Efficacy and durability (insecticidal and physical) of next generation insecticide-treated nets against pyrethroid resistant malaria vectors in Tanzania: A multi-faceted study.

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Declaration

I Jackline Martin confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Vector control interventions, particularly the scale-up of pyrethroid-treated bed nets (ITNs), have significantly reduced malaria transmission in sub-Saharan Africa over the past two decades. However, pyrethroid resistance threatens ITN effectiveness, especially as nets degrade over time. This thesis evaluates the bio-efficacy and durability of three new dual-active ingredient (A.I.) ITNs (Interceptor G2, Royal Guard, and Olyset Plus) compared to standard Interceptor nets.

A cluster-randomized controlled trial (cRCT) was conducted in Misungwi, Tanzania, in January 2019, distributing 40,000 nets of each type. Over 36 months, 3,072 ITNs of each type were monitored at 6–12 month intervals to assess survivorship and fabric integrity. Results showed a median functional survival of less than three years, with Olyset Plus having the shortest lifespan of 0.9 years.

Bio-efficacy studies at Kilimanjaro Christian Medical University College (KCMUCo) and the National Institute for Medical Research (NIMR) showed all ITNs met WHO bio-efficacy criteria, influenced mainly by blood-feeding inhibition rather than mortality. Against a resistant strain of *An. gambiae* s.s., new dual A.I. ITNs showed higher mortality than the reference net (Interceptor), with this advantage lasting 24 months. Fertility effects of pyriproxyfen in Royal Guard were observed up to six months in laboratory assays.

In experimental hut trials (EHTs), Royal Guard and Interceptor G2 demonstrated superior efficacy for entomological outcomes for one year, while Olyset Plus showed benefits only when new. In the cRCT, Royal Guard and Olyset Plus showed similar trends, while Interceptor G2 provided consistent protection for three years.

This study underscores the value of dual A.I. ITNs in community settings, emphasizing the need for continuous monitoring and research to develop longer-lasting ITNs and enhance malaria control in sub-Saharan Africa.

Acknowledgements

First and foremost, I extend my gratitude to Almighty God for granting me the courage to embark on this PhD journey.

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Special thanks to **Natacha Protopopoff** who ignited in me the passion for research since my master's degree until now and making my PhD life to be enjoyable.

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Lastly but not least, I extend heartfelt thanks to my daughter, **Sharon Martin**, for her patience and understanding throughout my research journey. May Almighty God grant you all your heart's desires.

Contents

List of figures

List of tables

List of Abbreviations

A.I.:	Active Ingredient
***An.*:**	*Anopheles*
CDC:	Centers for Disease Control and Prevention
CFP:	Chlorfenapyr
cRCT:	cluster Randomized Controlled Trial
EHT:	Experimental hut trial
EIR:	Entomological Inoculation Rate
GC7:	Grant cycle 7
GPS:	Global Positioning System
GTS:	Global Technical Strategy
HH:	Household
HI:	Hole Index
HPLC:	High-Performance Liquid Chromatography
ITN:	Insecticide-Treated Net
IVCC:	Innovative for Vector Control Consortium
KCMUCO:	Kilimanjaro Christian Medical University College
LLIN:	Long Lasting Insecticidal Net
IRS:	Indoor-Residual Spraying
LSHTM:	London School of Hygiene and Tropical Medicine
MRCC:	Medical Research Coordinating Committee
NIMR:	National Institute for Medical Research
ODK:	Open Data Kit
PBO:	Piperonyl butoxide
PPF:	Pyriproxyfen
Pyr:	Pyrethroid
QC:	Quality Control
SOP:	Standard Operating Procedure

SSA: Sub-Saharan Africa

WHO: World Health Organization

WHOPES: WHO Pesticide Evaluation Scheme

WHO-PQ: WHO Prequalification Programme

Glossary

Adverse event	Any outward medical condition in an individual exposed to a biological or chemical product, which does not necessarily have a causal relationship with the product
Anopheles, infective	Female *Anopheles* mosquitoes with sporozoites in salivary gland
Anthropophilic	Describes female *Anopheles* mosquitoes that prefer to feed on human host
Bioassays	Experimental testing that involves assessing the biological effectiveness of a treatment (such as infection, insecticide, pathogen, predator, or repellent) by deliberately exposing insects to it.
Discriminating dose/diagnostic dose	Refers to amount of insecticide (concentration) capable to differentiate between susceptible and resistance mosquitoes in a population and determine their proportion.
Endemic area	A region where there is a continuous, measurable incidence of malaria infection and mosquito-borne transmission over several years.
Endophily	Tendency of mosquitoes to rest indoor
Exophily	Tendency of mosquito to rest outdoor
Experimental hut trial	For vector investigations, a simulated house with entry and exit traps used to sample mosquitoes as they enter and exit, feed on blood indoors (when a host is present), and survive or die in each sub-sample, monitored daily or nightly.
Functional survival	Estimation of nets still in household in serviceable condition
Insecticide	Chemical products (natural or synthetic) that kills insects

Insecticide, cross-resistance	Resistance to one insecticide due to a mechanism that also provides resistance to another insecticide, even if the insect population has not been exposed to the latter.
Insecticide, mixture	An insecticide product composed of two or more active ingredients combined into a single formulation, and when applied mosquitoes come into contact with all ingredients simultaneously.
Insecticide resistance	The ability of mosquitoes to survive exposure to a standard dose of insecticide, which may result from physiological or behavioural adaptation.
Physical durability	The ability of the yarn/fabric of an ITN to resist wear and deterioration from continual use
Net attrition	This is opposite of survivorship; it refers to the proportion of nets no longer in use as intended after a defined period after their distribution to the households
LLIN	This is the WHO LLIN is an ITN that is specifically designed to maintain their insecticidal properties for an extended period without the need for re-treatment as they were permanently treated by the manufacturer. They are supposed to retain their efficacy for about 3-5 years or withstand 20 washes. In this thesis, the term LLINs is used throughout for all manufacturer-treated nets, instead of ITNs, which historically referred to nets that were manually re-treated.

Statement of contribution

The work described in the thesis was funded under the joint global health trial from the Department of Health and Social Care, the Department for International Development, the Medical Research Council and Wellcome and the Bill and Melinda Gates foundation through the Innovative vector control consortium (IVCC) (Grant #MR/R006040/1). All field work was conducted in Tanzania, and I was in charge of the overall management of the durability and bio-efficacy component of the project. My role and contribution were conceptualization, designing data collection tools, data curation, training field team and supervision, formal analysis and drafting the resulting manuscripts and thesis.

The description of thesis is as follows:

Chapter 1: Introduction /literature review

This chapter encompasses the introduction to the thesis as well as a review of the literature on vector control tools, including both historical and contemporary approaches to vector control.

PhD rationale

This chapter explain the reason why this research was conducted and its value in the context of vector control.

Objectives of the study

This chapter describes general and specific objectives of this thesis.

Chapter 2: Methodology

Durability of three types of dual active ingredient long-lasting insecticidal net compared to a pyrethroid-only LLIN in Tanzania: methodology for a prospective cohort study nested in a cluster randomized controlled trial.

Martin, J.L., Messenger, L.A., Mosha, F.W., Lukole,E., Mosha, J.F., Kulkarni, M., Churcher, S.T., Sherrard-Smith, E., Manjurano, A., Protopopoff, N., and Rowland, M.,. *Malar J* **21**, 96 (2022). https://doi.org/10.1186/s12936-022-04119-4

This chapter consists of a published paper describing the thesis methodology, which was nested in a cRCT in Misungwi district, Tanzania, evaluating three types of ITNs: Interceptor G2, Royal Guard and Olyset Plus compared to control standard Interceptor. The method describes procedures of assessing survivorship and fabric integrity of ITNs as well as bio-efficacy both in the laboratory and semi-field trial and chemical analysis. Mark Rowland and Natacha

Protopopoff designed the overall study, and I was involved in the development of sub-protocols for each study including sample size estimation and planning of activities. I led the writing of the manuscript.

Chapter 3: Monitoring of Fabric Integrity and Attrition Rate of Dual-Active Ingredient Long-Lasting Insecticidal Nets in Tanzania: A Prospective Cohort Study Nested in a Cluster Randomized Controlled Trial.

Martin, J., Lukole, E., Messenger, L.A., Aziz, T., Mallya, E., Bernard, E., Matowo, N.S., Mosha, J.F., Rowland, M., Mosha, F.W., Manjurano, A. and Protopopoff, N., *Insects*. 2024; 15(2):108. https://doi.org/10.3390/insects15020108.

This chapter consists of a published paper that evaluated fabric integrity and the attrition rate of a A.I. ITNs compared to standard LLINs in Tanzania. The study found that all net assessed has functional survival less than 3 years with Olyset Plus being the worst of all. I designed the study, conducted field work and supervision, data curation, analysis and led manuscript writing. It covers Objective one of the theses.

Chapter 4: Bio-efficacy of field aged novel class of long-lasting insecticidal nets, against pyrethroid-resistant malaria vectors in Tanzania: A series of experimental hut trials.

Martin JL, Messenger, L.A., Rowland, M., Mosha, F.W., Bernard, E., Kisamo, M., Hape, P., Limbe, S., Thickstun, C., Steven, C., Moshi, O., Shirima, B., Matowo, N.S., Mosha, J.F., Dee, D.P., Churcher, S.T., Kulkarni, M.A., Manjurano, A., and Protopopoff, N. Accepted by PLoS Global Heath July 2024.

In this chapter the efficacy of Interceptor G2, Royal Guard and Olyset Plus sampled after 0, 12, 24 and 36 months of use on the mortality, blood-feeding inhibition and reproduction inhibition (only Royal Guard) of host-seeking wild *Anopheles* in EHT (Objective 2) was reported. It also covers the assessment of the intensity of resistance to permethrin, alpha-cypermethrin, PPF and CFP and investigate the mechanisms of resistance of wild *Anopheles* mosquitoes in the experimental hut study area (Objective 3).

I designed the study, conducted the pilot study for mosquito density, selected the site for experimental hut construction, trained technicians and volunteers, designed data collection tools in open data kit (ODK), randomization using Latin square design, supervision for resistance testing, data analysis and led the writing of the manuscript.

Chapter 5: Evaluation of bio-efficacy of field aged novel long-lasting insecticidal nets (PBO, chlorfenapyr or pyriproxyfen combined with pyrethroid) against *Anopheles gambiae* s.s in Tanzania

Martin, J., Messenger, L.A., Bernard, E., Kisamo, M., Hape, P., Sizya, O., Festo, E., Matiku, W., Marcel, V., Malya, E., Aziz, T., Matowo, S.N., Mosha, J.F., Manjurano, A., Mosha, F.W., Rowland, M. and Protopopoff, N.

This chapter reported the bio-efficacy of dual ITNs sampled from community at 12, 24, 30 and 36 months. The main outcomes were mortality, blood feeding inhibition and sterility effect for Royal Guard ITNs using colony mosquitoes. This manuscript was submitted to current research in vector-borne diseases in July 2024 for possible publication.

I design data collection forms, supervision of laboratory work, quality check, data analysis and drafting the manuscript. This chapter covers objective 4 of the thesis.

Chapter 6: General discussion

This chapter explores the key findings of this thesis, referencing previous research to provide context and insights.

Funding

All objectives were mainly funded by the Bill and Melinda Gates Foundation through the Innovative Vector Control Consortium and under the global health trial, a jointly funded initiative from The Department of Health and Social Care, the Department for International Development, the Medical Research Council and the Wellcome Trust (Grant Ref: MR/R006040/1).

1 Chapter 1: Introduction, literature review and objectives

1.1 Introduction/literature review

Malaria transmission

Malaria is a deadly disease caused by *Plasmodium* parasites and transmitted by female *Anopheles* mosquitoes (1). There are six different *Plasmodium (P)* species affecting human populations: *P. ovale curtisi, P. ovale wallikeri*, *P. falciparum*, *P. malariae*, *P. vivax* and *P. knowlesi* with *P. falciparum* being the leading cause of malaria cases and death in Sub-Saharan Africa (SSA) (2).

African malaria vectors and behaviour

In SSA there are two major groups of malaria vectors which includes species from the *Anopheles (An.) gambiae* sensu lato (s.l) complex, such as *An. gambiae* sensu stricto (s.s), *An. coluzzii* (3) *An. arabiensis* , *An. melas, An. Merus* (4) and *An. bwambae* (5) as well as species from the *An. funestus* s.l. complex which consists of at least eleven sub species: *An. funestus* s.s, *An. vaneedeni, An. rivulorum, An. rivulorum*-like, *An. leesoni, An. confuses, An. parensis, An. brucei, An. aruni, An. fuscivenosus* and *An. fluviatilis* (6). The widespread distribution of these species across diverse geographical areas demonstrates the significant adaptability of mosquitoes across the African continent (7). *An. gambiae* s.s., *An. coluzzii*, and *An. funestus* s.s. prefer to feed on humans and rest indoors. However, the large-scale deployment of insecticide-based vector control interventions, such as LLINs, ITNs, and IRS, has led to adaptive changes in mosquito behaviour. These changes may include a shift in biting behaviour from midnight to early evening and/or late morning (8). Additionally, *An. gambiae* s.l., *An. coluzzii*, and *An. funestus* have been observed to rest outdoors and feed on animals (9-11). In Kenya and Tanzania for the *An. gambiae* complex there was a shift from predominantly *An. gambiae* s.s. before interventions to predominantly *An. arabiensis* after interventions. *An. arabiensis* another vector, expresses mixed behaviours showing exophilic and zoophagic tendencies (12). A study done in Tanzania assessing host preferences for malaria vectors reported that in rural settings there was no statistical significance in host preference between cattle and humans for both *An. gambiae* and *An. arabiensis* while in urban settings *An. arabiensis* preferred to feed on cattle than humans compared to rural settings (13). Both *An. gambiae* and *An. arabiensis* occupy a similar ecological habitat however, *An. gambiae* prefer more humid areas while *An. arabiensis* are highly tolerant to dry environments (14, 15). In Kenya, after 20 years of effective malaria vector control (*An. gambiae* and *An. arabiensis*), there was significant high reduction in genetic diversity of *An. gambiae* but not *An. arabiensis* (16). The plastic behaviour of *An. arabiensis* plays a critical role in the adaptation of this species. A recent report highlights the shift in the

contributions of various malaria vectors to transmission in the eastern and southern regions of Africa since 2010 to date. *An. funestus* has emerged as a more significant vector in malaria transmission compared to *An. gambiae* during this period (17).

What makes female *Anopheles* efficient malaria vectors?

The saliva of a malaria vector if infected, contain *Plasmodium* sporozoites which are the infective stage for humans. When a female *Anopheles* mosquito carrying malaria-causing parasites feeds on a human, it injects the parasites in the form of sporozoites into the bloodstream. These sporozoites travel to the liver of the human host and invade liver cells. Within each liver cell, the sporozoites undergo growth and division, producing tens of thousands of merozoites. The merozoites develop into sexual forms of the parasite known as male and female gametocytes, which circulate in the bloodstream (figure 1:1) and the only stage transmitted to mosquito. The maturation process of *P. falciparum* gametocytes involves transitioning through five distinct morphological stages, typically spanning a duration of 8 to 10 days (18).

The transmission of gametocytes from humans to mosquitoes depends on several factors, including the age, density, and sex ratio of the gametocytes, as well as antimalarial drug treatment and hosts immunity (19). When a mosquito bites an infected human, it ingests mature gametocytes, which develop into sporozoites. The extrinsic incubation period of the parasite refers to the duration from gametocyte ingestion to sporozoite development (20). If a mosquito does not survive longer than this extrinsic incubation period, it will not be able to transmit any malaria parasites. This parameter is crucial in vector control, as the longer a mosquito survives, the greater the likelihood of parasite transmission. Other factors contributing to vector efficiency include the relative density of the vector, human biting frequency, number of bites per person per day, human blood index (proportion of blood meals taken from humans), intervals between blood meals, and the life expectancy of the female mosquito (21). Together, these parameters determine the vectorial capacity of a malaria vector. Vectorial capacity is greatly influenced by the female mosquito's ability to feed on a human host (22). Mosquito species such as *An. gambiae* s.s., *An. coluzzi*, and *An. funestus*, which exhibit strong anthropophilic (preference for human blood), are considered the most efficient malaria vectors worldwide.

Although it is not feasible to directly measure the lifespan of these vectors in nature, indirect estimates of daily survivorship have been made for several *Anopheles* species. For instance, in Tanzania, estimates of daily survivorship for *An. arabiensis* was 0.76 and 0.86 for *An. funestus,* translating into average life expectancy of 3.6 days for *An. arabiensis* and 6.5 days for *An. funestus* (23). Assuming constant survivorship throughout the adult life of a mosquito, less than

10% of female *Anopheles* mosquitoes would survive longer than the 14-day extrinsic incubation period. However, if daily survivorship increased to 0.9, over 20% of *Anopheles* mosquitoes would survive (24).

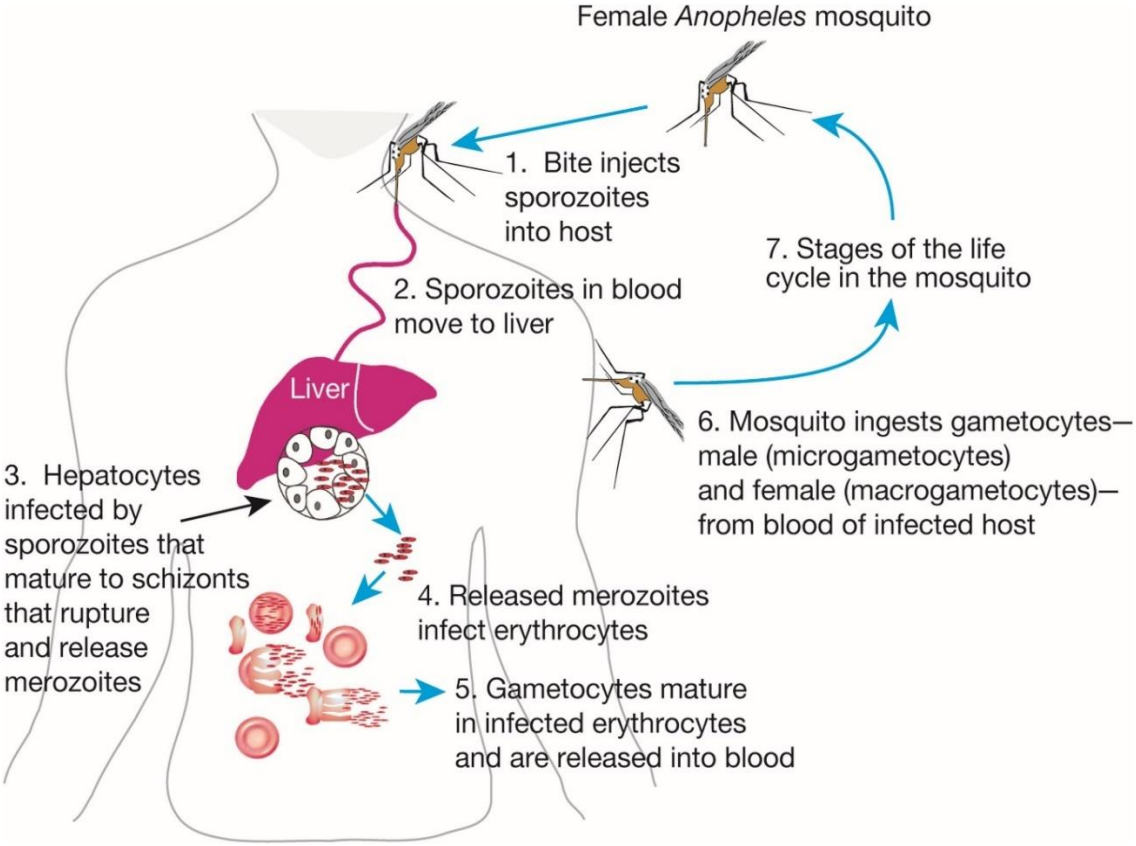

Figure 1:1: Life cycle of *Plasmodium* parasite.

Source ©2022; The consortium of Glycobiology editors, La Jolla, California

Malaria epidemiology

Globally, between 2000 to 2015, malaria death declined from 864,000 to 586,000 before increasing to approximately 249 million cases and 608,000 deaths in 2022 (25). Of these, 76% of malaria deaths were among children under five years old (25). The incidence of malaria increased from 244 million in 2021 to 249 million cases in 2022, while the estimated number of deaths (610,000 and 608,000 death in 2021 and 2022 respectively) remained nearly the same, showing a slight decrease (26). Sub-Saharan African countries bore a disproportionately high burden in 2022, accounting for 94% (233 million) of malaria cases and 95% (580,000) of malaria deaths (Figure 1:2). Four African countries:-Nigeria (31%), the Democratic Republic of Congo (12%), Niger (6%), and United Republic of Tanzania (4%) accounted for more than half of the global malaria deaths (25). It is unlikely that these countries will achieve the Global Technical Strategy (GTS) targets set by World Health Assembly (WHA) (27) of reducing malaria cases and deaths by 75% by 2025 and 90% by 2030. Factors contributing to this

stagnation include limited access to healthcare, ongoing conflicts and emergency, insecticide resistance, the impact of the COVID-19 pandemic, inadequate funding, and poor implementation capacity.

Despite the plateauing of funding and the challenges posed by the insecticide resistance and COVID-19 pandemic, the GTS goals remain unchanged. Modelling data indicates that if malaria interventions remain at their current level, the incidence could increase moderately (28). To prevent this, a concerted effort is needed to optimize the use of available interventions, achieving coverage levels above 80% for at-risk populations and improving the quality of services. This could significantly reduce the incidence of and deaths due to malaria.

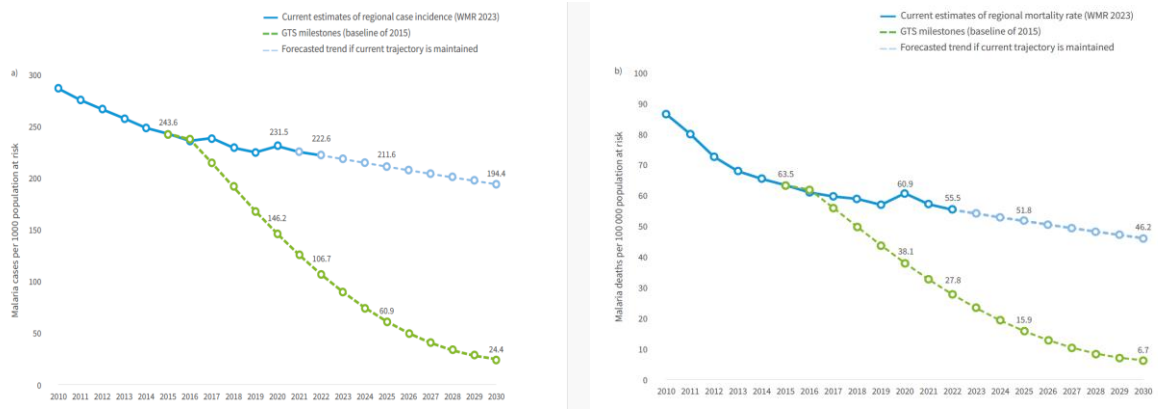

Figure 1:2: Comparison of progress in malaria case incidence (a) and mortality (b) with *Plasmodium falciparum* parasite rate (*pf*PR).
Source: GTS: Global technical strategies for malaria 2016-2030; WHO: world malaria report

Malaria Burden in Tanzania

In mainland Tanzania, a Demographic and Health Survey (DHS) conducted in 2015 and 2016 reported a 14% malaria prevalence across the country. However, these proportions vary geographically, with less than 1% reported in the highlands of Arusha and over 41% in the Lake Zone regions (29). This variation could be explained by species variation, altitude, social economic status (SES) as well as presence of different insecticide resistance mechanism as previously reported in the Lake Zone region that, malaria vectors exhibit both target site mutations (kdr East and West) and metabolic resistance mechanisms (30) which could among the factors attributed to high malaria prevalence around Lake zone.

In 2022, the DHS showed a malaria prevalence at 8% in children under 5 years old, which is lower than what was reported in 2015 (31) (Figure 1:3). In that year, more regions (Zanzibar,

Arusha, Kilimanjaro, Manyara, Dodoma, Singida, and Songwe) reported less than 1% malaria prevalence compared to the 2015/2016 survey, while Mtwara and Tabora reported much higher prevalence rates (20% and 23%, respectively) (31). Due to this variation, the NMCP stratified the country into four malaria epidemiological strata: very low, low, moderate, and high (30) to facilitate tailored malaria vector control interventions, optimizing resource allocation and maximizing the impact of interventions in the local context. However, the issue of insecticide resistance and outdoor malaria transmission threaten the interventions used for vector control (30). As a result, certain areas, continue to show higher malaria prevalence rates (61%) compared to the national average (32). A retrospective study carried out in Morogoro for six years (from 2014 to 2019) collecting data on malaria cases found that in two districts, Mkuyuni and Kiroka, nearly half of the tested population (n=35386, 46%) were positive for malaria (32).

To reduce the malaria burden in Tanzania, the Ministry of Health through the National Malaria Control Program (NMCP) in vector control define their goals, milestones and targets toward malaria vector control through mass replacement campaigns and school net program (SNP). Mass replacement campaigns based on accessibility and epidemiological risk, focusing on areas with less than 40% access, hotspots of moderate to high transmission, residual transmission areas, and emergency situations. SNP distribution ensure widespread coverage. LLINs are also distributed through Reproductive and Child health (RCH) clinics to protect vulnerable groups like infants and pregnant women, ensuring continued coverage. Special delivery systems target specific groups such as refugee camps, prisons, selected workforce sectors, young children with severe malaria, people living with Human Immunodeficient Virus (HIV), economically vulnerable elderly, mobile populations, socio-economically vulnerable neighbourhoods, hospitals, boarding schools, and orphanages, with a need for high community engagement for effective implementation (33).

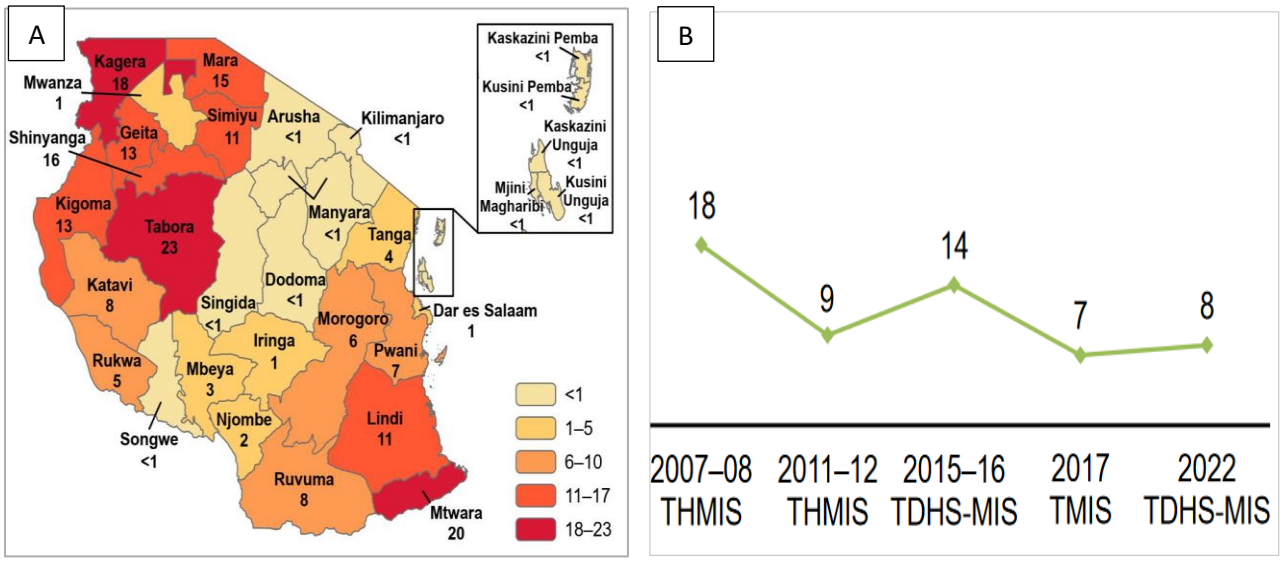

Figure 1:3: Tanzania Map showing A- Prevalence of malaria per region in 2015/2016 and B is trend in malaria prevalence to children aged 6-59 months.

Source; Tanzania demographic survey (DHS).

Existing tools for malaria vector control

The current methods for controlling malaria include ITNs and LLINs, Indoor Residual Spraying (IRS) (34), larval source management (LSM), biological control, genetic control, antimalaria medicines, malaria rapid diagnosis tests (RDTs) and vaccines (25). However, the primary contributors to malaria vector control are ITNs/LLINs and IRS.

ITNs interventions

The World Health Organisation's prequalification assessment process for vector control products, like insecticide-treated nets, has evolved to include a more thorough evaluation of their effectiveness. Previously, decisions about product prequalification—determining if a product meets the standards for safety, quality, and performance—were largely based on laboratory bioassays. These bioassays primarily involve controlled tests that measure the product's ability to kill or repel mosquitoes under specific lab conditions. However, lab tests alone don't always reflect how well a product will perform in real-world settings over time.

Now, prequalification decisions use a "weight-of-evidence" approach. This means that instead of relying only on lab results, the assessment also considers data from more rigorous studies, including randomized controlled trials (RCTs). RCTs are field studies conducted in actual settings where the product is intended to be used. By analyzing how the product performs in real environments and under diverse conditions, these trials provide a better indication of its long-term effectiveness and durability.

This comprehensive approach helps ensure that products not only meet initial performance standards but are also likely to provide adequate protection and effectiveness throughout their entire intended lifespan, giving users a "reasonable expectation" of consistent product performance in the field. Within the framework of prequalification (PQ), the term "bed net" is used to denote a material capable of offering protection to individuals within an enclosed space defined by the fabric, irrespective of its placement—be it outdoors, indoors, as a hammock, or in any other configuration (35). ITNs are bed nets treated with active ingredients (A.I.) aimed at repelling, killing, or knocking down malaria vectors (36), thereby augmenting both personal and community protection. This intervention exert influence on vector population dynamics, biting frequencies, and rates of sporozoite infection, thereby bolstering the overall effectiveness of malaria prevention efforts (35).

ITNs rely on three classes of insecticides: pyrethroids, pyrroles, and hormone growth regulators, and synergist PBO with pyrethroids being the most commonly used until recently (37). Pyrethroids (Pyr) bind to the voltage-gated sodium channel and lead to the rapid paralysis of insects (knockdown effect) and death (36). Pyriproxyfen (PPF) functions as a juvenile hormone mimic, disrupting the development and maturation of eggs. Chlorfenapyr (CFP), a pyrrole compound, impacts energy utilization by disturbing proton gradients in flight muscle (38). It achieves this by interfering with oxidative phosphorylation, thus causing a short circuit in mitochondrial respiration through the inner mitochondrial membrane (39). Consequently, the production of adenosine triphosphate (ATP) is hindered, depriving insects of essential energy and leading to their demise (39, 40).

Formerly, the presence of bed nets with intact fabric (i.e., undamaged) provided a physical barrier that prevented human–vector contact and reduced human blood-feeding (41, 42); treating the nets with insecticide provided additional protection by adding a toxic, repellent barrier (43). Sleeping under ITNs in endemic region contribute to 50% or more reduction in malaria transmission (44-46). Population based surveys across Sub-Saharan Africa reported that children sleeping under ITNs had 21% lower odds of malaria infection; however, the results depend on the net age with less than 1 year having the strongest protection. The protection by bed nets decreases as the net get older (47).

The Cochrane review done of five trials measuring mortality reported a significant reduction in malaria deaths among children under five years of age by 50% in the Sub-Saharan region, highlighting the benefits of ITNs in malaria-endemic areas (48). Additionally the review shows that ITN contribute to the reduction of severe malaria and its associated cost for both patient and health workers (49). Despite all the effort in vector control interventions, the World Health Organization's report in 2021 highlighted a plateau in the LLINs coverage and financial support in the fight against malaria (50).

IRS intervention

IRS is one of the major malaria vector interventions which uses long-acting insecticide on the walls and roofs of all houses and sometimes domestic animal shelters in a given area (51). It is effective against malaria vectors such as *An. gambiae* s.s., *An. arabiensis* and *An. funestus* in SSA (52). The aim of using IRS is to kill adult vector mosquitoes that land and rest on the sprayed surface. When mosquitoes contact with the sprayed wall, their life span are significantly reduced in such a way that they no longer transmit malaria parasites. Other insecticides repel mosquitoes hence reduce the number of mosquitoes entering the sprayed room and thus human vector contact (51). IRS relies on six classes of insecticide namely

pyrethroids, carbamates, organochlorines, organophosphates, pyrroles and neonicotinoids (37). Pyrethroid and Organochlorines bind to the voltage-gated sodium channel and lead to the rapid paralysis of insects (knockdown effect) and death (36). Organophosphates and carbamates deactivate acetylcholinesterase, resulting in an excess in acetylcholine leading to neuronal over-stimulation (53). Clothianidin, classified as a neonicotinoid, targets the nicotinic acetylcholine receptor (nAChR) in the insect central nervous system and has been employed in IRS operations.

The proportion of people at risk of malaria protected by IRS in endemic countries decreased from 5.5% in 2010 to 1.8% in 2022 globally. Since 2016, the percentage of the population covered by IRS has remained stable, with less than 6% protected in each WHO region (25). IRS campaign in four countries (Malawi, Madagascar, Zambia and Rwanda) contributed in protecting 4.8 million people from malaria. In Mainland Tanzania, about 2 million people were protected from malaria in 2021 (54). However, the number of districts selected for IRS was reduced from six district to two districts in 2022 with plan to discontinue from 2023 financial year (55). The fund for IRS was allocated to procure dual-ITNs and distribute in area with high pyrethroid resistance.

Comparison between ITN/LLIN and IRS interventions for malaria vector control

ITNs/LLINs and IRS are the primary methods of malaria control in Sub-Saharan Africa (12, 56), averting an estimated 2.1 billion cases (82% in sub-Saharan Africa) and 11.7 million deaths (94% in Sub-Saharan Africa) between 2000 and 2022, with LLINs being a major contributor (25). In United Republic of Tanzania, the number of people protected by IRS decreased by half (from 2,510,463 to 1,144,624) between 2020 to 2022 compared to ITNs which decreased from 19,684,506 in 2020 to 10,189,596 in 2022 (25). While studies comparing the cost-effectiveness and efficacy of these interventions offer different perspectives, some research suggests that they are equivalent in effectiveness (48). A study examining the impact of vector control on *Plasmodium falciparum* in Africa from 2000 to 2015 found that ITNs averted 68% of malaria cases, whereas IRS contributed to 10% of cases averted (57). In terms of cost-effectiveness, IRS is generally more expensive than ITNs due to the need for repeated applications and insecticide rotation to prevent resistance (58). When both interventions are used together, studies show no additional reduction in malaria prevalence compared to ITNs alone (59) but this depend on the insecticide mode of action as well as resistance mechanism of mosquitoes.

ITN Coverage and deployment

From 2004 – 2022 manufactures deliver about 2.9 billion ITNs globally of which 2.5 billion (86%) were supplied to Sub-Saharan Africa (25). This effort increase ownership of at least one ITN per household to 70% in 2022 compared to just 5% in 2000. The proportion of households owning at least one ITN for every two people increased from 1% in 2000 to 40% in 2022. Additionally, population access (the proportion of individuals with access to a net) rose from 3% in 2000 to 56% in 2022 (25), As a result, there was a reduction of case incidence in 2022 compared to 2000 (figure 1:4).

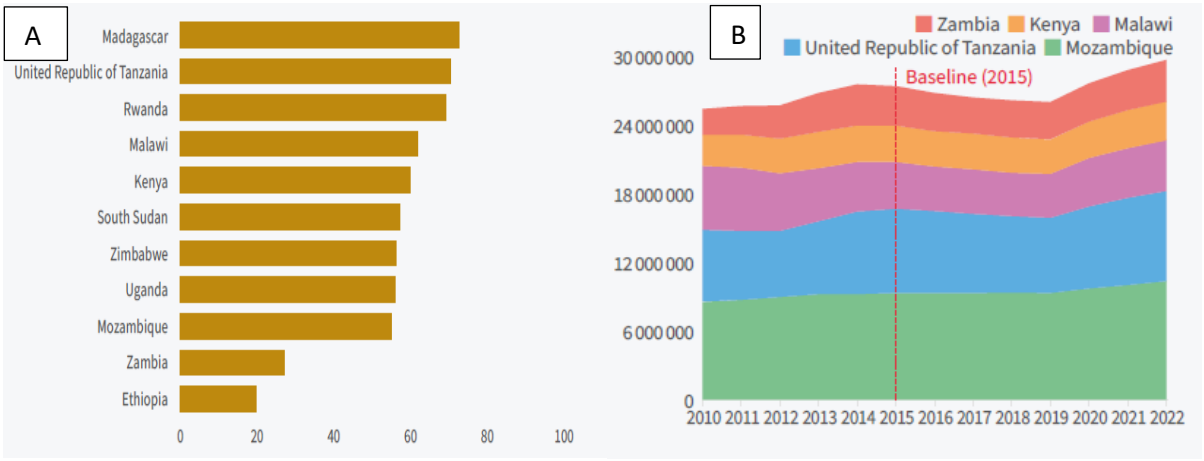

Figure 1:4: Percentage of population with access to an ITN in year 2022 (A) and estimated number of cases in countries that reduced case incidence by <55% in 2022 compared to 2015 (B).
Source: ITN coverage from Malaria Atlas Project (MAP) and WHO malaria report 2023.

Threats to effectiveness and durability of ITNs

The progress of global malaria vector control efforts has been jeopardized by the emergence of insecticide resistance, inadequate access to control measures, net attrition, and shifts in mosquito behaviour (26). Changes in mosquito behaviour include a tendency for mosquitoes to feed earlier in the evening and rest outdoors, which reduced their exposure to ITNs (26). While ITNs may provide personal protection even after nets become holed due to their insecticidal and excito-repellency effects (60), the challenge remains that more resistant mosquito phenotypes can penetrate these holes to feed (61) and be found resting on inner surfaces (62, 63). According to the WHO, a population of malaria vectors is considered as phenotypically resistant if less than 90% of its individuals are killed 24 hours after exposure to a discriminating dose of insecticides (64). Of the 88 malaria-endemic countries in Africa, 78 have confirmed resistance to at least one insecticide. Additionally, 29 countries have reported resistance to all four classes of insecticides: pyrethroids (in 87% of the countries), organophosphates (in 60%), carbamates (in 69%), and organochlorines (in 82%)) (Figure 1:5) (25). Resistance to pyrethroid

insecticides was first reported in 1993 in Ivory Coast (65), with high intensity of pyrethroid resistance frequently observed in west Africa compared to other subregions (66).

Data extracted from the insecticide resistance (IR) mapper database in 2021 show an important decrease in mortality in *An. gambiae* s.s., 24 hours post-exposure to a discriminating dose of pyrethroids since 1995 in SSA (67). This indicates a rapid expansion in insecticide resistance in SSA over the past 10 to 20 years.

Several studies have demonstrated that ITNs are becoming less effective at killing mosquitoes in areas of high resistance compared to when resistance was less prevalent (62, 68). In mainland Tanzania, insecticide resistance was reported to increase from 0% pyrethroid resistance (100% mortality) in 2004 to 80% (20% mortality) pyrethroid resistance in 2020 nationwide (69). Similar results, with mortality ranging from 12% to 23% in *An. gambiae* s.l., were reported in northwest Tanzania during a cRCT (70). Resistance to all insecticide classes was identified in *An. gambiae* s.l., while *An. funestus* s.l. exhibited resistance specifically to pyrethroids and DDT. *An. gambiae* s.l. demonstrated resistance through both target site mutations and metabolic mechanisms (71), whereas target site mutation in *An. funestus* was reported recently (72). Following the selection of high pyrethroid resistance arising from a combination of *kdr* and mono-oxygenase metabolic mechanisms in Northwest Tanzania, even new standard ITNs may not reduce malaria transmission substantially (70, 73, 74).

It has been documented that protection offered to users is reduced when the ITNs develop holes (75, 76) in area with insecticide resistance and may lead to ITNs being discarded, and therefore reduction in coverage (77, 78). In Zambia a study showed that the poor fabric integrity of standard pyrethroid nets affected their effectiveness against *An. arabiensis* (75). A similar study in Tanzania demonstrated that increased hole area was associated with higher numbers of *An. gambiae* inside the net (76). In contrast, studies conducted in same country (Tanzania), one looking at protective efficacy of PBO-Pyr nets reported no an association between hole area and malaria infection (79). Second study assessing the impact of textile durability on efficacy of dual ITNs reported no association between hole size/area and malaria prevalence, but association was observed in malaria incidence for all dual ITNs assessed (80). Washing and drying ITNs have been reported to be among the factors that contributed to reduce ITN insecticide concentration and the development of holes in the community (81). In Bouaké, Côte d'Ivoire, social and economic status was among the factors affecting net handling i.e (tucking it in bed, washing and drying) (82). In such conditions protective efficacy from malaria may be reduced when physical condition of the net deteriorates (83).

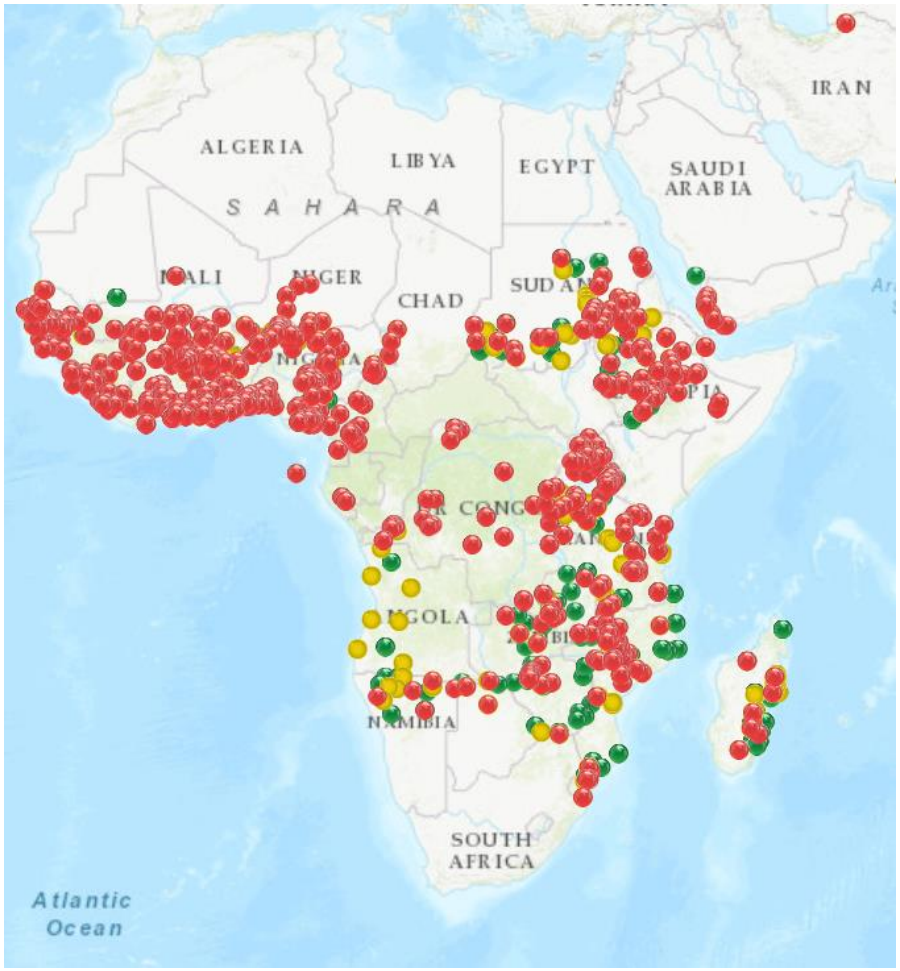

Figure 1:5: Insecticide resistance in Africa from 2014 to 2024.

Red dots represent the area where insecticide resistance has been confirmed, yellow dot represent the area where detection of insecticide resistance has been reported and green dot is area where mosquitoes are still susceptible. **Source** https://anopheles.irmapper.com/.

Insecticide resistance mechanisms

Several insecticide resistance mechanisms have been identified that contribute to phenotypic resistance in SSA: -

1/ Target site insensitivity involves point mutations in genes which encode for insecticide binding sites, e.g. knockdown resistance (*kdr*) in *An. gambiae* s.l.. *Kdr* mutation occurs in two different classes of insecticide (pyrethroid and DDT) due to cross-resistance since both pyrethroids and DDT targets the insects central nerve system (CNS) sodium channel. So far, two distinct mutations of the sodium channel protein sequence at position 1014 have been identified in *An. gambiae*, leading to amino acid residue changes from a leucine to a phenylalanine in West Africa (84), and a leucine to a serine in East Africa (85). Recently, another mutation of the sodium channel linked to pyrethroid resistance phenotype in *An. arabiensis* has been identified. In *An. funestus* mutation of the voltage gated sodium channel at position L976F linked to DDT resistance but not pyrethroid (deltamethrin) has been identified

(72). *Kdr* mutations have also been identified in other disease vectors, such as *Ae. aegypti (86)*, *Cx. pipiens* (87) and more recently, *Ae. albopictus* (88). A Multi-country study conducted across 13 sub-Saharan African countries, examining the progression of resistance mutations in the *dieldrin resistance locus* (*Rdl*) targeting the GABA receptor insecticide *Rdl* in African *Anopheles*, revealed that the *Rdl* resistance mutations arising post the initial introduction of dieldrin in the 1950s are projected to remain significant in the near term (89). Other target site mutation is resistance of *An. gambiae* to organophosphates and carbamates based on a reduced sensitivity of acetylcholinesterase was first detected in West Africa (90). This resistance is caused by a single mutation in the ace-1 gene at position 119, changing a Glycine in Serine within the active "gorge" of acetylcholinesterase (91).

2/ Metabolic resistance occurs when detoxification enzymes, such as carboxyl-esterases, cytochrome P450s, and glutathione-S-transferases, are over-expressed, enabling them to break down insecticides before they can reach their target (92, 93). Over-expression of enzymes capable of detoxifying insecticides or amino acid substitutions within these enzymes, which alter the affinity of the enzyme for the insecticide, can result in high levels of insecticide resistance (94).

One of the most common metabolic resistance mechanisms is that of elevated levels, or activity, of esterase enzymes which hydrolyze ester bonds or sequester insecticides and they have the ability to metabolize pyrethroids (95). The homologue enzyme carboxylesterase (CCEs) role in pyrethroid resistance has not been validated rather it's probable (96), they have been mostly associated to organophosphate resistance in mosquitoes.

Cytochrome P450-dependent monooxygenases are Phase I detoxification heme-thiolate enzymes catalysing various reactions, but are best known for their monooxygenase activity, introducing reactive or polar groups into xenobiotics like insecticides (97). These enzymes are well known in their ability to digest insecticide at a higher rate (98). A total of 111 P450 enzymes have been identified however not all of them are capable of detoxifying insecticides. Cytochrome P450 belong to six families and increased transcription of genes belonging to the CYP4, CYP6, and CYP9 has been observed in various insecticide-resistant species from different taxa (97). The research carried out in the Democratic Republic of Congo, investigating novel resistance markers in *An. gambiae* and *An. funestus,* revealed the identification of a triple mutant consisting of CYP4J5, G119S-ace1, CYP6P9a, and CYP6P9b, indicative of resistance to key insecticide classes (99). This mutation (CYP6P9a, and CYP6P9b) has been linked to failure of standard pyrethroid bed net in Cameroon (100).

Glutathione transferases (GSTs) are phase II multifunctional enzymes involved in the detoxification of many endogenous and xenobiotic compounds. Around thirty GST genes from different subfamilies have been identified in mosquitoes (101). Elevated GST activity has been implicated in resistance to at least four classes of insecticides in insects and it confer cross-resistance to pyrethroid and DDT insecticide. At least six classes of insect GSTs have been identified in *An. gambiae* (96). The Delta and Epsilon classes found exclusively in insects are the largest classes of insect GSTs. Members of both classes have been implicated in resistance to all the major classes of insecticide.

3/ Cuticular resistance is characterized by a modification of the insect cuticle associated proteins (e.g., cplcg3 and cplcg4) or metabolic enzymes which are localised to the cuticle (e.g., CYP4G16 and CYP4G17) (102) leading to a slower penetration of the insecticide reducing the amount of insecticide molecules within the insect thereby enhancing the efficiency of detoxification systems (96). Cuticular resistance in mosquitoes is usually characterized along with other types of resistance. Cuticular resistance was associated with increased thickness of the cuticle in *An. funestus* (103). Genes encoding cuticle proteins were also found over-transcribed in *An. gambiae* populations resistant to pyrethroids (104).

4/ Behavioural avoidance, particularly extensive exposure to insecticides, can result in behavioural changes that act as a contributing factor for resistance. For example changes in host seeking behaviour of *An. funestus* due to universal coverage of ITNs in Benin (105). Vector populations in Tanzania have been observed to shift their behaviour from late (midnight) biting to early in the evening, and increase outdoor biting and animal feeding in the presence of ITNs in different parts of Tanzania (9, 106, 107). Another example is the recent observation of a rising number of *An.funestus* being collected in the early morning, during school hours, in western Kenya (8). These shifts in behaviours can allows vector to reduce their contact with ITNs and diminish their impact on control. For example, a study in Uganda found that, behavioural avoidance by vectors impacted the effectiveness of vector control interventions (108).

5/ Microbiota associated resistance: Mosquitoes harbour a diverse community of microbes, encompassing bacteria, algae, fungi, and viruses. These microorganisms coexist closely, exerting a collective influence on mosquito physiology and metabolic functions (109). In Kenya, *An. gambiae* s.l. were observed to possess a range of bacterial taxa, spanning from resistant to susceptible, indicating a potential microbial-mediated mechanism contributing to insecticide resistance in mosquitoes (110). Similarly, in Cote d'Ivoire, the presence of *Asaia* and *Serratia* bacteria was associated with resistance to deltamethrin, underscoring the need for the

identification of novel microbial markers for surveillance purposes and the exploration of innovative control strategies to curb the further spread of resistance (111).

Next generation of ITNs and evaluation processes

To address the challenge of pyrethroid insecticide resistance, various new classes ITNs have been recommended by WHO. This include combinations of a pyrethroid insecticide with a synergist PBO (112) or a second insecticides (CFP or PPF) (25) as they show superior efficacy in improving malaria outcomes (prevalence or/and incidence) compared to standard pyrethroid LLINs (59, 113-115).

The evaluation of novel classes of vector control tools without WHO policy recommendations involves 1/assessing the product bio-efficacy again entomological outcome, safety and quality to obtain a WHO Prequalification listing and 2/ demonstrating efficacy through RCTs against epidemiological outcomes to evaluate public health value (figure 1:6.)

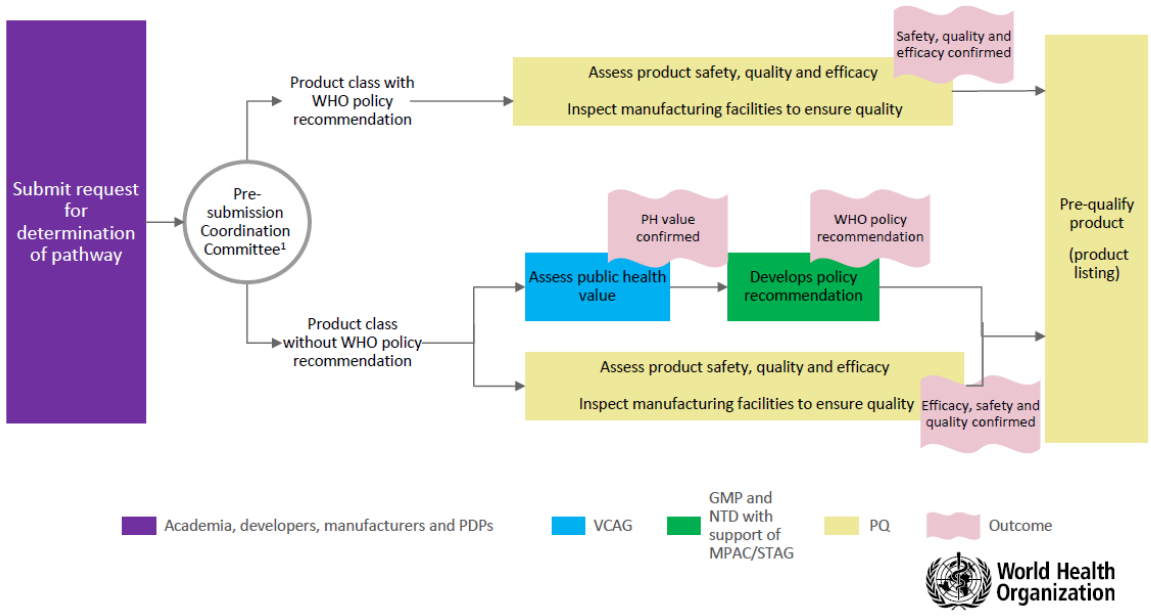

Figure 1:6: Demonstration of public health impact for new class of product (RCTs with epidemiological and entomological endpoint).

Source WHO evaluation process for vector control interventions 2017.

Prequalification of vector control product assessing safety, quality and bio-efficacy)

For ITN, evaluation will be done using WHO guidelines in phase I (laboratory), phase II (semi field condition) and phase III under field conditions and submitted to the WHO pre-qualification team. The guideline recommends assessing three elements: insecticidal bio-efficacy, physical or fabric integrity and survival/attrition (116). According to the WHO, insecticide in LLIN should demonstrate bio-efficacy over 3 years (117).

The ITN assessment in Phase I aims to determine the insecticidal activity, wash-resistance, and regeneration time of ITNs in a laboratory setting. This experiment does not aim to simulate field washing conditions but instead provides a standardized protocol to enable consistent comparisons across laboratories and different ITN products (118). The bio-efficacy and safety of these nets are evaluated against host-seeking mosquitoes in the presence of human occupants, under realistic household conditions, in EHTs (Phase II) (119, 120). Experimental huts are ideal for measuring the natural behaviour and killing of mosquitoes in the house as there is no manipulation of the host-seeking mosquito and no interference with the resting time of the blood-fed stage. A total of 8 brand of PBO-Pyr ITNs, 2 brands CFP-Pyr and one PPF-Pyr have been PQ listed based on various phase I and II studies carried out (121).

The first two brands of nets incorporating a pyrethroid insecticide and the synergist PBO recommended were PermaNet 3.0 (122) and Olyset Plus (123). Phase I studies showed that Olyset Plus and PermaNet 3.0 (PBO-Pyr ITNs) exhibited superior efficacy compared to the standard Olyset net when tested against the susceptible Kisumu strain (124, 125). This finding was corroborated in West Africa, where similar results were observed against wild, free-flying mosquitoes in experimental huts (124). Olyset Plus demonstrated significant mortality in malaria vector species compared to control nets, as documented in another study (100). Furthermore, consistent results were reported in Burkina Faso, where Olyset Plus outperformed pyrethroid-only LLINs in experimental field trials (126). A Cochrane review, pooling results from 10 experimental hut studies, indicated improved performance of PBO-Pyr nets (4 brands) over standard LLINs in terms of mortality and blood feeding inhibition, although these results exhibited heterogeneity. In areas with high mosquito resistance, PBO-Pyr nets were found to reduce mosquito blood feeding, hence better personal and community protection. However, this impact diminished after the nets underwent 20 washes (127, 128). Additionally, the efficacy of PBO-Pyr nets decreased in regions with high levels of mosquito resistance; the initially high mortality observed when these nets were new and unwashed was not sustained after multiple washes in the community. High resistance was defined with less than 30% mortality in standard bioassays (128).

Three more additional dual-active ingredient (A.I.) ITNs brand (Royal Guard, Interceptor G2 and PermaNet dual) have undergone evaluation in WHO Phase I and II trials, showing significant promise compared to standard LLINs in combating pyrethroid-resistant vectors. In Phase I studies, Royal Guard (PPF-Pyr ITNs) met the WHO criteria with 95% knockdown and more than 80% mortality for up to 20 washes when susceptible *An. gambiae* s.s. (Kisumu strain) were exposed in cone assays (129). It also met the WHO criteria in tunnel tests, with mortality exceeding 80% after 20 washes using the same strain. In a Phase II EHT conducted against wild, pyrethroid resistant *An. gambiae* s.l., Royal Guard demonstrated an 83% reduction in oviposition and a 95% reduction in offspring/hatching before washing. These values decreased to 25% and 50%, respectively, after 20 washes (130). Another dual-ITN (Olyset Duo), which combines permethrin and pyriproxyfen, was reported to be superior to the standard Olyset net in terms of mortality and sterilization of surviving blood-fed, insecticide-resistant *Anopheles* mosquitoes in southern Benin (131). Despite its performance against resistant *Anopheles* strains, it did not receive the WHO PQ recommendation.

Interceptor G2 combines chlorfenapyr and alpha-cypermethrin insecticides. Chlorfenapyr is a non-irritant insecticide, which requires a longer contact time between mosquitoes and the insecticide to induce mortality (132, 133). The study assessing the suitable concentration of chlorfenapyr to induce LC_{95} against susceptible and resistance strain were conducted in 16 countries in Sub-Saharan Africa. Of the concentration test, there were no significant difference between mortality (100%) induced by 100 µg/ml chlorfenapyr and 200 µg/ml of chlorfenapyr / bottle, 72 hours post exposure (134, 135).

The study conducted in Moshi insectary assessing the activity of pyrrole in mosquito bioassay reported that, in cone and cylinder tests, chlorfenapyr failed to meet the WHO standard within the three-minute exposure timeframe. However, it met the criteria when mosquitoes were exposed overnight in tunnel tests (136). The mechanism underlying chlorfenapyr's efficacy could elucidate why Interceptor G2 failed in cone tests but performed better in tunnel tests and EHTs. In *An. gambiae*, the expression of cytochrome P450s involved in oxidative metabolism is under circadian control, with stronger expression observed during the night (137). Evidently, all activities of *Anopheles*, such as flight and host-seeking, are high-energy and high-respiratory behaviours occurring during the night under circadian control (138, 139).

In EHT, Interceptor G2 was able to induce 71% mortality against free flying *An. gambiae* s.l. compared to an alpha-cypermethrin-only net (20% mortality) (140). Notably, the performance of dual-A.I. ITNs remained superior even after 20 washes compared to reference nets in

experimental huts and laboratory settings (130, 140, 141). In March 2023, the WHO recommended another mixture of chlorfenapyr and deltamethrin net (Permanent dual) to be used in area of pyrethroid resistance as it shows improved efficacy against pyrethroid resistance mosquitoes in southern Benin (142).

Some malaria transmission models utilize ITN Phase II experimental hut parameters, including vector knock-down, mortality, blood feeding inhibition, and exiting, to forecast malaria incidence and prevalence over time (143). These models also help assess the impact of nets on malaria transmission. However, the WHO has stated that such modelling alone is insufficient for evaluating new classes of ITNs given the current state of knowledge (144).

During literature review, few studies conducted in Phase III using dual A.I ITNs were found as follows: The study conducted in Kenya looking on bio-efficacy and durability of Olyset Plus compared to standard Olyset net reported that, Olyset Plus was superior than standard Olyset up to two years and functional survival was less than three years (145). Similar findings was reported in different parts of Tanzania (79) and in this thesis (146). In Burkina Faso, Olyset Duo were reported to have no differences in fabric integrity compared to standard net with poor survivorship reported in both nets after 36 months (147).

Public health values of new intervention class

New product needs to demonstrate efficacy against epidemiological outcomes (malaria prevalence or incidence) in two cRCTs to receive a public health recommendation (148). The Trial, protocol, (149) and results are reviewed by the WHO vector control advisory group (VCAG) to insure appropriate design and quality of data. Following the results review VCAG provides a report. Another committee, the WHO guideline review committee, reviews all the available evidence and formulate specific recommendations in the malaria guideline (150). PBO-Pyr, CFP-Pyr and PPF-Pyr combination ITNs have all received public health recommendations following results generated by cRCTs (59, 114, 115, 151, 152).

For PBO-Pyr ITNs, two cRCTs were conducted in two different countries, Tanzania and Uganda. The initial study compared the prevalence of parasites in children who used Olyset Plus nets versus standard Olyset nets in an area of Tanzania with significant pyrethroid resistance. Results showed a 60% decrease in parasite prevalence 21 months after the distribution of nets (59). In a subsequent trial conducted across East and West Uganda, where mosquitoes exhibited high resistance to pyrethroids, researchers compared parasite prevalence among children using either Olyset Plus or PermaNet 3.0 nets with those using Olyset or PermaNet 2.0 nets. The findings revealed a 17% reduction in parasite prevalence 25 months

post-distribution for PBO-Pyr nets (115). These results led the WHO to recognize the public health importance of PBO-Pyr ITNs, prompting the issuance of a conditional recommendation for their utilization as an innovative form of vector control (112).

Two trials, one in Tanzania and the other in Benin, were conducted to evaluate the efficacy of Interceptor G2 against standard Interceptor nets in areas with high pyrethroid resistance. The results showed a 55% lower odds of malaria infection among children aged 6 to 14 years and a 44% reduction in malaria case incidence in children aged 6 to 10 years after two years of using Interceptor G2 nets (151). Moreover, the entomological inoculation rate (EIR) was significantly lower (by 85%) in the Interceptor G2 arm compared to the standard pyrethroid-only arm (151). In the same trial, Olyset Plus exhibited a shorter protective effect of 12 months compared to a prior cRCT conducted in a different part of Tanzania, where PBO-Pyr ITNs remained effective for 24 months (59, 151). After 36 months of community use, Interceptor G2 continued to outperform Interceptor nets in reducing prevalence (152). A second cRCT conducted in Benin showed similar results, with Interceptor G2 reducing malaria incidence by 46% in children aged 6 to 10 years compared to pyrethroid-only LLINs, while Royal Guard did not significantly reduce malaria outcomes (114).

Laboratory and experimental hut data from studies conducted in Benin (130) and Cameroon (153) did not report a difference in mortality between Royal Guard and standard LLINs. Similar result was reported in cRCT in Benin where Royal Guard nets does not seem to contribute in malaria reduction significantly (114). In contrast, another trial conducted in Burkina Faso in 2018 (154) assessed Royal Guard, showing a small but significant reduction in malaria outcomes in the PPF arm compared to the standard LLIN arm one month post distribution.

Based on evidence from these two trials, WHO issued a strong recommendation for CFP-Pyr ITNs, while PPF-Pyr ITNs, review considered the trial conducted using another PPF-Pyr net (Olyset Duo) in Burkina Faso (147) to issue conditional recommendation.

1.2 PhD Rationale

This is the first study assessing the bio-efficacy and durability of the novel dual-A.I. ITNs (Royal Guard and Interceptor G2) and the PBO synergist net, Olyset Plus. It is designed to support the development of bio-efficacy and physical durability criteria for partner A.I. in relation to the cRCT efficacy outcomes and refine preferred product characteristics developed by the WHO. EHT done in the vicinity of the cRCT, with similar vector population characteristics using nets sampled from the main trial at intervals will allow us to understand the impact of field conditions, wear-and-tear and insecticidal deterioration on the efficacy of

the dual-A.I. ITNs on entomological outcomes, and to relate these to the cRCT epidemiological and entomological outcomes.

1.3 General objectives

This thesis aims to assess the bio-efficacy and durability of three types of ITNs Royal Guard, Olyset Plus, Interceptor G2 compared to standard Interceptor LLIN over 3 years of use in the community,

1.4 Specific objectives

1. To assess physical durability of netting materials and attrition rates of Interceptor G2, Royal Guard and Olyset Plus relative to standard pyrethroid only (Interceptor) over three years of use in the community.

2. To evaluate efficacy of Interceptor G2, Royal Guard and Olyset Plus sampled after 0, 12, 24 and 36 months of use on the mortality, blood-feeding inhibition, and reproduction inhibition (Royal Guard) of host-seeking wild *Anopheles* in experimental hut trials.

3. To assess the intensity of resistance to permethrin, alpha-cypermethrin, pyriproxyfen and chlorfenapyr and investigate the mechanisms of resistance of wild *Anopheles* mosquitoes in the experimental hut study area. This objective is combined with objective 2 in chapter 6.

4. To assess insecticidal activity (bio-efficacy) and chemical content of Interceptor G2, Royal Guard and Olyset Plus relative to standard LLIN over three years of use in the community.

2 Chapter 2: Material and Methods: Durability and bio efficacy of three types of dual active ingredient long-lasting insecticidal net.

The work in this chapter has been published as:

Martin JL, Messenger LA, Mosha FW, Lukole E, Mosha JF, Kulkarni M, Churcher TS, Sherrard-Smith E, Manjurano A, Protopopoff N, Rowland M. Durability of three types of dual active ingredient long-lasting insecticidal net compared to a pyrethroid-only LLIN in Tanzania: methodology for a prospective cohort study nested in a cluster randomized controlled trial. Malar J. 2022 Mar 19;21(1):96. doi: 10.1186/s12936-022-04119-4.

Abstract

Background: Progress achieved by long-lasting insecticidal nets (LLINs) against malaria is threatened by widespread selection of pyrethroid resistance among vector populations. LLINs with non-pyrethroid insecticides are urgently needed. This study aims to assess the insecticide and textile durability of three classes of dual-active ingredient (A.I.) LLINs using techniques derived from established WHO LLIN testing methods to set new standards of evaluation.

Methods: A WHO Phase 3 active ingredients and textile durability study will be carried out within a cluster randomized controlled trial in 40 clusters in Misungwi district, Tanzania. The following treatments will be evaluated: 1/ Interceptor®G2 combining chlorfenapyr and the pyrethroid alpha-cypermethrin, 2/ Royal Guard® treated with pyriproxyfen and alpha-cypermethrin, 3/ Olyset^{TM} Plus which incorporates a synergist piperonyl butoxide and the pyrethroid permethrin, and 4/ a reference standard alpha-cypermethrin only LLIN (Interceptor®). 750 nets will be followed in 5 clusters per intervention arm at 6-, 12-, 24- and 36-months post distribution for survivorship and hole index assessment. A second cohort of 1950 nets per net type will be identified in 10 clusters, of which 30 LLINs will be withdrawn for bio-efficacy and chemical analysis every 6 months up to 36 months and another 30 collected for experimental hut trials every year. Bio-efficacy will be assessed using cone bioassays and tunnel tests against susceptible and resistant laboratory strains of *Anopheles gambiae sensu stricto*. The efficiency of field-collected nets will be compared in six experimental huts. The main outcomes will be *Anopheles* mortality up to 72 hours post exposure, blood feeding and egg maturation using ovary dissection to assess impact on fecundity.

Conclusions: Study findings will help develop bio-efficacy and physical durability criteria for partner A.I., in relation to the cRCT epidemiological and entomological outcomes, and refine preferred product characteristics of each class of LLIN. If suitable, the bioassay and hut outcomes will be fitted to transmission models to estimate correlation with cRCT outcomes.

2.1 Background

Long-lasting insecticidal nets (LLINs) are the primary method of malaria control in sub-Saharan Africa. The World Health Organization (WHO) estimates that over 50% of the population now sleeps under LLINs. This has helped to reduce malaria incidence by 42% and mortality by 66% in Africa over the last 15 years (155). Until recently, pyrethroids were the only type of insecticide used routinely on LLINs. The rapid spread of pyrethroid resistance in vector populations threatens to reverse the success achieved so far (143). Several studies have demonstrated that LLINs are becoming less effective at killing mosquitoes in areas of high resistance compared to before (62, 68).

The first new type of LLIN developed to control resistant mosquitoes is a combination LLIN containing permethrin and the synergist piperonyl butoxide (PBO), which inhibits cytochrome P450 oxidases responsible for metabolic resistance (156). A community-based cluster randomized controlled trial (cRCT) conducted in north-western Tanzania (Kagera region) demonstrated a reduction in the prevalence of malaria by 44% in the pyrethroid-PBO LLIN arm (Olyset ^{TM} Plus) compared to the standard pyrethroid LLIN after one year and by 33% after two years (157, 158). Based on this study, the WHO recognized the improved public health value of the pyrethroid-PBO LLIN in areas of high resistance and provided interim recommendation for pyrethroid PBO LLINs (159). Since then, two dual-active ingredient (dual-A.I.) LLINs (Royal Guard® and Interceptor® G2) have been evaluated in WHO Phase I and II trials (116) and have shown promise compared to standard LLINs against pyrethroid resistant vectors. Each of these putative first-in-class LLINs are required by the WHO to undergo cRCTs versus standard LLINs to demonstrate, unequivocally, evidence of improved malaria control effect (144). Two such community cRCTs are currently underway in Misungwi, Tanzania (160) and in Cove, Benin (161).

For each first-in-class LLIN, in addition to cRCTs, assessment of the quality, long lasting entomological efficacy and safety is also required for each putative vector control product submitted to the WHO (144). Whether the LLIN product is a novel first-in-class LLIN, or a generic second-in-class LLIN, the LLIN should be evaluated in three different phases (116). After LLIN assessment for insecticidal activity and wash-durability in the laboratory (Phase I), the bio-efficacy and safety of these nets are evaluated against host-seeking mosquitoes in the presence of human occupants, under realistic household conditions, in experimental hut trials (Phase II) (119, 120). Thereafter, bio-efficacy, attrition and physical durability of nets are monitored in the community over 3 years in large-scale field trials requiring nets to be sampled from households and evaluating them for net integrity (hole index) and attrition (Phase III)

(116). Collectively, the data from the three phases are then reviewed by WHO for pre-qualification decision (144).

While community cRCTs provide definitive epidemiological evidence for the establishment of new product classes of LLIN, questions remain whether cRCTs should be the primary mechanism to generate malaria vector control evidence (162). It has been proposed that entomological evidence generated by experimental hut trials, if used to parameterize malaria transmission models, may be adequate to make to this judgement (143), as the cost of EHTs are much lower than cRCTs and much shorter in duration than the two years needed for cRCT which may delay the introduction of new tools and disincentivize investment in new active ingredients (163).

In some respects, experimental huts are ideal for measuring the natural behaviour and killing of mosquitoes in the home as there is no manipulation of the host-seeking mosquito and no interference with the resting time of the blood-fed stage. Some malaria transmission models make use of LLIN Phase II experimental hut parameters (e.g. vector knock-down, mortality, blood feeding inhibition, and exiting) to predict malaria incidence/prevalence over time (143) and the impact of nets on malaria transmission. The WHO has determined that current modelling is insufficient to evaluate new classes of LLINs given the present state of knowledge (144). Malaria transmission dynamics simulated by such models have been used to predict the public health impact of different vector control interventions (164). With further corroborative evidence, these models simulate transmission infection in populations of humans and mosquitoes and could be used to extrapolate the results of cRCTs to other sites with different epidemiology, entomology, or mixtures of control interventions. At present, it is unclear whether models parameterized with local hut trial data, which capture the behavior and survivorship of the local mosquito vector, would be more accurate in predicting local epidemiology than the current modelling approach that uses meta-analysis data from mosquito populations in disparate locations. A further drawback, these models have typically used data from LLINs subjected to 20 standardized washes to simulate the ageing process. As a result, their ability to predict is limited by the accuracy of standardized washing to reflect real life wear-and-tear and insecticidal durability under field conditions. In our studies, nets will be sampled from the community trials (at Phase III) for evaluation in experimental huts to parameterize the models.

The main aim of the study is to assess the insecticidal and physical durability of new dual-A.I. LLINs, Interceptor®G2, Olyset^{TM} Plus and Royal Guard® in the community over three years embedded within a cluster randomized controlled trial (cRCT). This is the first study assessing the durability of the novel dual-A.I. LLINs (Royal Guard® and Interceptor®G2) and the

synergist net, Olyset^{TM} Plus. It is designed to support the development of bio-efficacy and physical durability criteria for partner A.I. in relation to the cRCT efficacy outcomes and refine preferred product characteristics developed by the WHO. Experimental hut trials done in the vicinity of the cRCT, with similar vector population characteristics using nets sampled from the main trial at intervals will allow us to understand the impact of field conditions, wear-and-tear and insecticidal deterioration on the efficacy of the dual-A.I. LLINs on entomological outcomes, and to relate these to the cRCT epidemiological and entomological outcomes.

A secondary aim is to establish whether entomological outcomes generated during the WHO product evaluation process (adapted experimental hut trials and supporting bio-efficacy testing) from nets sampled from the community can provide a proxy for epidemiological outcomes of cRCTs via transmission modelling.

2.2 Methods

Study area

The WHO Phase III durability study is part of a four-arm cRCT carried out in Misungwi district (2°51'00.0"S, 33°04'60.0"E), on the Southern border of Lake Victoria, Tanzania. The cRCT study area includes 72 villages, 42,314 households and a population of 251,155 based on a census done in 2018 as part of the study. A detailed description of the cRCT is provided elsewhere (160). Six experimental huts are constructed in Magu district, Mwanza region, Tanzania (2°34.673'S, 33°07.170'E). The hut study site is north of the cRCT study area (Figure 4:1). The main vectors in the study area are *Anopheles funestus sensu stricto* (*s.s.*), *Anopheles arabiensis* and *Anopheles gambiae s.s.*, with *An. gambiae* s.s being the predominant species (165).

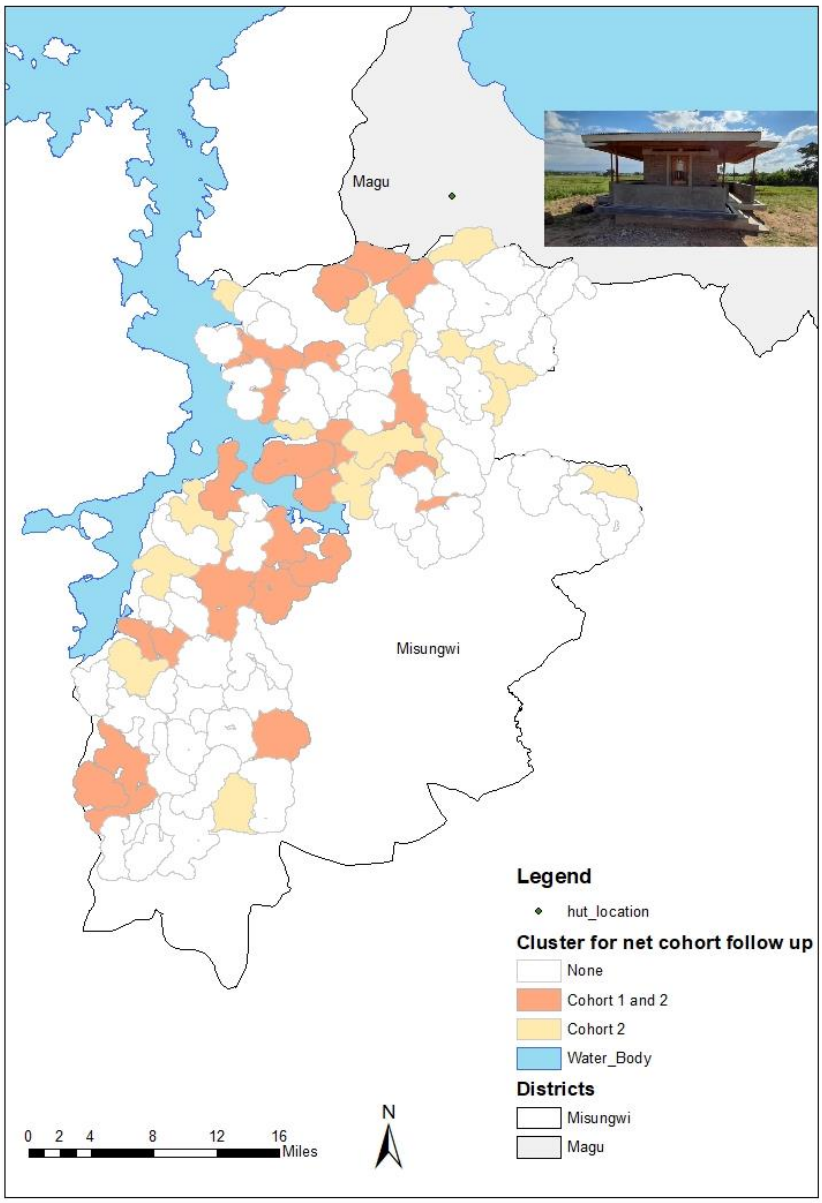

Figure 2:1.Map showing cRCT and experimental hut area

Descriptions of the interventions

The four LLINs under evaluation are 1/ Royal Guard®, a net combining pyriproxyfen (PPF) known to disrupt female reproduction and fertility of eggs and the pyrethroid alpha-cypermethrin; 2/ Interceptor®G2, a mixture net incorporating two adulticides with differing modes of action: chlorfenapyr and the pyrethroid alpha-cypermethrin; 3/ Olyset^{TM} Plus, a LLIN which incorporates a synergist piperonyl butoxide (PBO) to enhance the potency of pyrethroid insecticides; 4/ Interceptor® an alpha-cypermethrin only LLIN and the reference intervention (table 2:1).

Table 2:1: Summary of the bioassay testing plan and outcomes per net type and mosquito strain.

		Interceptor G2®	**Royal Guard®**	**Olyset ™ Plus,**
Susceptible strain: Assessment of pyrethroid				
	Treatment	1/Untreated, 2/Interceptor, 3/Interceptor G2®	1/Untreated, 2/Interceptor, 3/Royal Guard®	1/Untreated, 2/Interceptor, 3/Olyset ™ Plus
Cone	Outcomes	Kd*, mortality 24, 48 and 72 h**	Kd, mortality 24, 48 and 72 h	Kd, mortality 24, 48 and 72 h
	Exposure time	3 minutes	3 minutes	3 minutes
	Total nets	30 nets (t0 to t30), 50 nets (t36)	30 nets (t0 to t30), 50 nets (t36)	30 nets (t0 to t30), 50 nets (t36)
	Total pieces	5 (t0) and 4 subsequent follow ups (position 2 to 5)	5 (t0) and 4 subsequent follow ups (position 2 to 5)	5 (t0) and 4 subsequent follow ups (position 2 to 5)
	Total replicates / piece	4	4	4
	Total Mosquito per test	5	5	5
	Total mosquitoes	Total between 4800 to 6000 per time point	Total between 4800 to 6000 per time point	Total between 4800 to 6000 per time point
Tunnel	Outcomes	Immediate mortality and delayed mortality 24, 48 and 72 h, blood feeding	Immediate mortality and delayed mortality 24, 48 and 72 h, blood feeding	Immediate mortality, and delayed mortality 24, 48 and 72 h, blood feeding
	Exposure time	12 to 15 h	12 to 15 h	12 to 15 h
	Total nets	All failed net with cone	All failed net with cone	All failed net with cone
	Total pieces	1 per net	1 per net	1 per net
	Total replicates / piece	2 replicates	2 replicates	2 replicates
	Total Mosquito per test	50	50	50
	Total mosquitoes	Determined by number of failing nets	Determined by number of failing nets	Determined by number of failing nets

Resistant strain: Assessment of partner A.I. or synergist				
Cone	Outcomes		Kd, mortality 24, 48 and 72 h, ovarial development	Kd, mortality 24, 48 and 72 h
	Exposure time		3 minutes	3 minutes
	Total nets		30 nets (t0 to t30), 50 nets (t36)	30 nets (t0 to t30), 50 nets (t36)
	Total pieces		5 (t0) and 4 (t6 to t36)	5 (t0) and 4 (t6 to t36)
	Total replicates / piece		4	4
	Total Mosquito per test		5	5
	Total mosquitoes		Total between 4800 to 6000 per time point	Total between 4800 to 6000 per time point
Tunnel	Outcomes	Immediate mortality, and delayed mortality 24, 48 and 72 h, blood feeding	Immediate mortality and delayed mortality 24, 48 and 72 h, Blood feeding, ovarian development	Immediate mortality and delayed mortality 24, 48 and 72 h, blood feeding
	Exposure time	12 to 15 h	12 to 15 h	12 to 15 h
	Total nets	30 nets (t0 to t30), 50 nets (t36)	All failed net with cone	All failed net with cone
	Total pieces	1 per net (position 2)	1 per net	1 per net
	Total replicates / piece	2 replicates	2 replicates	2 replicates
	Total Mosquito per test	50	50	50
	Total mosquitoes	30 (50) nets x 1-piece x 2 replicates x 50 mosquitoes x 3 treatments = 9000	Determined by number of failing nets	Determined by number of failing nets
	Net Specificity	slow killing effect 72 hours; mortality main outcome	Ovarial development by dissection at 72 h post exposure	Colony mosquito's resistance strain (kdr*** and kdr+MFO****)

* Knockdown, ** hours, ***knockdown resistance, **** cytochrome P450 mono-oxygenase mechanisms

The four types of LLINs will be distributed to 84 clusters in the cRCT. Each household will receive one net for every two people. For odd numbers of occupants, the number of nets will be rounded up to cover the sleeping places. All nets distributed are rectangular (180 cm length × 160 cm width ×180 cm height) and dyed blue during manufacture. Forty of those clusters are selected for the durability study.

Net durability assessment

The efficacy and physical durability of the nets will be evaluated by means of a prospective cohort study (Figure 2:2). A census/enumeration of the household in the hamlet is completed as part of the cRCT and for each house, name and GPS coordinates are available.

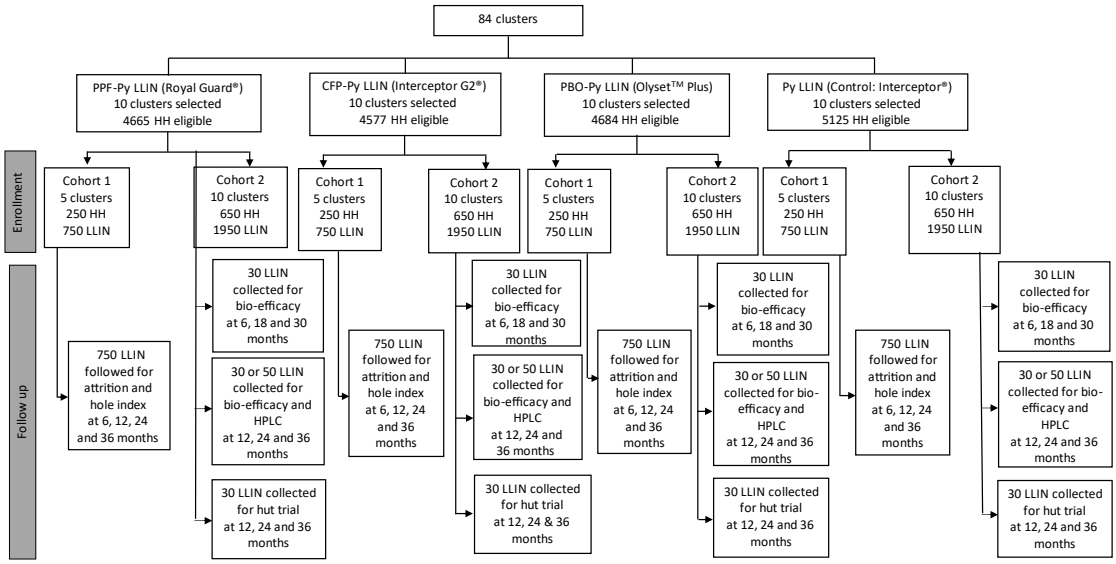

Figure 2:2: The number of clusters, household and LLINs selected per arm

Net attrition and fabric integrity (cohort 1)

A total of 250 households (HHs) in 5 clusters per study arm will be followed for LLIN physical integrity and attrition. Assuming an average of 3 LLINs per HH based on the average number of sleeping places, this will yield 750 LLINs. All 750 LLINs will be followed for attrition and for hole index. In each cluster HHs will be selected close to each other. During the first visit one month post distribution, in all the households willing to participate, their used LLIN will be identified with a unique identification number.

Household information on quality of housing and other socio-demographic characteristics will be collected (annex 4). The physical presence of all LLINs in the 250 HHs will be recorded at each visit; 6, 12, 24 and 36 months. If the net is still present in the HH, the investigator will record whether the net is being used for its intended purpose. Nets that are not used anymore will also be recorded. If the net is no longer in the house, the investigator will determine how it was lost. All LLINs followed for attrition and in current use will be inspected for number and size of holes on the side net panels divided into four areas from top to bottom and one on the roof position(166). Size will be classified into four categories: smaller than a thumb (0.5–2 cm), larger than a thumb but smaller than a fist (2–10 cm), larger than a fist but smaller than a head (10–25 cm) and larger than a head (> 25 cm). Evidence of repairs to the net fabric and the type of repair will be recorded. Hole counts will be made by removing each net and arranging it over a frame and returning the nets after measuring physical integrity.

Net withdrawal for insecticidal residual activity assessment and experimental hut trials (cohort 2)

LLINs will be withdrawn at 6-month time intervals up to 36 months and used for bio-efficacy testing and for once-per-year experimental hut trials. To reduce the impact of withdrawal on the cRCT outcomes, 10 clusters (5 clusters for attrition and integrity and 5 additional clusters for durability) per arm will be selected at random. A total of 650 HHs selected at random will give approximately 1950 LLINs per arm to follow up (Figure 2:2). Each net collected will be replaced with a new net of the same type, though these new nets will not be part of the study. Households will remain part of the cohort until no cohort nets are available in the households. The unit of observation will be the individual net. As for cohort 1, selected nets for cohort 2 will be labelled and associated household information collected. From time t0 to t30, 30 nets per survey will be collected for bio-efficacy and 50 nets will be collected at t36 months. From each of the LLINs selected for bio-efficacy, one net piece measuring 30 cm x 30 cm will be cut from each side at baseline (t0 month), and in subsequent follow-ups only positions 2 to 5 will be cut, since position 1 situated at the bottom of the net may be disproportionately exposed to extreme abrasion when tucked under the bed, as per WHO guideline (116). At each position, 3 samples adjacent to each other will be removed. The first will be used for chemical assay using High-Performance Liquid Chromatography (HPLC), the second for bio-efficacy on *An. gambiae s.s.* susceptible strain (Kisumu) and the third for bioassay on a resistant strain.

Adverse events

For 200 HHs per intervention arm (20 HHs per cluster), perceived adverse effects from users or guardians of users will be recorded. The HH will be selected at random from all enrolled in cohorts 1 and 2.

Net bio-efficacy assessment

In the context of bioassay testing of dual-A.I. LLINs, WHO guidelines will be followed (116, 167). As the former guideline pre-dates this protocol by several years and was focused mainly on pyrethroid LLIN, modifications are permissible to generate new evidence and were discussed with the WHO in advance. Bioassay testing of dual-A.I. LLINs will follow WHO guidelines (116, 167). As the former published guideline pre-dates this protocol by several years and was focused mainly on pyrethroid LLIN, WHO-sanctioned modifications were permitted in advance to generate new evidence.

Mosquito strains

A susceptible *An. gambiae s.s.* strain (Kisumu) will assess the bio-efficacy of the pyrethroid in each of the dual-A.I. LLINs. To assess the durability (bioavailability) of the partner insecticide, it will be necessary to use a pyrethroid resistant strain or species ideally with resistance intensity great enough to withstand the effect of the pyrethroid. A*n. gambiae s.s.* Muleba-Kis pyrethroid resistant strain (characterized by both *kdr*-East L1014S and mixed-function-oxidase based resistance) (168) will be used to assess the partner A.I. The resistant strain will be kept under constant pyrethroid selection pressure and phenotypic (using CDC bottle assay) and genotypic (molecular analysis by TaqMan assays) resistance will be monitored in every generation to assess changes in resistance frequency and intensity. The selection will be done once per generation at larval stage (168) using 0.08 µg/ml of alpha cypermethrin. Control larval bowls will be treated with 1ml of ethanol.

Rationales for the four A.I.

In the dual-A.I. LLIN, Interceptor®G2, alpha-cypermethrin is fast-acting, and following a short exposure in contact bioassays, susceptible mosquitoes are knocked down within 1h and dead within 24h. The pyrrole chlorfenapyr requires longer exposure, is slower acting taking up to 72h to kill following contact bioassay; for monitoring delayed mortality, the WHO has proposed that mosquitoes may be held for 72h with mortality reported every 24h. There is clear evidence that cone bioassays with 3 min exposures fail to predict field efficacy of Interceptor®G2 (140). WHO has stated that tunnel tests may be more appropriate to estimate the field durability of chlorfenapyr (169).

In the dual-A.I. LLIN Royal Guard®, PPF impacts egg development, while alpha-cypermethrin will induce mortality. The reproductive effects of PPF on blood-fed female mosquitoes are 3-fold. The first effect of PPF exposure is to disrupt the maturation of eggs and oviposition by females 2-3 days after blood-feeding. The second observable effect is reduction in the mean number of eggs per ovipositing female. The third effect is reduction in the hatch rate of laid eggs or in production of viable larvae. Conventionally, the effects on reproductive outcomes are assessed by observation of oviposition rate and hatch rate in mosquitoes exposed to PPF compared to unexposed mosquitoes (129, 170). The problem encountered with oviposition as an indicator of fertility/sterility is the low oviposition rate in the pyrethroid-resistant control unexposed mosquitoes (171). Furthermore, direct observations require a long follow-up, appropriate infrastructure, and can be laborious. An alternative approach is to dissect mosquitoes after exposure when eggs should normally have become fully mature (2-3 days post-blood meal). During the normal gonotrophic cycle, after taking a blood meal, the mosquito's oocytes change in size and shape, and finally reach Christopher's stage V which are a distinctive crescent shape (172). Previous work on PPF-treated females has shown morphological defects on oocyte maturation in the ovaries following exposure, with development of PPF-affected oocytes arrested before reaching stage V (173).

In the dual-A.I. LLIN Olyset ™ Plus, PBO inhibits the cytochrome P450 oxidases responsible for metabolic pyrethroid resistance while permethrin will induce mortality. Cone tests have been used effectively to assess the performance of Olyset ™ Plus (174); however, there is a need to use a resistant strain with oxidase-based mechanisms for these bioassays to assess the field durability of PBO.

Cone test

WHO cone tests will be performed on each of the pieces of the 30 (t0-t30) and 50 LLINs (t36) for the following products: Royal Guard® and Olyset ™ Plus (both with susceptible and resistant mosquito strains) and Interceptor®G2 (only against susceptible strain) (116). For each net sampled, all pieces cut will be tested. Cone will be set at an angle of 45° (175), four replicates will be done per net piece using 5 mosquitoes per cone and a total of 20 mosquitoes per piece, and 80 mosquitoes per net (100 for the baseline including position 1). For the control, an untreated net will be tested in parallel as well as 2 pieces of the standard LLIN collected at the same time point. Standard Interceptor® LLIN will be used as a control. The estimated number of mosquitoes to be tested is detailed in table 2:1.

Per bioassay, five unfed 2-5 days old *An. gambiae s.s.* will be introduced into each cone. After 3 min exposure, mosquitoes will be transferred into labelled paper cups covered with untreated netting with access to 10% sugar solution. The bioassays will be carried out at 75±10% RH and at 27±2°C. Any net which fails the cone criteria, i.e. mortality <80% and sterility <60% with pyriproxyfen exposure will be re-tested using the tunnel test.

Tunnel test

To assess the residual bio-efficacy of the chlorfenapyr component of Interceptor®G2, the tunnel test using resistant mosquitoes shall be specifically used as the first choice bioassay in preference to the cone test owing to its unusual mode of action on flight muscle function (140). The piece of net in position 2 of each of the 30 Interceptor®G2 will be systematically tested in the tunnel (table 2:1). To assess the pyrethroid component of Interceptor®G2, the cone test is suitable using the pyrethroid susceptible strain as the first-choice bioassay.

The tunnel test will be also used to assess the residual bio-efficacy of any LLIN pieces (Royal Guard® and Olyset ™ Plus) that do not meet the criteria of ≥95% knockdown (KD) after 60 minutes or mortality of ≥80% after 24 hours in cone bioassays. For failing nets of Royal Guard® and Olyset ™ Plus, the net piece that produces mortality closest to the mean mortality during the cone test will be used in the tunnel test.

The procedure for use of guinea pigs will be compliant with criteria laid down in EC Directive 86/609/ECC concerning protection of animals used for experimental purposes. Animal ethics approval has been sought from LSHTM with reference: 2019-14. The glass tunnel is 25 cm^2 and 60 cm long, divided at one third of the length by a disposable cardboard frame to which the LLIN netting piece is attached. The surface of netting "available" to mosquitoes is 400 cm^2 (20 cm x 20 cm). Nine holes, each 1 cm in diameter, (one at the center of the square and the other eight equidistant at 5 cm from the border) will be made in the netting to allow for passage of mosquitoes. Netting-covered cages at both ends provide easy access to add and remove mosquitoes. In one cage, a guinea pig will be restrained. Fifty unfed 5-8 days old mosquitoes will be introduced at the opposite end of the tunnel from where the guinea pig is restrained. The experiment will begin at 18:00 and end at 08:00 the following morning. Mosquitoes will be scored according to whether they passed through the netting, whether they successfully blood fed and whether they survived the exposure period.

Cylinder test

The cone and tunnel tests may prove inadequate for evaluating the durability of partner A.I.s over 1-3 years once the insecticidal content starts to decrease. The standard cone test exposes mosquitoes for 3 min only, which has been shown to underestimate contact time and the exposure time and mortality attained in experimental huts using resistant mosquitoes; 30 min exposure is probably more realistic when using resistant mosquitoes (136). Mortality generated with free-flying resistant mosquitoes in experimental huts correlates well with mortality attained in 30 min bioassay for some insecticides tested, e.g., chlorfenapyr and Interceptor®G2 (Rowland and Kirby unpublished data). While the contact time may differ for other insecticides or when the concentration decreases during 3 years in the field; now that the precedent is established for one type of dual-A.I. LLIN, the average contact time of free flying mosquitoes is likely to be longer than 3 min for other nets too. The WHO cylinder test will be performed for Olyset^{TM} Plus, Royal Guard® and Interceptor®G2 on a sub-sample of nets at each time point and compared to tunnel and cone results.

The netting will be stapled to WHO control test papers measuring 15 cm x 12 cm to facilitate rolling and fitting into the test cylinder in the same way as an insecticide test paper would be fitted. Holding rings are inserted to hold back the netting (176). Bioassays will follow the same procedure as insecticide susceptibility testing except that exposure time will be 3, 15, 30 and 60 min as necessary and this will be recorded as knockdown. Before exposure, 10 mosquitoes will be aspirated into the holding cylinder of the kit and then blown into the exposure cylinder according to standard procedures. After exposure, the test insects are blown back into the holding cylinder and 10% sugar solution provided. Ten mosquitoes per cylinder test would ensure a density per unit area of netting similar to that of five mosquitoes per cone.

Experimental hut design

The experimental huts in Magu are a modified version of the standard East African hut (177) featuring four brick walls, a wooden ceiling lined with hessian sackcloth, an iron sheeting roof, two baffled eave gaps above each wall, and a window trap on each wall. The huts are built on concrete plinths and surrounded by a water-filled moat to deter entry of scavenging ants. In the modified design, the four verandas are open; the baffled eave gaps above all four sides allow unimpeded entry of mosquitoes and minimal mosquito exiting. Mosquitoes are restricted to exiting through the window traps on the four walls of the hut. In each hut, cloth sheets are laid on the floor each night to ease the collection of knocked-down mosquitoes in the morning. Sugar solution is provided at night in the window traps to reduce mosquito mortality.

The nets will be evaluated using experimental huts for their effects on free-flying, wild *An. gambiae sensu lato* (*s.l.*), *An. funestus s.l.* and *Culex quinquefasciatus* mosquitoes for their ability to deter entry, repel mosquitoes, induce mortality and inhibit blood-feeding.

Adapted experimental hut study

Each of the 30 individual nets per product type collected from community at t12, 24 and 36 months will be tested in experimental huts. The following treatments shall be assessed at each time point:

1. Control: untreated polyethylene net with 6 holes
2. Standard LLIN: new Interceptor® washed one time with 6 holes.
3. Interceptor® at t12/t24/t36
4. Interceptor®G2 at t12/t24/t36
5. Royal Guard® at t12/t24/t36
6. Olyset^{TM} Plus at t12/t24/t36

The study will be done over 6-week periods. Sleepers will be rotated between huts on successive nights to account for individual attractiveness and net treatments rotated every week following a random Latin square design. Every week, collections shall be performed over 6 days and on the last day huts will be cleaned and aired before the next treatment rotation. Six replicates of untreated net and of new standard LLIN will be tested per hut treatment and will be swapped every day within each week of the trial. Field collected nets will be changed every day and tested for one night only per trial. Because there are 36 day/night collection per treatment for a complete Latin square rotation (sleepers and treatments) and only 30 field collected individual nets per product type an additional 6 new LLIN from each treatment will be evaluated (treatment 3 to 6). These new nets will act as a positive control and variation in outcomes over time in those nets will be accounted for in the analysis comparing efficacy of field net between time points. Six holes of 4 x 4 cm will be cut in the untreated and new LLINs used in each treatment arm following WHO guidelines (116). Hole size will be counted for the field collected nets as per cohort 1 nets and followed for fabric integrity. A hut trial study for each time point will be repeated 2 to 4 times to account for vector composition seasonality.

Mosquito processing

All mosquitoes collected in experimental huts will be monitored for three days and mortality recorded after 24, 48 and 72h post collection. Blood fed mosquitoes collected from Royal Guard®, Interceptor® and untreated nets, will be dissected after 72h for observation of

fertility/sterility. Mosquitoes will be recorded as fertile if the eggs are fully developed into Christopher's stage V (annex 6).

After dissection the first reader will read the slide and enter the results in a form, then a second reader will record the results in separate forms. All information will be entered into a database in Access and compared for consistency. If there is variation between readings, the third reader will be assigned to read the slide and record the results. All slides will be kept in fridge until the results has been confirmed by data manager.

A subset of live and dead mosquitoes from each hut will immediately be killed (if still alive) and stored in RNAlater® at -80ºC for species identification and resistance gene expression analysis. Following molecular species identification, presence/absence of resistance alleles will be compared between individuals of assumed resistant (alive) and susceptible (dead) phenotypes and changes in allele frequency will be compared between hut conditions and between baseline characterization and post-intervention.

Modelling of experimental hut trial entomological surrogates

While experimental hut trials are the gold standard for assessing LLIN efficacy against susceptible and resistant mosquitoes, mathematical modelling (143, 178) can be used to predict the public health impact of factors such as pyrethroid resistance on LLIN efficacy and malaria transmission (143). The models are calibrated to the local area using site-specific entomological and epidemiological data collected from the cRCT site (such as baseline mosquito bionomics, history of LLIN use and baseline malaria prevalence). Two sets of parameters are used to characterize the efficacy of trial dual-A.I. LLINs. The first uses estimates of the proportions of mosquitoes dying, blood-feeding and outcomes such as deterrence, exiting and repellence estimated using experimental hut trials conducted in the region of the cRCT. The second uses estimates for the same metrics derived from a meta-analysis of all currently available experimental hut trial data for the same dual-A.I. LLINs from across Africa. Models parameterized with these two sources of data are used to predict changes in malaria prevalence over time. These two models are statistically compared to the observed results of the cRCT at different time points following the mass campaign to investigate the benefit of local LLIN efficacy information. WHO discriminating dose bioassays are used to quantify the frequency of resistance in the mosquito populations in the vicinity of the experimental hut site to provide a link between the outcomes of the trial and widely used assays for assessing the frequency of resistance.

There is considerable uncertainty in how the efficacy of nets changes over time. It can be estimated using the WHO proxy of standardized washing, as described by experimental hut

outcomes from trials evaluating LLINs washed 0 and 20 times. The number of washes taken to halve the killing activity of the LLIN can be estimated and converted into predictions of the insecticidal half-life in years considering 20 washes to represent the decay expected in an LLIN over three years of use in the field (179). Estimates of the actual duration of insecticidal activity in the field is one of the more uncertain features of experimental hut trials and consequently for models evaluating the public health impact of novel LLINs. This is because of the uncertainty in the relationship between the number of washes and durability of insecticide in the field over time for non-pyrethroid A.I.s. Under field conditions, nets are subject to many environmental factors affecting durability in addition to washing, such as friction, wood smoke, and everyday wear and tear. This uncertainty is important to include as the average age of LLINs in Africa is over one year old and small changes in LLIN-induced mortality over time can have a large epidemiological impact as population LLIN coverage falls two or three years after the last mass distribution campaign. This problem seems particularly acute for Interceptor®G2 as chlorfenapyr cannot be evaluated in simple cone bioassays and nets washed 20 times (180). Evaluation of naturally aged LLINs collected from the field in experimental hut trials may allow more precise predictions of the longevity of a LLIN (i.e. half-life), allowing the decay in insecticidal activity to be directly estimated instead of having to rely on proxy measures. This is anticipated to improve the accuracy of epidemiological predictions made from entomological data which can be evaluated by comparing model predictions to the results of the main cRCT. The utility of incorporating other entomological data collected as part of the trial into the modelling framework shall be investigated.

Outcomes

The study outcomes are summarized in table 2:2.

Table 2:2. Study outcomes measurements

Outcomes	**Measurements**	**Collection**	**Frequency**
Bio-efficacy with susceptible and resistant *Anopheles* strains.	Mortality recorded immediately (60 minutes) and after 24, 48 and 72 h post exposure. Blood feeding inhibition recorded in tunnel test. Fecundity/fertility for Royal Guard® compared to standard LLIN and untreated net: proportion of abnormal ovaries.	Cone or tunnel test on 4/5 pieces per net from 30 nets per type per time point and 50 nets at 36 months	At 0, 6*, 12, 18*, 24, 30*, 36 months post distribution. *Test only performed when dual A.I. does not show superior

	Blood feeding inhibition (%): the reduction in blood feeding of mosquitoes in the treatment compared with % feeding in the control tunnel.	destructively sampled from cohort 2.	efficacy compared to standard LLIN against resistant *Anopheles* at the yearly time point.
Net attrition rate (survival).	Household visit and observation of study LLIN presence: % of study nets that are lost (no longer in use for sleeping under) in the receiving household at each time point.	Prospective cohort study cohort 1.	At 6, 12, 24 and 36 months.
Fabric integrity.	Number of holes and hole size in study LLIN to calculate HI.	Prospective cohort study cohort 1.	At 6, 12, 24 and 36 months.
Chemical content.	HPLC of pyrethroid and partner A.I.: amount of active ingredients in fiber.	bio-efficacy	At 0, 12, 24 and 36 months.
Adverse effect.	Household visit and questionnaire to report skin itching, facial burning, sneezing, nose running, headache, nausea, eye irritation, other.	Prospective cohort study cohort 1 and 2 nets.	At one month post distribution.
Efficacy against free flying mosquitoes.	Immediate mortality (%) recorded and after 24, 48 and 72 h. Blood feeding inhibition (%): the reduction in blood feeding of mosquitoes in the treatment compared with % feeding in the control huts. Deterrency (%): reduction in hut entry relative to the control huts with untreated nets. Exophily (%): The proportion of mosquitoes that exit early and are found in exit traps compared with the untreated control. Personal protection (%): the reduction in the number of mosquitoes blood-fed	Adapted experimental hut at each time point. Standard wash resistance experimental hut (0 and 20 wash nets).	At, 0, 12, 24 and 36 months. Once during the course of the trial.

	compared to number of mosquitoes blood-fed in the untreated control. Fecundity reduction (%): the reduction in fecundity per blood-fed female alive at 72h after exposure (using dissection methods) in Royal Guard® compared to standard LLIN and untreated net.		

h= hours, HI= Hole Index, HPLC=High-Performance Liquid Chromatography

Sample size calculation

Sample size calculations for prospective LLIN study of net survivorship were performed using the power log rank command in Stata v.15.1. A total of 750 LLINs per type from 5 clusters per arm (i.e. 150 per cluster) will allow detection of a 9.4% absolute difference (hazard ratio = 0.8651) in LLIN attrition rate assuming an attrition rate in the control arm of 70% over the 3 years. This is assuming an intra-cluster correlation coefficient (ICC) of 0.03. A hazard ratio=0.7951 (i.e. 14.3% absolute difference) can be detected, assuming an ICC=0.01, and a hazard ratio=0.7478 (i.e. 17.7% absolute difference) for an ICC=0.02.

Data management

All data on LLIN physical conditions, washing and household characteristics will be collected using Open Data Kit (ODK) forms. Bioassay data will be recorded on standardized forms and double entered into an Access file and linked to the database via the net identification number and time interval. Consistency checking will be done by running algorithms especially designed to identify sources of error. This database will be sent by the data manager to the project manager after each time-interval. The project manager will keep an updated master list of the location and status of each net. Data from bioassay and chemical residue analysis will be entered separately. Hut data will be entered directly in electronic forms prepared in ODK. Standard Operating Procedures (SOPs) for data collection will be developed and field staff will be appropriately trained to ensure rigorous data collection. This will include quality control (QC) of their own performance by checking for missing data or implausible responses. Further QC will be conducted by a supervisor who monitors the performance of field staff by checking for completeness and internal consistency of responses within hours of data collection. To maintain participant confidentiality, all consents forms will be kept in a locked cabinet only accessible by an authorized staff. Statistical analysis will be performed using Stata software v.15.1.

Data analysis

Fabric integrity (hole index)

Physical integrity of each net will be measured by the Hole Index (HI) as per WHO guideline (116). Holes will be counted and only the hole size >0.5 -2 cm will be recorded as size 1, 2-10 cm will be size 2, 10-25 cm size 3 and >25 cm in diameter will be size 4 (116). HI will be calculated using formula HI = (1 × no. of size-1 holes) + (23 × no. of size-2 holes) + (196 × no. of size-3 holes) + (576 × no. size-4 holes). Based on the HI the nets will be divided into 3 categories 1/ Good: HI <64 (hole surface area <79 cm^2 2/ Acceptable: HI = 64 -642 (80-789 cm2), 3/ too torn; HI >642 (hole surface area >790 cm^2 (181). Mean and median hole index and surface will be reported by type of net as well as proportion nets in each hole category (good, acceptable and too torn). Negative binomial regression models will be used to compare hole surface area between net types including co-variates such as socio-economic status, housing and sleeping conditions.

Attrition/survival

The rate of attrition will be first calculated as the proportion of study nets lost among all study nets originally received and further divided into reasons of net loss. To estimate functional survival only the attrition due to destruction, discarding or use for other purpose will be included (MPAC recommendation) (182). The functional survival rate (pX) of each type of study net at each time point will be calculated as:

pX=% surviving to time X **=** (number of study net present and "serviceable" at time X **/** number of study net originally received and not given away at time X) * 100

Descriptive statistics will be used to present the proportion surviving for each study net at each time point and compare the dual-A.I. LLINs to the standard LLIN. Cluster effect will be accounted for to estimate the 95% confidence interval.

Median survival time

Median survival time is the time point at which 50% of the study nets received are still present and in serviceable condition. First the functional survival rate to time X will be compared against a reference survival curve provided by WHO in the VCTEG report (182) The functional survival rate to time X will also be compared between the dual-A.I. LLINs against the standard LLIN. A Kaplan–Meier survival analysis will be used to estimate the median survival time of each study net and will consider the design effect.

Efficacy and long-lasting effect of the pyrethroid

The dual-A.I. net will meet WHO criteria (116) for efficacy and long-lasting effect of the pyrethroid if, after 36 months of use, at least 80% of sampled nets tested against a pyrethroid

susceptible mosquito strain are effective in WHO cone tests (≥ 95% knockdown or ≥ 80% mortality after 24 hours) or tunnel tests (≥ 80% mortality or ≥ 90% inhibition of blood-feeding). Thresholds for the second A.I. of each dual-A.I. LLIN are not known yet and would need to be established in relation to the cRCT findings. However, dual-A.I. LLIN efficacy outcomes against the resistant *Anopheles* strain will be compared to those of standard LLIN to assess superior effect at each time point.

Experimental hut trial analysis

Proportional outcomes (blood-feeding, deterrence, exiting mortality, and fertility) related to each experimental hut treatment will be assessed using binomial generalized linear mixed models (GLMMs) with a logit link function. A separate model will be fitted for each outcome. In addition to the fixed effect of each treatment and hole index categories, each model will include random effects to account for the following sources of variation: between the huts; between the sleepers; between the weeks of the trial; between trial rounds; and finally, an observation-level random effect to account for variation not explained by the other terms in the model (over dispersion). Location of the holes (zone 1 to 5) in relation of mosquito blood feeding and net entry will also be explored.

Entomological data from the entomological surrogate studies (experimental hut trials and supporting efficacy bioassays) will be integrated into a meta-analysis and modelling of disease outcomes to investigate possible relationships between epidemiological and entomological data from the cRCT.

Hole count data will be weighted as per WHO guidelines and the results compared with the recorded mortality per net type.

Comparisons of the changes in levels of gene expression will be performed between live mosquitoes collected from experimental huts containing treated interventions and experimental huts with no treatment/control treatment (relative to a susceptible laboratory control colony). The assumptions are that comparing between these two experimental groups will allow us to differentiate between metabolic genes which are constitutively over-expressed in the field populations and may contribute to resistance (i.e. expression levels observed in the control hut which contains a mixture of ‘resistant’ and ‘susceptible’ vectors of unknown phenotype) and those genes which are over-expressed in our field populations in response to intervention/insecticide exposure (i.e. expression levels observed in the treated huts compared to the control huts).

2.3 Discussion

The WHO Phase I, II and III is a gated process which makes assumptions about the relationship between durability over time and artificial washing as done in the laboratory. It's important to note that the 20 washes in Phase II are not meant to simulate the wear of a net used for 36 months in the field. Instead, this threshold is used as a benchmark or cut-off point to assess whether a net qualifies as a long-lasting insecticidal net (LLIN). For example, under field conditions the nets are subject to levels of abrasion which standardized washing using a bucket and pole cannot match [8]. Nets used in standardized Phase II studies are deliberately cut to make 6 holes; the average number of holes in field collected nets may exceed 20 after less than 2 years.

In large-scale field trials, physical durability is affected by net care and repair, frequency of use and maintenance practices, duration of transmission season, as well as textile physical features such as fibre material, knitting or weaving pattern (183). The WHO assumes a good LLIN will demonstrate a physical life span of 3 years, but this duration will vary between product, endemic regions and condition of use (184, 185). In the WHO guidelines, LLIN survivorship and fabric integrity are monitored in the community at 6, 12, 24 and 36 months (116). The pyrethroid component is expected to remain effective for 3 years (116) while the residual efficacy of other active ingredients are not yet known (184, 185). The proposed work was designed to address those assumptions and uncertainties, to establish the true correlations between WHOPES Phase I, II and III and improve the fit of transmission models to cRCT outcomes.

The data will help develop bio-efficacy and physical durability criteria for partner A.I.s, in relation to the cRCT epidemiological and entomological outcomes, and refine preferred product characteristics of each class of LLIN. Data generated by the study will be used to parameterize transmission models which in turn will be used to predict epidemiological outcomes after various intervals of use. Comparison of predicted outcomes with actual cCRT outcomes will determine the accuracy of the models and whether experimental hut testing of nets sampled during longitudinal modelling could serve as a surrogate for cCRTs. Since cCRT cannot be replicated in every location, the outcome of the study may show minimum standards the study net should meet in other places using experimental hut and modelled data.

Data generated by this study may elaborate the criteria or new thresholds of performance for new edition of the LLIN guidelines to help evaluate second in-class Dual A.I. LLIN products in these categories (144).

Declarations

Ethical approval and consent to participate.

This methodology has received ethical approval from the Medical Research Coordinating Committee (MRCC) of the National Institute for Medical Research (NIMR) (NIMR/HQ/R.8a/Vol.IX/2743), Kilimanjaro Christian Medical University College (KCMUCO), and the London School of Hygiene and Tropical Medicine (LSHTM) (**Ref:** 16524).

For the community Phase III durability activity written informed consent will be obtained from an adult in the household (annex 2). Written consent forms will also be obtained from volunteers (sleepers) who agree to participate in the hut study (annex 3). Only adults of 18 years or older will be recruited, excluding pregnant women. Volunteers will be offered daily chemoprophylaxis, and the risks of malaria explained (willingness to take will be an inclusion criterion). By sleeping under a mosquito net, they will obtain protection not dissimilar to exposure they would normally obtain against mosquitoes in their own home should they use a pyrethroid LLIN. Because an untreated net is used in one arm, all volunteers will be monitored each day for signs of fever. Confirmed falciparum parasitaemia will be treated with Coartem (artemether 20 mg/lumefantrine 120 mg) by a local physician. No side effects are expected from the LLIN except possibly some irritation to mucous membranes; these will be monitored and reported.

Guinea pigs will be used in the partner Institutions in Tanzania to feed the colony mosquitoes. Restrained guinea pigs are also used in WHO tunnel test as bait. KCMUCo and NIMR laboratories in Tanzania have obtained approval from the Animal Welfare and Ethical Review Board of LSHTM (reference: 2019-14) for the use of animals for this study.

3 Chapter 3: Physical durability of netting materials and attrition rates over three years of use in the community.

This work in this chapter has been published as:

Martin J, Lukole E, Messenger LA, Aziz T, Mallya E, Bernard E, Matowo NS, Mosha JF, Rowland M, Mosha FW, et al. Monitoring of Fabric Integrity and Attrition Rate of Dual-Active Ingredient Long-Lasting Insecticidal Nets in Tanzania: A Prospective Cohort Study Nested in a Cluster Randomized Controlled Trial. *Insects*. 2024; 15(2):108. https://doi.org/10.3390/insects15020108

Abstract:

Pyrethroid-treated long-lasting insecticidal nets (LLINs) have been the main contributor to the reduction in malaria in the past two decades in sub-Saharan Africa. The development of pyrethroid insecticide resistance threatens the future of LLINs, especially when nets become holed and pyrethroid decays. In this study, three new classes of dual-active ingredient (A.I.) LLINs were evaluated for their physical durability: (1) Royal Guard, combining pyriproxyfen, which disrupts female fertility, and a pyrethroid, alpha-cypermethrin; (2) Interceptor G2, which combines the pyrrole chlorfenapyr and a pyrethroid (alpha-cypermethrin); (3) Olyset Plus, which incorporates the pyrethroid permethrin and the synergist piperonyl butoxide, to enhance the pyrethroid potency; and Interceptor, a reference net that contains alpha-cypermethrin as the sole active ingredient. About 40,000 nets of each type were distributed in February 2019 to different villages in Misungwi. A total of 3072 LLINs were followed up every 6–12 months up to 36 months to assess survivorship and fabric integrity. The median functional survival was less than three years with Interceptor, Interceptor G2, and Royal Guard showing 1.9 years each and Olyset Plus showing 0.9 years. After 36 months, 90% of Olyset Plus and Royal Guard and 87% of Interceptor G2 were no longer in use (discarded) due to wear and tear, compared to 79% for Interceptor. All dual-A.I. LLINs exhibited poor textile durability, with Olyset Plus being the worst.

3.1 Introduction

Pyrethroid-only insecticide-treated nets (ITNs) and long-lasting insecticidal nets (LLINs) were the cornerstone for malaria vector control until comparatively recently when pyrethroid resistance emerged and now threatens malaria control. Apart from resistance, other factors threaten the future of LLINs, including net fabric durability, insecticide efficacy and retention, net usage, and wear and tear by daily handling (43). In areas with intense pyrethroid resistance, if LLINs are damaged, mosquitoes may penetrate the net holes and feed on human hosts, potentially transmitting malaria.

Formerly, the presence of LLINs with intact fabric (i.e., undamaged) provided a physical barrier that prevented human–vector contact and reduced human blood-feeding (41, 42); treating the nets with pyrethroid provided additional protection by adding a toxic, repellent barrier (43).

When mosquito populations become resistant and pyrethroid-only nets develop holes, users may perceive them as unprotective and discard them, leading to a reduction in coverage and usage (77, 78).

Other studies have reported that when insecticide in the netting material decreases and nets acquire holes, users have no or minimal protection as the mosquitoes can penetrate and ingest blood (75, 76). A study conducted in Zambia showed that the poor fabric integrity of standard pyrethroid nets affected their effectiveness against *Anopheles arabiensis* (75), while another in Tanzania demonstrated that increased hole area was associated with higher numbers of *An. gambiae* inside the net (76).

Washing and drying LLINs have been reported to be among the factors that contribute to reduced LLIN insecticide concentration and the development of holes in the community (81). Generally, social and economic status are two of the factors affecting net handling. A study conducted in Bouaké, Côte d'Ivoire, found that household owners with primary/higher education had better knowledge about how to manage (tuck in on the bed, washing, drying) nets than those who reported having received limited health information and education (82).

New classes of ITNs have been recommended by the WHO recently as they showed superior protection against malaria compared to standard LLINs in various cluster-randomized controlled trials (cRCTs) in Tanzania (59, 151), Benin (114), and Uganda (186). ITNs combining synergists piperonyl butoxide (PBO) and pyrethroid have been recommended and deployed routinely since 2018. In 2023, two other ITNs, combining two insecticides, dual-active ingredient (A.I.), pyrethroid and either chlorfenapyr in Interceptor G2 or pyriproxyfen in Royal Guard, received WHO approval (150). As these nets are being scaled up, net durability including fabric integrity and survivorship (attrition) (184) should be assessed to understand the epidemiological outcomes and how these interventions should be incorporated into vector

control programs. As part of the cRCT in Tanzania, this study assessed the survivorship/attrition rate and fabric integrity of cohorts of three dual-A.I. ITNs (Royal Guard, Olyset Plus, and Interceptor G2) over 3 years of community use, compared to pyrethroid-only ITNs.

3.2 Methodology

Characteristics of the Long-Lasting Insecticidal Nets (LLINs) Tested

The current investigation was embedded within a comprehensive cluster-randomized controlled trial (cRCT) carried out in the Misungwi district, Tanzania (151). In this cRCT, 84 clusters (21 clusters per intervention arm) received the distribution of four distinct types of long-lasting insecticidal nets (LLINs) in February 2019. The LLINs subjected to evaluation were as follows: (1) Royal Guard® (Disease Control Technologies, LLC, Greer 29650, USA), a dual-A.I. LLIN comprised of polyethylene containing alpha-cypermethrin (261 mg/m^2) and pyriproxyfen (225 mg/m^2) known for its capability to disrupt female reproduction and fertility of eggs; (2) Interceptor® G2 (BASF Corporation, Germany)), a dual-insecticide LLIN made of polyester coated with wash-resistant formulations of chlorfenapyr (200 mg/m^2) and pyrethroid (alpha-cypermethrin) (100 mg/m^2); (3) Olyset™ Plus (Sumitomo Chemicals, Tokyo, Japan), a LLIN that incorporates the pyrethroid permethrin (800 mg/m^2) and the synergist piperonyl butoxide (400 mg/m^2), which enhances the potency of permethrin; (4) Interceptor® (BASF Corporation, 15588 Ansan, Korea), an alpha-cypermethrin-treated LLIN at a target dose of 200 mg/m^2 coated onto polyester filaments as the reference intervention.

Study Area

Misungwi district covers an area of 2579 km^2. The estimated total population in the area is 467,867 found in 78 villages. Notably, there has been a consistent 2.9% annual population growth observed from 2012 to 2022 (187). The previous malaria control intervention in the area was a standard LLIN mass campaign conducted in 2015, indoor residual spraying (IRS) using pirimiphos-methyl from 2013 to 2017, and larvicide using Bti in 2018. The major malaria vector species found in the area are *An. funestus* complex, *An. gambiae* sensu stricto, and *An. arabiensis.* Details of the Misungwi cluster-randomized controlled trial (cRCT) have been previously published (188, 189), providing comprehensive information on households and the number of nets distributed per arm. For the current study, a subsample of 20 study clusters out of the total 84 utilized in the cRCT were randomly chosen for the assessment of LLIN attrition and fabric integrity (see Figure 3:1). The complete protocol has been previously documented (188).

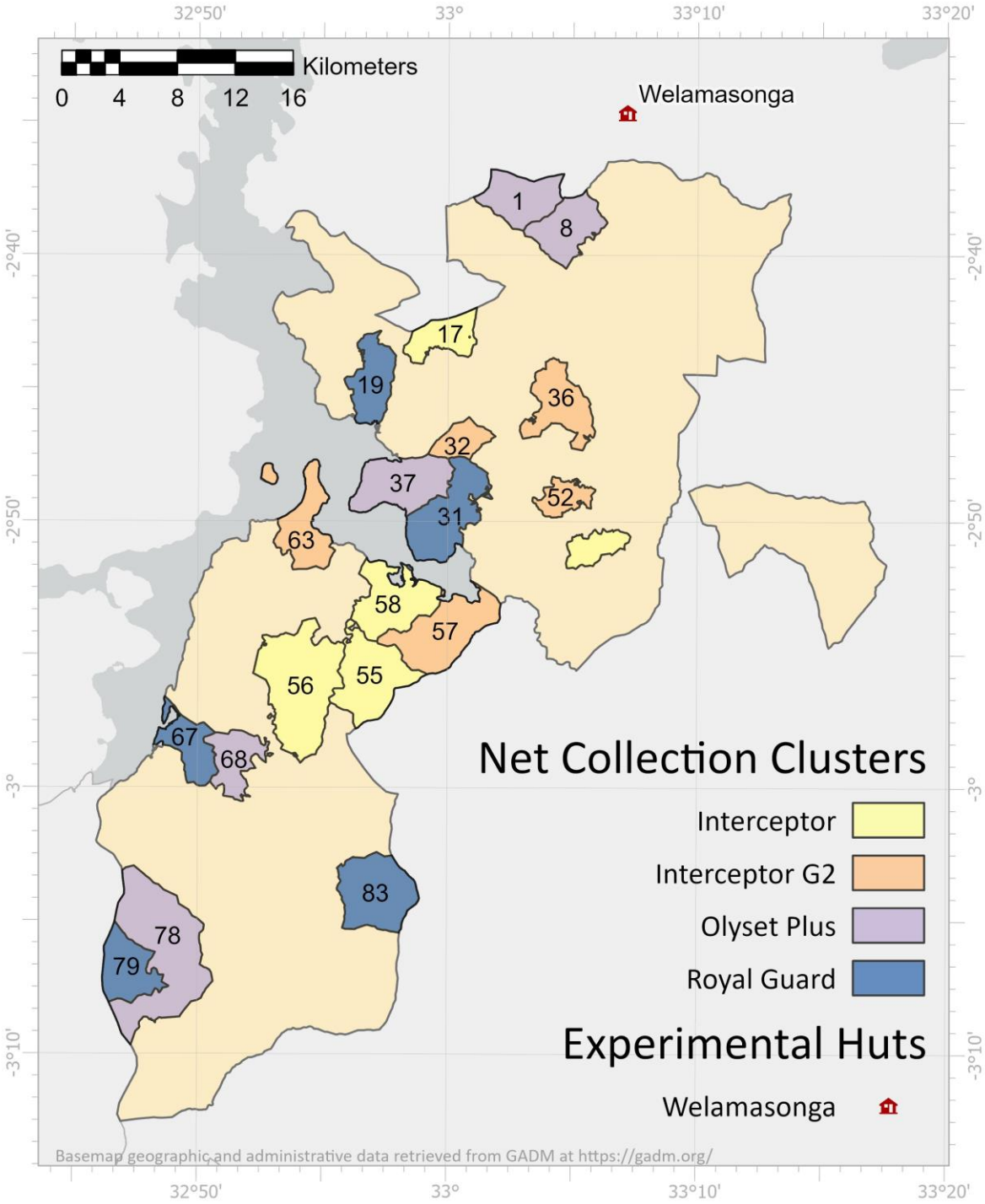

Figure 3:1: The 20 clusters randomly selected for net follow-up across Misungwi district. Olyset Plus (purple), Interceptor G2 (orange), Interceptor (yellow), and Royal Guard (blue). The numbers represent cluster numbers per arm where the nets were assessed. The map was created with ArcGIS software and all geographical and administrative data were sourced from GADM: https://gadm.org/license.html.

Study Design

This study adopted a prospective cohort design, tracking nets over three consecutive years to evaluate the survivorship/attrition and fabric integrity of potential dual-active ingredient (dual-A.I.) LLINs in comparison to standard LLINs. After LLIN distribution, a census/enumeration of households in the hamlet was completed as part of the cRCT, and each household was given unique identification numbers. Selected study LLINs were recorded and labeled with a household number and net number one month post-distribution.

Sample Size and Sampling

Sample size calculations were conducted using the power log-rank command in Stata v.15.1. A total of 750 LLINs per net type from 5 clusters per arm (equivalent to 150 per cluster) allowed for a detection rate with a 9.4% absolute difference (hazard ratio = 0.8651) in LLIN attrition rates, assuming an attrition rate in the control of 70% over the 3 years. This calculation takes into account an intra-cluster correlation coefficient (ICC) of 0.05.

Following distribution, all selected nets were labeled with the household number and a net number to generate a master list. In each arm, up to three nets from each selected household (HH) (with a total of 250 HHs selected) were assessed in 5 clusters per arm (20 clusters in total). The study nets (750 per arm) were randomly sampled from the master list and evaluated for survivorship/attrition and fabric integrity at 6, 12, 24, 30, and 36 months post-distribution. The objective of the study was explained to the head of the household before net inspections and those who agreed to participate in the study were interviewed about their socioeconomic status, housing materials, and the condition of the net through a structured questionnaire (annex 4 & 5) and templates for hole assessment.

Attrition Rate

In this study, attrition rate was defined as the number of nets that were not present in the household due to wear and tear or other causes (118). The reverse of the attrition rate was survivorship, which included all nets present in the household during the survey. All causes of attrition were assessed using a structured questionnaire (annex 4 & 5). A structured questionnaire was employed during the survey and questions were asked in Swahili or the regional language depending on the preference of the participants. The physical presence of the nets was observed by field technicians. Probing questions were utilized to inquire about the net's location, enabling owners to specify whether the net was discarded, given away, or used in another location. The procedure adhered to the WHO guidelines for the laboratory and field assessment of LLINs in 2013.

Differences in attrition rate were assessed as per WHO guidelines (190) using descriptive statistics. The attrition rate was assessed in 750 study nets per arm and measured by physical observation of the net in each room. All observed nets were recorded, and the householder was asked if the net was used for its intended purpose.

Fabric Integrity

Fabric integrity was defined as the physical state of the net to estimate bite protection. During surveys, the structured questionnaire (annex 5) was administered to each household and thereafter, each net was taken outside the room and hung in the frame by a trained technician. The nets were split into four different zones and holes were assessed using a hole template. The number and size of holes including tears in the netting and split seams by location and size were classified into four categories: smaller than a thumb (diameter of 0.5–2 cm, hole size 1), larger than a thumb but smaller than a fist (2–10 cm, hole size 2), larger than a fist but smaller than a head (10–25 cm, hole size 3), and larger than a head (>25 cm, hole size 4). Hole sizes greater than 0.5 cm were recorded (166). The holes were counted from zone one (bottom part of the net), upwards to the roof section. All data were recorded in an Open data kit (ODK) version 1.26, San Diego, USA), and thereafter, the net was returned to the room and the user was instructed to use the net until the next visit.

Data Analysis

All analyses were conducted using Stata version 18. Household characteristics were computed using proportional statistics. There were an additional 6 to 12 houses visited during the survey period that were not initially selected, and while these nets were included in the analysis of consent results, they were not considered in the assessment of functional survival.

Hole size was weighted to calculate the proportionate hole index (pHI) using the formula pHI = (1 × number of size 1 holes) + (23 × number of size 2 holes) + (196 × number of size 3 holes) + (576 × number of size 4 holes). The pHI was categorized based on recommended cut-off points into three categories (good 0-64cm^2, damaged 65 -642 cm^2 and torn >643 cm^2) (191) (table 3:S1). The sum of the pHI in the good and damaged categories was presented as serviceable LLINs, while those in the “too torn” category were termed as unserviceable. Furthermore, the proportion of nets with at least one hole of any size was calculated per net brand per time point. The attrition rate was calculated as the proportion of study nets not present in the household during the survey period due to wear and tear and other reasons, divided by all study nets originally received, excluding nets lost to follow-up (118). Reasons

for net loss were also investigated (191). The 95% confidence intervals (CIs) for this estimate were calculated by projecting the corresponding proportion's 95% CI based on the attrition rate.

For functional survival, nets present at each time point in serviceable conditions were considered, while survivorship was defined as nets present in the household during the survey period, regardless of the pHI category. Cox proportional regression models were fitted to predict the median functional survival and survivorship of each net and its hazard ratio. Functional survival was defined as a net still in serviceable condition, with a hole area <643 cm^2, that was still in possession during the time of the survey. Survival time was calculated as the duration between the start of follow-up and when the event occurred (net loss) in years. For all physically inspected nets, the survey time was taken at the time of the event. If the net was not observed, the respondent was asked to estimate when the net was lost, disposed of, or given away.

Secondary analysis was done to assess the association between net type, age and social economic status using survival analysis.

Summary for analysis plan Table 3:1.

Table 3:1: Summary table describing structure of the statistic model used in analysis (additional information not published)

Outcome variable	Fixed effects	Random effects	Model
Analysis presented in the publication			
Attrition	Net type + net age + survey time	Cluster	Survival analysis
Durability	Net type + net age+survey time	Cluster	Proportion (pHI)
Functional Survival	Net type+age+time	Cluster + structure of the house	Cox proportional hazard model
Secondary analysis			
Attrition	Net type + net age + survey time + SES + HH type + Education + number occupant	Cluster	Survival analysis

Ethical Statement

This study was nested in a larger cRCT conducted in Misungwi. The cRCT received ethical approval from Kilimanjaro Christian Medical Collage, the National Institute for Medical Research (NIMR/HQ/R.8a/Vol.IX/2743), and the London School of Hygiene and Tropical Medicine (**Ref:** 16524). Informed consent to explain the purpose (objective) and nature of the study was read in Swahili and the local language if the household head did not understand Swahili. For those who consented, a signature or fingerprint was taken.

3.3 Results

Study LLIN and Household Enrollment

A total of 1154 households were enrolled for follow-up. Amongst these houses, 3072 study nets were labeled of which 767 were standard Interceptor, 772 Interceptor G2, 766 Olyset Plus, and 767 Royal Guard (see figure 3:2, table 3:1). The total number of households selected for one-month post-distribution for net durability assessment was 1154. However, additional houses were visited (unintentionally) during each survey round: 12 houses at 6 and 24 months (totaling 1166 houses), 11 houses at 12 months (totaling 1165 houses), 6 houses at 30 months (totaling 1160 houses), and 10 houses at 36 months (totaling 1164 houses) (see Figure 3:2). The average number of people and sleeping places per household was similar across study arms, as was the population age distribution (see table 3:1). More than half of the household heads had primary education, and this was consistent across study arms. House structures and characteristics were similar, with burnt brick walls, mud floors, and metal sheet roofs being the most common materials, while over 90% of income in all study arms came from fishing or farming (refer to table 3:1). At each cross-sectional survey, consent was given in 938 (80%), 1071 (92%), 1039 (89%) 1160 (83%), and 882 (76%) households at 6, 12, 24, 30, and 36 months, respectively (see Figure 3:2). The remaining dwellings were either not found, vacant, householders refused, or interviewers asked to return later.

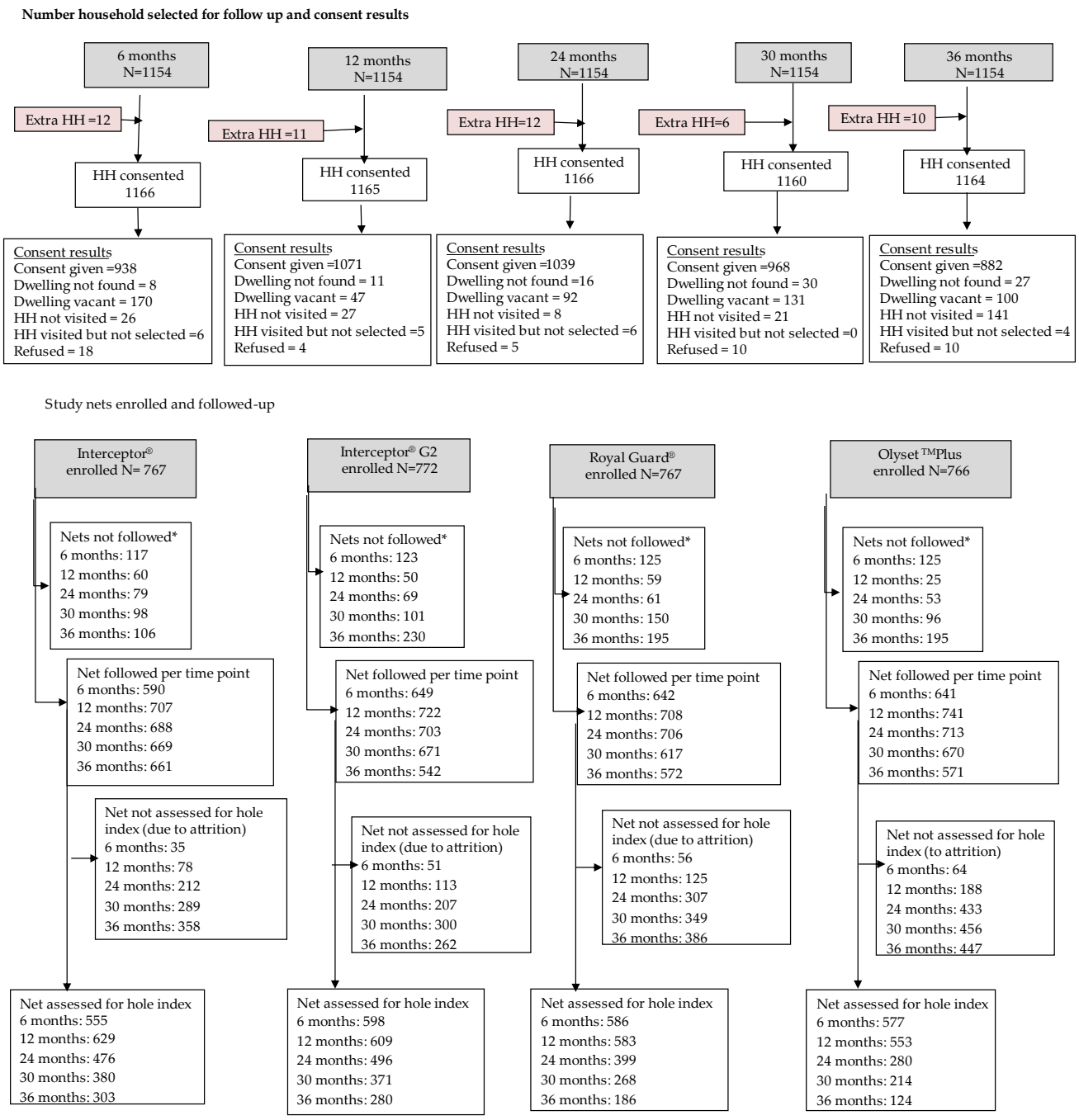

Figure 3:2. Number of households enrolled for follow-up and number of study nets assessed**.**

Table 3:2: Household, social, and economic characteristics across 5 clusters per study arm.

Characteristics	Interceptor	Interceptor G2	Olyset Plus	Royal Guard
Clusters	5	5	5	5
Number of participants	6624	6743	6604	6466
Average number of people per household	7.5	7.1	7.6	7.7
Mean number of sleeping places per household	3.7	3.5	3.5	3.6
Mean nets per household	2.76	2.61	2.71	2.57
Age distribution of household members % (95% CI)				
5 years	18.8% (17.9–19.5)	17.6% (16.8–18.5)	18.6% (17.5–19.7)	17.3% (16.4–18.2)
5–15 years	33.3% (32.3–34.4)	33.3% (32.1–34.6)	37.3% (34.6–40.1)	35.9% (34.6–37.2)
>15 years	47.9% (46.9–48.9)	49.0% (47.7–50.4)	44.1% (41.9–46.2)	46.8% (45.5–48.1)
Highest level of education of household head % (95% CI)				
No education	30.7% (27.6–34.1)	25.8% (22.8–29.0)	28.2% (25.1–31.5)	32.6% (29.5–36.1)
Primary education	66.6% (63.3–69.9)	69.3% (65.9–72.4)	69.7% (66.4–72.9)	64.6% (61.1–67.9)
Housing materials % (95% CI)				
Walls: burned brick	99.4% (98.5–99.7)	97.5% (96.2–98.4)	98.6% (97.5–99.2)	98.9% (97.9–99.5)
Floor: mud	61.2% (57.7–64.6)	62.4% (58.9–65.8)	72.0% (68.8–75.1)	69.9% (66.6–73.1)
Roof: metal sheet	76.9% (73.8–79.7)	70.7% (67.4–73.8)	72.4% (69.2–75.5)	72.4% (69.1–75.4)
Source of income % (95% CI)				
Fishing/farming	98.7% (97.6–99.3)	90.4% (88.2–92.3)	98.6% (97.5–99.2)	98.9% (97.9–99.5)

Attrition

During longitudinal surveys, all causes of net attrition rate and losses were assessed (figure 3:3 and table 3: S2). At six months, the majority of the nets lost were either given away to relatives (39% (95% CI: 23–58) for Interceptor; 33% (95% CI: 20–47) for Interceptor G2 and 15% (95% CI: 8–27) for Royal Guard) or used in another location (43% (95% CI: 26–61) for Interceptor; 26% (95% CI: 15–4) for Interceptor G2, and 42% (95% CI: 29–55) for Royal Guard) except for Olyset Plus, where most of the nets were discarded (69%, 95% CI: 59–77) at six months.

At twelve months, LLINs were given away to relatives, used in another location, and used for other purposes were almost half of lost nets for Interceptor and Royal Guard, while for Interceptor G2 and Olyset Plus, the majority (66% of each net type) were lost because they were discarded. From 24 to 36 months, discarding the net was the main reason for attrition with the highest (87% (95% CI: 84–89) and 90% (95% CI: 87–92)) for Olyset Plus and 74% (95%

CI: 70–78) and 90% (95% CI: 87–92) for Royal Guard, respectively (see figure 3:3, table 3: S2).

Overall attrition rate (all-cause net loss) at 6 months post-distribution was lowest (6.3%, 95% CI: 5–9) for Interceptor nets compared to dual-A.I. LLINs (Interceptor G2 9.1% (95% CI: 7–12), Olyset Plus 17.9% (95% CI: 15–21), and Royal Guard 10.1% (95% CI: 8–13)). There was a drastic increase in attrition in Olyset Plus of which half of the nets were no longer present in the houses compared to Interceptor nets, which was not the case for Interceptor G2 and Royal Guard at 12 months. At the 24-month survey, 81.9% (95% CI: 79–85) of Olyset Plus and 60.1% (95% CI: 56–64) of Royal Guard were no longer present, compared to Interceptor nets. Overall attrition rates increased until 36 months with Olyset Plus being significantly worse (90.5%, 95% CI: 88–93; $p < 0.001$) compared to the standard Interceptor (table 3:2).

Table 3:3: Percent attrition of LLINs surveyed and hazard ratio per net type and net age.

Net Type	% Attrition (95% CI)					Hazard Ratio
	6 Months	12 Months	24 Months	30 Months	36 Months	
Interceptor	6.3% (5–9)	15.9% (13–19)	40.6% (37–44)	52.8% (49–57)	62.9% (59–67)	1
Interceptor G2	9.1% (7–12)	21.1% (18–24)	43.2% (40–47)	57.9% (54–62)	63.3% (59–67)	1.4 (0.9–2.1), $p = 0.121$
Olyset Plus	17.9% (15–21)	50.7% (47–54)	81.9% (79–85)	85.2% (82–88)	90.5% (88–93)	2.8 (1.8–4.4), $p < 0.001$
Royal Guard	10.1% (8–13)	29.9% (27–33)	60.1% (56–64)	72.6% (69–76)	81.9% (79–85)	1.5 (0.9–2.4), $p = 0.078$

Secondary analysis for risk factors for attrition (not published)

Factors such as using the Olyset Plus net, having lower education, using certain mattress types (like grass or foam), and increasing the number of people (adults or children) contribute significantly to increased hazard. In contrast, higher education appears to offer some protective effect, though its significance is borderline (table 3:4).

Table 3:4: Risk factors for attrition.

Variable	Hazard ratio	P-value	95% CI
Net type			
Interceptor	1		
Interceptor G2	1.339	0.057	0.9 - 1.8
Olyset Plus	2.701	<0.001	1.9 - 3.8
Royal Guard	1.451	0.024	1.0 - 2.0
Bed type			
Standard bed	1		
Bed with stick	1.228	0.351	0.8 - 1.9
No bed	1.16	0.048	1.0 - 1.3
Matress			
Reed mat	1		
Grass	4.073	0.01	1.4 -11 .9
Foam mattress	2.059	0.002	1.3 -3.2

Other (no, clothes)	1.883	0.011	1.2 -3.1
Education			
Higher	0.651	0.053	0.4 -1.0
Secondary	1.729	0.004	1.2 -2.5
No/primary	2.252	<0.001	1.9 -2.7
Number of people			
Total adult	1.063	<0.001	1.0 - 1.1
Total children	1.061	0.007	1.0 - 1.1

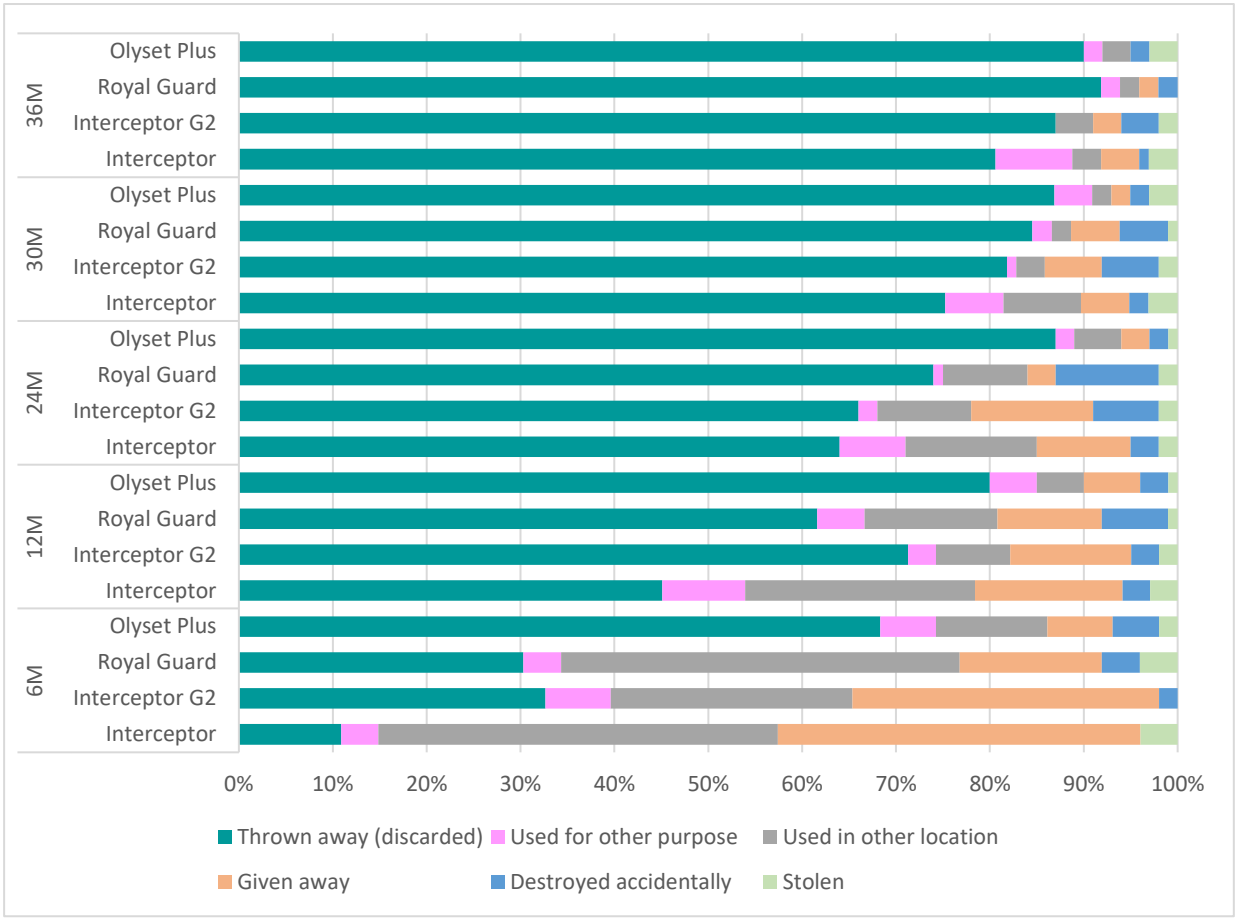

Figure 3:3: All causes of attrition by net type per survey.

Physical Integrity

Over the six months, over 90% of nets distributed were still in serviceable condition, except for Olyset Plus with 75% of nets discarded. These proportions decreased with time, with only 39% (95% CI: 35–44) of Olyset Plus in moderate or good condition at 12 months compared to 80% (95% CI 76–83) for control nets (Interceptor). Of the different dual-A.I. LLINs, Olyset Plus performed the poorest; 82% (95% CI: 74–88) were categorized as too torn 36 months post-distribution, compared to Interceptor nets at 52% (95% CI: 46–58), while 58% (95% CI: 51–63) of Interceptor G2 and 68% (95% CI: 61–74) of Royal Guard were too torn (Figure 3:4).

The proportion of nets with at least one hole increased from 6 months to 24 months but no difference was observed in holes between 30 and 36 months. There was a significant difference

in the proportion of standard Interceptor with at least one hole and Olyset Plus (OR: 1.5, 95% CI: 1.2–1.8, $p < 0.001$) at 6 months and 12 months (OR: 1.3, 95% CI: 1.1–1.6, $p = 0.002$). For the Royal Guard, the proportion of nets with at least one hole was only significant at 6 months (OR: 0.7, 95% CI: 0.6–0.9, $p = 0.010$) compared to Interceptor.

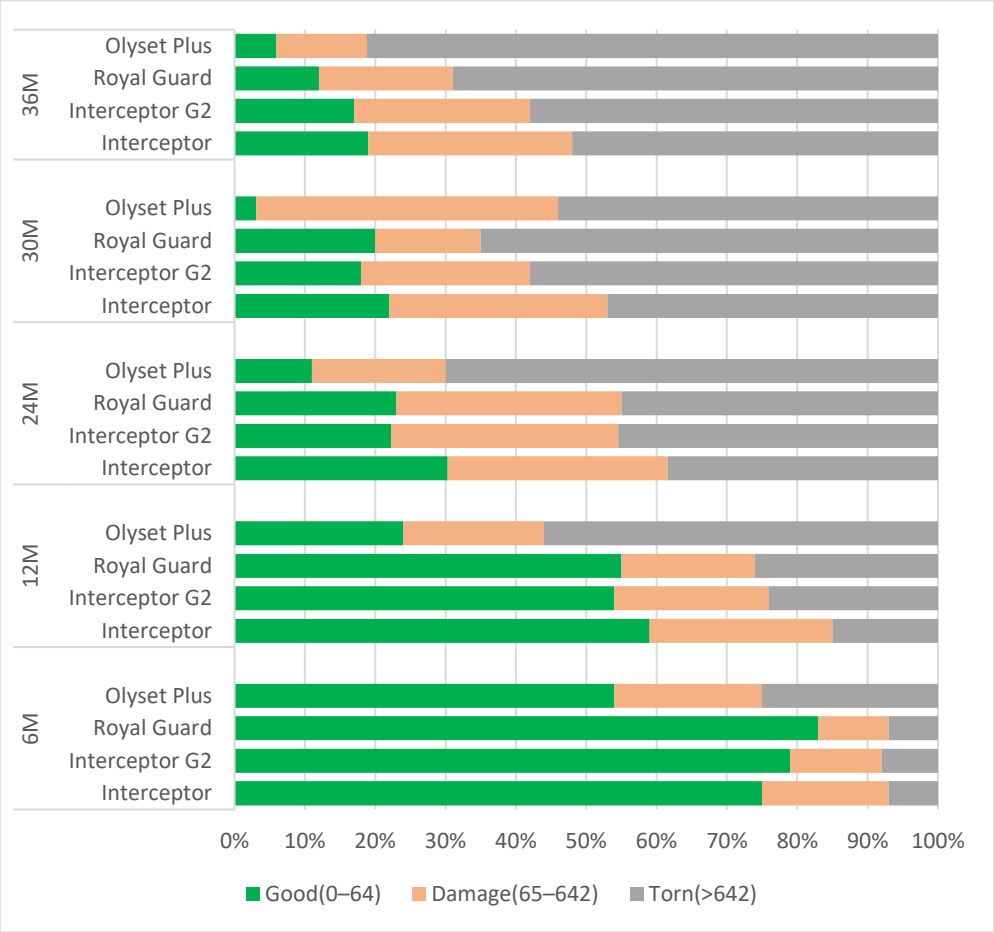

Figure 3:4: Physical condition of nets remaining in the household at the time of survey.

Green shows proportion of nets in good condition (pHI 0–64), light pink shows proportion of nets in damaged condition (pHI 65–642), and grey shows proportion of nets in torn condition (pHI > 643). Nets in category "torn" are generally too torn to be useable, whereas nets in the categories good and damaged may still be used to good effect.

Function survival and survivorship of the assessed LLIN

The median functional survival for Interceptor, Interceptor G2, and Royal Guard were 1.9 years each, while for Olyset Plus, the median functional survival was 0.9 years (see table 3:3). More than 80% of the study LLINs were still in the houses (survivorship) regardless of the size of holes after 6 months of use, and the proportion of survivorship decreased as net age with 37% survivorship for Interceptor G2, 18% for Royal Guard, and 10% for Olyset Plus compared to 37% for Interceptor nets after 36 months of use (see table 3: S3a).

After 3 years of net use, only 21.8% (95% CI: 19–25) of Interceptor nets were still in serviceable condition compared to 19.7% (95% CI: 16–23) for Interceptor G2, 3.9% (95% CI: 3–6) for Olyset Plus, and 8.6% (95% CI: 7–11) for Royal Guard (see figure 3:5, table 3: S3b).

Table 3:5: Median survivorship and functional survival of surveyed LLINs in years.

Net Type	Median Survivorship with 95% CI	Median Functional Survival with 95% CI
Interceptor	2.4 [2.4–2.7]	1.9 [1.9–2.0]
Interceptor G2	2.4 [2.4–2.5]	1.9 [1.9–1.9]
Olyset Plus	1.9 [1.8–1.9]	0.9 [0.9–1.0]
Royal Guard	1.9 [1.9–2.4]	1.9 [1.9–1.9]

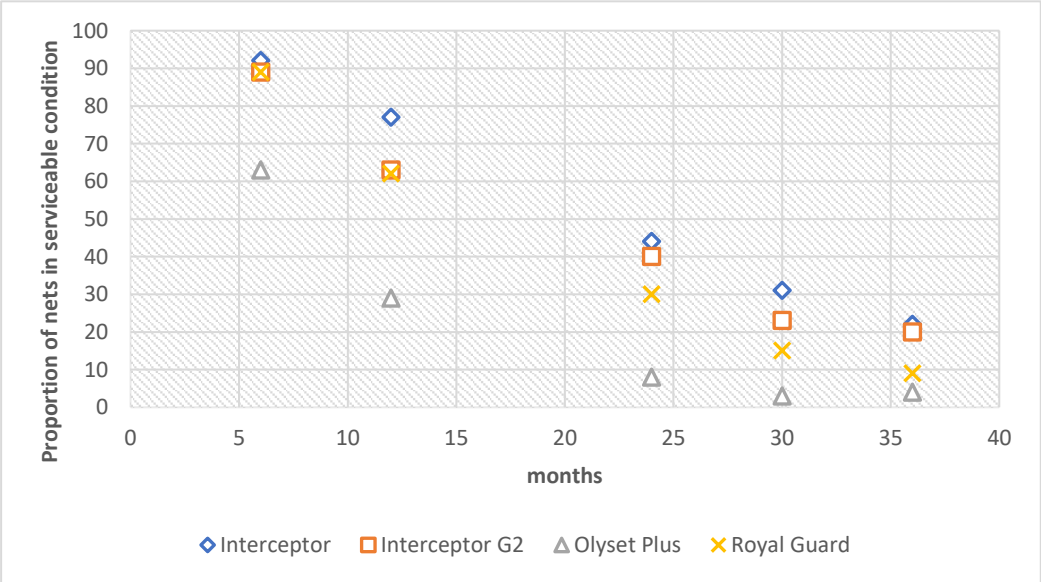

Figure 3:5: Estimated percentage of functionally surviving LLINs per time point.

3.4 Discussion

This study provides a comprehensive evaluation of fabric integrity and survivorship of dual-A.I. LLINs in the Misungwi district, Tanzania. The reported net life spans (functional survival) from the present study fell below the WHO-recommended threshold of three years in operational settings, which was consistently observed across all four LLIN brands of net. Specifically, Interceptor, Interceptor G2, and Royal Guard each exhibited a life span of 1.9 years, while Olyset Plus showed the shortest life span with 0.9 years. The survivorship, considering all nets observed in households regardless of hole size, followed a similar trend, with Interceptor and Interceptor G2 at 2.4 years and Olyset Plus and Royal Guard at 1.9 years each. The main reason reported for the shorter functional survival was attrition due to the wear

and tear of net fabrics. Half of Olyset Plus nets were in very poor condition (unserviceable) at 12 months while the other nets crossed this threshold after 30 months of follow-up. According to the WHO, unserviceable nets would provide little to no protection to the sleeper (192).

The overall functional survival of all LLINs evaluated in the present study was found to be less than three years. In the study area, we discovered additional mosquito nets still in their original packaging, obtained from various sources. In every household, there were new nets from a different brand than the ones we distributed. This might contribute to the swift discarding of nets, as residents had spare nets available to replace the ones provided in the study. Several other studies also reported shorter functional survival than that recommended by the WHO. For instance, more recently, a trial in Tanzania assessing several standard pyrethroid (Olyset, PermaNet 2.0et, and NetProtect) LLINs side by side across different districts reported a median functional survival of 2.0 for Olyset, 2.5 years for PermaNet 2.0et, and 2.6 years for NetProtect (193). There was variation between LLINs in the rate of damage, lost bio-efficacy, and number discarded by households. Of all the nets assessed, Olyset nets were discarded in a higher proportion than PermaNet 2.0et and NetProtect as they were perceived to provide no protection when torn (193). Another study in Zanzibar reported a median survival of 2.9 years in Unguja and 2.7 in Pemba of PermaNet 2.0 vs. Olyset nets (194). Similarly, in Ethiopia, a median survival of 19 months was reported for standard LLINs (195). In contrast, a study conducted in Nigeria reported higher functional survival rates in three areas surveyed (3.0 years in Nasarawa, 4.5 years in Cross River, and 4.7 years in Zamfara), and the difference between states was influenced by social–economic status and housing materials, rather than netting materials (196). In the present study, the functional survival of Olyset Plus was by far the lowest, much shorter than what has been reported in any other studies and lower than a study conducted in different settings in Tanzania that evaluated the same brand of net (79). For example, in Muleba, the functional survival was 1.6 years for Olyset Plus and 1.9 years for standard Interceptor nets. Multiple factors could account for the disparity between the two studies. First, in the current study, in the households we observed other new non-study nets, potentially leading to the replacement of LLINs with new, non-study nets, even within the damaged category. The evidence of using other LLINs has been documented in the main cRCT, of which 76.5% to 82.6% of other nets were being used in 24 months, which was not the case in a previous study. In the present study, the decrease in net survivorship over time for each net brand aligned with the reduction in net usage during the main RCTs, with Olyset Plus net usage being the lowest at 36 months (11%), while Interceptor usage was slightly higher (>30%) (152). Secondly, differences in user behavior (79, 166, 197, 198) may also explain those differences.

After three years of LLIN use, Olyset Plus, Royal Guard, and Interceptor G2 generally exhibited slightly higher attrition rates compared to the standard LLIN, Interceptor. The questionnaire assessing all causes of attrition highlighted that the majority of LLINs being discarded were due to wear and tear and this proportion was increasing over time. The other causes of loss, especially at the beginning of the follow-up, were used in other locations or given away to family or others. Finally, LLINs sold, stolen, and destroyed accidentally represented only a small proportion compared to other causes of net loss. A similar finding was reported in Ethiopia, where the attrition rate of a sub-sample was 48.8% after three years, with the reason being that the nets were too torn (physically damaged) for use, while 13% were used in other locations, and 12.8% were used for other activities (195). The increased attrition rate due to the loss of fabric integrity has impacted malaria transmission in malaria-endemic regions in Kenya, where 40% of nets were extremely damaged after 12 months post-distribution (199). In Tanzania, attrition was even higher, with fewer than 83% of bed nets distributed for daily use no longer present in households after 3 years, giving a median survival rate of 1.6 and 1.9 years for Olyset Plus and Olyset net, respectively (79). This is comparable to a research initiative undertaken in Burkina Faso to evaluate permethrin–pyriproxyfen nets, where the study findings reveal that merely 13% of the distributed nets remain in households after a span of 36 months (147). In Senegal, where Interceptor nets were lost mainly due to wear and tear (200), users reported that nets were disposed of as they believed they did not offer protection due to the accumulation of holes (200). These findings contrast with the World Health Organization (WHO)'s former assumption of nets being present and functional for 3 to 5 years in the community (118). The secondary analysis examining risk factors for attrition revealed an association between attrition rate and socioeconomic status, with higher education appearing to have a protective effect on net handling.

The physical integrity of all distributed LLINs deteriorated with time, with 50 to 80% of the nets considered extremely torn after 36 months, according to brands. Olyset Plus were the most damaged nets followed by the dual A.I. LLIN, Royal Guard, Interceptor G2, and Interceptor, the pyrethroid LLIN. In contrast, longitudinal monitoring conducted in north-west Tanzania reported that 37% of Olyset net and 55% of Olyset Plus were considered extremely damaged (unserviceable according to WHO categories) (79). A cross-sectional community survey conducted in Uganda reports that, after 25 months, there were no discernible differences in the physical durability when comparing long-lasting insecticidal nets with and without PBO (piperonyl butoxide) (115). In all surveys, Interceptor G2 had a lower proportion of "too torn" nets compared to Olyset Plus. No significant differences in the proportion of holes were observed between Interceptor and Interceptor G2 at any timepoint. The results from a structured

questionnaire administered during a survey in Zambia reported that the nets developed holes quickly due to the size (small nets compared to bed size) and material of the net (75). The most significant explanatory factor for survival has been reported to be the combination of a better attitude to net care and exposure to messages related to nets (185). Additionally, the study in Benin reported user preference of the net fabric (polyester vs polyethylene) impacted the functional survival of the net (201). The multifaceted evaluation provides valuable insights into the challenges and dynamics of LLIN durability, aiding in the ongoing efforts to optimize malaria prevention strategies.

3.5 Conclusions

The median functional survival for all classes of LLIN was less than two years, with Olyset Plus median survival of less than 1 year compared to the 3-year survival formerly assumed by WHO. The main reason for net loss was attrition due to wear and tears. Ranking the nets, Interceptor, the standard pyrethroid (reference net), and Interceptor G2 seem to display better physical integrity than the other two dual-A.I. nets. More development from manufacturers, oversight of quality, and donor investment are needed to enhance the textile durability of next-generation mosquito nets.

4 Chapter 4: Bio-efficacy of field aged novel class of long-lasting insecticidal nets, against pyrethroid-resistant malaria vectors in Tanzania: A series of experimental hut trials.

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Abstract

New classes of long-lasting insecticidal nets (LLINs) have been recommended by the World Health Organization (WHO) to control malaria vectors resistant to pyrethroid insecticides. This study was nested in a large-scale cluster-randomized controlled trial conducted (cRCT) in Tanzania. A series of experimental hut trials (EHTs) aimed to evaluate the bio-efficacy of trial LLINs on mosquito indicators most pertinent to malaria transmission over 3 years of use in the community to better understand the outcomes of the cRCT.

The following field-collected LLINs were assessed: 1) Olyset Plus (combining piperonyl butoxide synergist and permethrin), 2) Interceptor G2 (chlorfenapyr and alpha-cypermethrin), 3) Royal Guard (pyriproxyfen and alpha-cypermethrin), 4) Interceptor (alpha-cypermethrin only) conducted in parallel with 5) a new Interceptor, and 6) an untreated net. Thirty nets of each type were withdrawn from the community at 12, 24, and 36 months after distribution and used for the EHTs. Pre-specified outcomes were 72-hour mortality for Interceptor G2, 24-hour mortality for Olyset Plus, and fertility based on egg development stage for Royal Guard.

Overall, Interceptor G2 LLINs induced higher 72-hour mortality compared to standard LLINs of the same age up to12 months (44% vs 21%, OR: 3.5, 95% CI: 1.9 – 6.6, p-value < 0.001), and 24-hour mortality was only significantly higher in Olyset Plus when new (OR: 13.6, 95%CI: 4.4 – 41.3, p-value < 0.001) compared to standard LLINs but not at 12 months (17% vs 13%; OR: 2.1, 95% CI: 1.0 – 4.3; p-value = 0.112). A small, non-significant effect of pyriproxyfen on *Anopheles* fertility was observed for Royal Guard up to 12 months (75% vs 98%, OR: 1.1, 95% CI: 0.0 – 24.9, p-value = 0.951). There was no evidence of a difference in the main outcomes for any of the new classes of LLINs at 24 and 36 months compared to standard LLINs.

Interceptor G2 LLINs showed superior bio-efficacy compared to standard LLINs only up to 12 months, and the effect of Olyset Plus was observed when new for all species and 12 months for *An. gambiae* s.l. only. The pyriproxyfen component of Royal Guard had a short and limited effect on fertility. The decrease in effectiveness of Olyset Plus and Royal Guard LLINs in the EHTs aligns with findings from the cRCT, whereas efficacy of Interceptor G2 lasted for a longer period in the cRCT compared to the EHT. Further investigations are needed to understand the complete scope of chlorfenapyr mode of action. Additional EHT in various contexts will help confirm the residual efficacy of the dual active ingredient LLINs and support the development of longer-lasting nets.

4.1 Introduction

Malaria persists as a significant global challenge, with an estimated 241 million cases and 627,000 deaths in 2020. The African region, contributes to 96% of all malaria cases and associated fatalities (202). In an effort to alleviate the malaria burden, 2.3 billion insecticide-treated nets, specifically long-lasting insecticidal nets (LLINs), were distributed globally between 2004 and 2022, with a substantial 86% delivered to sub-Saharan Africa (202). In 2020, malaria endemic countries received an estimated 229 million LLINs, with 19.4 million of them being pyrethroid-piperonyl butoxide (PBO)-treated nets (202). LLINs and other vector control interventions are predicted to have averted 1.7 billion malaria cases and 10.6 million deaths between 2000 and 2020 (202). Coincident with the scaling up of LLINs and other insecticidal tools across sub-Saharan Africa, mosquito vector populations have developed resistance to pyrethroids, which until recently were the only insecticide class recommended for use in LLINs. Despite the physical and chemical barrier provided by intact LLINs, offering a high degree of personal protection even against resistant mosquitoes, the development of holes or a decline in insecticide efficacy with net ageing could confer a significant competitive advantage to resistant mosquitoes (203). Multiple studies have illustrated a diminishing effectiveness of LLINs in eliminating mosquitoes in regions characterized by high pyrethroid resistance (131, 143). As a results reduction of malaria has come to a halt due to the emergence of both biological and non-biological challenges. This has led to a swift increase in insecticide resistance across all *Anopheles* species (204, 205), and poses a significant threat to the efficacy of vector control interventions. The influence of insecticide resistance on malaria transmission varies across different geographical locations (206, 207).

To tackle the spread of resistance, new malaria vector control tools were needed. The first new class of LLINs developed to control resistant mosquitoes was a combination net containing a pyrethroid insecticide and the synergist PBO (123). In experimental field trials, these LLINs induced significant mortality in vector species (100). The study done in Burkina Faso reported Olyset Plus outperformed pyrethroid-only LLINs (126). This class of LLINs was also evaluated in a cluster-randomized control trial (cRCT) in Tanzania and Uganda. In Tanzania, the study found 44% reduction malaria prevalence in the pyrethroid-PBO LLIN arm (Olyset Plus brand) compared to the standard pyrethroid LLIN arm (Olyset net) after one year and a 33% reduction after two years (59). In a Ugandan cluster-randomized controlled trial (cRCT) conducted over 18 months, the pyrethroid-PBO LLINs (PermaNet 3.0 and Olyset Plus) demonstrated a 13% and 14% reduction in parasite prevalence after 12 and 18 months respectively. These findings prompted the World Health Organization (WHO) to acknowledge the public health significance

of pyrethroid-PBO LLINs and issue a conditional recommendation for their use as a novel class of vector control product(112).

Since then, two additional dual- active ingredient (A.I.) ITNs (Royal Guard and Interceptor G2) have undergone evaluation in WHO Phase I and II trials, showing significant promise compared to standard LLINs in combating pyrethroid-resistant vectors. In Phase I studies, Royal Guard (containing the pyrethroid alpha-cypermethrin, and the juvenile growth hormone inhibitor pyriproxyfen) met the WHO criteria with 95% knockdown and more than 80% mortality for up to 20 washes when susceptible *Anopheles (An.) gambiae* s.s. (kisumu strain) were exposed in cone assays (129). It also met the WHO criteria in tunnel tests, with mortality exceeding 80% after 20 washes using the same strain. In a Phase II experimental hut trial conducted against wild, pyrethroid resistant *An. gambiae* s.l., Royal Guard demonstrated an 83% reduction in oviposition and a 95% reduction in offspring/hatching before washing. These values decreased to 25% and 50%, respectively, after 20 washes. Interceptor G2 (containing alpha-cypermethrin and the pyrrole chlorfenapyr) was able to induce 71% mortality against free flying *An. gambiae* s.l. in an experimental hut trial compared to an alpha-cypermethrin-only net (20% mortality) (140). In a large-scale cRCT conducted in Tanzania, Royal Guard, Interceptor G2 and Olyset Plus LLINs were evaluated against standard Interceptor nets in the context of pyrethroid resistant malaria vectors. The results revealed 55% lower odds of malaria infection in children aged 6 to 14 years and 44% reduction in malaria case incidence in children aged 6 to 10 years after two years of net use in the Interceptor G2 arm. Additionally, the entomological inoculation rate (EIR) was also significantly lower (by 85%) in the Interceptor G2 arm compared to the standard pyrethroid-only arm (151). In the same trial, Olyset Plus showed a shorter protective effect of 12 months compared to a previous cRCT conducted in a different part of Tanzania, where pyrethroid-PBO LLINs remained effective for 24 months (59, 151). A second cRCT conducted in Benin showed similar results, with Interceptor G2 reducing malaria incidence by 46% to children aged 6 to 10 years compared to pyrethroid-only LLINs, while Royal Guard did not significantly reduce malaria outcomes (114). Based on evidence from these two trials, Interceptor G2 received a strong recommendation from WHO, while Royal Guard received a conditional recommendation. For Royal Guard, a third trial conducted in Burkina Faso (154) was considered by WHO. It was the only trial showing a small but significant reduction in malaria outcomes in the PPF arm compared to the standard LLIN arm.

New products must also undergo WHO pre-qualification to demonstrate efficacy in phase II-controlled EHT evaluation as well as phase III community study to assess efficacy over the intended life span of the product (3 years for LLIN) in normal condition of use.

During the Phase II semi field evaluations of LLINs efficacy, it is anticipated that each net should maintain insecticidal activity through at least 20 standardized washes, considering vector knock-down, mortality, and blood feeding inhibition as per WHO guidance (116). Standard phase II studies are conducted using unwashed nets and nets being washed 20 times as a proxy for a 3-year net's use in the community. Olyset Plus, Royal Guard and Interceptor G2 have all completed the standard phase II studies and have received temporary pre-qualification (208).

Alongside a standard phase III study (188) embedded in the cRCT in Tanzania, the present paper reports a series of adapted phase II experimental hut (EHT) study conducted near the cRCT. The EHT study area had similar vector population and was designed to assess nets obtained from the main trial at regular intervals, which will enable to understand the impact of field conditions, wear-and-tear, and insecticidal deterioration on the bio-efficacy of the dual-AI LLINs on entomological outcomes (188). This approach aimed to link the study's bio-efficacy outcomes with the epidemiological and entomological results observed in the cRCT, while also setting new evaluation standards for these innovative products.

4.2 Methodology

Experimental Hut Study Site

The experimental hut (EH) study was conducted in Welamasonga village (2°34.673' 33°07.170'), Magu district Mwanza, Tanzania, between 2020 to 2022. In this village, six east African experimental huts were constructed in the north part of the Misungwi cRCT area (188) (see Figure 4:1). A detailed description of the cRCT area can be found in the protocol published elsewhere (151).

Magu experiences the same climatic conditions as the cRCT site, characterized by two rainy seasons (March – May and October – December) separated by a long dry season (June – August or September) and a short dry season (December or January – February) (188). The area surrounding the experimental huts consists of open fields used for cultivating rice and vegetables, with irrigation from ponds. The main vector species in this area are *An. funestus* sensu stricto (s.s), *An. arabiensis* and *An. gambiae* sensu stricto (s.s). The composition of these species varies with the season, with *An. gambiae* senso lato (s.l) dominating during the rainy season and *An. funestus* s.l. during the dry season.

Baseline resistance monitoring using CDC bottle assay reported a 69% mortality (24 hours post exposure) in *An. gambiae* s.l. exposed to the diagnostic dose of alpha-cypermethrin and 12% mortality for those exposed to permethrin. While a 25% mortality was reported for *An. funestus* s.l. after exposure to permethrin.

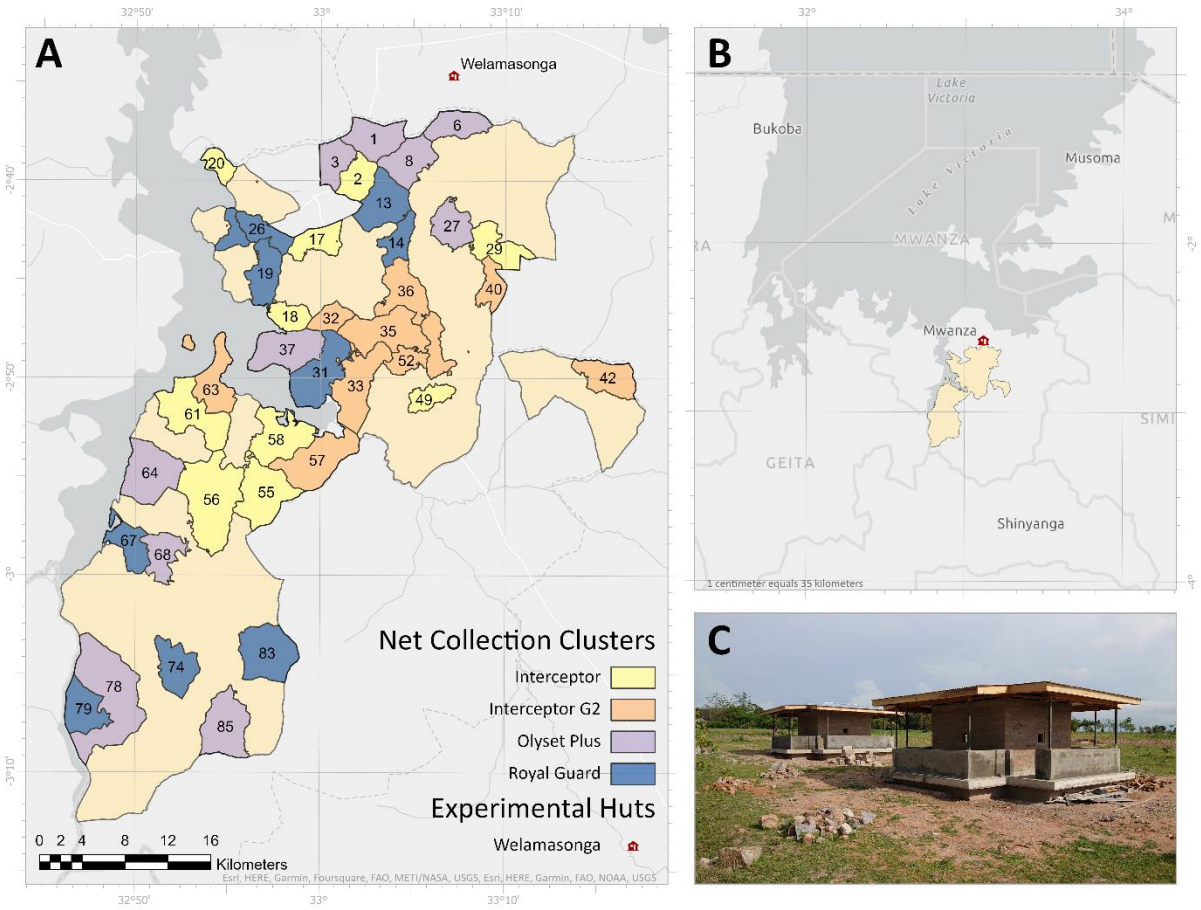

Figure 4:1: Map showing the location of experimental huts in the north part (B &C) of the main cRCT area and the clusters where the net was collected (A).

The map was created by ArcGIS software and all geographical and administrative data was sourced at GADM: https://gadm.org/license.html.

Net distribution and its characteristics.

The nets rotated in the experimental hut were collected from Misungwi district, Mwanza, Tanzania where the cRCT was conducted. The cRCT spanned between 2019 and 2022 and aimed to assess the effectiveness of two dual-A.I. LLINs and a pyrethroid-PBO LLIN, compared to standard pyrethroid-only nets against malaria infection (151). In January 2019, study nets were distributed across the study arms in 84 clusters within the study area. Before distribution, six new nets from each brand were retained to be tested in the experimental huts. Characteristics of the four LLIN distributed were as followed: 1/ Royal Guard, a polyethylene net combining 225 mg/m^2 of pyriproxyfen, which is known to disrupt female reproduction and fertility of eggs, and 261 mg/m^2 pyrethroid alpha-cypermethrin; 2/ Interceptor G2, a polyester material coated with two adulticides with differing modes of action; 200 mg/m^2 of chlorfenapyr (a pyrrole) and a 100 mg/m^2 of pyrethroid (alpha-cypermethrin;); 3/Olyset Plus, a polyethylene material which incorporates a synergist (400 mg/m^2) PBO, to enhance the potency of pyrethroid insecticides and 800 mg/m^2 of pyrethroid permethrin; 4/ Interceptor, a polyester filament coated

with 200 mg/m^2 of alpha-cypermethrin as a comparator to highlight the effect of the partner A.I. (table 4:1).

Sample size and sampling/withdrawal of LLIN for experimental hut trials

A total of six treatments were rotated during each EHT study. In addition to the four types of nets collected in the community, two other treatments were included, new standard LLIN (Interceptor as positive control) and untreated nets (negative control) were included (116) as shown in table 4:1 below.

Table 4:1: EHT treatments evaluated.

Treatment	Status of the net	Number of nets
1. Olyset Plus	Field collected	30 nets (tx*)
	New unwashed with 6 holes	6 nets (t0**)
2. Interceptor G2	Field collected	30 nets (tx)
	New unwashed with 6 holes	6 nets (t0)
3. Royal Guard	Field collected	30 nets (tx)
	New unwashed with 6 holes	6 nets (t0)
4. Interceptor	Field collected	30 nets (tx)
	New unwashed with 6 holes	6 nets (t0)
5. Positive control	New Interceptor with 6 holes	6 nets (t0)
6. Negative control	New untreated with 6 holes	6 nets (t0)

*tx are nets collected at 12, 24, and 36 months. ** new unwashed net.

This study collected 30 LLINs per arm from the community at each time point. Since each EHT study was conducted over 36 nights and each intervention net was tested for one night, an additional six new LLINs of each type (Royal Guard, Interceptor G2, Olyset Plus, and Interceptor) were added to treatment 1 to 4 to complete the rotation and assess the efficacy of those different net types when they are new.

One month after distribution, 1950 nets in 5 clusters per treatment arm were labelled with a unique number. Thirty of these labelled nets were collected at intervals of 6, 12, 24, 30 and 36 months. Nets collected at 6 and 30 months were not included in the experimental hut rotation due to time constraint and limited number of huts. The sampling design is explained in more detail elsewhere (188). Each collected net in the community was replaced by a new net of the same type. However, these replacement nets were not included in the study. Households remained part of the eligible cohort until no enrolled nets were available.

Number and size of holes in field collected nets were assessed to estimate the total hole area in each net brand, following WHO guideline (116). The nets were categorized based on the total area: "good" if the area was less than 79 cm^2, "damaged" hole area ranged from 80 to 789 cm^2, and "torn" if the hole area was greater than 790 cm^2 (191).

Experimental hut study

The six experimental huts used for the study were built following an east African design but without veranda. Each EHT study was done over a 6-week period. Sleepers were rotated between huts on successive nights to account for individual attractiveness, and treatments were rotated every week following a random Latin square design. For each hut trial, during the 36 nights, each net (30 tx and 6 t0) from each treatment (Olyset Plus, Interceptor G2, Royal Guard and Interceptor) was rotated every night. For all new intact LLINs, six holes of 4 x 4 cm were cut following WHO guidelines (116).

During each night collection, cloth sheets were laid on the floor of each huts and sugar solution was provided at night in the window traps to reduce mosquito mortality (209). The day after net installation in the hut, mosquitoes were collected using standard mouth aspirators from the room (floor, walls and ceilings), inside net, and window trap. The mosquitoes were packed in paper cups labelled with the collection date, hut number, net type, collection area (window/room/net) and collector initials. All mosquitoes were sent to the National Institute for Medical Research (NIMR) insectary. Mosquitoes were identified morphologically to species (210) and categorized by gonotrophic status (i.e., unfed, freshly fed, semi-gravid and gravid).

Mosquito mortality was monitored everyday up to 72 hours in a controlled environment with 75±10% RH and temperature of 27±2°C at NIMR insectary. The effect of pyriproxyfen on reproductive outcomes was assessed on mosquitoes collected from the huts deployed with Royal Guard, Interceptor and untreated nets by dissecting gravid *Anopheles* 72-hours after collection when eggs should normally have fully matured (172, 173).

After a 72-hour holding period, *An. gambiae* s.l. were further identified to species-level using a TaqMan PCR assay to distinguish *An. gambiae* s.s. from *An. arabiensis* (211) and the same was done for *An. funestus* s.l. to distinguish between *An. funestus* s.s and *An. parensis* (212). Half of the blood fed and unfed, alive and dead mosquitoes were packed in RNAlater for determination of species and cytochrome P450 expression levels using qPCR (213, 214).

Unexposed mosquitoes were homogenized using a Qiagen TissueLyser II (Qiagen, Hilden, Germany) with 5 mm stainless steel beads. RNA was extracted using the Qiagen RNeasy 96 kit, following the manufacturer's protocol. Approximately 2 µg of RNA from each sample was

treated with RQ1 RNase-free DNase (Promega, Southampton, UK). Then, 1 µg of DNase-treated RNA from each mosquito was used to synthesize cDNA, utilizing the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA), as per the manufacturer's guidelines. Quantitative real-time reverse transcription PCR (qRT-PCR) was used to measure expression of eight metabolic genes (CYP6M2, CYP6P3, CYP6P4, CYP6Z1, CYP4G16, CYP9K1, CYP6P1 and GSTE2), commonly overexpressed in vector populations in African countries (215-217). Standard curves of Ct values for each gene were generated using a five-fold serial dilution of cDNA to assess PCR efficiency. Each 10µL reaction volume contained 2µL cDNA, 10µM of each primer and 5µL 2× Roche FastStart Essential DNA green master mix. Reactions were performed in technical triplicate using a StepOnePlus real-time PCR system (Applied Biosystems, UK). Reaction conditions were 10 min at 95°C; 35 cycles of 10 s at 95°C, 22 s at 60°C, and 10 s at 72°C, followed by a melt curve. Fold Change (FC) of each target metabolic gene from field samples, relative to a species-specific, susceptible laboratory strain (*An. gambiae* Kisumu), were calculated using the $2^{-\Delta\Delta CT}$ method (218), incorporating PCR efficiency. The housekeeping gene ribosomal protein S7 was used for normalization.

Nets collected at each time point were assessed in the same year, with those collected after 12 months of use tested in 2020, those collected after 24 months in 2021, and those collected after 36 months in 2022. To account for vector seasonality and estimate net efficacy against different *Anopheles* species, four hut studies were conducted for nets collected at each time point over a year (188).

Resistance monitoring

Wild *An. gambiae* s.l. and *An. funestus* s.l. were collected from houses adjacent to the experimental hut site using standard mouth aspirator around 6:00am to 7:00am in parallel of EHT study. Mosquitoes were morphologically identified to species and kept for three days to allow digestion of blood meal before bioassay testing. Resistance intensity to the insecticides contained in each LLIN was assessed using WHO/CDC bottle bioassays every year. Mosquitoes were exposed to the diagnostic doses of alpha-cypermethrin or permethrin and increased to 2, 5 and 10 times of diagnostic concentration for 30 minutes, for chlorfenapyr (100 µg/ml), pyriproxyfen (100 µg/ml) for 60 minutes; PBO pre-exposure was also performed using WHO tube bioassays followed by CDC exposure to pyrethroid.

Outcome measures

The primary outcomes vary for each product and are on the mode of action of the active ingredient or synergist being evaluated following WHO recommendation (112, 116) as follows:-

Primary outcomes were 1/ 72-hour mortality for Interceptor G2, 2/24-hour mortality for Olyset Plus and 3/fertility for Royal Guard, calculated as the proportion of blood fed females alive at 72-hours with fully mature eggs. Secondary outcomes were: 1/ 24-hour mortality for Royal Guard and for all the nets 2/ blood feeding: proportion of blood fed mosquitoes collected, 3/deterrence: percentage reduction in density of *Anopheles* in treatment huts compared to negative control huts (fitted with untreated nets), 4/ exophily: proportion of mosquitoes that exited early and were found in window traps compared with the untreated control huts.

Data analysis

Data analysis was performed using STATA software version 17. After data cleaning, four nights of collections were removed due to reporting errors across all three years of rotation. The nature of error was mismatch between field and laboratory data. Descriptive statistics were used to estimate the proportion of mosquito species collected each year.

Proportional outcomes (blood-feeding, deterrence, exiting mortality, and fertility) related to each experimental hut treatment was assessed using binomial generalized linear mixed models (GLMMs) with a logit link function. A separate model was fitted for each outcome. In addition to the fixed effect of each treatment and hole index categories, each model includes random effects to account for the following sources of variation: between the huts; between the sleepers; between the weeks of the trial; between trial rounds; and finally, an observation-level random effect to account for variation not explained by the other terms in the model (over dispersion). Location of the holes (zone 1 to 5) in relation of mosquito blood feeding and net entry was assessed. The interaction between treatment and net age was reported using odds ratios with their 95% confidence intervals (CI) and p-values were used to assess statistical significance at the 0.05 level for comparisons of mortality and blood-feeding. Mortality and blood feeding graphs were plotted using ggplot in R software. A secondary analysis was conducted using separate models to examine the interaction between mosquito species and net age, employing Generalized Linear Mixed Models (GLMMs) with a logit link function. However, these results were not included in the publication.

For resistance data, lethal dose values (LD25, LD50, LD95 and LD99) were calculated using a probit model with log-10 transformed data in IBM SPSS v28 software. The curve estimation was based on the probability of mosquito death as a function of the total number of mosquitoes

and insecticide dose (113). Point estimates of LDs and 95% CIs were then back transformed to their original scale to obtain the reported values, indicating the difference in diagnostic dose of an insecticide required to kill 25%, 50%, 95% or 99% of tested mosquitoes. Comparisons of LD50 values among clusters and/or years were statistically estimated using the Relative Median Potency (RMP), calculated as the ratio of point estimates with simultaneous 95% CIs. Comparisons of "potency" in this context are median lethal concentrations/doses. A ratio of "1" is considered insignificant, meaning LD50 was equal among comparison groups. Reduction in fertility was calculated as ((proportion fertile in control -proportion fertile in treatment)/ proportion fertile in untreated net control) *100. P-value less than 0.05 was considered as significant.

4.3 Results

Species composition and outcomes in negative and positive control huts over the 3 years

A total of 12 experimental hut trials (4 rotation round per timepoint (t12, t24 and t36)) were conducted between 2020 and 2022, resulting in 2,588 collection nights. During this period, 17,040 male and female mosquitoes were collected with 87% (14,841/17,040) of them being female. Among the female mosquitos, 26% (3,925/14,841) were identified as *Anopheles,* while the remaining were *Culex quinquefasciatus*.

For all collection years combined, 63.4% (2488/3925) of the *Anopheles* were identified as *An. gambiae* s.l. while the remaining 36.6% (1437/3925) were *An. funestus* s.l.. Among the *An. gambiae* complex identified to species-level, 56.8% (673/1185) were determined to be *An. gambiae* s.s. while the rest were classified as *An. arabiensis*. Notably, the proportion of *An. gambiae* s.s. was highest in 2020 and subsequently decreased over the years (as shown in table 4:2).

Table 4:2: Hut trials entomological characteristics (density and species composition) over the three years of follow-up.

	Year 1: 2020		Year 2:2021		Year 3:2022	
Total hut/night collection	863		864		864	
Total mosquitoes collected (female)	4320 (3869)		7424 (6612)		5296 (4360)	
Total *Anopheles* collected (female)	1974 (1611)		1904 (1457)		1204 (857)	
Anopheles vectors: N, mean [95%CI]	1611	1.9 [1.7 - 2.0]	1457	1.7 [1.5 - 1.8]	857	0.9 [0.9 - 1.1]
Culex species: N, mean [95%CI]	2270	2.6 [2.4 - 2.8]	5155	5.9 [5.2 - 6.7]	3503	4.1 [3.7 - 4.4]

Species composition n/N, % [95%CI]			
*An. gambiae*s.l./*Anopheles*	1161/1611, 72.1%, [68.9 - 75.1]	842/1457, 57.8% [51.9 - 63.4]	485/857, 56.6% [50.4 - 63.5]
*An. funestus*s.l./*Anopheles*	450/1611, 27.9% [24.4 - 30.7]	615/1457, 42.2% [36.6 - 48.1]	372/857, 43.4% [36 - 49.6]
*An. gambiae*s.s/*An.gambiae*s.l	347/410, 85.0% [80.7 - 87.8]	181/334, 54.0% [48.8 - 59.4]	143/435, 33.0% [29 – 37]
*An. funestus*s.s/*An.funestus*s.l	151/153, 99.0% [94.8 - 99.6]	436/460, 95.0% [92.3 - 96.5]	321/368, 87.0% [83 – 90]

Anopheles mortality for untreated nets (negative control huts) was less than 5% after 24-hours which meets the WHO recommended threshold, while the exophilic rate ranged between 62% and 68% depending on the year. Blood feeding was between 18% and 21% in huts fitted with a new standard pyrethroid LLIN Interceptor (positive control huts); 24-hour mortality was low and ranged from 8% to 12%. The mortality against *An. funestus* complex for the whole period of study varied between 4% to 10% while it ranged between 7% to 18% for *An. gambiae* complex (see Figure 4: S1).

EHT results on mortality and blood-feeding

There was higher 24-hour mortality in *Anopheles* collected in huts fitted with new (0 month) Olyset Plus compared to a new standard pyrethroid LLIN (raw data 38% vs 6%), adjusted odds ratio (OR): 13.6, 95%CI: 4.4 – 41.3, p-value<0.001 (Figure 4:2). Mortality in Olyset Plus dropped over time and while it remained slightly higher compared to standard pyrethroid LLINs of the same age, the difference was not significant after nets were used for 12 months or more (12 months: 17% vs 13%; OR: 2.1, 95%CI: 1.0 – 4.3; p=0.112, 24 months: 12% vs 11%, OR: 1.4, 95%CI: 0.6 – 3.3; p=0.310 and 36 months: 10% vs 7%, OR: 1.0, 95%CI: 0.3 – 3.5; p=0.890) (see figure 4:2 & table 4: S4a). By species, 24-hour mortality remained significantly higher in Olyset Plus compared to the standard pyrethroid LLIN in *An. gambiae* s.l. (23% vs 13%, OR: 2.6, 95%CI: 1.0 – 6.4; p=0.045) at 12 months but no effect was observed on the *An. funestus* complex at this time point (see table 4: S4b). Blood feeding for both species was lower in Olyset Plus compared to Interceptor LLINs up to 12 months, but the difference was not significant at any of the following time points (see Figure 4:4).

Interceptor G2 LLINs provided higher 72-hour mortality compared to Interceptor LLINs when new (raw data 58% vs 14%), (OR: 11.9, 95%CI: 4.8 – 29.7 p<0.001) and after 12 months of use (43% vs 21%, OR: 3.5, 95%CI: 1.9 – 6.6, p<0.001) (see Figure 4:3)The effect was observed for both *An. gambiae* s.l. and *An. funestus* (see table 4: S4b & S4c). At 24 and 36 months, the difference in mortality was no longer significantly higher. Similar findings were observed for 24-hour mortality (see Figure 4:2). In terms of blood feeding inhibition, there were no

differences between Interceptor G2 compared to Interceptor LLINs at any time point (see Figure 4:4).

For Royal Guard, the primary outcome was effect on fertility. In Royal Guard LLINs huts, a total of 707 female *Anopheles* were collected, of which 138 were blood fed among them, 133 were alive after 72hrs. Since half of the alive mosquitoes were preserved in RNA for gene expression, only 79 were dissected (see table 4:3). The fertility rate was 67% when Royal Guard was new and increased to 75% after 12 months of use. Meanwhile, it varied between 94% and 100% for *Anopheles* collected from untreated nets and standard pyrethroid Interceptor LLINs. At 24 and 36 months, all mosquitoes were found with fully mature eggs in Royal Guard and categorized as fertile. Interestingly, despite small numbers being dissected, a reduction in fertility was only observed for *An. gambiae* s.l. and not *An. funestus* s.l.. Mortality outcomes were also monitored and were found significant at 0 month and 12 months compared to Interceptor LLINs. Blood feeding was lower at 0, 12 and 36 months in Royal Guard compared to standard pyrethroid LLINs but the difference was not significant and decreased with time, while at 24 months more blood fed *Anopheles* were found in the Royal Guard huts (as shown in figure 4:4 and table 4:3) than in standard pyrethroid LLINs.

EHT results on deterrence, exiting and net penetration

There was no clear pattern with deterrence for *An. gambiae* s.s.. For nets aged 0 and 24 months there was no deterrence effect in the treatment huts relative to huts fitted with untreated nets while at 12 and 36 months there was some reduction of *An. gambiae* s.s. entered in huts with treated nets (table 4: S5). For *An. funestus* complex, regardless of net age there was some hut entry reduction in huts fitted with Interceptor G2 and standard pyrethroid Interceptor LLINs while that was not observed at any time point for Olyset Plus and Royal Guard (except at 36 months for the latter). Overall, the highest exit rate for *Anopheles* was found in treatment huts compared to untreated huts, but there were no significant differences between Interceptor G2, Royal Guard or Olyset Plus compared to Interceptor of the same age. Overall, penetration of nets by *Anopheles* was consistently lower for all the insecticide treated nets compared to untreated nets (Table 4: S5).

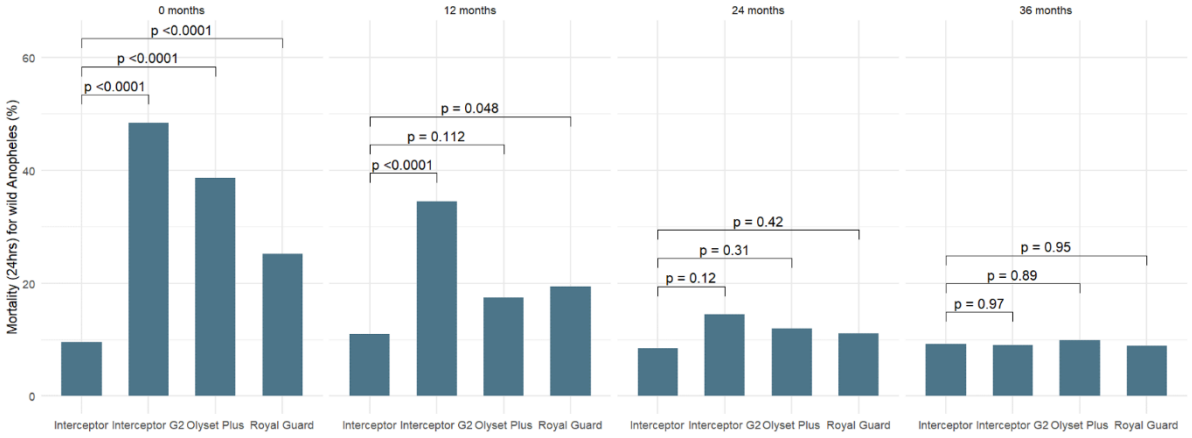

Figure 4:2: Model output mortality (24-hrs) of wild free flying female *Anopheles* in experimental hut by net type and age.

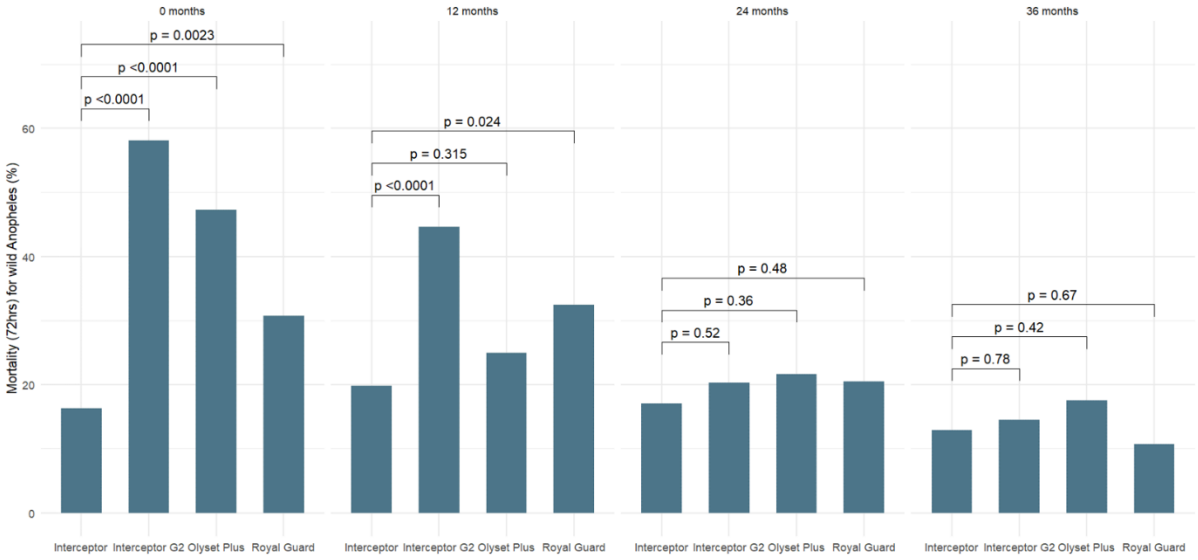

Figure 4:3: Model output mortality (72-hrs) of wild free flying female *Anopheles* in experimental hut by net type and age.

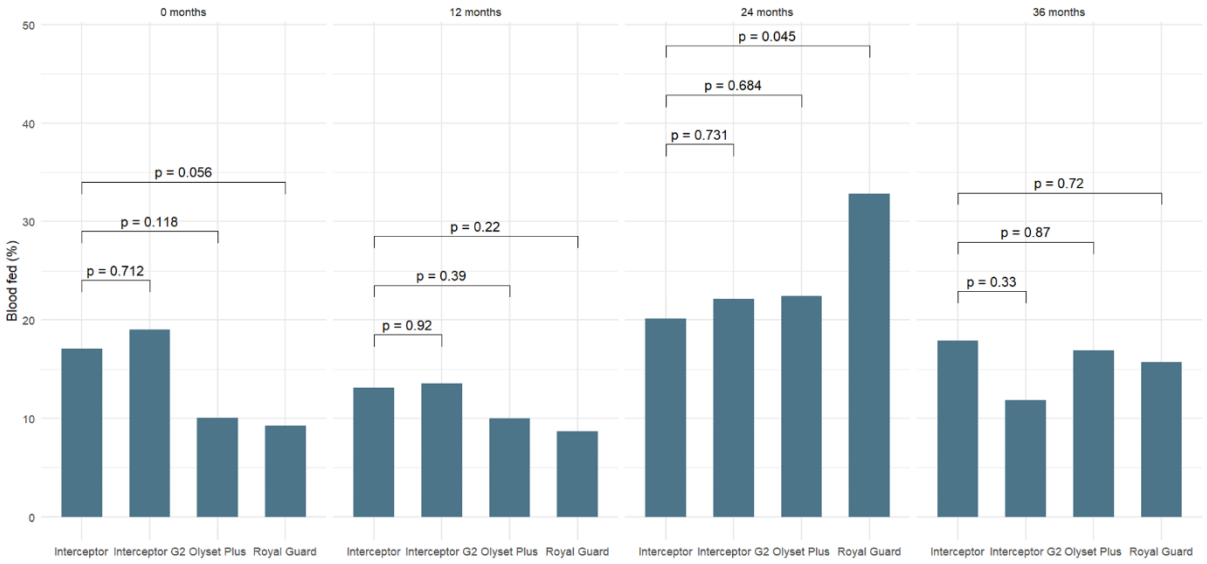

Figure 4:4: Model output blood feeding in wild free flying female *Anopheles* in experimental hut by net type and age.

Table 4:3: Fertility in wild free flying female *Anopheles* in experimental hut fitted with Interceptor, untreated net and Royal Guard by age.

Net type	Total collected	Total BF*	Total alive 72hrs post collection	Total BF* alive 72hrs post collection	Number dissected	Total fertile	Percentage (%)	95CI
0months								
Untreated	104	32	100	32	26	26	100	-
Interceptor	113	23	98	23	16	15	94	62 - 99
Royal Guard	139	15	96	14	6	4	67	36 - 99
12 months								
Untreated	230	73	213	72	57	56	98	89 - 100
Interceptor	181	28	143	27	17	17	100	-
Royal Guard	222	24	152	23	12	9	75	41 - 93
24 months								
Untreated	156	57	150	57	37	37	100	-
Interceptor	170	38	136	37	30	30	100	-
Royal Guard	225	79	174	76	51	51	100	-

36 months								
Untreated	133	34	127	34	28	28	100	-
Interceptor	91	17	78	17	17	17	100	-
Royal Guard	121	20	107	20	20	20	100	-

*Blood fed

Hole characteristics of nets sampled from the community and effect of hole and net age on vector blood feeding

The mean hole area for Interceptor G2 and Olyset Plus LLINs aged 12 months for the good category was 26.5cm^2 and 27.9 cm^2 respectively, while the mean hole area in Royal Guard was 16.2 cm^2. On average, hole size increased with net age (see table 4: S6). Overall, mortality and blood feeding were not impacted by the size or number of holes in any of the nets.

Insecticide resistance in malaria vectors.

Longitudinal changes in vector insecticide resistance intensity were assessed to determine their influence on vector mortality in the EHTs. Permethrin resistance intensity was high in *An. funestus* in year one (LD50 = 292.9 [52.7–3906.3]) but decreased over time, with LD50 values of 33.7 [20.5–81.7] in year two and 19.1 [13.5–36.2] in year three. By comparison, levels of alpha-cypermethrin resistance intensity in *An. funestus* increased significantly during the EHTs (year two LD50 = 22.5 [12.9 – 65.1] and year three LD50 = 181.6 [61.8 – 1499.9]). In *An. gambiae* s.l., a similar decline in permethrin resistance intensity was evident (year one LD50 = 3417 [4.0 – 7319224.4], year two LD50 = 24.1 [15.5 – 48.3] and year three LD50 = 43.6 [26.4 – 100.0]). However, there was no parallel increase in alpha-cypermethrin resistance (year one LD50 = 0.24 [0.0 – 0.6], year two LD50 = 0.52 [0.2 – 0.9] and year three LD50 = 0.79 [0.3 – 1.3]). In both vector species complex, there was limited change in permethrin resistance intensity following PBO pre-exposure.

Following chlorfenapyr exposure, high 72-hour mortality was evident in both species complexes at year one (92% [95% CI: 87 – 97] and 91% [95% CI: 87 – 96] for *An. gambiae* s.l. and *An. funestus* s.l., respectively); complete (100%) mortality was observed for both *An. gambiae* s.l. and *An. funestus* s.l, in year two and three.

During pyriproxyfen testing, there was no impact on fertility in *An. funestus* s.l. in year one while an 8.1% [8/98] reduction was observed in *An. gambiae* s.l. in year one. For year two and

three, only *An. gambiae* s.l. were tested and 8.8% [5/57] and 6.7% [1/15] reduction in fertility was observed respectively. Overall, sterility effect was less than 10%.

Dynamic changes in expression of eight metabolic genes (CYP6P4, CYP6Z1, CYP4G16, CYP9K1, CYP6M1, CYP6P1, CYP6P3 and GSTF2) were observed in PCR-confirmed *An. gambiae* s.s. between trial years one and two (Figure 4:5). CYP9K1, CYP6M2, and GSTE2 displayed consistently minimal overexpression relative to the susceptible colony strain. Compared to the untreated net arm, significant declines in CYP6P4 expression were observed in mosquitoes exposed to Interceptor (washed and unwashed) and Olyset Plus, while no significant decrease in CYP6Z1 expression was noted in mosquitoes exposed to Royal Guard and Interceptor G2 between years one and two. However, a significant increase in CYP6P1 expression was observed in vectors collected from Royal Guard EHTs. In figure 4:5 error bars represent 95% confidence intervals. Statistically significant differences in expression levels between trial years are indicated as follows: ns=not significant; *= p-value <0.05; **=p-value <0.01; ***= p-value< 0.001.

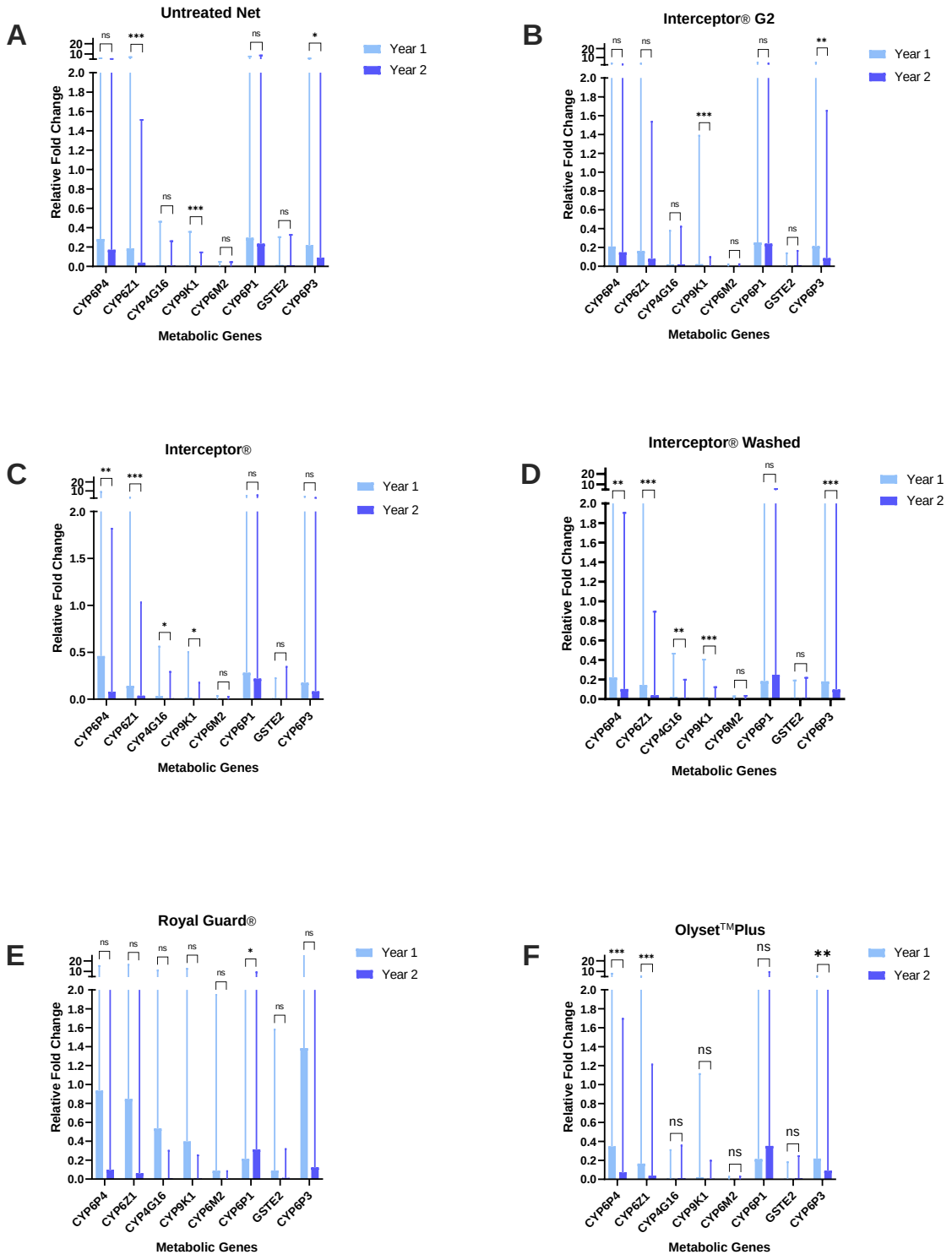

Figure 4:5: Gene expression in wild field collected *An. gambiae* s.l. relative to colony susceptible population over two years.

Untreated net (A), Interceptor G2 (B), standard Interceptor (field collected) (C), standard Interceptor (washed once) (D), Royal Guard(E) and Olyset Plus (F).

4.4 Discussion

These series of experimental hut trials were conducted to assess the impact of interventions (dual-A.I. LLINs) on mortality and blood-feeding rates in resistant *An. gambiae* s.l. and *An. funestus* s.l. vector populations and to better understand the associated cRCT outcomes. When new, Interceptor G2 and Olyset Plus provided a significantly higher killing effect compared to the reference net (Interceptor) against wild-resistant *Anopheles* mosquitoes found in the Magu district. Mortality was also generally higher for these two nets at 12 months, but there was only strong evidence for a difference for Interceptor G2. Royal Guard did not have a significant impact on fertility outcomes at any of the time points.

The highest effect of Interceptor G2 on vector mortality was observed for both vectors *An. gambiae* s.l. and *An. funestus* up to 12 months, with no observable difference after 24 and 36 months of net use compared to Interceptor. Previous experimental hut trials done in Tanzania (141) assessing new unwashed Interceptor G2 reported a 72-hour mortality around 50% against *An. funestus* similar to our findings, while mortality was in general higher in Benin (71%) and in Cote d'Ivoire (87%) against *An. gambiae* s.l.. Interestingly, in these EHTs, after 20 standardized washes, 72-hour mortality was 52% in Tanzania, 65% in Benin(140) and 82% in Cote d'Ivoire (219), all much higher than what we observed in our EHT at any given time point. In our study, the insecticidal content over time depleted quicker compared to those exposed to laboratory washing procedures; indeed, the residual concentration of chlorfenapyr was only 8% of the initial content after 36 months of operational use while the chlorfenapyr retention observed in nets washed 20 times were 32% in Tanzania (141) and 37% in Benin (140, 141).

Overall mortality induced by field-collected Olyset Plus ITNs were significantly higher than that of the pyrethroid-only net (Interceptor) when the nets were new, but not at any other time point. When analysing the effect by species, mortality was also significantly higher in *An. gambiae* s.l. at 12 months, which was not the case for *An. funestus*. In the cRCT conducted as part of this study (151), Olyset Plus LLIN was only effective for a year while a previous cRCT conducted in Muleba-Tanzania showed that Olyset Plus LLIN arm had lower malaria than Olyset Net (59) and provided personal protection (76) up to 21 months of use in an operational setting. The EHT results from the present study suggest that Olyset Plus may control *An. gambiae* s.s. better than *An. funestus*, which could explain the difference between the two cRCTs. In Misungwi, the main vector was *An. funestus* (220) while in Muleba it was *An. gambiae* s.s. (59). The effectiveness of Olyset Plus declined as the net aged, which aligns with the observed reduction in both permethrin and PBO content after 36 months of community use (8.3 g/kg vs 20 g/kg and ~0.7 g/kg vs 10 g/kg, respectively, when new). Similar findings were reported in Uganda (115) where there was a low retention (3.7 g/kg) of PBO content 25 months

after the distribution of Olyset Plus LLINs. In Kenya (145), 87% of PBO and 52% of permethrin were lost after 36 months of community use. Notably, PBO retention was higher when nets were subjected to 20 laboratory washes (2.0 g/kg) in Benin (221).

For secondary analysis, Interceptor G2 shows statistically significant effects for both *An. gambiae* and *An. funestus*, though the protective effect (mortality) up to one year, however, Olyset Plus and Royal Guard exhibit potentially protective effects, but the p-values indicate the results are not statistically significant at any time point at the conventional threshold ($p < 0.05$).

In the case of Royal Guard, sterility effect was low over the three-year period. Only 33.3% of *Anopheles* were deemed sterile after exposure to new, unwashed Royal Guard nets, and this figure decreased to 25% after one year of use. There was no observed sterility effect at 24 and 36 months. The rapid decrease of sterility effect in Royal Guard could partly be due to pyriproxyfen resistance as it was observed already in some populations of *An. gambiae s.l.* of Tanzania (113). Notably, the impact of Royal Guard on mortality was noticeable only up to 12 months. The limited number of blood-fed *Anopheles* still alive at 72-hours suggests that the combination of pyrethroid and pyriproxyfen in the first year may have had a greater impact on mortality and blood feeding than on fertility. A study in Benin (129) using Royal Guard LLINs indicated a reduction in the reproductive ability of mosquitoes up to 20 washes in a laboratory setting. However, the effect was lower (25%) in experimental huts. Another pyriproxyfen net brand, Olyset Duo, evaluated in Moshi, Tanzania, against *An. gambiae* s.l. reported 34.6% fertility after exposure in tunnel tests following 20 washes (222). Nonetheless, an entomological assessment of this brand in a cRCT in Burkina Faso revealed that the sterility effect was only observed for one month after LLIN distribution (147). The difference in performance between laboratory and community studies could be explained by more stringent washing methods and abrasion during daily use. In our study only 28% of pyriproxyfen and 62% of alpha-cypermethrin remained on the nets after 36 months while insecticide retention was higher when washed in the laboratory (57% and 76% for pyriproxyfen and alpha-cypermethrin, respectively) (129).

It is important to note that all these studies were carried out using unwashed nets, with the nets washed 20 times. Our results highlight that a net washed 20 times under Phase II conditions do not simulate a net used in the field for 36 months, as community washing process can be more intense and the nets more subject to friction during general use (17). In addition, hole size in aged nets in community may differ from the 4x4 cm standardized holes created in washed nets. Data from experimental hut trials conducted with ‘real-life’ field nets can help explain why

Royal Guard LLINs had a more moderate effect on mosquito density and transmission in community trials, and why Olyset Plus LLINs did not last for more than 24 months in recent prequalify trials (151, 152).

To prequalified new LLIN products, WHO recommends different phases of evaluation including phase II laboratory wash resistance which is assumed to correlate with 36 months of community use (116). Furthermore, all the previous studies (wash resistance studies) were done on a small batch of nets while this study was carried out in the larger cRCT. The WHO could review guidelines for evaluation (phases I, II and III) and increase number of washes and recommend a more abrasive washing method to mimic 36 months LLIN in a field setting. Others have speculated that the quality of nets has reduced over the years (43). This could also be the case in this study; all previous phase II studies were conducted on a small batch of nets made specifically for the study's purpose, whereas here, a subsample taken from 45,000 nets distributed was used. Additional standard EHTs with unwashed and 20-wash nets could be conducted using nets purchased in bulk for distribution by the malaria control program to verify this assumption.

Resistance intensity monitoring demonstrated high resistance to permethrin in *An. funestus* during the first year, which diminished in the second and third years. In contrast, alpha-cypermethrin resistance intensity increased significantly over the trial years in *An. funestus*, which aligns with the resistance monitoring results from the main cRCT (113). 'The variation in insecticide resistance over time may also be due to changes in species composition with relative proportion of *An. arabiensis* increasing, as *An. gambiae* and *An. arabiensis* may exhibit different levels of resistance intensity, as observed in another area of Tanzania (70). *An.arabiensis* tends to feed on animal and rest either indoor or outdoor but this depend on the season and host availability. In this thesis high number of *An. arabiensis* were observed resting indoor during dry season assuming that they fed on other host and come to rest in the huts. Blood meal analysis could help to explain blood meal source for *An. arabiensis* but this was not done in this thesis.

Chlorfenapyr demonstrated high killing effect in all three years of resistance monitoring without evident selection to this A.I., while pyriproxyfen failed to induce significant sterility effect against *An. gambiae* s.l. and *An. funestus* complex throughout the same period. Pyriproxyfen resistance results is consistent with those observed in cRCT study site and could explain the relatively small effect observed in both cRCT and EHT presented (113). The molecular analysis for the detoxification enzymes revealed over-expression of genes (CYP6P3) in Interceptor, Interceptor G2 and Olyset Plus which strongly metabolize pyrethroid insecticide, as it was

observed in the study examining the functional genetic keys that confer resistance in African malaria vectors, *An. gambiae* s.l. (223-225).

One of the limitations of this study was the variation in species composition over time, which could explain why some of the nets did not perform as anticipated. Future studies may evaluate nets of different ages side-by-side to control for this heterogeneity across years. A second limitation is that a relatively small number of blood fed *Anopheles* alive at 72-hours were available for dissection and therefore the impact of Royal Guardon sterility might have been slightly underestimated. The third limitation is that, the study only used 30 nets sampled from five clusters per arm out of the 21 clusters, so they are probably not representative of the overall community in Misungwi.

Overall, the reduction in efficacy of Olyset Plus and Royal Guard LLINs observed in the EHTs seems to match the entomological and epidemiological findings in the cRCT conducted in Misungwi. Indeed, Olyset Plus provided superior protection against malaria and vectors outcomes compared to Interceptor up to 12 months only (151) while there were no significant differences for Royal Guard. The difference in performance between laboratory and community studies could be explained by the high resistance of *An. gambiae* s.l. and *An. funestus* s.l. to pyriproxyfen observed using WHO/CDC bottle assays (< 10% sterility effect) and by more stringent washing methods and abrasion during daily use. However, this was less clear for Interceptor G2. While in the current EHTs, Interceptor G2 outperformed Interceptor only up to 12 months, the cRCT outcomes reported significant reductions in malaria prevalence and vector densities in the Interceptor G2 arm compared to the standard LLIN at 24 months (114, 151) and even up to 36 months (152). Differences in species composition in the EHT and cRCTs area could explain the difference as there was a majority of *An. funestus* found in the cRCTs while *An. arabiensis* was the predominant species in the EHT when the 24- and 36-months nets were tested. In the cRCT, it was reported that *An. arabiensis* was not well controlled by any of the dual active ingredient LLINs, including Interceptor G2, due to its exophilic behaviors (220). Given that chlorfenapyr is a multifaceted insecticide, differences between cRCT and EHT outcomes may be explained by potential effects of chlorfenapyr on parasite development in mosquitoes (135, 226). This could explain the stronger and longer lasting effect observed in the community cRCT which may not be captured in EHT. What the result of the present EHT also highlighted is that the reduction in effect observed in the cRCT in Tanzania over the three years might not only be due to reduction in net usage but also to the sharp decrease in partner A.I. or PBO resulting in lower killing effect as the net aged.

Modelling of EHT data have been used to parameterize mechanistic models for malaria vectors and predict the epidemiological efficacy of LLINs (227). The use of EHT data which better correlates with cRCT outcomes as observed in the present study may improve the fit of these models and could be sufficient for the evaluation of second in class products.

4.5 Conclusion

Olyset Plus LLINs exhibited higher mortality rates, and Royal Guard LLINs demonstrated greater sterility effect compared to Interceptor LLINs, but only when newly introduced.. In contrast, Interceptor G2 LLINs exhibited superiority against *An. gambiae* s.l. and the *An. funestus* complex compared to Interceptor LLINs, but this advantage was observed only for up to 12 months. These findings align with the results of the cluster-randomized controlled trial (cRCT) in some ways but differ in others because the EHT findings suggest a much shorter period (up to 12 months) of efficacy for Interceptor G2 than found in the cRCT (36 months prevalence reduction). Further investigation is needed to explore additional effects, such as the effect of chlorfenapyr on parasite reduction, to fully comprehend its impact on malaria transmission. Additionally, conducting standard and adapted EHT in various contexts will help confirm the residual efficacy of the dual active ingredient LLINs and support the development of longer-lasting nets.

5 Chapter 5: Evaluation of bio-efficacy of field aged novel long-lasting insecticidal nets (PBO, chlorfenapyr or pyriproxyfen combined with pyrethroid) against *Anopheles gambiae* s.s in Tanzania

Martin, J., Messenger, L.A., Bernard, E., Kisamo, M., Hape, P., Sizya, O., Festo, E., Matiku, W., Marcel, V., Malya, E., Aziz, T., Matowo, S.N., Mosha, J.F., Manjurano, A., Mosha, F.W., Rowland, M. and Protopopoff, N. Evaluation of bio-efficacy of field aged novel long-lasting insecticidal nets (PBO, chlorfenapyr or pyriproxyfen combined with pyrethroid) against *Anopheles gambiae* s.s in Tanzania, 2024.

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Abstract

Next-generation of long-lasting insecticidal nets (LLINs) combining two insecticides or an insecticide with synergist are vital in combating malaria, especially in areas with pyrethroid-resistant mosquitoes where standard pyrethroid LLIN may be less effective. A WHO Phase III net durability study was conducted in Misungwi, Tanzania during a randomized controlled trial. This study assessed the bio-efficacy of three net brands combining a pyrethroid insecticide and either a synergist PBO for Olyset Plus, a second insecticide pyriproxyfen for Royal Guard, and chlorfenapyr for Interceptor G2 used in the community during three years in Tanzania. Those nets were compared to standard pyrethroid only net Interceptor. A total of 1950 nets were enrolled across 10 clusters in each treatment arm. A total of 30 nets per type were collected every 6 months up to 30 months, with 50 nets sampled at 36 months. WHO cone bioassays and tunnel tests were performed at 0, 12, 24, 30 and 36 months. Both susceptible *An. gambiae* s.s. Kisumu strain and resistant *An. gambiae* s.s. Muleba-Kis strain were exposed. Over 80% of the nets tested against the susceptible Kisumu strain met WHO criteria after 3 years of use in the community. In the tunnel test, mortality (at 72 hours) of the resistant *Anopheles* varied between 52% and 20% in Interceptor G2 and was higher than that of the standard Interceptor net of the same age up to 24 months. Olyset Plus mortality (at 24 hours) ranged between 84% and 33% in the tunnel, with superior efficacy compared to the standard net observed at 0, 24, and 36 months. Sterility effects in Royal Guard were significantly higher when these nets were new and at 6 months compared to Interceptor net but decreased to less than 10% after 12 months. Royal Guard consistently induced higher mortality compared to interceptor up to 30 months. Overall, next-generation LLINs demonstrated a higher effect on mortality compared to standard LLINs. However, the superior bio-efficacy did not last for 3 years and varied according to net brands.

5.1 Introduction

Vector control interventions have played a crucial role in reducing malaria, averting an estimated 2.1 billion cases (82%) and 11.7 million deaths (94%) in Sub-Saharan Africa (SSA) between 2000 and 2022, with long-lasting insecticidal nets (LLINs) being a major contributor (1). Standard LLIN has faced different challenges including wide spread of insecticide resistance which may have contributed to the increase of malaria case globally since 2015. In 2022, there were 5 million more cases reported in the sub-Saharan region, compared to 2021 (1).

In an area where mosquitoes develop resistance to pyrethroids, user protection are reduced as mosquitoes can penetrate through holes and successfully feed on human host hence increasing malaria transmission (2). To address this challenge, various insecticide nets (ITNs) treated either with two insecticides or with a pyrethroid insecticide and a synergist have been recommended by World Health Organisation (WHO) (1) as they show superior efficacy on malaria outcomes compared to standard LLIN (3-7). In the context of WHO Prequalification Insecticide treated bed nets (ITNs) are defined as mosquito nets treated with active ingredients (A.I.) aimed at repelling or killing Anopheles, thereby providing both personal and community protection (8).

Olyset Plus, a net combining PBO and the pyrethroid permethrin, outperformed standard Olyset net against multi-resistance wild *Anopheles* mosquitoes in experimental hut (9). Similar results were reported in Cameroon after using the F1 from the crossing between highly resistant *An. funestus* (FUMOS) and susceptible *An. funestus* colony (FANG) (10) and Burkina Faso with significant mortality in resistant malaria vector species compared to standard pyrethroid nets (10, 11) in experimental hut trials (EHT).

Royal Guard (containing the pyrethroid alpha-cypermethrin, and the juvenile growth hormone inhibitor pyriproxyfen) met the WHO criteria with 95% knockdown and more than 80% mortality for up to 20 washes against resistant *An. gambiae* s.s. (Kisumu strain) in cone assays (12). It also met the WHO criteria in tunnel tests, with mortality exceeding 80% after 20 washes using the same strain. They were also more effective against wild pyrethroid-resistant vectors (increase in sterility and mortality) in EHT (12, 13) in Benin.

Interceptor G2 (containing alpha-cypermethrin and the pyrrole chlorfenapyr) was able to induce 71% mortality against resistant free flying *An. gambiae* s.l. in an EHT compared to an alpha-cypermethrin-only net (20% mortality) (13). When Interceptor G2 was assessed in other studies using wash nets, the performance of dual-A.I. ITNs remained superior even after 20 washes compared to reference nets in experimental huts and laboratory settings (12-14).

In order to assess the effective life span of the net under routine use, insecticide bio-efficacy and physical durability of naturally aged nets need to be monitored in a community study setting conducted for a minimum of 3 years (8, 12). Standard pyrethroid-only nets have demonstrated 3-year long lasting efficacy in various studies; however, there is less evidence of the long-lasting bio-efficacy of the second partner insecticide or PBO when net aged. Bio-efficacy is measured by the ability of ITNs to induce mosquito mortality, knockdown, prevent blood feeding or sterility for some insecticide in laboratory bioassays using susceptible and resistant mosquito strains (15).

This study aims to assess the bio-efficacy against entomological outcomes of dual-A.I. ITNs and PBO-Pyr ITN compared to standard pyrethroid LLINs collected from the community over three years.

5.2 Methodology

Study design

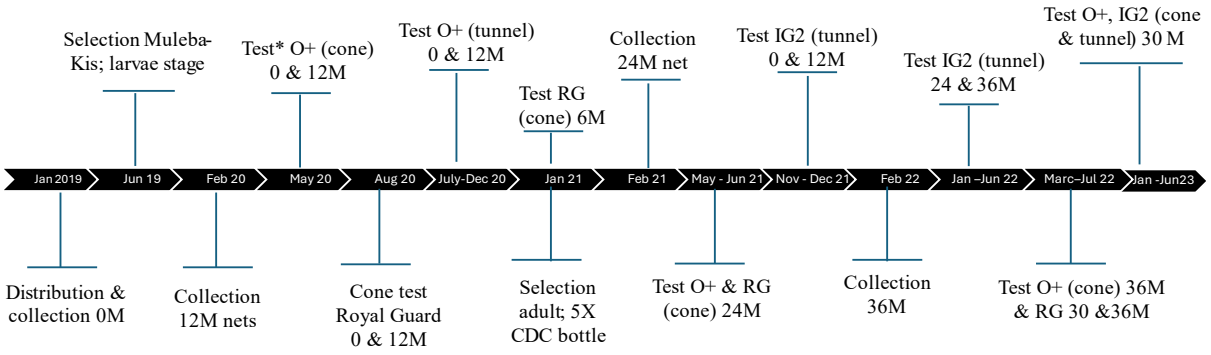
The laboratory cone and tunnel assays were performed using natural aged nets sampled from Misungwi community and new unwashed nets against susceptible Kisumu strain (*An. gambiae* s.s.) and resistant strain Muleba-Kis (*An. gambiae* s.s.) (16).

The following ITNs were assessed: 1/ Royal Guard (Disease Control Technologies, LLC, Greer, SC, USA), a net combining 225 mg/m2 pyriproxyfen (PPF), which is known to disrupt female mosquito reproduction and fertility of eggs, and 261 mg/m2 of pyrethroid alpha-cypermethrin; 2/ Interceptor G2 (BASF Corporation), a mixture ITN made of polyester incorporating two adulticides with different modes of action 200 mg/m2 chlorfenapyr and 100 mg/m2 alpha-cypermethrin. The chlorfenapyr disrupts insect ability to convert energy. 3/ Olyset Plus (Sumitomo Chemicals, Tokyo, Japan) is an ITN which incorporates a synergist, 400 mg/m2 piperonyl butoxide (PBO), to enhance potency of the partner pyrethroid insecticide and 800 mg/m2 of permethrin; 4/ Interceptor (BASF Corporation, Ansan, Republic of Korea) net, (positive control net) which contain alpha-cypermethrin at a target dose of 200 mg/m2 on polyester fabric. Untreated nets were purchased from local market and used as negative control.

Sample size and sampling of ITNs

In January 2019, over 147,000 ITN (Interceptor, Interceptor G2, Royal Guard or Olyset Plus) were distributed across 84 clusters separated in 4 arms in Misungwi district. The description of the Misungwi study area and net sampling is detailed elsewhere (16, 17). This study was conducted in 10 of the 21 clusters in each arm. For each arm 1950 ITNs were enrolled, and 30 nets sampled at 12, 18, 24, and 30 months, and 50 nets collected at 36 months (16). Testing

was done in priority for time points 0, 12, 24, and 36 months (Figure 5:1). Intermediate time points at 6, 18, and 30 months were tested if annual time point did not meet WHO threshold with ≥ 95% knockdown or ≥ 80% mortality after 24 hours in cone assays or tunnel tests ≥ 80% mortality or ≥ 90% inhibition of blood-feeding



* RG= Royal Guard, O-plus= Olyset Plus, IG2= Interceptor G2

The figure show time of net distribution, collection from the field and time test was conducted. For 0, 12 and 24 months total of 120 LLINs (30 from each arm) were sampled while at 36 months a total of 200 LLINs (50 from each arm) were collected

Figure 5:1: Sampling and testing frame of ITNs collected from the field.

The ITNs sampled (188) were prepared in accordance with WHO guidelines (118). Each net was given a unique identification number based on household and net numbers. For new unwashed nets, 3 adjacent pieces of 30 cm x 30 cm each were collected in each side of the nets which mean 15 pieces per net (118). At subsequent time point the bottom pieces of one of the sides was excluded due to potential abrasion (4 sides x 3 pieces). From the 3 adjacent pieces collected in each side, one underwent chemical analysis via high-performance liquid chromatography (HPLC) at Singapore accredited laboratory, another was tested against susceptible mosquitoes, and the third against resistant mosquitoes. Samples were labelled with household number, net type, net number, net position, time point, and preparation date, then stored in a refrigerator.

Testing was conducted at the Pan African Malaria Vector Research Consortium (PAMVERC) facility in the National Institute for Medical Research (NIMR), Mwanza, with susceptible *Anopheles* (*An.*) *gambiae* sensu stricto (s.s.) Kisumu strain and at the Kilimanjaro Christian Medical University College (KCMUCo) facility in Lower Moshi with the resistant strain *An.*

gambiae s.s. Muleba-Kis. The tests were conducted according to WHO LLIN testing guidelines 2013 (118).

Characterization of mosquitoes

The study employed both susceptible *An. gambiae* s.s. and resistant *An. gambiae* s.s. Muleba-Kis. The Muleba-Kis colony was established by mating F1 wild male *An. gambiae* mosquitoes with susceptible female *An. gambiae* s.s., followed by larval selection using pyrethroid (alpha-cypermethrin) insecticide at varying concentrations (228). This strain exhibited both target-site resistance mainly kdr-East L1014S and metabolic resistance i.e. mixed function oxidases-based resistance, serving as a model to assess the efficacy of the partner active ingredient (A.I.). The colony was regularly exposed to pyrethroid selection pressure, with monitoring of phenotypic and genotypic resistance to track changes in resistance frequency and intensity.

For the mosquitoes used to test Olyset Plus and Royal Guard dual-ITNs, selection was done once per generation at the larval stage, using 0.08 µg/ml of alpha-cypermethrin. To increase resistance as the difference in mortality between Interceptor G2 and standard pyrethroid LLIN with the Muleba-Kis selected at 0.08 µg/ml was not large enough, additional selection was done at the adult stage using a 5-fold diagnostic dose of alpha-cypermethrin in Centre for Disease Control (CDC) bottles. Throughout the study, all mosquitoes were maintained in a controlled environment with a temperature range of 25°C-29°C and relative humidity maintained between 60%-95%.

Table 5:1: Test plan for the active ingredients in study ITNs

Net type	**Active ingredient**	**Strain**	**Primary test method**	**Primary outcomes**
Interceptor G2	Chlorfenapyr	Muleba-Kis	Tunnel	72 hrs mortality and blood feeding inhibition
	Alpha-cypermethrin	Kisumu	Cone*	60 min Knock down (kd) and 24 hrs mortality
Royal Guard	Pyriproxyfen	Muleba-Kis	Cone*	Sterility assessed by dissection of ovary after 72hrs

	Alpha-cypermethrin	Kisumu	Cone*	60min Kd and 24 hrs mortality
Olyset Plus	Piperonyl-Butoxide	Muleba-Kis	Cone*	60min Kd and 24 hrs mortality
	Permethrin	Kisumu	Cone*	60min Kd and 24 hrs mortality

*Tunnel test was done if cone test did not meet the WHO threshold with ≥ 95% knockdown or ≥ 80% mortality after 24 hours, or tunnel tests were to have ≥ 80% mortality or ≥ 90% inhibition of blood-feeding

Testing procedures

For cone test, each 30 cm x 30 cm sample of netting was fixed to a cone frame positioned at a 45^{o} to 60^{o} angle during exposure (229). Mosquitoes (either susceptible or resistant) aged between 2-5 days were exposed for 3 minutes and then transferred into paper cups with access to 10% sugar solution. An untreated net was run in parallel during each test. The bioassays were carried out at 75±10% relative humidity (RH) and temperature of 27±2°C. The temperature was controlled using a room heater while a humidifier was employed to maintain humidity levels. Room conditions were recorded in the environmental condition chart three times a day (i.e morning, afternoon and evening). Knock-down (60 minutes) and mortality at 24, 48 and 72 hours was reported for all ITNs tested (table 5:1). The effect of pyriproxyfen was assessed by blood feeding mosquitoes before exposure to treatment. Egg development stage determined by ovarian dissection was reported at 72 hours for Royal Guard ITNs, positive control (standard Interceptor LLIN) and negative control (untreated net) (222).

If the mortality rate in the negative control was over 10%, the experiment was repeated. For each ITN sample, between 80 to 100 mosquitoes were exposed. The pyrethroid component was assessed for all the net samples (Royal Guard, Olyset Plus, Interceptor G2) using susceptible *An. gambiae* Kisumu strain. To assess the partner insecticide, cone tests were conducted for Royal Guard and Olyset Plus while tunnel tests were used for Interceptor G2 against the Muleba-Kis resistant colony (188). Of the three treatments, Interceptor G2 net was the only net tested directly in tunnel tests due to it's slow mode of action that need longer exposure (39).

Otherwise tunnel tests were carried out on the nets that did not meet the 80% mortality in the cone bioassays as per the WHO guideline (118). Two replicates on the net piece that yielded mortality closest to the mean mortality observed during the cone test was tested in the tunnel test (188). Nine holes of 1 cm diameter were cut in each net piece. The net was fitted in the

tunnel holding frame available for mosquitoes. A guinea pig was sedated using ketamine and xylazine and restrained in a cage at the shorter part of the tunnel (118) as a blood meal source for mosquitoes. A total of 50 non-blood fed mosquitoes aged 5-8 days were introduced at the opposite end of the tunnel to where the guinea pig was (release chamber). The experiment began at 18:00 and ended at 08:00 the following morning. Testing was conducted at 80±20% RH and 27±2°C. In the morning, the mosquitoes were collected using a mouth aspirator and placed in a separate paper cup per physiological status (i.e. blood fed, unfed) from each chamber, and supplied with 10% glucose. Blood feeding rate was recorded in the morning of mosquito collection and mortality was recorded after 24, 48 and 72 hours post exposure. If mortality in control was >10% or blood feeding <50%, then the test was repeated.

The colony mosquitoes for Royal Guard were blood fed on guinea pigs before exposure in cone assays and only successfully blood-fed (full engorged) were exposed. After exposure, mosquitoes were monitored for 72 hrs to allow egg maturation. During the normal gonotrophic cycle, after taking a blood meal, the mosquito's oocytes change in size and shape, and finally reach Stage V, which are a distinctive crescent shape (230). The effect of pyriproxyfen on reproductive outcome was assessed by dissecting gravid *An. gambiae* s.s. after exposure when eggs should normally have been fully matured (230).

Chemical analysis (additional information not published)

Chemical analysis was done by TÜV SÜD PSB Pte Ltd, Micro contamination Diagnosis, Singapore.Net pieces from each five position at 0 month (5*30*4=600 pieces) at the beginning of the trial (0 month) were analyzed to ensure that the target dose of the insecticide has been achieved. In subsequent follow up (at 12,24 and 36 months) only 4 pieces from 4 position (4*30*4=480pieces per time point) were analysed to facilitate interpretation of bioassay data. Secondary outcome was average chemical residue of each A.I per net at each time point of observation.

Resistance intensity assays for alpha-cypermethrin and permethrin

To monitor insecticide resistance heterogeneity in the colony of mosquitoes used for cone and tunnel tests, *An. gambiae* s.s. Muleba-Kis were subjected to different concentrations (1X, 2X and 5X) of alpha-cypermethrin and permethrin for 30 minutes in CDC bottle assays (table 5: S7 & S8). If the mortality rate at 5X was below 97%, further testing at 10X was conducted. Knock-down observations were documented at 15, 30 and 60 minutes. Following the 30-minute exposure, surviving *An. gambiae* s.s. from treated and control containers were transferred into paper cups with cotton wool soaked in a 10% glucose solution. The knock-down or dead *An.*

gambiae s.s. were also separated into cups with the same glucose solution in case of potential revival. Mortality/revival assessments were conducted at 24, 48, and 72 hours.

Data analysis

The bioassay data were recorded on standardized forms and double entered into an Access file to ensure accuracy. The analysis was done using Stata software version 18. Proportional knockdown (KD), 24-hour mortality, and blood feeding inhibition (BFI) were reported along with their respective 95% confidence intervals (CIs). Nets were classified as pass or fail based on WHO thresholds, requiring ≥ 95% knockdown or ≥ 80% mortality after 24 hours. For tunnel tests, the criteria were ≥ 80% mortality or ≥ 90% inhibition of blood feeding. Since there is no thresholds set up for dual A.I. LLIN against resistant *Anopheles*, at each time point we compared mortality (24 or 72 hours) or sterility (for Royal Guard) between standard pyrethroid LLIN Interceptor and the dual A.I. or synergist nets. The comparison of mortality from different treatments to the control was conducted using multilevel mixed effects generalized linear models, with test number as a random effect and net type as a fixed effect. Odds ratios and p-values were reported.

Resistance mortality was expressed as the proportion of dead mosquitoes across all exposure replicates of the total number of exposed mosquitoes. A parallel calculation was executed to derive the percentage of control mortality. The mortality of 5–20% observed in control assays was adjusted using Abbott's formula. Dose-response analysis involved the utilization of a log-probit statistical model to estimate lethal doses (LD50 and LD90) along with their corresponding 95% confidence intervals.

5.3 Results

Bio-efficacy results against susceptible strain

WHO criteria were only met for the cone test over all time points (with susceptible strain), but for the tunnel test - WHO criteria were not met from 12 months on in all 4 brands of net (see figure 5:2).

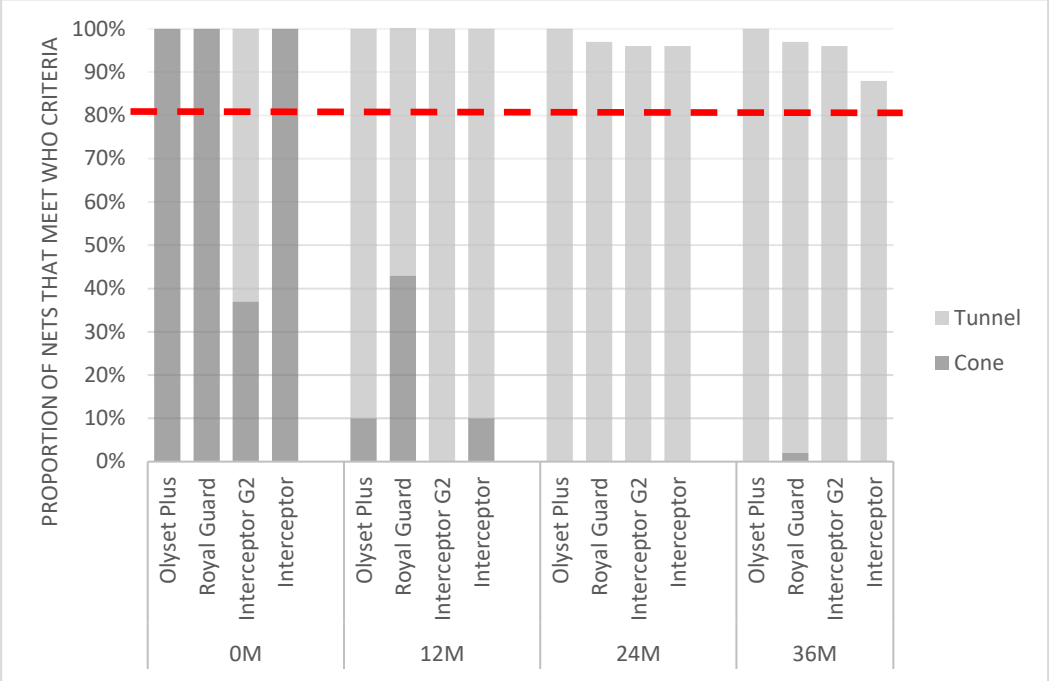


Figure 5:2. Proportion of net that passed WHO threshold, 24hrs post exposure in cone and tunnel tests against susceptible *An. gambiae* s.s Kisumu strain.

Bio-efficcay results against resistant *An. gambiae*.s.s. Muleba-Kis

When Olyset Plus was new, 24-hour mortality against resistant *An. gambiae* s.s. Muleba-Kis recorded in the cone was 67% (95% CI: 71 – 78) compared to 7% (95% CI: 6 – 11) for Interceptor nets (Figure 5:3A). At the subsequent time points most of the Olyset Plus nets had to be tested in the tunnel. In the tunnel tests, mortality was 84% when new, decreasing to 46% at 12 months, 44% at 24 months, and 33% at 36 months. Mortality was significantly higher in Olyset Plus compared to standard Interceptor nets, at 0, 24 and 36 months (figure 5:3B). In tunnel, mortality for Interceptor nets varied between 17% and 53% with no trend related to net aged. However, the highest mortality was reported at 12 and 30 months, which might explain the lack of difference between Olyset Plus and Interceptor at this time point.

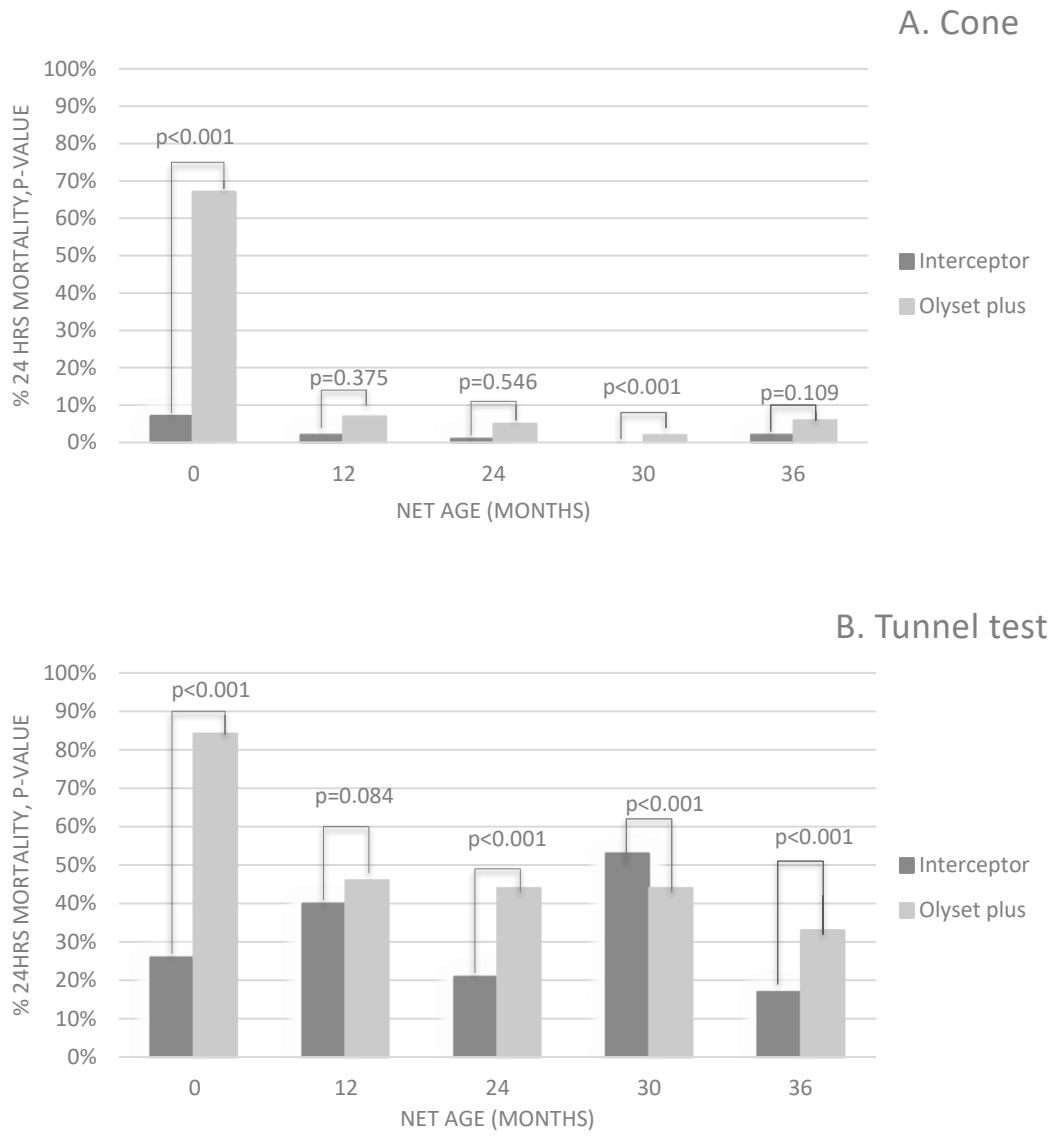


Figure 5:3 : 24-hours mortality recorded in Olyset Plus against *An. gambiae* s.s. resistance strain in cone (A) and tunnel tests (B).

In both cone and tunnel tests, the 24 hours mortality of resistant *An. gambiae* s.s. was significantly higher after exposure to Royal Guard compared to standard interceptor at all time points. In the cone tests, mortality was 83% (95% CI: 82 – 87) when Royal Guard was new and decreased to 20% (95% CI: 16 – 24) after 36 months (figure 5:4A). Mortality in tunnel was generally higher than in cone tests at the same time points (figure 5:4B. There was a small proportion of *Anopheles* surviving (8%) after 72-hour post-exposure period, (table 5:S9), resulting in a limited number of mosquitoes available for dissection at zero month (Table 5:2). The sterility effect in cone tests was 88% with new unwashed Royal Guard nets (figure 5:5), 46% at 6 months, and subsequently ranged from 5% to 2%. Given the fact that the sterility

effect did not increase with longer exposure in tunnels test up at to 24 months (<5%), tunnel tests were not performed at 36-month.

Table 5:2: Number of mosquitoes exposed and dissected per each time point

Dissection	Month	Total exposed	Total alive after 72hrs	Tot dissected	%fertile
Royal Guard	0	2992	210	210	12(4 - 19)
Interceptor		960	756	178	100
Untreated		1000	938	503	100
Royal Guard	6	2398	653	446	54(48-59)
Interceptor		799	670	394	100
Untreated		798	736	398	100
Royal Guard	12	2324	1297	678	96(94 - 98)
Interceptor		780	646	90	100
Untreated		760	727	376	100
Royal Guard	24	2422	1540	801	95 (93 - 97)
Interceptor		800	697	196	100
Untreated		601	547	299	100
Royal Guard	30	1607	602	340	78 (72 - 84)
Interceptor		474	424	216	100
Untreated		401	365	203	100
Royal Guard	36	3302	2239	1107	93 (91 - 95)
Interceptor		820	758	410	100
Untreated		820	790	411	100

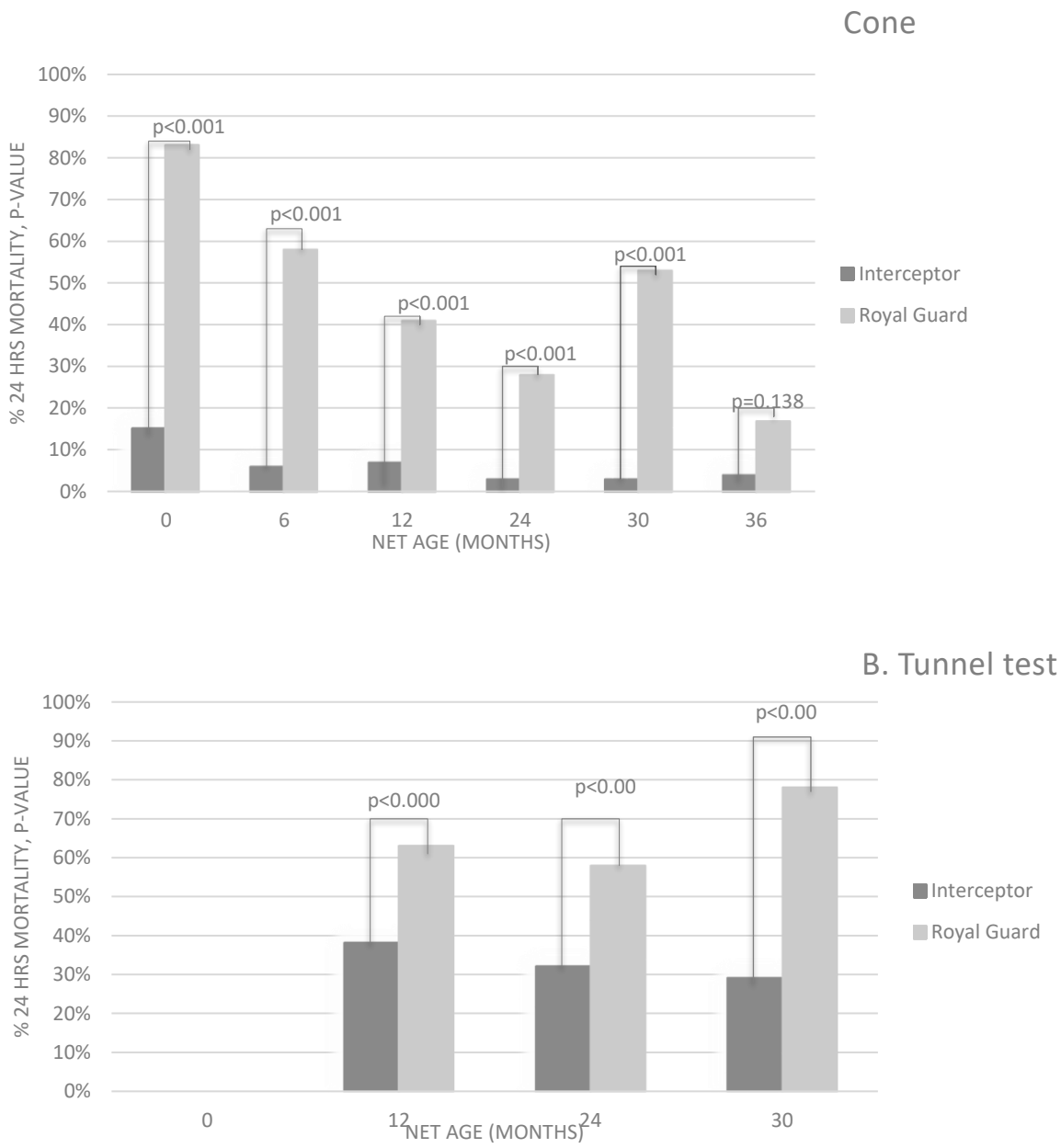


Figure 5:4: 24-hours mortality recorded in Royal Guard against resistance strain in cone (A) and tunnel tests (B).

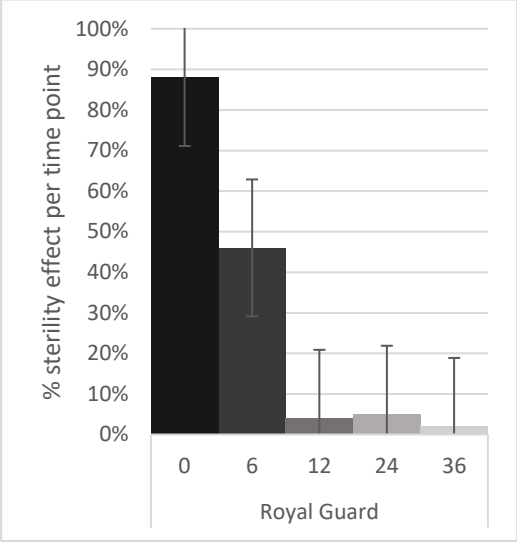


Figure 5:5: Sterility effect of PPF on egg development in cone tests

Mortality (72 hours) in the Muleba-Kis resistance strain exposed to Interceptor G2 ranged from 52% (95% CI: 45 – 58) when new to 20% (95%CI 17 - 24) at 36 months. Mortality was significantly higher in Interceptor G2 compared to standard LLIN up to 24 months time point, with the largest difference observed when the net was new (Figure 5:6).

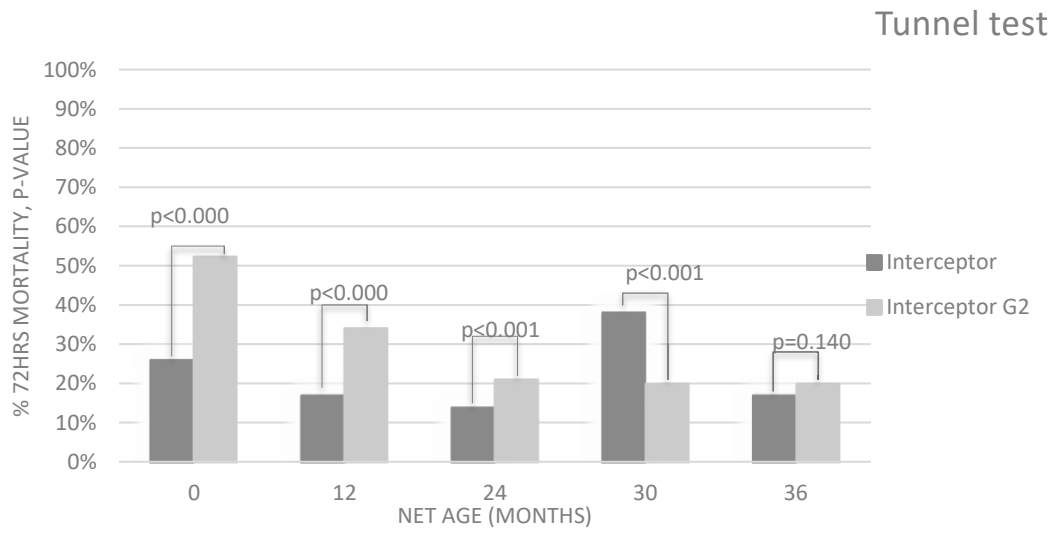


Figure 5:6: 72-hours mortality recorded in Interceptor G2 against resistance strain in tunnel test.

The blood feeding rate in resistant Muleba-Kis strain was 94% for untreated net exposed in tunnel tests. The highest blood feeding inhibition (BFI) was observed for Olyset Plus and Royal Guard net across all time points. This could be due to high content of pyrethroid available in these nets. BFI varied between 71% and 92% for Olyset Plus and between 76% and 87% in Royal Guard. Similar to mortality BFI was higher at 0, 24 and 36 months for Olyset Plus

compared to standard LLIN. For Royal Guard when tunnel tests were performed at 12, 24, and 30 months, BFI was also higher than standard LLIN, with the largest difference observed at 24 months. For Interceptor G2, BFI was 75% when new and decreased to 29% after 36 months, remaining similar to interceptor at all time point (figure 5:7).

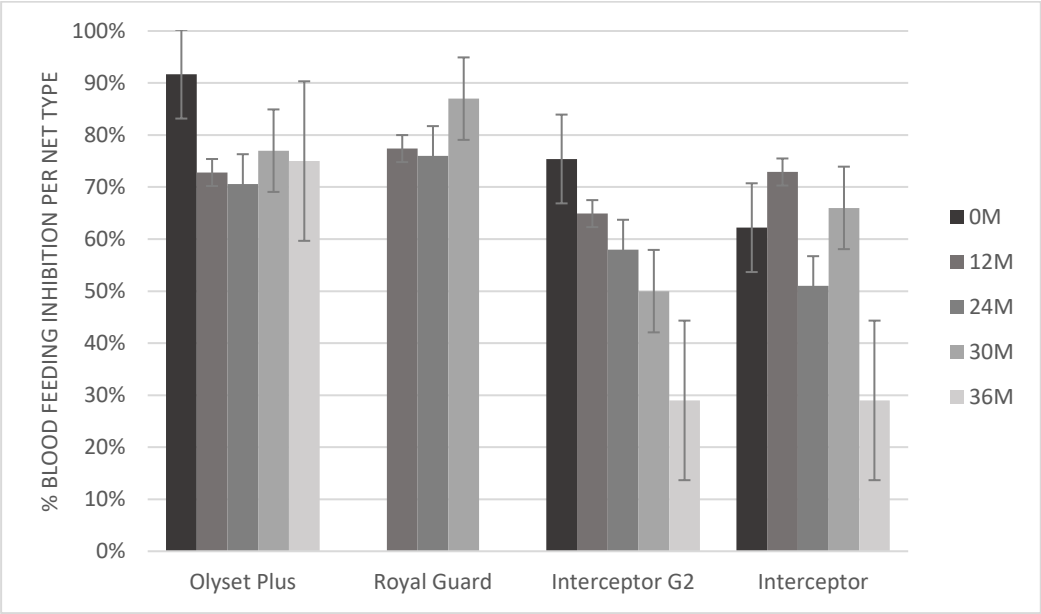


Figure 5:7: Blood feeding inhibition in the resistance strain induced by each net at different time points

Concentration of active ingredient (insecticide and PBO synergist).

All net met specification when new. Concentration of pyrethroid was reduced each year with 83%, 72%, 57%, 38% reduction off pyrethroid content for standard pyrethroid, Interceptor G2, Olyset Plus and Royal Guard respectively at 36month compared to initial concentration. For Interceptor G2 CFP decline by 92%%, PBO in Olyset Plus by 93% and PPF in Royal Guard by 72 (table 5:3)

Table 5:3: Shows the concentration of active ingredient (insecticide and PBO synergist) in the study nets when new and after 12, 24 and 36 months of use in the community.

Net type	Active ingredient	Concentration g/kg				
		Specification g/kg (+/-25%)	New net mean (Sd)	12 months mean (Sd)	24 months mean (Sd)	36 months mean (Sd)
Standard Pyr ITN	**α**-cypermethrin	5.0 (3.75-6.25)	4.7 (0.4)	2.3 (1.3)	1.6 (1.2)	0.8 (0.8)
Pyriproxyfen ITN	**α**-cypermethrin	5.5 (4.125-6.875)	5.3 (0.2)	4.3 (0.8)	3.0 (1.5)	3.3 (1.0)
	Pyriproxyfen	5.5 (4.125-6.875)	5.4 (0.2)	2.3 (0.8)	1.5 (0.7)	1.5 (0.8)
	α-cypermethrin	2.4 (1.8-3.0)	2.5 (0.3)	1.3 (0.5)	0.7 (0.4)	0.7 (0.4)

Chlorfenapyr ITN	Chlorfenapyr	4.8 (3.6-6.0)	5.0 (0.6)	1.4 (1.1)	0.9 (0.7)	0.4 (0.6)
PBO ITN	permethrin	20 (15-25)	19.4 (0.4)	12.9 (3.2)	10.5 (3.3)	8.3 (2.8)
	PBO	10 (7.5-12.5)	9.6 (0.3)	4.0 (1.4)	2.9 (1.7)	0.7 (0.8)

Insecticide resistance intensity

In the first year, concentration (LD50) of permethrin needed to kill 50% of Muleba-Kis strain (mosquitoes strain selected at larvae stage using 0.08 µg/ml of alpha-cypermethrin) was 31.1 µg/ml (95% CI [22.4–39.7]). By the third year, the LD50 was 96.2 µg/ml (95% CI [86.9–105.9]), indicating an increase in insecticide resistance in the colony (see Table 5: S10). For alpha-cypermethrin, the LD50 was 12.4 µg/ml (95% CI [9.7–15.1]) during the first year, decreasing to 5.9 µg/ml (95% CI [4.9–6.9]) in the third year and 5.6 µg/ml (95% CI [3.6–7.5]) in the fourth year. Mosquitoes from the first and second year were used to test 0, 12 and 24 months Olyset plus and Royal Guard, while mosquitoes from the third and fourth years were used for testing 30- and 36-months nets. The decrease in alphacypermethrin resistance over time might explain the increase in mosquitos' mortality exposed to interceptor. This coupled with the increase in permethrin resistance, could explain why the difference in mortality between interceptor and Olyset plus was not as pronounced toward the end of the testing in years 3 and 4.

Mosquitoes selected at the adult stage with 5 times diagnostic dose of alpha-cypermethrin showed no mortality after exposure to a diagnostic dose (12.5 µg /ml) of alpha-cypermethrin during the first year of testing. They were not tested further for resistance in year 3 to 4, so variation in resistance during these years is not known.

5.4 Discussion

The bio-efficacy of Interceptor G2, Royal Guard, and Olyset Plus was compared to a standard pyrethroid LLIN (Interceptor) over three years under community use conditions. Overall, the killing effects of these nets were superiors to the standard pyrethroid LLIN against resistant vectors over time. Olyset Plus demonstrated higher mortality than the standard LLIN when new, and at 24 and 36 months in tunnel tests. Royal Guard also consistently showed higher mortality and delayed mortality (72 hours) but had limited sterility effects only for the first 6 months compared to the standard LLIN. Interceptor G2 was superior to Interceptor up to 2 years.

All nets met the WHO bio-efficacy criteria in cone or tunnel tests against the susceptible colony strain. They achieved WHO criteria primarily through blood feeding inhibition in tunnel tests against the susceptible An. *gambiae* s.s. rather than mortality or knockdown. In this study, the

bio-efficacy of dual AI ITNs against susceptible mosquitoes was sustained for 3 years, meeting WHO expectations.

In the present study, the stronger and longer lasting impact observed with Royal Guard was on mortality rather than sterility. Royal Guard induced mortality was significantly higher than that of the standard Interceptor net over time. These findings contradict laboratory and experimental hut data from studies conducted in Benin (130) and Cameroon (153) which did not report a difference in mortality between Royal Guard and standard LLINs. In Benin, mortality in cone tests were very high for both nets (over 80%). In EHT in Benin and for the study in Cameroon mortality were around 20% for both nets (depending on species and study area). Interestingly, in the Benin study (130), one of the treatment was an ITN with only pyriproxyfen, which induced some mortality (ranging from 47% when new to 28% after 20 washes). The impact of PPF on mosquito longevity has also reported elsewhere (231). This could explain the superior killing effect observed in the present study if there was some additive effect of the pyrethroid and pyriproxyfen. Differences between the studies could be attributed to insecticide resistance and the fact that the reference net (Interceptor) in the present study uses a different treatment technology than Royal Guard, which could result in different insecticide surface concentrations and thus varying killing effects. Another notable difference with other studies was the impact of Royal Guard on sterility. In the present study, a significant sterility effect (88%) was observed in surviving mosquitoes 72 hours after exposition to Royal Guard in cone assays, and this effect were reduced by half after 6 months. In Benin, a reduction in offspring was observed with new and after 20 washes Royal Guard compared to the control net (130). Another study with a different brand reports a longer lasting sterility effect (131). These discrepancies could be explained by the washing methods of the nets, as nets in the laboratory are not exposed to the abrasive washing conditions found in community settings.

Olyset Plus outperformed the control (standard Interceptor net) when new, and then again at 24 and 36 months of use. In another Phase III durability study conducted in Uganda, the superior bio-efficacy of field Olyset Plus nets compared to standard LLIN was reported for up to 2 years (115). Similarly, a study in Kenya, assessing the bio-efficacy and durability of PBO-based nets found that Olyset Plus nets performed better or close to WHO critical threshold for about two years in the field (145).

Interceptor G2 demonstrated superiority over Interceptor net up to 24 months. At 30 months, Interceptor seemed to perform better than Interceptor G2, which could be explained by the reduced alpha-cypermethrin resistance intensity in the colony strain observed when those net were tested. After 36 months, no statistical difference was observed between Interceptor G2 and

Interceptor. The decreased bio-efficacy and chlorfenapyr content in Interceptor® G2 ITNs may partially explain the reduction in its enhanced personal protection effect compared to the Interceptor® ITN observed in the third years of the laboratory study. Similar findings were reported in Benin(201). Studies conducted in Cameroon (22), Tanzania (141), and Benin (140) using washed nets usually reported higher mortality than the present study, with Interceptor G2 showing superior bio-efficacy even after 20 washes. The difference in mortality observed could be due to varying level of resistance intensity and the colony species strain used in these countries. In the present study, it was particularly challenging to select a colony of *An. gambiae* s.s. that was resistant enough to see a difference in mortality between Interceptor G2 and the reference standard LLIN, and secondly to maintain the resistance intensity constant over the 3 years of testing. Standard operating procedures (SOPs) (232, 233) have been developed for guidance, but more experiments are necessary to determine the best methods to reduce the testing period and variation in mosquito strains use in this testing.

In the present laboratory study, Royal Guard and Olyset Plus outperformed the standard LLIN in terms of entomological bio-efficacy in tunnel, maintaining efficacy the longest, while the superior efficacy of Interceptor G2 ended after 2 years. Interestingly, in terms of impact on malaria in the associated community cRCT, Interceptor G2 was the most effective against epidemiological and entomological outcomes for 3 years. Olyset Plus outperformed the standard LLIN for one year, and Royal Guard did not show a superior effect in the cRCT (151).

Ideally, supplementary evidence on the entomological mode of action of the partner active ingredient is needed to provide greater insight on the class of net's capacity to control malaria transmission. For Interceptor G2, there may be growing evidence that, in addition to killing vectors, the chlorfenapyr component also affects *Plasmodium* inside the vector (135, 226). For Olyset Plus, there may be a residual repellence effect at low concentration, evident in the tunnel tests but not in the community where PBO net usage was low due to quality issues (234). With Royal Guard, the net is designed to control the F1 larval population, which may also be regulated by density dependent competition for planktonic food resources which undermines control.

Malaria transmission models that rely only on experimental hut trials may not be sufficient to parameterise the full transmission model and may need supplementary entomological laboratory outcomes to provide insight into what information may be missing. There is a continuing need for cRCT trials to provide definitive epidemiological evidence on the level of effect and mode of entomological action.

5.5 Conclusion

All nets tested met the WHO criteria for up to 3 years against a susceptible strain of *Anopheles*. Overall, next-generation ITNs demonstrated a higher effect on mortality compared to standard LLINs; however, the superior efficacy did not last for 3 years and varied according to net brands. Our results provide the first evidence on the bio-efficacy of Interceptor G2 and Royal Guard after use in the community, and these nets could be used as reference for testing other product within the same classes.

6 Chapter 8: General discussion

Malaria is a deadly disease transmitted by female *Anopheles* mosquitoes. Different interventions to combat its spread, including ITNs, IRS, proper diagnostics and artemisinin-based combination therapy (ACT) has been put in place for community protection in Africa endemic areas. These measures significantly reduced malaria incidence, with ITNs and IRS alone preventing 78% of cases between 2000 and 2015 (57). However, progress has stalled due to widespread insecticide resistance, suboptimal coverage and use, financial constraints, net durability issues, and challenges with community acceptance; as a result, an increase of 5 million cases was reported in 2022 compared to 2021 (25). We are now at a critical moment, where we urgently need new vector control tools to tackle insecticide resistance effectively. With the availability of malaria vaccines (RTS, S and R21), new vector control tools could put malaria control back on track. However, this requires increased financial support, development and assessment of innovative tools, and improved bed net quality and performance to enhance protection and reduce the malaria burden.

To support efforts toward malaria control and elimination, particularly in combating insecticide resistance, manufacturers have developed several novel ITNs with different modes of action. Between 2004 and 2022, approximately 2.9 billion bed nets were distributed worldwide, with 86% of these being deployed in Sub-Saharan Africa (25). In 2022 alone 282 million ITNs were delivered worldwide in malaria endemic countries with 92% delivered in sub-Saharan regions. Among these, 51% were PBO-Pyr ITNs and 8% were dual A.I. ITNs (25).

This thesis focuses on assessing the efficacy and durability of dual-A.I. ITNs, deployed in the community in Tanzania, in the laboratory and experimental huts against various entomological indicators.

Overview of the key findings per net type and overall interpretation

The functional survival of Interceptor G2 was 1.9 years in our setting. Maximum impact of Interceptor G2 on delayed mortality (72 hours) was 52% when new. Mortality was superior to the standard pyrethroid net up to 24 months (21% vs 14%) when tested in the laboratory against resistant colony vectors. Against free flying *An. gambiae* s.l. and *An. funestus* in experimental huts, mortality was 58% at 0 months and was superior to standard LLIN up to 1 year (45% vs 19.5%). After 3-year pyrethroid content was reduced to 28% of the initial content and 7% for chlorfenapyr.

The functional survival of Olyset Plus was 0.9 years after 36 months of use in the community. The majority (90%) of ITNs were discarded at 36 months due to excessive wear and tears. The efficacy of Olyset Plus was observed when these ITNs were new (mortality 67% vs 7%) and then at 24 and 36 months of used compared to pyrethroid only net in cone assays. In experimental huts mortality was only significantly higher against free flying mosquitoes at 0 month with 39% mortality for Olyset plus vs 9% in standard LLIN. The concentration of pyrethroid was reduced to 43% of initial content while, the remaining concentration of PBO were 7% at 36-months.

For Royal Guard, the functional survival was 1.9 years. Thirty-three per cent of mosquitoes collected in experimental hut were sterile when Royal Guard was new and 25% at 12 months. In contrast, 88% sterility was observed in cone assays when these nets were new and reduced to 46% at 6 months. Royal Guard seems to have more pyrethroid content remaining (62%) than other net brands while 28% of pyriproxyfen remained after 36 months. The most important impact of Royal Guard was on mortality rather than sterility. In cone tests, Royal Guard had a higher killing effect than standard LLIN up to 30 months in contrast to experimental hut where it performed better up to 12 months.

All the objectives of the thesis were achieved. In summary, all nets had functional survival below the WHO recommended threshold of 3 years durability (**objective 1**). This thesis highlights poor fabric integrity and user behaviour as contributing factors, with nets being discarded due to perceived lack of protection as holes accumulate. In general, the bio-efficacy results in the laboratory (**objective 4**) and experimental huts (**objective 2**) were lower than those reported in studies examining wash resistance with 20 washes. Superiority against resistant *Anopheles* (both colony and free-flying) was not observed for the full 3-year period. The monitoring of insecticide resistance (**objective 3**) in free-flying and colony mosquitoes was not always consistent but still showed variation in resistance over the period of the project. This variation could explain some of the differences in bio-efficacy observed over the testing years between the dual A.I. net/PBO net and the standard net. This thesis also highlights discrepancies between net performance in laboratory and semi-field conditions versus community settings, emphasizing the need for more robust evaluation methodologies that better reflect field conditions. Moreover, the study suggests potential revisions to WHO evaluation guidelines to better simulate real-world usage and improve predictive accuracy.

Study successes

Standard Phase III durability and entomological efficacy study of ITNs are usually done in a small scale, in 1-3 villages with 250 ITNs per treatment (190). The evaluation of ITNs can be

done either through retrospective cross-sectional surveys or prospective longitudinal studies. These studies assess and monitor insecticidal activity, fabric integrity and survivorship when used in field conditions. Often these villages have been involved in several studies over the year (191) and the number of nets distributed are coming from small batches produced specifically for the study. Our study was nested within a cRCT which comes with several benefits but also some limitations. One of the first advantages is the fact the nets followed for our study were selected from 40,000 nets per arm distributed for the trials, giving a more realistic idea of the performance and quality of the nets when produced at scale. Secondly, the cohort of nets were taken from a larger number of villages, for durability (5 cluster/village per net type) and for bio-efficacy (10 clusters/villages), providing more realistic data on their performance. The results are therefore more representative of the target population and diverse community.

During the project, between 2020 and 2023, a total of 178,557 *Anopheles* mosquitoes (both Kisumu and resistant strains) were exposed to complete the bio-efficacy testing. This was a significant achievement in terms of logistics and capacity, demonstrating KCMUCO and NIMR ability to handle large-scale testing and data collection. Coordinating and managing such a high volume of mosquito exposures required meticulous planning, resource allocation, and collaboration among the project teams. In addition, a total of 12 EHTs were conducted between 2020 and 2022, resulting in 2,588 collection nights and 17,040 *Anopheles* mosquitoes collected. Due to the relatively low density of *Anopheles* in the EHTs, we conducted four EHT studies for each time point. This approach was challenging but led to a more robust dataset due to the replicates. It also allowed us to observe the impact of each type of net on various vectors, *An. gambiae* s.s., *An. arabiensis* and *An. funestus*, which have different seasonal pattern.

Challenges, limitations and way forward

There were however some constraints, combining a durability study with a cRCT increases the complexity of the study design, and workload for the researchers and implementation personnels. Priority was also given initially to the cRCTs and field collections which led to a delay in starting the bioassays on the nets in the laboratory and experimental hut trial. While it also increased cost and logistics of the project, there was some cost saving by training the cRCTs temporary personnel and using materials (GPS, Smart phone, car) for various field activities.

An important challenge the study faced in the field was bed bug infestation. A significant number of bed bugs were found on nets especially the part tucked under the bed, leading households to eradicate them by hanging the nets in sunlight and soaking them in detergent overnight. These methods, however, may reduce the ITNs' efficacy and durability. Bed bug was also an important contributing factor reported by the community for not using the nets

(publication in preparation). For ITNs used in bio-efficacy testing; to prevent bed bug spread, all nets were stored in a freezer at -20°C for three days before experimental hut rotations.

Another important challenge during the project was the change in insecticide resistance in both the laboratory-reared colony and the wild *Anopheles* for EHT. First, we had difficulty with the initial selection and maintenance of resistance in the colony mosquitoes over the three years of testing. For the mosquitoes used to test Olyset Plus and Royal Guard dual-ITNs, selection was done once per generation at the larval stage, using 0.08 µg/ml of alpha-cypermethrin. For Interceptor G2, the same mosquitoes (0.08 µg/ml selected) were re-selected at the adult stage using a 5-fold diagnostic dose of alpha-cypermethrin in CDC bottles to increase resistance intensity in the Muleba-Kis *An. gambiae* s.s. colony. There was some variation in resistance intensity over time, which might explain the variation in mortality in the standard pyrethroid LLIN. This led to smaller differences in mortality observed between the intervention of ITNs and the standard pyrethroid LLIN when mortality in the latter was high. This was observed for Olyset Plus tested at 12 and 30 months and for Interceptor G2 at 30 months, where the absence of difference could be explained by a higher mortality of Muleba-Kis exposed to Interceptor at this time point. The EHT studies spanned over a 3-year period and there were changes in the species composition and resistance intensity over time that could also have influenced the results at some point of time. During the analysis the timing (rotation round number) of the EHT was accounted for to reduce the impact of this variation in the study. Testing was consistent over time, but there were some changes to the initial protocol based on results or availability of mosquitoes. One was for Royal Guard ITNs where intermediate time point (6 months) was tested for sterility outcome. Priority was given to cone assays when results suggested that longer term exposure to tunnel did not increase the sterility, therefore tunnel test for 36 months net pieces were not conducted. During the EHT, adult mosquitoes were collected in the neighbouring houses for resistance monitoring. Due to low precipitation in some seasons, we failed to collect enough mosquitoes to perform testing and consistently monitor the trend in resistance for some species.

When the study was designed in 2018, there were no detailed guidelines and SOPs on how to evaluate the efficacy of the various new in-class dual A.I. ITNs. The available guideline was for monitoring efficacy of pyrethroid with some suggestion on how to test new dual A.I. ITNs or PBO-Pyr ITNs. For slow acting insecticide with different mode of action, the guideline recommend additional of epidemiological data for proof of principle (118). For example, Interceptor G2, manufactured by BASF, incorporates chlorfenapyr, which is classified under group 13 in the Insecticide Resistance Action Committee (IRAC) Mode of Action (MoA) (39). This means it is a slow acting chemistry that acts as an uncoupler of oxidative phosphorylation

by disrupting the proton gradient. This disruption impedes mitochondrial respiration through the inner mitochondrial membranes of insect cells, preventing ATP synthesis and depriving insects of energy, ultimately causing their death (40). Novel slow-acting chemistries may not meet the benchmark standards set for conventional pyrethroid ITN exposures, but they can be highly effective when tested according to their specific modes of action. Given its unique mode of action, this thesis employed modified testing methods using tunnel tests for chlorfenapyr, as previous studies indicated that a 3-minute cone assay exposure did not produce significant mortality in resistant mosquitoes (136). Royal Guard, manufactured by Disease Control Technology LLC, incorporates a mixture of alpha-cypermethrin and pyriproxyfen. Assessment of the sterility impact was usually done by exposing blood fed *Anopheles* to the PPF-Pyr ITN and allowing them to lay eggs, monitor hatching rate and counting first larvae instar (235). The procedure is labour-intensive and time consuming (236). In addition, only a small proportion of the blood-fed females laid eggs in colony conditions, even for those exposed to an untreated net (236). This challenge is even more significant for blood-fed wild mosquitoes. In this thesis, the effect of pyriproxyfen was assessed through dissection, as it is an alternative method for evaluating sterility in *Anopheles* mosquitoes (222). Indeed, the development of eggs to stage 5 is an indication of the fertility of the *Anopheles* and does not depend on the oviposition conditions necessary for *Anopheles* to lay eggs. Others (130, 147) have evaluated the efficacy of pyriproxyfen on fertility by leaving blood fed exposed mosquitoes to lay eggs naturally (oviposition) and monitoring the eggs hatching rate and surviving rate (table 6:1). The choice of method depends on the available human resources, the number of mosquitoes to be exposed, and the mosquito species. The study conducted in Benin looking at the suitable method to evaluate sterility effect of Royal Guard reported that, the specificity and sensitivity of ovary dissection was higher compared to oviposition (237). Additionally, *An. funestus* faces challenges in laying eggs under insectary conditions, making oviposition difficult (238).

Comparisons between testing methods

Later WHO guideline (150), WHO Prequalification of Vector Control Products and other initiatives such as Innovation To Impact (I2I), have developed adapted protocol and SOPs (239, 240) for the testing of new ITNs classes which included some of the indicators we have used for our study. Recently, we partnered with I2I and a group of experts on a project to develop consensus SOPs for evaluating first-in-class ITNs, with the goal of establishing uniform evaluation criteria. SOPs were gathered from 13 stakeholders, including the Pan African Malaria Vector Research Consortium (PAMVERC). This evidence was consolidated, resulting in the formulation of a single SOP to standardize testing methods for new dual A.I. ITNs (233).

Summary of the differences between our study, WHO guidelines and the consensus SOPs is presented in table 6:1 below. In the consensus SOP, it was agreed to assess all pyrethroid-PBO ITNs using WHO standard guideline (118). For ITNs that fail the cone test (KD60 < 95% or 24-hour mortality < 80%), a tunnel test should be conducted. All other parameters, such as exposure time, the number of mosquitoes per cone, and the age of mosquitoes, remain consistent with WHO guidelines (118). However, instead of cutting one piece from the roof and three from the sides, the new SOP suggests taking two samples from the roof and two from the side panels. This adjustment accounts for potential variation in manufacturing batches between the roof and side panels. The number of replicates were reduced to two replicates and not four as previously stated in WHO guideline (118), resulting in a total of 8 replicates per net, with a 24-hour mortality endpoint. The SOP does not set a cutoff point or threshold for a net to pass or fail, as it depends on the *Anopheles* species used for testing and the intensity of resistance. The SOPs, report the important to store net samples in a refrigerator or a cool, dry place with a temperature below 5°C. A minimum sample of 30 ITNs per treatment as per WHO guideline was recommended (233).

For the PPF-Pyr ITN, all parameters remain consistent with WHO guidelines (118) except for the mosquito age, which is adjusted to 3-5 days instead of 2-5 days. This age range allows extra days for mating, increasing the likelihood of insemination. Additionally, the suggested blood-feeding time ranges from 3 to 9 hours before exposing mosquitoes to the insecticide, depending on the source of the blood meal. The suggested outcomes (endpoints) include oviposition inhibition and egg laying for sites assessing the effects of PPF through oviposition and fertility inhibition, egg development stage, and fertility rate for sites conducting dissections. All sites should also record KD, 24-hour, and 72-hour mortality. The SOP advises not to include pyrethroid results in the reporting since pyrethroids are not expected to induce any sterility effects. An untreated net is suggested for control/comparison (233). Setting thresholds for the sterility effect of Royal Guard ITNs was not achieved because the operational data on how these ITNs performed were yet to be generated. This gap needs to be addressed to maintain uniformity in evaluating the PPF component of Royal Guards ITNs (233).

For CFP-Pyr, it was agreed to use tunnel test and whenever tunnel seem not to work, other available methods such as I-ACT could be deployed. Similar to the experimental hut, the I-ACT assay uses whole nets and human hosts to assess the bio-efficacy of field-used ITNs. However, it is conducted under controlled conditions with laboratory-reared mosquitoes. In this setup, mosquitoes are released into net chambers where a volunteer sleeps beneath the test net, and all mosquitoes are recaptured the following morning. However, subsequent reports indicated that the I-ACT method yielded similar results to the tunnel test (241). The number of net pieces to

be tested was increased from one, as stated by WHO (118), to two, justifying that the roof part should be tested since studies report more mosquito activity occurs on the roof part than the side part (241, 242). The SOP suggests that CFP-Pyr nets should be tested at night (in darkness) when mosquitoes' circadian rhythm is at its peak (233).

Table 6:1: Comparison of testing method in cone and tunnel test for dual A.I. nets in WHO guidelines (118) Consensus SOPs (233) and the methods of my PhD study.

	Comparison of testing plan			
	Parameters	**WHO Guideline 2013**	**PhD Study**	**Consensus SOP**
Cone test	Net pieces to sample per net	5 pieces (t0) and 4 (t6 to t36): 1 piece per net side and 1 roof part	Same*	4 pieces (2 from roof part and 2 side part)
	Time exposure	3 minutes	Same*	Same*
	Species/strain	Susceptible to test pyrethroid component and resistant strain for partner insecticide	Same*	Same*
	Number mosquito exposed	5 mosquito per replicate	Same*	Same*
	Mosquito age	2-5 days	Same*	2-5 days PBO-Pyr 3-5 PPF-Pyr
	Total mosquito	50 mosquito/piece	40 mosquitoes/piece	40 mosquitoes/net
	Blood feeding status before exposure	Unfed	Unfed for all type of net Fed 1 hours before (for PPF assessment in net)	3-9 hrs before exposure to the net (only for PPF-Pyr)
	Number replicates	4 replicates per piece (20 to 16 replicates per net)	Same*	2 replicates per piece (8 replicate/ net)
	Time net collection	0, 6, 12, 18, 24, 30 and 36 months	Same*	N/A

	Test priority	0, 12, 24 and 36 months	0, 12, 24 and 36 months	N/A
	Method to assess effect of PPF	No guidance	Dissection for sterility effect	Oviposition and dissection
	Outcome measured	60 min KD 24hrs Mortality	Mortality (KD: 60 min, 24 & 72hrs), sterility (Egg development stage) for PPF-Pyr nets	Mortality (KD: 60 min. 24 & 72hrs), Oviposition inhibition, Fertility inhibition & Egg development stage.
Tunnel test	Net pieces to sample per net	1 piece per net	Same*	CFP-Pyr 2 pieces (1 roof and 1 side).
	Time exposure	Overnight (12-15 h)	Overnight	12-15 hrs
	Number mosquito exposed	100 mosquito/net	50 mosquito per replicate	50 mosquito per replicate
	Mosquito age	5 - 8 days	Same*	Same*
	Total mosquito	100 mosquito per net	Same *	100 per net for CFP-Pyr 200 nets for other net
	Number replicates	1 replicate	2 replicates per piece	1 replicate per piece
	Time net collection	0, 6, 12, 18, 24, 30 and 36 months	Same*	N/A
	Test priority	For the net which fail cone and those with excito-repellent; 20 washed or more	0, 12, 24 and 36 months	N/A

	Outcome measured	Mortality (Immediate mort, 24hrs, 48 hrs and 72hrs) and blood feeding inhibition	Mortality (Immediate mort, 24hrs, 48 hrs and 72hrs), blood feeding inhibition and Sterility (egg development) for PPF-Pyr	Mortality (Immediate mort, 24hrs, 48 hrs and 72hrs) and blood feeding inhibition, Oviposition inhibition Fertility inhibition Egg development stage.
EHT	Total net per treatment	minimum 30 per treatment except 36 month 50 ITN tested	Same*	N/A
	Time net collection	0 and 20 wash	0, 6, 12, 18, 24, 30 and 36 months	
	Rotation priority	N/A	1, 12, 24 and 36 months	
	Rotation	Random Latin square design, 36 nights (depend on number of net); 2 rotation/treatment if few mosquitoes collected	Random Latin square design, 36 nights; 4 rotation/treatment/timepoint	
	Outcome measured	Mortality (Immediate mort and 24hrs), blood feeding inhibition, Deterrence and Exophily	Mortality (Immediate mort, 24hrs, 48 hrs and 72hrs), blood feeding inhibition, sterility (egg development for PPF-Pyr nets), Deterrence and Exophily,	

*Same as WHO guideline 2013; PBO-Pyr= Olyset Plus; CFP-Pyr= Interceptor G2; PPF-Pyr= Royal Guard

The work in this thesis was instrumental in developing adapted SOPs for the evaluation of next-generation nets. In the context of PQ, the updated version of guideline (243) aim to maintain data consistence and improved policy outcomes. Additionally, it emphasize aligning data

requirements with the intended use and duration of effect of the product rather than simply meeting guideline criteria (243).

It is important to note that this thesis does not compare the non-inferiority between new candidate dual A.I. net and the first in-class ITNs, as the new modified guideline for evaluating and reporting the primary outcome became available only by the end of this study (243) and the thesis establishes the efficacy of the first-in-class intervention. According to the later guideline, all new second-in-class ITNs should ideally be compared to the first-in-class comparator that has demonstrated epidemiological efficacy. For example, Olyset Plus or PermaNet 3.0 will be the comparator for all new PBO-Pyr net and Interceptor G2 for new CFP-Pyr LLIN. This comparison should be presented using odds ratios with their respective 95% confidence intervals (CIs), as well as percent blood feeding rates with 95% CIs and p-values, to assess the non-inferiority of these second-in-class ITNs relative to first-in-class LLINs. The candidates should then be compared to the comparators using a non-inferiority margin of 0.7 for the odds ratio, meaning that the lower boundary of the 95% CI should not fall below 0.7 (243).

The guideline assumes that results from entomological indicators of new insecticide-treated nets in laboratory and EHTs will align with epidemiological outcomes from large-scale RCTs. However, this was not entirely the case, especially for Interceptor G2, which appeared to perform better in the community than in the bio-efficacy and EHT studies reported. This suggests that the starting point for second in class determination should be the results of the cRCT rather than laboratory or experimental hut results.

Comparing efficacy findings: Laboratory and EHTs versus cRCT results.

Different cRCTs conducted in Tanzania (59, 151, 152), Benin (114), Uganda (115) and Burkina Faso (171) evaluated dual A.I. and PBO-Pyr ITNs and contributed to the decision-making process by WHO in recommending these ITNs over pyrethroid-only nets in areas with higher or moderate levels of pyrethroid resistance (150).

Testing dual A.I. in an EHT could serve as a proxy for a cRCT if the efficacy observed in hut trials correlates with the cRCT results. This approach could reduce the costs associated with conducting cRCTs and expedite data generation and decision-making processes (34, 244). Results from our EHT, along with another study (245) were shared with Imperial College to validate this assumption. However, the performance of the nets in the laboratory, EHT, and during the cRCTs does not appear to be fully aligned.

For Interceptor G2, in EHT settings conducted in different countries, mortality varied across different geographical locations (146, 153, 220), possibly due to the design of the experimental huts or the species composition in those areas (figure 6:1).

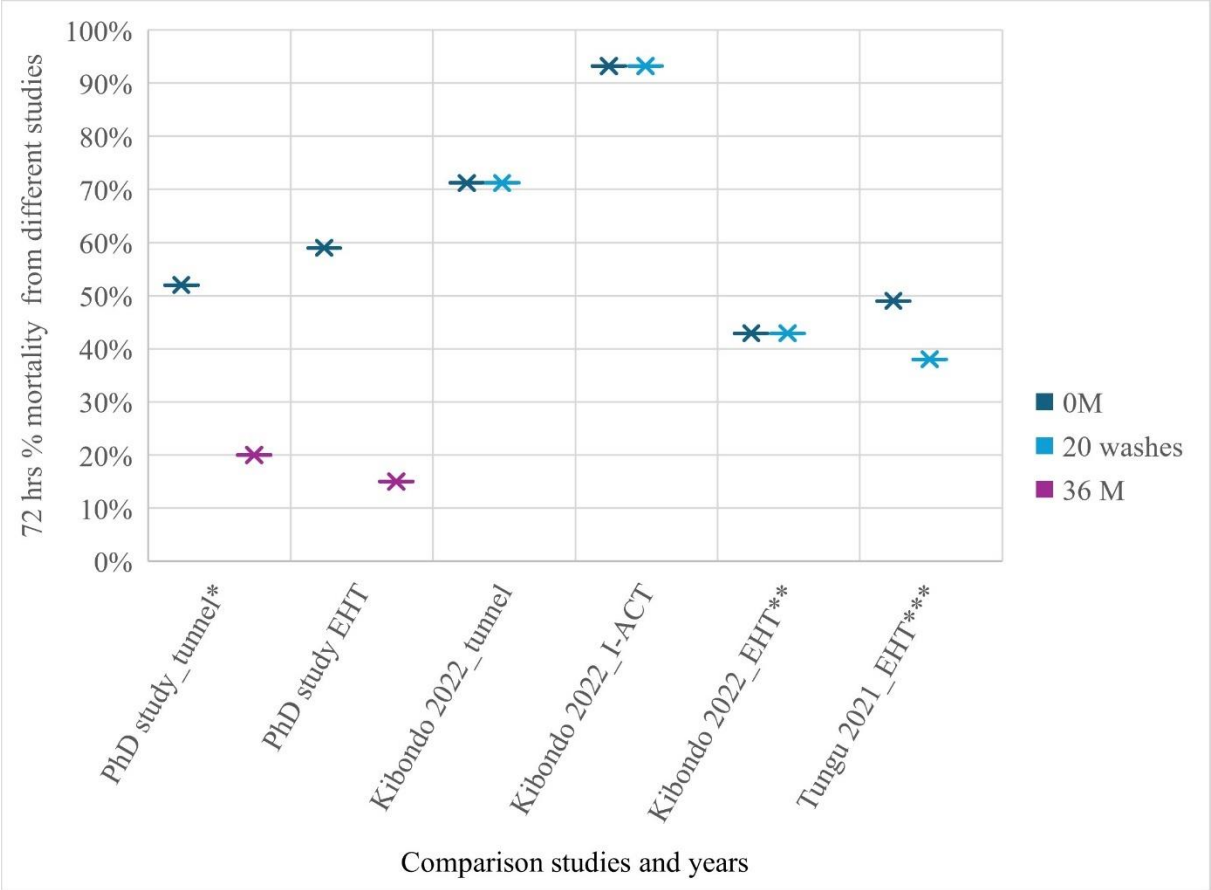


Figure 6:1: Comparison in mortality induced by interceptor G2 against different malaria vectors.

*The study was done using field collected nets at different time point against wild (*An.gambiae* and *An.funestus*) and colony mosquitoes (*An.gambiae* s.s), ** the study use wash resistance nets against *An. arabiensis* (DOI: 10.1186/s13071-022-05207-9) and *** the study use wash resistance nets against *An.funestus* (DOI: /10.1186/s12936-021-03716-z).

In the current EHT and laboratory study, superiority of Interceptor G2 lasted for 12 and 24 months respectively only against resistance *Anopheles* mosquitoes. The findings in this thesis differ from findings reported in cRCT (152). In cRCTs conducted in Tanzania, the efficacy of Interceptor G2 against malaria transmission (EIR) and prevalence was observed for up to 36 months. In Benin, its superiority was observed for up to 24 months (114, 151, 152). The reason for this discrepancy could be due to differences in species composition. In Tanzania, where *An. funestus* was the predominant species in the cRCT study area, Interceptor G2 was reported to work better against *An. funestus* than *An. arabiensis*. No information was provided for the

impact of Interceptor G2 on *An.gambiae* s.s. density (220). In the EHT study area in Tanzania, over half (63%) of the mosquitoes were *An. gambiae* s.s. and may explain the lower performance of Interceptor G2. In contrast, the study in Benin reported *An. coluzzii* to be predominant species during trial (114). Furthermore, Interceptor G2 has been reported to reduce *Plasmodium falciparum* Oocysts development in affected mosquitoes, which may reduce transmission (135). This additional effect might not be detected in the EHT and laboratory testing (151).

Results for Olyset Plus seemed to more closely match the trial conducted in Misungwi (113, 151), where it provided superior protection in epidemiological and entomological outcomes for only one year. In contrast, studies in Muleba (Tanzania) and Uganda showed longer-lasting effects in RCTs, suggesting differences in species composition, resistance and underlying resistance mechanisms may explain these variations (59, 115).

The reduction in efficacy in the current study could be attributed to the poor quality of fabric material, which deteriorated in operational settings, leading to most nets being discarded by users within a year, as reported in this thesis (146). In our project, there were extra non study nets in households, which could have led people to discard the study nets more quickly as they had replacement nets. This effect may have been more significant with Olyset Plus, which develops holes more rapidly. In contrast, earlier studies in phases I and II indicated that Olyset Plus met WHO criteria even after 20 washes and demonstrated significant mortality against resistant *Anopheles* mosquitoes in experimental huts (124) (figure 6:2). While some studies reported efficacy of Olyset Plus for up to 24 months in reducing mosquito density and human contact (76), functional survival was found to be less than three years in a previous study conducted in another part of Tanzania (79) similar to the findings reported in this thesis (146).

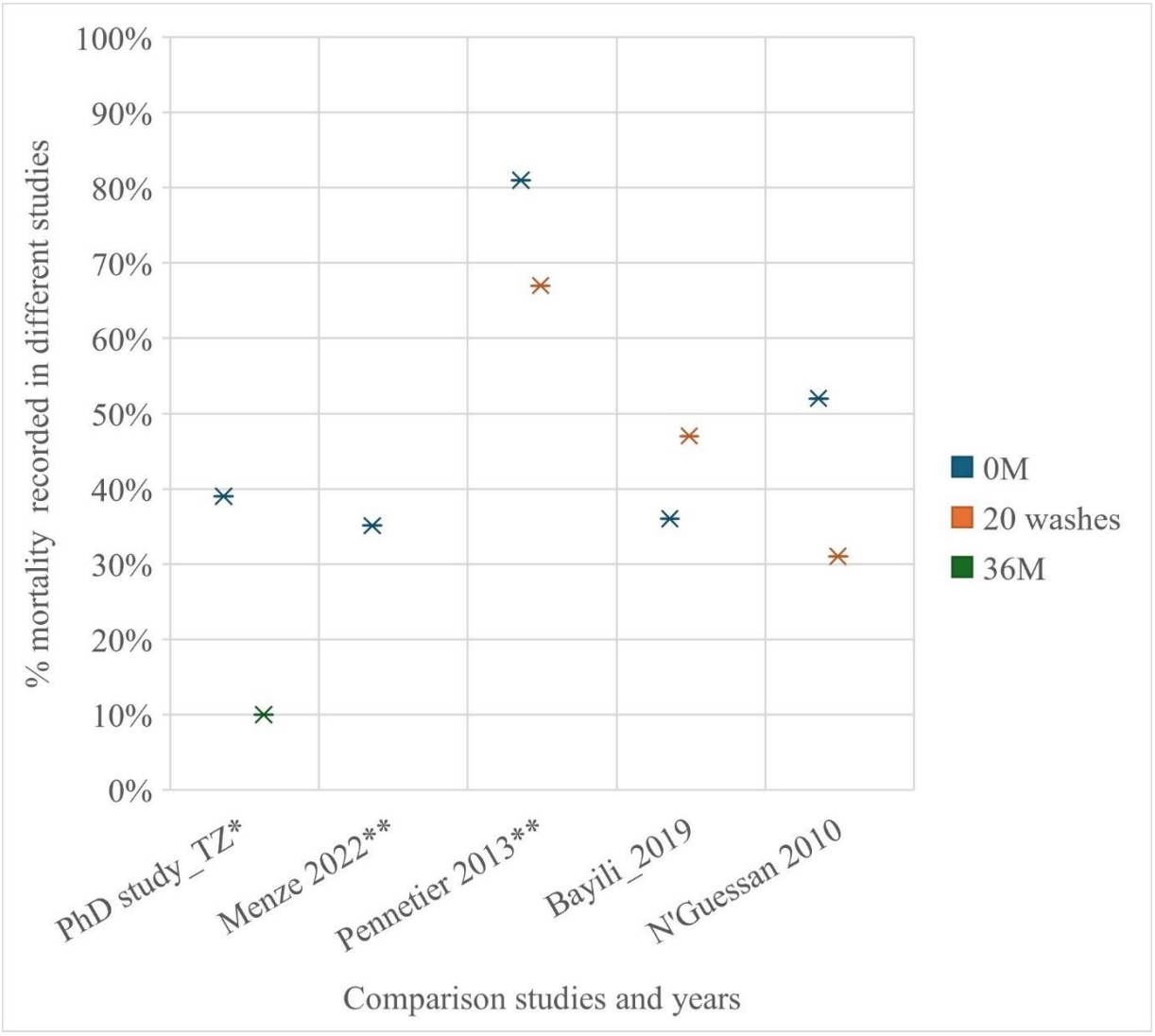


Figure 6:2: Comparison in mortality recorded induced by Olyset Plus in EHTs.

*The study use field collected nets tested against free flying malaria vectors, ** the study use new nets with six hole tested against *An.funestus* and *** the study use new and 20 washed nets tested against *An.gambiae*.

Royal Guard did not show an impact on epidemiological indicators in Tanzania or Benin (114, 151) and was only effective for the first year in reducing mosquito density in Benin (114). In this thesis, sterility effects were limited and observed only when the nets were new up to 6 months in the laboratory and only when new in EHT. However, mortality was consistently higher than standard LLIN for 30 months in laboratory and up to 24 months in EHT. Given that Royal Guard induced more mortality than sterility, there was reduced number of mosquitoes available for oviposition assessment. In the cRCT conducted in Burkina Faso, the effect of pyriproxyfen content against resistant mosquitoes was detected one month post distribution (figure 6:3). No sterility effect was detected thereafter (147).

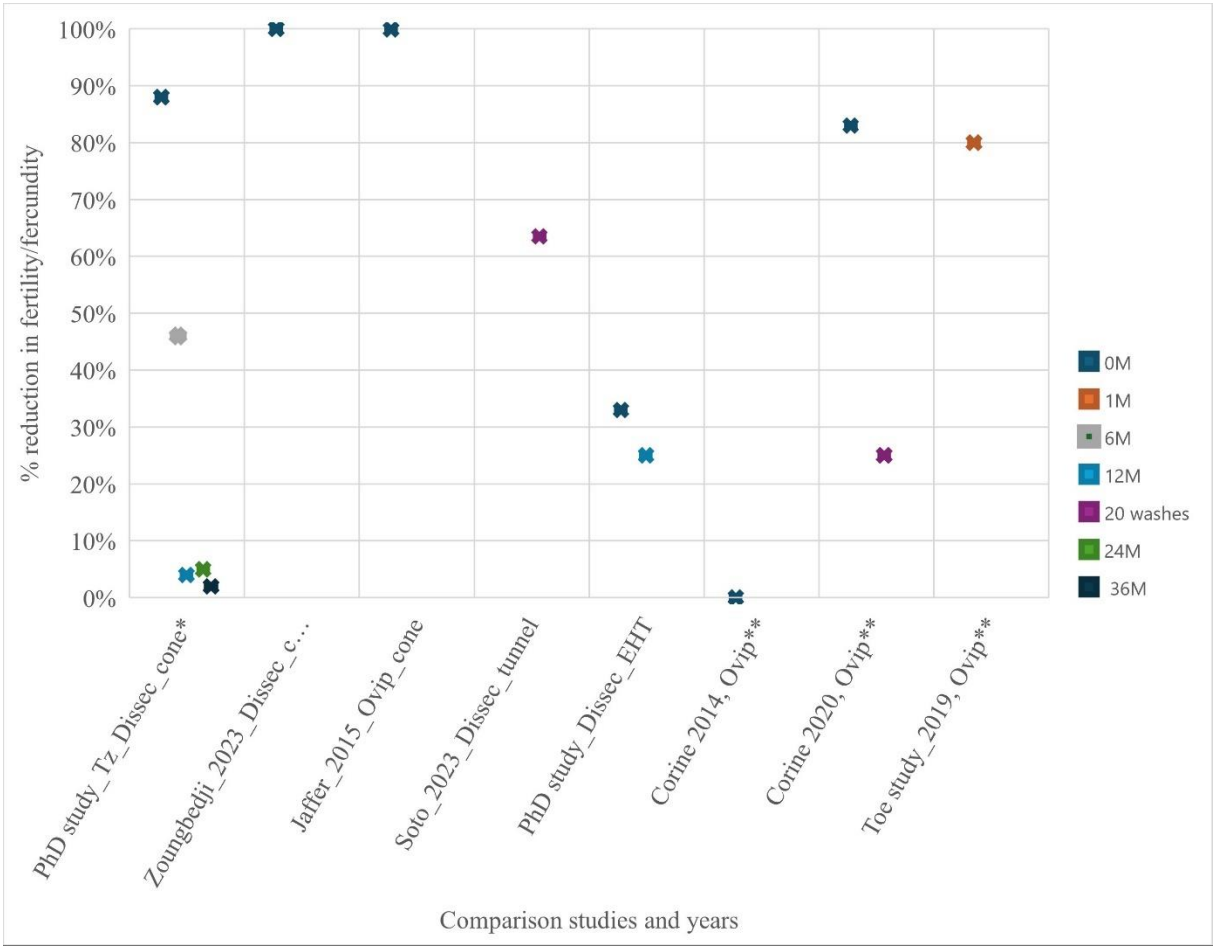


Figure 6:3: Comparison in fertility rate recorded in Royal Guard nets.

* The study uses colony resistance strain for cone assays and wild free flying mosquitoes for EHT; ** the study uses wild mosquitoes**.**

Implication of the results and future direction.

All dual A.I. ITNs have shown superior performance against resistant vectors compared to standard LLINs. However, the bio-efficacy was not maintained for three years, and fabric integrity varied significantly among different brands. Based on this evidence, countries should carefully monitor the durability of these nets in their specific contexts and develop appropriate replacement strategiesImplementation studies were conducted in six countries looking at net replacement campaign for Interceptor G2 and Olyset Plus versus standard Interceptor. The study reported the efficacy of Interceptor G2 and Olyset Plus in malaria case reduction was higher in the first year and reduced significantly after three years using either Interceptor G2 or Olyset Plus. This aligns with the finding reported in Tanzania cRCT (151) and in this thesis which reported functional survival of Interceptor G2 to be 1.9 years (figure 6:4). Similar findings were reported from other part of Tanzania evaluating PBO-Pyr ITN compared to pyrethroid alone (79).

ITN replacement after two years of community use will increase the efficacy in malaria reduction and provide better protection to the community. However, cost and improving net durability will also be an important avenues to consider. In this thesis, notably, Olyset Plus exhibited the shortest lifespan, with an alarming functional survival of only 0.9 years, significantly lower than any other ITN brand studied. This finding underscores the urgent need for improved LLIN durability to ensure sustained malaria prevention efforts and sustain the millennium development goal of reduction of malaria by 90% in 2030.

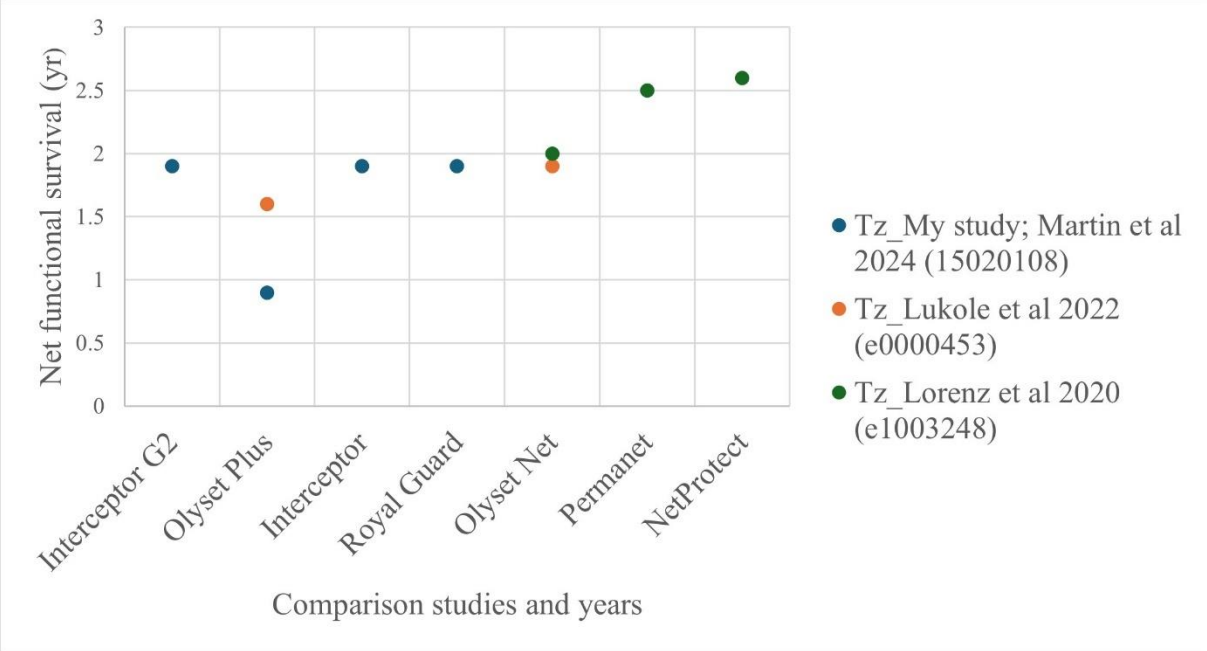


Figure 6:4: Comparison of different studies in functional survival of dual A.I. nets

National Malaria control program in Tanzania deployed pyrethroid-PBO nets and distributed them in area where malaria vectors are highly resistant since 2018 to date through yearly primary school distribution campaign. Recently, there has been a plan to procure dual A.I. nets (Interceptor G2) for distribution in high malaria districts, while distributing PBO-Pyr in areas with moderate insecticide resistance and standard nets in areas with pyrethroid-susceptible mosquito populations. This approach will enhance overall coverage and net usage, leading to a decrease in malaria transmission. To meet WHO's 2030 malaria targets, Tanzania must also consider evaluating and potentially adopting additional interventions. By elucidating the factors influencing ITN performance, this thesis contributes valuable insights to the ongoing efforts to optimize malaria prevention strategies and reduce the burden of disease in affected communities.

Challenges in Deploying Dual A.I. ITNs for Malaria Control

1. **Increased cost of dual A.I. ITNs**

The dual A.I ITNs are important to combat the widespread of insecticide resistance and reduction of malaria case incidence and prevalence, however, increased costs to procure second in-class ITNs challenges the effort for malaria elimination by 2030. Globally the cost to deliver these ITNs to the end users is high (3.02$US per net) compared to 2.07$US per net for pyrethroid only LLINs (151) of which the countries in sub-Saharan region (endemic countries) is highly impacted due to country economies. As of 2024, there remains a significant funding gap for insecticide-treated nets (ITNs), with grant cycle 7 (GC7) acquiring only $310 million out of the initial $475 million required. However, only $165 million has been secured to date, leaving a substantial deficit in funding for ITN distribution and implementation efforts especially in sub-Saharan Africa (personal communication: Global fund). This calls for more effort to find additional resource to ensure their roll out continues. Local governments should locate the budget to keep the distribution of these new ITNs in areas with moderate or high resistance when donors stop supporting the intervention otherwise progress will stall.

2. **Reduce susceptibility status in chlorfenapyr against *An. gambiae*.**

The susceptibility of malaria vectors, particularly *An. gambiae* to chlorfenapyr has been extensively investigated across several countries, including Ghana, Cameroon, the Democratic Republic of Congo, Uganda, and Malawi. While varying levels of susceptibility have been reported, notably higher susceptibility status has been observed in *An. funestus* populations (246). In a related study examining the potential association between cytochrome P450 enzymes and chlorfenapyr metabolism, researchers assessed the expression levels of eight genes known to confer resistance to pyrethroid insecticides in *An. gambiae* mosquitoes (247). The findings revealed that three out of eight genes, namely CYP6P3, CYP9J5, and CYP9K1, were overexpressed in some of the countries, indicating their potential involvement in the metabolism of chlorfenapyr (248). Such metabolic pathways pose a significant challenge in the fight against malaria vectors, as they may contribute to the development of resistance to chlorfenapyr in future. Further research is warranted to fully elucidate the range of cytochrome P450 enzymes that interact with chlorfenapyr. This knowledge is essential for the development of effective strategies to overcome resistance mechanisms and optimize the use of chlorfenapyr in malaria vector control efforts.

3. Mosquitoes and human behaviour.

Changes in mosquito behaviour such as early biting and late time spent outdoors contributes to reducing the effectiveness of ITNs because mosquitoes can blood feed in host late evening before they go to bed. In Kenya, assessing late morning biting behaviours as a risk factor for malaria transmission in Siaya school revealed that, *An. funestus* bite between 06:00 and 07:00 hours in the morning and extend till 11:00 am when the children are outside for learning purpose. This student might receive potential bite because they spend more time outdoor during school hours (8). This calls for additional intervention which will target outdoor mosquitoes for full protection.

Conclusion

Olyset Plus LLINs exhibited higher initial mortality rates, and Royal Guard LLINs demonstrated greater sterility effects compared to Interceptor LLINs, though only when new. Interceptor G2 LLINs outperformed Interceptor LLINs against *An. gambiae* s.l. and the *An. funestus* complex for up to 12 months in EHT and 24 months in laboratory assays against *An. gambiae* s.s and provided extended malaria protection for up to 3 years in associated cRCTs. While all tested nets met WHO criteria against susceptible *Anopheles* strains for up to 3 years, the superior efficacy of next-generation ITNs over standard LLINs diminished within 12 months and varied by brand. Further research is needed to explore additional effects, such as chlorfenapyr's impact on parasite reduction, and to confirm the residual efficacy of dual active ingredient LLINs through standard and adapted EHTs in various contexts. Our results provide the first evidence on the bio-efficacy of Interceptor G2 and Royal Guard after community use, setting a reference for testing other products in the same classes. Overall, the findings from this thesis emphasize the complexity of ITN durability, efficacy, and resistance dynamics in malaria-endemic regions. Addressing these challenges requires a multifaceted approach, including continuous product improvement, rigorous surveillance, and evidence-based policy decisions.

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7 Annex 1: Supplementary (S) tables

7.1 Chapter 3: Supplementary files for durability study

Table 3: S1: pHI categories as per WHO guideline 2013.

Category	pHI value range	Total hole surface area in cm^2
Good	0-64	<100
Damage	65 – 642	100 – 1,000
Too torn	643+	>1,000

Table 3: S2: Reason for attrition per net type and net age

		Interceptor® % (95%CI)	Interceptor® G2 % (95%CI)	Olyset™ Plus % (95%CI)	Royal Guard® % (95%CI)
Thrown away	6M	11% (3 - 29)	33% (20 - 48)	69% (59 - 77)	30% (19 - 44)
	12M	31% (22 - 41)	66% (58 - 73)	66% (60 - 72)	39% (31 - 47)
	24M	64% (59 - 70)	66% (61 - 71)	87% (84 - 89)	74% (70 - 78)
	30M	73% (68 - 78)	81% (77 - 85)	86% (83 - 89)	82% (78 - 85)
	36M	79% (75 - 83)	87% (83 - 90)	90% (87 - 92)	90% (87 - 92)
Used for other purpose	6M	4% (0 - 22)	7% (2 - 20)	6% (3 - 12)	4% (0 - 14)
	12M	11% (6 - 19)	4% (2 - 9)	9% (6 - 14)	8% (5 - 14)
	24M	7% (4 - 10)	2% (1 - 4)	2% (1 - 4)	1% (0 - 3)
	30M	6% (4 - 10)	1% (0 - 3)	4% (3 - 6)	2% (1 - 4)
	36M	8% (6 - 11)	0	2% (1 - 4)	2% (1 - 4)
used in other location	6M	43% (26 - 61)	26% (15 - 41)	12% (7 - 19)	42% (29 - 55)
	12M	32% (23 - 42)	10% (6 - 15)	8% (5 - 12)	23% (17 - 31)
	24M	14% (11 - 19)	10% (7 - 14)	5% (4 - 7)	9% (7 - 12)
	30M	8% (6 - 12)	3% (1 - 5)	2% (0 - 3)	5% (3 - 8)
	36M	3% (2 - 5)	4% (2 - 6)	3% (1 - 4)	2% (1 -4)
Given away to relatives	6M	39% (23 - 58)	33% (20 - 49)	7% (3 - 14)	15% (8 - 27)
	12M	18% (11 - 27)	13% (8 - 19)	10% (6 - 14)	16% (10 - 22)
	24M	9% (6 - 13)	9% (7 - 13)	2% (1 - 4)	2% (1 - 4)

	30M	5% (3 - 7)	6% (4 - 9)	2% (1 - 4)	4% (2 - 6)
	36M	3% (2 - 5)	3% (1 - 5)	0	2% (1 - 4)
Destroyed accidentally	6M	0	2% (0 - 15)	5% (2 - 11)	4% (0 - 14)
	12M	3% (1 - 10)	3% (1 - 8)	5% (3 - 9)	11% (7 - 9)
	24M	3% (1 - 5)	7% (5 - 11)	2% (1 - 4)	11% (8 - 14)
	30M	2% (1 - 4)	6% (4 - 9)	2% (1 - 3)	5% (3 - 7)
	36M	1% (0 - 3)	4% (2 - 6)	2% (1 - 4)	2% (1 - 4)
Stolen	6M	4% (0 - 22)	0	2% (0 - 7)	4% (0 - 14)
	12M	3% (1 - 10)	2% (0 - 6)	1% (0 - 4)	1% (0 - 5)
	24M	2% (0 - 4)	2% (0 - 4)	1% (0 - 2)	2% (0 - 3)
	30M	3% (2 - 6)	2% (1 - 4)	3% (2 - 5)	1% (0 - 3)
	36M	3% (2 - 6)	2% (0 - 4)	3% (2 - 5)	0

Table 3: S3a: Survivorship of the LLIN per time point

Net type	% Survival, 95%CI				
	6month	12month	24month	30month	36month
Interceptor®	93.7% [91 - 95]	84.0% [81 - 87]	59.4% [56 - 63]	47.2% [43 - 52]	37.1% [33 - 41]
Interceptor® G2	90.9% [88 - 93]	78.9% [76 - 82]	56.8% [53 - 60]	42.0% [38 - 46]	36.7% [33 - 41]
Olyset™ Plus	82.1% [79 - 85]	49.2% [46 -52]	18.1% [15 - 21]	14.8% [12 - 18]	9.5% [7 - 12]
Royal Guard®	89.9% [87 - 92]	70.1% [67 - 73]	39.9% [376 - 44]	27.4% [24 - 31]	18.0% [15 - 21]

Table 3: S3b: Functional survival of the LLIN per time point

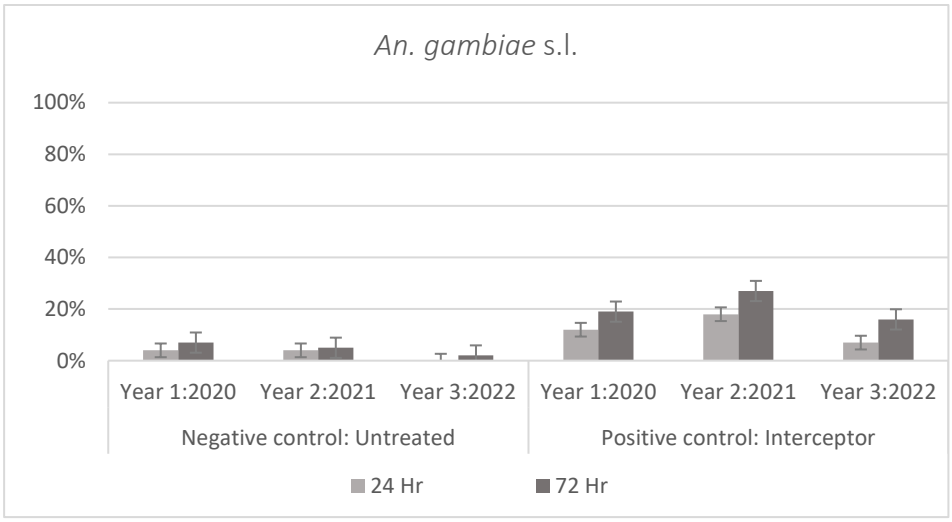
Net type	% function survival, 95%CI				
	6month	12month	24month	30month	36month
Interceptor®	91.7% [88 - 94]	76.7% [73 - 80]	44.3% [40 - 48]	30.5% [27 - 34]	21.8% [19 - 25]
Interceptor® G2	88.5% [85 - 91]	63.2% [59 - 67]	39.9% [36 - 44]	23.1% [20 - 27]	19.7% [16 - 23]
Olyset™ Plus	62.6% [58 - 67]	29.4% [26 - 33]	8.3% [6 - 11]	3.2% [2 - 5]	3.9% [3 - 6]
Royal Guard®	88.6% [85 - 91]	62.2% [58 - 66]	29.9% [26 - 34]	15.4% [13 - 19]	8.6% [7 - 11]

Table 3: S4. Secondary analysis to assess if SES and housing structure impacted attrition in different net type

Variable	Hazard ratio	P-value	95% CI
Net type			
Interceptor	1		
Interceptor G2	1.339	0.057	0.9 - 1.8
Olyset Plus	2.701	<0.001	1.9 - 3.8
Royal Guard	1.451	0.024	1.0 - 2.0
Bed type			
Standardbed	1		
Bedwith stick	1.228	0.351	0.8 - 1.9
No bed	1.16	0.048	1.0 - 1.3
Matress			
Reed mat	1		
Grass	4.073	0.01	1.4 -11 .9
Foam mattress	2.059	0.002	1.3 -3.2
Other (no, clothes)	1.883	0.011	1.2 -3.1
Education			
Higher	0.651	0.053	0.4 -1.0
Secondary	1.729	0.004	1.2 -2.5
No/primary	2.252	<0.001	1.9 -2.7
Number of people			
Total adult	1.063	<0.001	1.0 - 1.1
Total children	1.061	0.007	1.0 - 1.1

7.2 Chapter 4: Supplementary files for Experimental hut study

Figure 4: S1: Control mortality for *An. Gambiae* s.l and *An.funestus* complex (separately) collected from experimental hut trial.



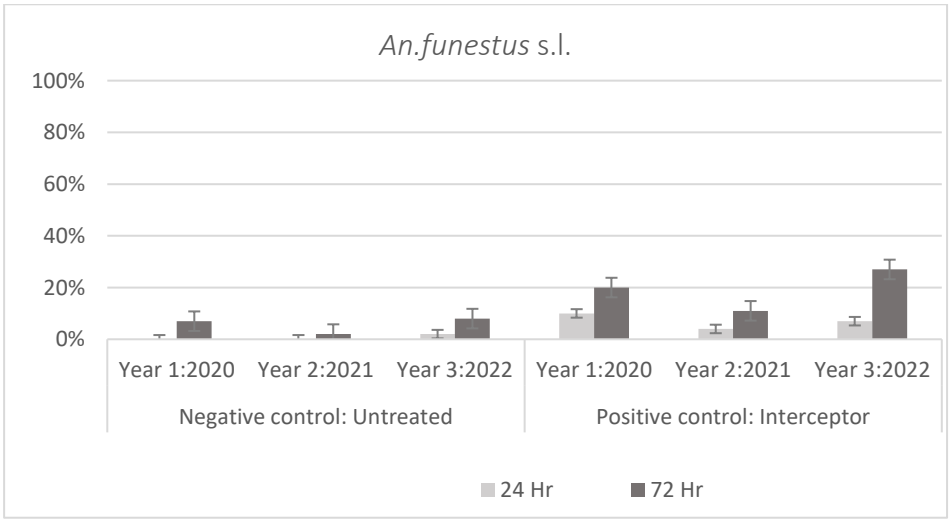


Table 4: S4a: Percent mortality and blood feeding with their odds ratio and 95%CI for Anopheles mosquitoes collected from the huts.

		24 hours mortality % (n/N)				72 hours mortality % (n/N)				BF % (n/N)		
	Total collection	mortality % (n/N)	OR*	95%CI	p value	mortality % (n/N)	OR*	95%CI	p value	BF (n/N)	OR*	95%CI
0 month												
Interceptor (reference)	72	6% [7/111]	1			14% [15/110]	1			21% [23/111]	1	
Interceptor G2	72	48% [49/102]	19.9	6.5 - 61.6	<0.001	58% [59/102]	11.9	4.8 - 29.7	<0.001	28% [29/102]	1.1	0.4 - 2.7
Royal Guard	72	25% [35/139]	7.0	2.3 - 20.9	<0.001	31% [43/139]	3.7	1.5 - 8.7	0.0023	9% [13/139]	0.5	0.2 - 1.2
Olyset Plus	72	38% [43/112]	13.6	4.4 - 41.3	<0.001	46% [52/112]	7.0	2.8 - 17.1	<0.001	10% [11/112]	0.5	0.2 - 1.2
12 month												
Interceptor (reference)	120	13% [23/181]	1			21% [38/181]	1			15% [28/181]	1	
Interceptor G2	120	35% [68/193]	4.6	2.3 - 9.5	<0.001	44% [84/193]	3.5	1.9 - 6.6	<0.001	18% [34/193]	0.9	0.4 - 1.9
Royal Guard	120	19% [43/222]	2.1	1.0 - 4.4	0.048	33% [73/222]	2.1	1.1 - 3.8	0.315	8% [18/222]	0.6	0.3 - 1.3
Olyset Plus	119	17% [44/252]	2.1	1.0 - 4.3	0.112	27% [67/252]	1.5	0.8 - 2.9	0.212	10% [32/252]	0.6	0.3 - 1.4
24 month												
Interceptor (reference)	120	11% [18/170]	1			20% [34/170]	1			22% [38/170]	1	
Interceptor G2	120	15% [27/180]	1.9	0.8 - 4.2	0.12	20% [36/180]	1.3	0.7 - 2.6	0.52	24% [43/180]	1.1	0.5 - 2.3
Royal Guard	120	11% [25/225]	1.3	0.6 - 3.1	0.42	20% [46/225]	1.1	0.6 - 2.2	0.48	36% [80/225]	1.8	0.9 - 3.6
Olyset Plus	120	12% [25/208]	1.4	0.6 - 3.3	0.31	24% [49/208]	1.2	0.6 - 2.3	0.663	25% [52/208]	1.1	0.5 - 2.2
36 month												
Interceptor (reference)	120	7% [6/91]	1			14% [13/91]	1			19% [17/91]	1	
Interceptor G2	120	9% [9/95]	1.6	0.4 - 5.7	0.97	15% [14/95]	1.5	0.6 - 4.1	0.78	13% [12/95]	0.6	0.2 - 1.7
Royal Guard	120	8% [10/121]	1.0	0.3 - 3.6	0.95	11% [13/121]	0.8	0.3 - 2.3	0.67	17% [20/121]	0.9	0.3 - 2.2
Olyset Plus	120	10% [13/124]	1.0	0.3 - 3.5	0.89	18% [22/124]	1.5	0.6 - 3.8	0.430	17% [21/124]	0.9	0.3 - 2.3

Table 4: S4b: Percent mortality and blood feeding for *An. gambiae* s.l with their odds ratio and 95%CI for Anopheles mosquitoes collected from the huts

		24 hours mortality % (n/N)				72 hours mortality % (n/N)				BF % (n/N)		
	Total collection	mortality % (n/N)	OR*	95%CI	p value	mortality % (n/N)	OR*	95%CI	p value	BF (n/N)	OR*	95%CI
0 month												
Interceptor (reference)	72	7 [5/73]	1			11 [8/72]	1			19 [14/73]	1	
Interceptor G2	72	42 [28/66]	12.4	3.1 - 49.2	<0.001	55 [36/66]	15.2	4.6 - 50.9	<0.001	24 [16/66]	1.5	0.5 - 5.0
Royal Guard	72	34 [27/79]	10.1	2.7 - 37.8	<0.001	37 [29/79]	6.6	2.1 - 20.8	<0.001	13 [10/79]	0.6	0.2 - 1.9
Olyset Plus	72	44 [26/59]	17.3	4.3 - 69.5	<0.001	54 [32/59]	13.1	3.9 - 43.7	<0.001	12 [7/59]	0.4	0.1 - 1.7
12 month												

Interceptor (reference)	120	13 [16/128]	1			22 [28/128]	1			13 [17/128]	1	
Interceptor G2	120	35 [54/155]	4.9	2.1 - 11.8	<0.001	42 [65/155]	3.2	1.5 - 6.7	0.002	19 [29/155]	1.2	0.5 - 3.0
Royal Guard	120	22 [36/161]	2.5	1.0 - 6.0	0.04	34 [54/161]	2.1	1.0 - 4.3	0.058	11 [17/161]	0.7	0.2 - 1.7
Olyset Plus	119	23 [35/155]	2.6	1.0 - 6.4	0.045	30 [47/155]	1.7	0.8 - 3.8	0.174	9 [14/155]	0.4	0.1 - 1.1
24 month												
Interceptor (reference)	120	9 [11/120]	1			19 [23/120]	1			23 [28/120]	1	
Interceptor G2	120	18 [22/121]	2.3	0.8 - 6.7	0.13	23 [28/121]	1.3	0.5 - 3.2	0.578	28 [34/121]	1.1	0.4 - 2.9
Royal Guard	120	13 [17/126]	2.0	0.6 - 5.9	0.232	24 [30/126]	1.6	0.7 - 4.0	0.298	35 [44/126]	1.6	0.6 - 4.0
Olyset Plus	120	20 [19/97]	2.5	0.8 - 7.7	0.125	24 [23/97]	1.2	0.4 - 3.1	0.75	21 [20/97]	0.8	0.3 - 2.2
36 month												
Interceptor (reference)	120	11 [5/44]	1			16 [7/44]	1			25 [11/44]	1	
Interceptor G2	120	9 [5/55]	0.7	0.1 - 3.5	0.646	13 [7/55]	0.7	0.2 - 2.9	0.658	13 [7/55]	0.3	0.1 - 1.3
Royal Guard	120	8 [6/77]	0.6	0.1 - 2.9	0.545	16 [12/77]	0.9	0.3 - 3.5	0.982	21 [16/77]	0.8	0.2 - 2.8
Olyset Plus	120	10 [7/67]	0.8	0.2 - 3.7	0.791	22 [15/67]	1.6	0.5 - 5.6	0.457	16 [11/67]	0.5	0.1 - 1.8

Table 4: S4c: Percent mortality and blood feeding for *An. gambiae* s.l with their odds ratio and 95%CI for Anopheles mosquitoes collected from the huts

		24 hours mortality % (n/N)				72 hours mortality % (n/N)				BF % (n/N)		
	Total collection	mortality % (n/N)	OR*	95%CI	p value	mortality % (n/N)	OR*	95%CI	p value	BF (n/N)	OR*	95%CI
0 month												
Interceptor (reference)	72	5 [2/38]	1			18 [7/38]	1			24 [9/38]	1	
Interceptor G2	72	53 [19/36]	56.0	6.2 - 504.1	<0.001	56 [20/36]	11.1	2.4 - 49.8	0.002	17[6/36]	0.6	0.1 - 2.6
Royal Guard	72	10 [6/60]	3.5	0.4 - 30.7	0.252	23 [14/60]	1.6	0.4 - 6.6	0.499	8 [5/60]	0.3	0.1 - 1.3
Olyset Plus	72	26 [14/53]	13.4	1.7 - 108.5	0.015	37 [19/53]	3.2	0.8 - 12.8	0.099	15 [8/53]	0.5	0.1 - 2.1
12 month												
Interceptor (reference)	120	13 [7/53]	1			19 [10/53]	1			21 [11/53]	1	
Interceptor G2	120	37 [14/38]	5.9	1.4 - 25.3	0.016	50 [19/38]	5.1	1.5 - 17.8	0.01	11 [4/38]	0.4	0.1 - 1.6
Royal Guard	120	16 [10/61]	1.4	0.3 - 5.7	0.734	28 [17/61]	1.9	0.6 - 6.2	0.27	11 [7/61]	0.4	0.1 - 1.5
Olyset Plus	119	15 [15/97]	1.2	0.3 - 4.6	0.811	21 [20/97]	1.1	0.3 - 3.3	0.93	19 [18/97]	0.8	0.3 - 2.6
24 month												
Interceptor (reference)	120	14 [7/50]	1			22 [11/50]	1			20 [10/50]	1	
Interceptor G2	120	17 [10 / 59]	1.4	0.3 - 5.8	0.686	25 [15/59]	1.3	0.4 - 4.3	652	25 [15/59]	0.9	0.3 - 3.3
Royal Guard	120	14 [14/99]	0.8	0.2 - 3.2	0.734	21 [21/99]	0.9	0.3 - 2.7	0.806	35 [35/99]	1.9	0.6 - 5.8
Olyset Plus	120	10 [11/111]	0.7	0.2 - 2.8	0.6	23 [26/111]	1.2	0.4 - 3.6	0.73	26 [29/111]	1.3	0.4 - 3.9

36 month												
Interceptor (reference)	120	2 [1/47]	1			13 [6/47]	1			13 [6/47]	1	
Interceptor G2	120	13 [5/40]	7.2	0.5 - 111.1	0.156	28 [11/40]	2.9	0.6 - 13.4	0.181	18 [7/40]	1.1	0.2 - 5.9
Royal Guard	120	5 [2/44]	1.7	0.1 - 32.7	0.728	5 [2/44]	0.2	0.02 - 1.7	0.136	9 [4/44]	0.6	0.1 - 3.5
Olyset Plus	120	4 [2/56]	1.6	0.1 - 26.6	0.754	12 [7/56]	0.9	0.2 - 4.3	0.921	19 [11/56]	1.5	0.3 - 6.4

Table 4: S5: Total collected mosquitoes per treatment per time point inside net and exit traps with percent deterrence

	An.gambiae s.l.					*An.funestus* s.l.			
Treatment	**Total Caught**	**% Deterrence**	**% Exit (95%CI)**	**% Net (95%CI)**		**Total Caught**	**% Deterrence**	**% Exit (95%CI)**	**% Net (95%CI)**
0 month									
Untreated net	63	ref	68 (58 - 79)	11 (4 - 18)		41	ref	68 (54 - 83)	17 (4 - 30)
Interceptor	75	-19%	81 (71 - 91)	1 (0 - 4)		38	7%	76 (63 - 90)	8 (0 - 15)
Interceptor G2	66	-5%	67 (56 - 78)	9 (3 - 15)		36	12%	61 (39 - 84)	11 (1 - 21)
Royal Guard	79	-25%	78 (68 - 89)	3 (0 - 6)		60	-46%	77 (61 - 93)	3 (0 - 8)
Olyset Plus	59	6%	71 (52 - 90)	5 (0 - 11)		53	-29%	74 (63 - 85)	6 (0 - 12)
12 months									
Untreated net	176	ref	65 (57 - 73)	22 (16 - 28)		54	ref	54 (38 - 70)	20 (7 - 33)
Interceptor	128	27%	82 (74 - 90)	5 (1 - 8)		53	2%	89 (80 - 98)	2 (0 - 6)
Interceptor G2	155	12%	72 (63 - 82)	7 (2 - 12)		38	30%	74 (58 - 89)	3 (0 - 8)
Royal Guard	161	9%	79 (71 - 86)	5 (0 - 9)		61	-13%	85 (77 - 94)	3 (0 - 8)
Olyset Plus	153	13%	79 (72 - 86)	5 (1 - 9)		97	-80%	82 (73 - 92)	1 (0 - 3)
24 months									
Untreated net	78	1	68 (58 - 78)	13 (5 - 20)		81	1	59 (45 - 73)	23 (13 - 34)
Interceptor	120	-54%	74 (65 - 84)	6 (1 - 10)		50	38%	80 (69 - 91)	8 (0 - 16)
Interceptor G2	121	-55%	65 (55 - 75)	14 (7 - 22)		59	27%	73 (55 - 91)	8 (1 - 16)
Royal Guard	126	-62%	60 (51 - 70)	4 (0 - 9)		99	-22%	79 (68 - 89)	4 (0 -9)
Olyset Plus	97	-24%	75 (66 - 85)	1 (0 - 3)		111	-37%	84 (76 - 91)	2 (0 - 4)
36 months									
Untreated net	81	1	57 (45 - 69)	11 (5 - 17)		52	1	69 (55 - 84)	12 (3 - 20)
Interceptor	44	46%	73 (57 - 88)	2 (0 - 7)		47	10%	57 (38 - 77)	13 (0 - 24)
Interceptor G2	55	32%	62 (50 - 74)	5 (0 - 11)		40	23%	65 (44 - 86)	13 (0 - 30)
Royal Guard	77	5%	78 (68 - 87)	1 (0 - 4)		44	15%	84 (75 - 93)	0
Olyset Plus	67	17%	85 (77 - 93)	1 (0 - 4)		57	-10%	79 (65 - 92)	4 (0 - 8)

Table 4: S6: Effect of hole on blood feeding in *Anopheles* mosquitoes collected.

Net age		Interceptor G2					Olyset Plus					Royal Guard				
	Hole area (per cm2)	mean hole area	Blood feeding				mean hole area	Blood feeding				mean hole area	Blood feeding			
			% (n/N)	OR*	95%CI	p value		% (n/N)	OR*	95%CI	p value		% (n/N)	OR*	95%CI	p value
12	< 79	26.5	13.8 (17/123)	1			27.9	7.1(3/42)				16.2	8 (9/107)			
	80 to 789	242.5	20.8 (10/48)	1	0.3 - 3.1	0.962	390.8	10.9(8/73)	0.6	0.2 - 1.6	0.319	260.3	12.7 (8/63)	0.9	0.4 - 2.5	0.968
	>790	1744.8	27.3(6/22)	1.5	0.4 - 1.6	0.592	3243.8	15.5(21/135)	1.2	0.4 - 3.3	0.714	1641.1	13.5 (7/52)	1.2	0.4 - 3.6	0.802
24	< 79	28.7	27.0 (10/37)				29.8	11.1 (2/18)				19.8	32.2 (28/87)			
	80 to 789	255.2	22.7 (10/44)	0.6	0.2 - 1.7	0.306	475.7	8.3 (2/24)	0.4	0.1 - 1.6	0.197	530.5	0 (0/3)	0.5	0.2 - 1.6	0.272
	>790	2064.5	29.3 (29/99)	0.6	0.2 - 1.7	0.369	2890.1	27.1 (45/166)	1.1	0.4 - 3.5	0.806	2587.8	37.8 (51/135)	1.1	0.6 - 2.1	0.813
36	< 79	35.4	11.1 (2/18)				17.2	27.3 (3/11)				35.9	14.3 (3/21)			
	80 to 789	402.6	23.3 (7/30)	4.1	0.2 - 75.4	0.34	305.7	3.8 (1/26)	0.6	0.1 - 5.3	0.614	397.8	14.3 (4/28)	1.9	0.3 - 12.4	0.493
	>790	1951.6	10.6 (5/47)	0.7	0 - 12.9	0.822	3592.9	20.7 (18/87)	0.6	0.1 - 4.9	0.672	2430.6	18.1 (13/72)	1.5	0.3 - 8.4	0.626

Table 4: S7A: Secondary analysis model showing interaction between vector species, net type and time point.

The results show that time point and net type have a significant effect on the mortality observed per net.

Term in Model	X2	P value
Time point	61.55	<0.001
Net type	76.86	<0.001
Time point*net type	27.41	0.026

7.3 Chapter 5: Supplementary files for bio-efficacy laboratory study

Table 5: S8: Total replicates and number of *Anopheles* to be exposed in α -cypermethrin for one resistance frequency test

Replicates	Insecticide	Concentration	Number of *Anopheles* per bottle
Bottle 1 (1X)	α -cypermethrin	12.5 µg /ml	15-20
Bottle 2 (2X)	α -cypermethrin	25 µg /ml	15-20
Bottle 3 (5X)	α -cypermethrin	62.5 µg /ml	15-20
Bottle 4 (10X)	α -cypermethrin	125 µg /ml	15-20
Bottle 5	Control (Acetone)	1ml	15-20

Table 5: S9: Total replicates and number of *Anopheles* to be exposed in permethrin for one resistance frequency test

Replicates	Insecticide	Concentration	Number of Anopheles per bottle
Bottle 1 (1X)	permethrin	21.5 µg /ml	15-20
Bottle 2 (2X)	permethrin	43 µg /ml	15-20
Bottle 3 (5X)	permethrin	107.5 µg /ml	15-20
Bottle 4 (10X)	permethrin	215 µg /ml	15-20
Bottle 5	Control (Acetone)	1ml	15-20

Table 5: S10: Cone and tunnel test results conducted for each type of net (Interceptor, Interceptor G2, Royal Guard, and Olyset Plus) against the susceptible *An. gambiae s.s.* strain at 0, 12, 24, and 36 months post-distribution. Only nets that failed WHO criteria in cone bioassays were further tested in tunnel assays.

Bio-efficacy of Alphacypermethrin: Cone test with susceptible kisumu strain																
Treatments	Interceptor				Interceptor G2				Royal Guard				Olyset Plus			
Net age	0	12	24	36	0	12	24	36	0	12	24	36	0	12	24	36
N ITNs tested	30	30	30	50	30	30	30	50	30	30	30	50	30	30	30	50
N pieces tested	150	120	120	200	150	120	120	200	150	120	120	200	150	120	120	200
N exposed	2868	2347	2349	7242	2527	2350	2356	4021	2968	2365	2343	3970	2841	2302	2374	3896
N KD	2834	1589	911	1554	2287	1011	645	532	2956	2004	1107	2012	2841	1612	936	587
N dead	2749	1224	596	873	971	713	490	262	2959	1729	614	1242	2817	1275	669	494
% KD	99 (98 - 99)	68 (63 - 72)	39 (34 - 43)	21 (20 - 23)	91 (88 - 93)	43 (37 - 49)	27 (23 - 31)	13 (10 - 17)	100 (99 - 100)	85 (80 - 89)	47 (42 - 52)	51 (45 - 57)	100	70 (65 - 75)	39 (34 - 44)	15 (11 - 19)
% dead	96 (94 - 98)	52 (46 - 58)	25 (22 - 29)	12 (11 - 13)	38 (33 - 44)	30 (25 - 36)	21 (19 - 23)	6 (4 - 8)	100 (99 - 100)	73 (66 - 80)	26 (22 - 31)	31 (25 - 37)	99 (98 -100)	55 (50 - 61)	28 (25 - 31)	13 (9 - 16)
N passed cone	30	3	0	0	11	0	0	0	30	13	0	1	30	3	0	0
% passage	100	10	0	0	37	0	0	0	100	10	0	3	100	10	0	0
Tunnel test with failled nets (<80% mort or KD<95%) from cone bioassay																
Net age	0	12	24	36	0	12	24	36	0	12	24	36	0	12	24	36
N ITNs tested	15	27	30	50	19	30	30	50	NA	27	30	49	NA	27	30	50
N pieces tested	15	27	30	93	19	30	30	50	NA	27	30	49	NA	27	30	50
N exposed	939	2398	2608	4496	2802	2763	2238	4817	NA	1561	2755	3704	NA	2495	2809	4649
N dead	640	1770	1560	2963	1924	2152	1306	3063	NA	1175	1563	2491	NA	1908	1809	3194
N blood-fed	10	28	37	71	40	22	20	64	NA	9	28	37	NA	42	7	18
% dead	68 (63 - 73)	74 (71 - 77)	60 (55 - 64)	66 (64 - 68)	69 (66 - 72)	78 (74 - 82)	58 (55 - 62)	64 (62 - 66)	NA	75 (72 - 78)	57 (52 - 62)	67 (65 - 69)	NA	76 (75 - 78)	64 (60 - 69)	69 (66 - 71)
% blood-fed	1 (0 - 2)	1 (0 - 2)	1 (0 - 2)	2 (0 - 2)	1 (0 - 2)	1 (0 - 1)	0 (0 - 2)	1 (0 - 2)	NA	0 (0 - 1)	1 (0 - 2)	0 (0 - 2)	NA	2 (0 - 3)	0	0
%BFI	NA	98.1	97.3	96.9	97.3	98.1	98.1	97.5	NA	98.1	98	98	NA	96.9	100	100
N passed Tunnel	NA	27	30	50	19	30	30	50	NA	27	30	49	NA	27	30	50
% passage	NA	100	100	100	100	100	100	100	NA	100	100	100	NA	95	100	100

All mortality is reported in 24 hours post exposure

Table 5: S11: Results from multilevel mixed effects generalized linear models comparing mortality of **susceptible** *An. gambiae s.s.* between each dual AI/PBO net and standard LLINs of the same age, measured in cone and tunnel test.

	Cone test					Tunnel test			
	Net age	24hrs % Mortality	Odds ratio	95%CI	P-value	% Mortality	Odds ratio	95%CI	P-value
Olyset Plus Vs Interceptor, 24hrs mortality	0M	99% vs 91%	5.22	2.9 - 9.1	<0.001	-	-	-	-
	12M	55% vs 48%	1.24	1.1 - 1.4	0.011	76% vs 75%	1.08	0.4 - 0.9	0.384
	24M	28% vs 24%	1.21	o.9 - 1.5	0.068	64% vs 64%	0.99	0.8 - 1.2	0.872
	36M	13% vs 10%	1.25	0.9 - 1.6	0.064	68% vs 66%	1.09	0.2 - 0.9	0.171
Royal Guard Vs Interceptor, 24hrs mortality	0M	100% vs 99%	4.09	1.7 - 9.7	0.002	-	-	-	-
	12M	73% vs 57%	2.09	1.7 - 2.5	<0.000	75% vs 70%	1.28	1.0 - 1.6	0.023
	24M	26% vs 29%	0.82	0.7 - 0.9	0.035	57% vs 58%	0.96	0.8 - 1.1	0.628
	36M	31% vs 10%	4.69	3.7 - 5.9	<0.000	67% vs 63%	1.14	1.0 - 1.3	0.044
Interceptor G2 Vs Interceptor, 72hrs mortality	0M	59% vs 97%	0.03	0.0 - 0.1	<0.000	92% vs 91%	1.02	0.9 - 1.2	0.767
	12M	49% vs 75%	0.28	0.2 - 0.4	<0.000	91% vs 89%	1.22	1.0 - 1.5	0.032
	24M	43% vs 48%	0.83	0.7 - 0.9	0.023	73% vs 72%	1.09	0.9 - 1.3	0.322
	36M	18% vs 22%	0.8	0.7 - 0.9	0.012	72% vs 73%	0.88	0.8 - 0.9	0.039

Table 5: S12: Results from multilevel mixed effects generalized linear models comparing mortality of **resistant** *An. gambiae s.s.* between each dual AI/PBO net and standard LLINs of the same age, measured in cone and tunnel test.

		Cone test				Tunnel test			
	Net age	24hrs % Mortality	Odds ratio	95%CI	P-value	% Mortality	Odds ratio	95%CI	P-value
Olyset Plus Vs Interceptor, 24hrs mortality	0M	67% vs 7%	35.4	10.9 - 115.0	<0.001	84% vs 26%	15.9	10.8 - 23.4	<0.001
	12M	7% vs 2%	2.2	0.4 - 12.6	0.375	46% vs 40%	1.3	0.9 - 1.7	0.084
	24M	5% vs 1%	0.3	0 - 14.1	0.546	44% vs 21%	3.1	2.3 - 4.2	<0.001
	30M	2% vs 0%	1527981	210996.1	<0.001	44% vs 53%	0.7	0.5 - 0.8	0.001
	36M	6% vs 2%	3.5	0.8 - 16.4	0.109	33% vs 17%	2.8	2.2 - 3.3	<0.001
Royal Guard Vs Interceptor, 24hrs mortality	0M	83% vs 15%	215.6	26.5 - 1753.4	<0.001	-	-	-	-
	06M	58% vs 6%	44.3	8.7 - 225.5	<0.001	-	-	-	-
	12M	41% vs 7%	19.1	4.9 - 73.9	<0.001	63% vs 38%	3.4	2.4 - 4.9	<0.001
	24M	28% vs 3%	14.7	3.1 - 70.9	0.001	58% vs 32%	3.3	2.6 - 4.1	<0.001
	30M	53% vs 3%	154.8	7.6 - 3138.5	0.001	78% vs 29%	8.5	4.7 - 15.4	<0.001
	36M	17% vs 4%	3.6	0.7 - 19.1	0.138				
Interceptor G2 Vs Interceptor, 72hrs mortality	0M					52% vs 26%	3	2.3 - 4.0	<0.001
	12M					34% vs 17%	2.6	1.9 - 3.5	<0.001
	24M					21% vs 14%	1.6	1.2 - 2.1	0.001
	30M					20% vs 38%	0.4	0.3 - 0.5	<0.001
	36M					20% vs 17%	1.2	0.9 - 1.4	0.14

Table 5: S13: Mortality of resistance *An.gambiae* s.s (Muleba-Kis) against Royal Guard

		0M	6M	12M	24M	30M	36M
Cone	Royal Guard	84%	63%	34%	24%	50%	20%
	Interceptor	10%	9%	10%	7%	2%	2%
Tunnel	Royal Guard			63%	58%	78%	
	Interceptor			38%	32%	29%	

Table 5: S14: Percentage mortality induced by pyrethroid (permethrin and alpha-cypermethrin) against resistance (Muleba-Kis) strain

		1X		2X		5X		10X	
		24 hrs	72hrs	24 hrs	72hrs	24 hrs	72hrs	24 hrs	72hrs
Year 1	Permethrin	43%	68%	64%	86%	60%	74%	86%	91%
	Alpha-cypermethrin	58%	71%	64%	80%	85%	97%	94%	97%
Year 3	Permethrin	14%	18%	18%	29%	49%	59%	83%	90%
	Alpha-cypermethrin	66%	69%	81%	83%	87%	89%	96%	97%

Table 5: S15: Relative media potency estimate has been added as supplementary file.

Alpha-cypermethrin against *An.gambiae* s.l								
	Years	Interaction btw years	95% Confidence Limits			95% Confidence Limits with LOG Transform[a]		
			Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	1	2	0.440	0.112	1.171	-0.356	-0.952	0.068
		3	0.298	0.067	0.766	-0.525	-1.171	-0.116
	2	1	2.272	0.854	8.964	0.356	-0.068	0.952
		3	0.678	0.294	1.349	-0.169	-0.532	0.130
	3	2	1.474	0.741	3.406	0.169	-0.130	0.532
		1	3.350	1.305	14.820	0.525	0.116	1.171

Alpha-cypermethrin against *An.funestus* complex								
	Years	Interaction btw years	95% Confidence Limits			95% Confidence Limits with LOG Transform[a]		
			Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	1	2	0.124	0.029	0.284	-0.907	-1.544	-0.546
	2	1	8.075	3.517	34.974	0.907	0.546	1.544

Permethrin against *An.gambiae* s.l								
	Years	Interaction btw years	95% Confidence Limits			95% Confidence Limits with LOG Transform[a]		
			Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	1	2	141.801	0.164	285674.044	2.152	-0.784	5.456
		3	78.301	0.090	142982.225	1.894	-1.046	5.155
	2	1	0.007	3.500E-06	6.083	-2.152	-5.456	0.784
		3	0.552	0.327	0.837	-0.258	-0.485	-0.077
	3	2	1.811	1.195	3.058	0.258	0.077	0.485
		1	0.013	6.994E-06	11.126	-1.894	-5.155	1.046

Permethrin against *An.funestus* complex								
	Years	Interaction btw years	95% Confidence Limits			95% Confidence Limits with LOG Transform[a]		
			Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	1	2	8.682	1.633	84.609	0.939	0.213	1.927
		3	15.335	2.801	186.040	1.186	0.447	2.270
	2	1	0.115	0.012	0.612	-0.939	-1.927	-0.213
		3	1.766	1.140	3.310	0.247	0.057	0.520
	3	2	0.566	0.302	0.878	-0.247	-0.520	-0.057
		1	0.065	0.005	0.357	-1.186	-2.270	-0.447

8 Annex 2: Net Study informed consent agreement

Introduction

Good morning. My name is _____. I work with PAMVERC Malaria Prevention Trial Missungwi. We work together with the Missungwi District Health office, the National Institute for Medical Research, the Kilimanjaro Christian Medical College, London School of Hygiene and Tropical Medicine.

General purpose of the survey

We would like to include you in a study to find information on quality and durability of the LLIN (net) you have been given earlier.

We would like to confirm how long the insecticide will last on the net. This is done by testing the nets in many households in several villages to find out how long they remain effective against malaria-transmitting mosquitoes. The strength of the netting material will be also looked.

Procedure

If you are willing to participate in the study:

- we expect you to not give away or sell the study net;
- you are free to stop using the nets at any time but we would expect you to let investigators know the reasons why you stopped using them during the follow up survey and to allow inspection of the nets;

Your household has been selected for repeated follow up (every 6 months for 3 years). We could take your net away for analysis. These nets will be replaced at that time. I would therefore like to have your consent to be interviewed; this will last about 20-30 minutes. During the interview, I will ask you some questions about your household, the status of the nets given to you or your family members and how you use your net and side effect if any. I will ask you to show the net to me, so I can assess its quality by counting holes in the net. I will not damage the net, and after the interview, I will return it.

Adverse effects, risks and participant protection

There is a remote possibility that you may get malaria even while using nets. This might be possible due to biting of mosquitoes outdoors or early in the night while your family was not sleeping under the net. Thus, if you suffer from fever, you should immediately approach the health staff available in your nearby health centre for treatment of possible malaria, as detailed below.

We are aware that pyrethroid insecticides are being used to treat the nets in the malaria control programme. Permethrin, the insecticide used on the nets, has been tested before and has not been found to have any undue adverse effects in most people at the dose found on the nets. Transitory tingling or runny nose has been recorded when nets are used for the first time when taken from its package. There is no cause for alarm as these effects pass within a day or two

Voluntariness and confidentiality

It is entirely your choice to take part in or not take part in this survey as I have just described it. If you agree to take part, you can also decide not to answer any of the questions that you do not want to. If at any point in time during the study you take the decision not to participate any further, you are free to do so immediately and it will have no consequences, for example, your net will not be taken back from you. However, we will want to ask you question to find out the reason why you decided to no longer use the net. We would also expect you to retain the net until our next visit so we can inspect its condition. Your individual information will be kept private.

Costs and compensation for participating in the study

You will not be asked to pay anything for you to participate in this study. The study will not reimburse you with any payment for taking part in the study.

The London School of Hygiene and Tropical Medicine is the Sponsor and hold insurance policies which apply to this study

Thank you very much for your time. Would you like to take part in this survey?

Consent section

- The study has been explained to me,
- I have been given the opportunity to ask questions concerning this study. Any such questions have been answered to my full satisfaction.
- I understand participation is voluntary and I may revoke this consent at any time without penalty or loss of benefits,

- I agree that the data generated from this study can be used in the future for other malaria related research. Yes |___| No |___|

I agree to take part.

Name of guardian/parent..... Signature/Thumb print .....

Name of the witness.....Signature……

Name of interviewer……...Signature.....

If you have any questions or clarification pertaining to this survey, please feel free to ask the field workers or you may contact Study staff; Mr Eliud Lukole, PAMVERC, 0766240101; Dr Jackline Mosha, NIMR Mwnza, 0754404140; Dr Alphaxard Manjurano, 0756026661;

If you have any questions about your rights as a study patient, or if you think your child has been injured because of this study, please contact the Chairman of the National Health Research Ethics Committee (NatHREC) on 0222 121 400/390]

PROJECT COPY

Cluster Number: ___ ___Household Number: ___ ___ ___ Date: __ __/__ __/__ __

Net Study Informed Consent agreement

Purpose of the survey

You received new LLIN and we would like to confirm how long the insecticide will last on the net. This is done by testing the nets in many households in several villages to find out how long they remain effective against malaria-transmitting mosquitoes. The strength of the netting material will be also looked.

Consent section

- The study has been explained to me,
- I have been given the opportunity to ask questions concerning this study. Any such questions have been answered to my full satisfaction.
- I understand participation is voluntary and I may revoke this consent at any time without penalty or loss of benefits,
- I agree that the data generated from this study can be used in the future for other malaria related research. Yes |___| No |___|

I agree to take part.

Name of guardian/parent.....…….....

Signature/Thumb print .....

Name of the witness.....Signature.....

Name of interviewer…….....Signature.....

If you have any questions or clarification pertaining to this survey please feel free to ask the field workers or you may contact Study staff; Mr Eliud Lukole, PAMVERC, 0766240101; Dr Jackline Mosha, NIMR Mwnza, 0754404140; Dr Alphaxard Manjurano, 0756026661;

If you have any questions about your rights as a study patient, or if you think your child has been injured because of this study, please contact the Chairman of the National Health Research Ethics Committee (NatHREC) on 0222 121 400/390]

9 Annex 3: Volunteers information sheet and consent agreement (Hut trial)

V2.0: 17/02/2020

Introduction

Good morning. My name is _____. We work together with the Magu District Health office, the National Institute for Medical Research, the Kilimanjaro Christian Medical College, London School of Hygiene and Tropical Medicine.

Purpose of the survey

We would like to include you in a study to find out if different new nets products are effective against malaria. Malaria is transmitted by mosquitoes that carry the malaria parasite. The control interventions reduce the number of infected mosquitoes. We want to find out whether the LLINs reduce the number of mosquitoes flying in houses. It will provide information on which new LLINs works best to reduce mosquito numbers and malaria.

Participant Selection

We shall test the net in normal use in our huts. You and other volunteers will sleep under the net from 7 pm to 6 am from Monday to Friday. We can tell if the net is working by looking for dead mosquitoes in the hut in the morning. This evaluation will continue for seven weeks. We shall be regularly changing the nets. Sometimes the net will have been left unwashed, sometimes it will have been washed many times and sometimes nets will have been collected from the community. In this way we can work out if the nets are still working.

We are looking for local village volunteers to participate in the study. As a local you will have been exposed to biting by these mosquitoes. We are wanting to recruit adult males or females who are able to understand the purpose of the study. The persons have to be responsible, and we would like them to help us collect the mosquitoes from the huts in the morning. We are looking for individuals who are able in principle to be available for the entire weeks of the study. You have to be willing to stay in the huts all night long. During the study you would be

expected to sleep away from their families for a full 3 months. If there are more suitable volunteers than positions, we shall select individuals by lot.

Risks and Benefits:

By sleeping under a mosquito net, you will obtain protection similar to what you have in your house if you use a insecticide treated net. But because in one of the huts you will also be sleeping under an untreated net we will monitor your fever every day. In the event that you contract malaria you must be aware of the signs and symptoms (which we shall tell you about), and inform us if you acquire any kind of fever so we can take other measures to treat you. If you have fever or suspected malaria a rapid diagnostic test will be taken for confirmation of parasites, and if positive you will be treated for free with an effective antimalarial drug.

You might also experience some reaction to the insecticide on the net, we request that you tell us about any sensations, side effects, or symptoms that may be due to sleeping under the treated net, so that we can take appropriate medical action. These symptoms may include headache, dizziness, sneezing, itching, tiredness. These side effects, if they occur at all, are temporary and are known to have no long-term consequence. A PAMVERC physician will be on hand to examine you. Any possible allergies to the insecticide formulation pointed out by the volunteers will be treated by the PAMVERC clinical team. You will receive a prophylaxis against malaria during the study.

Other possible discomforts include scratching caused by mosquito bites biting though the net, and blisters, redness or skin irritation caused by the insecticide itself.

Voluntariness and confidentiality

It is entirely your choice to participate in the activities I have just described it. You will not be penalized in any way if you refuse and will still receive all the services you currently receive if you choose not to participate. If at any point in time during the activities you take the decision not to participate any further, you are free to do so immediately, and it will have no consequences. Your individual information will be kept private. You will be given a code number and so any data is in reference to the code number rather than your name. This helps to protect your anonymity.

Costs and compensation for participating in the study

You will be paid a sum of 5000 Tz shilling a night for participating in the study as transport allowance.

Thank you very much for your time. Would you like to participate in the activities?

If you have any questions or clarification pertaining to this project please feel free to ask the field workers or you may contact Study staff; Ms. Jackline Martin, PAMVERC, 07574571391; Dr Jackline Mosha, NIMR Mwanza, 0754404140; Dr Alphaxard Manjurano, 0756026661;

If you have any question of this study, please contact the Chairman of the National Health Research Ethics Committee (NatHREC) on 0222 121 400/390.

Volunteer COPY Date: _____/_____/_____

Volunteers Informed Consent agreement

Purpose of the survey

We are looking for volunteers to participate in a study to find out if different new nets products are effective against malaria. Malaria is transmitted by mosquitoes that carry the malaria parasite. We want to find out whether the new mosquito net reduce the number of mosquitoes entering in houses. It will provide information on which new LLINs works best to reduce mosquito numbers and malaria.

Consent section

- The study has been explained to me,
- I have been given the opportunity to ask questions concerning this study. Any such questions have been answered to my full satisfaction.
- I understand participation is voluntary and I may revoke this consent at any time without penalty or loss of benefits,
- I agree that the data generated from this study can be used in the future for other malaria related research. Yes |___| No |___|

I agree to take part.

Name of participant..... Signature/Thumb print .....

Name of the witness..... Signature……

Name of interviewer…….....Signature.....

If you have any questions or clarification pertaining to this project please feel free to ask the field workers and nurse or you may contact Study staff; Ms. Jackline Martin, PAMVERC, 07574571391; Dr Jackline Mosha, NIMR Mwanza, 0754404140; Dr Alphaxard Manjurano, 0756026661;

If you have any question of this study, please contact the Chairman of the National Health Research Ethics Committee (NatHREC) on 0222 121 400/390.

PROJECT COPY

Cluster Number: ___ ___Household Number: ___ ___ ___ Date: __ __/__ __/__ __

Volunteers Informed Consent agreement

Purpose of the survey

We are looking for volunteers to participate in a study to find out if different new nets products are effective against malaria. Malaria is transmitted by mosquitoes that carry the malaria parasite. We want to find out whether the new mosquito net reduce the number of mosquitoes entering in houses. It will provide information on which new LLINs works best to reduce mosquito numbers and malaria.

Consent section

- The study has been explained to me,
- I have been given the opportunity to ask questions concerning this study. Any such questions have been answered to my full satisfaction.
- I understand participation is voluntary and I may revoke this consent at any time without penalty or loss of benefits,
- I agree that the data generated from this study can be used in the future for other malaria related research. Yes |___| No |___|

I agree to take part.

Name of guardian/parent..... Signature/Thumb print .....

Name of the witness.....Signature……...

Name of interviewer…….....Signature.....

If you have any questions or clarification pertaining to this project please feel free to ask the field workers and nurse or you may contact Study staff; Ms. Jackline Martin, PAMVERC, 07574571391; Dr Jackline Mosha, NIMR Mwanza, 0754404140; Dr Alphaxard Manjurano, 0756026661; If you have any question of this study, please contact the Chairman of the National Health Research Ethics Committee (NatHREC) on 0222 121 400/390.

10 Annex 4: Household questionnaire baseline and adverse events

Malaria Prevention Trial: Misungwi (MRC/WT/DFID)

Household and adverse effect form phase III study durability nets (English) v1.0

Date (dd/mm/yy)	__ __/ __ __/ __ __
Interviewer Initials	|_____|
Identification	
Hamlet Code	|____|____|____|
Ward name	
Village Name	
Hamlet Name	
Cluster Number	|____|_____|
Household number/Unique address	|___|____|___|____|____|____|

READ THE CONSENT

Interview Information

Question description	Options	Answer
Consent result	Consent given	1
	ineligible (does not have PAMVERC net)	2
	Return later	3
	Refused	4
	Dwelling vacant for survey duration	5
	Dwelling not found	6
	Dwelling not visited	7
Total number of visits	|___|	
Comments		

Number of PAMVERC project LLIN

No.	Question description	Options	Answer	If	Goto
6	How many PAMVERC project LLIN that can be used for sleeping does your household have? (Probe for any nets currently not in used, stored, saved, still in packaging)	Number Nets	_ _	**Net >0** **Net=0 or DNK**	**7** **End quest**
7	Date of receipt of LN?	Saturday 26 January 2019	26/01/2019		
		Sunday 27 January 2019	27/01/2019		
		Monday 28 January 2019	28/01/2019		
		Other (free date)	__/__/____		
		Don't know	99		

Socio Economical Status

No.	Question description	Options	Answer	If	Goto
7b	**Name of the household head**	**Text**			
8	Who is responding to the questions?	**Check box**			
		Head of the household / Wife	1		
		Other adult in the household	2		
8b	How many people slept in your household last night? (99 if don't know)	Adults > 15 years	|__|__|		
		5-15 years	|__|__|		
		< 5 years	|__|__|		
9	Has the head of the household ever attended school?	Yes	1		
		No	0	0	**11**
		Don't Know	98	98	**11**
10	What is the highest level of school the head of the household attended:	Primary	1		
		Secondary/technic	2		
		Higher	3		
		Don't Know	98		
11	Has the head of the household WIFE ever attended school?	Yes	1		
		No	0	0	**13**
		Not Applicable	2	2	**13**
		Don't Know	98	98	**13**
12	What is the highest level of school the head of the household WIFE attended:	Primary	1		
		Secondary/technic	2		
		Higher	3		
		Don't Know	98		
13	How many rooms are there in this household? >>***Include all structures (huts etc)***	Number (Don't Know = 98)	_ _		
14	How many rooms in this household are used for sleeping?	Rooms number	|_____|		
15	How many sleeping places were used last night in your households (beds, mattresses or mats)? ***>>Ask for both inside the hut and outside***	Number	_ _		
16	What is the main material of the roof? (observed)	Grass/Papyrus/leaves	1		**17**
		Metal sheets	2		
		Metal sheets & Grass/Papyrus/leaves	3		
		Other	4	4	**16b**
16b	If Other type of roof specify	Free text			
17	What is the main material of the floor?	earth/sand	1	1	**18**
		cement	2	2	
		Earth/sand & Cement	3	3	

17b	If Other type of floor specify	Free text			
18	What is the main material of the walls?	Grass/leaves	1	1	**18**
		Mud	2	2	
		brick	3	3	
		Brick and Mud	4	4	
		wood	5	5	
		plastic sheeting	6	6	
		Other	7	7	**18b**
18b	If Other type of wall specify	Free text			
19	Are the walls plastered?	yes completely	1	1	
		partially/damaged	2	2	
		no	0	0	
20	Are eaves open? (Is there is a gap between the top of the wall and the roof?)	Yes	1	1	
		No	0	0	
21	Does the house have a ceiling?	Intact	1	1	
		Damage/Partial/traditional	2	2	
		No ceiling	0	0	
22	Where does the Households main income come from?	Fishing/Farming/Selling cash crops	1	1	**23**
		Mining	2	2	
		Buisness/Shop	3	3	
		Medical/Teacher/Goverment	4	4	
		Other	5	5	**22b**
22b	If Other kind of income specify	Free text			
23	Does the household (any member) have any of the following	**Check box**			
		Electricity	|__|		
		Radio	|__|		
		mobile phone	|__|		
		Bicycle	|__|		
		Motorbike	|__|		
		Car or truck	|__|		
		Canoe or boat with motor	|__|		
		Sewing machine	|__|		
		Livestock	|__|		
		Television	|__|		
		Canoe or Boat without motor	|__|		
24	What is the main source of drinking water for members of your household? *(choose only one)*	**Piped water**			
		Piped into dwelling	1		
		Piped to yard/plot	2		
		Piped to neighbor	3		
		Public tap/standpipe	4		
		Dug well			
		Protected well	5		
		Unprotected well	6		
		Water From Spring	7		
		Rainwater	8		
		Surface Water (River/Dam/Lake/Pond/Stream	9		
		Other, Specify_____			

25	What kind of toilet facility do members of your household usually use?	Flush Toilet	1		
		Ventilated Inmproved Pit Latrine	2		
		Traditional Pit Latrine	3		
		None/bush	4		
		Other, Specify_____			
26	What type of fuel does your household mainly use for cooking?	Firewood/straw	1		
		Charcoal	2		
		LPG/Natural gas	3		
		Biogas	4		
		Dung	5		
		Electricity	6		
		Paraffin	7		
		Other, Specify_____			
27	Does the household own land used for farming?	Yes	1		
		No	0		

FIELD WORKER TO LABEL NET (HOUSEHOLD ID + NET ID (2 DIGITS))

Number of PAMVERC project LLIN selected for phase III

No.	Question description	Options	Answer	If	Goto
30	How many PAMVERC project LLIN does the field worker selected for the study? (PAMVERC LLIN in use)	Number Nets	_ _	Net >0 Net=0 or DNK	31 End quest

GO TO NET SUB_FORM

Information on all selected net (each net)

No.	Question description	Options	Answer	If	Goto
31	What is the net identification?	uniq id (2 digits)	|___|___|		
32	What is the LLIN code *(look at the label)*	P8X01MS-LNBE	1		
		58147523	2		
		58574747	3		
		1.CL88190.1.BL.08.18	4		
33	How does the loop look like? (*observed*)	White piece of netting	1		
		Blue piece of netting	2		
		Blue ribbon	3		
		Pink ribbon	4		
34	Is the net selected for adverse effects?	Yes	1	1	35
		No	0	0	Go to next net or end questionnaire

Adverse effects (for each PAMVERC LLIN selected for the study)					
No.	**Question description**	**Options**	**Answer**	**If**	**Goto**
35	Who is responding to the questions about adverse events? *(If children under 10 ask guardian parents, between 11 and 18 asked children in presence of parents/guardian)*	**Check box (Yes)**			
		Head of the household	|__|		
		User of the net	|__|		
		Parent or guardian of users of nets	|__|		
		Other adult in the household	|__|		
36	Number of people sleeping under this net	Number	__ __		
37	Is the net used every night since the nets was received?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
38	Any itching of the skin or paraesthesia?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
39	Any facial burning?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
40	Any sneezing?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
41	Any liquid discharge from the noze?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
42	Feeling headache?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
43	Any nausea?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
44	Eye irritation?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
45	Tears coming from the eyes?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
46	Experienced bad smell using nets?	Yes	1		
		No	0		
		Don't Know/ no answer	98		
47	Any other symptoms? Please specify	Free text			
48	Have you reported this symptoms to a physician/nurses?	Yes	1	If the respondent answers positively to 37 to 45b ask this question	
		No	0		
		Don't Know/ no answer	98		
GO TO NEXT PAMVERC LLIN LABELLED OR END QUESTIONNAIRE					

11 Annex 5: Net follow up survey

Malaria Prevention Trial: Misungwi (MRC/WT/DFID)

Net follow up study (English) v2.0 08/02/2019

Questions on each net

LN Status									
No.	**Question**	**Mosquito net 1**		**Mosquito net 2**		**Mosquito net ….**	
26	**Cluster**	|___|___|		|___|___|		|___|___|	
26b	**Hamlet code**	|___|___|___|		|___|___|___|		|___|___|___|	
27	**Household Number**	|___|___|___|		|___|___|___|		|___|___|___|	
28	Net identification number (LOOK ON THE MASTER LIST)	|___|___|		|___|___|		|___|___|	
29	Is this net still in possession of the household?	Yes	1	Yes	1	Yes	1
		No	0	No	0	No	0
		Yes goto	**32**				
30	If No, what was the reason for the loss?	Net was damaged and thrown away	1	Net was damaged and thrown away	1	Net was damaged and thrown away	1
		Net was given away to others	2	Net was given away to others	2	Net was given away to others	2
		Net was stolen	3	Net was stolen	3	Net was stolen	3
		Net was sold	4	Net was sold	4	Net was sold	4
		Net is being used in another location	5	Net is being used in another location	5	Net is being used in another location	5
		Other, specify	7	Other, specify	7	Other, specify	7
30b	If the response to 29 is other specify	Text		Text		text	
31	How many months ago did this net get lost?	Months		Months		Months	
	Enter "00" for months if less than one month	Don't remember	98	Don't remember	98	Don't remember	98
		GO TO NEXT NET					

LN Use and Handling							
No.	**Question**	**Mosquito net 1**		**Mosquito net 2**		**Mosquito net 3**	
32	Was the net observed by the FW	Yes	1	Yes	1	Yes	1
		No	0	No	0	No	0
		IF NO GO TO NEXT NET					
33	Which type of Nets	P8X01MS-LNBE	1	P8X01MS-LNBE	1	P8X01MS-LNBE	1
		58147523	2	58147523	2	58147523	2
		58574747	3	58574747	3	58574747	3
		1.CL88190.1.BL.08.18	4	1.CL88190.1.BL.08.18	4	1.CL88190.1.BL.08.18	4
34	How many nights has this net been used in the last week (the last 7 days)?	Every night (7 nights)	1	Every night (7 nights)	1	Every night (7 nights)	1
		Most nights (5-6)	2	Most nights (5-6)	2	Most nights (5-6)	2
		Some nights (1-4)	3	Some nights (1-4)	3	Some nights (1-4)	3
		Not used last week	4	Not used last week	4	Not used last week	4
		Net is not used at all	5	Net is not used at all	5	Net is not used at all	5
		Don't know	98	Don't know	98	Don't know	98
		if net not used last week or at all go	**38**			**Otherwise got to**	**35**
35	During which periods of the year is this net used to sleep under?	All year	1	All year	1	All year	1
		Only the rainy season	2	Only the rainy season	2	Only the rainy season	2
		Only the dry season	3	Only the dry season	3	Only the dry season	3
		Don't know	98	Don't know	98	Don't know	98
36	Was this net used by any person last night?	Yes	1	Yes	1	Yes	1
		No	0	No	0	No	0
		Don't Know	98	Don't Know	98	Don't Know	98
		No goto	**38**	**DNK goto**	**40**		
37	Who used this net last night?	Line number of users (Q01)		Line number of users (Q01)		Line number of users (Q01)	
		Sex: F / M Age (yrs): ------ Pregnancy: Y/N		Sex: F / M Age (yrs): ------ Pregnancy: Y/N		Sex: F / M Age (yrs): ------ Pregnancy: Y/N	
	>> probe for any additional person using this net last night	Sex: F / M Age (yrs): ------ Pregnancy: Y/N		Sex: F / M Age (yrs): ------ Pregnancy: Y/N		Sex: F / M Age (yrs): ------ Pregnancy: Y/N	
		Sex: F / M Age (yrs): ------ Pregnancy: Y/N		Sex: F / M Age (yrs): ------ Pregnancy: Y/N		Sex: F / M Age (yrs): ------ Pregnancy: Y/N	

38	Why was the net not used	**I can't use it answer**		**I can't use it answer**		**I can't use it answer**	
		No space	1	No space	1	No space	1
		No sleeping place to cover	2	No sleeping place to cover	2	No sleeping place to cover	2
		Being washed	3	Being washed	3	Being washed	3
		Usual user(s) did not sleep here	4	Usual user(s) did not sleep here	4	Usual user(s) did not sleep here	4
		Don't know how to use it answer		**Don't know how to use it answer**		**Don't know how to use it answer**	
		Don't know to hang or use	5	Don't know to hang or use	5	Don't know to hang or use	5
		Don't want to use it answers		**Don't want to use it answers**		**Don't want to use it answers**	
		No mosquitoes now	6	No mosquitoes now	6	No mosquitoes now	6
		Too hot	7	Too hot	7	Too hot	7
		Feel "closed in" or afraid	8	Feel "closed in" or afraid	8	Feel "closed in" or afraid	8
		Net too old or torn	9	Net too old or torn	9	Net too old or torn	9
		Net too dirty	10	Net too dirty	10	Net too dirty	10
		The house has been sprayed	11	The house has been sprayed	11	The house has been sprayed	11
		Afraid of Fire	12	Afraid of Fire	12	Afraid of Fire	12
		Net not hung	13	Net not hung	13	Net not hung	13
		Used for other purpose	14	Used for other purpose	14	Used for other purpose	14
		Don't know	98	Don't know	98	Don't know	98
		Other	15	Other (specify below)	15	Other (specify below)	15
		If Other goto 38b		**Otherwise Go to 39**			
38b	If the response to why net not used is other specify	Text		Text		text	
39	Where did you found the net not used (observe)	Still in the package (never used)	1	Still in the package (never used)	1	Still in the package (never used)	1
		Hang	2	Hang	2	Hang	2
		Store away	3	Store away	98	Store away	98
		If still in package "END NET QUEST"		**If Hang goto**	**40**	**If Store away goto**	**46**
40	Do you tuck the net in at night?	Yes	1	Yes	1	Yes	1
		No	2	No	2	No	2
		Don't know	98	Don't know	98	Don't know	98
41	How is the net found? (observe)	Hanging loose over sleeping place	1	Hanging loose over sleeping place	1	Hanging loose over sleeping place	1
		Hanging tied in knot	2	Hanging tied in knot	2	Hanging tied in knot	2
		Hanging folded	3	Hanging folded	3	Hanging folded	3
		Visible but not hung up	4	Visible but not hung up	4	Visible but not hung up	4
		Store away	5	Store away	5	Store away	5
42	What type of bed is the net hanging over? (observe)	Wooden or iron bedframe (improved) [mbao, chuma, kimetengenezwa na fundi]	1	Wooden or iron bedframe (improved) [mbao, chuma, kimetengenezwa na fundi]	1	Wooden or iron bedframe (improved) [mbao, chuma, kimetengenezwa na fundi]	1
		Stick bedframe [mjiti, kimetengenezwa huko]	2	Stick bedframe [mjiti, kimetengenezwa huko]	2	Stick bedframe [mjiti, kimetengenezwa huko]	2
		No bedframe	3	No bedframe	3	No bedframe	3
		Other, specify	4	Other, specify	4	Other, specify	4
42b	If the response to "what type of bed is the net hanging over" is other specify	Text		Text		text	
43	What type of mattress/sleeping material is used with this net? (observe)	Reed mat (mkeka)	1	Reed mat (mkeka)	1	Reed mat (mkeka)	1
		Grass	2	Grass	2	Grass	2
		Foam/spring mattress	3	Foam/spring mattress	3	Foam/spring mattress	3
		Bare flour or ground	4	Bare flour or ground	4	Bare flour or ground	4
		Clothes/other net/material	5	Clothes/other net/material	5	Clothes/other net/material	5
		Other, specify	6	Other, specify	6	Other, specify	6
43b	If the response to "what type of bed is the net hanging over" is other specify	Text		Text		text	
44	Do you use an open flame for cooking, heating or lighting where the net is found?	Yes	1	Yes	1	Yes	1
		No	0	No	0	No	0
		Don't Know	98	Don't Know	98	Don't Know	98
		Yes goto 45		**No & DNK goto 46**			
45	If yes which type of open flame are you using	Wood fire	1	Wood fire	1	Wood fire	1
		Charcoal fire	2	Charcoal fire	2	Charcoal fire	2
		Wax candle	3	Wax candle	3	Wax candle	3
		Oil lamp with a glass	4	Oil lamp with a glass	4	Oil lamp with a glass	4
		Oil lamp without a glass	5	Oil lamp without a glass	5	Oil lamp without a glass	5
		Other, specify	6	Other, specify	6	Other, specify	6
46	Has the net ever been washed?	Yes	1	Yes	1	Yes	1
		No	2	No	2	No	2
		Don't know	98	Don't know	98	Don't know	98
		Yes goto 47		**No & DNK goto 52**			

47	When was the last time you washed the net	less than 1 week ago	1	less than 1 week ago	1	less than 1 week ago	1
		1 week to 1 month ago	2	1 week to 1 month ago	2	1 week to 1 month ago	2
		1-3 months ago	3	1-3 months ago	3	1-3 months ago	3
		3-6 months ago	4	3-6 months ago	4	3-6 months ago	4
		> 6 months ago	5	> 6 months ago	5	> 6 months ago	5
		Don't know	98	Don't know	98	Don't know	98
48	What type of soap was used?	None	1	None	1	None	1
		Local bar soap	2	Local bar soap	2	Local bar soap	2
		Detergent powder (e.g. OMO)	3	Detergent powder (e.g. OMO)	3	Detergent powder (e.g. OMO)	3
		Mix (bar and detergent)	4	Mix (bar and detergent)	4	Mix (bar and detergent)	4
		Bleach	5	Bleach	5	Bleach	5
		Don’t know	98	Don’t know	98	Don’t know	98
49	How long did the net soak for?	Did not soak the net	1	Did not soak the net	1	Did not soak the net	1
		< 1 h	2	< 1 h	2	< 1 h	2
		> 1 h	3	> 1 h	3	> 1 h	3
		Don’t know	98	Don’t know	98	Don’t know	98
50	Was the net scrubbed hard or beaten on a hard surface (e.g. rocks, with sticks)?	Yes	1	Yes	1	Yes	1
		No	2	No	2	No	2
		Don't know	98	Don't know	98	Don't know	98
51	Where was the net dried?	Outside in the sun	1	Outside in the sun	1	Outside in the sun	1
		Outside in the shade	2	Outside in the shade	2	Outside in the shade	2
		Inside	3	Inside	3	Inside	3
		Don’t know	98	Don’t know	98	Don’t know	98
LN Condition							
52	Aspect of the net (observe)	Clean	1	Clean	1	Clean	1
		A bit dirty	2	A bit dirty	2	A bit dirty	2
		Dirty	3	Dirty	3	Dirty	3
		Very dirty	4	Very dirty	4	Very dirty	4
53	In the past month, have any new holes appeared in the net that you are aware of?	Yes	1	Yes	1	Yes	1
		No	0	No	0	No	0
		Don’t Know	98	Don’t Know	98	Don’t Know	98
		No / Don't know goto	**55**				
54	What caused these new holes?	Tore or split when caught on an object	1	Tore or split when caught on an object	1	Tore or split when caught on an object	1
		Was burned	2	Was burned	2	Was burned	2
		Was caused by animals	3	Was caused by animals	3	Was caused by animals	3
		Children	4	Children	4	Children	4
		Don't know	5	Don't know	5	Don't know	5
		In another way, specify	6	In another way, specify	6	In another way, specify	6
54b	If the response to "What caused these new hole" is other specify	Text		Text		text	
55	Does the net has any hole (observe)	Yes	1	Yes	1	Yes	1
		No	0	No	0	No	0
		IF No GO TO NEXT NET QUESTIONNAIRE					
56	ZONE 1 (TOP PANNEL) Number of holes size 1	Not larger than a finger		Not larger than finger		Not larger than a finger	
57	ZONE 1 (TOP PANNEL) Number of holes size 2	Larger than finger but not larger than hand width		Larger than finger but not larger than hand width		Larger than finger but not larger than hand width	
58	ZONE 1 (TOP PANNEL) Number of holes size 3	Larger than hand width but smaller than head		Larger than hand width but smaller than head		Larger than hand width but smaller than head	
59	ZONE 1 (TOP PANNEL) Number of holes size 4	Larger than head		Larger than head		Larger than head	
60	ZONE 2 Number of holes size 1	Not larger than a finger		Not larger than finger		Not larger than a finger	
61	ZONE 2 Number of holes size 2	Larger than finger but not larger than hand width		Larger than finger but not larger than hand width		Larger than finger but not larger than hand width	
62	ZONE 2 Number of holes size 3	Larger than hand width but smaller than head		Larger than hand width but smaller than head		Larger than hand width but smaller than head	
63	ZONE 2 Number of holes size 4	Larger than head		Larger than head		Larger than head	
64	ZONE 3 Number of holes size 1	Not larger than a finger		Not larger than finger		Not larger than a finger	
65	ZONE 3 Number of holes size 2	Larger than finger but not larger than hand width		Larger than finger but not larger than hand width		Larger than finger but not larger than hand width	
66	ZONE 3 Number of holes size 3	Larger than hand width but smaller than head		Larger than hand width but smaller than head		Larger than hand width but smaller than head	

67	ZONE 3 Number of holes size 4	Larger than head		Larger than head		Larger than head	
68	ZONE 4 (Bottom pannel) Number of holes size 1	Not larger than a finger		Not larger than finger		Not larger than a finger	
69	ZONE 4 (Bottom pannel) Number of holes size 2	Larger than finger but not larger than hand width		Larger than finger but not larger than hand width		Larger than finger but not larger than hand width	
70	ZONE 4 (Bottom pannel) Number of holes size 3	Larger than hand width but smaller than head		Larger than hand width but smaller than head		Larger than hand width but smaller than head	
71	ZONE 4 (Bottom pannel) Number of holes size 4	Larger than head		Larger than head		Larger than head	
72	ROOF Number of holes size 1	Not larger than a finger		Not larger than finger		Not larger than a finger	
73	ROOF Number of holes size 2	Larger than finger but not larger than hand width		Larger than finger but not larger than hand width		Larger than finger but not larger than hand width	
74	ROOF Number of holes size 3	Larger than hand width but smaller than head		Larger than hand width but smaller than head		Larger than hand width but smaller than head	
75	ROOF Number of holes size 4	Larger than head		Larger than head		Larger than head	
76	What types of hole are observed?	Hole cause by tears	1	Hole cause by tears	1	Hole cause by tears	1
		Holes at hanging points	2	Holes at hanging points	2	Holes at hanging points	2
		Open seams	3	Open seams	3	Open seams	3
		Burn holes	4	Burn holes	4	Burn holes	4
		Holes from rodents	5	Holes from rodents	5	Holes from rodents	5
		Whole section missing	6	Whole section missing	6	Whole section missing	6
77	Number of holes repaired (observe)	Total		Total		Total	
78	Have you tried to fix any holes in the net	Yes	1	Yes	1	Yes	1
		No	2	No	2	No	2
		Don't know	98	Don't know	98	Don't know	98
		IF No GO TO	**80**	**If yes GOTO**	**79**	**DON'T KNOW GOTO NEXT NET OR END**	
79	How did you repair the hole?	Stitched	1	Stitched	1	Stitched	1
		Knotted/tied	2	Knotted/tied	2	Knotted/tied	2
		Patched	3	Patched	3	Patched	3
		Other way, specify	4	Other way, specify	4	Other way, specify	4
79b	If the response to How did you repair the hole is other way specify	Text		Text		text	
80	If not, what was the main reason?	Too busy/no time	1	Too busy/no time	1	Too busy/no time	1
		Not necessary, the net is still good	2	Not necessary, the net is still good	2	Not necessary, the net is still good	2
		Don’t know how to fix	3	Don’t know how to fix	3	Don’t know how to fix	3
		Too damaged to fix	4	Too damaged to fix	4	Too damaged to fix	4
		Other, specify	5	Other, specify	5	Other, specify	5
80b	If the response to "If not, what was the main reason" is other way specify	Text		Text		text	
		AFTER ANSWER GO TO NEXT NET or END QUESTIONNAIRE					

12 Annex 6: SOP ovary dissection and estimation of oviposition inhibition

Standard Operation Procedure (SOP) for dissection and estimation of oviposition inhibition in resistance *Anopheles* colony strain after exposure to Pyriproxyfen/pyrethroid treated nets

Protocol Code:	
Revision number:	01
First edition prepared by:	Jackline Martin and Nancy Matowo
First edition approved by:	Natacha Protopopoff
Date first released:	
Date last revised:	06/07/2019
Protocol effectives from:	
Protocol expires on:	
For use by:	
For usein (Distribute to):	
Related forms:	
Related documents:	2014 Methods in Anopheles Research (MR4)

1. Aim

Assess the efficacy of pyriproxyfen incorporated in dual insecticide treated nets on the fertility of resistant Anopheles strain.

2. *Anopheles* tested

Adult female mosquitoes of the *An.gambiae* s.s. Muleba kis (kdr east & MFO) will be obtained from a laboratory culture maintained at KCMUCo. Two to five day old blood-fed female mosquitoes will be used in bioassays.

3. Treatment

- LN Royal Guard
- Untreated Net
- Standard LN: Interceptor G2

4. Outcomes

- Kd60, mortality 24, 48, 72 hours post exposure
- Percentage of dissected females with under-developed ovaries at 72 hours post feeding (i.e. the follicles failed to completely develop from previtellogenic resting stage I to maturity stage V).
- Proportion of dissected females with deformed eggs (eggs not fully developed, at stage II-IV)
- Average number of eggs in the ovaries at 72 hours' posts feeding.

5. Materials for dissection

First prepare all the material for dissection

- Dissecting microscope
- Slides
- Marker pen
- Dissecting kit
- Distilled water
- Compound microscopy
- Lamp

- Record form
- Pen and pencil
- Slide box
- Chloroform
- Cotton wool
- Plastic pipette
- Beaker/paper cup

6. Methods

Cone bioassay will be performed according standard WHO procedure. Five freshly blood fed *Anopheles* RSP will be introduced into each cone. Twenty to twenty-five replicates of five mosquitoes will be tested. After a 3 minutes exposure, mosquitoes will be transferred into labelled paper cups covered with untreated netting. When the testing is finished mosquitoes will be provided with 10% sugar solution. Knock down after 60 minutes and mortality at 24, 48, 72 hours will be recorded.

All alive gravid female Anopheles mosquitoes at 72hrs post-esposure will be anesthetized at -20°C freezer condition for five up to ten minutes before dissection. Mosquitoes found dead and gravid after scoring mortality will also be dissected. Prior to dissection, female Anopheles species will be morphologically identified by taxa i.e. separating *An. gambiae* sl from *An. funestus* group as per the identification key procedures by Gillies & Coetzee (refer SOP for identification of Anopheles mosquitoes by Gillies and Coetzee).

The investigators conducting dissection should be blinded from the treatment. Individual gravid female Anopheles mosquitoes will be dissected by gently pulling out the last two segments of the abdomen under a stereoscopic microscope at 0.7x magnification as described previously on the SOP MiS004 and procedures by Detinova et al (249). Follicular ovaries and eggs development stages will be further examined and observed under a compound microscope at 4x or 10x magnification power. Detailed procedures for ovarian dissection of female mosquitoes are as follow;

a. <u>Procedure</u>

- Prepare and label the dissecting slide.

- Record the status and age of the mosquitoes at dissection in a dissection form. All mosquitoes at 72hrs post-collection should be gravid at the time of dissection. However, any dead mosquito before 72hrs post-collection should be also dissected
- Mount a freshly killed gravid female mosquito on either its left or back side on a slide with its abdomen pointing to the right
- Use a dissecting needle on the thorax to hold the mosquito stationary while separate the abdomen from the head and thorax
- Add a drop of distilled water on the last two segments, 6^{th} and 7^{th} sternites of the mosquito abdomen (figure 1)
- Place the slide under a stereoscopic dissecting microscope (at a low magnification power 0.7x), using a dark background
- Gently pulling off the last two segments of the mosquito abdomen using a dissecting needle on the right
- Use the needle to separate the ovaries from other internal material. For better visualization of the ovarian follicles, it is important that while separating the ovaries to cut on the common oviduct instead of separating from lateral oviduct
- Wash off fat and other debris by rinsing the ovaries with distilled water
- Leave the slide with dissected ovaries and eggs to air dry

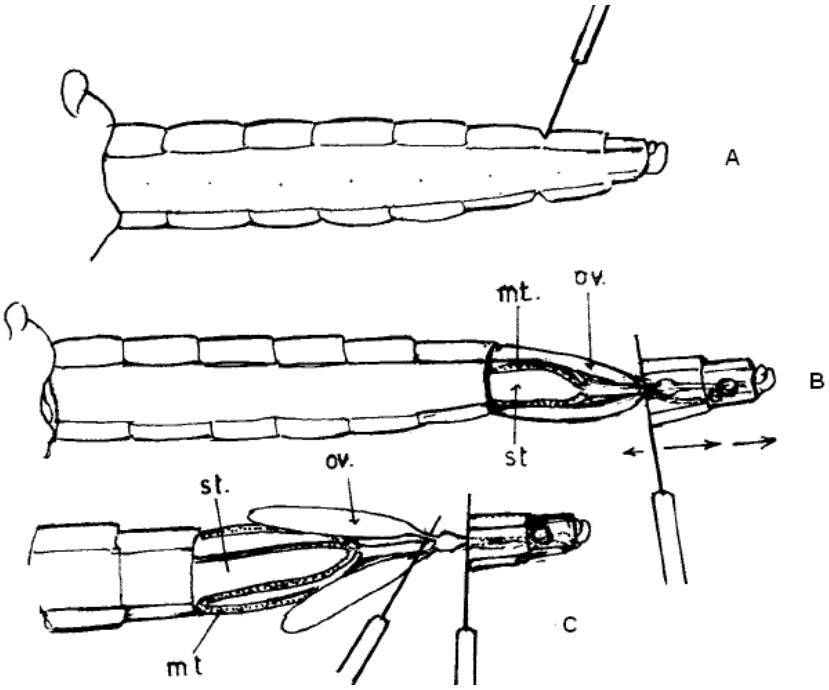


Figure 1. Extraction of ovaries. *ov* = *ovary*, *st* = *stomach*, *mt* = *malpigian tubules*

b. Scoring Egg maturation and development

The standard Christopher scale will be used to score the development stage of the eggs (figure 2)

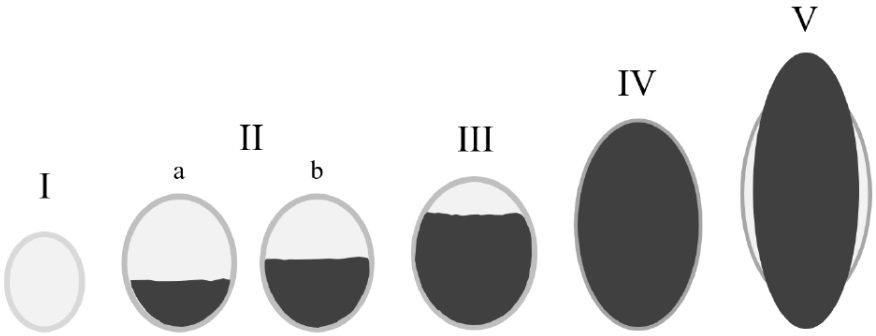


Figure 1. Christophers' Stages of Egg Development. Stage I: the primary follicle. Stage II: a) the follicle gains yolk protein, b) the follicle is approximately half comprised of yolk. Stage III: the follicle fills with yolk. Stage IV: the follicle elongates. Stage V: complete maturation of the egg, with floats.

Figure 2: Showing Christopher's phases of egg development in mosquito ovary (250)

- You can observe the ovaries and eggs under the dissecting microscope but it is recommended to examining and viewing the mosquito ovaries eggs and under a finer compound microscope at either 4x or 10x magnification power. Sometimes you may need to add a small drop of water for better visualization
- Record the image showing developmental status of ovaries and eggs (Figure 2 and 3) in a mosquito dissection form**.** Take photographs with a camera microscope and save the images into a tablet PC. Please remember to rename each image corresponding to the label on the dissecting slide. Second readings should be done separately by a different investigator

- Classify and interpret the developmental status of ovarian follicles/eggs according to Christopher's stage of egg development (235, 250). Females Anopheles mosquitoes are categorized as **"fertile"** if the eggs have fully developed (normal elongated, boat-shaped/sausage shape with floats) and **"infertile"** if the eggs do not undergone fully development (less elongated/spherical shape) (Figure 1 and 2)

- Using counter to score and record the number of underdeveloped eggs which will be found retained in the follicular ovaries. Also the number of developed eggs should be scored and recorded. Second count should be done separately by a different investigator

- After dissection, the remaining mosquito parts including wings, legs, head and thorax will be discarded.

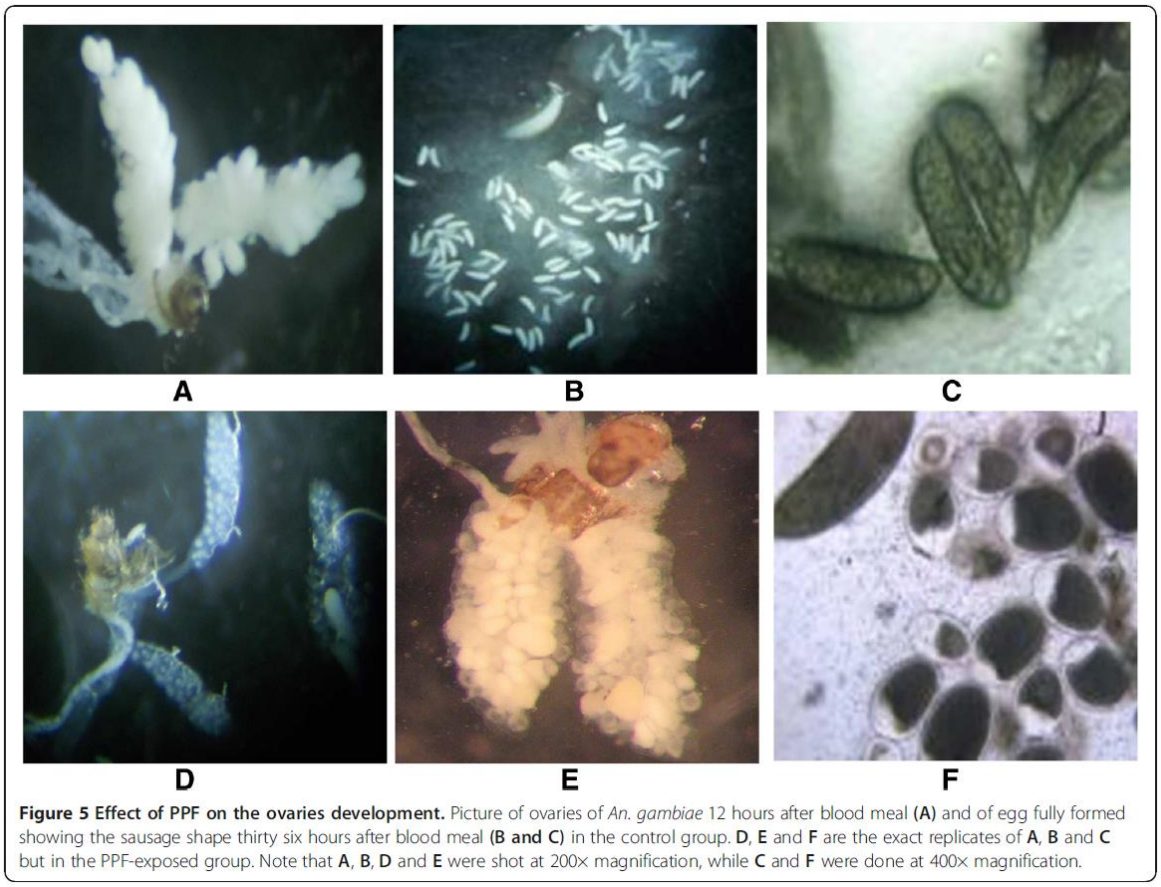


Figure 5 Effect of PPF on the ovaries development. Picture of ovaries of *An. gambiae* 12 hours after blood meal **(A)** and of egg fully formed showing the sausage shape thirty six hours after blood meal **(B and C)** in the control group. **D**, **E** and **F** are the exact replicates of **A**, **B** and **C** but in the PPF-exposed group. Note that **A**, **B**, **D** and **E** were shot at 200× magnification, while **C** and **F** were done at 400× magnification.

Figure 3: Pictorial presentation of the status of ovarian follicles and eggs development in female *Anopheles gambiae* exposed to PPF compare to unexposed mosquitoes (adapted from Koama et al (235))

13 Annex 7: Scientific papers that I co-author during my PhD

Lukole et al. Malaria Journal (2024) 23:199
https://doi.org/10.1186/s12936-024-05020-y

Malaria Journal

RESEARCH Open Access

Will a lack of fabric durability be their downfall? Impact of textile durability on the efficacy of three types of dual-active-ingredient long-lasting insecticidal nets: a secondary analysis on malaria prevalence and incidence from a cluster-randomized trial in north-west Tanzania

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Abstract

Background The Dual-Active Ingredient long-lasting insecticidal nets (Dual-AI LLIN) have been developed to counteract the reduced efficacy of pyrethroid (PY)-only nets due to widespread pyrethroid insecticide resistance in malaria vector mosquitoes. They constitute half of the nets distributed in sub-Saharan Africa between 2022 and 2024. However, their effectiveness once they develop holes is unclear, particularly in pyrethroid-resistant settings. This study evaluates the textile integrity of three dual- AI LLINs compared to standard PY LLN, over 3 years of use in a community in Tanzania and the associated impact on malaria prevalence and incidence.

Methods A secondary analysis of data from a randomized controlled trial (RCT) in North-western Tanzania was conducted to evaluate the effectiveness of α-cypermethrin only; pyriproxyfen and α-cypermethrin (PPF-PY); chlorfenapyr and α-cypermethrin (chlorfenapyr-PY); and the synergist piperonyl butoxide and permethrin (PBO-PY) LLINs on malaria infection prevalence and case incidence. The association between the net textile condition and 1/ malaria prevalence over 3 years of use between 2019 and 2022, and 2/malaria case incidence in a cohort of children over 2 years of follow-up was assessed between 2019 and 2021.

Results There was no significant association between damaged (OR 0.98, 95% CI 0.71–1.37, p-value =0.655) and too-torn (OR 1.07, 95% CI 0.77–1.47, p-value = 0.694) compared to intact nets on malaria prevalence for all net types.

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Tropical Medicine and Infectious Disease

MDPI

Article

Evaluation of Durability as a Function of Fabric Strength and Residual Bio-Efficacy for the Olyset Plus and Interceptor G2 LLINs after 3 Years of Field Use in Tanzania

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Abstract: Long-lasting insecticidal nets (LLINs) are prone to reduction in insecticide content and physical strength due to repeated washes and usage. The significant loss to these features jeopardizes their protection against bites from malaria vectors. Insecticide washout is attributed to routine use, friction, and washing, while fabric damage is associated with routine use in households. To maintain coverage and cost-effectiveness, nets should maintain optimal bio-efficacy and physical strength for at least 3 years after distribution. In this study, the bio-efficacy and fabric strength of Olyset plus (OP) LLINs and Interceptor G2 (IG2), that were used for 3 years, were assessed in comparison to untreated and new unwashed counterparts. Both IG2 and OP LLINs (unused, laboratory-washed, and 36 months used) were able to induce significant mortality and blood feeding inhibition (BFI) to mosquitoes compared to the untreated nets. Significantly higher mortality was induced by unused IG2 LLIN and OP LLIN compared to their 36-month-old counterparts against both pyrethroid resistant and susceptible *Anopheles gambiae* sensu strito. The physical strength of the IG2 LLIN was higher than that of the Olyset Plus LLIN with a decreasing trend from unwashed, laboratory-washed to community usage (36 months old). Malaria control programs should consider bio-efficacy and physical integrity prior to an LLINs' procurement and replacement plan.

Keywords: long-lasting insecticidal nets; fabric strength; bio-efficacy; fabric damage; chemical and physical protection

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1. Introduction

Long-lasting insecticidal nets (LLINs) are the main vector control tools for malaria prevention, and are responsible for significant malaria reduction in sub-Saharan Africa over the past two decades [1,2]. Between 2000 and 2019, WHO reported 1.5 billion malaria cases and 7.6 million deaths being averted [3]. The LLINs provide personal protection by preventing human–vector contact through a physical barrier [4], and providing a killing effect, repelling, sterilizing or inhibiting blood feeding through their insecticide component [5]. It is evident that the LLIN maintains its bio-efficacy (chemical protection) even when the LLIN is physically damaged [5]. Consequently, LLINs have been regarded as a main vector control tool against malaria vectors [6,7]. As a result, more than 1.9 billion Insecticide-treated nets (ITNs) were deployed in sub-Saharan Africa between 2004 and 2019 [3]. However, malaria reduction has stalled in the past few years [8] due, in part, to

Effects of next-generation, dual-active-ingredient, long-lasting insecticidal net deployment on insecticide resistance in malaria vectors in Tanzania: an analysis of a 3-year, cluster-randomised controlled trial

Louisa A Messenger, Nancy S Matowo, Chad L Cross, Mohamed Jumanne, Natalie M Portwood, Jackline Martin, Eliud Lukole, Elizabeth Mallya, Jacklin F Mosha, Robert Kaaya, Oliva Moshi, Bethanie Pelloquin, Katherine Fullerton, Alphaxard Manjurano, Franklin W Mosha, Thomas Walker, Mark Rowland, Manisha A Kulkarni, Natacha Protopopoff

Summary

Background Insecticide resistance among malaria-vector species is a pervasive problem that might jeopardise global disease-control efforts. Novel vector-control tools with different modes of action, including long-lasting insecticidal nets (LLINs) incorporating new active ingredients, are urgently needed to delay the evolution and spread of insecticide resistance. We aimed to measure phenotypic and genotypic insecticide-resistance profiles among wild *Anopheles* collected over 3 years to assess the longitudinal effects of dual-active-ingredient LLINs on insecticide resistance.

Methods For this analysis, data nested in a 3-year, four parallel-arm, superiority cluster-randomised controlled trial (cRCT) in Tanzania, collected from 84 clusters (39 307 households) formed of 72 villages in the Misungwi district, were used to measure insecticide-resistance profiles among female *Anopheles* mosquitoes via insecticide-resistance bioassays and quantitative RT-PCR of metabolic-resistance genes. Wild, blood-fed, indoor-resting mosquitoes were collected annually during the rainy seasons from house walls in clusters from all four trial groups. Mosquitoes were morphologically identified as *An gambiae* sensu lato (SL) or *An funestus* SL before separate bioassay testing. The primary outcomes were lethal-dose values for α-cypermethrin, permethrin, and piperonyl butoxide pre-exposure plus permethrin-resistance intensity bioassays, mortality 72 h after insecticidal exposure for chlorfenapyr bioassays, fertility reduction 72 h after insecticidal exposure for pyriproxyfen bioassays, and fold change in metabolic-enzyme expression relative to an insecticide-susceptible laboratory strain. All primary outcomes were measured in *An funestus* SL 1 year, 2 years, and 3 years after LLIN distribution. Primary outcomes were also assessed in *An gambiae* SL if enough mosquitoes were collected. The cRCT is registered with ClinicalTrials.gov (NCT03554616).

Findings Between May 24, 2019, and Oct 25, 2021, 47 224 female *Anopheles* were collected for resistance monitoring. In the pyrethroid (PY)-LLIN group, there were significant increases in α-cypermethrin-resistance intensity (year 1 LD50=9·52 *vs* year 2 76·20, p<0·0001) and permethrin-resistance intensity (year 1 13·27 *vs* year 2 35·83, p=0·0019) in *An funestus* SL. In the pyriproxyfen PY-LLIN group, there was similar increase in α-cypermethrin-resistance intensity (year 1 0·71 *vs* year 2 81·56, p<0·0001) and permethrin-resistance intensity (year 1 5·68 *vs* year 2 50·14, p<0·0001). In the piperonyl butoxide PY-LLIN group, α-cypermethrin-resistance intensity (year 1 33·26 *vs* year 3 70·22, p=0·0071) and permethrin-resistance intensity (year 1 47·09 *vs* year 3 2635·29, p<0·0001) also increased over time. In the chlorfenapyr PY-LLIN group, there were no effects on α-cypermethrin-resistance intensity (year 1 0·42 *vs* year 3 0·99, p=0·54) or permethrin-resistance intensity (data were not estimable due to nearly 100% mortality). There were also minimal reductions in chlorfenapyr susceptibility. However, in the chlorfenapyr PY-LLIN group, a significant decline in piperonyl-butoxide synergy was seen by year 3 (year 1 0·02 *vs* year 3 0·26, p=0·020). Highly over-expressed detoxification enzymes showed dynamic patterns of selection throughout the trial.

Interpretation Our phenotypic data supports trial epidemiological findings; chlorfenapyr PY-LLINs provided superior protection from malaria across multiple transmission seasons, with few effects on insecticide-resistance selection. Rapid pyrethroid-resistance intensification in the piperonyl butoxide PY-LLIN group and pre-existing tolerance of pyriproxyfen in vector populations might explain the poorer performance of these two interventions regarding malaria outcomes. Further work is required to elucidate the potential mechanisms driving cross-resistance between pyrethroids and novel active ingredients to better inform the design of pre-emptive resistance-management strategies.

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Effectiveness of long-lasting insecticidal nets with pyriproxyfen–pyrethroid, chlorfenapyr–pyrethroid, or piperonyl butoxide–pyrethroid versus pyrethroid only against malaria in Tanzania: final-year results of a four-arm, single-blind, cluster-randomised trial

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Summary

Background New classes of long-lasting insecticidal nets (LLINs) containing two active ingredients have been recently recommended by WHO in areas where malaria vectors are resistant to pyrethroids. This policy was based on evidence generated by the first 2 years of our recently published trial in Tanzania. In this Article, we report the final third-year trial findings, which are necessary for assessing the long-term effectiveness of new classes of LLIN in the community and the replacement intervals required.

Methods A third year of follow-up of a four-arm, single-blind, cluster-randomised controlled trial of dual active ingredient LLINs was conducted between July 14, 2021, and Feb 10, 2022, in Misungwi, Tanzania. Restricted randomisation was used to assign 84 clusters to the four LLIN groups (1:1:1:1) to receive either standard pyrethroid (PY) LLINs (reference), chlorfenapyr–PY LLINs, pyriproxyfen–PY LLINs, or piperonyl butoxide (PBO)–PY LLINs. All households received one LLIN for every two people. Data collection was done in consenting households in the cluster core area with at least one child between 6 months and 15 years of age who permanently resided in the selected household. Exclusion criteria were householders absent during the visit, living in the cluster buffer area, no adult caregiver capable of giving informed consent, or eligible children who were severely ill. Field staff and study participants were masked to allocation, and those analysing data were not. The primary 24-month endpoint was reported previously; here, we present the secondary outcome, malaria infection prevalence in children at 36 months post LLIN distribution, reported in the intention-to-treat analysis. The trial was registered with ClinicalTrials.gov (NCT03554616) and is now complete.

Findings Overall usage of study nets was 1023 (22·3%) of 4587 people at 36 months post distribution. In the standard PY LLIN group, malaria infection was prevalent in 407 (37·4%) of 1088 participants, compared with 261 (22·8%) of 1145 in the chlorfenapyr–PY LLIN group (odds ratio 0·57, 95% CI 0·38–0·86; p=0·0069), 338 (32·2%) of 1048 in the PBO–PY LLIN group (0·95, 0·64–1·42; p=0·80), and 302 (28·8%) of 1050 in the pyriproxyfen–PY LLIN group (0·82, 0·55–1·23; p=0·34). None of the participants or caregivers reported side-effects.

Interpretation Despite low coverage, the protective efficacy against malaria offered by chlorfenapyr–PY LLINs was superior to that provided by standard PY LLINs over a 3-year LLIN lifespan. Appropriate LLIN replacement strategies to maintain adequate usage of nets will be necessary to maximise the full potential of these nets.

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Introduction

Pyrethroid (PY) long-lasting insecticidal nets (LLINs) are the primary method of malaria control in sub-Saharan Africa.[1] These nets have contributed considerably to the decline in malaria morbidity and all-cause mortality across the region. However, the rapid spread of PY resistance in malaria vector populations[2] is a contributing factor to the stagnation of decline in malaria cases and mortality in recent years.

To counter the challenge of PY resistance, several new classes of LLINs have been developed that include a mixture of a PY insecticide and the synergist piperonyl butoxide (PBO) or a second insecticide with a differing mode of action to PYs. The most recent

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See **Comment** page 11

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Effectiveness and cost-effectiveness against malaria of three types of dual-active-ingredient long-lasting insecticidal nets (LLINs) compared with pyrethroid-only LLINs in Tanzania: a four-arm, cluster-randomised trial

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Summary

Background Long-lasting insecticidal nets (LLINs) have successfully reduced malaria in sub-Saharan Africa, but their effectiveness is now partly compromised by widespread resistance to insecticides among vectors. We evaluated new classes of LLINs with two active ingredients with differing modes of action against resistant malaria vectors.

Methods We did a four-arm, cluster-randomised trial in Misungwi, Tanzania. Clusters were villages, or groups of hamlets, with at least 119 households containing children aged 6 months to 14 years living in the cluster's core area. Constrained randomisation was used to allocate clusters (1:1:1:1) to receive one of four types of LLIN treated with the following: α-cypermethrin only (pyrethroid-only [reference] group); pyriproxyfen and α-cypermethrin (pyriproxyfen group); chlorfenapyr and α-cypermethrin (chlorfenapyr group); or the synergist piperonyl butoxide and permethrin (piperonyl butoxide group). At least one LLIN was distributed for every two people. Community members and the field team were masked to group allocation. Malaria prevalence data were collected through cross-sectional surveys of randomly selected households from each cluster, in which children aged 6 months to 14 years were assessed for *Plasmodium falciparum* malaria infection by rapid diagnostic tests. The primary outcome was malaria infection prevalence at 24 months after LLIN distribution, comparing each of the dual-active-ingredient LLINs to the standard pyrethroid-only LLINs in the intention-to-treat population. The primary economic outcome was cost-effectiveness of dual-active-ingredient LLINs, based on incremental cost per disability-adjusted life-year (DALY) averted compared with pyrethroid-only LLINs, modelled over a 2-year period; we included costs of net procurement and malaria diagnosis and treatment, and estimated DALYs in all age groups. This study is registered with ClinicalTrials.gov (NCT03554616), and is ongoing but no longer recruiting.

Findings 84 clusters comprising 39 307 households were included in the study between May 11 and July 2, 2018. 147 230 LLINs were distributed among households between Jan 26 and Jan 28, 2019. Use of study LLINs was reported in 3155 (72·1%) of 4378 participants surveyed at 3 months post-distribution and decreased to 8694 (40·9%) of 21 246 at 24 months, with varying rates of decline between groups. Malaria infection prevalence at 24 months was 549 (45·8%) of 1199 children in the pyrethroid-only reference group, 472 (37·5%) of 1258 in the pyriproxyfen group (adjusted odds ratio 0·79 [95% CI 0·54–1·17], p=0·2354), 512 (40·7%) of 1259 in the piperonyl butoxide group (0·99 [0·67–1·45], p=0·9607), and 326 [25·6%] of 1272 in the chlorfenapyr group (0·45 [0·30–0·67], p=0·0001). Skin irritation or paraesthesia was the most commonly reported side-effect in all groups. Chlorfenapyr LLINs were the most cost-effective LLINs, costing only US$19 (95% uncertainty interval 1–105) more to public providers or $28 (11–120) more to donors per DALY averted over a 2-year period compared with pyrethroid-only LLINs, and saving costs from societal and household perspectives.

Interpretation After 2 years, chlorfenapyr LLINs provided significantly better protection than pyrethroid-only LLINs against malaria in an area with pyrethroid-resistant mosquitoes, and the additional cost of these nets would be considerably below plausible cost-effectiveness thresholds ($292–393 per DALY averted). Before scale-up of chlorfenapyr LLINs, resistance management strategies are needed to preserve their effectiveness. Poor textile and active ingredient durability in the piperonyl butoxide and pyriproxyfen LLINs might have contributed to their relative lack of effectiveness compared with standard LLINs.

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See Comment page 1202

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Differential impact of dual-active ingredient long-lasting insecticidal nets on primary malaria vectors: a secondary analysis of a 3-year, single-blind, cluster-randomised controlled trial in rural Tanzania

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Summary

Background Gains in malaria control are threatened by widespread pyrethroid resistance in malaria vectors across sub-Saharan Africa. New long-lasting insecticidal nets (LLINs) containing two active ingredients (dual active-ingredient LLINs) have been developed to interrupt transmission in areas of pyrethroid resistance. We aimed to evaluate the effectiveness of three dual active-ingredient LLINs compared with standard pyrethroid LLINs against pyrethroid-resistant malaria vectors in rural Tanzania.

Methods In this study, we did a secondary analysis of entomological data from a four-group, 3 year, single-blind, cluster-randomised controlled trial carried out between Feb 18, 2019, and Dec 6, 2021. We conducted quarterly indoor mosquito collections using the Centers for Disease Control and Prevention light trap, in eight houses in each of the 84 study clusters in the Misungwi district, northwestern Tanzania. *Anopheles* vectors were then tested for malaria parasites and identified at species level, to distinguish between sibling species of the *Anopheles gambiae* and *Anopheles funestus* groups, using molecular laboratory techniques. The primary outcomes were density of different malaria vector species measured as the number of female *Anopheles* collected per household per night, the entomological inoculation rate (EIR), an indicator of malaria transmission, and sporozoite rate. Entomological outcomes were assessed on the basis of intention to treat, and the effect of the three dual active-ingredient LLINs was compared with the standard pyrethroid LLINs at household level.

Findings Dual active-ingredient LLINs had the greatest effect on *Anopheles funestus* sl, the most efficient vector in the study area, with comparatively weak effect on *An arabiensis*. *An funestus* density was 3·1 per house per night in the pyrethroid LLIN group, 1·2 in the chlorfenapyr pyrethroid LLIN group (adjusted density ratio [aDR]=0·26, 95% CI 0·17–0·14, p<0·0001), 1·4 in the piperonyl-butoxide pyrethroid LLIN group (aDR=0·49, 0·32–0·76, p=0·0012), and 3·0 in the pyriproxyfen pyrethroid LLIN group (aDR=0·72, 0·47–1·11, p=0·15). Malaria transmission intensity was also significantly lower in the chlorfenapyr pyrethroid group, with 0·01 versus 0·06 infective bites per household per night in the pyrethroid LLIN group (aDR=0·21, 0·14–0·33, p<0·0001). Ecological niche models indicated that vector-species distribution was stable following LLIN intervention despite the reductions observed in *An funestus* sl density.

Interpretation Chlorfenapyr pyrethroid LLINs were the most effective intervention against the main malaria vector *An funestus* sl over 3 years of community use, whereas the effect of piperonyl-butoxide pyrethroid LLIN was sustained for 2 years. The other vector, *An arabiensis*, was not controlled by any of the dual active-ingredient LLINs. Additional vector control tools and strategies targeted to locally prevalent vector species evading dual active-ingredient LLINs should be deployed to further reduce malaria transmission and achieve elimination.

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Introduction

The primary malaria-vector control tools, consisting of pyrethroid long-lasting insecticidal nets (LLINs) and indoor residual spraying, have substantially reduced malaria morbidity and mortality across sub-Saharan Africa.[1] Among the challenges faced by malaria control strategies, the most important are widespread pyrethroid resistance in malaria-vector populations[1] and insufficient funding for malaria control, which have led to intervention prioritisation by national malaria control programmes.

To address the biological challenge of insecticide resistance while sustaining malaria control gains, novel next-generation dual active-ingredient LLINs are under

Article

Developing Consensus Standard Operating Procedures (SOPs) to Evaluate New Types of Insecticide-Treated Nets

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Simple Summary: Malaria control relies on insecticide-based tools which target the mosquito vector. Predominantly, a group of insecticides called pyrethroids are used in these tools. Globally, however, mosquitoes are increasingly developing resistance to pyrethroids. Subsequently, new products, such as insecticide-treated nets (ITNs), which contain combinations of insecticides from different classes, or chemicals that work synergistically with pyrethroids, are being developed. Several of these new net types are being rolled out for testing and use. However, standardized methods to measure how long these nets remain active against mosquitoes are lacking, which makes evaluating the long-term efficacy of these products challenging. In this publication, we propose a pipeline used to collate and interrogate several different methods to produce a singular 'consensus standard operating procedure (SOP)', for monitoring the residual efficacy of three new net types: pyrethroid + piperonyl butoxide (PBO), pyrethroid + pyriproxyfen (PPF), and pyrethroid + chlorfenapyr (CFP).

Abstract: In response to growing concerns over the sustained effectiveness of pyrethroid-only based control tools, new products are being developed and evaluated. Some examples of these are dual-active ingredient (AI) insecticide-treated nets (ITNs) which contain secondary insecticides, or synergist ITNs which contain insecticide synergist, both in combination with a pyrethroid. These net types are often termed 'next-generation' insecticide-treated nets. Several of these new types of ITNs are being evaluated in large-scale randomized control trials (RCTs) and pilot deployment schemes at a country level. However, no methods for measuring the biological durability of the AIs or synergists on these products are currently recommended. In this publication, we describe a pipeline used to collate and interrogate several different methods to produce a singular 'consensus standard operating procedure (SOP)', for monitoring the biological durability of three new types of ITNs: pyrethroid + piperonyl butoxide (PBO), pyrethroid + pyriproxyfen (PPF), and pyrethroid + chlorfenapyr (CFP). This process, convened under the auspices of the Innovation to Impact programme, sought to align methodologies used for conducting durability monitoring activities of next-generation ITNs.

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An increasing role of pyrethroid-resistant *Anopheles funestus* in malaria transmission in the Lake Zone, Tanzania

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Anopheles funestus **is playing an increasing role in malaria transmission in parts of sub-Saharan Africa, where *An. gambiae* s.s. has been effectively controlled by long-lasting insecticidal nets. We investigated vector population bionomics, insecticide resistance and malaria transmission dynamics in 86 study clusters in North-West Tanzania. *An. funestus* s.l. represented 94.5% (4740/5016) of all vectors and was responsible for the majority of malaria transmission (96.5%), with a sporozoite rate of 3.4% and average monthly entomological inoculation rate (EIR) of 4.57 per house. Micro-geographical heterogeneity in species composition, abundance and transmission was observed across the study district in relation to key ecological differences between northern and southern clusters, with significantly higher densities, proportions and EIR of *An. funestus* s.l. collected from the South. *An. gambiae* s.l. (5.5%) density, principally *An. arabiensis* (81.1%) and *An. gambiae* s.s. (18.9%), was much lower and closely correlated with seasonal rainfall. Both *An. funestus* s.l. and *An. gambiae* s.l. were similarly resistant to alpha-cypermethrin and permethrin. Overexpression of *CYP9K1, CYP6P3, CYP6P4* and *CYP6M2* and high L1014S-*kdr* mutation frequency were detected in *An. gambiae* s.s. populations. Study findings highlight the urgent need for novel vector control tools to tackle persistent malaria transmission in the Lake Region of Tanzania.**

The widespread deployment of primary vector control interventions, principally long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS), has substantially reduced malaria incidence across sub-Saharan Africa[1,2]. Between 2000 and 2015, 68% of the 1.5 billion malaria cases averted can be attributed to LLINs alone[1]. However, current estimates indicate the rates of decline have begun to stagnate[2]. Tanzania is among the 10 sub-Saharan African countries where malaria burden is concentrated[3], contributing to 5% of global malaria deaths[2]. Malaria infection varies nationwide with an average prevalence of 7.3% in children under 5 years of age in 2017[4]. Vector control by the National Malaria Control Programme (NMCP) is based on sustaining high LLIN access and use[5], via universal coverage campaigns supplemented with continuous distribution from school net programmes, antenatal care campaigns and the expanded programme for immunization; and targeted IRS in high transmission areas in the North–West[6]. Effective and sustainable malaria vector control is plagued by a number of challenges, including the evolution of vector behavioural and physiological resistance to current control interventions[7]. In the majority of sentinel districts across Tanzania, *Anopheles* mosquitoes have demonstrated reduced susceptibly to at least one public health insecticide[8,9].

Continued use of insecticide-based malaria control tools has been linked with changes in *Anopheles* feeding and resting behaviors and relative species composition[10–13]. In some countries, *Anopheles funestus* sensu stricto (s.s.) has historically played a significant role in malaria transmission[14–17] largely due to its predominantly

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