of the National Academy of Sciences,* 113(32), pp. 8975-8980.

Vinit, R. *et al.* (2020) 'Decreased bioefficacy of long-lasting insecticidal nets and the resurgence of malaria in Papua New Guinea', *Nature Communications*, 11(1), pp. 1–7. Available at: https://doi.org/10.1038/s41467-020-17456-2.

WHO (1957) "Expert Committee on Insecticides: seventh report' *Switzerland,* CID: 20.500.12592/tf0scs, https://policycommons.net/artifacts/573805/expert-committee-on-insecticides/1552363/ (Accessed 3 February 2024).

WHO (2012) 'Global Plan for Insecticide Resistance Management in malaria vectors', *WHO Global Malaria Programme,* Geneva, Switzerland.

WHO (2013) 'Guidelines for Laboratory and Field-Testing of Long-Lasting Insecticidal Nets', *World Health Organization*, Geneva, Switzerland.

WHO (2015) 'World Malaria Report 2015', *The World Health Organization*, Geneva, Switzerland.

WHO (2016) 'Malaria prevention through insecticide treated nets', *The World Health Organization*, Geneva, Switzerland.

WHO (2017) 'World Malaria Report 2017', *The World Health Organization*, Geneva, Switzerland.

WHO (2018) 'Test procedures for insecticide resistance monitoring in malaria vector mosquitoes', 2 ed., *World Health Organization,* Geneva, Switzerland.

WHO (2019a) 'Compendium of WHO malaria guidance: Prevention, diagnosis, treatment, surveillance and elimination', *World Health Organization,* Geneva, Switzerland.

WHO (2019b) 'Guidelines for malaria vector control', *World Health Organization*, Geneva, Switzerland.

WHO (2020) 'World Malaria Report 2020: 20 years of global progress and challenges', *World Health Organization,* Geneva, Switzerland.

WHO (2021a) 'Insecticide-treated nets for malaria transmission control in areas with insecticide-resistant mosquito populations: preferred product characteristics*', World Health Organization,* Geneva, Switzerland, p. 5.

WHO (2021b) 'World malaria report 2021', *World Health Organization,* Geneva, Switzerland.

WHO (2023a) 'List of prequalified vector control products', https://extranet.who.int/pqweb/vector-control-products/prequalified-product-list (Accessed 1 February 2024).

WHO (2023b) 'World malaria report 2023' *World Health Organization,* Geneva, Switzerland, Licence: CC BY-NC-SA 3.0 IGO.

Wondji, C. S., Coleman, M., Kleinschmidt, I., Mzilahowa, T., Irving, H., Ndula, M., Rehman, A., Morgan, J., Barnes, K. G. and Hemingway, J. (2012) 'Impact of pyrethroid resistance on operational malaria control in Malawi', *Proceedings of the National Academy of Sciences,* 109(47), pp. 19063-19070.

Wondji, C. S., Irving, H., Morgan, J., Lobo, N. F., Collins, F. H., Hunt, R. H., Coetzee, M., Hemingway, J. and Ranson, H. (2009) 'Two duplicated P450 genes are associated with pyrethroid resistance in *Anopheles funestus*, a major malaria vector', *Genome Research,* 19(3), pp. 452-459.

World Health Organization, (2010) 'WHO pesticide evaluation scheme: 50 [fifty] years of global leadership (No. WHO/HTM/NTD/WHOPES/2010.2)', *World Health Organization.*

Wood, O., Hanrahan, S., Coetzee, M., Koekemoer, L. and Brooke, B. (2010) 'Cuticle thickening associated with pyrethroid resistance in the major malaria vector *Anopheles funestus'*, *Parasites & Vectors,* 3(1), pp. 1.

Yang, G.-G., Kim, D., Pham, A. and Paul, C. J. (2018) 'A Meta-Regression Analysis of the Effectiveness of Mosquito Nets for Malaria Control: The Value of Long-Lasting Insecticide Nets', *International Journal of Environmental Research and Public Health,* 15(3), pp. 546.

Ye, Y. and Andrada, A. (2020) 'Estimating Malaria Incidence through Modeling Is a Good Academic Exercise, but How Practical Is It in High-Burden Settings?', *The American Journal of Tropical Medicine and Hygiene,* 102(4), pp. 701-702.

Yewhalaw, D., Asale, A., Tushune, K., Getachew, Y., Duchateau, L., & Speybroeck, N. (2012) 'Bio-efficacy of selected long-lasting insecticidal nets against pyrethroid resistant *Anopheles arabiensis* from South-Western Ethiopia', *Parasites & Vectors*, 5(1), pp. 1-9, https://doi.org/10.1186/1756-3305-5-159

Zamawe, C.O., Nakamura, K., Shibanuma, A. and Jimba, M. (2016) 'The effectiveness of a nationwide universal coverage campaign of insecticide-treated bed nets on childhood malaria in Malawi', *Malaria Journal*, 15, pp.1-8.

Zengenene, M.P., Munhenga, G., Chidumwa, G. and Koekemoer, L.L. (2021) 'Characterization of life-history parameters of an *Anopheles funestus* (Diptera: Culicidae) laboratory strain', *Journal of Vector Ecology*, 46(1), pp.24-29.

Appendices

I. Original Paper

II. Certificate of Ethical Clearance (Wits)

III. Certificate of Ethical Clearance (CoMREC

Parasites & Vectors

RESEARCH Open Access

Intensity of insecticide resistance in the major malaria vector *Anopheles funestus* from Chikwawa, rural Southern Malawi

Justin Kumala[1,2,3], Lizette L. Koekemoer[2,3], Maureen Coetzee[2,3] and Themba Mzilahowa[1*]

Abstract

Background: Malaria vector control using insecticide-based approaches has proven to be an effective strategy. However, widespread insecticide resistance among malaria vector populations across sub-Saharan Africa threatens to derail control efforts. This study was conducted in Chikwawa district, an area in rural southern Malawi characterised by persistent malaria transmission and reports of insecticide resistance in the local mosquito population. The aim of the was to characterise the intensity of insecticide resistance within a population of *Anopheles funestus* sensu lato (s.l.), a major vector of malaria in this district.

Methods: Live adult females belonging to the *An. funestus* group were collected from households by indoor aspiration. The CDC bottle assay was used for phenotypic quantification of resistance to deltamethrin, permethrin and alpha-cypermethrin at 1×, 2.5×, 5× and 10× the recommended diagnostic dose for each of these insecticides. WHO tube assays were used to determine susceptibility to bendiocarb, dichlorodiphenyltrichloroethane (DDT) and pirimiphos-methyl insecticides at diagnostic concentrations.

Results: *Anopheles funestus* s.l. exposed to 10× the recommended diagnostic dose was highly resistant to alpha-cypermethrin (mortality 95.4%); in contrast, mortality was 100% when exposed to both deltamethrin and permethrin at the same dose. Despite showing susceptibility to deltamethrin and permethrin at the 10× concentration, mortality at the 5× concentration was 96.7% and 97.1%, respectively, indicating moderate resistance to these two insecticides. WHO susceptibility assays indicated strong resistance against bendiocarb (mortality 33.8%, $n = 93$), whereas there was full susceptibility to DDT (mortality 98.9%, $n = 103$) and pirimiphos-methyl (mortality 100%, $n = 103$).

Conclusions: Strategies for managing resistance to insecticides, particularly against pyrethroids, must be urgently implemented to maintain the effectiveness of insecticide-based vector control interventions in the area. Such strategies include the wide-scale introduction of third-generation synergist insecticide-treated bed nets (ITNs) and next-generation dual active ingredient ITNs. The use of effective non-pyrethroids, such as pirimiphos-methyl, clothianidin and potentially DDT, could provide a window of opportunity for indoor residual spraying across the district. This strategy would support the current Malawi Insecticide Resistance Management Plan which aims at rotating insecticides to minimise selection pressure and slow down the evolution of resistance to approved insecticides. These actions will help to prevent malaria vector control failure and improve progress towards malaria elimination.

*Correspondence: tmzilahowa@mac.kuhes.ac.mw

[1] Malaria Alert Centre-Communicable Diseases Action Centre (MAC-CDAC), Kamuzu University of Health Sciences, Blantyre, Malawi
Full list of author information is available at the end of the article

Keywords: Pyrethroids, Susceptibility test, Bioassay, Insecticide-treated nets, Indoor residual spraying, Malaria transmission, Vector control, Sub-Saharan Africa

Background

Good progress had been made in reducing the burden of malaria in the African region over the past two decades, despite stalls in progress and an increase in cases between 2020 and 2021 associated with disruptions in the implementation of control programs due to the COVID-19 pandemic [1]. In the African region, it was estimated that *Plasmodium falciparum* prevalence declined by 50% while incidence decreased by 42% between 2000 and 2019 [2]. In Malawi, the implementation of evidence-based policies, including the provision of indoor residual spraying (IRS), insecticide-treated bed nets (ITNs) and intermittent preventive therapy for pregnant women (iPTp), has contributed to a decline in malaria prevalence from 44% in 2010 to 22% by 2017 [3]. Mean modelled prevalence rates of *P. falciparum* declined from 29% in 2010 to 15% in 2017, with the percent decline ranging from 3 to 79% in some areas [4]. However, these gains are threatened by increases of insecticide resistance in malaria vector populations, both locally and across sub-Saharan Africa [5–9].

In Malawi, resistance to at least one insecticide has been reported in many areas of the country, including resistance to multiple insecticides in Chikwawa district. Chikwawa is an area along the Lower Shire Valley. Malaria transmission is persistent in this area, and it is known to be among the highest transmission risk areas, with *P. falciparum* prevalence rates as high as 15% ($n = 9646$) among participants, as reported in a recent study [10]. *Anopheles funestus* sensu stricto (s.s.), the primary malaria vector in this district, has shown reduced susceptibility to pyrethroids and carbamates, with studies indicating that this resistance is enzyme-mediated [11–13]. *Anopheles arabiensis*, a secondary vector in the district, has also shown reduced levels of susceptibility to pyrethroids [11, 12, 14]. However, the extent to which the rise of insecticide resistance might be hampering malaria control efforts in the district remains under-explored.

The level of pyrethroid resistance in Malawian *An. funestus* sensu lato (s.l.) has slowly risen since it was first reported in 2008 by Hunt et al. [15]. In 2015, reports from Chikwawa district indicated an increase in multiple resistance mechanisms in the local *An. funestus* population, namely over-expression of the pyrethroid resistance genes *CYP6Pa/b* and *CYP7M7* and presence of the dieldrin resistance mutation (A296S-RDL) [13, 16, 17]. Such reports suggest that insecticide-based vector control tools (such as ITNs and IRS) may slowly lose their effectiveness over time, especially in the wake of multiple resistance, as recently reported in neighbouring Mozambique where the same resistance mechanisms in the local *An. funestus* population have induced a loss of efficacy of piperonyl-butoxide (PBO)-based ITNs [18], indicating other uncharacterised mechanisms at play. In Cote d'Ivoire, PBO pre-exposure of resistant *An. gambiae* s.l. did not yield full restoration of susceptibility to pyrethroids and neither was there full susceptibility to chlorfenapyr, a novel insecticide used for public health purposes in combination with pyrethroids in the newer generation ITNs [19].

There is a lack of compelling evidence-based data in Malawi on the nature of the relationship—if any—between insecticide resistance and the effect of ITNs on mosquito mortality in the field. Specifically, it is unknown whether malaria transmission control is compromised in people using ITNs and whether there is any relationship between mosquito resistance and epidemiological profiles of clinical malaria in the district. These factors are important in establishing the real impact of resistance to operational malaria control [20–22]. In this context, the aim of this study focused on determining the intensity of resistance of mosquitoes to deltamethrin, permethrin and alpha-cypermethrin (pyrethroids) and alternative non-pyrethroid insecticides. The study was part of a larger study which aimed at investigating the impact of insecticide resistance on vector control efforts in the district. The main driver of this study was the need to determine the operationally significant level of resistance that may serve as an early warning signal of potential loss of effectiveness in order to guide the local vector control intervention policy and minimise or prevent control failure.

Methods

Study design

This entomological study was conducted after the rainy season, between April and July 2018, across two malaria sentinel sites, Chakanira (15°58′54.1″ S, 34°48′09.1″ E) and Sisewu (16°04′39.9″ S, 34°49′39.9″ E) villages in Chikwawa district, southern Malawi (Fig. 1). Malaria

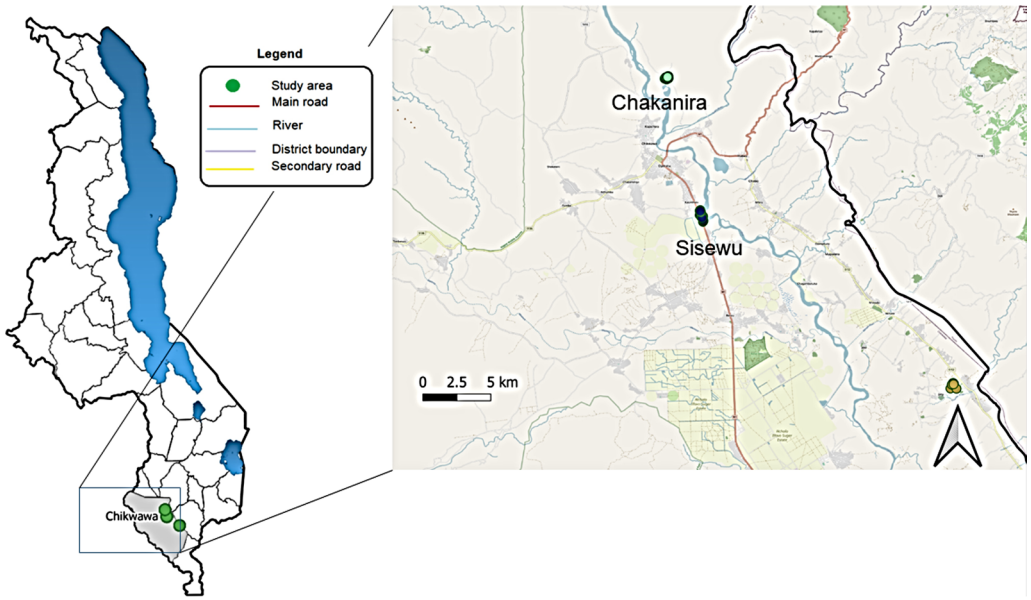


Fig. 1 Map of Malawi, showing the location of Chikwawa district and study sites (Source: QGIS 3.18.3)

transmission is perennial in this area, but cases peak at the height of the rainy season, usually between November and April [23].

Mosquito collections

Collections of live adult mosquitoes were carried out inside houses using a battery-powered Prokopak aspirator (John W Hock Co., Gainesville, FL, USA)). As this method collects resting mosquitoes, there was a chance that some of the captured mosquitoes would had a blood meal or a partial blood meal. Hence, following capture, all mosquitoes were held for 1–2 days to allow them to rest and also digest any existing blood meals, if any [24], ensuring that the mosquitoes used in bioassays were free from any blood meals. The absence of any blood meal was also verified visually prior to the bioassay. Following capture in the field, mosquitoes were placed in paper cups which were placed in cooler boxes and provided with a sugar solution prior to transport to the laboratory in Blantyre. The local vectors are *An. funestus* s.s. (most abundant) and *An. arabiensis* [25]. All mosquitoes used in this study were wild-caught female *An. funestus* group mosquitoes of unknown age; no specimens were reared in the laboratory or insectary for the insecticide susceptibility tests. The WHO protocol allows for the direct use of wild-caught mosquitoes for bioassays as they are operationally relevant [26].

CDC bottle assays

Tests were performed on three replicates of 20–25 wild-caught adult females of unknown age. These mosquitoes were exposed for 1 h in 250-ml Wheaton glass bottles coated with a pyrethroid insecticide (permethrin, deltamethrin or alpha-cypermethrin) at a concentration of 1×, 2.5× DC, 5× DC and 10× of the respective diagnostic concentration (DC). Final concentrations were: (i) deltamethrin: DC = 12.5 µg/ml, 2.5× DC = 31.3 µg/ml, 5× DC = 62.5 µg/ml, 10× DC = 125 µg/ml; (ii) permethrin: DC = 21.5 µg/ml, 2.5× DC = 53.8 µg/ml, 5× DC = 107.5 µg/l, 10× DC = 215 µg/ml; and (iii) alpha-cypermethrin: DC = 12.5 µg/ml, 2.5× DC = 31.3 µg/ml, 5× DC = 62.5 µg/ml, 10× DC = 125 µg/ml. Negative controls were bottles coated with acetone, the solvent used to dilute the test insecticides. Knockdown was recorded at 10-min intervals from the time of exposure until 1 h post-exposure, at which time mosquitoes were then transferred to holding cages. A small piece of cotton wool moistened with 10% sugar solution was provided to the mosquitoes during the recovery period after the 1-h exposure, until 24 h post-exposure [26]. Mortality was recorded at 24 h post-exposure. The strength of resistance, also called intensity, was characterised by comparing the mortality at 24 h for each of the insecticides at the

Table 1 CDC bottle assays for Chakanira and Sisewu villages

Village	Insecticide	Dose	No. mosquitoes exposed[a]	Unadjusted % mortality	Adjusted % mortality[b]
Chakanira	Control	0×	133	9.0	0.0
	Deltamethrin	1×	38	84.2	82.6
		2.5×	90	82.2	80.5
		5×	75	94.7	94.1
		10×	71	100.0	100.0
	Permethrin	1×	73	93.2	92.5
		2.5×	62	93.5	92.9
		5×	68	97.1	96.8
		10×	77	100.0	100.0
	Alpha-cypermethrin	1×	52	84.6	83.1
		2.5×	53	86.8	85.5
		5×	74	95.9	95.5
		10×	65	95.4	94.9
Sisewu[c]	Control	0×	25	8.0	0.0
	Deltamethrin	1×	21	90.5	89.6
		2.5×	25	92.0	91.3
		5×	38	94.7	94.3
		10×	17	100.0	100.0

[a] Low sample numbers could have affected these tests

[b] Adjusted mortality using Abbott's formula as described by WHO [26]

[c] Due to limited mosquito numbers collected from Sisewu village, susceptibility tests were only carried out on one insecticide, deltamethrin

various multiples of the diagnostic dose (Table 1). Resistance intensity was classified as low, moderate or high using a threshold of 90% for determining a resistant population at the diagnostic dose (1× DC) [27, 28]. The CDC bottle assays were used to determine resistance intensity as they allow for evaluation of customised multiple concentrations of an insecticide [24].

WHO susceptibility tests

Wild-caught live female *An. funestus* mosquitoes were tested for phenotypic resistance to permethrin (0.75%), dichlorodiphenyltrichloroethane (DDT) (4%), bendiocarb (0.1%) and pirimiphos-methyl (0.25%) using standard WHO insecticide susceptibility assays [26]. The mosquitoes were exposed to papers treated with the respective insecticide for 1 h, knockdown was then assessed and the mosquitoes were transferred to holding tubes and provided with 10% sugar solution. Knockdown was assessed every 10 min from exposure to 1 h post-exposure, then mortality was recorded at 24 h post-exposure

Species identification by PCR

Following exposure the insecticides in the bioassay, a sample of the *An. funestus* population was subjected to species identification using PCR. Samples were analysed using standard operating procedures for identifying *An. funestus* group members [29, 30]. The PCR cycling conditions were: 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 45 °C for 30 s, 72 °C for 40 s, with a final extension at 72 °C for 5 min.

Data analysis

Minitab statistical software 20 was used to analyse data. Most data were summarised using descriptive statistics, including mosquito counts and mean mortalities. Mosquito mortality was calculated by dividing the number of dead/moribund mosquitoes over the total of mosquitoes exposed to a given insecticide/dose, then expressed as a percentage. Group means of mortality rates for different insecticides and doses were compared using analysis of variance (ANOVA). F-test *P*-values were reported from the ANOVA tests. The Tukey's post-hoc test was then used for pairwise comparisons of group means at a confidence level of 95%, with the aim to look for differences in mean mortality rates among the insecticides and given doses, and determine by how much they differed.

Results

A total of 1332 live wild *An. funestus* s.l. females were collected, of which 1153 came from Chakanira village and 179 from Sisewu village. These were used for the

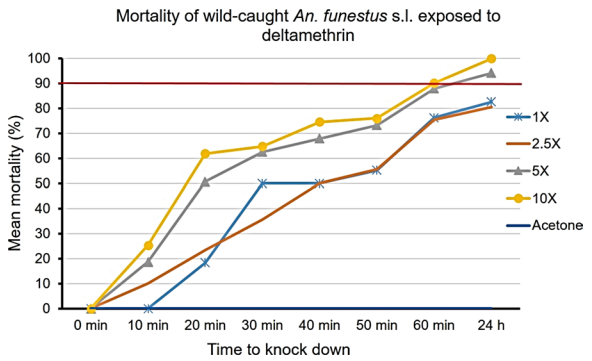


Fig. 2 Knockdown time and 24-h mortality rates of wild-caught *Anopheles funestus* s.l., after exposure to various concentrations of deltamethrin. The horizontal red line represents the 90% mortality threshold for confirmation of resistance

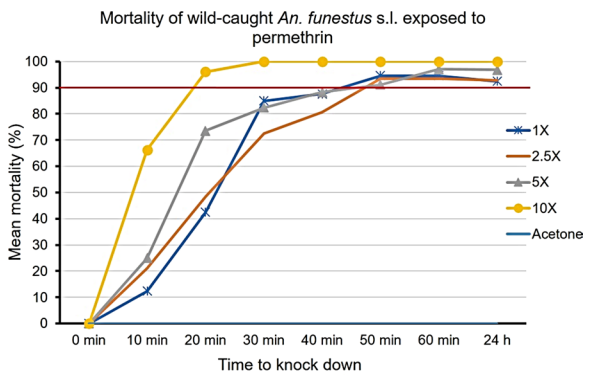


Fig. 3 Knockdown time and 24-h mortality rates of wild-caught *An. funestus* s.l., after exposure to various concentrations of permethrin. The horizontal red line represents the 90% mortality threshold for confirmation of resistance

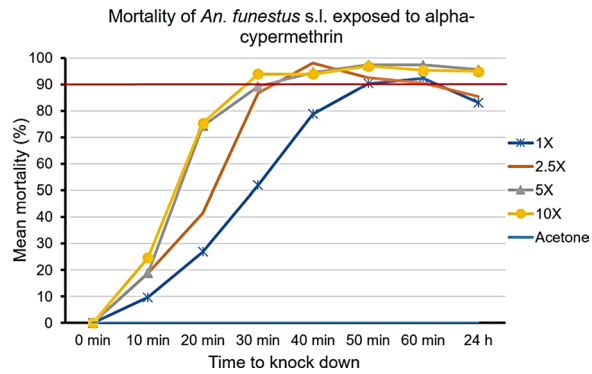


Fig. 4 Knockdown time and 24-h mortality rates of wild-caught *An. funestus* s.l., after exposure to various concentrations of alpha-cypermethrin. The horizontal red line represents the 90% mortality threshold for confirmation of resistance

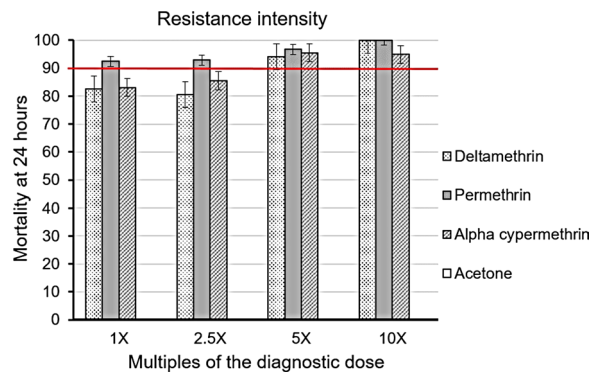


Fig. 5 The response of wild *An. funestus* s.l. to different doses of the three pyrethroids tested. The horizontal red line at 90% represents the WHO threshold for a resistant population. Error bars represent standard error of mean mortality rates at 24 h post-exposure

insecticide susceptibility tests (CDC bottle assays and WHO tube assays). A subsample of 400 (75 from Sisewu and 325 from Chakanira) was then selected for molecular identification. A small number of *An. gambiae* s.l. was also collected (1 specimen from Chakanira and 12 from Sisewu), but these were not processed further.

CDC bottle assays

The results of the pyrethroid intensity tests for the two villages are summarised in Table 1, which shows the numbers of mosquitoes tested per insecticide concentration (dose), percent mortality rates and adjusted mortality rates using Abbott's formula [26].

Figures 2, 3, 4 show the percentage knockdown of *An. funestus* s.l. from Chakanira village to the four concentrations of each of the three pyrethroids. Mortalities were adjusted using Abbott's correction formula as per the WHO test procedures [26].

Resistance intensity

Observed mortalities at the diagnostic dose (1× DC) were 82.6% ($n=38$) for deltamethrin, 92.5% ($n=73$) for permethrin and 83.1% ($n=52$) for alpha-cypermethrin. These results confirm resistance to both deltamethrin and alpha-cypermethrin and suspected resistance to permethrin (Table 1; Fig. 5). For deltamethrin and alpha-cypermethrin, mortalities were still under the 90% threshold at 2.5× DC while they were in the range 90–98% at 5× DC in the Chakanira population [28].

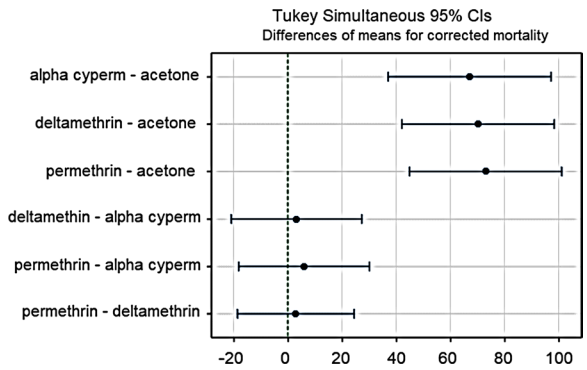


Fig. 6 Pairwise comparisons of mean mortalities for deltamethrin, permethrin, alpha-cypermethrin and acetone negative control

For the given doses, ANOVA test results were as follows: 1× DC (ANOVA, $F=0.71$, $df=2$, $P=0.543$); 2.5× DC (ANOVA, $F=0.49$, $df=2$, $P=0.636$); 5× DC (ANOVA, $F=1.83$, $df=2$, $P=0.240$; 10× DC (ANOVA, $F=4.77$, $df=2$, $P=0.087$. Tukey's post-hoc test was then used for pairwise comparisons of group means at a confidence level of 95% (Fig. 6). These test probabilities were all above the significance threshold (alpha = 0.05), which indicated that the test could not find an instance where the group mean for each insecticide at a given dose was statistically different from the others. In other words, the mean mortalities between the insecticides at each given dose belonged to the same population (were similar) and there was no evidence that any one of the insecticides performed better than the other at a given dose. This result was also indicated in the interval plots by the overlapping 95% confidence intervals of the means (Fig. 7).

WHO susceptibility tests

From the total live collections, 408 specimens were used for WHO susceptibility tests against DDT, bendiocarb, pirimiphos methyl and permethrin (Table 2). Only a single WHO susceptibility test was done for Sisewu village on permethrin, due to low numbers of mosquitoes collected.

Molecular species identification

The molecular study for species identification on 400 wild females revealed that 98% ($n=392$) were *An. funestus* s.s., followed by *Anopheles parensis* (1.5%, $n=6$) and *Anopheles rivulorum* (0.5%, $n=2$). While the majority of these specimens were from Chakanira village (81.2%, $n=325$), 18.8% ($n=75$) were from Sisewu village and were identified as follows: 72 *An. funestus* s.s., two *An. rivulorum* and one *An. parensis*.

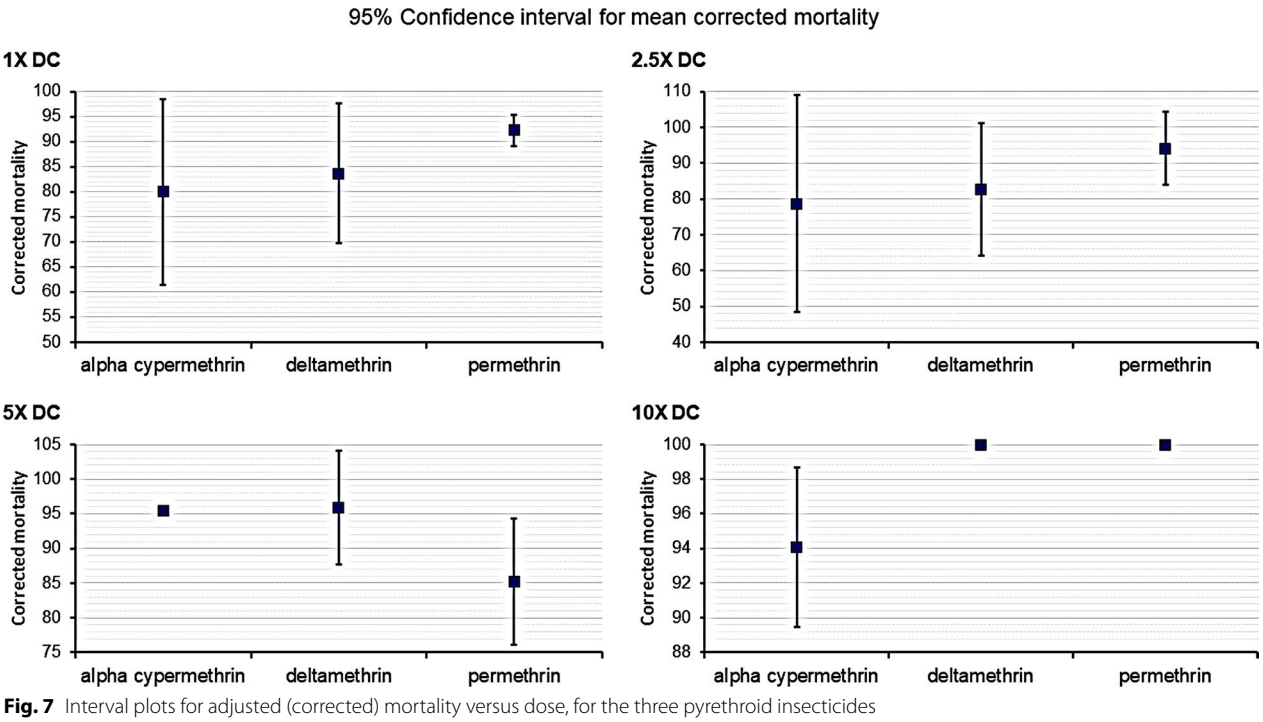


Fig. 7 Interval plots for adjusted (corrected) mortality versus dose, for the three pyrethroid insecticides

Table 2 WHO susceptibility tube assays

Village	Insecticide	Dose	No. of mosquitoes exposed[a]	% Mortality
Chakanira	Control	0×	29	0
	Dichlorodiphenyl-trichloroethane (DDT)	1×	103	98.9
	Control	0×	27	0
	Bendiocarb	1×	93	33.8
	Pirimiphos-methyl	1×	103	100
Sisewu[b]	Control	0×	28	0.0
	Permethrin	1×	25	35.4

[a] Low sample numbers could have affected these tests

[b] Due to limited mosquito numbers collected from Sisewu village, susceptibility tests were only carried out on permethrin

Discussion

The results of the present study highlight moderate to high resistance intensities in *An. funestus* from Chikwawa village against pyrethroid insecticides. Resistance to deltamethrin was confirmed in *Anopheles funestus* s.l. mosquitoes exposed to this insecticide at 1× DC, while in assays of these mosquitoes exposed to 2.5× DC and 5× DC, the mortality was < 98%. Susceptibility to deltamethrin was only restored when a 10× dose was used; hence, the classification of moderate intensity [28]. Similar results were observed for permethrin, with the only difference being that mortality at 1× DC was 92.5%, indicating possible resistance, which was confirmed when higher doses were used: at 2.5× and 5× DC, mortalities ranged from 90 to 98%. Susceptibility was also restored at 10× DC, similar to deltamethrin, indicating moderate resistance. Mortality to alpha-cypermethrin was < 90% at 1× DC and 2.5× DC, while it fell to 90–98% at 5× and 10× DC, indicative of a high intensity of resistance to this pyrethroid.

In addition to observing resistance against all three pyrethroids, we observed a low mortality (33.8%, $n = 93$) of *An. funestus* to the carbamate bendiocarb. However, intensity assays were not carried out in this study, and this observation warrants further study. Previous studies carried out in southern Malawi reported that *An. funestus* mortality to bendiocarb dropped from 60% in 2009 to 30.1% in 2014, then fell further to 19% in 2015 [16]. Unlike previous studies which reported moderate resistance or variable responses to DDT in the *An. funestus* s.s. population [13, 16], complete susceptibility to DDT (mortality 98.9%, $n = 103$) was observed in the current study. The present study also corroborates previous observations of full susceptibility to organophosphates, with an observed mortality of 100% ($n = 103$) to pirimiphos-methyl [11–14, 16].

One of the limitations of using wild mosquitoes is the possibility that they could have been exposed to various field conditions, including varying degrees of insecticide doses (e.g. on nets, wall surfaces or in agricultural fields), which potentially increases the risk of overestimating resistance in the given mosquito population [26]. Older mosquitoes may tend to be weaker and hence more susceptible, risking an under-estimation of resistance [26]. However, this scenario more closely represents the actual conditions that any given intervention would encounter in the field; in addition, that wild-collected specimens are also the epidemiologically relevant cohort of vectors [23]. Changes in insecticide susceptibility in wild-caught vectors will thus more closely reflect changes in intervention efficacy [26, 31]. Another limitation of the current study is that we could not test many insecticides at all doses due to low sample numbers. However, even given the low numbers, we were still able to document the problem and its severity. Advanced molecular or biochemical tests for resistance mechanisms were performed as these were previously described in the study area by Riveron et al. [15].

The Malawi National Malaria Control Programme (NMCP) recently adopted an Integrated Vector Control Strategy (IVCS) 2020–2024 and the Insecticide Resistance Management Plan (IRMP) 2019–2022; these two programmes currently form the core of the malaria control programme in the country. For vector control, universal coverage of all at-risk populations with ITNs is the main approach together with complementary IRS in selected districts. In Malawi, ITNs are distributed via mass campaigns every 3 years, as well as through routine distribution in clinics to pregnant mothers and children under the age of 5 years, and IRS is implemented yearly in four districts [11, 32]. However, the implementation of IRS over the years has not been consistent in all the selected districts due to logistical and financial constraints and the rise in pyrethroid resistance. It was this increase in pyrethroid resistance that prompted the development of the strategic plan for monitoring and managing resistance (the IRMP) to counter the potential detrimental impact of resistance [11, 33]. The current study highlights the extent of the problem of resistance in Chikwawa village, warranting immediate action to safeguard progress in malaria control.

Although DDT is still effective against local mosquitoes and is recommended in the current vector control strategy, the potential use of this insecticide is hampered by concerns surrounding its use [34–37]. In 2006, the WHO made a call for the use of DDT for vector control and provided clear and strict guidelines which it adopted from

the Stockholm Convention, stipulating how countries can incorporate DDT for malaria control in their malaria management programmes while strongly mitigating against any potential risks. Following the 2006 WHO recommendation for the use of DDT in highly endemic settings, Malawi announced that it would pilot a DDT IRS programme [36, 38]. Evidence shows that countries that continue both timely and correct use of DDT for IRS can reduce malaria transmission by up to 90% [39]. When South Africa reintroduced DDT spraying in 2000, they reduced the number of malaria cases and deaths drastically, managed to keep levels under control and, by 2012, set the goal to eliminate the disease entirely [40, 41]. The number of countries in Africa adopting DDT spraying for malaria control has risen, and since 2005, the list includes Malawi's eastern and southern neighbour Mozambique, where *An. funestus* s.s. is a also key vector [42].

However, it has been argued that because Malawi has largely an agricultural economy, the benefits of using DDT for the fight against malaria may be outweighed by the potential detrimental effects on the agricultural industry and the economy at large [43]. This debate is also common in other African countries, and some studies have even suggested that the potential benefits of DDT for malaria vector control would be in the same order of magnitude as its detrimental effects on infant mortality [44]. Compounds such as clothianidin and deltamethrin/clothianidin combinations thus offer a good option for a rotation strategy with organophosphates in the IRS programme. The current vector control strategy also recommends PBO-nets and combination ITNs for areas with high levels of pyrethroid resistance [11, 33]. The present study supports and highlights the critical importance of this strategy as resistance intensity data have predictive value for making informed insecticide choices, to prevent compromised transmission control [27, 28, 31].

Clearly, a vector control strategy such as IRS that incorporates DDT and pirimiphos-methyl or other organophosphates may be a preferred means to manage the current situation of resistance. Such a strategy would serve as an interim solution as novel compounds are being developed or await registration and WHO approval and recommendation. Also, strategies that incorporate insecticides with new generation modes of action, or those that do not involve the use of insecticides at all, should complement ongoing interventions to avoid further risks that resistance may pose to the control programme. These findings also highlight the importance of making new generation ITNs (such as PBO-nets) readily and widely accessible to communities as they have demonstrated to have greater entomological and epidemiological impact than standard long-lasting insecticidal nets (LLINs) in settings of high insecticide resistance [31]. Such a multi-pronged approach will aid the country as it transitions towards elimination.

In this study, *An. funestus* s.s. was found to be the most abundant mosquito species, as also previously observed [12]. *Anopheles parensis* and *An. rivulorum* were also detected, and continuous surveillance and monitoring of these species needs to be routinely incorporated into control programmes, as these two species have been shown elsewhere to be contributing to transmission and also found biting humans indoors [45, 46]. Very few *An. gambiae* s.l. were collected ($n=13$) in this study and no further investigations were done on these specimens.

Conclusion

The level of resistance in Chikwawa village has grown over the years, and current intensity determined in the present study warrants an urgent solution. Our results highlight the importance of a resistance management strategy to cushion against the potential negative impact of resistance. The use of third-generation synergists or dual active ingredient ITNs, and pirimiphos-methyl- or DDT-based IRS would help to control the current resistance intensity in *An. funestus.* Such steps will prevent control failure and safeguard the control gains that have been achieved so far. More research needs to be done to further validate the intensity of resistance against carbamates.

Acknowledgements

We would like to thank all study participants, Health Surveillance Assistants (HSAs), village chiefs and all people who supported us during the study. We thank the Wits Research Institute for Malaria, Centre for Emerging Zoonotic & Parasitic Diseases at the National Institute for Communicable Diseases, and MAC Communicable Diseases Action Center for institutional space and support.

Author contributions

JK, MC, and TM designed the study. JK conducted the field collection of specimen and bioassays. LK led the molecular laboratory work, JK drafted the original manuscript. MC, TM and LK led the interpretation of findings. All authors read and approved the final manuscript.

Funding

This research was supported by the Consortium for Advanced Research Training in Africa (CARTA). CARTA is jointly led by the African Population and Health Research Center and the University of the Witwatersrand, and funded by the Carnegie Corporation of New York (Grant No–B 8606.R02), SIDA (Grant No:54100029) and the DELTAS Africa Initiative (Grant No: 107768/Z/15/Z). The statements made and the views expressed are solely the responsibility of the researchers. LLK is supported by the DST/NRF South African Research Chairs Initiative (Grant No. 64763).

Availability of data and materials

Datasets used for the analysis of findings of this study are available upon request via the authors' email addresses.

Declarations

Ethics approval and consent to participate

Ethical clearance for the study was obtained from the Human Research Ethics Committee at the University of the Witwatersrand in South Africa (Clearance number: M170662) and from the College of Medicine Research Ethics Committee (CoMREC) in Malawi (Clearance number: P.12/17/2324). Study participants were recruited as volunteers from the study area and informed consent was obtained before participation.

Consent for publication

All authors read and approved the final manuscript, and consented for publication.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Malaria Alert Centre-Communicable Diseases Action Centre (MAC-CDAC), Kamuzu University of Health Sciences, Blantyre, Malawi. [2]Wits Research Institute for Malaria, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa. [3]Centre for Emerging Zoonotic & Parasitic Diseases, National Institute for Communicable Diseases, Johannesburg, South Africa.

Received: 12 January 2022 Accepted: 26 April 2022
Published online: 21 June 2022

References

1. WHO. World malaria report 2021. Switzerland: World Health Organization; 2021. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021. Accessed 31 May 2022 .
2. WHO. World malaria report. 20 years of global progress and challenges. Switzerland: World Health Organization; 2020. https://www.who.int/publications/i/item/9789240015791. Accessed 31 May 2022.
3. Mwendera CA, De Jager C, Longwe H, Kumwenda S, Hongoro C, Phiri K, et al. Challenges to the implementation of malaria policies in Malawi. BMC Health Serv Res. 2019;19:194. https://doi.org/10.1186/s12913-019-4032-2.
4. Chipeta MG, Giorgi E, Mategula D, Macharia PM, Ligomba C, Munyenyembe A, et al. Geostatistical analysis of Malawi's changing malaria transmission from 2010 to 2017. Wellcome Open Res. 2019;4:57. https://doi.org/10.12688/wellcomeopenres.15193.2.
5. Tizifa TA, Kabaghe AN, McCann RS, van den Berg H, Van Vugt M, Phiri KS. Prevention efforts for malaria. Curr Trop Med Rep. 2018;5:41–50. https://doi.org/10.1007/s40475-018-0133-y.
6. Choi KS, Christian R, Nardini L, Wood OR, Agubuzo E, Muleba M, et al. Insecticide resistance and role in malaria transmission of *Anopheles funestus* populations from Zambia and Zimbabwe. Parasit Vectors. 2014;7:464.
7. Moyes CL, Athinya DK, Seethaler T, Battle KE, Sinka M, Hadi MP, et al. Evaluating insecticide resistance across African districts to aid malaria control decisions. Proc Natl Acad Sci USA. 2020;117:22042–50. https://doi.org/10.1073/pnas.2006781117.
8. Owuor KO, Machani MG, Mukabana WR, Munga SO, Yan G, Ochomo E, et al. Insecticide resistance status of indoor and outdoor resting malaria vectors in a highland and lowland site in Western Kenya. PLoS ONE. 2021;16:0240771. https://doi.org/10.1371/journal.pone.0240771.
9. Pinda PG, Eichenberger C, Ngowo HS, Msaky DS, Abbasi S, Kihonda J, et al. Comparative assessment of insecticide resistance phenotypes in two major malaria vectors, *Anopheles funestus* and *Anopheles arabiensis* in south-eastern Tanzania. Malar J. 2020;19:408. https://doi.org/10.1186/s12936-020-03483-3.
10. Andronescu LR, Buchwald AG, Coalson JE, Cohee L, Bauleni A, Walldorf JA, et al. Net age, but not integrity, may be associated with decreased protection against *Plasmodium falciparum* infection in southern Malawi. Malar J. 2019;18:329. https://doi.org/10.1186/s12936-019-2930-8.
11. Abeku T, Mzilahowa T, Beard J, Yihdego Y, Dandalo L, Mokuena M, et al. Insecticide resistance management plan, Malawi, 2019 – 2022. Lilongwe: Malaria Consortium. 2019.
12. PMI VectorLink Project. Malawi entomological monitoring annual report. Blantyre: Malaria Alert Centre. 2019.
13. Mzilahowa T, Chiumia M, Mbewe RB, Uzalili VT, Luka-Banda M, Kutengule A, et al. Increasing insecticide resistance in *Anopheles funestus* and *Anopheles arabiensis* in Malawi, 2011–2015. Malar J. 2016. https://doi.org/10.1186/s12936-016-1610-1.
14. Abeku T, Mzilahowa T, Yihdego Y, Dandalo L, Burnett J, Mokuena M. Integrated vector control strategy for malaria control, Malawi, 2020–2024. Lilongwe: Malaria Consortium; 2020.
15. Hunt R, Edwardes M, Coetzee M. Pyrethroid resistance in southern African *Anopheles funestus* extends to Likoma Island in Lake Malawi. Parasit Vectors. 2010;3:1.
16. Riveron JM, Chiumia M, Menze BD, Barnes KG, Irving H, Ibrahim SS, et al. Rise of multiple insecticide resistance in *Anopheles funestus* in Malawi: a major concern for malaria vector control. Malar J. 2015;14:1–9.
17. Riveron JM, Ibrahim SS, Chanda E, Mzilahowa T, Cuamba N, Irving H, et al. The highly polymorphic CYP6M7 cytochrome P450 gene partners with the directionally selected CYP6P9a and CYP6P9b genes to expand the pyrethroid resistance front in the malaria vector *Anopheles funestus* in Africa. BMC Genom. 2014;15:1.
18. Riveron JM, Huijben S, Tchapga W, Tchouakui M, Wondji MJ, Tchoupo M, et al. Escalation of pyrethroid resistance in the malaria vector *Anopheles funestus* induces a loss of efficacy of piperonyl butoxide–based insecticide-treated nets in Mozambique. J Infect Dis. 2019;220:467–75. https://doi.org/10.1093/infdis/jiz139.
19. Kouassi BL, Edi C, Tia E, Konan LY, Akré MA, Koffi AA, et al. Susceptibility of *Anopheles gambiae* from Côte d'Ivoire to insecticides used on insecticide-treated nets: evaluating the additional entomological impact of *piperonyl* butoxide and chlorfenapyr. Malar J. 2020;9(1):454. https://doi.org/10.1186/s12936-020-03523-y.
20. Kleinschmidt I, Bradley J, Knox TB, Mnzava AP, Kafy HT, Mbogo C, et al. Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: a WHO-coordinated, prospective, international, observational cohort study. Lancet Infect Dis. 2018;18:640–9. https://doi.org/10.1016/s1473-3099(18)30172-5.
21. Kleinschmidt I, Mnzava AP, Kafy HT, Mbogo C, Bashir AI, Bigoga J, et al. Design of a study to determine the impact of insecticide resistance on malaria vector control: a multi-country investigation. Malar J. 2015;14:282. https://doi.org/10.1186/s12936-015-0782-4.
22. Wondji CS, Coleman M, Kleinschmidt I, Mzilahowa T, Irving H, Ndula M, et al. Impact of pyrethroid resistance on operational malaria control in Malawi. Proc Natl Acad Sci USA. 2012; 109:19063–70. http://www.pnas.org/content/109/47/19063.full.pdf.
23. Kabaghe AN, Chipeta MG, Gowelo S, Mburu M, Truwah Z, McCann RS, et al. Fine-scale spatial and temporal variation of clinical malaria incidence and associated factors in children in rural Malawi: a longitudinal study. Parasit Vectors. 2018;11:1–11.
24. Brogdon W, Chan A. Guideline for evaluating insecticide resistance in vectors using the CDC bottle bioassay. Atlanta:Centers for Disease Control and Prevention; 2010.
25. Mzilahowa T, Hastings IM, Molyneux ME, McCall PJ. Entomological indices of malaria transmission in Chikhwawa district. Southern Malawi Malar J. 2012;11:1–9.
26. WHO. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, 2nd ed; Geneva, Switzerland: World Health Organisation; 2018. https://apps.who.int/iris/bitstream/handle/10665/250677/9789241511575-eng.pdf.Accessed 31 May 2022.
27. Bagi J, Grisales N, Corkill R, Morgan JC, N'Falé S, Brogdon WG, et al. When a discriminating dose assay is not enough: measuring the intensity of insecticide resistance in malaria vectors. Malar J. 2015;14:1–9.
28. Venter N, Oliver SV, Muleba M, Davies C, Hunt RH, Koekemoer LL, et al. Benchmarking insecticide resistance intensity bioassays for *Anopheles* malaria vector species against resistance phenotypes of known epidemiological significance. Parasit Vectors. 2017;10. https://doi.org/10.1186/s13071-017-2134-4. https://parasitesandvectors.biomedcentral.com/track/pdf/10.1186/s13071-017-2134-4.
29. Cohuet A, Simard F, Toto JC, Kengne P, Coetzee M, Fontenille D. Species identification within the *Anopheles funestus* group of malaria vectors

in Cameroon and evidence for a new species. Am J Trop Med Hyg. 2003;69:200–5.
30. Koekemoer L, Kamau L, Hunt R, Coetzee M. A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: culicidae) group. Am J Trop Med Hyg. 2002;66:804–11.
31. Gleave K, Lissenden N, Chaplin M, Choi L, Ranson H. Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. Cochrane Database Syst Rev. 2021;5:012776. https://doi.org/10.1002/14651858.CD012776.pub3.
32. Ye Y, Andrada A. Estimating malaria incidence through modeling is a good academic exercise, but how practical is it in high-burden settings? Am J Trop Med Hyg. 2020;102:701–2. https://doi.org/10.4269/ajtmh.20-0120.
33. WHO. Insecticide-treated nets for malaria transmission control in areas with insecticide-resistant mosquito populations: preferred product characteristics. Geneva: World Health Organization; 2021. https://www.who.int/publications/i/item/9789240018730. Accessed 31 May 2022.
34. News24: Malawian MPs in bizzare debate over use of DDT to fight malaria. 2010. https://www.news24.com/news24/Archives/City-Press/Malawian-MPs-in-bizarre-debate-over-use-of-DDT-to-fight-malaria-20150430. Accessed 31 Jul 2021.
35. Kabange SC. African countries debate using DDT in anti-malaria efforts. 2011. https://www.voanews.com/africa/african-countries-debate-using-ddt-anti-malaria-efforts. Accessed 31 Jul 2021.
36. van den Berg H. Global status of DDT and its alternatives for use in vector control to prevent disease. Environ Health Perspect. 2009;117:1656–63. https://doi.org/10.1289/ehp.0900785.
37. Coetzee M, Koekemoer LL. Molecular systematics and insecticide resistance in the major African malaria vector *Anopheles funestus*. Annu Rev Entomol. 2013;58:393–412. https://doi.org/10.1146/annurev-ento-120811-153628.
38. Lancet T. DDT for malaria control: the issue of trade. Lancet. 2007;369:248. https://doi.org/10.1016/s0140-6736(07)60119-6.
39. Rehwagen C. WHO recommends DDT to control malaria. BMJ. 2006;333:622. https://doi.org/10.1136/bmj.333.7569.622-b.
40. Hargreaves K, Koekemoer L, Brooke B, Hunt R, Mthembu J, Coetzee M. *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. Med Vet Entomol. 2000;14:181–9. http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2915.2000.00234.x/abstract.
41. Maharaj R, Morris N, Seocharan I, Kruger P, Moonasar D, Mabuza A, et al. The feasibility of malaria elimination in South Africa. Malar J. 2012;11:423.
42. Coleman M, Casimiro S, Hemingway J, Sharp B. Operational impact of DDT reintroduction for malaria control on *Anopheles arabiensis* in Mozambique. J Med Entomol. 2008;45:885–90.
43. Chanda E, Mzilahowa T, Chipwanya J, Mulenga S, Ali D, Troell P, et al. Preventing malaria transmission by indoor residual spraying in Malawi: grappling with the challenge of uncertain sustainability. Malar J. 2015;14:1. https://doi.org/10.1186/s12936-015-0759-3.
44. Chen A, Rogan WJ. Nonmalarial infant deaths and DDT use for malaria control. Emerg Infect Dis. 2003;9:960–4. https://doi.org/10.3201/eid0908.030082.
45. Burke A, Dahan-Moss Y, Duncan F, Qwabe B, Coetzee M, Koekemoer L, et al. *Anopheles parensis* contributes to residual malaria transmission in South Africa. Malar J. 2019. https://doi.org/10.1186/s12936-019-2889-5.
46. Kawada H, Dida GO, Sonye G, Njenga SM, Mwandawiro C, Minakawa N. Reconsideration of *Anopheles rivulorum* as a vector of *Plasmodium falciparum* in western Kenya: some evidence from biting time, blood preference, sporozoite positive rate, and pyrethroid resistance. Parasit Vectors. 2012;5:230. https://doi.org/10.1186/1756-3305-5-230.

Publisher's Note

R14/49 Mr Justin Kumala et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170662

NAME: (Principal Investigator)	Mr Justin Kumala et al
DEPARTMENT:	Wits Research Institute for Malaria, School of Pathology Malaria Alert Centre, Blantyre, Malawi
PROJECT TITLE:	The Effect of Insecticide Resistance on Malaria Vector Control in Chikwawa District, Southern Malawi
DATE CONSIDERED:	30/06/2017
DECISION:	Approved unconditionally
CONDITIONS:	South African Human Research Ethics Committees (HRECs) have no standing outside South Africa. Ethics approval is also required from local HRECs in Malawi
SUPERVISOR:	Prof Maureen Coetzee
APPROVED BY:	Professor CB. Penny Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	14/09/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review July and will therefore be due in the month of July each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

18th September, 2017

Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the College of Medicine Research and Ethics Committee (COMREC) has reviewed and approved a study entitled:

P.12/17/2324 - The effect of insecticide reistance on malaria vector control in Chikwawa, Southern Malawi version 2 dated 10 January 2018 by Justin Kumala

On 19-Feb-18

As you proceed with the implementation of your study, we would like you to adhere to international ethical guidelines, national guidelines and all requirements by COMREC as indicated on the next page

19-Feb-18

Dr. YB. Mlombe - Chairperson (COMREC)

Date

REQUIREMENTS FOR ALL COMREC APPROVED RESEARCH PROTOCOLS

1. Pay the research overhead fees as required by the College of Medicine for all approved studies.

2. You should note that the COMREC Sub-Committee on Research Participants' Safety will monitor the conduct of the approved protocol and any deviation from the approved protocol may result in your study being stopped.

3. You will provide an interim report in the course of the study and an end of study report.

4. All COMREC approvals of new applications and progress reports are valid for one year only. Therefore all approved studies running for more than one year are subject to continuing review annually. You are required to submit a progress report to COMREC within 90-30 days before the expiration date. Your current expiration date is 19-Feb-19. Studies shall be considered lapsed and inactive if continuing review application is not received one month after the expiry of the previous approval. In that case, all study related operations should cease immediately except those that are necessary for the welfare of subjects.

5. All investigators who are Medical Practitioners must be fully registered with the Medical Council of Malawi.