

The durability of long-lasting insecticidal nets
treated with and without piperonyl butoxide:
implications for bioefficacy and personal
protection

By

Frank Mehan

Student number: 00117295

June 2022

Supervisors:

Dr Lisa Reimer

Prof. Martin Donnelly

Prof. Philip McCall

Thesis submitted in accordance with the requirements of the Liverpool
School of Tropical Medicine for the degree of Doctor of Philosophy

Abstract

Long-lasting Insecticidal nets (LLINs) are a fundamental component of malaria control strategies, yet they accumulate physical damage and lose insecticide over time. However, the impact of this deterioration on bioefficacy and bloodfeeding is poorly described. There is growing evidence that the operational lifespan of LLINs is less than the expected three years in some settings. Consequently, there is a need to assess the durability of LLIN products to determine the appropriate timescale of distribution. The emergence of pyrethroid-resistance in sub-Saharan Africa has motivated the development of LLIN designs that contain the synergist piperonyl butoxide to restore susceptibility. The aim of this study was to quantify the durability of LLINs with and without piperonyl butoxide in operational conditions.

The nets assessed in this study were provided from collections performed as part of a randomised control trial to compare pyrethroid-only and pyrethroid-PBO nets. Each cluster received a pyrethroid-PBO net (‘Olyset Plus’ or ‘PermaNet 3.0’) or pyrethroid-only equivalent (‘Olyset Net’ or ‘PermaNet 2.0’). Samples were assessed at baseline, 12 months, and 25 months post distribution. The chemical content of pyrethroid and PBO of nets was assessed using high-performance liquid chromatography. The number, size, and location of holes on each net was assessed visually. Bioefficacy was assessed by exposing pyrethroid-resistant *Anopheles* to samples using WHO cone and wireball bioassays. To evaluate the impact of hole location on protective effect, behavioural experiments with free-flying pyrethroid-resistant *Anopheles* around human-occupied holed nets were conducted. Bloodfeeding success, 1hr knockdown, and 24hr mortality were compared for nets with no hole, hole in the top, or hole in the side.

Pyrethroid content remained relatively stable across timepoints. However, the PBO content of both Olyset Plus and PermaNet 3.0 declined over the same period, falling by 55% ($P < 0.001$) and 58% ($p < 0.001$) respectively after 25 months. Both PBO nets were highly effective against pyrethroid-resistant *An. gambiae* when new but declined over time. After 25 months, 24hr mortality was 22.92% for Olyset Plus and 46.6% for PermaNet 3.0. There was a strong correlation between PBO content and mortality. There was no difference in any physical durability metric between any of the LLIN products evaluated, at any timepoint. In behavioural assays, holes on the top of the net had a much greater risk of bloodfeeding

compared to holes on the side (30.65% compared to 4.13%, $P=0.021$) after one hour. There was no difference in bloodfeeding success between Olyset Plus and Olyset Net ($p=0.076$). Very few bloodfed mosquitoes survived the assay with Olyset Plus, with 96.1% of all bloodfed mosquitoes dying after 24 hours despite very low mortality for those that did not bloodfeed (<5%). Finally, when attempting to escape the net after bloodfeeding, mosquitoes were twice as likely to get out of a net with a hole on the top than on the side.

These findings indicate that pyrethroid-PBO bed nets were highly effective against pyrethroid-resistant mosquitoes in Uganda when new but efficacy declined sharply over time. For both Olyset Plus and PermaNet 3.0, this rapid reduction in bioefficacy correlated with a steep decline in PBO content. Given that pyrethroid-PBO nets are becoming widespread, this finding is highly concerning and requires further investigation in other settings. Moreover, the rapid reduction in bioefficacy indicates a distribution cycle shorter than three years may be prudent. Physical integrity outcomes were very similar for the PBO nets and their pyrethroid-only equivalents. Current WHO durability assessment guidelines consider all holes equally when evaluating serviceability for use, yet here it was observed that holes on the top of the net were a 10x greatest risk for mosquito entry and bloodfeeding compared to the side. Consequently, guidelines for assessing survivability should be updated to appropriately weight holes on the top.

Acknowledgements

The work presented here could not have been completed without the support of many people.

First, I want to thank my primary supervisor Dr Lisa Reimer for her continuing guidance, support, and patience. It took a long time to settle on a project but Lisa helped turn my hare-brained schemes into cohesive and feasible research plans, and gave me the freedom to try out new ideas. Thank you for everything.

A huge thank you to my secondary supervisors Professor Martin Donnelly and Professor Philip McCall. Thank you Martin for trusting me to be part of the LLINEUP project and for your ongoing guidance on seeing the big picture and making this work the most impactful it can be. Philips's for your wealth of knowledge on mosquito behaviour and insights into the world of bed nets.

A massive thank you to Amy Lynd, without whom I quite simply would not have got through this PhD. Amy's huge wealth of knowledge, guidance, and willingness to tell me off when I've done something stupid was incredibly influential to my own development and growth. The vast majority lab and insectary skill needed for this project was taught to me by Amy.

To Agaba, Violet and all of the field team that performed the fieldwork in Uganda that provided samples and data that made so much of this work possible. When my fieldwork was cancelled in 2020 and 2021 it seemed like curtains for my PhD plans, I'm so incredibly grateful for all of your work.

A big thank you to Kath Gleave and Jeff Jones for helping me getting set up with the behavioural working and their guidance in turning experimental plans on paper into practical routines. Additionally, another big thank you to Kath for detailed feedback on my behavioural chapter.

To Emma Reid for helping look after the mozzies and covering weekends. I can't even imagine how many hours we spent in the insectary, but you still kept us in good spirits all the way though.

To Jonathan Thornton for helping with rearing mosquitoes and testing bed nets, our chats about Star Trek made those long days in the insectary much more bearable.

A massive thank you to Julie-Anne Tangena for being a fantastic mentor, colleague, and friend through all of the ups and downs of this PhD. I like to think I've matured a quite a bit these past few years and you were a great role model in helping me become more focussed and professional.

Dr Rosemary Lees and Professor Steve Torr for being an excellent PAP panel, providing advice and insight that came in very useful, particularly on time management and increasing the practical impact of my work.

A big thank you to all of the fantastic mates I made along the way in my time at LSTM. A particular shout out to Charlotte, Anna, and Dan for keeping me sane along the way.

To my wonderful family for supporting me in this journey for as long as I can remember! Mum and Dad I'm so grateful for everything, it was a big step moving to Liverpool for this PhD but you were so supportive and encouraged me to follow my dreams. To my lovely sisters for always being there and keeping me laughing every day with your nonsense. I love you all so much.

To my Liverpool family. Thank you Jane, Ryan, Tash, and Luis for welcoming me into your family and making me feel like one of you right from the start. The pandemic was a difficult time for us all, but I'll never forget how it brought us all together. Nine-Nine! Love you all

To Xan for being the wee dug. Thank you for helping me get up early in the morning by booting me in the face.

To my lovely Emma for your unending love and support. I don't know what I'd do without you. It's been a long road getting through this PhD but you've been there for me all the way. Love you always x

A huge thank you to you all!

Table of Contents

Chapter One: Introduction.....	12
1.1 Background	12
1.1.1 Global importance of malaria	12
1.1.2 Life cycle.....	14
1.2 Interventions to reduce human malaria prevalence	17
1.2.1 Overview of malaria control strategies.....	17
1.2.2 Case management	17
1.2.3 Mass drug administration	18
1.2.4 Vaccines for malaria.....	19
1.2.5 Control of malaria vectors	20
1.3 Insecticide treated nets for malaria control	24
1.3.1 Evidence of bed net efficacy in reducing malaria outcomes	24
1.3.2 Personal Protection.....	25
1.3.3 Community protection.....	26
1.3.4 LLIN coverage and use	28
1.3.5 LLIN retention	31
1.4 Emergence of Insecticide resistance.....	32
1.4.1 Overview of insecticides for malaria control	32
1.4.2 Target site resistance	35
1.4.3 Metabolic resistance.....	35
1.4.5 Impact of pyrethroid resistant vectors on malaria outcomes	38
1.4.6 Next generation tools for targeting pyrethroid resistant mosquitoes	40
1.5 Assessing the durability of long-lasting Insecticidal Nets	42
1.5.1 Purpose of durability assessment	42
1.5.2 Definition of durability.....	43
1.6 Behaviour of host-seeking <i>An. gambiae</i> around LLINs	54
1.6.1 Mosquito detection of humans	54
1.6.2 Behaviour of <i>An. gambiae</i> around insecticidal nets	56
1.6.3 Interaction between <i>An. gambiae</i> and holed LLINs.....	58
1.7 Aims and objectives	60
Chapter Two: Chemical content and bioefficacy of Long Lasting Insecticidal Nets treated with and without piperonyl butoxide across two years of operational use in Uganda.....	62
2.1 Introduction	62
2.1.1 Background	62
2.1.2 Assessing LLIN durability	64

2.1.3 Uganda LLIN evaluation project.....	66
2.1.4 Aim	67
2.1.5 Objectives.....	68
2.1.6 Study site and context	69
2.2 Methods.....	70
2.2.1 LLIN description.....	70
2.2.2 Sample size.....	71
2.2.3 Chemical Analysis.....	72
2.2.2 Bioefficacy	76
2.2.3 Data Analysis	83
2.3 Results	85
2.3.1 Chemical integrity	85
2.3.2 Mosquito characterisation.....	89
2.3.3 Bioefficacy	93
2.4 Discussion.....	99
2.4.1 Chemical integrity	99
2.4.2 Bioefficacy	101
2.4.3 Use of the WHO wireball assay.....	104
2.5 Conclusion.....	106
Chapter Three: Physical Integrity of LLIN in operational conditions	107
3.1 Introduction	107
3.1.1 Background	107
3.1.3 Aim	109
3.1.4 Objectives.....	110
3.2 Methods.....	111
3.2.1 LLIN description.....	111
3.3.2 Sample size.....	111
3.3.3. Hole measurement and damage categorisation.....	111
3.3.4 Socioeconomic predictors.....	113
3.3.5 Data analysis	114
3.3 Results	116
3.3.1 Proportion of nets with at least one hole.....	116
3.3.2 Total surface area of holes.....	119
3.3.3 Proportion of nets in each pHI category.....	121
3.3.4 Socioeconomic Indicators	122
3.3.4 Relationship between physical integrity and chemical content.....	126

3.4 Discussion.....	128
3.5 Conclusion.....	132
Chapter Four: Impact of hole location on entry rate of <i>Anopheles</i> mosquitoes into a host-baited bed nets treated with permethrin and piperonyl butoxide (PBO)	133
4.1 Introduction	133
4.1.1 Background	133
4.1.2 Aim	137
4.1.3 Objectives.....	138
4.2 Materials and Methods.....	139
4.2.1 LLIN description and preparation	139
4.2.2 Mosquito characteristics	140
4.2.3 Experimental design.....	141
4.2.5 Sample size.....	149
4.2.6 Data analysis	151
4.2.7 Participant recruitment.....	151
4.2.7 Measures of protective effect.....	153
4.2.7 Ethical considerations and Research Ethics Committee approval.....	154
4.3 Results.....	156
4.3.1 Benchtop bioefficacy outcomes.....	156
4.3.2 Personal protection.....	159
4.3.3 Bioefficacy	165
4.4 Discussion.....	171
4.4.1 Limitations.....	174
4.4.2 Future work.....	176
Chapter Five: General Discussion.....	177
5.1 summary of key findings	177
5.2 Implication for the evaluation and deployment of LLINs	178
5.3 Hole location and personal protection	182
5.4 Predictors of LLIN bioefficacy	187
5.5 Future work and next steps	190
Chapter Six: References	192
Appendix I: Comparison of WHO Cone and WHO Wireball:.....	203
Appendix II: Ethical approval documentation.....	204
Appendix III: Peer-reviewed publication of Chapters Two & Three	239
Appendix IV: Additional relevant works published during this PhD studentship	255

Table of Figures

Figure 1.1 Life cycle of Plasmodium spp. parasite in human and anopheline hosts	15
Figure 1.2 A family sleeps within an ITN	21
Figure 1.3 Estimated access and use rate of insecticide treated nets across sub-Saharan Africa	29
Figure 1.4 Predicted mean mortality of Anopheles gambiae (s.l.) across sub-Saharan Africa.....	34
Figure 1.5 Example of WHO cone bioassay..	46
Figure 1.6 A heavily damaged LLIN sampled from a household in western Uganda	51
Figure 1.7 Mosquito flight tracks around an unbaited and baited LLIN.....	57
Figure 2.1. Diagram of serial dilutions performed to prepare insecticide standards for HPLC.....	75
Figure 2.2 Example of WHO cone bioassay.	78
Figure 2.3 Image of cube variant of WHO wireball assay in use	81
Figure. 2.4 Mean concentration of deltamethrin, permethrin, and PBO detected in net samples	88
Figure 2.5. Adjusted mortality of An. gambiae strain 'Busia' after 60 minute WHO tube exposures .	90
Figure 2.6. Frequency of resistance markers Cyp4J5, Cyp6P4, and Vgsc-L1014S for 'Busia' strain	92
Figure 2.7. Mean knockdown and adjusted mortality in cone bioassays.....	94
Figure 2.8. Mean knockdown and adjusted mortality in WHO wireball assays	96
Figure 2.9. Relationship between total chemical content and bioefficacy	98
Figure 3.1. Diagram of 'Top', 'Upper', and 'Lower' net sectors used to categorise hole location	113
Figure 3.2. Proportion of each LLIN with at least one hole at each timepoint.....	116
Figure 3.3. Proportion of nets with holes in each sector	117
Figure 3.5 Total holed area across all LLIN products at 12 months and 25 months.....	119
Figure 3.6. Total area of holes in each sector for all LLIN products	120
Figure 3.7. Proportion of collected nets in each pHI category	121
Figure 3.9. Mean total hole area in households with and without windows.....	124
Figure 3.10. Proportion of collected nets in each pHI category at 12 months and 25 months	125

Figure 3.11. Modelled relationship between PBO content and Total hole area	127
Figure 4.1. Diagram of experimental setup showing top and side hole position.....	144
Figure 4.2. Bioefficacy of Olyset Net and Olyset Plus in WHO wireball bioassay.....	156
Figure 4.3. Outcomes associated with Olyset Net and Olyset Plus in benchtop arm-feeding assay .	158
Figure 4.4. Bloodfeeding success with each combination of LLIN Product	160
Figure 4.5. Predicted population personal protection against bloodfeeding of LLINs.....	162
Figure 4.6. Predicted number of infected bites in each geographic context	164
Figure 4.7 1hr knockdown and 24hr mortality for each combination of LLIN and Hole Position	166
Figure 4.8. Percentage of mosquitoes that both bloodfed and survived.....	168
Figure 4.9. Mean percentage of mosquitoes found outside net the at the end of the exit assay	169
Figure 5.1 Total hole area for every net assessed.	188

Table of Tables

TABLE 1.1. WHO GUIDANCE FOR ESTIMATING THE SIZE OF HOLES IN LLINS	52
Table 2.1. Specifications of LLIN products assessed in study.....	70
Table 2.2. Sample size of nets used for each outcome	71
Table 2.3. Mean chemical content (in g/kg) for each active ingredient in each LLIN product.....	85
Table 3.1. Sample size of nets used for each outcome.	111
Table 3.2. Household Indicators included in analysis.....	114
Table 3.3. Relative risk ratio of at least one hole in each sector across all nets collected.....	118
Table 3.4. Relationship between household indicators and total surface area of all holes.....	123
Table 4.1. Manufacturer specifications of LLIN products assessed in study.....	139
Table 4.2. Number of observations performed for each free-flying experiment.	150
Table 4.3 Allocation of participants to arms of the free-flying bloodfeeding assay.....	152
Table 4.4. Odds Ratio of bloodfeeding success between LLIN Products for each hole position.....	159
Table 4.5. Odds ratio of bloodfeeding success between hole positions for each LLIN product	159
Table 4.6 <i>An. gambiae</i> indoor biting density and <i>Plasmodium</i> sporozoite rate across regions	163
Table 4.7 Odds Ratio of one hour knockdown between LLIN products across all hole positions.....	165
Table 4.8 Odds Ratio of 24 hour mortality between LLIN products	166
Table 4.9. Odds Ratio of bloodfeeding and surviving. Comparison of LLIN products	167
Table 4.10. Odds Ratio of bloodfeeding and surviving. Comparison of hole position	167
Table 4.11. Odds Ratio of a mosquito being found outside the net at the end of the exit assay	169
Table 4.12. Odds Ratio of one hour knockdown of mosquitoes in the exit assay.....	170
Table 4.13. Odds Ratio of 24hr mortality of mosquitoes in the exit assay.....	170

Chapter One: Introduction

1.1 Background

1.1.1 Global importance of malaria

Malaria is one of the world's oldest recognised diseases, with genetic, archaeological, and written evidence of malaria impacting a number of ancient cultures (Neghina et al. 2010, Gelabert et al. 2017,). Today, malaria remains a leading causes of morbidity and mortality globally, with an estimated 241 million cases and 627,000 associated deaths occurring worldwide in 2020 across the 85 countries where it is endemic (WHO 2020, WHO 2021b). It is estimated that in 2020 approximately half of the world's population were at risk of malaria.

In 2020, sub-Saharan Africa accounted for approximately 95% of all global malaria cases, with Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%), Mozambique (4%), Angola (3.4%), and Burkina Faso (3.4%) accounting for the majority of cases (WHO 2020). The high intensity of malaria transmission in sub-Saharan Africa compared to other regions is associated with the presence of highly anthropophilic mosquito vectors and climates favourable to this species. In many respects, malaria is a disease of poverty with countries and communities in sub-Saharan Africa often lacking access to wealth and resources that would fund interventions and allow for the general economic development associated with reducing public health impact. Malaria is the most common cause of infection-associated morbidity in Africa and is often cited as a core contributor to slow economic development in the region. Young children are particularly susceptible to developing severe malaria symptoms, with children under the age of five representing the vast majority (77%) of malaria deaths (WHO 2020). Additionally, pregnant women are at greater risk of infection, with 20%

of all low-birthweight babies in sub-Saharan Africa the result of *Plasmodium* infection during pregnancy (Bardají et al. 2008, Bauserman et al. 2019).

The onset of malaria is associated with recurrent fevers and chills, which may be mild and mistaken for other infections that cause flu-like symptoms (Bartoloni and Zammarchi 2012). However, if left untreated there is a high risk of progressing to severe anaemia and ultimately death within a matter of days (Sypniewska et al. 2017). However, while acknowledging the clear moral imperative to avert malaria mortality there is a growing awareness that afebrile cases, which would be expected to account for the vast majority of cases, also have important implications for health and society in endemic countries (Lindblade et al. 2013). These afebrile cases may not result in treatment seeking behaviour, yet the recurrence of these infections is associated with chronic fatigue, impaired cognitive function and school performance in children, and concurrent bacterial infections (Bousema et al. 2014, Chen et al. 2016). Consequently, malaria transmission has profound social implications beyond mortality and has been identified as an important impediment to socioeconomic development in sub-Saharan Africa. Hereafter the term 'chronic malaria' is used to refer to these sub-clinical cases that do not result in treatment seeking behaviour but have an insidious effect.

1.1.2 Life cycle

Malaria is the result of infection with protozoan parasites of the genus *Plasmodium*, though only a small number cause disease in humans (Howarth 1988, Meuwissen and Ponnudurai 1988). The primary cause of malaria worldwide is infection with parasite *Plasmodium falciparum*, accounting for approximately 95% cases in 2020 (WHO 2021b). Four other *Plasmodium* species together account for the remaining 5% of overall malaria infections each year; *P. vivax* (the dominant parasite outside of sub-Saharan Africa), *P. ovale*, *P. knowlesi*, and *P. malariae* (Price et al. 2020, WHO 2020).

The *Plasmodium* life cycle involves a complex series of stages in both vertebrate and invertebrate hosts (Tuteja 2007, Howick et al. 2019)(**Figure 1.1**). The invertebrate component of this life cycle, mosquitoes from the genus *Anopheles*, are the vector that transmits infection from one human to the next through their bites (Ross 1898). In brief, when an *Anopheles* mosquito infected with *Plasmodium* sporozoites bites a human, these sporozoites develop into merozoites that invade red blood cells and reproduce (causing the recurrent fevers associated with malaria)(Venugopal et al. 2020). The sexual stage gametocytes that burst from red blood cells are then ready to be ingested by mosquitoes and restart the cycle.

Development of the *Plasmodium* parasite in the midgut and salivary glands of infected *Anopheles* mosquitoes to the infective stage takes a number of days. This period of time is referred to as the Extrinsic Incubation Period (EIP). Typically, the EIP for *P. falciparum* is assumed to be 12-14 days however there is a growing evidence base that it is influenced by biotic and abiotic factors (Ohm et al. 2018, Childs and Prosper 2020). The EIP is important for malaria control programmes as it is the minimum period of time an *Anopheles* mosquito must survive to become infectious (Paaijmans et al. 2012, Stopard, Churcher and Lambert

2021). If malaria interventions can reduce the numbers of mosquitoes that live long enough to become infectious, the number of infectious bites individuals receive will be reduced (Smith et al. 2021).

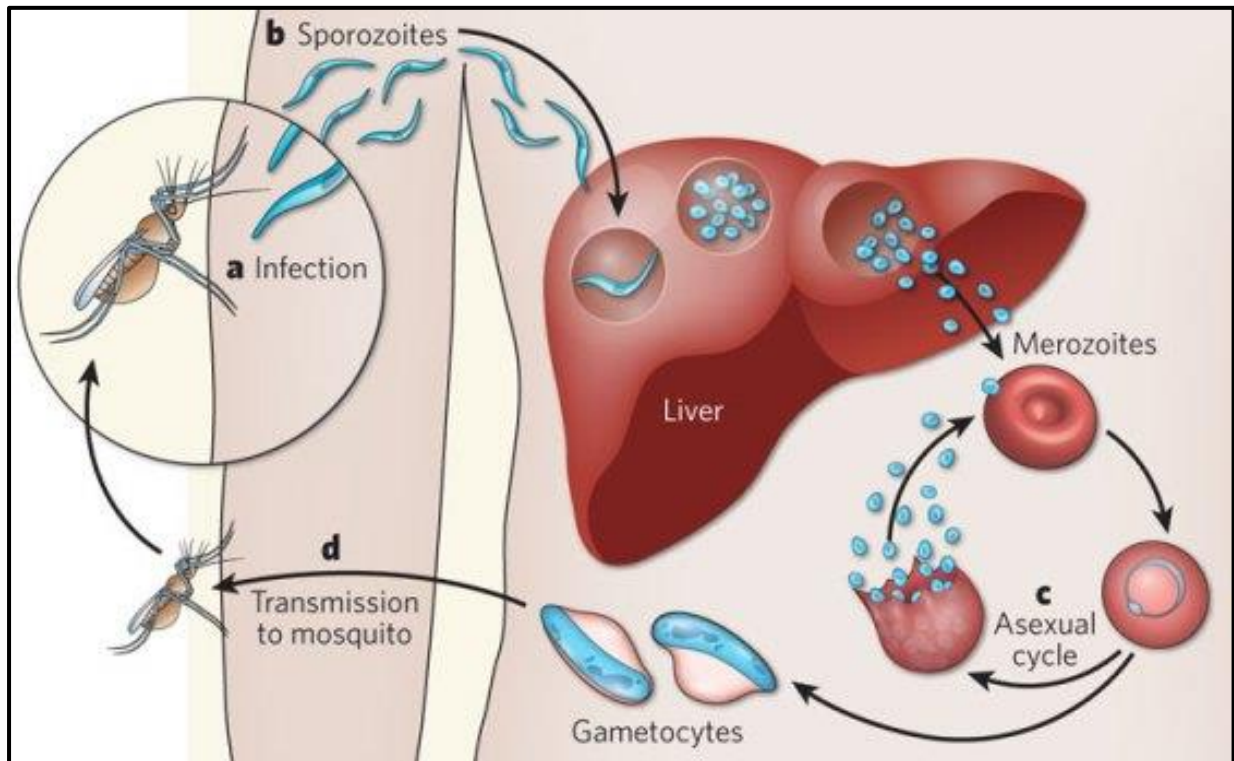


Figure 1.1 Life cycle of *Plasmodium* spp. parasite in human and anopheline hosts (Harvard University press)

The high malaria morbidity and mortality in sub-Saharan Africa is directly associated with the presence of highly efficient mosquito vectors of infection. Malaria vectors in sub-Saharan Africa are primarily *An. gambiae*, *An. coluzzii*, *An. funestus*, and *An. arabiensis* (Wiebe et al. 2017). They are highly specialised for tracking and bloodfeeding on humans (Dekker et al. 2002, Hawkes et al. 2017, Meza et al. 2019). These species thrive in the rural and agrarian landscapes meaning their densities are typically low in urbanised areas however the growing awareness of the suitability of urban landscapes for *An. stephensi* is an emerging issue (Coetzee 2004, Sinka et al. 2010, Sinka et al. 2020).

1.2 Interventions to reduce human malaria prevalence

1.2.1 Overview of malaria control strategies

Global malaria control strategy is a complex integration of multiple approaches which target either the *Plasmodium* parasite itself or limit exposure to the bites of mosquito vectors to reduce malaria morbidity and deaths. Due to the highly adaptive nature of both *Plasmodium* parasites and *Anopheles* vectors, malaria control strategy is constantly adjusting to maintain levels of protection (Hemingway et al. 2016).

1.2.2 Case management

The most direct malaria intervention is effective case management of individual patients with potential malaria symptoms (Galactionova et al. 2015). Effective diagnosis and clearing of *Plasmodium* parasites from the patient limits the risk of onwards transmission, preventing future malaria cases. The development of rapid diagnostics tests (RDTs) which detect *Plasmodium*-specific antigens in the blood to diagnose plasmodium infection allow malaria to be quickly differentiated from other infections in low-resource settings. Standard treatment for RDT positive malaria cases is with artemisinin combination-therapy (ACT), a combination of a fast-acting (artemisinin) and a slow-acting (amodiaquine, mefloquine, pyrimethamine, lumefantrine, or piperazine) anti-malaria compound (Van der Pluijm et al. 2021). The use of two different drugs in combination helps to slow the rate at which the *Plasmodium* parasite develops resistance (Ouhi et al. 2021).

1.2.3 Mass drug administration

In addition to treating individual malaria cases, strategies that target *Plasmodium* infection on a population level have been implemented in a number of settings. Seasonal Malaria Chemoprevention (SMC) is the pre-emptive administration of antimalaria drugs to children prior to and during the rainy months of a year when the abundance of malaria vectors is highest. SMC was endorsed by the WHO in 2012 (WHO 2012), yet despite the success of small pilot programmes large scale implementation was slow due to shortages of drugs. However, a recent large scale observational study in west and central Africa estimated a 57% and 42% reduction in hospital deaths in The Gambia and Burkina Faso respectively due to SMC (Baba et al. 2020). Mass drug administration (MDA) is the deployment of antimalaria drugs to every member of a defined population within a geographic area in order to reduce *Plasmodium* prevalence (Webster et al. 2014). A key benefit of MDA is that it targets both symptomatic and chronic malaria infection, clearing *Plasmodium* infection from those that may not otherwise have sought treatment. In addition to being deployed seasonally as part of routine malaria intervention strategies, MDA may be deployed as part of an emergency response during malaria epidemics, or in settings with very low malaria transmission as a final push towards elimination. However, a 2021 Cochrane review of 13 studies that investigated the impact of MDA for malaria control concluded that MDA has no impact on health outcomes in areas where more than 10% of the population is infected with Plasmodium parasites (Shah et al. 2021). Additionally, the same review concluded that in areas where <10% of the population are infected, MDA causes infections to drop quickly but rebounds to original levels within approximately four months.

1.2.4 Vaccines for malaria

Following pilot programmes in Ghana, Kenya, and Malawi, in 2021 the WHO recommended a vaccine for the first time as a malaria control intervention (WHO 2021a). The RTS,S/AS01 vaccine contains proteins normally secreted by the sporozoite stage of *Plasmodium* parasites, triggering an immune response that primes the individual for future *Plasmodium* infection (Dimala et al. 2018). This vaccine is intended to be administered to children from the age of five months, as a schedule of four doses. Vaccines are not intended to be an alternative to more established malaria control approaches, instead supplementing case management and vector control as part of an integrated strategy. Initial findings from randomised control trials are promising, with a 30% reduction in severe childhood malaria for one year after administration (Tinto *et al.* 2019). An associated modelling study estimated that 5.3 million cases and 24,000 deaths could be averted if this vaccine were deployed throughout sub-Saharan Africa to regions where *Plasmodium* population prevalence was >10% (Hogan, Winskill and Ghani 2020). However, the WHO highlights that further studies are needed to assess efficacy in different settings and quantify longevity of the protective effect. The RTS,S vaccine is currently the only vaccine approved for malaria control however there are a number of other products in development.

1.2.5 Control of malaria vectors

Malaria vector control (sometimes referred to as vector management) are strategies that reduce human prevalence by interrupting transmission of *Plasmodium* in mosquito populations. Malaria transmission by mosquitoes can be reduced through a number of approaches, from directly protecting individuals from potentially infectious bites to efforts to reducing mosquito lifespan and thereby preventing the parasite from developing in the mosquito to its infectious stage.

Historically, malaria transmission in sub-Saharan Africa was dominated by *An. gambiae* which seeks hosts indoors, targeting humans as they sleep at night. This led to the development of vector control tools that prevent indoor biting and kill those that attempt to. The primary method of preventing indoor biting remains insecticide treated nets (ITNs) and indoor residual spraying. However, there is evidence that some degree of outdoor biting occurs and has become more important for malaria transmission due to successes in preventing indoor biting techniques (Russell et al. 2011, Govella and Ferguson 2012, Sougoufara, Ottih and Tripet 2020,)

The first two decades of the 21st century saw a profound reduction in estimated malaria cases, falling from approximately 1.5 million annual cases in 2000 to 0.65 million in 2020. The dramatic reduction in malaria cases in the past two decades have been linked, in large part but not exclusively, to mass distributions of insecticide treated nets (Strode et al. 2014, Bhatt et al. 2015, Pryce, Richardson and Lengeler 2018). The massive scale up in ITN distributions in this century has been funded by billions of dollars of global aid, with approximately two billion bed nets were delivered across Sub-Saharan Africa between 2004 and 2020. Insecticidal bed nets (ITNs) interrupt the transmission of *Plasmodium* parasites by reducing the number of

infectious bites individuals in an endemic area will receive (Strode et al. 2014)(**Figure 1.2**). Designed to be hung over sleeping spaces, insecticidal bed nets provide a physical and chemical barrier against bloodfeeding by *Anopheles* mosquitoes as their occupant's sleep. The protective effect of an LLIN is a combination of two mechanisms, a physical barrier through densely weaved fabric and a chemical barrier through impregnated insecticide. The physical barrier forces the host-seeking mosquito to make contact with the insecticide, then impedes access to the sleeping individual while the insecticide takes effect (Parker et al. 2015). The chemical effect of the insecticide intoxicates the mosquito on contact, preventing blood-feeding through paralysis followed by death.



Figure 1.2 A family sleeps within an ITN to protect against host-seeking *Anopheles* mosquitoes (image credit: The Carter Center/L. Gubb)

Indoor Residual Spraying (IRS) is the application of insecticide to households (and sometimes surrounding structures) to target mosquitoes that rest on them. Deploying IRS to households is considered to be more challenging than distributing ITNS due to the need for well trained staff to prepare the household for spraying and deploy the insecticide (Tangena et al. 2020). To minimise the impact of cross-resistance with the pyrethroid insecticides used in bed nets, 11 insecticides with a variety of modes of action are approved for use in IRS (Global Fund, 2022). Currently, the WHO recommends IRS only be targeted to areas of low to moderate endemic transmission rather than deployed widely on a country scale (WHO, 2018). Due to the widespread use of insecticidal bed nets for malaria control, evidence for the beneficial impact of IRS on malaria outcomes is invariably assessed in addition to bednets rather than in isolation. A systematic review of IRS for preventing malaria, conducted by Choi et al. (2019), concluded that pyrethroid IRS provided zero to marginal protective effect compared to ITNs only in terms of clinical malaria incidence (OR: 1.07), parasite prevalence (OR: 1.11), or anaemia prevalence (OR: 1.12). However, evidence for the benefit of non-pyrethroid IRS was stronger, with clinical incidence overall lower with IRS though highly variable between insecticides and locations. While IRS is often described as a major part of vector control strategy for malaria, in practice only a very small minority of individuals are protected by the technique, with only 2.6% of the worlds at risk population estimated to be protected by IRS in 2020 (a substantial decline from the 5.8% protected by IRS in 2010)(WHO 2021b).

An emerging tool for vector control is the use of endectocides. Endectocides are systemic drugs administered to hosts which are toxic to insects. The benefit of endectocides is that they are safe, long-lasting (remaining toxic months after administration), relatively inexpensive and easier to administer. However, the public health impact and cost effectiveness of endectocides is not well documented. Additionally, there is concerns of

endectocides compounds entering ecosystems, with documented examples of degradation of soil and impact on non-target invertebrates.

1.3 Insecticide treated nets for malaria control

1.3.1 Evidence of bed net efficacy in reducing malaria outcomes

Studies conducted prior to the emergence of insecticide resistance in sub-Saharan Africa provide strong evidence that use of bednets is associated with reductions in malaria prevalence. Of the 663 million clinical cases (credible interval 542–753 million) estimated to have been averted between 2000 and 2015, ITN distributions were responsible for 68% of this reduction (Bhatt et al. 2015). A comprehensive meta-analysis of 23 randomised control trials estimated the impact of pyrethroid bed nets on malaria morbidity and mortality (Pryce et al. 2018). The authors concluded that compared to no nets, pyrethroid bed nets reduce child mortality by 17% and reduce the incidence of clinical malaria by approximately half. When comparing pyrethroid nets to untreated nets, the benefit of insecticides is clear with child mortality reduced by 33% and clinical malaria by cases reduced by 23% compared to untreated nets. At a population level, they concluded that pyrethroid nets reduce *P. falciparum* prevalence by approximately 10% compared to untreated nets.

1.3.2 Personal Protection

When a susceptible *Anopheles* mosquito approaches a bed net, it picks up a dose of pyrethroid insecticide which causes it to lose motor coordination and results in paralysis. The combination of this chemical effect and protective barrier prevent biting on the occupant inside. In addition to a direct insecticidal effect, there is evidence that pyrethroids provide personal protection by inciting an irritant ('excito-repellent') response in the mosquito on contact, resulting in avoidance behaviour away from the net. Studies in experimental hut trials conducted before the emergence of insecticide resistance in sub-Saharan Africa provide evidence of a deterrent effect of bed nets. An early investigation in The Gambia in 1991 observed fewer *Anopheles* mosquitoes entered huts with a baited pyrethroid net than a hut with a baited untreated net (Lindsay et al. 1991), with the deterrent proportional to the concentration of pyrethroid. However, where these mosquitoes are diverted to is not well described.

The physical barrier of a bed net provides a degree of protection against the bites of *Anopheles* mosquitoes, even if no insecticide is present. Historical data indicates that untreated nets reduce *P. falciparum* by approximately 51% compared to no nets (Clarke et al. 2001). However, robust modern data on this comparison is not available as randomised control trials with no net would not be ethically permissible (or necessary) given the strong evidence of the protective benefit of bed nets. However, there is some evidence that both susceptible and pyrethroid-resistant *Anopheles* mosquitoes can bite directly through the netting of a bed net (treated or untreated) if the host is pressed against the net (Hauser, Thiévent and Koella 2019).

1.3.3 Community protection

Community protection is the indirect benefit of insecticidal bednets to those nearby which do not sleep under one. As mosquitos that are knocked down or killed by one net cannot go on to bite individuals at another house, non-users gain a level of protection. Additionally, there is evidence that even when a mosquito survives interaction with a pyrethroid bed net their longevity is reduced (reducing the probability that they will survive long enough to become infectious) with laboratory based experiments conducted by Barreaux *et al.*, (2022) observing that mean survival time of *An. gambiae* post exposure reduced from 18 days to 15 days.

The quantitative impact of community protection is difficult to mathematically disentangle from personal protection, as a randomised control trial with an untreated group would be ethically untenable. The existence of the community protection of insecticidal bed nets is supported by studies that measure malaria outcomes against individual and community level net use. Investigations in the Democratic Republic of Congo (Levitz *et al.* 2018), Liberia (Stebbins, Emch and Meshnick 2018), and a meta-analysis of case data across 17 sub-Saharan countries (Larsen *et al.* 2014) indicate that high net use in a population provides a protective benefit for an individual even if they themselves do not use a net. Given the challenges in investigating community protection in the field, *in-silico* modelling exercises that compare human simulated populations with treated and untreated bed nets can provide insight. Transmission modelling by Unwin *et al.* (2022) supports the concept of community protection, estimating that when 80% of a population sleep under a pyrethroid net the remaining population were exposed to 30% less infectious bites per year (than if nobody slept under a net). They predict that the impact of community protection scales positively with coverage. However, they note that community effect is highly dependent on the susceptibility of

mosquitoes to insecticide, with the level of indirect protection greatly reduced when mosquitoes are resistant.

1.3.4 LLIN coverage and use

Initial bed net distributions involved the use of a simple cotton net that was dipped in insecticide, providing a physical layer of protection and an insecticidal effect against mosquitoes, albeit for only a matter of months before the insecticide was depleted and required retreating. Gradually, these self-treated bed nets have given way to Long-lasting Insecticidal Nets (LLINs), designed with their own internal reservoir of insecticide intended to continually regenerate surface levels to maintain insecticidal effect over multiple years. By providing longer lasting protection than standard ITNs, LLINs are intended to maintain consistent levels of protection in the years between distributions.

Initially LLINs were targeted to the most vulnerable groups in a population, such as children under the age of five and pregnant women. However, with the acknowledgment that bed nets may have community protective effects and consistent funding for national malaria control programmes, the WHO now recommends that all individuals in endemic areas are given access to an LLIN. While providing every individual with access to a bed net may not be logistically feasible due to the constraints of geography and demography, the WHO sets the target of providing access to 80% of at-risk individuals (WHO, 2013). Furthermore, the WHO recommends that national distributions of LLINs occur every three to five years however the specific timeline of a country's distribution programmes will be dictated by financial and logistical considerations.

The period from 2000 to 2017 represented a massive scale up in bed net distributions in sub-Saharan Africa. The numbers of nets distributed each year rose every year during this period, resulting in a record 56.3% (95% CI: 54.1–58.8) of at risk individual having access to a bed net in 2016 (with a total net crop of 380 million) (Bertozzi-Villa et al. 2021). However, this

achievement would represent a plateau in net coverage, decreasing to 51.0% (95% CI: 48.8–54.8) in 2019, for a total net crop of 337 million. Coverage metrics for 2020 are shown in **Figure 1.3**. This reversal in coverage is typically attributed to stalling support for vector control programmes, with stable levels of global funding made available for national control programmes despite growing populations and development of more expensive vector control tools. Consequently, despite the great strides forward compared to 2000, coverage levels in sub-Saharan Africa remain below WHO targets. In 2020, only five out of forty African countries (for which data are available) were predicted to reach the target of 80% coverage (Benin, Mali, Niger, Togo, and Uganda). Additionally, Bertozzi-Villa *et al.* estimate that as national coverage levels exceeded 50%, allocation tended to become less efficient with regions already at high coverage receiving more nets and regions with lower coverage continuing to have poor access (typically those where the population is more diffuse and difficult to reach).

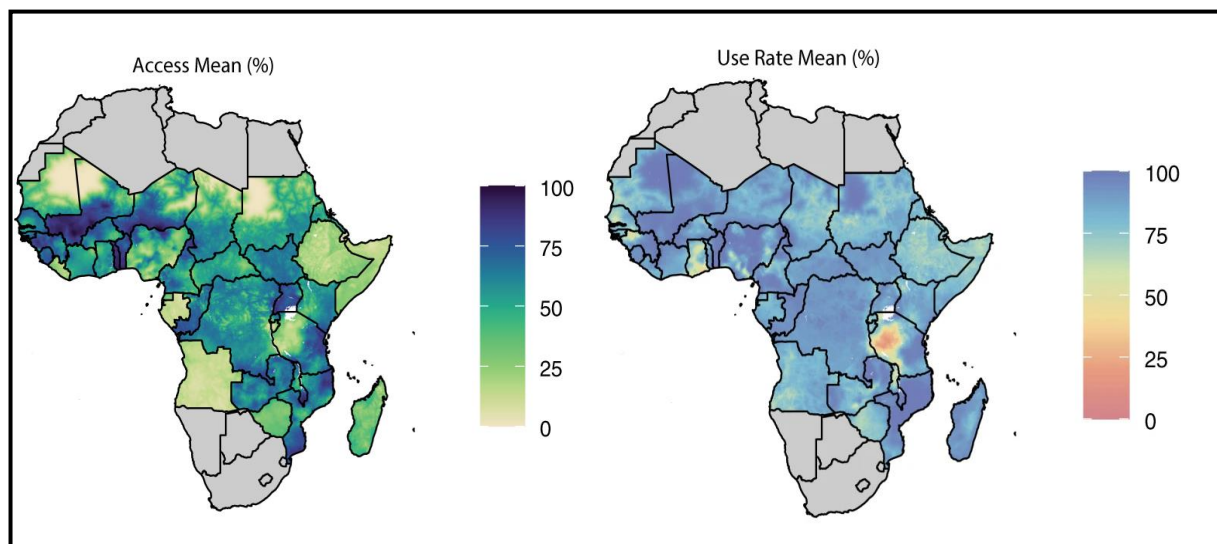


Figure 1.3 Estimated access and use rate of insecticide treated nets across sub-Saharan Africa in 2020. Note variability in metrics both between and within countries. (Adapted from Bertozzi-Villa et al. 2021)

An unavoidable weakness of bed nets as a vector control tool is they require their user to choose to use them. The owner of a net must perceive it to be useful and choose to keep it draped over their sleeping space each night despite the frequent finding from sociological surveys that nets are perceived to trap heat, resulting in discomfort. However, a 2021 meta-analysis of net coverage and use concluded that use of LLINs was typically high, with owners generally using a net if they had one, with 87.1% of those with access self-reporting that they used it on a regular basis (Bertozzi-Villa et al. 2021). However, estimating true rates of net use is difficult as it is documented that net owners tend to over-report the frequency at which they use their nets, with a meta-analysis of different survey methods estimating that self-reported use is 8% higher than objectively measured use (Krezanoski, Bangsberg and Tsai 2018).

1.3.5 LLIN retention

Current WHO guidance to national malaria control programmes (NMCPs) on distributing LLINs recommends nets are replaced every three to five years. However, there is strong and consistent evidence that LLIN retention time across sub-Saharan Africa is well short of three years. Bertozzi-Villa *et al.*, (2021) calculated that across the 40 countries assessed, median retention time was just 1.64 years. Only Cameroon, Guinea, and Niger, and were found to have median retention times at or above three years, with Mozambique and South Sudan amongst the poorest at only one year (though the modelling approach used set one year as the minimum value, meaning it could possibly be lower). These findings indicate that even where mass distributions are carried out every three years, large proportions of the population have no personal protection from the bites of *Anopheles* mosquitoes for extended periods of time. This is particularly alarming given that the use of alternative vector control techniques such as IRS have declined (WHO, 2021) meaning in many cases an LLIN is the only line of defence against mosquito bites. Previous studies that investigate the motivations behind a net owner's decision to discard their net report that owners chose to throw their net away when it is perceived to be too torn (Batisso et al. 2012, Gnanguenon et al. 2014, Koenker et al. 2014). However, this perceived physical damage may not bear any relationship with the personal protection of that net, with users instead highlight the visual element.

1.4 Emergence of Insecticide resistance

1.4.1 Overview of insecticides for malaria control

Pyrethroids are a class of fast-acting synthetic insecticides derived from pyrethrum, a naturally occurring insecticide found in the flowers of *Chrysanthemum* species plants (Ensley 2018). Pyrethroid insecticides have a number of characteristics that make them useful for mass deployment in insect control, including favourable safety profile for humans, high specificity for invertebrates, and rapid paralysing effect on target species even at low concentrations (Hougard et al. 2003, Briët et al. 2013). Furthermore, a key advantage of pyrethroids in terms of widespread use is their good ecological safety profile (compared to other insecticides used historically for insect control such the organochloride DDT) with low toxicity to birds and mammals however pyrethroids do have high toxicity to fish if water is polluted (Zaim, Aitio and Nakashima 2000, Kolaczinski and Curtis 2004). Additionally, as pyrethroids are degraded by sunlight and air they have low environmental persistence (Spurlock and Lee 2008, Tang et al. 2018).

The target site of pyrethroid insecticides are the voltage-gated sodium channels (VGSCs) of mosquito neurons, which play a critical role in moderating the neurochemical signals that coordinate a mosquito's organ function and movement (Silva, Santos and Martins 2014). When these VGSCs open to allow sodium ions to enter into the nerve cell, the resulting action potential (movement of charged ions) across the membrane creates an electrical signal that activates surrounding cells. Pyrethroid insecticides work by interfering with the activity of these VGSCs. When pyrethroids bind to open sodium channels, they prolong opening thereby blocking incoming signals and preventing coordination of the mosquito's nervous system. The result of this loss of nerve function is paralysis ('knockdown') and death.

There are two broad categories of synthetic pyrethroid insecticides: type I and type II (Ensley 2018). Both Type I and Type II target the VGSCs causing persistent opening and onset of paralysis, which may result in death. Type I pyrethroids make the nervous system hypersensitive to incoming stimuli, resulting in the rapid firing of mosquito neurons. Examples of type I pyrethroids include allethrin and permethrin. Type II pyrethroids differ from type I by the addition of a cyano group. The mode of action of type II pyrethroids is less well understood than Type I but it is thought that they may bind to different secondary target sites. Examples of type II pyrethroids include cypermethrin and deltamethrin. Prior to 2017, synthetic pyrethroids were the only class of insecticides approved for use in insecticidal nets (ITNs and LLINs). However, the WHO has given an interim recommendation for the use of pyrrole insecticides for use as a secondary active ingredient in pyrethroids nets (WHO 2017b).

A growing challenge in vector control programmes is the highly adaptive nature of vector populations (Killeen and Ranson 2018). The progress in reducing malaria morbidity and mortality achieved in the past two decades is threatened by the widespread rise of pyrethroid resistance in *An. gambiae* populations (Hemingway *et al.*, 2016)(**Figure 1.4**). Pyrethroid insecticides are the primary active ingredient in all WHO prequalified LLINs, yet mosquito populations throughout sub-Saharan Africa are now less susceptible to these compounds than ancestral population. Due to the growing frequency of target site mutations and metabolic resistance, *An. gambiae* mosquitoes are less likely to die as a result of a bloodfeeding attempt and more likely to achieve onwards transmission by biting a second individual (Moyes *et al.* 2020).

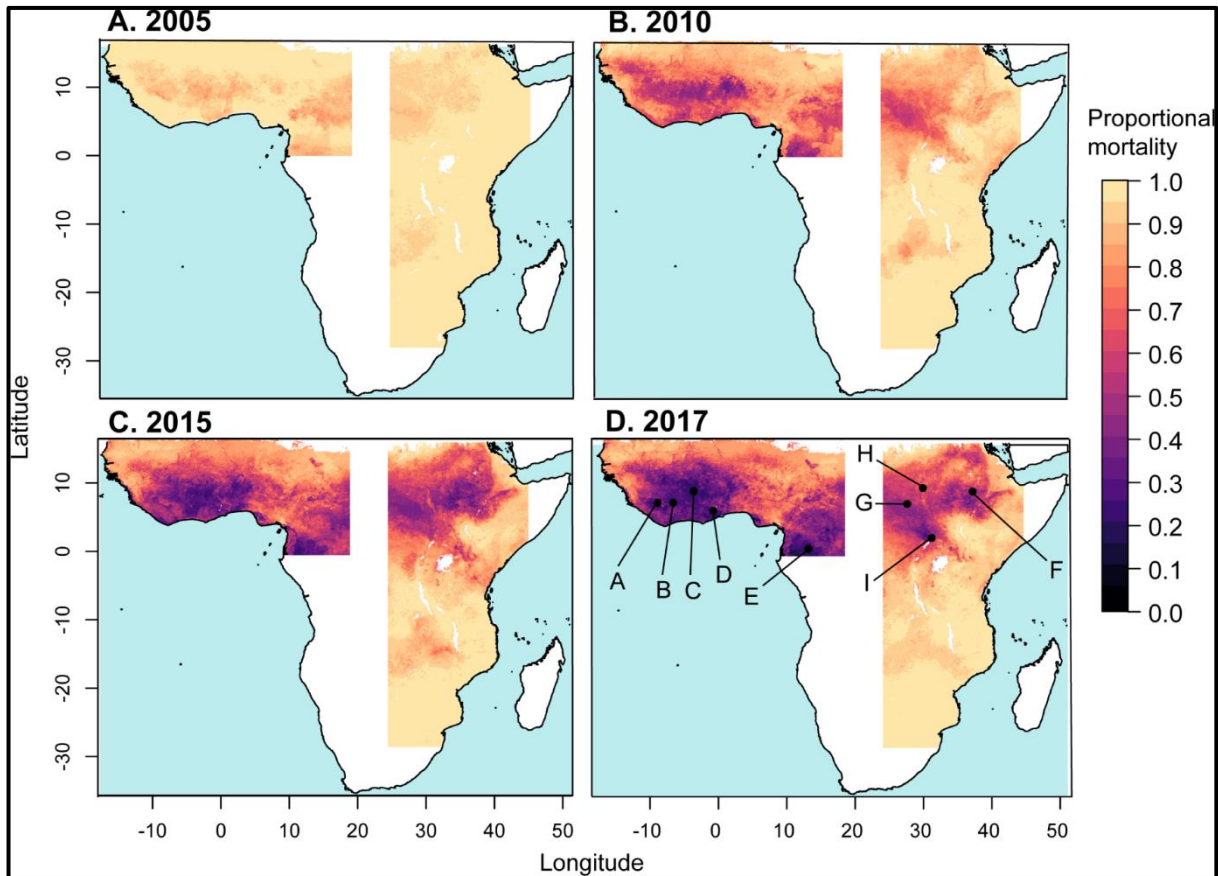


Figure 1.4 Predicted mean mortality of *Anopheles gambiae* (*s.l.*) After 1hr exposure to 0.05% deltamethrin across western and eastern sub-Saharan Africa, from 2005 to 2017. Note declining mortality with time, indicating growing pyrethroid resistance. (Adapted from Hancock et al. 2020)

1.4.2 Target site resistance

The widespread deployment of pyrethroid insecticides for malaria control and crop pests has created a selective pressure for mosquitoes that are less susceptible to their action. The emergence of target site mutations in sodium channels that limit binding by pyrethroids are now widespread in sub-Saharan Africa (Moyes et al. 2020). In *An. gambiae*, Single Nucleotide Polymorphisms (SNPs) at the L1014 locus result in amino acid substitutions that change the structure of the VGSCs. Two resistance alleles have been identified at this locus; the substitution of leucine with phenylalanine (*L1014F*, historically associated with West Africa) and the substitution of leucine with serine (*L1014S*, historically associated with East Africa). These alterations at the target site for pyrethroids are associated with *An. gambiae* tolerating increased exposure without being paralysed, referred to as a 'knockdown resistance' (*kdr*) (Reimer et al. 2014).

1.4.3 Metabolic resistance

Metabolic resistance in mosquitoes is the overexpression of detoxifying enzymes that evolved to break down steroids, fatty acids, and foreign compounds into nontoxic products for excretion. The genetic mechanisms of metabolic resistance vary between settings, involving mutations across multiple gene families. Given the varied genetic background between different mosquito strains and individuals, it is difficult to conclusively link phenotypic resistance to specific genetic mutations. Thus, metabolic resistance is not as well described as target site resistance.

Metabolic resistance to pyrethroids is associated with over-expression of three groups of enzymes: Cytochrome P450s, glutathione S-transferases (GST), and esterases. Cytochrome P450s are a superfamily of enzymes found across taxonomic kingdoms that are responsible for oxidising and clearing steroids, fatty acids, and foreign compounds. Polymorphisms in copy number variation (CNV), the duplications or deletions of genomic sequences, of genes associated with P450s have been linked to metabolic resistance. In laboratory experiments with *An. gambiae* the upregulation of genes associated with the expression of the Cytochrome P450s, *Cyp6m2* and *Cyp6p3*, have been linked with resistance to pyrethroids (Edi *et al.* 2014). The importance of these genes is supported by (Lucas *et al.* 2019) which identified high levels of CNVs in *Cyp6m2* and *Cyp6p3* amongst wild populations in regions where phenotypic resistance is widespread.

The fitness costs of metabolic resistance to pyrethroids have been documented in both *Aedes* and *Anopheles* mosquitoes, though they are poorly characterised thus there are substantial knowledge gaps on the extent to which energy demanding over-expression of metabolic proteins negatively effect on life-history traits (Gleave, Mechan and Reimer 2022). However, there is emerging evidence that these metabolic changes have a deleterious effect on the reproductive output of malaria vectors under laboratory conditions. The GST mutation L119T-GSTe2 in *An. funestus* is associated with reduced lifetime fecundity but longer longevity (Tchouakui *et al.* 2018). It is reported that GSTs protect mosquitoes against oxidative stress, which may account for the increased longevity associated with over-expression. Over-expression of *Cyp6P9* in *An. funestus* was associated with reduced fecundity (Mugenzi *et al.* 2019, Tchouakui *et al.* 2020) and slower larval development (Tchouakui *et al.* 2021). Tchouakui *et al.*, 2021 hypothesise that over-expression of *Cyp6P9* results in decreased locomotive performance in larvae, resulting in poorer feeding in larval habitats compared to

larvae that lack this mutation. Laboratory based assessments indicate that pyrethroid-resistant *An. gambiae* reverts back to susceptibility in the absence of selection pressure by pyrethroids, estimated at 15 generations or 1.3 years in a typical malaria endemic-setting (Machani et al. 2020).

1.4.5 Impact of pyrethroid resistant vectors on malaria outcomes

Despite the unprecedented gains for malaria control at the beginning of this century, the second decade of this century has seen stalling progress (Noor and Alonso 2022; Rosenthal 2022). There is widespread concern that growing pyrethroid resistance is undermining the global gains in reducing morbidity and mortality (Hemingway et al. 2016; Ranson et al. 2016; Killen et al. 2018). However, while the decrease in susceptibility of wild populations to pyrethroids across sub-Saharan Africa is not in doubt, the impact of pyrethroid resistance in a mosquito population on clinical malaria outcomes is difficult to assess. The link between pyrethroid susceptibility and malaria transmission is complicated by the sub-lethal effects of insecticides and fitness costs of resistance, both of which place pressure on mosquito populations even if they survive immediate contact (Viana et al. 2016; Tchouakui et al 2020; Gleave et al. 2021). Additionally, limited but growing evidence of outdoor biting by *Anopheles* mosquitoes allows vectors to circumvent indoor based insecticides (Sougoufara et al. 2020, Musiba et al. 2022, Sangbakembi-Ngounou et al. 2022).

Laboratory based research on *An. gambiae* with both target site and metabolic mutations indicate that even when LLINs have little immediate effect on contact, a resistance mosquito may still suffer delayed mortality several days later. Viana *et al.*, (2016) observed that the life span of pyrethroid resistant *An. gambiae s.s.* was cut by 25-60% (across different exposure regimes) following exposure to deltamethrin. This reduction in lifespan would be expected to impact malaria transmission, with mosquitoes less likely to survive the Extrinsic Incubation Period needed to become infectious

Malaria modelling studies conducted alongside experimental hut trials by Churcher *et al.* (2016) indicate that pyrethroid resistance in *Anopheles* populations is associated with increased malaria risk, with mortality in bioassays being a good predictor of bloodfeeding success in huts. They demonstrated that pyrethroid-resistant mosquitoes were less deterred from entering huts and observed that probability of blood-feeding only increased when a high proportion of mosquitoes are resistant. Overall, the transmission dynamic models predicted that higher frequency of pyrethroid resistant mosquitoes has a positive correlation with the number of clinical cases. In the model, this simulated impact on malaria outcomes occurs as the result of increased bloodfeeding probability alongside decreased probability of dying due to contact with the net, thereby resulting in an increase in mosquitoes that survive to become infectious (with the effect most pronounced in areas of high net coverage).

1.4.6 Next generation tools for targeting pyrethroid resistant mosquitoes

A key difficulty in addressing pyrethroid resistance in malaria vector populations is the lack of alternative insecticidal chemistries available. Synthetic pyrethroid insecticides were widely used in agricultural pest control before being adapted for use in malaria control strategies. A lack of suitable candidate chemistries with the same attributes as pyrethroids, that do not have cross-resistance with pyrethroids, has led to the interim solution of supplementing pyrethroids with a second chemistry (WHO 2017a, Toe et al. 2018). The synergist piperonyl butoxide (PBO) was quickly identified as a potential partner compound. Developed to maintain the effectiveness of agricultural control programmes following resistance to pyrethroids in crop pests, PBO enhances the potency of pyrethroid insecticides despite having no insecticidal activity of its own. When PBO enters the body of a mosquito it inhibits the activity of Cytochrome P450 enzymes preventing them detoxifying or sequestering pyrethroid compounds, increasing the susceptibility of resistant mosquitos (Hodgson and Levi 1999; Edi et al 2014)). However, it should be noted that only metabolic resistance mechanisms are inhibited by PBO meaning target site alterations still provide a protective effect against pyrethroids. Nonetheless, pyrethroid LLINs supplemented with PBO were given an interim endorsement for use in the field in 2017 (WHO 2017a).

LLINs containing piperonyl butoxide (PBO-LLINs) have been deployed in a number of countries with moderate-high pyrethroid resistance to determine their effectiveness in reducing malaria prevalence compared to pyrethroid-only LLINs. An 18 month randomised control trial (RCT) comparing PBO-LLINs and standard LLINs in Uganda identified a 25% reduction in parasite prevalence in children 2-10 years old after six months, which was sustained to the end of the trial period (Staedke et al. 2020). Similarly, a RCT in neighbouring Kenya observed a 33% reduction in malaria prevalence with PBO-LLINs in children 2-10 years old after six

months, falling slightly to a 26% reduction after 12 months (Minakawa et al. 2021). A 2021 systematic review of PBO-LLINs concluded that they had improved epidemiological and entomological outcomes compared to standard pyrethroid LLINs for up to 25 months in areas where resistance was moderate to high (Gleave et al. 2021). Additionally, the authors highlighted that there was little evidence of increased entomological efficacy of PBO-LLINs in areas where pyrethroid resistance was low. Importantly, this review stressed the lack of durability data for PBO-LLINs, with a need to build up an evidence base of the physical and chemical durability of these nets in the field.

1.5 Assessing the durability of long-lasting Insecticidal Nets

1.5.1 Purpose of durability assessment

In order for national malaria control programmes to make informed decisions on what LLIN products are suitable for use in their setting and on the appropriate time between national distributions, they require context-specific information on the operational lifespan of nets. As LLIN designs vary in active ingredients, fabric used, and the mechanism by which insecticide is stored and released from the fibres, one LLIN product may perform better in a given setting compared to another. For this reason, it is recommended that programs distribute multiple LLIN products at each distribution to provide comparable data on which designs perform best within that setting.

Current WHO guidelines expect that LLINs retain their biological activity for at least 20 washes (under laboratory conditions) and provide protection for at least three years when used appropriately. Consequently, national mass distributions are typically conducted at three year intervals (WHO 2013a). However, there is a growing evidence base that the operational lifespan is below three years in many settings and is not uniform either within or between countries (Gnanguenon et al. 2014, Toé et al. 2019, Lorenz et al. 2020, Bertozzi-Villa et al. 2021). In response to emerging evidence that the operational lifespan is poorer than expected and variable between settings, WHO Pesticide Evaluation Scheme (WHOPES) guidance was developed to provide a universal framework by which the durability of LLIN products could be assessed. These guidelines lay out clear targets by which the quality and performance of LLIN products can be assessed at timepoints after distribution, with full WHO recommendation reserved until large-scale evidence is accumulated indicating these targets are met.

1.5.2 Definition of durability

The broad description of a durable bed net design is one that is retained by its owner, maintains insecticidal effect against susceptible *Anopheles* mosquitoes for three years, and is sufficiently physically robust to prevent excessive holes which a mosquito may enter through (WHO 2011). This WHOPEs LLIN durability assessment framework provides a clear methodology for monitoring and evaluating the survivorship, chemical integrity, bioefficacy, and physical integrity of LLIN products.

1.5.2.1 Survivorship

Survivorship is defined as the proportion of nets distributed that are still present in a household and suitable for use, monitored at timepoints after distribution. The causes of nets no longer being present in a household are not well described. Owners may choose to discard their nets if they perceive them to no longer be useful (which may bear no relationship with the personal and community effect of that net) (Batisso et al. 2012, Gnanguenon et al. 2014). However, it should be noted that survivorship is complicated by movement of individuals between households. As survivorship is defined as a specific enumerated net remaining in a specific household, a net that was taken with its owner when they moved to a different household is indistinguishable from a net that was thrown away for the purposes of survivorship (Guglielmo *et al.* 2021). Consequently, while survivorship is important to measure, it is difficult to disentangle the physical priorities of an LLIN product from socioeconomic factors and human behaviour. The inverse of survivorship is 'attrition' which is the proportion of nets distributed that are no longer available for use. Nets that appear to have never been used are excluded from survivorship and attrition calculations.

Net survivorship is highly variable between settings, meaning that the attrition rate with a given LLIN product is context-specific to the country in which it was assessed. In a three-year cross-section study in Tanzania, Lorenz *et al.* (2020) observed that only 54% of nets distributed were still present after three years. Furthermore, they observed that survivorship varied between LLIN Products after three years; with 45% of PermaNet 2.0 and 58% of Olyset Net still present. In Kenya, 86.4% of Olyset Net LLINs and 91.2% of Olyset Plus LLINs were still present after three years (Gichuki *et al.* 2021). In a cross-section study in a semi-arid region of Ethiopia with the LLIN PermaNet 2.0, 67% of nets were no longer present in households after two years (Solomon *et al.* 2018). In a particularly extreme example, a randomised control trial in the cascades region of Burkina Faso, just 12% of Olyset Net LLINs were still present in households after three years (Toé *et al.* 2019). Evidently, the assumption of a three-year service life is not supported by these observations.

The loss of nets across the course of a durability trial may confound the reporting of durability outcomes (Toé *et al.* 2019, Batisso *et al.* 2012, WHO 2011). When nets are thrown away, they are unable to be assessed for chemical or physical integrity and therefore censored from the final dataset. This phenomenon is known as survivorship bias, which is a common confounder in randomised control trials (RCTs) (Keiding *et al.* 2019, van Eekelen *et al.* 2021). If nets were discarded at random then there would be no confounding effect however if the decision to discard is associated with the outcomes being assessed, then the data may be distorted. For example, if a net owner chose to discard their net due to the perception that it was too physically torn then that net is censored from the data and physical integrity outcomes are downwardly biased as a result (the equivalent in a RCT of a pharmaceutical product would be the most seriously ill patients dying and the health outcomes of the remaining cohort improving on average as a result).

1.5.2.2 Chemical Integrity

Chemical integrity is the quantity of active ingredient(s) remaining in sampled nets at timepoints after distribution (expressed as a proportion of the total net in g/kg or mg/m²). It is expected that the chemical content of insecticidal nets will be lost over time with routine use such as washing and handling thus LLINs are designed with a sufficient reservoir of insecticide that the content available to the mosquito will remain sufficient across multiple years of use. Previous investigations of the chemical integrity of LLINs across a number of settings (including Tanzania, Burkina Faso, and Benin) have observed that pyrethroid levels tend to remain relatively stable over time (Lorenz *et al.* 2014, Massue *et al.* 2016, Toé *et al.* 2019, Lorenz *et al.* 2020, Ngufor *et al.* 2020). However, current methods for assessing the chemical integrity of LLINs using High-Performance Liquid Chromatography (HPLC) can only measure the total insecticide content of a homogenised sample from a net thus cannot quantify the amount of insecticide that is on the surface and is bioavailable to a mosquito that contacts it. To address this, techniques that use mass spectroscopy to quantify surface chemistry are under development but are not yet available for use. Additionally, chemical assessment of LLIN products is complicated by the development of novel designs with multiple active ingredients. If the active ingredients within an LLIN product bleed out of the fibres at different rates, as has been observed for the pyrethroid-pyriproxyfen net Olyset Duo (Toé *et al.* 2019), then the result may be variable ratios of compounds at the surface of the net across time which complicates the interpretation of durability data.

1.5.2.3 Bioefficacy

LLIN Bioefficacy is the insecticidal effect of a net against *An. gambiae* mosquitoes. Square pieces (30cm x 30cm) are cut from nets sampled from the field and tested in benchtop assay to assess the extent to which bioefficacy has been retained relative to a brand new sample of that LLIN product. The key bioefficacy outcomes of the WHOPES durability guidance are knockdown after one hour (the proportion of mosquitoes incapacitated) and 24 hour mortality (the proportion of mosquitoes dead)(WHO 2011). These outcomes are assessed using WHO cone bioassays (**Figure 1.5**), a benchtop setup where mosquitoes are held in close proximity to a net sample for three minutes(WHO 2013a).



Figure 1.5 Example of WHO cone bioassay. Note that the mosquito is avoiding contact with the insecticidal net by resting on the untreated cotton wool.

Current guidance outlines thresholds that nets are expected to meet to be considered effective, defined as knockdown $\geq 95\%$ or killing $\geq 80\%$ of susceptible *An. gambiae* (WHO 2013a, WHO 2013b). As LLIN products with mosquito repellent properties may not achieve these thresholds despite functioning as intended (due to mosquitoes avoiding the net surface) it is recommended that a confirmatory assay with rodents as bait is performed to assess blood-feeding inhibition. This assay of a bed net sample's blood-feeding inhibition, the WHO Tunnel Test, consists of two chambers with a holed net sample obstructing the connecting tunnel between them. The rodent is restrained in one chamber and mosquitoes released into the other, the net is assessed based on the proportion of mosquitoes that are prevented from crossing through the net to bloodfeed on the bait overnight. If a net in the tunnel prevents $\geq 90\%$ of susceptible mosquitoes from bloodfeeding and/or kills $\geq 80\%$ then it is considered to have passed. However, in practice the WHO Tunnel Test is not widely used due to ethical considerations regarding the welfare of the animal used, which may suffer both psychological and physiological distress due to confinement, dehydration, and biting by mosquitoes. As a result, obtaining approval for these experiment from Research Ethics Committees is a major barrier to their use. Additionally, the use of non-human bait to assess the behaviour of the highly anthropophilic *An. gambiae* limits the interpretation of result in terms of bloodfeeding inhibition on human occupants. Consequently, there is a need for alternative bioassay methods for assessing the performance of LLINs with repellent properties.

The bioefficacy of LLINs after three years in operational conditions against pyrethroid-susceptible *An. gambiae* females (as measured in benchtop exposures) is highly variable between settings. In a retrospective study of the bioefficacy of the permethrin LLIN Olyset Net nets sampled in Tanzania, 100% of nets sampled after three years passed the WHO bioefficacy criteria outlined above after three years (Massue *et al.* 2016). However, in a subsequent randomised control trial in the same country with the same product, 75.0% of Olyset nets sampled passed (Lorenz *et al.* 2020). Additionally, the pass rate for the Olyset Net sampled after three years in randomised control trials in Burkina Faso and Kenya was only 58.3% and 42.0% respectively (Toé *et al.* 2019, Gichuki *et al.* 2021).

In a retrospective study of the bioefficacy of the deltamethrin LLIN PermaNet 2.0, 90.0% of nets passed the bioefficacy criteria after 32 months (Anshebo *et al.* 2014). Furthermore, a randomised control trial conducted in Zanzibar reported that 100% of PermaNet 2.0 nets sampled after 36 months passed the bioefficacy criteria (Haji *et al.* 2020). Finally, in a randomised control trial in Tanzania, 85% of PermaNet 2.0 nets sampled after three years passed bioefficacy criteria (Lorenz *et al.* 2020). The variability in bioefficacy outcomes between durability studies for the same LLIN product in different settings highlights the context dependence of these results and that they are not readily comparable between countries.

1.5.2.4 Gaps in WHO bioefficacy assessment guidelines

Current WHO durability guidelines for evaluating the bioefficacy of sampled nets do not include a methodology for assessing performance against pyrethroid resistant mosquito colonies. As the current guidelines predate the widespread emergence of pyrethroid-resistance in sub-Saharan Africa, it is not required that nets demonstrate bioefficacy against pyrethroid-resistant mosquitoes regardless of resistance levels in the setting that net was used. Given the challenges of pyrethroid resistance for vector control programmes, developing a methodology for demonstrating efficacy against them should be a high priority. Developing such guidelines is complicated by variation in the strength of phenotypic resistance, limiting direct comparisons between studies with different resistant colonies. To address this gap, some existing durability studies that have assessed bioefficacy against pyrethroid resistant populations have taken the initiative to collect wild mosquitoes from the study site and rear them for use in testing, thereby making the findings informative of performance in that context (Toé *et al.* 2019).

A further gap in durability monitoring guidelines of LLINs sampled from the field is the lack of methodology for assessing 'next-generation' products with secondary AIs supplementing pyrethroids. For products where the pyrethroid is supplemented by piperonyl butoxide (such as 'Olyset Plus' and 'PermaNet 3.0') a similar methodology to the current guidelines may be appropriate (albeit with pyrethroid-resistant mosquitoes) but for emerging products with completely different mechanisms of action additional assays are required. For example, the dual AI LLIN 'Interceptor G2' (IG2) supplements pyrethroid with the pyrrole class insecticide chlorfenapyr which is designed to be much slower acting and inflict mortality days after exposure. It would appear self-evident that the 1hr and 24hr bioefficacy outcomes outlined above would be inappropriate to fully assess the performance of IG2, with a longer

time delay such as 72hr or 96hr mortality providing additional insight. As dual-AI LLIN products become more common and varied, it could be argued that a single universal methodology for assessing the performance of all LLINs is no longer appropriate, with guidelines instead tailored to classes of products with specific approaches to interrupting transmission (such as distinguishing between products designed to restore susceptibility to pyrethroid resistant mosquitoes and products with slow-acting insecticides).

1.5.2.5 Physical integrity

Physical integrity is the condition of the fabric of bed nets sampled from the field. As holes accrue in nets over time as a result of handling and use, these gaps in the fabric may provide an entry point for mosquitoes to bite the occupant (**Figure 1.6**). Thus, even if a net retains sufficient chemistry to ultimately kill mosquitoes that approach, the mosquito may be able to obtain a bloodmeal before it dies. Consequently, it is important to monitor the extent of physical damage on nets to identify LLIN products which are the least susceptible to physical damage.

A recent meta-analysis by Wheldrake *et al.*, (2021) identified mechanical damage as the primary cause of hole formation (63.14% of all holes), as opposed to burning or animal damage (though 27.87% of holes occurred due to rodents). Furthermore, mechanical damage was responsible for 81.50% total damage by area. They outlined a general pattern of damage accumulation, with small holes occurring due to abrasion with rough materials (such as straw) and these smaller holes later catching on an anchor point as the net is moved to cause a tear.



Figure 1.6 A heavily damaged LLIN sampled from a household in western Uganda (photograph taken by Amy Lynd, reproduced with permission).

Current WHO durability guidelines quantify net damage using proportionate Hole Index (pHI)(WHO 2011, WHO 2013b). The pHI system takes an approximation of total damaged area on a net and categorises it into bands of 'serviceability', with nets identified as either 'good', 'damaged', or 'too torn' (with 'too torn' indicating that a net is unsuitable for use). The total damage on a net is approximated by observing each hole and comparing it with body parts to estimate its size, summing these estimated values for all holes on the net, as described in Table 1.1. This methodology for approximating hole size is intended to accelerate the process of assessing physical integrity, as the number of nets and holes assessed may make direct measurement onerous.

TABLE 1.1. WHO GUIDANCE FOR ESTIMATING THE SIZE OF HOLES IN LLINS

Hole category	Reference to body part	Estimated diameter (cm)	Estimated area (cm ²)
1	'smaller than a thumb'	0.5–2	1
2	'larger than a thumb but smaller than a fist'	2-10	23
3	'larger than a fist but smaller than a head'	10-25	196
4	'larger than a head'	> 25	578

Holes less than 0.5cm (category one) are not considered in the calculation of pHI. The hole index is then calculated using the following calculation:

$$\text{Hole index} = (\text{number of size 1 holes} \times 1) + (\text{number of size 2 holes} \times 23) + (\text{number of size 3 holes} \times 196) + (\text{number of size 4 holes} \times 579)$$

Once the Hole Index has been calculated for a net, this approximation of hole area is categorised into the following categories: 'good' = $\leq 64\text{cm}^2$, 'damaged' = $65\text{--}643\text{cm}^2$, 'too torn' $\geq 644\text{cm}^2$. These categories are extrapolated from a small number of early behavioural studies with pyrethroid bed nets that observed that the bloodfeeding inhibition against susceptible *An. gambiae* decreases from 100% when fully intact to between 69-75% when total damaged area is 96cm^2 (Curtis, Myamba and Wilkes 1996, Malima et al. 2008), and that bloodfeeding inhibition is greatly diminished when total damaged area is greater than 1000cm^2 . However, a recent evaluation of methods for evaluating hole size indicated that the WHO estimates of hole area tend to overestimate the size of holes on nets by approximately 100% due to most holes in practice tending to be being elliptical rather than a circle (Vanden Eng et al. 2017).



The interaction between holes in an insecticidal net and the host-seeking behaviour of *Anopheles* mosquitoes is not well described, with the WHO outlining the impact of hole location on net entry a priority to be addressed by future durability monitoring guidelines. Currently, the guidance states that the location of each hole (top or side) should be reported but this information is not factored into any of the reported outcomes.

1.6 Behaviour of host-seeking *An. gambiae* around LLINs

1.6.1 Mosquito detection of humans

Mosquitoes require sense organs to navigate their environment in order to locate food, mates, oviposition sites, and bloodmeal sources. A number of previous investigations have identified that olfactory chemical cues are the primary means by which female mosquitoes identify appropriate bloodmeal hosts (Raji and DeGennaro 2017), supplemented by thermal and visual cues as the mosquito approaches the target. The malaria vector *An. gambiae* has evolved a strong preference for humans (anthropophily), which contributes to their high capacity to transmit *Plasmodium* infection in human populations.

It has been observed in a number of studies that a potent initial cue to activate host-seeking behaviour is carbon dioxide (CO₂), detected by the maxillary palps, which induces sustained flight towards the source. The ubiquitous presence of CO₂ in animals' breath makes it a poor indicator of specific hosts thus a combination of cues are used. Even when rendered completely unable to detect CO₂ (by inactivating the genes associated with development of the olfactory neurons that are sensitive to changes in CO₂ concentration) host-seeking is diminished but not completely stopped (McMeniman et al. 2014). The host-seeking of *An. gambiae* is greatly enhanced by the detection of human odour, a complex mixture of volatile compounds, including lactic acid, ketones, sulphides, ammonia, and carboxylic acids that together form a distinct signature that distinguishes us from other animals (Zwiebel and Takken 2004). In isolation, lactic acid has been shown to be a strong attractant for Anophelines, with the addition of lactic acid to animal odour making it attractive to *An. gambiae* and *An. coluzzii* (Dekker et al. 2002).

Once the mosquito has navigated along the concentration gradient of CO₂ and human odours towards its approximate source (such as into the home of a sleeping human) it uses heat to identify host bodies. There is evidence from laboratory studies that at close range *An. gambiae* is attracted to land on objects that are 37⁰C, indicating that their thermal receptors have evolved to identify humans (McBride 2016). Additionally, visual cues are thought to contribute to the host seeking behaviour of *An. gambiae* however it's mechanism and importance are not well described. However, more recent behavioural experiments indicate it is the combination of these cues that motivates *An. gambiae* to land on human, as demonstrated by the weak response to exposed human skin in bloodfeeding assays where the hosts breath cannot reach the mosquito (Webster, Lacey and Cardé 2015).

1.6.2 Behaviour of *An. gambiae* around insecticidal nets

While informal observations of mosquitoes clustering around the top of bednets were noted since the earliest years of their use, explicit investigation of the spatial dynamics of host-seeking activity has occurred only relatively recently. Semi-field experiments conducted by Lynd and McCall (2013) using untreated nets coated in an adhesive observed that 74-87% of pyrethroid-susceptible *An. gambiae* mosquitoes (3-7 day old unfed females) released were caught on the top of the net when occupied by human bait (clustering towards the head and chest). In contrast, when nets were unoccupied the mosquitoes caught were much more evenly distributed over the net surface. While the use of adhesives meant only initial contact could be quantified, preventing subsequent behaviour from being expressed, this initial finding supported the hypothesis that mosquitoes are attracted to the top surface of the net by a rising plume of chemical attractants expelled by the occupant. These initial findings were supported by subsequent experiments by Sutcliffe and Yin (2014) using human-baited untreated nets arrayed with sticky panels, also observing that initial contact by pyrethroid-susceptible *An. gambiae* was strongly focused on the top of the net. Given the limitations imposed by using adhesives and that untreated nets may not be representative of behaviour around insecticidal nets, Parker *et al.* (2015), conducted infrared video tracking experiments to observe the behaviour of pyrethroid-susceptible *An. gambiae* s.s around the deltamethrin LLIN PermaNet 2. Parker *et al.* observed that total activity across one hour sessions was strongly focussed on the roof of human occupied nets, with 78.3% of activity on the roof with an LLIN and 74.7% with an untreated net (**Figure 1.7**). Additionally, they hypothesise that *An. gambiae* are unable to visually detect the presence of the LLIN, given evidence of similar approach velocities between an LLIN and no LLIN in wind tunnels, instead speculating that navigate towards the host using chemical cues. A lack of visual detection when navigating

around LLINs would have implications for damaged nets, suggesting *An. gambiae* mosquitoes would not perceive them and navigate accordingly.

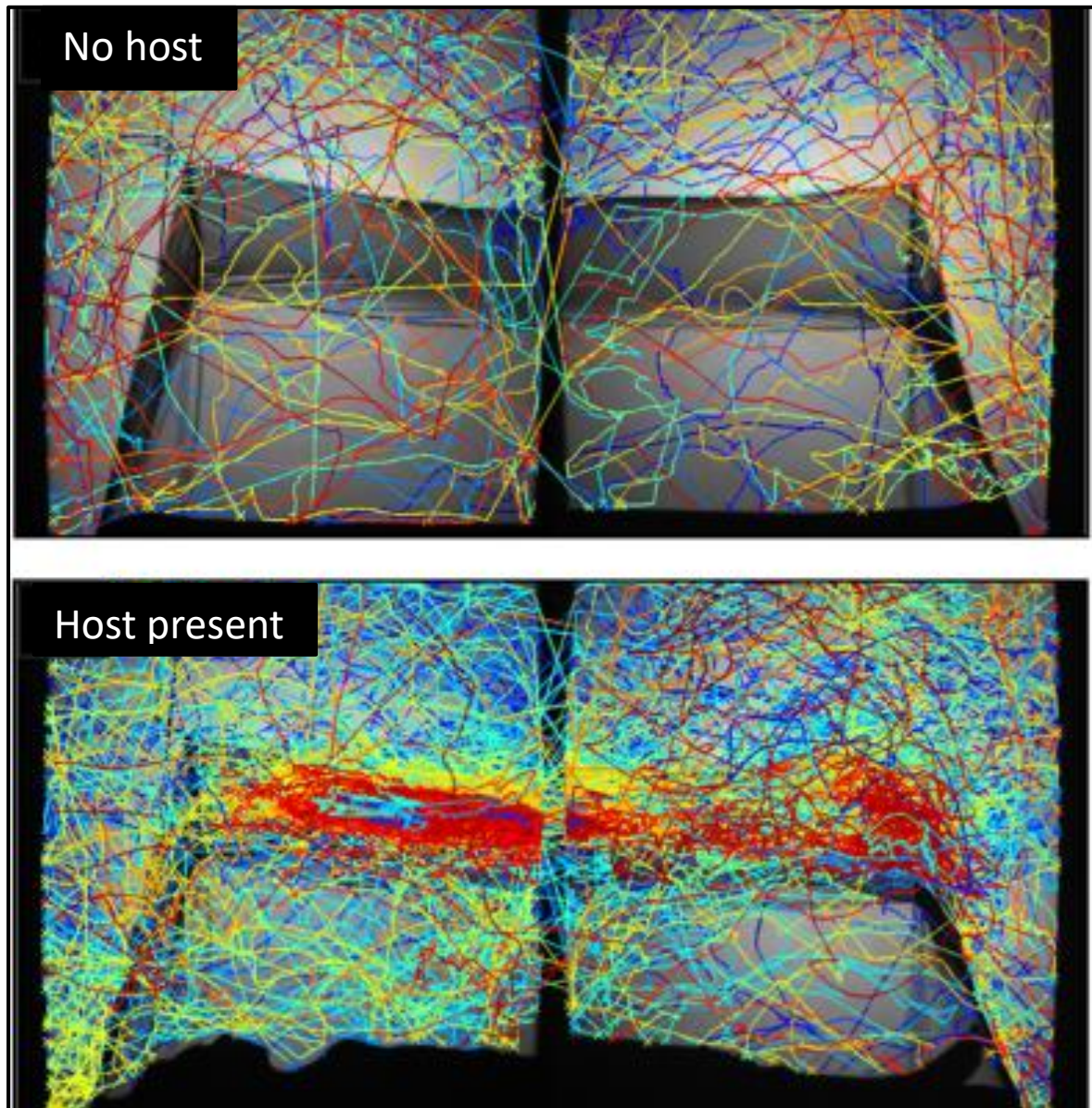


Figure 1.7 Mosquito flight tracks around an unbaited and baited LLIN. Each coloured track represents a single mosquito, note concentration of activity on top panel of net in baited (host present) image. Adapted from Parker et al. 2015.

1.6.3 Interaction between *An. gambiae* and holed LLINs

LLINs provide a physical barrier against the bites of host-seeking *Anopheles* mosquitoes yet the consequences when holes develop in this barrier are not well understood. Following the WHO identification in 2011 of mosquito entry into holed LLINs as a priority knowledge gap to be addressed, the earliest study to comprehensively investigate mosquito entry into damaged nets was conducted by Randriamaherijaona and colleagues (2015). Using release recapture experiments in experimental huts Randriamaherijaona *et al.* assessed bloodfeeding inhibition and insecticidal effect of pyrethroid nets (PermaNet 2.0) with different extents of physical damage (with total holed area ranging from 15cm² to 22,500cm²). They observed that the probability that an *An. gambiae* female would bloodfeed on occupants increased exponentially with the total holed surface area on the net. Interestingly, bloodfeeding success was independent from insecticidal outcomes with no difference in bloodfeeding rates between susceptible and resistant mosquitoes. However, this study did not investigate the impact of the location of these holes on bloodfeeding success. Investigations of the impact of hole location on mosquito entry into LLINs is a slowly emerging topic. As quantifying bloodfeeding success requires human participants to potentially expose themselves to bites, many laboratory based studies chose to instead visually monitor hole entry as a proxy for bloodfeeding.

A 2017 investigation by Sutcliffe *et al.*, used video recording to compare the probability that 4-8 day old unfed *An. gambiae* females would pass through equally sized holes on the top and side of an untreated net. In addition to confirming prior findings that mosquito activity was heavily focused on the top of the net, they observed that mosquitoes arriving near a hole on the top were 20% more likely to pass through than when at a hole on the side. Taken together, they concluded that holes on the top were a much greater risk for hole entry with a hole on

the side. They developed a model which estimated that a 1cm diameter hole in the top is equivalent to a 70cm diameter hole in the side. While these findings certainly challenge the current WHO guidance that all holes are weighted equally in terms of assessing physical damage, regardless of where they are located on the net, the use of an untreated net rather than an insecticide treated net limits interpretation in terms of an LLIN in operational conditions

The mosquito behaviour studies outlined above conducted their investigations at standard insectary conditions ($27^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and 80% relative humidity) however there is emerging evidence that the patterns of mosquito behaviour around an LLIN is influenced by temperature. Sutcliffe and Yin (2021) observed that overall mosquito activity around an untreated net in a 'warm' $27\text{--}30^{\circ}\text{C}$ room was lower and less focussed on the roof than in a 'cool' $23\text{--}25^{\circ}\text{C}$ room.

1.7 Aims and objectives

The aim of this study is to investigate the physical and chemical changes of LLINs with time in operational use. While it is established that LLINs generally become physically damaged and lose insecticide from the fibres over time, the implications of this for their protective effect is poorly described. Furthermore, given recent advancements in characterising *An. gambiae* behaviour around bednets, this study aims to investigate if the location of holes on a net impacts the risk that mosquito will bloodfeed on the occupant and successfully escape to survive the encounter. Additionally, given the development and growing deployment of 'next-generation' PBO-LLINs designed to combat insecticide resistance in vector populations, this study will address these aims for this new LLIN class.

To address these aims, the following objectives were devised.

- 1) Quantify the chemical content of both conventional pyrethroid-only LLINs and their 'next-gen' PBO equivalents at timepoints after distribution to the field.**
- 2) Quantify the bioefficacy of both conventional pyrethroid-only LLINs and their 'next-gen' PBO equivalents at timepoints after distribution to the field against**
 - a. Pyrethroid-susceptible *An. gambiae***
 - b. Pyrethroid-resistant *An. gambiae***
- 3) Quantify the relationship between chemical content and bioefficacy**
- 4) Quantify total damage to LLINs across timepoints to:**
 - a. Investigate differences between LLIN products**
 - b. Investigate spatial trends in the location of damage on the nets**
 - c. Quantify the relationship between total damage and bioefficacy**
- 5) Investigate the relationship between hole location and personal protection of LLINs**
- 6) Compare the personal protection of pyrethroid-only and pyrethroid-PBO LLINs**

Chapter Two: Chemical content and bioefficacy of Long Lasting Insecticidal Nets treated with and without piperonyl butoxide across two years of operational use in Uganda

Statement of contribution

The findings presented in this chapter were made possible by the contributions of a number of individuals. While the preparation of net samples and all subsequent assessments were performed by myself, these samples were sent to me by field collections made by colleagues at the Infectious Diseases Research Collaboration in Kampala, Uganda. The collections were performed alongside a larger randomised control trial into the epidemiological and entomological impact of the addition of PBO to pyrethroid bed nets.

2.1 Introduction

2.1.1 Background

Long Lasting Insecticidal Nets (LLINs) are the cornerstone of global malaria control strategy, forming a physical and chemical barrier against the bites of *Anopheles* mosquitoes (Churcher *et al.*, 2016). Progress in interrupting malaria transmission in sub-Saharan Africa in the past two decades has been attributed in large part to mass distributions of LLINs (Bhatt *et al.* 2015, Pryce, Richardson and Lengeler 2018). To achieve high levels of coverage, the World Health Organisation (WHO) recommends that countries distribute nets through both national programmes and antenatal services (WHO, 2013). The pyrethroid insecticides in LLINs are intended to maintain sufficient concentrations for at least three years, thus distributions are typically planned to occur at three-year intervals. However, recent durability studies suggest that in some countries the operational lifespan of LLINs is less than three years (Gnanguenon *et al.* 2014, Toé *et al.* 2019, Lorenz *et al.* 2020, Bertozzi-Villa *et al.* 2021). To sustain the impact of malaria control, National Malaria Programmes (NMPs) must identify LLIN products that are

durabile within the cultural and environmental context of their country (WHO 2011, WHO 2013). However, existing WHO LLIN durability assessment guidelines were not intended for the growing diversity of net classes that have been designed in response to growing pyrethroid resistance in mosquito populations. To allow NMPs to evaluate the performance of LLIN products in their country, there is a need to adapt the current WHO guidelines to include testing against pyrethroid-resistant mosquitoes.

LLINs interrupt the transmission of *Plasmodium* parasites by reducing the number of infectious bites individuals will receive (Churcher *et al.*, 2016). The chemical effect of the insecticide intoxicates the mosquito on contact, inhibiting motor function to prevent blood-feeding and eventually death (Rehman *et al.*, 2014). Additionally, there is evidence that pyrethroids provide their protective effect inciting an ‘excito-repellent’ effect, resulting avoidance away from the net (Lindsay *et al.* 1991, Faulde and Nehring 2012). However, it remains unclear if this repellency undermines the broader community protective effect by diverting mosquitoes towards unprotected individuals.

The WHO currently only recommends the use of pyrethroid and, more recently, pyrrole insecticides for use on LLINs (WHO 2017). However, the continued use of LLINs for controlling malaria transmission in sub-Saharan Africa is threatened by the widespread rise of pyrethroid resistance in *Anopheles* vectors across the region (Churcher *et al.* 2016, Hemingway *et al.* 2016, Ranson and Lissenden 2016). The development of target site alterations and metabolic resistance enables mosquitoes to tolerate exposure, increasing the chance they will obtain a bloodmeal and survive the encounter (Irish *et al.* 2008, Asidi *et al.* 2012, Strode *et al.* 2014). While there is evidence that pyrethroid LLINs retain a degree of protection against pyrethroid resistant mosquito populations by imposing fitness costs that reduce lifespan and

reproductive success, stalling progress in reducing infection has motivated the development of LLIN classes (Alout *et al.* 2016, Viana *et al.* 2016,). Due to the limited alternatives to pyrethroids, efforts to maintain the impact of LLINs have focused on secondary chemistries that increase the susceptibility of pyrethroid-resistant mosquitoes. Piperonyl butoxide (PBO) is a synergist that inhibits the Cytochrome P450 enzymes of the mosquito metabolism that neutralise pyrethroids (Darriet and Chandre 2011). In 2017, the WHO announced an interim recommendation of pyrethroid LLINs containing PBO in areas of moderate pyrethroid resistance (WHO, 2017). While evidence on the efficacy pyrethroid-PBO LLINs is still emerging, a 2021 Cochrane review concluded that they reduce blood-feeding and increase mortality in moderately resistant *An. gambiae* populations compared to conventional pyrethroid-only nets (Gleave *et al.*, 2021). However, the same review emphasised that the durability of these new designs incorporating PBO under operational conditions has not yet been assessed. In order for pyrethroid-PBO LLINs to obtain full WHO recommendation, it must be demonstrated that they maintain their insecticidal effect for the full three-year distribution cycle.

2.1.2 Assessing LLIN durability

LLINs distributed to endemic areas are acknowledged to lose insecticide content as a result of routine operational use (WHO, 2011; WHO, 2013). As nets are washed and handled, the insecticide at the surface is depleted and gradually regenerated by the internal reservoir within or on the fibres (Gimnig *et al.* 2005). Pyrethroid LLINs are designed with sufficient insecticide content to regenerate for at least three years of appropriate use, with an expectation that they will be replaced before this time (WHO 2013). However, while there is evidence from multiple settings that pyrethroid levels remain high in conventional pyrethroid-only LLINs over the timescale of a distribution cycle, the durability of the secondary active

ingredients (such as PBO) is not established. To assess if the expectation of a three-year lifespan is met, WHO LLIN durability guidelines outline defined bioefficacy benchmarks that allows insecticidal performance to be compared between timepoints (WHO 2011, WHO 2013). Physical condition is not considered in the assessment of bioefficacy. However, there is growing evidence to suggest the bioefficacy of pyrethroid-LLINs in operational conditions varies substantially between products and may fall short of three years.

To date the number of full three-year durability studies for pyrethroid-only LLIN is limited. The variability in bioefficacy after three years between different studies and LLIN products highlights that each durability trial has limited interpretation beyond the context in which it was conducted. The differing environmental and socio-economic conditions in which LLINs are used would be expected to impact bioefficacy outcomes after three years. Consequently, for national malaria programmes to identify the optimal LLIN products for their context, durability studies must continue to be performed in different countries and settings.

The WHO LLIN durability assessment guidelines were not designed to assess the growing diversity of LLIN classes that have been developed in response to pyrethroid resistance. Currently, LLINs being assessed are only required to demonstrate efficacy against fully pyrethroid-susceptible mosquitoes. Consequently, to assess the durability of 'next generation' products with a second chemistry supplementing the pyrethroid such as pyrethroid-PBO LLINs, there is a need to expand the WHO durability guidelines to include testing against pyrethroid-resistant mosquitoes.

2.1.3 Uganda LLIN evaluation project

In Uganda, the country with the highest malaria burden in East Africa, progress in controlling transmission has stalled (Lynd *et al.* 2019). Despite national programmes from 2015 to 2017 to distribute pyrethroid LLINs and deploy indoor residual spraying (IRS) with pyrethroids, cases did not decrease. The declining efficacy of conventional control strategies coincides with emerging evidence of both high levels of knockdown (*kdr*) resistance and moderate levels of metabolic resistance in mosquito populations throughout the country (Lynd *et al.* 2019, Njoroge *et al.* 2021). In an effort to resume progress towards elimination, in 2017 the Ugandan Ministry of Health initiated a mass distribution of pyrethroid-only and pyrethroid-PBO LLINs. As each district received either LLINs with or without PBO, this mass distribution presented an opportunity to perform an evaluation of PBO LLINs on a national scale. The Long-Lasting Insecticidal Net Evaluation Uganda Project (LLINEUP) is an international collaboration between research institutions and the Ugandan Ministry of Health to assess the impact of the distribution of PBO-LLINs in Uganda on epidemiological, entomological, and durability outcomes. The LLINEUP project aims to provide insight into the efficacy of the addition of PBO to pyrethroid LLINs in interrupting malaria transmission compared to pyrethroid-only LLINs.

2.1.4 Aim

This chapter constitutes the bioefficacy and chemical durability component of the LLIN-EUP (Long-Lasting Insecticidal Net Evaluation in Uganda Project), a large field study covering approximately half of Uganda's land mass to evaluate the efficacy of pyrethroid/PBO LLINs. The aim of this chapter is to compare the chemical integrity and bioefficacy of LLINs with and without-PBO in the Uganda PBO trial. The durability of Olyset Duo and PermaNet 3.0, both dual-AI pyrethroid+PBO LLINs, will be assessed with comparison to their pyrethroid-only equivalents LLINs from the same manufacturer (Olyset and PermaNet 2.0 respectively). The purpose of this chapter is not to directly compare Olyset Plus and PermaNet 3.0 but to assess how the performance of each products changes over time.

2.1.5 Objectives

The objectives of this chapter are to quantify the chemical integrity of LLINs sampled at timepoints following distribution in a cluster randomised durability trial in Uganda. These objectives are derived from the WHO durability guidelines (WHO 2011, WHO 2013b).

The primary objective **(1)** of this chapter is to assess the impact of the addition of PBO on the bioefficacy of pyrethroid LLINs over the distribution cycle. This will be achieved by comparing the performance of two pyrethroid-PBO LLIN products with their pyrethroid-only equivalents in WHO cone bioassays at timepoints after distribution.

The secondary objectives of this chapter are to **(2)** describe the chemical integrity of pyrethroid-PBO and pyrethroid-only LLINs over the same time period by measuring insecticide content with high-performance liquid chromatography (HPLC)

2.1.6 Study site and context

This durability investigation was conducted using nets from a cluster randomised trial to evaluate the efficacy of pyrethroid LLINs containing the synergist piperonyl butoxide (with comparison to pyrethroid-only LLINs). The protocol for this trial has been published previously (Staedke *et al.* 2019). The study area covered approximately half of Uganda's land mass, included a range of sociological and ecological environments. Uganda's healthcare system is decentralised across 112 health districts that have local decision-making powers and a general hospital, further divided into health sub-districts (HSDs) that contain a health centre and each serve approximately 100,000 people. A total of 104 HSDs from both Western and Eastern Uganda were included in the trial.

Prior to the commencement of the study, a sensitisation programme was undertaken by Uganda's Ministry of Health to engage stakeholders at district and community level. Community leaders were consulted on their inclusion in the trial and leaflets were distributed on the purpose and proper use of LLINs. Household level surveys were conducted to estimate the numbers of nets required in each HSD. In line with WHO guidance, the number of nets to be allocated to a household was the total number of individuals divided by two (rounded up) (WHO, 2013).

Due to interim WHO guidance (since revoked) advising against the co-occurrence of Indoor Residual Spraying (IRS) with pirimiphos-methyl ('Actellic') and pyrethroid-PBO LLINs, areas of the country where pirimiphos-methyl IRS was due to be deployed were not included in the study [20].

2.2 Methods

2.2.1 LLIN description

Four LLIN products were distributed and assessed in this study, all of which have obtained WHO pre-qualification. This consisted of two pyrethroid-PBO nets (Olyset Plus and PermaNet 3.0) and two pyrethroid-only nets (Olyset and PermaNet 2.0). All nets were of the ‘special’ size, measuring 180cm long x 170 width x 170 height. The chemical specifications of each net included in the study are shown in Table 2.1.

Table 2.1. Specifications of LLIN products assessed in study. The target dose was defined as the amount of chemical per kg of fabric

Product name	Manufacturer	Weave	Insecticide target
Olyset Net	Sumitomo Chemical Ltd.	Polyethylene (150 denier)	Permethrin: 20g/kg (\pm 5.0)
Olyset Plus	Sumitomo Chemical Ltd.	Polyethylene (150 denier)	Permethrin: 20g/kg (\pm 5.0) PBO: 10g/kg (\pm 2.5g/kg)
PermaNet 2.0	Vestergaard Frandsen	polyester (100 denier)	Deltamethrin: 1.4g/kg (\pm 0.35)
PermaNet 3.0	Vestergaard Frandsen	Polyester (roof: 100 denier, Sides: 75 denier)	Deltamethrin: 4.0g/kg (\pm 1.0) roof 2.1g/kg (\pm 0.525) sides

Unused 'baseline' nets were obtained by randomly sampling from the shipment of nets intended for distribution in the field.

2.2.2 Sample size

Table 2.2. Sample size of nets used for each outcome.

Outcome	Timepoint	PermaNet	PermaNet	Olyset	Olyset	Total
		2.0 (n)	3.0 (n)	Net (n)	Plus (n)	
Chemical integrity (HPLC)	Baseline	5	5	5	5	20
	12 Month	38	35	34	31	138
	25 Month	29	30	30	30	119
WHO Cone assay	Baseline	5	5	5	5	20
	12 Month	7	7	7	7	28
	25 Month	7	7	7	7	28
WHO Wireball assay	Baseline	-	-	5	5	10
	12 Month	-	-	7	7	14
	25 Month	-	-	7	7	14

2.2.3 Chemical Analysis

To assess the chemical content of each net, the insecticide was extracted into solution and quantified using high-performance liquid chromatography using the methods outlined below.

2.2.3.1 Net processing

Two 25x25cm samples, cut from the top panel, were used to calculate the chemical content of each net. Each 25x25cm net sample was carefully unwrapped from its aluminium packing and placed into a die cutting machine (Sissix, UK) which cut out five 5cm diameter circles from the netting (the WHO durability guidelines do not specify the method by which these pieces should be cut thus a haberdasher style cutting press was identified as a straightforward yet reliable means of obtaining consistent samples). A separate cutting board was used for each LLIN product to prevent cross-contamination and each cutting board wiped down with 70% ethanol between every individual sample. All net cuttings were visually inspected for cutting errors. The remains of the net piece from which they were cut were then carefully repackaged in the same labelled aluminium foil piece.

The sum of the five net cuttings (total surface area 78.53cm²) were then weighed together, which was noted in an excel sheet. Following this, all five samples were placed into an Eppendorf tube (labelled with the net ID and LLIN product) and stored at 3-5°C until assessed for chemical content.

2.2.3.2 Insecticide extraction

To prepare net cuttings for chemical content assessment, the insecticide must first be released from the net fibres into solution. To dissolve the sample, an extraction solution was prepared containing 900ml of heptane and 100ml of 1-propanol, as per the methods of Ngufor *et al.*, 2022. Following this, 100mg of dicyclohexyl phthalate (DCP) was dissolved in the extraction solution to act as an internal standard at the analysis stage. Once prepared, the bottle containing the extraction solution was labelled with the date and stored at 3-5°C (and used within 4 weeks of preparation).

A Gibson pipette was used to transfer 5ml of the extraction solution to each Eppendorf tube containing a net sample. The tube was then capped with tinfoil and the lid closed tightly to minimise evaporation. Negative controls were prepared by transferring 5ml of the extraction solution into Eppendorf tubes containing no net sample

The insecticide extraction process was performed by placing each prepared Eppendorf tube into a heat block set at 85°C for 45 minutes, vortexing for three seconds every 15 minutes. To allow these extracted samples to be stored indefinitely, heptane and propanol were evaporated off. A Gibson pipette was used to transfer 1ml of extracted solution from each sample into a glass vial, with the vials then placed in a heat block set at 60°C for 10 minutes to evaporate. These evaporated samples were then placed into a fridge at 3-5°C, where they could be stored indefinitely if needed until chemical analysis.

2.2.3.3 Preparing samples for chemical analysis

Evaporated samples were removed from the fridge and resuspended in 1ml of acetonitrile. The resuspended sample was then vortexed for one minute and transferred to a labelled Eppendorf tube. Immediately before chemical analysis, the contents of each Eppendorf were transferred to a 300µl glass insert chromatography vial using a 200µl syringe with a 0.2µM filter. DCP controls were not filtered.

2.2.3.4 Preparing internal standards

As chemical analysis estimates the quantity of a compound by comparing light absorbance to that of a known quantity of the same compound, internal standard must be prepared which have a specific concentration of the given insecticide to be assessed. Given the LLIN products to be analysed in the study, internal standards were prepared for permethrin, deltamethrin and PBO. This was done by first weighing 10mg of the given insecticide into an Eppendorf tube and adding 1000µl of acetonitrile (the medium in which compounds are suspended in chemical analysis). Serial dilutions were then performed to prepare concentrations of the insecticide at 1000µg, 500µg, 250µg, 125µg, 62.5µg per ml (**Figure 2.1**). A Gibson pipette was then used to transfer 200µl of each concentration into a labelled glass vial. Additional four 'blank' vials containing only acetonitrile were prepared, to be used for calibrating absorbance (as these blanks are used to set relative absorbance level to zero, to which other compounds can then be compared).

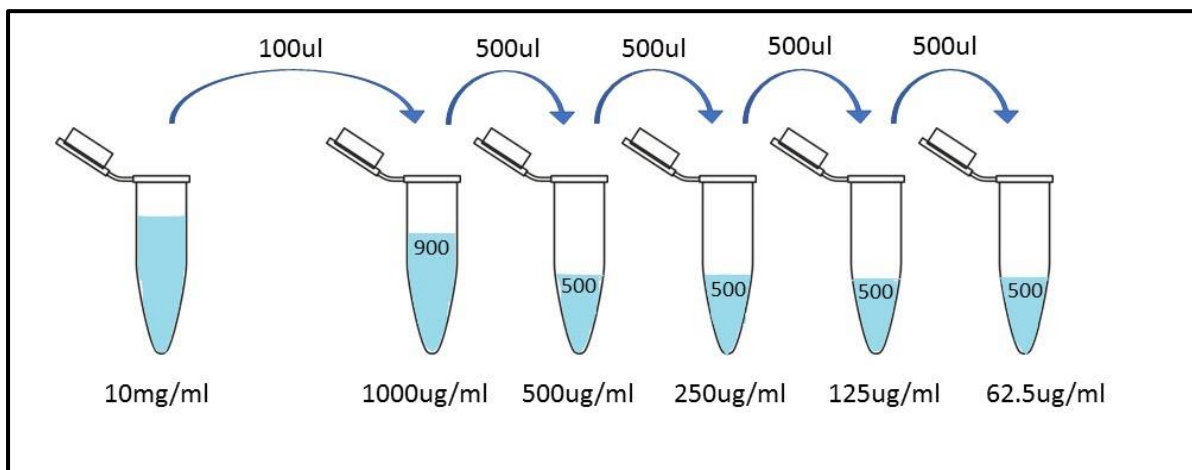


Figure 2.1. Diagram of serial dilutions performed to prepare insecticide standards for HPLC.

2.2.3.5 High performance Liquid Chromatography set-up and analysis

The HPLC analysis was performed on an Agilent 1100 Series machine (Agilent, California US).

The insecticide content the samples was determined by injecting 10 μ l aliquots from each chromatography vial through a reverse-phase Hypersil GOLD C18 column at room temperature. A mobile phase of 70% acetonitrile/30% water was used with a flow rate of 1ml/minute. Chromatographic peaks for each sample were observed at a wavelength of 226nm

Quantities of Permethrin, Deltamethrin, and Piperonyl Butoxide in each sample were calculated by comparison to standard curves of each compound (PESTANAL[®], analytical standard, Sigma-Aldrich, Missouri US) and corrected against internal standard DCP. HPLC data were analysed using OpenLAB software v2.1 (Agilent, California US). The weight of each sample before extraction was then used to calculate insecticide content (in grams per kilogram). The chemical content of each net was calculated as the average of the two samples assessed.

2.2.2 Bioefficacy

To assess bioefficacy, pyrethroid-resistant *Anopheles* mosquitoes were exposed to the net samples using standard WHO bioassays as outlined in the methods below:

2.2.2.1 Mosquito characterisation

All mosquitoes used are 3-5-day old unfed females, reared in a temperature and humidity-controlled insectary. Two different mosquito strains were used in the cone bioassays; Kisumu and Busia. Kisumu is a pyrethroid susceptible strain of *An. gambiae* established at LSTM in 1975 from field collections conducted in what is now Kisumu county (formerly Kisumu district), in Western Kenya. Busia is a strain established in November 2018 from mosquitoes collected in Busia, Eastern Uganda. Wild populations from Eastern Uganda been previously characterised as possessing resistance to pyrethroids through both target site alterations (*Vgsc*-1014F/S) and intermediate levels of metabolic mutations (*Cyp4j5* and *Coeae1d*) (Lynd *et al.*, 2019). To maintain the frequency of alleles associated with pyrethroid-resistance, Busia was selected with 0.05% deltamethrin every 3rd generation. Both phenotypic and genotypic characterisation were conducted on the same generation (G35).

The phenotypic resistance status of the Busia strain was assessed using WHO tube bioassays. The purpose of this assay is to confirm that a mosquito colony is resistant to pyrethroids by exposing them to paper treated with a WHO specified dose for a set amount of time. To ensure quality and comparability, insecticide papers are provided by the WHO. The concentration of pyrethroid determined by the WHO (discriminating dose) is that which systematically gives 100% mortality against susceptible strains after 1 hour of exposure. Any mosquito strain for which 24hr mortality is systematically <80% is considered to be resistant.

To characterise mosquitoes for this study, Busia was exposed to a range of insecticides in the WHO tube assay. Insecticides used were as follows: permethrin (0.75%), deltamethrin (0.05%), DDT (4.0%), and α -cypermethrin (0.05%). Mosquitoes were exposed to the insecticide for one hour, with the number of mosquitoes dead after 24 hours recorded. Additionally, to investigate the extent to which 'Busia's' susceptibility can be restored by PBO, the tube assays were repeated with the inclusion of prior exposure to PBO papers for one hour before insecticide exposure.

Genotypic characterisation was assessed by quantifying the frequency of single nucleotide polymorphisms (SNPs) linked to target site (*Vgsc*-L1014S, *Vgsc*-L1014F) and metabolic resistance (*Cyp6P4* and *Cyp4J5*) in east African *An. gambiae* by Weetman *et al.*, (2018). The protocol for detecting resistance-associated alleles was developed by Amy Lynd (unpublished internal SOP, 2020), utilising Locked Nucleic Acid (LNA) probes. A total of 92 3-5 day old Busia females were sampled for genotypic analysis. DNA extraction was achieved using "Nexttec" extraction plates (as per manufacturer's instructions), using two mosquito legs. Mosquito legs were placed in 20 μ l of 1x STE buffer (Sodium Chloride-Tris-EDTA, Fisher Scientific) then incubated for 30 minutes at 95 $^{\circ}$ C. Probes were ordered from Integrated Data Technology (eu.idtdna.com/site/order/qpcr/primetimeprobes/lna). Reactions were prepared in optical PCR tubes and run on an AriaMX qPCR machine. Reaction conditions were 95 $^{\circ}$ C for three minutes, followed by 40 cycles of 95 $^{\circ}$ C for five seconds and 60 $^{\circ}$ C for 30 seconds (for a total runtime of 52 minutes). Results were analysed on AriaMX software (v1.5).

2.2.2.2 Sampling preparation

Two 25cmx25cm pieces from the top of each individual bed net assessed were used to evaluate bioefficacy. These were the same net pieces from which cuttings were made for chemical assessment.

2.2.2.3 WHO Cone bioassay

To test the bioefficacy of nets collected in the study, WHO cone bioassays were performed on net samples using the protocol outlined in WHO durability monitoring guidelines (WHO 2011, WHO 2013). In the WHO cone assay, mosquitoes are held in a plastic cone (10cm diameter by 7cm height) against a bed net for three minutes. To secure cones to the net pieces, two Plexiglas panels with cut-outs the same diameter as the cones are used as substrates (**Figure 2.2**). The netting and the rim of a cone are sandwiched between the Plexiglas panels and screwed together. The full assembly is then mounted at a 45-degree angle to maximise the contact time between the mosquito and the net (Okumu et al., 2014).



Figure 2.2 Example of WHO cone bioassay. Testing backboard is angled at 45 degrees to discourage avoidance from the net.

As per standard insectary rearing and testing conditions, ambient conditions in the testing room ranged from 27 ± 2 °C and $80 \pm 10\%$ relative humidity. Prior to testing beginning, mosquitoes are placed in the testing room for one hour to acclimatise. To commence a cone test, seven mosquitoes are transferred into the cone through the open top using a mouth aspirator. Once transferred, the hole is immediately plugged with cotton wool and a timer started. After three minutes have passed, the mosquitoes are removed from the cone with a separate aspirator (to avoid contamination of the aspirator used for putting mosquitoes into the cone) and transferred to a paper cup labelled with the time, date, and sample ID. The mosquitoes were then offered sugarwater and left in the same room to recover. Knockdown and mortality were then scored at 1 hour and 24 hours post-exposure respectively.

The bioefficacy of an LLIN product under operational conditions is assessed by calculating the proportion of nets that meet WHO bioefficacy criteria at each timepoint. Individual nets are expected to achieve either 80% mortality or 95% knockdown against pyrethroid susceptible *An. gambiae s.s.* mosquitoes. An LLIN product is considered to have passed overall if 80% of nets of that type met these criteria at all timepoints up to 36 months. Chemical and physical integrity are not included in bioefficacy criteria.

2.2.2.4 WHO Wireball bioassay

Given previous literature indicating that WHO Cone bioassays are insufficient to assess the bioefficacy of LLIN products containing insecticides with high contact irritancy (Abdel-Mohdy et al 2009) , such as the permethrin in Olyset Net and Olyset Plus, supplemental WHO wireball assays were performed on the same nets used in WHO cone bioassays. However, while the same nets were evaluated in both the cone and wireball, different samples from that net were used due to the destructive nature of chemical analysis. Consequently, in WHO wireball assays samples from the sides of the net were used as these pieces were still fully intact. The use of side pieces meant that PermaNet 3.0 could not be meaningfully compared as there is no PBO on the side panels.

The WHO wireball bioassay is a method for exposing mosquitoes to a piece of netting (insecticide treated net, ITN or long lasting insecticidal net, LLIN). The netting sample is wrapped around a metal frame, creating a fully enclosed area (**Figure 2.3**). By surrounding the frame on all sides with the netting, it is assumed that mosquitoes released inside cannot avoid contact by flying away from the net surface. The current WHO methodology for the wireball assay describes two different acceptable frames which can be used to affix the net; either a 15 cm x 15cm x 15 cm cube or a sphere made up of two intersecting circles 15 cm in diameter. Here the cube variant was used.



Figure 2.3 Image of cube variant of WHO wireball assay in use. Image shows netting material wrapped around the metal cube frame and secured in place with elastic bands. Mosquitoes are released into the cube by a 'sleeve' of excess material.

As in the cone bioassay, seven 3-5 day old females were released into the wireball for three minutes then assessed for 1hr knockdown and 24 hour mortality. As before, ambient conditions in the testing room ranged from 27 ± 2 °C and $80 \pm 10\%$ relative humidity. These wireball assays were performed with the same pyrethroid-resistant 'Busia' strain.

The purpose of this secondary testing was to give LLIN products that performed poorly in the WHO Cone assay a second chance to demonstrate performance using an assay where there were no surfaces on which the mosquito could rest to avoid contact (such as the cone itself in the WHO Cone assay). While the WHO Tunnel test is recommended as a secondary assay for assessing nets with high contact irritancy, the current study could not undertake this technique due to the ethical issues surrounding the use of small mammals as bait.

2.2.3 Data Analysis

Data analyses were conducted using R (version 3.6.0), all graphs were produced using the ggplot2 package (version 3.2.1). Associations between outcomes and variables of interest were quantified using Generalized Linear Mixed Models (GLMMs) using the 'lme4' package (version 1.1-21). To account for unexplained variation between separate pieces from individual nets and between clusters, the net ID (a unique identifier for each net distributed) and HSD number were each included in the models as a random effect. Additionally, both the temperature and relative humidity of the testing room were included in initial model fitting but accounted for such little variance in the final model that they were removed for simplicity.

The model selection process used stepwise regression, working backwards from a maximally complex model to produce the most parsimonious fit. Variables that did not significantly increase explanatory power, as indicated by log-likelihood ratio tests (LRTs) ('lmtest' package version 0.9-37), were excluded from the final model. The p values reported are the output of these LRTs. Pairwise comparisons between levels within a categorical variable were performed using least square means with the 'lsmeans' package (version 2.30-0).

Unless otherwise stated, all mean values reported here are the predicted mean values obtained from the appropriate statistical model. The margin of error (95% confidence intervals) around predicted means are calculated by model-based bootstrapping. This bootstrapping technique is performed by resampling the data (with replacement) using the 'BootMer' package with 1000 resamples. This bootstrapping method is preferred over the 95% CIs calculated from raw standard errors of the mean (reported by the GLMM output) as rather than simply averaging over the random effect (clusters) it allows the distribution of

means to approach a normal distribution (due to the Central Limit Theorem) providing a margin of error that is less skewed.

2.3 Results

2.3.1 Chemical integrity

2.3.1.1 Standard of baseline nets

At baseline, all net samples tested met or exceeded the minimum target dose of AI as per their respective specifications (**Table 2.3**).

Table 2.3. Mean chemical content (in g/kg) for each active ingredient in each LLIN product at baseline, 12 months, and 25 months post-distribution. Values in bracket indicate 95% confidence interval

Active Ingredient	LLIN product	Timepoint		
		Baseline	12 months	25 months
Deltamethrin	PermaNet 2.0	1.3 (0.8-1.9)	1.1 (0.9-1.3)	0.7 (0.5-0.9)
	PermaNet 3.0	5.0 (4.1-5.9)	4.2 (4.0-4.5)	3.5 (3.2-3.8)
Permethrin	Olyset Net	19.5 (19.9-21.1)	17.0 (16.4-17.6)	18.2 (17.6-18.7)
	Olyset Plus	16.1 (13.6-18.5)	14.5 (13.7-15.4)	17.4 (16.5-18.3)
PBO	PermaNet 3.0	26.8 (22.9-30.7)	15.3 (13.7-16.9)	11.0 (9.4-12.7)
	Olyset Plus	8.2 (6.7-9.8)	5.0 (4.4-5.7)	3.7 (3.0-4.3)

2.3.1.2 Deltamethrin content

The deltamethrin content of PermaNet 3.0 decreased with each timepoint ($p < 0.001$, **Figure 2.4A**). In the period from 0 to 25 months, mean deltamethrin content of PermaNet 3.0 nets declined from 4.98g/kg (95% CI: 4.08-6.01) to 3.484g/kg (95% CI: 3.19-3.78). Despite this, the deltamethrin content of all PermaNet 3.0 nets collected at 25 months remained within the target dose range (3.0-5.0g/kg). For PermaNet 2.0, mean deltamethrin content after 25 months was not statistically different from baseline ($p = 0.071$).

2.3.1.3 Permethrin content

The permethrin content of Olyset Plus varied across the sampled timepoints ($p < 0.001$, **Figure 2.4B**) however pairwise comparison indicated no overall difference between baseline and the final timepoint ($p = 0.591$). Mean permethrin content in Olyset Plus at baseline was 16.08 (95% CI: 13.70-18.62), declining to 14.54 (95% CI: 13.64-15.35) after 12 months, then appearing to increase to 17.39 (95% CI: 16.53-18.22) after 25 months. A similar pattern was observed for Olyset Net, with permethrin content varying across timepoints overall ($p < 0.001$) yet pairwise comparison indicating no difference between baseline and the 25-months ($p = 0.327$).

2.3.1.4 Piperonyl butoxide content

The PBO content of PermaNet 3.0 declined across the sampled timepoints ($p < 0.001$, **Figure 2.4C**). PBO content for PermaNet 3.0 at baseline was 26.81g/kg (95% CI: 22.80-31.07), declining to 15.28g/kg (95% CI: 13.74-16.71) after 12 months ($p = 0.001$), then further to 11.03g/kg (95% CI: 9.35-12.67) at the 25 month timepoint ($p = 0.001$).

A similarly sharp decrease in PBO was observed for Olyset Plus between baseline and 25 months ($p < 0.001$, **Figure 2.4C**). At baseline mean PBO content for Olyset Plus was 8.17g/kg (95% CI: 6.51-9.82) before declining to 5.03g/kg (95% CI: 4.37–5.74) after 12 months ($p = 0.002$). From 12 months to 25 months post-distribution, PBO content further fell to 3.66g/kg (95% CI: 2.97-4.28, $p = 0.013$).

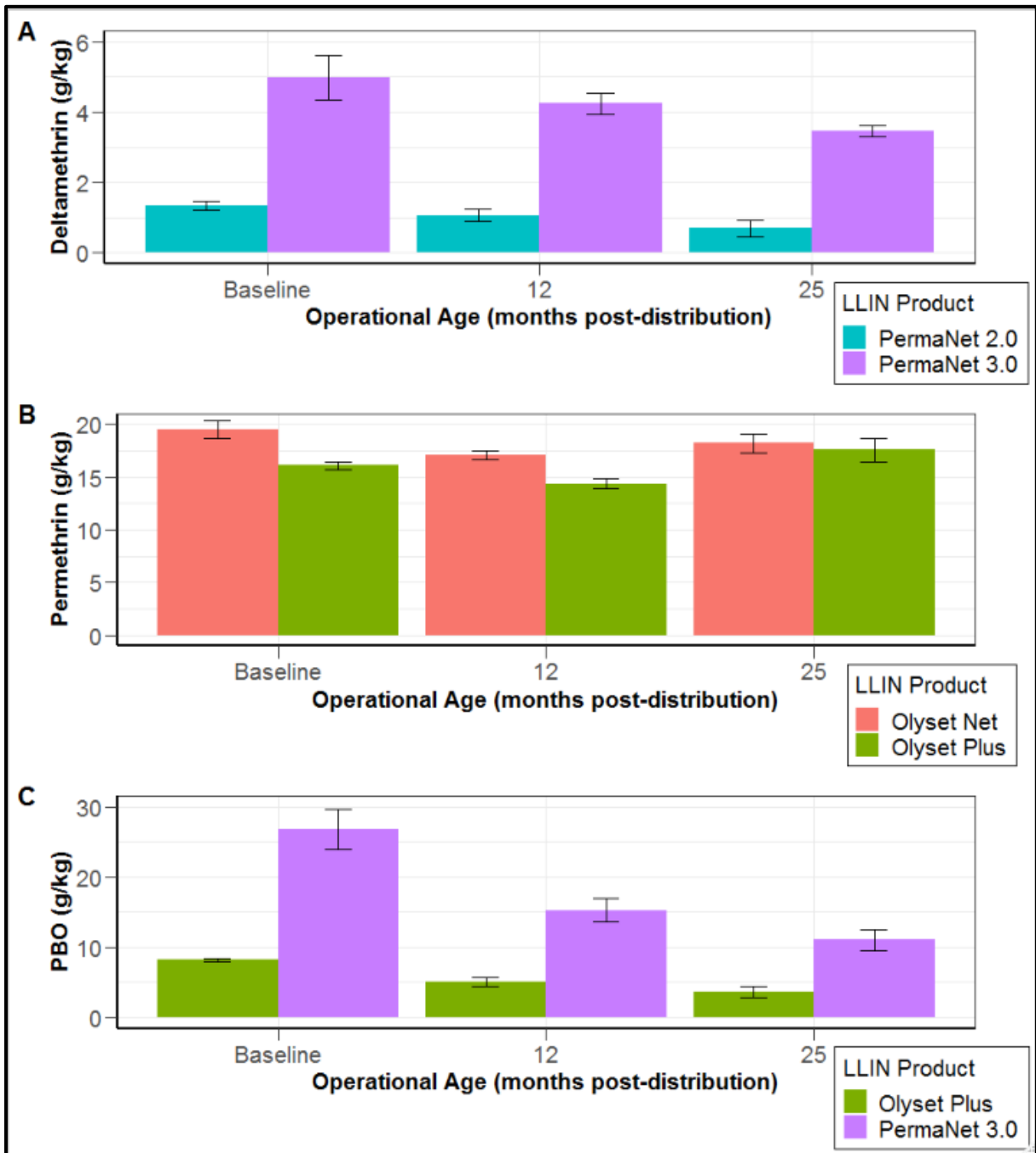


Figure. 2.4 Mean concentration of (A) deltamethrin, (B) permethrin, and (C) PBO detected in net samples at each sampled timepoint (measured using HPLC). Error bars indicate 95% CIs.

2.3.2 Mosquito characterisation

2.3.2.1 Phenotypic resistance

One hour exposures in WHO tube assays confirmed that the site-specific *Busia* strain was resistant to both 0.75% permethrin and 0.05% deltamethrin (by the WHO definition of <90% mortality)(**Figure 2.5**) , with mean 24hr mortality after a one hour exposure 21.76% (95% CI: 15.94-27.58) and 73.67 (95% CI: 69.45-77.89) respectively for each pyrethroid. When the same process was repeated with the addition of a one hour pre-exposure to PBO, adjusted mortality rose to 50.41% (95% CI: 29.70-71.12) and 99.00 (95% CI: 97.04-100) for permethrin and deltamethrin respectively. This increase in mortality after PBO pre-exposure indicates that susceptibility of *Busia* to pyrethroids can be at least partially, but not fully, restored by PBO.

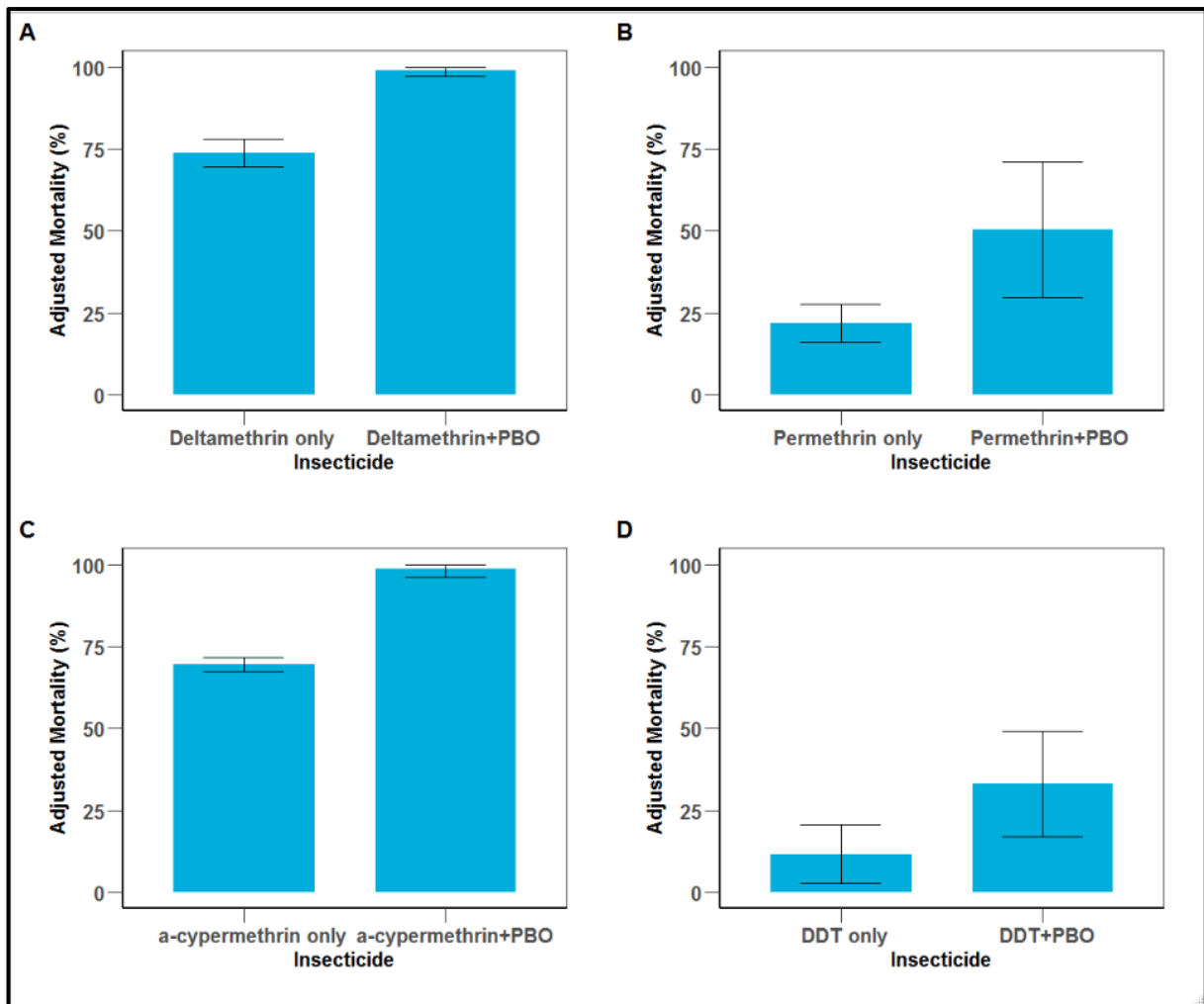


Figure 2.5. Adjusted mortality of *An. gambiae* strain 'Busia' after 60 minute WHO tube exposure to **(A)** 0.05% deltamethrin, **(B)** 0.75% permethrin, **(C)** 0.05% alpha-cypermethrin, **(D)** 4% DDT

2.3.2.1 Genotypic resistance

Resistance screening of the 'Busia' strain used for bioefficacy testing was performed for the resistance variants *Vgsc*-L1014S, *Vgsc*-L1014F, *Cyp6P4* and *Cyp4J5* using PCR based approaches. The marker for *Vgsc*-L1014F was not detected in any samples thus it is not shown here. The overall frequency of *Cyp4J5* in Busia was 45.5%, with heterozygotes dominating the sample at 69% of all mosquitoes sampled (**Figure 2.6A**). Only 11.1% of Busia were homozygous for the *Cyp4J5* mutation. For the *Cyp6P4* mutation, the overall frequency was 31.5%, with very few mosquitoes homozygous for the mutation (7.6%) and homozygous wild type mosquitoes slightly outnumbering heterozygotes(**Figure 2.6B**). Finally, Busia was at near fixation for the *Vgsc*-L1014S mutation (**Figure 2.6C**), with an overall frequency of 94.7% and no homozygous wild type mosquitoes.

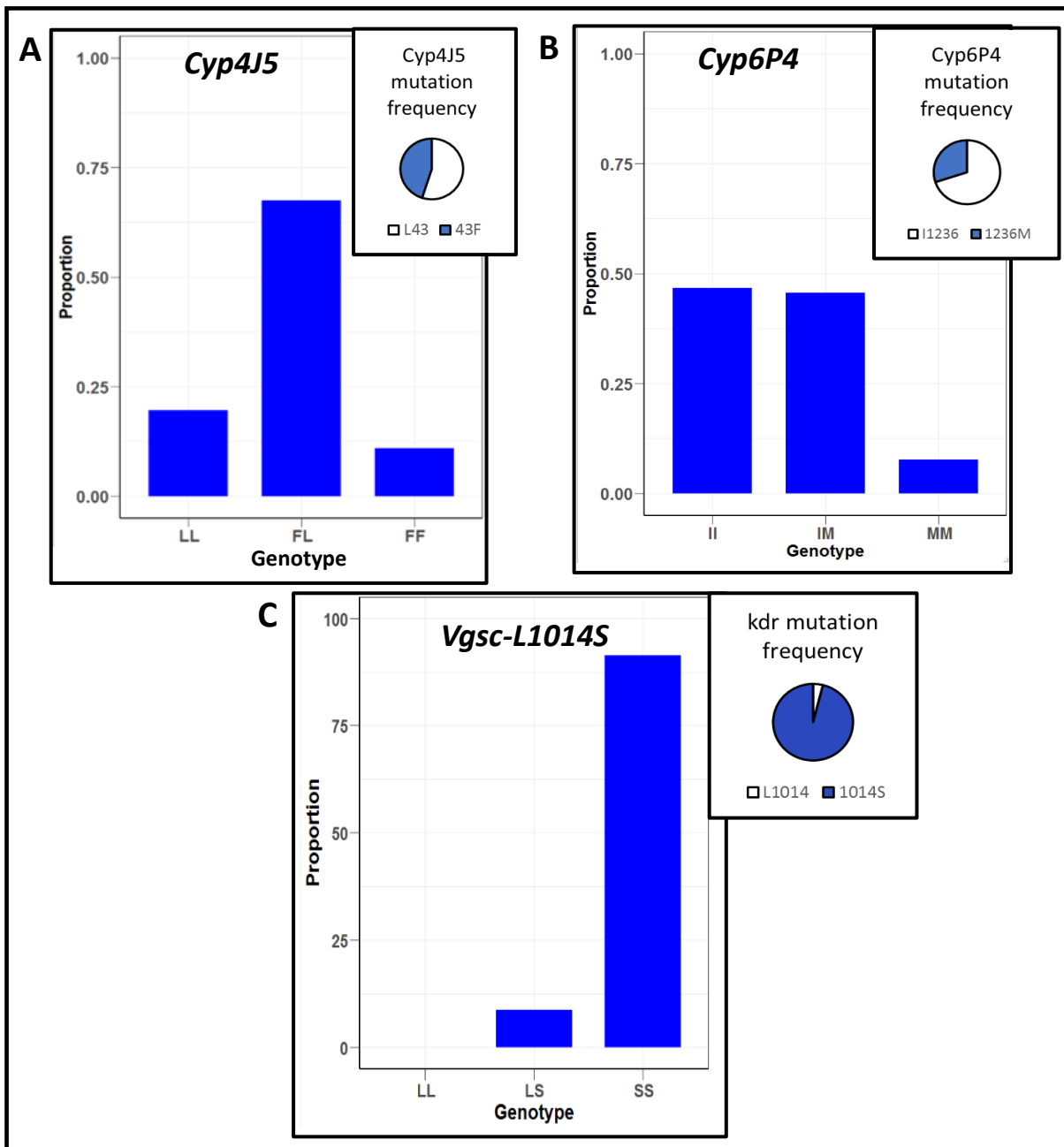


Figure 2.6. Frequency of resistance markers (A) *Cyp4J5*, (B) *Cyp6P4*, and (C) *Vgsc-L1014S* for 'Busia' strain *An. gambiae* (s.s) mosquitoes. Frequencies represent proportion of females assessed (n=92)

2.3.3 Bioefficacy

2.3.3.1 WHO Cone bioassay: Pyrethroid resistant *An. gambiae*

Knockdown for PermaNet 3.0 remained very high throughout, achieving 99.7% (95%CI: 97.26-99.65, **Figure 2.7A**) at baseline and remaining stable to 12 months ($p=0.441$), though declining to 78.57% (95% CI: 63.57-93.58, $p<0.001$) after 25 months. PermaNet 3.0 was fully lethal against the pyrethroid-resistant strain when new but mortality declined with operational use, falling by 26.8% (95%CI: 16.28-37.33) for each year in the field ($p<0.001$, **Figure 2.6B**). In contrast, both mortality and knockdown with PermaNet 2.0 against the pyrethroid-resistant strain was very low throughout the sampled timepoints. There was no difference in knockdown for PermaNet 2.0 between timepoints ($p= 0.278$), with overall mean knockdown 5.13% (95% CI: 2.23-9.97). Furthermore, there was no difference in adjusted mortality between timepoints for PermaNet 2.0 ($p=0.992$), with mean mortality across all timepoints 1.92% (95% CI: 0-11.8%)

Knockdown with Olyset Plus was 46.98% (95%CI: 18.55-79.13) when new but fell considerably to 3.54% (95%CI: 0.7-10.54) after two years ($p=0.005$, **Figure 2.7A**). Mortality with Olyset Plus in cone assays was low throughout, killing 12.19% (95%CI: 5.45-17.01) at baseline and 3.34% (95%CI: 0-8.71) after two years but with no significant difference between timepoints ($p=0.226$, **Figure 2.7B**). Knockdown for Olyset Net did not vary significantly between sampled timepoints ($p=0.207$), with mean knockdown 4.86% (95% CI: 1.30-11.36%) across all timepoints. Similarly, mortality for Olyset Net was not statistically different between timepoints ($p=0.447$), with mean mortality 3.60% (95% CI: 0-12.82) across all timepoints.

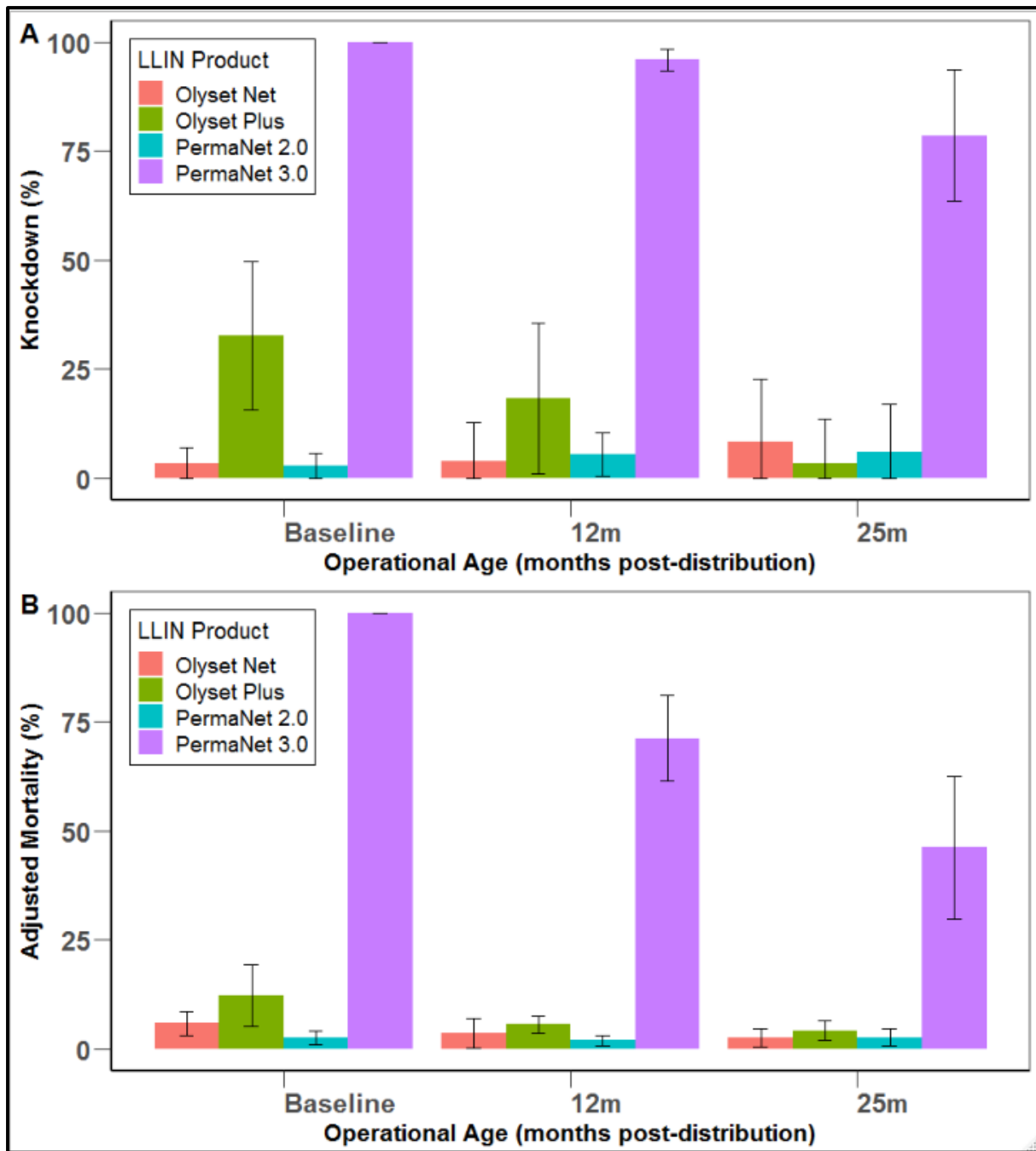


Figure 2.7 Mean (A) knockdown and (B) adjusted mortality in cone bioassays with pyrethroid resistant *An. gambiae s.s.* ('Busia') for each LLIN product tested at baseline, 12 months, and 25 months in the field.

2.3.3.2 WHO Wireball assay: Pyrethroid resistant *An. gambiae*

The performance of Olyset Plus in wireball assays was greatly improved compared to the same nets in the cone assay, knocking down 98.93% (95%CI: 94.43-100, **Figure 2.8A**) at baseline. After 12 months knockdown had not significantly reduced (73.92%, 95% CI: 54.88-92.97, $p=0.376$) however there was an overall decline to 45.72% (95% CI: 22.84-68.62, $p=0.021$) after 25 months. Mortality for Olyset Plus against the pyrethroid resistant strain in WHO wireball assays at baseline was similarly improved compared to the WHO cone assay, killing 87.72% at baseline (95%CI: 77.68-97.76, **Figure 2.8B**). However, after 12 months mortality had declined to 44.15% (95%CI: 29.32-58.98, $p=0.002$) though the subsequent decline to 25.92% (95%CI: 11.92-39.93) at 25 months was not statistically significant ($p=0.216$).

With Olyset Net in the WHO wireball assay, there was no difference in 1hr knockdown between timepoints ($p=0.125$). Overall mean knockdown for Olyset Net did not vary significantly across timepoints ($p=0.493$), with mean mortality overall 11.56% (95% CI: 9.08-14.04). A direct comparison between bioefficacy outcomes for Olyset Net and Olyset Plus in the WHO cone and wireball assays is shown in **Appendix I, supplementary Table 1 and 2**.

The one hour knockdown of PermaNet 2.0 was improved in the WHO wireball compared to the WHO cone. In the wireball, mean knockdown across timepoints was 28.64% (95% CI: 24.08-33.21) with no difference between timepoints ($p=0.317$). Similarly, mortality in the wireball was improved compared to the cone, killing 12.09% (95% CI: 8.43-15.74) at baseline then decreasing to 5.90% (95% CI: 3.09-8.71) after 25 months ($p=0.031$) however all other pairwise comparisons were not significantly different (Baseline:12m, $p=0.406$)(12m:25m, $p=0.431$).

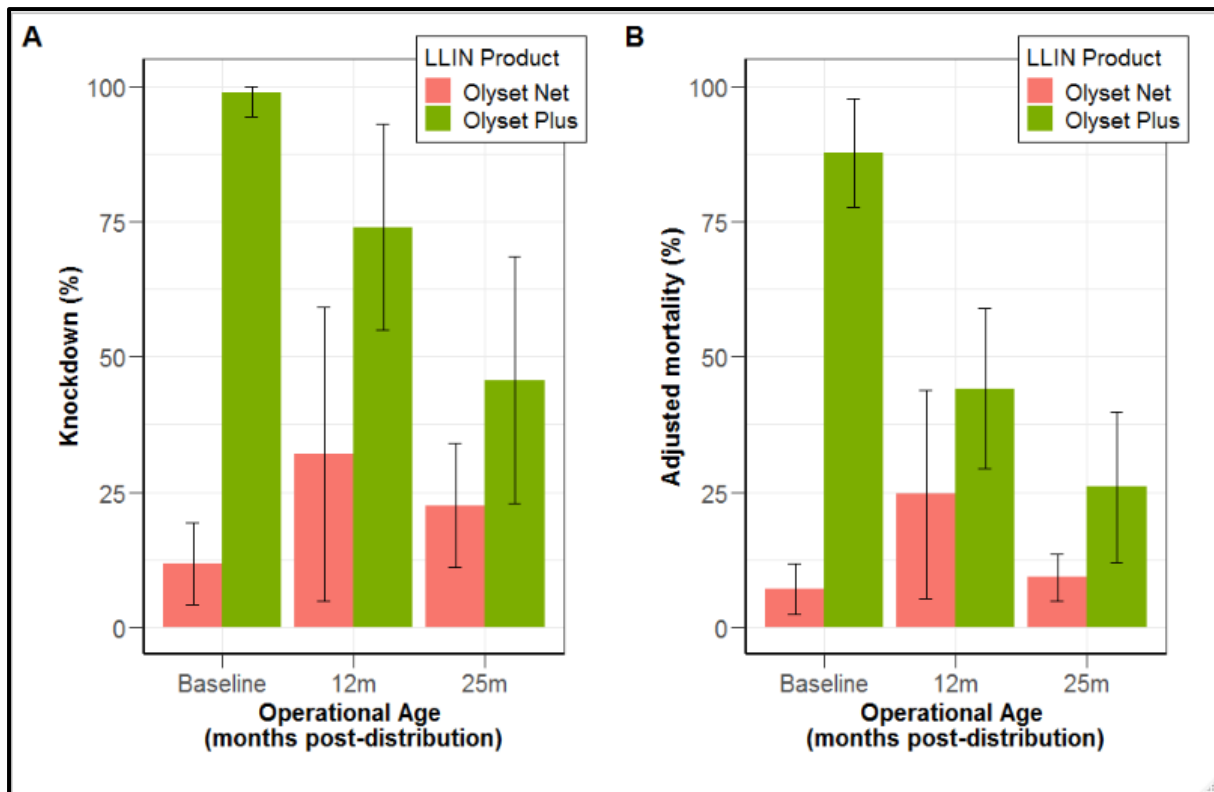


Figure 2.8. Mean **(A)** knockdown and **(B)** adjusted mortality in WHO wireball assays with pyrethroid-resistant *An. gambiae* strain ('Busia') for Olyset Net and Olyset Plus at baseline, 12 months, and 25 months in the field.

2.3.3.4 Relationship between chemical integrity and bioefficacy

The relationship between chemical integrity and predicted mortality for the pyrethroid-resistant *An. gambiae* s.s. Busia line is shown in **Figure 2.9**.

For PermaNet 3.0 in the WHO cone bioassay, mortality was dependent on both total deltamethrin content and total PBO content, as indicated by a significant interaction between the two variables ($p < 0.001$, **Figure 2.9A**). Modelling indicated there is a non-linear association between PBO content and mortality, with mortality falling more sharply with each consecutive g/kg of PBO that is lost (**Figure 2.9C**). When the deltamethrin value was fixed at the mean of the data (4.42g/kg) a reduction in PBO from 25g/kg to 15g/kg resulted in predicted mortality falling from 98% to 90%. Furthermore, a reduction in PBO content from 15g/kg to 5g/kg resulted in a decline in predicted mortality from 90% to 57%. Consequently, the model predicted that to achieve 80% mortality against this pyrethroid resistant mosquito strain a minimum of 11g/kg PBO was needed.

For Olyset Plus in the WHO wireball bioassay, mortality had no statistical relationship with total permethrin content ($p = 0.583$) and was instead directly correlated with total PBO content ($p < 0.001$, **Figure 2.9B**). Modelling indicated there was a linear association between PBO content and predicted mortality, with mortality falling by 11.12% for each g/kg PBO that is lost (**Figure 2.9D**). The model predicted that to achieve 80% mortality against this strain a minimum of 7.7g/kg PBO was needed.

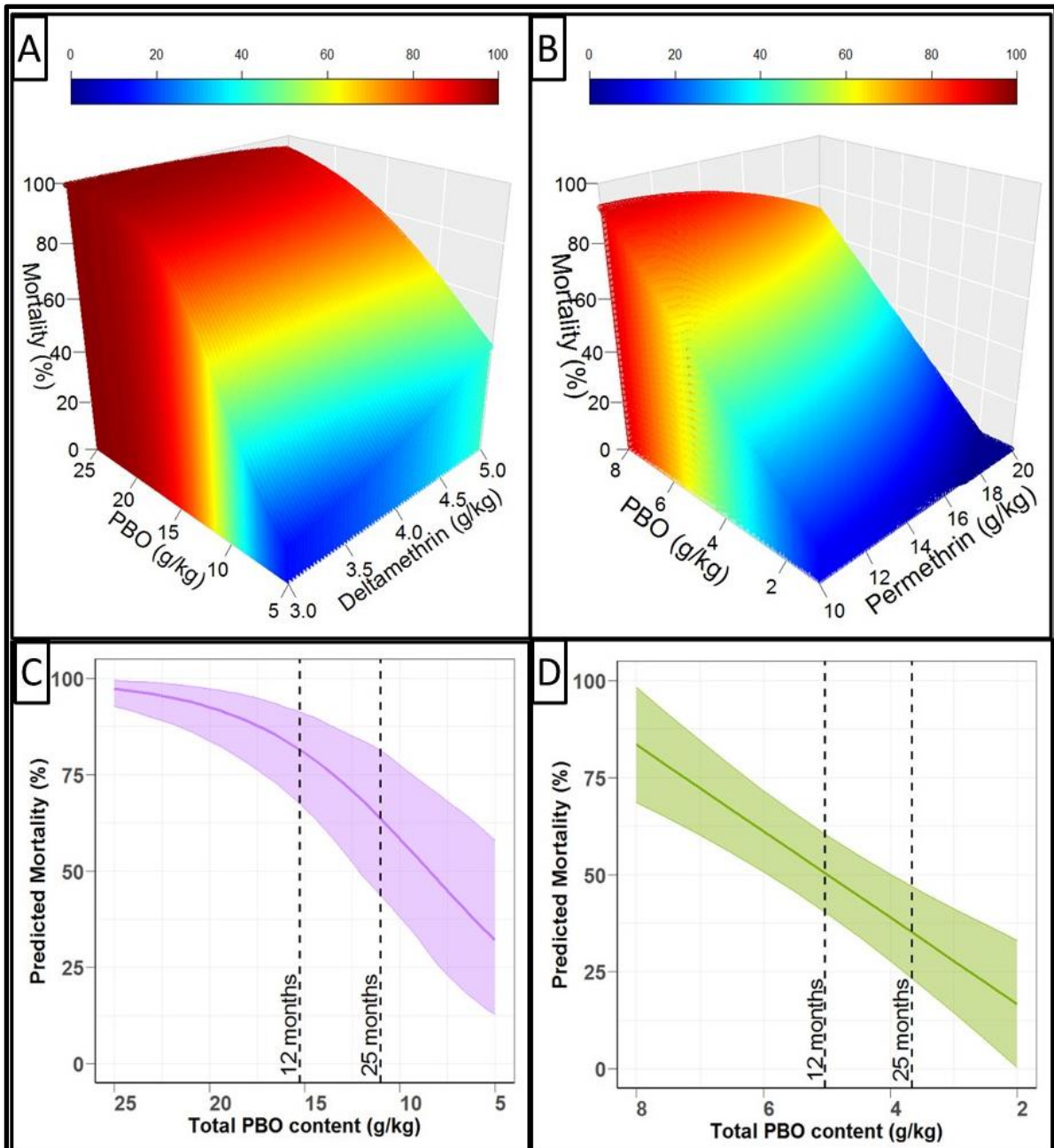


Figure 2.9. Relationship between total chemical content and bioefficacy against pyrethroid-resistant *An. gambiae* s.s. **(A)** PermaNet 3.0 in WHO Cone Bioassays **(B)** Olyset Plus in WHO Wireball Bioassays **(C)** PermaNet 3.0 in WHO Cone with deltamethrin value fixed at mean (4.42g/kg) **(D)** Olyset Plus in WHO Wireball with permethrin value fixed at mean (15.45g/kg).

2.4 Discussion

2.4.1 Chemical integrity

All nets met the manufacturers specified target dose for their respective chemistries, confirming that all LLIN products were both legitimate and of the minimum quality.

The pyrethroid content of the LLINs assessed was relatively stable across the two years of the study, with the exception of PermaNet 3.0 which declined by ~30% (yet was still within the manufacturer's target range). The stability of pyrethroids over two years observed here is consistent with studies from a range of settings (Toe *et al.* 2019, Lorenz *et al.* 2020, Gichuki 2021). In contrast, the PBO content of both PBO-LLINs declined more rapidly over the same time period, with under half of the initial content remaining after 25 months. This decline in PBO was also observed in a durability trial with Olyset Plus in neighbouring Kenya, which observed total concentration decreased by 40% after two years (Massue *et al.* 2021). Given the correlation between chemical content and bioefficacy observed here, this substantial decline in PBO content for PBO-LLINs raises concerns over the operational lifespan of these nets. However, despite this decline in PBO content it is important to note that the concurrent trial of epidemiological outcomes in the study site demonstrated that PBO-LLINs maintained superior protection over their conventional equivalents up to 25 months compared to pyrethroid-only LLINs (Staedke *et al.* 2020, Gleave *et al.* 2021) . Nonetheless, these data should raise concerns on the operational lifespan of Olyset Plus and Permanet 3.0.

While a strong correlation between total PBO content and bioefficacy was observed for both PBO-LLINS, this relationship may not be causal and total chemical content quantified by HPLC may not be fully representative of the concentration at the surface bioavailable to mosquitoes. It was observed that total pyrethroid was not statistically associated with

bioefficacy, this of course does not imply that the pyrethroids were irrelevant but instead that pyrethroid levels remained sufficient throughout. However, the clear collinearity of PBO content and mortality against the resistant strain indicates that the poor retention of PBO in the nets over time had implications for bioefficacy. This raises concerns over the PBO bleed rate of both Olyset Plus and PermaNet 3.0, suggesting that further development is needed to improve the retention of the secondary AI. Currently, there is a lack of available tools for quantifying the concentration of insecticide on the surface of LLINs, addressing this gap is important for future studies seeking to link chemical content to bioefficacy.

All netting samples used in HPLC were taken from the roofs of nets, this was done to ensure a fair comparison between Olyset Plus, which has PBO throughout, and PermaNet 3.0, which has PBO on the roof only. One implication of this methodology is that spatial variation in chemical decline across the net would go undetected. Consequently, the findings of the HPLC analysis in this study are representative of the roof but not necessarily the sides of the net. Future durability studies may wish to compare netting samples from different areas of the net surface when conducting chemical analysis.

2.4.2 Bioefficacy

Both Olyset Plus and PermaNet 3.0 were highly effective against the site-specific pyrethroid-resistant strain when new, demonstrating that these PBO-LLIN products do indeed restore its susceptibility to pyrethroids. This observation is consistent with the finding from associated epidemiological trials that these nets reduced childhood parasitaemia in the study area where these nets were collected (Staedke *et al.* 2020). However, while both PBO-LLINs tested were highly effective against the pyrethroid-resistant strain at baseline their bioefficacy diminished with operational use (with the mortality associated with Olyset Plus and PermaNet 3.0 decreasing to 26% and 46% respectively after two years). The diminishing differential in bioefficacy between PBO-LLINs and their pyrethroid-only equivalents is also consistent with the observation that differential impact on childhood parasitaemia narrowed over the same time. The steep reduction in bioefficacy with both PBO-LLINs against a study site specific pyrethroid-resistant strain is greatly concerning. LLINs are typically distributed with the expectation they will be replaced after three years yet in this context Olyset Plus and PermaNet 3.0 had a greatly diminished killing effect after the first two years. While the bioefficacy values themselves are specific to the Busia strain (and its associated resistance mechanisms) and not necessarily representative of other pyrethroid-resistant strains, there is an urgent need to investigate if this downwards trend is observed in other settings against other pyrethroid resistant populations. Given these findings there is an argument that, within the Ugandan context, LLINs should be distributed on a two rather than three-year cycle to maintain efficacy.

Current WHO bioefficacy criteria requires nets to achieve 80% mortality against pyrethroid susceptible mosquitoes but no such criterion exists for resistant mosquitoes. In part, this is due to the challenge of monitoring and maintaining consistent levels of pyrethroid resistance

in a mosquito colony over time. The pyrethroid-resistant *An. gambiae* colony used in this study was descended from mosquitoes collected in a field site in eastern Uganda. This locally derived colony was chosen to allow the LLINs to be tested against *An. gambiae* mosquitoes with similar resistance mechanisms to those in the study site. However, while colonies are subjected to regular exposures to maintain resistance conferring alleles, there is opportunity for the frequency of different mutations to fluctuate over the long timescales of a durability trial. Such fluctuations in resistance levels risk introducing a bias into comparisons of bioefficacy between timepoints. To address this, an alternative approach not performed here would have been to perform all cone bioassays in a short space of time after all nets have been collected. This approach would minimise variation in resistance levels between timepoints. However, this may not be logistically feasible in some circumstances due to the large numbers of mosquitoes needed for each timepoint and may risk introducing a different bias due to the different amount of time nets would have to be held in storage.

It is important to note that the bioefficacy outputs here are not necessarily a direct indicator of personal protection under operational conditions. The sustained, forced interaction between mosquitoes and net is unrepresentative of the interaction between an *An. gambiae s.l.* mosquito and an LLIN in practice, obscuring the complex behaviours exhibited at the net interface. Recent video tracking studies indicate that mosquito contact with the net surface is not continuous, instead consisting of numerous instantaneous encounters over the full duration of observation (or until the mosquito succumbs to the insecticidal effect)(Parker *et al.* 2015, 2017). Thus, three-minute benchtop bioassays with a small cutting from a net may be a poor indicator of the practical protection of the whole net in use.

One potential limitation of this work is that it did not include the WHO Tunnel Test, which is intended to directly assess blood-feeding inhibition as an output by using a rodent as bait. It is argued that the measurement of blood-feeding inhibition allows repellency to be relevant property in itself rather than as a confounding factor (as it is in the WHO Cone bioassay). However, there are ethical barriers to performing this assay as the animal inside suffers both the psychological stress of confinement and the physical harm of being bitten. Additionally, as *Anopheles* mosquitoes are typically highly anthropophilic the rodent bait may not provide a useful representation of the push/pull interactions of a repellent net and an attractive host, which weakens the argument for performing such a laborious experiment. Consequently, many institutes are unwilling or unable to perform the WHO Tunnel Test due to these ethical concerns and a lack of the associated animal licences required.

2.4.3 Use of the WHO wireball assay

A bed net in the field may exhibit excito-repellent properties (avoidance behaviour after coming into proximity to an insecticide) which allow it to prevent biting without the need to kill thus are not captured by these benchtop assays. The discrepancy in bioefficacy between the WHO Cone and Wireball with the same netting sample indicate the methodologies result in different interaction between the mosquito and the net. The low knockdown and mortality observed with Olyset Plus in the WHO cone bioassay was in stark contrast with the high bioefficacy observed with the same nets in the WHO wireball bioassay. This difference in outcomes between methodologies may be associated with the reputed excitorepellency of permethrin, manifesting as reduced contact with the net surface. As the wireball method surrounds the mosquito on all sides with netting, there is no insecticide-free surface to rest on and a greater insecticidal effect is observed. Consequently, future investigations with excito-repellent LLINs may wish to also include an assay that prevents avoidance from the net, such as the WHO wire-ball assay. However, it should be noted that this forced contact with a repellent net is not a realistic depiction of a free-flying mosquito approaching a sleeper under field conditions, where the mosquito may avoid contact and thus not receive a lethal dose of insecticide. Thus, bioefficacy outcomes in a WHO wireball is not indicative of personal protection from mosquito bites. Nonetheless, the WHO cone bioassay remains useful as it is an objective benchmark of knockdown and mortality across timepoints that is relatively straightforward to perform.

There are a number of challenges in implementing the WHO wireball assay that may discourage its use in future studies. Importantly, there is no consensus Standard Operating procedure (SOP) for the WHO wireball assay meaning it is not explicitly clear how the experimental setup is prepared (the methodology here was interpreted from a single short

paragraph in the 2006 guidance for assessing bioefficacy on nets in operational conditions. Additionally, the wrapping of the net samples around the wireball frame required substantial optimisation to arrive at a practical setup due to the lack of guidance in the lone formal document. Importantly, the WHO wireball guidance provides a choice of two suitable frames, either a 15cm diameter cube or a 15cm diameter sphere, with a markedly different internal volume (cube = 3375cm³, sphere = 1767cm³). This investigation utilised the cube method due to the ease of procurement, but it remains unaddressed if there is a difference in outcomes between the cube and sphere methodologies. Additionally, the insertion and removal of mosquitoes from the wireball frame proved to be much more challenging than the WHO cone setup, with the large internal volume of the wireball cube giving mosquitoes space to avoid the pipette used for collection (compared to the small internal volume of the WHO cone). Quickly removing all of the mosquitoes from the wireball proved to be challenging initially, requiring practice to perform efficiently and consistently to avoid adding to the exposure time. Future investigations may wish to consider using a mechanical aspirator to reliably remove mosquitoes from the wireball however this may introduce a bias between studies as not all labs may have access to them.

The WHO wireball method requires standardisation, with a resolution to the unusual latitude of allowing the user to choose between two different physical set-ups (ball or cube) with a large disparity in volume. However, given the need for bioassay methods that prevent mosquitoes from avoiding the net (due to the need to assess bioefficacy of products with excito-repellent properties) the wireball has potential to be a mainstream tool in net durability studies. There is a need for a detailed and unambiguous SOP for conducting the WHO wireball, with an argument to be made for choosing the wire or cube as the definite

method. Additionally, there is a lack of validation of the technique, with a need to determine the optimal number of mosquitoes and optimal exposure time.

2.5 Conclusion

Here, it is demonstrated that both Olyset Plus and PermaNet 3.0 had superior bioefficacy against pyrethroid-resistant *An. gambiae* s.s. mosquitoes from the trial site compared to their pyrethroid-only equivalents. However, the superiority of PBO-LLINs over conventional LLINs in bioassays narrowed with the operational life of the net, correlating with a sharp decline in PBO content. The diminished bioefficacy of PBO-LLINs against pyrethroid-resistant mosquitoes after just two years of operational use is of great concern and there is an urgent need to assess the durability of these LLIN products in other settings. Within the context of Uganda, these findings suggest a standard three-year distribution cycle would be inappropriate for Olyset Plus or PermaNet 3.0, with a shorter cycle of at most two years being more appropriate.

This chapter also demonstrates the differential bioefficacy outcomes observed with the WHO Cone and Wireball assays when assessing the same net sample. Unlike the cone, it appears that mosquitoes in the Wireball were unable to avoid contact thus providing a more reliable benchmark of knockdown and mortality. The contrasting performance of the same Olyset Plus nets in the WHO Cone assay and the WHO Wireball bioassay highlights that LLIN products with excito-repellent properties should be assessed with approaches that minimise avoidance from the net surface.

Chapter Three: Physical Integrity of LLIN in operational conditions

Statement of contribution

The findings reported here were made possible by data collections performed in the field by colleagues at the Infectious Diseases Research Collaboration, Uganda. The outcomes assessed were developed through meetings with leaders of the field team and formalised in SOPs written by myself and Dr Amy Lynd. The socioeconomic data utilised here were collected for the main epidemiological trial but I repurposed this for assessing durability outcomes by linking households IDs from which net were collected.

3.1 Introduction

3.1.1 Background

Long lasting Insecticidal Nets (LLINs) provide a physical barrier against the bites of host-seeking mosquitoes, preventing the mosquito from reaching the occupant while the insecticide takes effect (Bhatt *et al.* 2015, Parker *et al.* 2015). However, LLINs are acknowledged to accumulate physical damage with routine operational use, such as washing and handling (Gnanguenon *et al.* 2014, Lorenz *et al.* 2014, Toé *et al.* 2019). These holes may provide access to mosquitoes, allowing them to reach the occupant inside to feed and potentially transmit infection (Sutcliffe and Colborn 2015, Sutcliffe, Ji and Yin 2017). Thus, assessing the physical durability of emerging LLIN products is important to ensuring that individuals protection is sustained in the years between distributions (WHO 2013a).

Given that physical damage is thought to contribute to a reduction in protective effect over time, the World Health Organisation (WHO) recommends that LLINs are distributed at three to five years intervals to ensure adequate coverage (WHO 2013a). As new LLIN products with different active ingredients and textiles enter the market, the physical durability of these

products must be assessed in different countries to inform decision makers as to which demonstrate durability within their specific context. Even where a new LLIN design is a modification of an existing and well established product, such as the integration of a secondary compound, it is important that they are assessed completely on their own merits (rather than the assumption that a modified product with the same physical specifications will be equally durable).

The WHO and Vector Control Technical Expert Group (VCTEG) have published guidelines that describe universal methods to assess LLIN durability. The physical condition of an individual net is assessed by proportionate Hole Index (pHI) which involves the designation of the total damage on a net into three broad categories: "Acceptable", "Damaged", "Too Torn"; with the latter indicating that a net has its protective effect greatly reduced due to holes. Currently, the guidelines recommended that hole size is estimated by approximate comparisons to body parts ('thumb', 'fist', 'head'), which may be beneficial for quickly assessing a large number of nets but results in loss of resolution may make it more difficult to detect subtle changes over time. Furthermore, at present holes are considered equally regardless of where they occur on the net (e.g. on the top panel or at the bottom near the floor) yet recent evidence that mosquito activity is concentrated on the top of the net indicates that hole location may be relevant for the risk of a mosquito entering it (Lynd and McCall 2013, Sutcliffe and Yin 2014).

The causes of damage and hole formation in bed nets is not well understood but is broadly acknowledged to be a combination of human and environmental factors (Solomon *et al.* 2018). Existing studies are typically limited to a specific country or context, thus predictors of net damage are not readily generalisable between studies. There is a need for malaria control programmes to identify predictors of physical damage within their context in order to

maximise the protection of communities at greater risk to having damaged net, whether by programmes designed to improve net care or by supplemental net deliveries between national distributions. Generally speaking, low socioeconomic status within the context of sub-Saharan Africa is typically associated with poorer physical integrity outcomes. However, the specific causal factors of holes are difficult to identify (as this would require direct observation) thus the more feasible alternative is to identify socioeconomic predictors that correlate with the occurrence of damage.

In 2017, the Ugandan Ministry of Health initiated a mass distribution of pyrethroid-only and pyrethroid-PBO LLINs. As each district in the country received either LLINs with or without PBO, this mass distribution presented an opportunity to evaluate the physical durability of PBO LLINs on an unprecedented scale. Additionally, by identifying characteristics of households that correlate with heightened physical damage, this study is an opportunity to identify key indicators of communities that need to be prioritised for more frequent or supplemental net distribution efforts.

3.1.3 Aim

The aim of this chapter is to investigate longitudinal changes in the physical integrity of PBO-pyrethroid LLINs (Olyset Plus and PermaNet 3.0) in operational conditions in Uganda,

alongside their conventional pyrethroid-only equivalent LLIN products (Olyset net and PermaNet 2.0 respectively).

3.1.4 Objectives

The objectives of this chapter are to compare the physical integrity of LLINs sampled at timepoints following distribution in a cluster randomised durability trial in Uganda. These objectives are derived from the WHOPES LLIN durability guidelines (WHO 2011, 2013A).

The primary objectives of this chapter are to **(1)** quantify the proportion of nets of each LLIN product in each proportionate Hole Index (pHI) category at 12 and 25 months post-distribution, with an emphasis on the proportion of nets that are 'too torn' for use. The second primary objective is to **(2)** identify socioeconomic predictors of net damage.

The secondary objectives are to **(3)** quantify the proportion of nets with at least one hole for each LLIN product, **(4)** quantify the total surface area of damage on each LLIN product, and **(5)** model the relationship between physical integrity and chemical integrity (using the chemical assessment data reported in the previous chapter).

3.2 Methods

3.2.1 LLIN description

LLIN products assessed are as previously described in chapter 2.

3.3.2 Sample size

The sample size of each outcome assessed in this chapter is shown in **Table 3.1**.

Table 3.1. Sample size of nets used for each outcome.

Outcome	Timepoint	PermaNet 2.0 (n)	PermaNet 3.0 (n)	Olyset Net (n)	Olyset Plus (n)	Total
Proportionate Hole Index/ Hole Area/ Household indicators	12 Month	98	100	100	97	395
	25 Month	97	100	99	100	396
Relationship between chemical integrity and hole area	12 Month	38	35	34	31	138
	25 Month	29	30	30	30	119

3.3.3. Hole measurement and damage categorisation

Holes were measured on the sampled nets by colleagues from the Infectious Diseases Research Collaboration (based in Kampala, Uganda) at a field laboratory in Jinja, South-Eastern Uganda. To assess the physical integrity of the net fabric, nets were placed over a metal frame measuring W160 x L180 x H170 cm and any holes >0.5cm recorded (Lorenz *et al.* 2014). The size of a hole was defined by its length (the longest dimension) and width (measurement perpendicular to length measurement). As per WHOPES guidelines, holes smaller than 0.5cm (in length or width) and holes that had been repaired were noted but not included in the final dataset (WHO 2011). For each individual hole; the horizontal width, vertical length, and height from the ground were entered into a row of an excel sheet,

alongside the household ID and HSD number. This excel sheet with all holes included was sent to me for cleaning, formatting and data analysis.

Hole size was calculated using the formula for an ellipse ($\text{area}=\pi*\text{length}*\text{width}$). The total area of damage on a net was summed and used to categorise the net within the WHO proportionate Hole Index (pHI) categories; 'Good' (0-64cm²), 'Damaged' (65-642cm²), or 'Too torn' (643cm²+)(WHO 2013). Additionally, the proportion of nets of each LLIN product with at least one hole was calculated for each timepoint.

The vertical height of the hole on the net surface was measured as the distance between the ground and the centroid of the hole (no specific location measurements were taken for holes on the top surface). This height value was used to locate the hole within Top, Upper, and Lower sectors of an LLIN as defined by Sutcliffe and Yin (Sutcliffe and Yin 2014, Sutcliffe et al. 2017)(**Figure 3.1**).

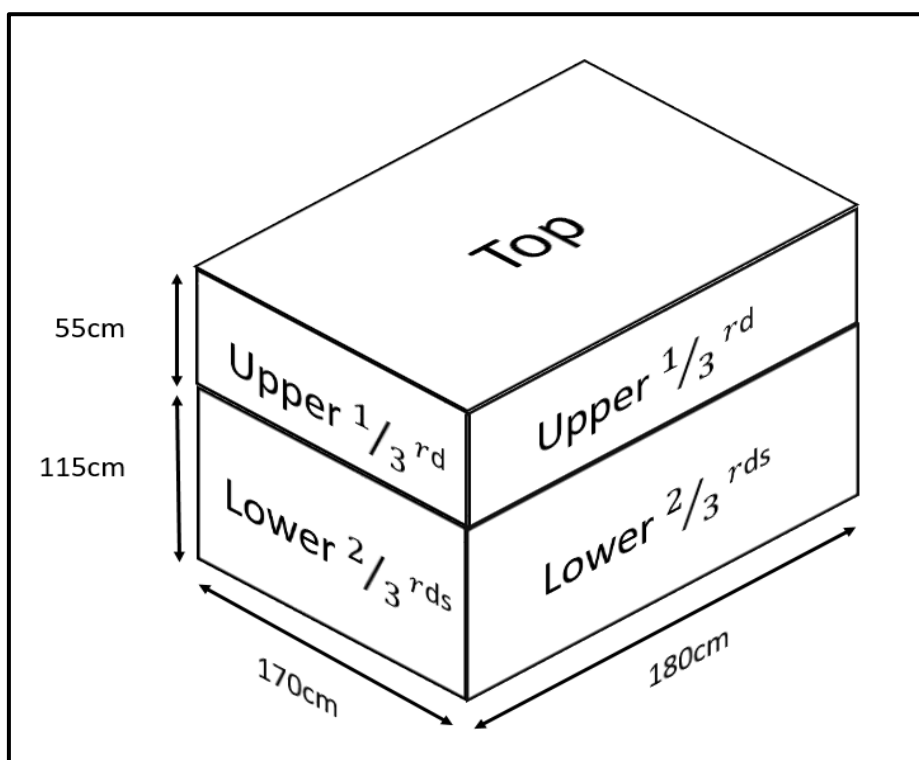


Figure 3.1. Diagram of 'Top', 'Upper', and 'Lower' net sectors used to categorise hole location (outlined by Sutcliffe and Yin, 2014)

3.3.4 Socioeconomic predictors

The socioeconomic factors assessed here were integrated from baseline surveys conducted for the main epidemiological trial of malaria outcomes. In the main epidemiological trial, baseline surveys were conducted on all enrolled houses. Thus, as the households from which nets were collected for durability assessment were a random subset of all households included in the epidemiological trial, baseline sociological data is available for all households from which nets were sampled. Data were obtained with permission from colleagues at the University of California, San Francisco and the household ID associated with each enrolled households used to integrate each indicator variables with the appropriate net samples. Household indicators included in the survey are shown in **Table 3.2.**

Table 3.2. Household Indicators included in analysis

Household indicator	Subcategories
Fuel type	Charcoal, Firewood
Wall material	Mud-pole, Unburnt bricks, Burnt bricks
Floor material	Earth-sand, earth-dung, Concrete
Roof material	Thatched, Iron
Windows	None, At least one
Eaves	Open, Closed

3.3.5 Data analysis

Data analyses were conducted using the R programming language (version 3.6.0), all graphs were produced using the ggplot2 package (version 3.2.1). Factors associated with outcome variables were identified using Generalized Linear Mixed Models (GLMMs) using the lme4 package (version 1.1-21). To account for unexplained variation between individual nets and between HSDs, the net ID (a unique identifier for each net distributed) and HSD number were each included in all models as a random effect. Additionally, to control for differences in the time each HSD received their nets the distributional ‘wave’ was included as a fixed categorical variable. The model selection process used stepwise regression, working backwards from a maximally complex model. Variables that did not significantly increase explanatory power (as indicated by log-likelihood ratio tests, ‘lmtest’ package version 0.9-37), were excluded from the final model. All potential interactions between fixed effects were considered. The p values reported here for fixed effects are the output of these LRTs. Pairwise comparisons between levels within a categorical variable were performed using least square means with the ‘lsmeans’ package (version 2.30-0).

To quantify the relationship between physical integrity and chemical integrity, the total hole area of each sampled LLIN was combined with the chemical assessment data reported in the previous chapter. As pyrethroid content was demonstrated to be stable for all LLIN products tested, this modelling focused on the total PBO content of each PBO-LLIN (PermaNet 3.0 and Olyset Plus). A GLM was then fit separately to the PermaNet 3.0 and Olyset Plus chemical assessment data with total hole (cm^2) fit as a fixed effect. The appropriate curve of the linear relationship, if any, was defined assessing the explanatory power of polynomial terms. Model selection and p value reporting was performed as above.

3.3 Results

3.3.1 Proportion of nets with at least one hole

The proportion of nets of each type with at least one hole at 12- and 25 months post distribution is shown in **Figure 3.2**. The overall proportion of nets with at least one hole after 12 months in operational conditions was 0.727, increasing to 0.829 after 25 months (OR: 1.821, 95%CI: 1.289-2.571, $p < 0.001$). There was no difference in the proportion of nets with at least one hole between the four LLIN products tested at any timepoint ($p = 0.306$).

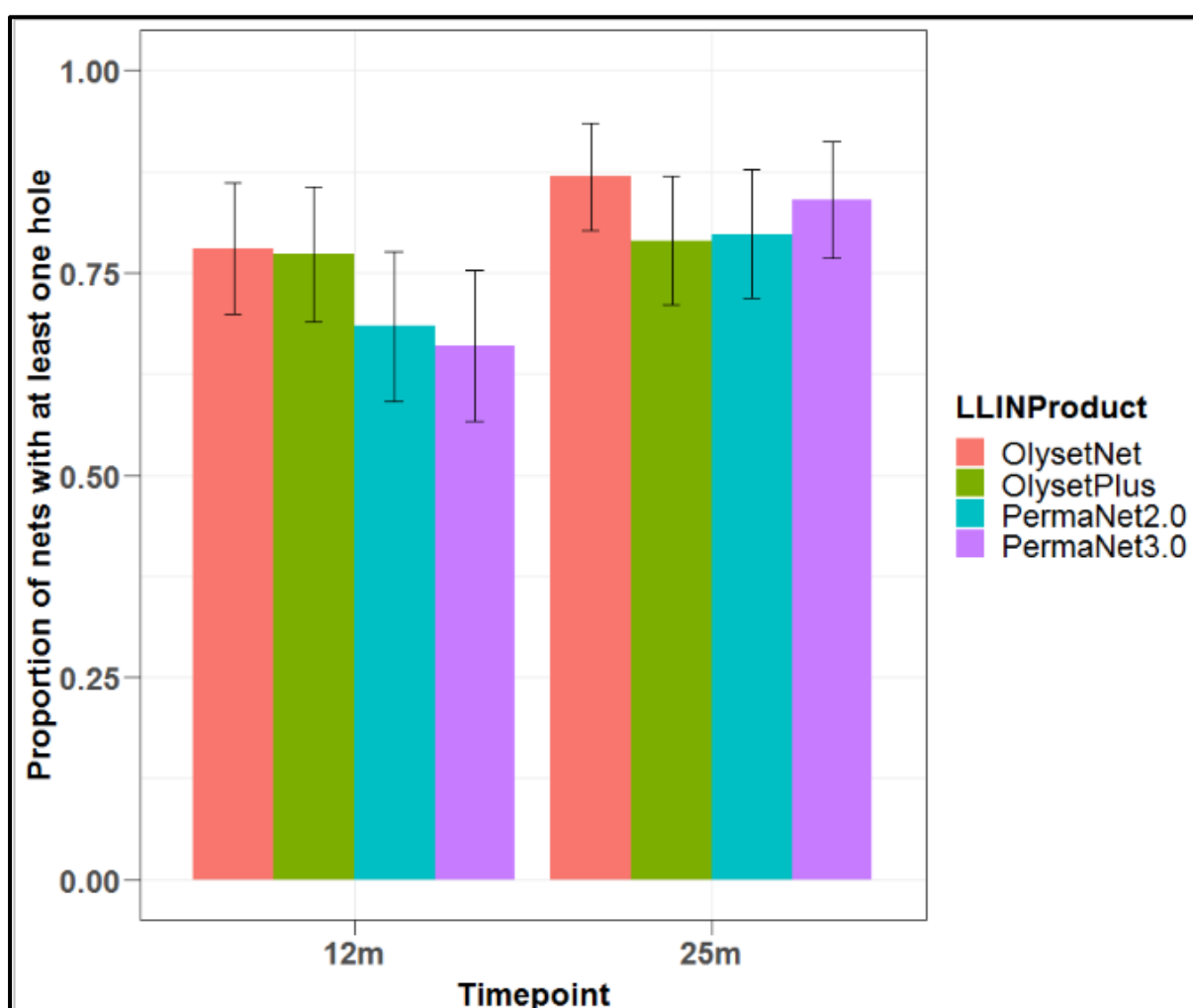


Figure 3.2. Proportion of each LLIN with at least one hole at each timepoint. Error bars indicate 95% CI

When hole probability was separated by net sector, there was no difference between LLIN products ($p=0.139$). Due to this lack of difference in hole probability between LLIN products, the data was combined to investigate overall trends in hole occurrence on different parts of the net. At both 12 and 25 months post distribution, there was a significant difference in the probability of at least one hole in a given sector (**Figure 3.3**).

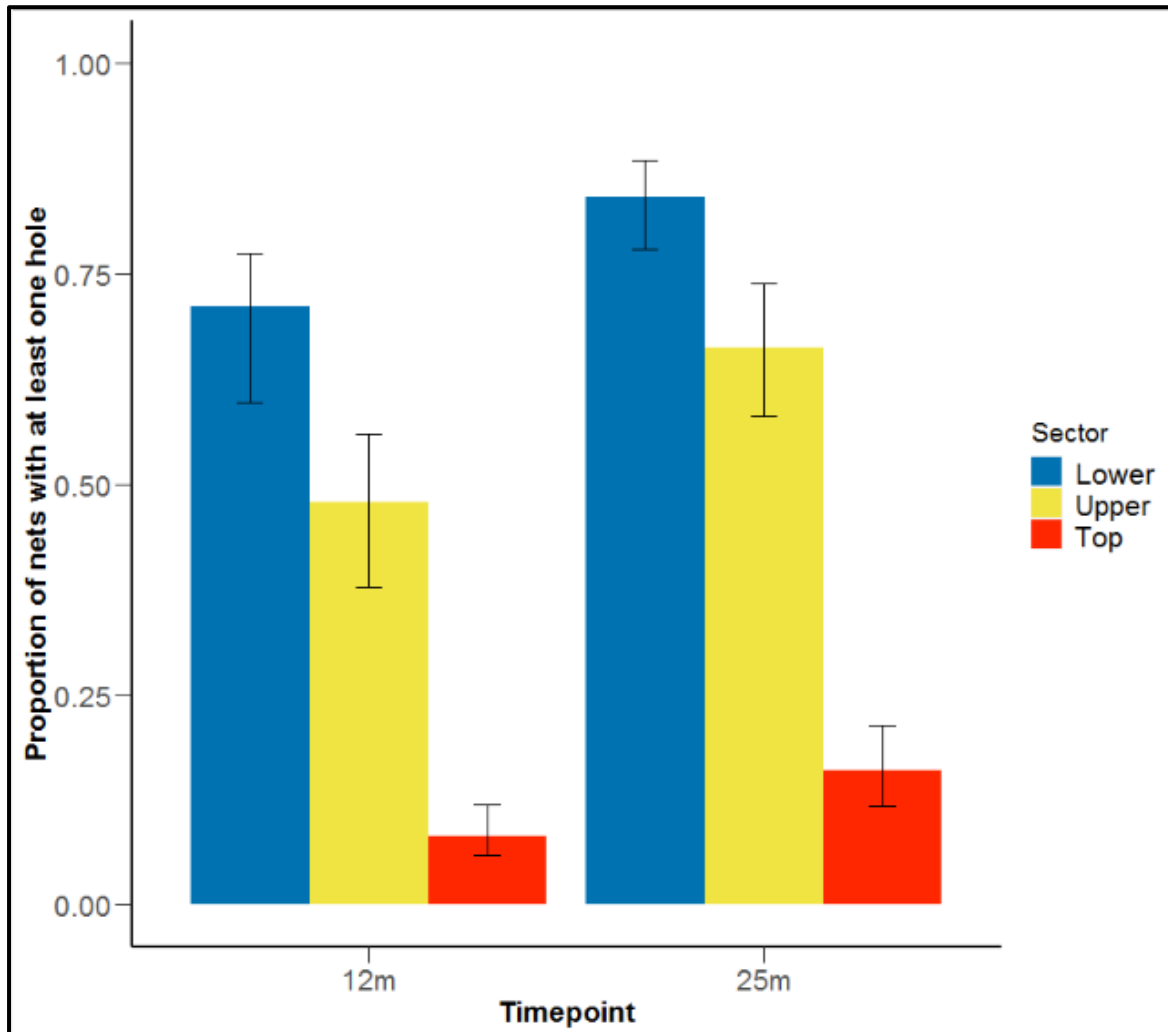


Figure 3.3. Proportion of nets with holes in each sector (lower 2/3rds, upper 1/3rd, top) at each timepoint. Aggregated across all four LLIN products. Error bars indicate 95% CIs.

The Relative Risk Ratios for the data as a whole across all LLIN products and timepoints combined are shown in **Table 3.3**.

Table 3.3. Relative risk ratio of at least one hole in each sector across all nets collected

Comparison	Relative Risk Ratio	Pairwise p value
Lower/Top	6.806	<0.001
Lower/Upper	4.988	<0.001
Upper/Top	1.354	<0.001

3.3.2 Total surface area of holes

There was no difference in total hole area between any of the four LLIN products tested ($p=0.270$). However, when all LLIN products were combined and only nets with $>1\text{cm}^2$ considered, there was an overall increase in total holed area from 12m post-distribution to 25m post-distribution ($p=0.0005$, **Figure 3.5**), which approximately doubled from 59.33cm^2 (95% CI: 45.08 - 78.25) to 105.49cm^2 (95% CI: 83.43 -136.86).

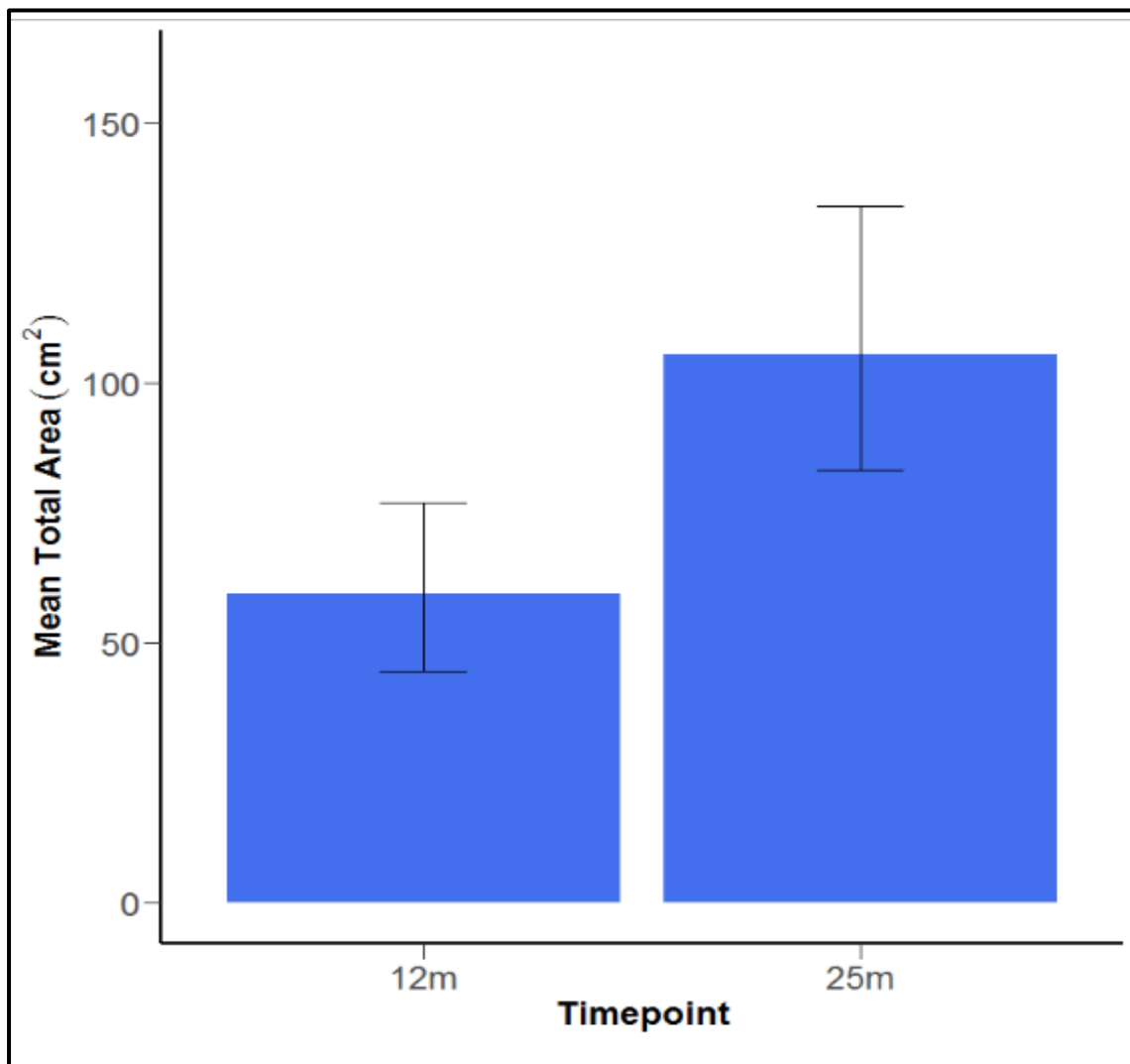


Figure 3.5 Total holed area across all LLIN products combined at 12 months and 25 months post distribution. Nets with no holes excluded. Error bars indicate 95% CIs.

There was a significant difference in the total surface area of holes in each sector of the net ($p < 0.001$, **Figure 3.6**), with the lower sector having the highest area of damage at both 12 and 25 months post distribution. At 25 months, the total hole area on the lower part of the net was 4.17 times higher than in the top. There was no difference in total surface area of holes in each sector between LLIN products ($p = 0.737$) indicating that the same trend was consistent across them.

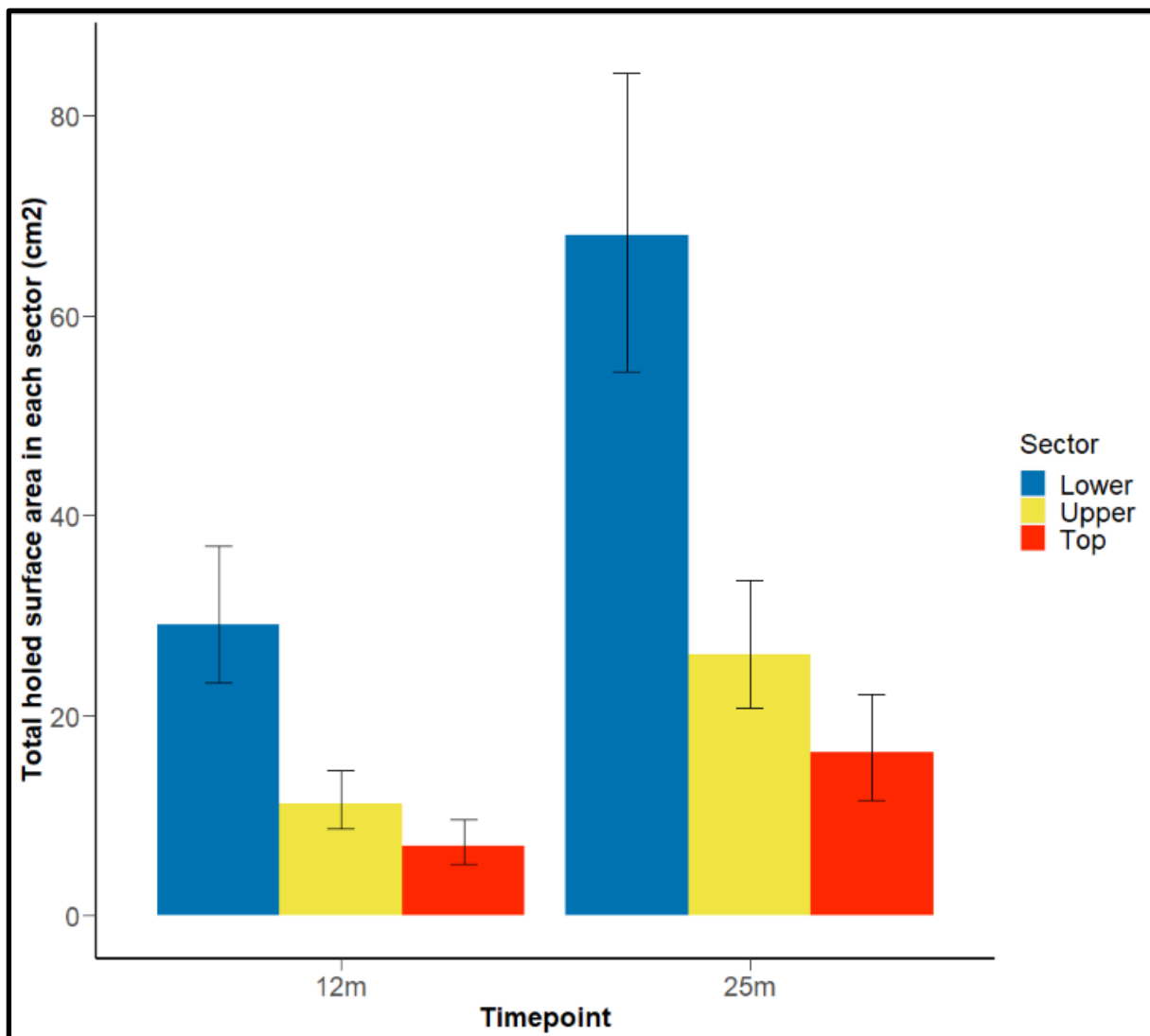


Figure 3.6. Total area of holes in each sector (lower 2/3rd, upper 1/3rd, top) for all LLIN products combined at 12 months and 25 months post distribution. Nets with no holes excluded. Error bars indicate 95% CIs.

3.3.3 Proportion of nets in each pHI category

At 12 months post-distribution, the proportion of nets classified as 'too torn' on the pHI scale was 0.066 (**Figure 3.7**), with this proportion approximately doubling after 25 months to 0.125 (OR: 2.017, 95%CI: 1.268-3.208, $p < 0.001$). There was no significant difference in the proportion of nets that were 'too torn' between LLIN products ($p = 0.644$).

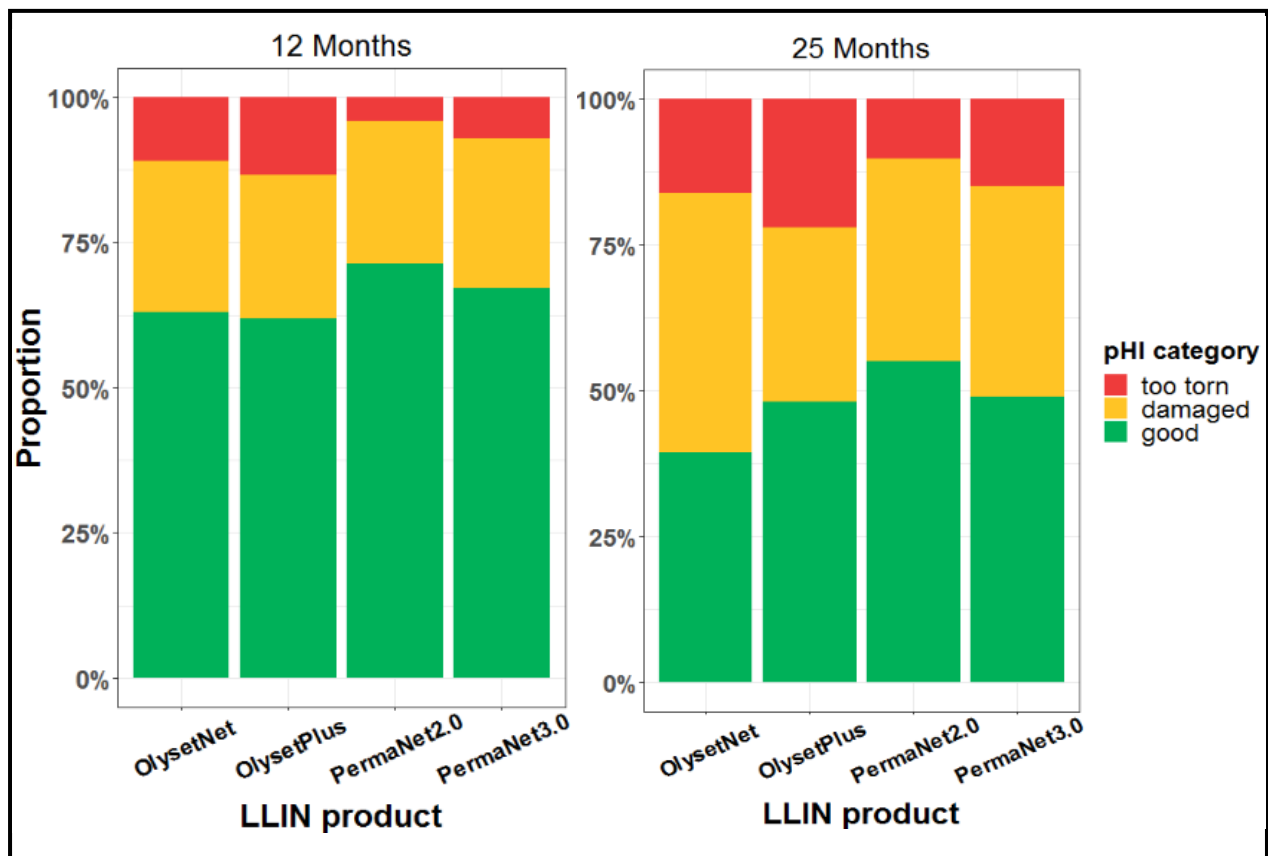


Figure 3.7. Proportion of collected nets in each pHI category ('Too torn', 'Damaged', 'Good') at 12 months and 25 months post distribution.

3.3.4 Socioeconomic Indicators

The association between various socioeconomic indicators and the total surface area of damage sampled from their household was assessed, along with the timepoint of collection. In the final parsimonious model with all non-significant predictors removed, only 'timepoint', 'windows', and 'eaves' were retained. The variables 'Region', 'Fuel type', 'Wall material', 'Floor material', and 'Roof material' did not significantly increase the explanatory power of the final model (**Table 3.4**).

The model estimated indicated that the presence of windows on a household was the strongest predictor of hole damage, with mean hole area 3.36 times higher in households with windows than those without when all other factors were controlled for (**Figure 3.8**). The presences of eaves was also found to be a strong predictor of total hole area, with the mean hole area of households with eaves 1.76 times higher than those without.

Table 3.4. Relationship between household indicators and total surface area of all holes for all LLIN products combined.

Predictor	Estimate	95% CI	p
<i>Timepoint</i>			
12m (reference)	1	-	-
25m	2.83	2.02-3.96	<0.001*
<i>Region</i>			
East (reference)	1	-	
West	0.72	0.46-1.12	0.331
<i>Fuel type</i>			
Charcoal (reference)	1	-	
Firewood	1.04	0.59-1.83	0.432
<i>Wall Material</i>			
Mud-pole (reference)	1	-	(0.903)
Unburnt bricks	0.86	0.46-1.63	0.899
Burnt bricks	1.24	0.73-2.11	0.702
<i>Floor Material</i>			
Earth-sand (reference)	1	-	(0.484)
Earth-dung	1.04	0.68-1.58	0.984
Concrete	0.84	0.47-1.47	0.786
<i>Roof Material</i>			
Thatched (reference)	1	-	
Iron	0.57	0.29-1.13	0.138
<i>Windows</i>			
None (reference)	1	-	
At least one	0.38	0.22-0.66	0.001*
<i>Eaves</i>			
Open (reference)	1	-	
Closed	0.58	0.39-0.87	0.019*

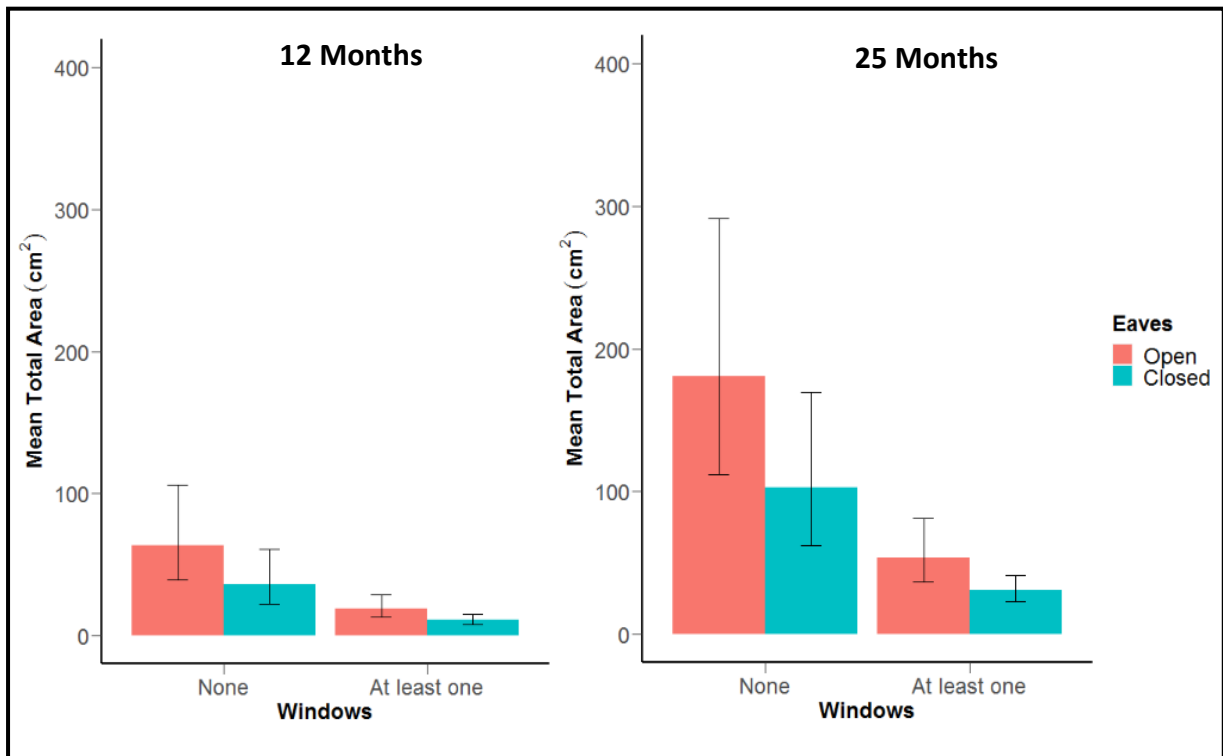


Figure 3.9. Mean total hole area in households with and without windows. Error bars indicate 95% Confidence Intervals.

Given the finding that the presence or lack of windows is a strong indicator of the physical condition of a sampled net, the proportionate Hole Index reported above was revisited with the data subset into these two categories (for all LLIN products combined). Across both timepoints, nets sampled from households with at least one window were 3.35 times more likely to be in the 'Too torn' category' (95% CI: 1.86-6.01)(Figure 3.10).

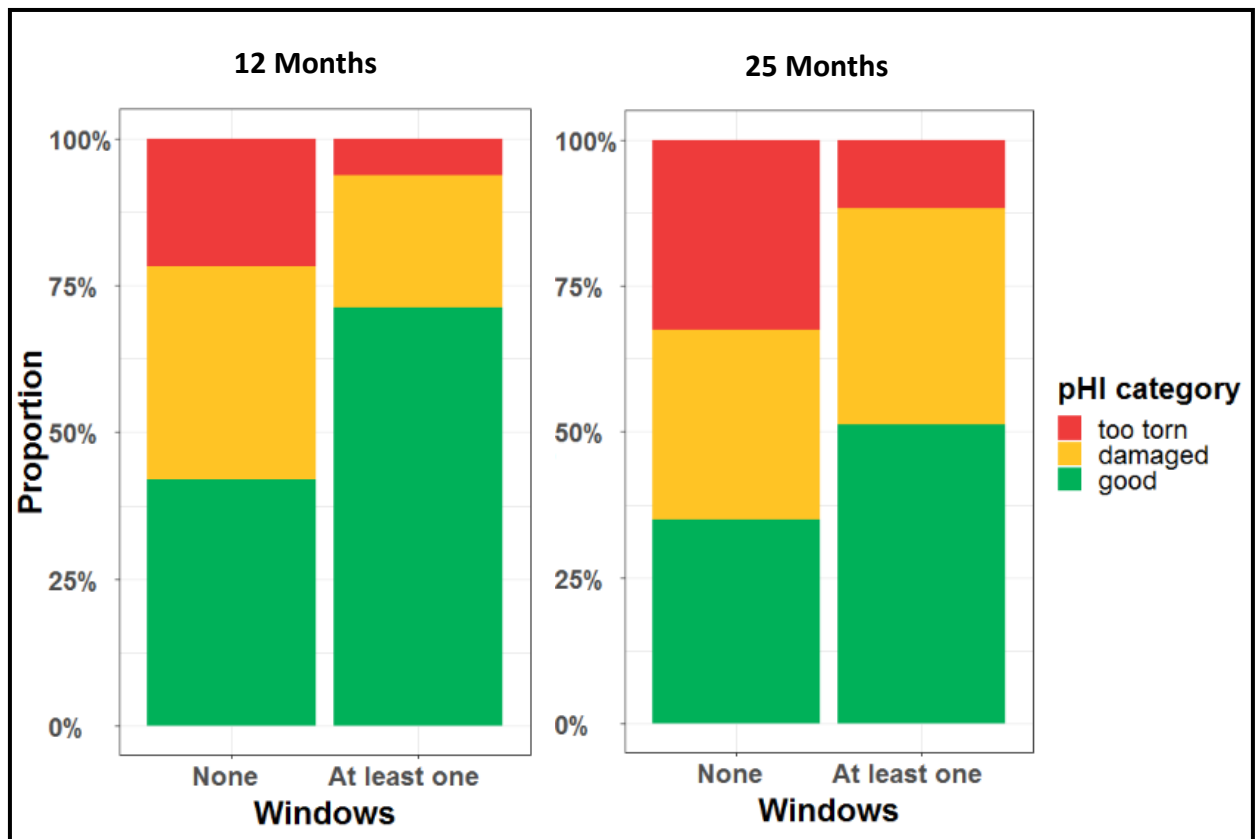


Figure 3.10. Proportion of collected nets in each pHl category ('Too torn', 'Damaged', 'Good') at 12 months and 25 months post distribution.

3.3.4 Relationship between physical integrity and chemical content

The relationship between predicted total PBO content and total hole area in nets sampled at 12 and 25 months post distribution is shown in **Figure 3.11**.

For Olyset Plus, hole area was predictive of total PBO content. The model predicted a weak linear association with the log of total hole area (estimate=-0.398, $R^2=0.294$, $p<0.001$), resulting in a non-linear relationship on the true scale (**Figure 3.11A**). In practical terms, this represents a relatively rapid reduction in total PBO content up to 125cm^2 (declining from a initial value of 5.82g/kg down to 3.90g/kg) but with a much more gradual reduction in total PBO content with each additional unit of damage beyond this.

A similar relationship was observed for the total PBO content of PermaNet 3.0 (**Figure 3.11B**), with the model predicting a relatively weak linear association with the log of total hole area (estimate=-1.0574, $R^2=0.231$, $p<0.001$, **Figure 3.12B**). On the true scale, there was a relatively rapid reduction in PBO content up to 160cm^2 (declining from an initial value of 16.51g/kg down to 11.14g/kg) with a gradual reduction thereafter.

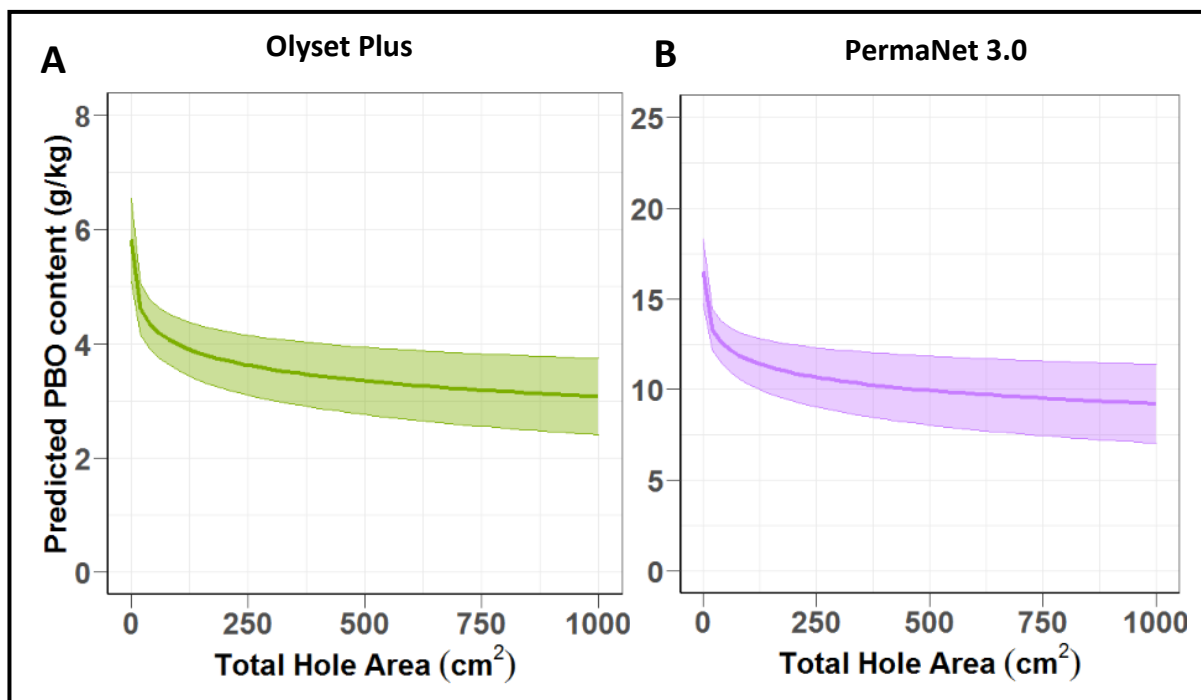


Figure 3.11. Modelled relationship between PBO content and Total hole area (combined surface area of all holes on a net) of **(A)** Olyset Plus and **(B)** PermaNet 3.0 (across all 12 and 25 months net samples). Errors bars indicate predicted 95% confidence interval of the mean. Note difference in y axis between each LLIN product (maximum value of y axis indicates manufacturers target dose when new).

3.4 Discussion

There was no difference in physical integrity outcomes between any of the four LLINs tested after 25 months in operational conditions. Thus, by these metrics PBO-LLINs nets were as physically durable as their pyrethroid-only equivalents. The observation here that there was no difference in physical outcomes between Olyset Plus and its conventional equivalent Olyset Net would follow from the equal fabric density (150 denier) of the two LLIN Products, yet this finding contrasts with a recent study in North-Western Tanzania which observed a higher proportion of Olyset Plus in the 'too torn' category (55% compared to 33% after three years)(Lukole *et al.*, 2022, in review). However, this inequity observed in Tanzania after three years was not evident at the two year mark thus there is agreement with the current study as it is possible this disparity would have emerged if extended to three years. Additionally, the authors of the Tanzania study highlight that this difference between PBO and non-PBO nets may be a survivorship bias as Olyset Plus was more likely to be retained than Olyset net even in poor physical condition (which they hypothesise to be due to the net users perception that Olyset Plus is effective in reducing biting).

Across all LLIN products assessed, general trends in physical integrity were observed across time with 50cm² of damage accumulating on the nets each year. Furthermore, it was observed that the majority (62%) of this damage occurred on the lower 2/3rds of the net where mosquito activity is expected to be low. Conversely, very little damage occurred on the top of the net where mosquito activity is expected to be high. However, it should be noted that greater mosquito activity on each part of a net is not necessarily indicative of the probability that a mosquito will successfully blood-feed, as the ability of *An. gambiae* to enter holes on an LLIN and successfully bloodfeed is poorly described. Nonetheless, the observation that

damage varies by location on the net and the implication that mosquitoes are more likely to chance upon top holes highlights the need for further research into mosquito behaviour around damaged bed nets.

It was observed that nets sampled from households with no windows were more than three times more likely to be in the most severe damage category than nets from households with at least one window. While it is possible that this disparity is directly associated with the housing structure itself (such as the presence of straw), housing type may in fact be an indirect indicator of other household variables such as the construction of the bed frame, the presence of animals indoors, or the type of cooking material used in the household as has been observed in Benin (Gnanguenon *et al.* 2014). Nonetheless, there may be an argument to distribute nets more frequently than three years in regions where traditional housing remains common. It should be noted that a concurrent study found that the net attrition rate for this distribution was high, with adequate coverage (one LLIN for every two residents) decreasing from 71% at baseline to 35% after 25 months (Maiteki-Sebuguzi, C. *et al.* in prep), indicating that LLIN attrition after distribution is an issue. If, as might be expected, households chose to discard damaged at a higher rate than nets in good condition, then the physical damage observed in the current study may be an underestimate. An important side note is that the estimate of roof material as a predictor of total damage was large, as is indicated in other previous studies, yet was not significant in the final model. This statistical ‘overshadowing’ indicates that the predictive power of roof material is more appropriately allocated to the presence or absence of windows (which serves as a reminder that these variables are indicators and not necessarily causally associated with net damage). A key caveat to these interpretations is that this socioeconomic data was not intended for this purpose, instead meant for identifying predictors of epidemiological outcomes in the same

households, thus did not include factors such as bedding type and materials that may be important for durability outcomes.

Given the importance of total PBO content reported previously and the concerning rapid rate at which PBO content declined over time, the relationship between physical integrity and PBO content was investigated. It was found that total surface area of holes was indeed a significant predictor of PBO content, albeit a relatively weak one, especially with high levels of damage. The initial stages of damage accumulation (for 125cm² Olyset Plus and 160cm² for PermaNet 3.0) correlated with loss of approximately 1/3rd of PBO content. However, after this initial damage the reduction in PBO content was extremely gradual to the extent that even an highly physically damaged nets would not be an indicator of low PBO content.

The current physical integrity outputs outlined in the WHO durability guidelines cannot be directly interpreted in terms of personal and community protection from mosquito bites. The pHI categories use cut-off values that are based on limited data, particularly for the threshold between 'Damaged' and 'Too torn'. Though any such categorisations will inevitably be somewhat arbitrary, there is a need to better understand the impact of declining physical integrity on both mosquito blood-feeding inhibition and mortality to better inform these guidelines. There is empirical evidence that damage to pyrethroid LLINs reduces personal protection from bites but that mortality is independent from holed surface area and instead dependent on resistance status (Randriamaherijaona *et al.* 2015). Consequently, damaged LLINs would be expected to retain some community effect against mosquito populations that are susceptible to their chemistry. Despite this, the median retention time of LLINs is well below three years in many settings (1.64 years across sub-Saharan Africa and 1.66 years for Uganda)(Bertozzi-Villa *et al.* 2021). Given evidence that perception of physical integrity is the

primary consideration in retention(Koenker *et al.* 2014), developing more durable LLIN products may have epidemiological impacts beyond what would be indicated by studies of mosquito behaviour, due to improved retention.

Despite the central importance of LLINs to vector control of malaria and the long standing acknowledgement that bed nets accumulate damage over time, our collective understanding of how mosquitoes interact with damaged bednets is surprisingly poor. The relationship between physical damage to an LLIN and the probability that *An. gambiae* mosquitoes will successfully enter and bloodfeed is poorly described but is an emerging area of research. Importantly, even if blood-feeding probability on damaged LLINs were to be better elucidated to inform how personal protection changes with hole size and location, a large knowledge gap would remain regarding community protection. As onwards transmission is dependent on a mosquito both entering a bed net to successfully feed then escaping back out of the net it just entered and survive the encounter, there are important knowledge gaps that must be addressed regarding mosquitoes' ability to exit damaged LLINs and survive the extrinsic incubation period. Particularly, there is a need to investigate the personal and community protection of novel dual-AI LLINs entering the market, such as PBO LLINs. Additionally, given the widespread emergence of insecticide resistance in sub-Saharan Africa, there is a need to assess how resistant mosquitoes interact with both conventional pyrethroid LLINs and with novel LLIN products designed to kill them.

3.5 Conclusion

Here, it is demonstrated that both Olyset Plus and PermaNet 3.0 were as physically durable as their conventional equivalents. Distinct trends in damage accumulation were observed across all LLIN products, with the majority of damage occurring in the lowest part of the net where little mosquito activity would be expected to occur. This disparity in where damage occurs, despite growing evidence that mosquito activity is strongly focused on the top of the net, highlights a need to reassess the current assumption of the durability guidelines that all holes count equally in categorising a sampled net as suitable for use.

It was observed that the lack of windows on a household was a strong indicator of nets being in poor physical condition, alongside the presence of open eaves. Given that these indicators can be readily assessed at a glance, there is an argument that the Ugandan Ministry of Health should use this information to identify communities that are a priority in future distributions. Additionally, similar research should be conducted in other settings to identify similarly strong indicators that a household may need nets replaced at more frequent intervals.

Chapter Four: Impact of hole location on entry rate of *Anopheles* mosquitoes into a host-baited bed nets treated with permethrin and piperonyl butoxide (PBO)

Statement of contribution

The experimental design, planning, volunteer recruitment and data collection in this chapter were conducted by myself. I received assistance in data collection from LSTM Research Technician Nicola Fletcher to release and recapture mosquitoes. I was one of the participants in this behavioural chapter.

4.1 Introduction

4.1.1 Background

Long-lasting insecticidal nets (LLINs) are a central component of global malaria control strategies, protecting against biting by *Anopheles* mosquitoes as their occupant sleeps to reduce exposure to malaria-causing parasites (Pryce, Richardson and Lengeler 2018). These LLINs provide both physical protection through tightly woven fabric and chemical protection through insecticide on the surface. The protective effect of a bed net is typically split into two broad categories: personal protection and community protection. Personal protection, the more easily quantified of the two, is the ability of a bed net to prevent bloodfeeding on the occupants (Lindsay et al. 1991, Sutcliffe and Colborn 2015). Community protection is the broader effect a net has on the surrounding human population (whether they use a net or not) as mosquitoes killed by a net in one household cannot go on to bite individuals in another household at a later time (Levitz *et al.*, 2018; Unwin *et al.*, 2022). Thus, in theory even a net that performs poorly in preventing bloodfeeding on its occupant has an important role in community protection if bloodfed mosquitoes die or have their lifespans reduced due to the interaction. However, over time LLINs become physically damaged through routine use and

washing resulting in holes which may provide access to mosquitoes (Gnanguenon et al. 2014, Toé *et al.* 2019). The implications of physical damage on the protection of a population and the resulting success of malaria control programmes is not well understood

Host-seeking *Anopheles* mosquitoes are initially attracted towards potential sources of a bloodmeal by the elevated CO₂ levels expelled, then follow the concentration gradient of CO₂ to its approximate source. The combination of heat and volatile chemicals from the skin attracts the mosquito to land on the hosts body, where it can obtain a bloodmeal. There is growing evidence that anopheline behaviour as they approach bed nets follows consistent patterns that influence the probability it will encounter holes. Early experiments using adhesive panels on bed nets indicated that anophelines first contact with a bed net was generally on the top panel (Lynd and McCall 2013, Sutcliffe and Yin 2014). These studies calculated that approximately 80% of initial contact was with the top panel, with the caveat that the use of adhesives to assess first contact prevented any further behaviour from being expressed. These findings were supported by subsequent studies without adhesives, instead using video analysis that observed approximately 80% of activity occurring on the top surface (Sutcliffe and Colborn 2015, Parker *et al.* 2015). Furthermore, Parker *et al.* demonstrated that this concentration of activity on the top surface was sustained for the duration of the testing period. The consistent finding that *Anopheles* host-seeking activity is strongly focused on the top of the net would suggest that these mosquitoes had a greater chance of encountering a hole on the top than elsewhere however the literature of investigations of hole entry around bed nets remains sparse. The same video analysis of susceptible *An. gambiae* around untreated nets described above (Sutcliffe and Colborn 2015) indicated that mosquitoes had an increased chance of encountering a hole in the top and a 20% greater chance of entering through a hole on the top once encountered. However, their use of an untreated net rather

than a functional pyrethroid treated net limits the insight for vector control. As the presence of insecticide would be expected to have implications for hole encounter and passage due to the repellent and incapacitating effects of insecticide on the mosquito (Abdel-Mohdy et al 2009), studies that use insecticide-treated nets are needed (Parker *et al.* 2015). Importantly, none of these studies address the core knowledge gap for assessing the impact of holes on bed nets in the context of vector control, specifically if bloodfeeding success varies with hole location and if mosquitoes die as a result of the attempt. Finally, there is a dearth of literature on the exit behaviour of bloodfed mosquitoes from the inside of a net. The fibres of a bed net are omnidirectional, delivering insecticide to mosquitoes that contact them from any direction, yet there is virtually no understanding of how much the escape attempt contributes to bioefficacy.

This knowledge gap in mosquito entry into nets is further compounded by the widespread rise of insecticide resistance in mosquitoes, allowing them to tolerate exposure to pyrethroids used in LLINs (Churcher *et al.* 2016, Ranson and Lissenden 2016). As mosquitoes become better able to withstand contact with LLINs, there is concern that they will be more likely to succeed in entering through holes to bite the occupant and survive. As pyrethroid resistance is now widespread in sub-Saharan Africa, the need to assess the behaviour of pyrethroid-resistant mosquitoes is clear.

In response to growing pyrethroid resistance, so called 'next generation' pyrethroid LLINs supplemented with the synergist piperonyl butoxide (PBO) have been developed to restore susceptibility (Gleave et al. 2021). These PBO-LLINs are now a major part of malaria control strategy in sub-Saharan Africa, with 42.8% of LLINs distributed in the region in 2021 of this type (Alliance for Malaria Prevention, 2021). As the deployment of this class of product is

targeted to areas of moderate-high pyrethroid-resistance, the result is an interaction with pyrethroid-resistant mosquito populations. However, the behaviour of pyrethroid-resistant mosquitoes around pyrethroid-PBO LLIN products is largely unaddressed at the time of writing. Previous research investigating mosquito entry into pyrethroid nets, observed no difference between pyrethroid-susceptible and pyrethroid resistant *An. gambiae*, concluding that bloodfeeding success was independent of bioefficacy (Randriamaherijaona et al. 2015). It remains unknown if this logic applies the bloodfeeding success of pyrethroid-resistant *An. gambiae* around PBO LLINs. Additionally, in the context of the findings reported in the previous chapter that damage on PBO-LLINs (and their conventional equivalents) occurred highly disproportionately on the side of the net, there is a need to compare the protective efficacy of PBO-LLINs with damage on the top and side of the net.

4.1.2 Aim

Recent studies have indicated that the host-seeking activity of *An. gambiae* is heavily focused on the top of an occupied bed net, here I hypothesise that holes on the top of the net pose a greater risk for bloodfeeding on the occupant compared to holes on the side.

The aim of this study is to investigate if the location of a hole on a net (top or side) impacts the personal protection of an LLIN (defined as the proportion of mosquitoes that successfully bloodfeed on the occupant). This study will address this question for both the conventional permethrin LLIN Olyset Net, and for its next generation counterpart Olyset Plus which is treated with both permethrin and piperonyl butoxide to target insecticide-resistant mosquitoes.

4.1.3 Objectives

Objective **(1A)** is to compare the blood-feeding success pyrethroid-resistant *An. gambiae* mosquitoes when exposed to each combination of net type (Olyset Net and Olyset Plus) and hole location (No hole, Side hole, Top hole)(each combination is hereafter referred to as ‘arms’ of the study). This objective is measured as the proportion of mosquito’s blood fed in each arm of the study (regardless of if they are dead or alive). Objective **(1B)** is to calculate the Population Personal Protection of a hypothetical community as the occurrence of LLINs with a hole in either the top or side increases as a proportion of all nets.

Objective **(2A)** is to compare the proportion of pyrethroid-resistant *An. gambiae* mosquitoes knocked down (i.e. incapacitated) for each arm of the study, and Objective **(2B)** is to compare the proportion of pyrethroid-resistant *An. gambiae* mosquitoes dead after 24 hours in each arm.

Objective **(3A)** is to compare the escape rate of bloodfed mosquitoes from Olyset Plus for each hole position and Objective **(3B)** is to compare the 24 hour mortality of these bloodfed mosquitoes.

Benchtop assays with the same LLINs were performed to assess bloodfeeding and bioefficacy and to investigate if they are indicative of the outcomes of free-flying behavioural experiments.

4.2 Materials and Methods

4.2.1 LLIN description and preparation

The LLIN products used in this chapter were a single sample each of Olyset Net and Olyset Plus. Both nets were in original packaging with appropriate labels on one corner, though not sealed at the time they were obtained. These nets were obtained by private 'off the shelf' purchases made by Dr Amy Lynd in retailers in Uganda. These nets were resized to fit the single bed used in the testing room by cutting excess material using scissors and connecting the resized pieces using a sewing machine. Nets were altered down from the 'special' size (measuring 180cm long x 170cm width x 170cm height) to custom dimensions of length 180cm, width 90cm, height 90cm. This resizing would not be expected to alter protective effect as the chemistry is unchanged and the height of the net remains the original size.

The manufacturers specifications for these products are shown in **Table 4.1**.

Table 4.1. Manufacturer specifications of LLIN products assessed in study.

Product name	Manufacturer	Weave	Insecticide target
Olyset Net	Sumitomo Chemical Ltd.	Polyethylene (150 denier)	Permethrin: 20g/kg (\pm 5.0)
Olyset Plus	Sumitomo Chemical Ltd.	Polyethylene (150 denier)	Permethrin: 20g/kg (\pm 5.0) PBO: 10g/kg (\pm 2.5g/kg)

Prior to any experimentation, both LLIN products used were hung up in a well-ventilated room for 24 hours to air out the net as recommended by the manufacturer. When not in use in a given days assays, LLINs were wrapped in aluminium foil and stored in a refrigerator at 5°C.

On each testing day, the LLIN product to be assessed was set up at least two hours prior to any behavioural assays to allow the net to reach room temperature.

4.2.2 Mosquito characteristics

The mosquito colony used for all experiments in this chapter was *Anopheles gambiae* s.s. strain 'Busia 6P4'. This strain is successor strain of the 'Busia' strain used in Chapter Two, with founding females individually selected for being homozygous for the *Cyp6P4* mutation by Amy Lynd (unpublished). Thus, the *Cyp6P4* mutation was fixed in the 'Busia 6P4' colony and all mosquitoes used were confirmed to be homozygous for this mutation. The fixing of *Cyp6P4* was done to avoid the experimental outcome being confounded by the variable frequency of this metabolic resistance allele. To characterise the phenotypic resistance of Busia 6P4, WHO tube assays with permethrin and PBO were performed using the methods described previously (WHO 2006). After a 60 minute exposure to 0.75% permethrin 1hr Knockdown and 24hr Mortality was 18.86% and 17.92% respectively, increasing to 61.6% and 55.3% with prior 60 minute exposure to 4% PBO.

Mosquitoes used in the experiment were 3-5 days old females, which had not previously fed on human blood prior to the experiment unless explicitly stated in the methodology for a given experiment. Mosquitoes were reared under standard insectary conditions ($27^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and 80% relative humidity). Throughout the experiment, the lighting conditions of the rearing insectary was set under a 12:12 light/dark cycle where the lights were off from 12pm to 12am to simulate the conditions of night. Thus, adult mosquitoes used in this experiment experienced night-time from 12pm to 12am throughout their entire lifespan from hatching. This reversed lighting cycle allowed experiments to be conducted with mosquitoes in the night phase of their circadian rhythms during normal working hours.

4.2.3 Experimental design

4.2.3.1 *benchtop assays*

WHO tube, cone, and wireball assays were conducted as described previously.

Benchtop bloodfeeding assays were performed to assess the proportion of mosquitoes that could successfully feed through the gaps in the netting of an untreated net, Olyset net, and Olyset Plus. Two net samples from each LLIN product were each sampled five times. Samples were they offcuts from each net obtained in the resizing process. Untreated negative control netting was tested at the beginning and end of each testing day.

The day prior to testing, the sugar pad on the cage of mosquitoes to be tested was replaced with water and two hours prior to testing the water source was removed completely. One hour prior to testing five 3-5 day old female *Busia* mosquitoes were placed into a paper cup using a mouth aspirator and this cup placed in the testing room for 15 minutes to allow the mosquitoes to acclimatise. The mouth of the cup was covered in untreated netting. Immediately before testing, the piece of netting to be assessed was wrapped around the operator's arm. To begin the assay, a three minute timer was set and the cup placed upside down on the operators net-covered arm. After the three minutes had ended, the cup was removed from the arm and the mosquitoes transferred using a mouth aspirator to a second cup when they were provided with a sugar source. This second cup was necessary as the exposure cup may have become contaminated with insecticide, which may continue to affect the mosquitoes after the three minute assay (especially if it contaminates the sugar source). The number of mosquitoes bloodfed was visually assessed immediately after testing, knockdown assessed after one hour, and mortality after 24 hours. If a mosquito was not bloodfed to repletion (i.e. appeared to be partially bloodfed) it was still recorded as bloodfed.

Optimisation notes

A prototype version of the benchtop bloodfeeding assay utilised an exposure cup with the netting sample affixed over the mouth however this methodology proved impractical due to the need remove mosquitoes immediately at the end of the assay to avoid additional exposure beyond three minutes. The large internal volume and elongated shape of the cup made it difficult to remove mosquitoes quickly and consistently, as it allowed mosquitoes space to evade being caught.

4.2.3.2 Free-flying bloodfeeding assay

To investigate the impact of hole location on bed net on the probability a host-seeking, pyrethroid-resistant *An. gambiae* mosquito will successfully bite a person inside and survive the encounter, the following experiment was devised. The circumstances of a mosquito approaching a sleeper under a damaged bed net were reconstructed, with each arm of the study representing human volunteers beneath a bed net with damage on different parts of the net. All combinations of the two LLIN products ('Olyset Plus' and 'Olyset Net') and the three damage statuses ('No hole', 'side hole', 'top hole') were assessed, for a total of six study arms. The effectiveness of each arm in preventing blood-feeding and killing the mosquito in each arm will be quantified. A human participant is necessary inside the net to provide the chemical cues that attract mosquitoes to approach. Multiple participants are needed as people vary in their attractiveness to mosquitoes.

The same sample of each LLIN product was used for all experiments. A circular hole 15cm in diameter (area = 176.71cm²) was cut into the centre of the top and one side panel of both the Olyset Net and Olyset Plus net. This hole size was chosen as it puts the net in the 'damaged' category of the proportionate Hole Index (pHI) system described previously. A piece of spare netting material from each net (obtained in the resizing process) was then used as a patch to cover the hole not being assessed in a given days assay.

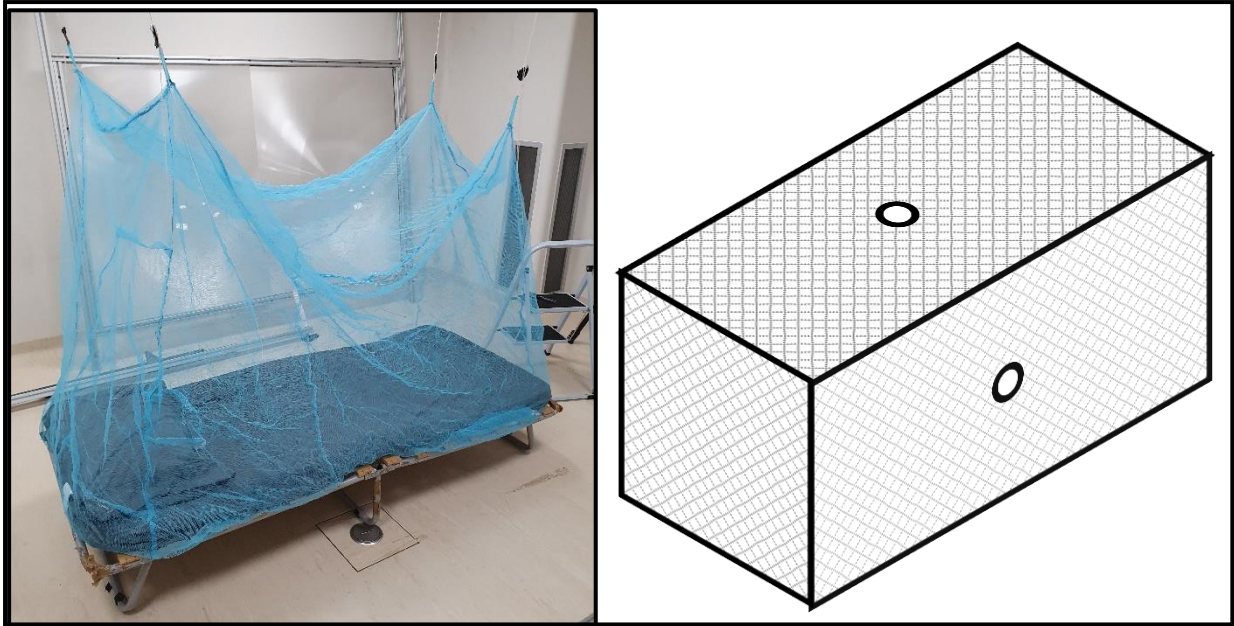


Figure 4.1. Diagram of experimental setup showing top and side hole position. The hole(s) not being assessed in a given assay are covered with net pieces cut from the same sample.

All experiments were conducted in a climate-controlled room purpose built for assessing the behaviour of free-flying mosquitoes in the LSTM Accelerator building, with dimensions; length 7m, width 5m, and a height of 2.5m. The airflow in the testing room was deactivated for the duration of the free-flying bloodfeeding assay to prevent interference with mosquito movement. The environmental conditions in the testing room were set to standard insectary conditions ($27^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and 80% relative humidity) and reversed 12:12 light cycle as described above, meaning the room was dark for the duration of each assay. All experiments were performed within the testing window of midday to 5pm, when an *An. gambiae* mosquito on a reversed day:night cycle would be expected to be in the host-seeking phase of their circadian rhythms. Mosquitoes used were 3-5 day old female *An. gambiae* 'Busia' colony. Twenty four hours prior to each experiment, mosquitoes to be used had their normal 10% sugar solution replaced with distilled water only. This water solution was then removed three hours before

release to minimise the chance that a mosquito would be too full to blood feed. One hour before release, the cup containing the twenty mosquitoes to be used were placed in the testing room to acclimatise.

In the study of mosquito entry into holed nets, a human volunteer was required to lie beneath the net in each experiment. The bed was orientated lengthways in the room, with the direction of the pillow-end of the bed reversed in each assay to account for random variation caused by orientation. This volunteer was asked to wear light clothing such as a t-shirt and avoiding wearing scented products the day of testing. Participants were asked to remain relatively still within the net, allowing for normal movements associated with comfortably resting. Importantly, participants were asked to avoid responding to mosquitoes that entered the net if possible.

Mosquitoes were released into the testing room by a simple yet well established 'cup and string' mechanism, allowing them to be released by an operator on the outside. After 60 minutes had elapsed, the operator carefully entered the room to prevent mosquitoes escaping then turned on the lights. Mosquitoes were collected with a mechanical aspirator and placed into one of two labelled cups depending on whether they were collected inside or outside the net.

Once all mosquitoes had been collected, the volunteer was assisted out of the net. The two collection cups were then placed in an adjoining climate-controlled room to allow the mosquitoes to be assessed. Blood-fed status and knockdown (mosquitoes in an incapacitated state) were visually assessed and counted immediately after the end of the assay. Bloodfed mosquitoes were transferred to a third and fourth cup labelled 'inside bloodfed' and 'outside bloodfed' as appropriate (This is done because mosquitoes which were bloodfed at collection

may not be visually apparent as bloodfed the following day). A 10% sugar solution was then provided to each cup and 24hr mortality assessed the following day.

The testing room was decontaminated when changing between LLIN products to avoid any residual insecticide from one net interfering with the outcome of subsequent assays with another. All surfaces (floor, wall, and ceiling) were decontaminated with 5% Decon90 solution (Decon laboratories, Sussex), followed by 70% ethanol. To confirm that the room was sufficient decontaminated of insecticide and that there was no residual insecticidal effects of cleaning solutions, WHO Cone assays with susceptible 'Kisumu' strain *An. gambiae* were performed on all four walls of the testing room (seven 3-5 day old females per cone, two cones per wall).

Optimisation notes

Optimisation of the free-flying bloodfeeding assay involved many iterations of different setups. The impact of airflow, bed orientation, collection tool and mosquito age were assessed. The powerful airflow of the environmental systems (located immediately above the net) was found to interfere with mosquito entry into the top of the net, preventing them from entering thus it was decided to deactivate the airflow for the duration of each assay. Furthermore, consistently recapturing mosquitoes at the end of the assay in a timely manner was initially difficult and required multiple practice sessions to become proficient. Additionally, in initial optimisation experiments mosquitoes were sometimes able to escape into the adjoining observation room (due to the holes in the hole for equipment cabling) thus efforts were made to plug all of these gaps, allowing consistent 100% recovery to be achieved. Initially, a manual mouth aspirator was used for mosquito collection however this proved to be time consuming and difficult to achieve (as it has relatively poor suction through a very small opening) thus optimisation assays were performed with a mechanical aspirator (which has much higher suction through a wide opening). The mechanical aspirator was found to greatly simplify and speed up collection without any impact on bioefficacy outcomes.

4.2.3.3 Free-flying escape assay

To assess the ability of blood-fed *An. gambiae* to escape an LLIN once inside and to quantify the contribution of the exit behaviour to bioefficacy, an experiment was devised to release bloodfed mosquitoes from inside of the nets. The same testing room, mosquito colony and cleaning protocol outlined above was used.

As in the previously described free-flying experiment, 3-5 day old *An. gambiae* 'Busia 6P4' females were used, which were starved of sugar for 24 hours prior to testing and deprived of a moisture source two hours prior to testing. Each assay utilised 20 mosquitoes. Thirty minutes prior to testing, the cup was placed against the operator arm to allow mosquitoes to arm-feed through the untreated netting. The cup was visually inspected periodically to assess how many had blood fed, with the operator exhaling over the mouth of the cup to encourage feeding. It was intended that if not all mosquitoes had bloodfed after 30 minutes had elapsed then these unfed females would be removed however this did not prove necessary.

No human host was included in the free-flying escape assay; thus, the room was unoccupied for the duration of the assay. Each assay lasted five hours, as this was the longest period of time that was logistically feasible due to the fixed lighting cycle and availability of facilities staff to restore airflow. The previously described string-pull mechanism for releasing mosquitoes was not suitable for releasing mosquitoes inside of the net, instead the elasticated netting of the cup was loosened until it was only being held by hand pressure and the cup placed in into the net. The operator then promptly left the room.

After five hours had elapsed, mosquitoes were collected from the testing room into one of two cups labelled 'inside' and 'outside' depending on whether they had been found inside or outside the bed net. Cups were assessed for 1hr knockdown and 24hr mortality.

4.2.5 Sample size

The sample size calculation for the primary outcome (proportion bloodfed) is based on a previous behavioural study by Randriamaherijaona *et al.* (2015) that investigated mosquito passage through holes in LLINs. The comprehensive reporting of explanatory power associated with each variable tested (pyrethroid or untreated, mosquito resistance status, hole area) allowed informed power analysis for the current study. While their study investigated only conventional pyrethroid nets (i.e. did not include PBO-LLINs), the effect size of LLIN type (insecticide vs non-insecticide) and mosquito resistance status (resistant vs susceptible) are informative for the current study. They report that net type and resistance status together explain a total of 34% of variation in the data, rising to 92% with the inclusion of hole size (in cm²). However, as their study included holes sizes much larger than the proposed current study, I have chosen to be conservative in these estimates by using the lower value of 34% in this sample size calculations.

The R package ‘pwr’ to calculate the necessary sample size. Using the approaches developed by Cohen (1998) the method took an assumed effective size for hole size (here **0.34**) and calculated the total number of samples needed to differentiate between treatment groups (assuming a balanced experimental design). The number of treatment groups is specified here by the number of ‘degrees of freedom’ (effectively the number of parameters in the model that can vary). Here the total number of degrees of freedom in the model is four:

$$1 \text{ (for net type)} + 2 \text{ (for hole location)} + 1 \text{ for the model intercept} = 4$$

As is convention, I aimed for a type 1 error probability (significance level) of **0.05** and a type 2 error probability of **0.2** (equivalent to 80% power).

Effect size: 0.34

Degrees of freedom: 4

Type I error prob: 0.05

Type II error prob: 0.20

The output of this power calculation indicated the total samples needed (assuming a fully balanced experimental design) was 36, allowing six observations of all pairwise net type and hole location combinations (**Table 4.2**).

Table 4.2. Number of observations performed for each free-flying experiment.

Outcome	Damage category	Olyset Net (n)	Olyset Plus (n)
Hole entry assay	No holes	6	6
	15cm diameter hole in side panel	6	6
	15cm diameter hole in top panel	6	6
Hole exit assay	15cm diameter hole in side panel	-	5
	15cm diameter hole in top panel	-	5

4.2.6 Data analysis

Data analyses were conducted using R (version 3.6.0), all graphs were produced using the ggplot2 package (version 3.2.1). Associations between outcomes and variables of interest were quantified using Generalized Linear Models (GLMs) using the 'stats' package (version 4.3.0). The model selection process used stepwise regression, working backwards from a maximally complex model to produce the most parsimonious fit. Variables that did not significantly increase explanatory power, as indicated by log-likelihood ratio tests (LRTs)('lmtest' package version 0.9-37), were excluded from the final model. The p values reported are the output of these LRTs. Pairwise comparisons between levels within a categorical variable were performed using least square means with the 'lsmeans' package (version 2.30-0).

4.2.7 Participant recruitment

A total of four human participants were included in this study. The allocation of participants to each replicate of the free-flying bloodfeeding assay is shown in **Table 4.3**. I was the sleeper on days in which no participants were available thus am overrepresented in the study (participant D).

Table 4.3 Allocation of participants to arms of the free-flying bloodfeeding assay. Each letter (A-D) represents a unique participant.

Olyset Plus						
Replicate	1	2	3	4	5	6
No hole	A	B	C	D	D	D
Side hole	A	B	C	D	D	D
Top hole	A	B	C	D	D	D
Olyset Net						
Replicate	1	2	3	4	5	6
No hole	A	B	D	D	D	D
Side hole	A	B	D	D	D	D
Top hole	A	B	D	D	D	D

4.2.7 Measures of protective effect

Population personal protection, denoted as $\bar{\phi}_c$, is the average bloodfeeding success across a community when a given proportion of the human population is protected by a bed net. It is calculated using the following equation, as per the methods of Briet *et al.*, 2012:

$$\bar{\phi}_c = \frac{c(1-\delta)(1-\rho_t)\bar{\phi}_t + (1-c)(1-\rho_o)\bar{\phi}_o}{c(1-\delta)(1-\rho_t) + (1-c)(1-\rho_o)}$$

However, as deterrence from entering households (δ) is not a variable here this can be simplified to:

$$\bar{\phi}_c = \frac{c(1-\rho_t)\bar{\phi}_t + (1-c)(1-\rho_o)\bar{\phi}_o}{c(1-\rho_t) + (1-c)(1-\rho_o)}$$

Where:

c = coverage

ρ_o = proportion of females prevented from feeding in the control arm

ρ_t = proportion of females prevented from feeding in the treatment arm

$\bar{\phi}_o$ = proportion of mosquitoes that fed in the control

$\bar{\phi}_t$ = proportion of mosquitoes that fed in the treatment

4.2.7 Ethical considerations and Research Ethics Committee approval

It was understood from the outset that participants may be bitten by mosquitoes in the course of this study. Typically, mosquito bites result in only minor discomfort and itching yet in rare cases some individuals may exhibit a more pronounced response, including severe swelling at the site of the bite and more serious allergic reactions. To minimise the risk of a severe reaction to mosquito bites occurring to participants in this study, selection criteria was devised to include only individuals which were at low risk. In this study, only individuals that had previously worked in an insectary and are approved by LSTM to arm-feed mosquitoes were considered for participation (as such individuals are routinely bitten in the course of their work thus will be aware of their body's response to a bite). Additionally, participants may suffer minor discomfort due to the hot and humid environment of the room. However, this too is mitigated by only including individuals familiar with working in insectary conditions and limiting the time inside to 60 minutes.

This study of mosquito behaviour around human baited LLINs was approved by the Liverpool School of Tropical Medicine Research & Ethics Committee (**LSTM Ref 21-065, Appendix II**), which is the sponsoring institute. The full ethics documentation for this study can be found in **Appendix II**.

Potential participants were approached for involvement in the study via email (using language approved by the LSTM REC, **Appendix II**). If a potential participants expressed interest in taking part in this study they were asked to consider an information sheet, and return a consent form signed if they wished to confirm themselves as a participant (**Appendix II**). Care was taken to avoid any potential participants feeling pressured to volunteer, as made clear in the consent form. Potential participants were made aware that being bitten by a mosquito

during the course of the study is likely and made aware of the potential side effects associated with mosquito bites. Participants were asked to return for multiple testing days. Specifically, participants were asked to volunteer on three occasions: one session for each of the three damage patterns (top hole, side hole, fully intact) for a given net design. However, if a volunteer was willing to participate in more than three sessions, the additional sessions were included in a different arm.

No human participants were involved in the investigation of mosquitos exit behaviour from holed LLINs thus REC approval was not sought.

4.3 Results

4.3.1 Benchtop bioefficacy outcomes

4.3.1.1 WHO wireball assay

In the WHO wireball assay, there was a large difference in one hour knockdown between LLIN Products ($p < 0.001$, **Figure 4.2A**), with Olyset Net knockdown down only 9.75% (95% CI: 2.46-16.67) yet Olyset Plus knocking down 86.46% (95%CI: 79.36-93.57). A similar difference between LLIN Products seen for adjusted 24 hour mortality ($p < 0.001$, **Figure 4.2B**), with 3.19% killed by Olyset Net (95% CI: 0-13.97) and 66.23% killed by Olyset Plus (95% CI: 55.44-77.00).

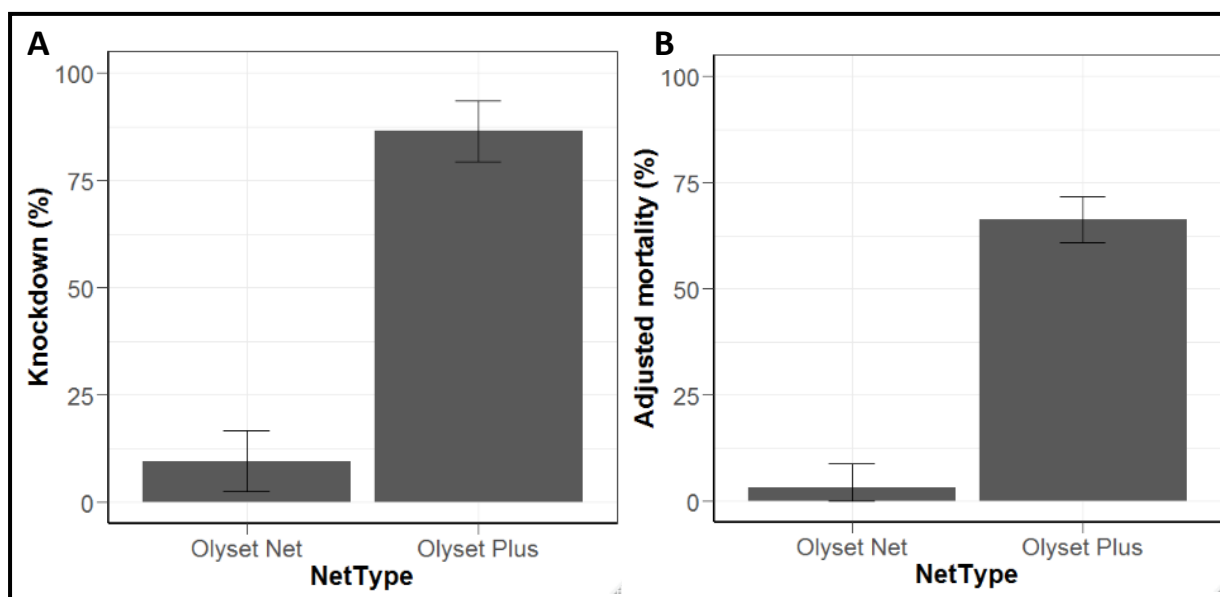


Figure 4.2. Bioefficacy of Olyset Net and Olyset Plus in WHO wireball bioassay. **(A)** 1hr knockdown **(B)** 24hr Mortality. Errors bars indicate 95% confidence intervals.

4.3.1.2 Arm-feeding assay

Only a minority of mosquitoes were able to successfully feed through netting in assays with Olyset Net and with Olyset Plus (**Figure 4.3A**). There was no difference in bloodfeeding success between Olyset Net and Olyset Plus ($p=0.562$). The raw mean bloodfeeding success was higher for both Olyset Net and Olyset Plus than the untreated net however, the only pairwise comparison that was statistically significant was that between Olyset Plus and the untreated net ($p=0.021$), with 38.93% of mosquitoes able to feed directly through Olyset Plus (95% CI: 30.58-47.28).

The proportion of mosquitoes knocked down after the arm-feeding assay was very low, with zero knockdown for the untreated net and Olyset Net (**Figure 4.3B**). Mean 1hr knockdown for Olyset Plus was 4.61% (95% CI: 0-13.53) however, there was no difference in knockdown between Olyset Net and Olyset Plus ($p=0.999$). Mortality for the arm-feeding assay after 24 hours was also very low (**Figure 4.3C**), with zero mosquitoes dead after exposure to the untreated net and Olyset Net. Mean 24hr mortality for Olyset Plus in the arm feeding assay was 9.09% (95% CI: 3.09-15.09), significantly higher than that of Olyset Net ($p=0.042$).

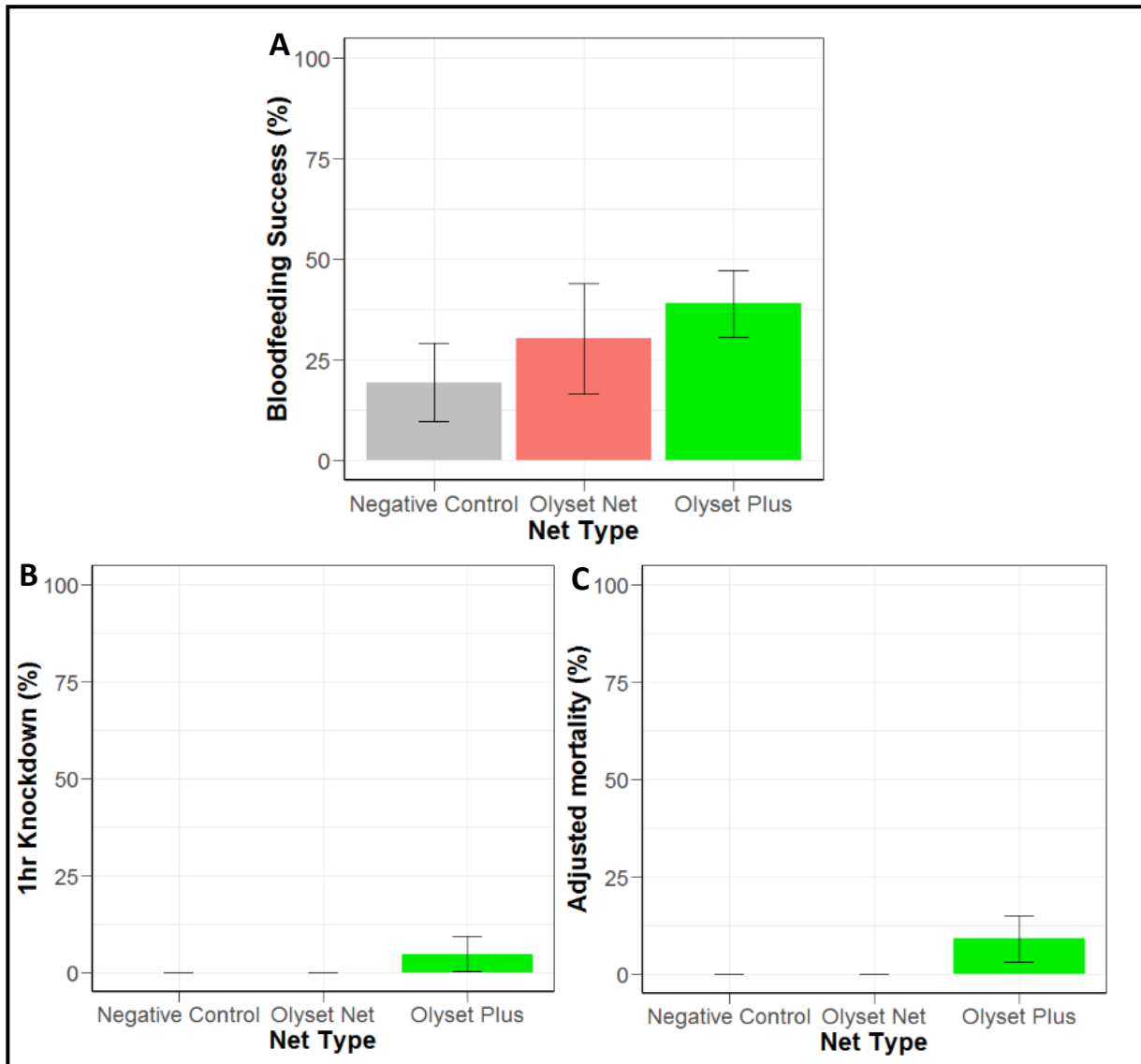


Figure 4.3. Outcomes associated with Olyset Net and Olyset Plus in benchtop arm-feeding assay. **(A)** Bloodfeeding success **(B)** 1hr knockdown **(C)** 24hr Mortality. Errors bars indicate 95% confidence intervals.

4.3.2 Personal protection

4.3.2.1 Bloodfeeding success

There was no difference in bloodfeeding success of free-flying pyrethroid-resistant *An. gambiae* mosquitoes around holed nets between Olyset Net and Olyset Plus, for any of the hole positions assessed (**Table 4.4**)

Table 4.4. Odds Ratio of bloodfeeding success between LLIN Products for each hole position assessed.

Comparison (OlysetPlus/OlysetNet)	Odds Ratio	95% CI	P value
Top	1.61	0.76-2.46	0.076
Side	0.62	0-1.38	0.446
None	1	NA	1.000

It was observed that bloodfeeding success varied by hole position for both LLIN products tested (**Figure 4.4**), with mosquitoes more than four times more likely to obtain a bloodmeal if there was a hole in the top than a hole in the side (**Table 4.5**). There was no difference in bloodfeeding success between a hole in the side and no hole for either Olyset Net ($p=0.999$) or Olyset Plus ($p=0.999$).

Table 4.5. Odds ratio of bloodfeeding success between hole positions for each LLIN product assessed.

Net Type	Pairwise comparison	Odds Ratio	95% CI	P value
Olyset Net	Top/Side	4.15	2.04-6.26	<0.001
Olyset Plus	Top/Side	10.71	5.95-15.47	<0.001

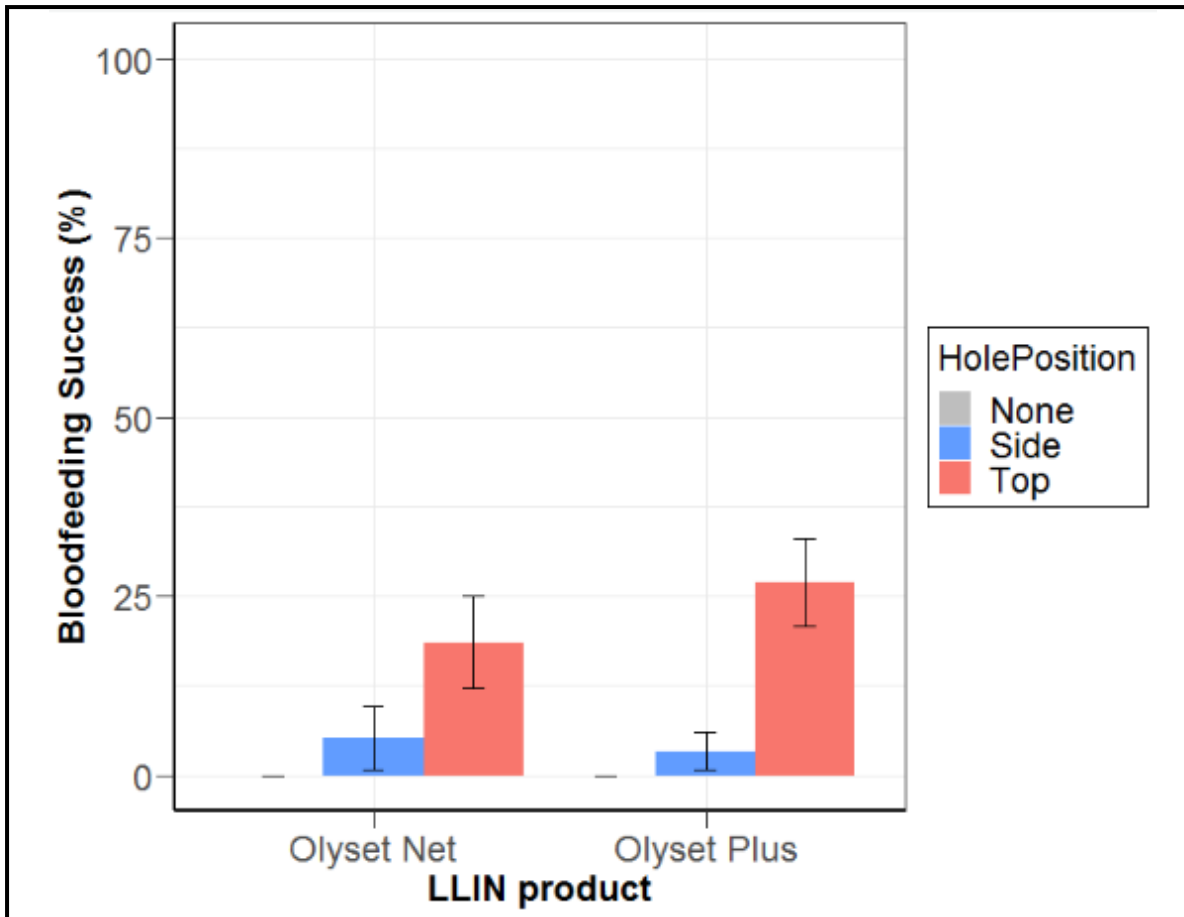


Figure 4.4. Bloodfeeding success with each combination of LLIN Product (Olyset Net and Olyset Plus) and Hole Position (None, Side, Top). All holes were circles 15cm in diameter. Error bars indicate 95% CIs.

4.3.2.2 Estimated impact of hole occurrence on population protective efficacy

To demonstrate the relative impact of top holes and side holes on personal protection, using the observed bloodfeeding success of pyrethroid-resistant *An. gambiae* mosquitoes described above, the notional population protection under different frequencies of net damage was calculated for Olyset Plus (Briet *et al.*, 2012). As no mosquitoes were able to successfully feed on an occupant under Olyset Plus in free-flying assays when no holes were in the net, predicted population protection at full coverage was calculated to be 1 (complete protection). Therefore, estimated population protection at a more reasonable estimate of 80% coverage, when all nets were fully intact, was 0.8 (**Figure 4.5**). The change in population protection as more nets develop a hole in either their top or side is shown along the x axis. A key output from this model is that the increasing frequency of nets with a large (15cm²) hole in the side is predicted to have a minimal effect on population protection, with the total proportion of bites prevented decreasing to only 0.78 when 50% of nets had such a hole. The increasing frequency of nets with a hole in the top is predicted to have a larger impact on population protection, with the total proportion of bites prevented declining to 0.69 when 50% of nets has a hole in the top.

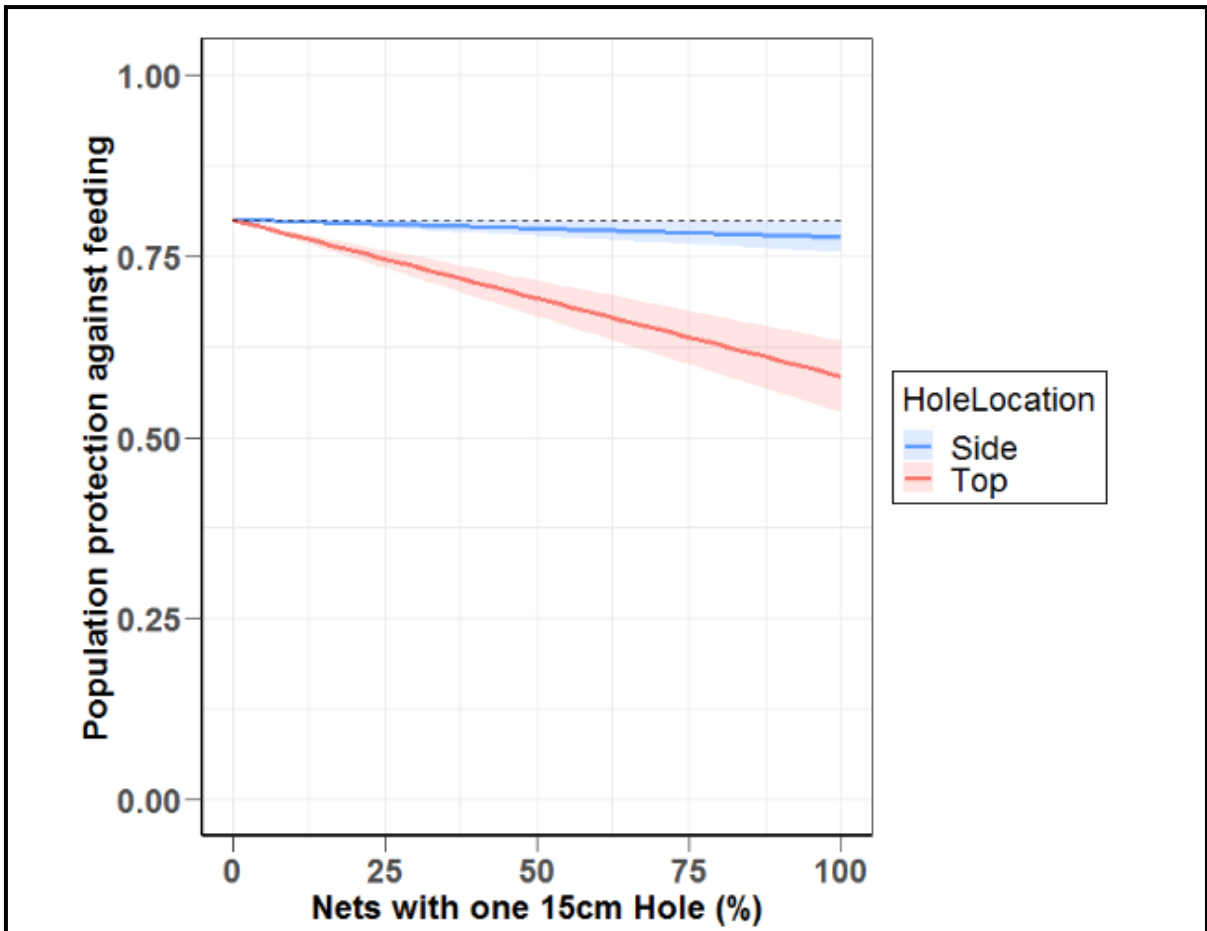


Figure 4.5. Predicted population personal protection against bloodfeeding of LLINs with a 15cm diameter hole in the top of side. Coverage of LLINs is assumed to be 80% (dashed horizontal line represents population protection when nets are fully intact).

4.3.2.3 Results in context: Predicted personal protection in Uganda

To place the findings of this chapter in a practical context, the bloodfeeding inhibition estimates were applied to baseline entomological data from the site of the PBO LLIN durability trial in Uganda, collected previously by Lynd *et al.* (2019). By combining mosquito density data from collections with sporozoite rates, I was able to estimate the number of infectious bites a person in that setting would be expected to receive each year had the slept under the LLINs evaluated here. These estimates represent only the direct personal protection of the net's occupants. The baseline mosquito density and sporozoite rates reported by Lynd *et al.* (2019) are presented in **Table 4.6**, alongside infected bites per year calculated here from these values. However, as shown in **Figure 4.6**, the large variation in nightly biting density obscured any practical difference between hole position and LLIN product.

Table 4.6 mean *An. gambiae* indoor biting density and *Plasmodium* sporozoite rate across regions at baseline in the Uganda PBO trial (values from Lynd *et al.* 2019).

Region	Nightly biting density (95% CI)	Sporozoite rate	Infected bites per year (95% CI)
Mid-Eastern	8.57 (2.34-30.11)	0.043	134.51 (36.72-472.57)
North-Eastern	0.42 (0.09-2.22)	0.043	6.59 (1.42-34.84)
Mid-Western	1.02 (0.25-4.29)	0.043	16.00 (3.92-67.33)
East-Central	7.28 (1.54-33.16)	0.043	114.25 (24.17-520.46)

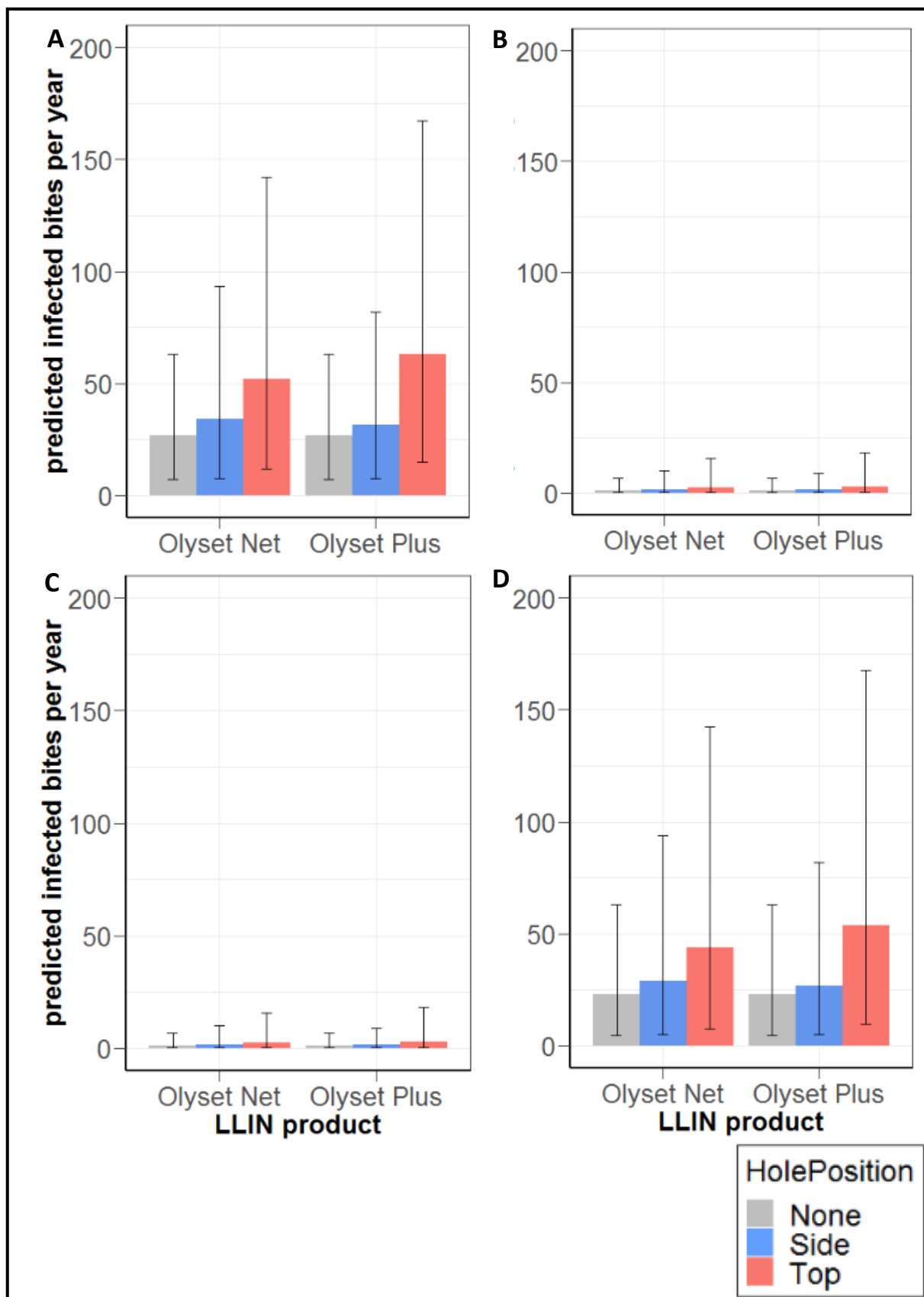


Figure 4.6. Predicted number of infected bites an occupant would receive per year in each geographic context if they were to sleep under an LLIN assessed in this study. **(A)** Mid-Eastern region, **(B)** North-Eastern Region, **(C)** Mid-Western Region, **(D)** East-central Region.

4.3.3 Bioefficacy

4.3.3.1 1hr Knockdown and 24 hr Mortality

Hole position made no statistical contribution to the model of 1hr knockdown ($df=2$, $\chi^2=1.187$, $p=0.552$), indicating that there was no difference in the probability of a free-flying pyrethroid-resistant *An. gambiae* being knocked down after exposure to a net with no hole, side hole, or top hole. The lack of a significant interaction between Hole Position and Net Type ($df=2$, $\chi^2=0.0618$, $p=0.969$) indicated that indifference to hole position was the case for both Olyset Plus and Olyset Net. Knockdown with Olyset Plus against free-flying pyrethroid-resistant *An. gambiae* was superior to Olyset Net across all hole positions assessed (**Table 4.7**), with Olyset Plus knocking down 37.88% (95% CI: 30.78-44.98) compared to 11.80% (95% CI: 8.27- 15.23) for Olyset Net (**Figure 4.7A**).

Table 4.7 Odds Ratio of one hour knockdown between LLIN products across all hole positions.

Comparison	Odds Ratio	95% CI	p value
OlysetPlus/OlysetNet	4.65	2.91-6.38	<0.001

Hole position did not contribute to the model of 24 hour mortality ($df=2$, $\chi^2=1.997$, $p=0.101$), indicating that there was no difference in the probability of a mosquito being killed between a net with no hole, a side hole, or a top hole. There was no significant interaction between Hole Position and Net Type ($df=2$, $\chi^2=1.99$, $p=0.368$). Mortality with Olyset Plus against free-flying pyrethroid-resistant *An. gambiae* was superior to that of Olyset Net for all hole positions assessed (**Table 4.8**), killing 36.40% (95% CI: 29.28-43.52) and 8.68% (95% CI: 2.54-14.81) respectively (**Figure 4.7B**).

Table 4.8 Odds Ratio of 24 hour mortality between LLIN products

Comparison	Odds Ratio	95% CI	p value
OlysetPlus/OlysetNet	6.02	3.45-8.58	<0.001

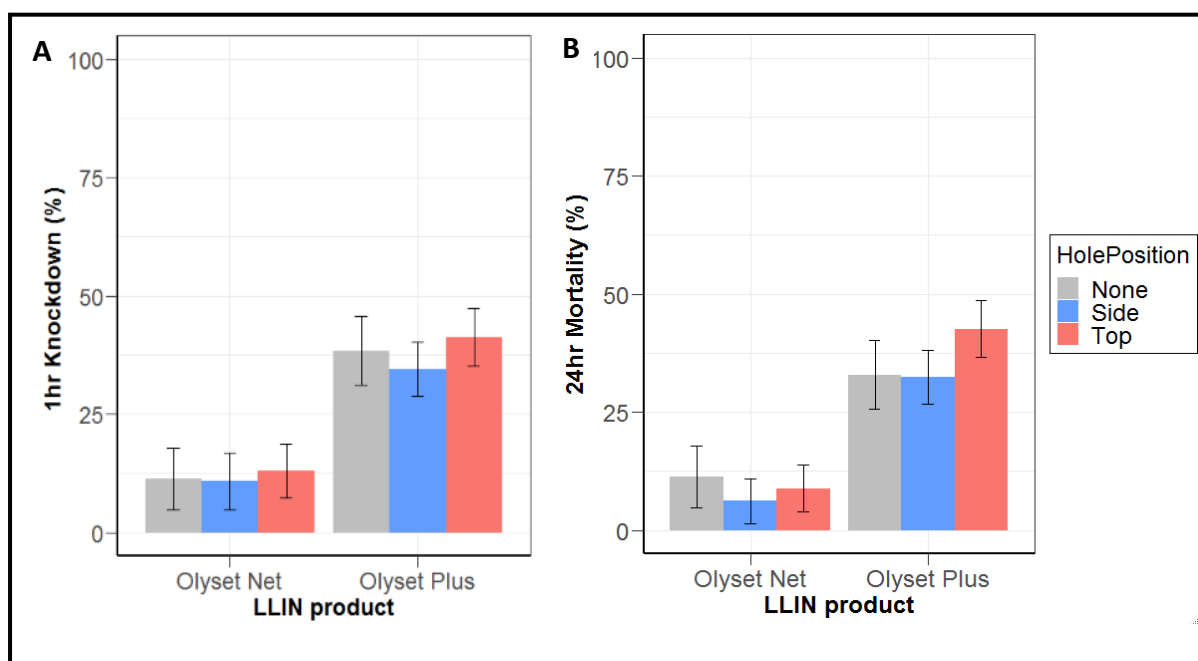


Figure 4.7 (A) 1hr knockdown and **(B)** 24hr mortality with each combination of LLIN Product (Olyset Net and Olyset Plus) and Hole Position (None, Side, Top). All holes were circles 15cm in diameter. Error bars indicate 95% CIs.

4.3.3.2 Proportion of mosquitoes that both bloodfed and survived

Both Net Type (df=1, $\chi^2=24.836$, $p < 0.001$) and Hole Position (df=2, $\chi^2=18.238$, $p < 0.001$) were significant predictors of the probability that a mosquito would be able to both bloodfeed and survive the assay. No mosquitoes were able to both bloodfeed and survive in assays with no holes, as none were able to bloodfeed.

The pyrethroid resistant mosquitoes' chances of bloodfeeding and surviving were lower with Olyset Plus than with Olyset Net (**Table 4.9**). Additionally, a higher proportion of mosquitoes were able to successfully bloodfeed and survive if the net had a hole in the top compared to a hole in the side (**Table 4.10**). However, inhibition of mosquitoes successfully bloodfeeding and surviving was high for all combinations of LLIN Product and Hole Position with even the worst performing group, Olyset Net with a hole in the top, preventing 83.95% of occurrences (**Figure 4.8**).

Table 4.9. Odds Ratio of bloodfeeding and surviving. Comparison of LLIN products

Comparison	Odds Ratio	95% CI	p value
OlysetPlus/OlysetNet	0.13	0.06-0.21	0.001

Table 4.10. Odds Ratio of bloodfeeding and surviving. Comparison of hole position

Comparison	Odds Ratio	95% CI	p value
Top/Side	5.99	2.71-9.27	0.001

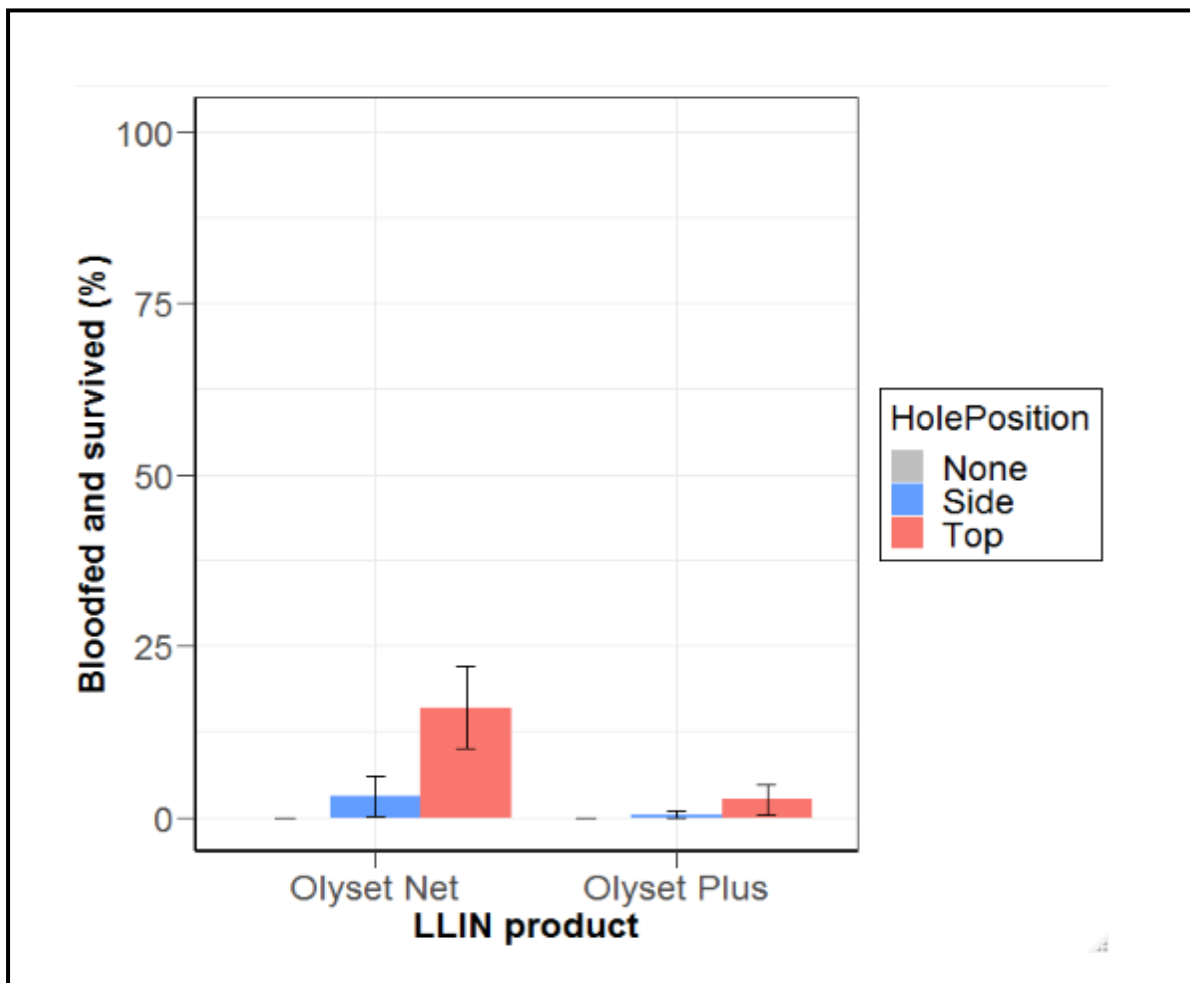


Figure 4.8. Percentage of mosquitoes that both bloodfed and survived for each LLIN product with a hole in each position. All holes were circles 15cm in diameter. Error bars indicate 95% CIs.

4.3.3.3 Ability of bloodfed mosquitoes to exit nets and survive

Mosquitoes had a greater chance of escaping to the outside of Olyset Plus by the end of the five hour exit assay if the net had a hole in the top than in the side (**Table 4.11**). When there was a hole in the top of the net, 62.94% (95% CI: 54.17-71.71) were found outside the net compared to 33.25% (95% CI: 19.14-47.36) when there was a hole in the side (**Figure 4.9**).

Table 4.11. Odds Ratio of a mosquito being both bloodfed and found outside the net at the end of the exit assay, comparison of top and side holes.

Comparison	Odds Ratio	95% CI	p value
Top/Side	1.88	1.37-2.39	0.021

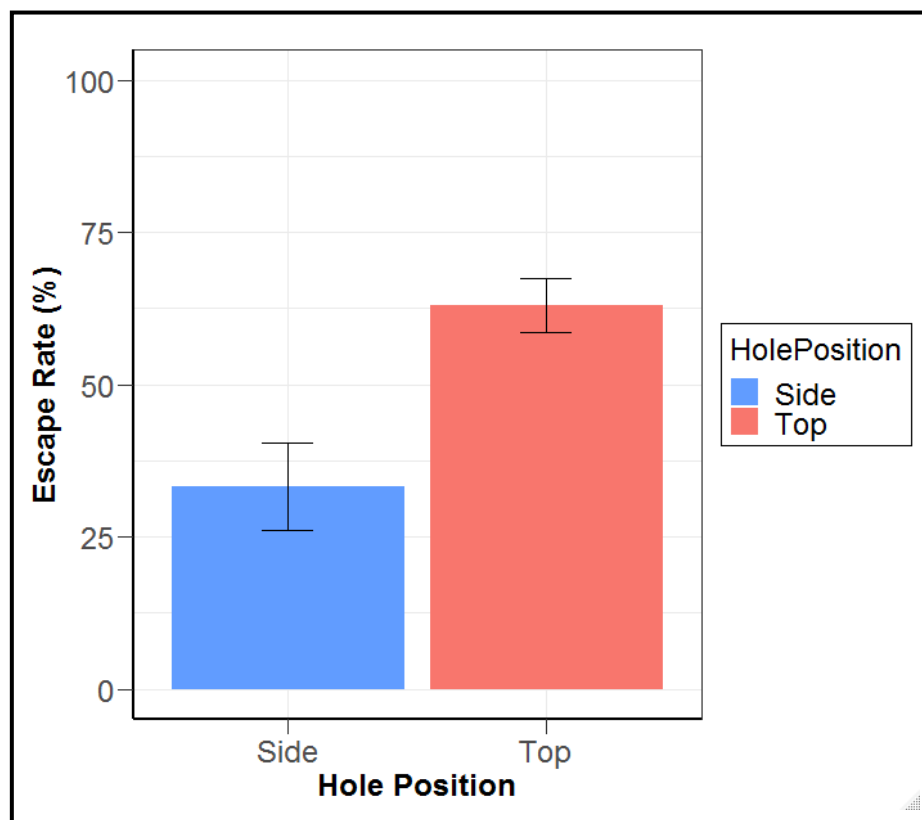


Figure 4.9. Mean percentage of mosquitoes found outside net the at the end of the exit assay (escape rate) for Olyset Plus with a 15cm diameter circular hole in the side or top. All mosquitoes blood-fed immediately prior to release inside the net. Error bars indicate 95% confidence intervals.

Bioefficacy outcomes for Olyset Plus against blood-fed mosquitoes were very high. There was no difference in 1hr knockdown between the net with the top hole and the net with a side hole (**Table 4.12**). Overall, 1hr knockdown for blood-fed mosquitoes released inside nets, for top and side holes combined, was 94.68% (95% CI: 77.13-100). Similarly, there was no difference in 24hr mortality between the net with the top hole and the net with a side hole (**Table 4.13**). Overall, 24 hour mortality for blood-fed mosquitoes released inside nets, for top and side holes combined, was 94.1% (95% CI: 76.67-100) .

Table 4.12. Odds Ratio of one hour knockdown of mosquitoes in the exit assay, comparison of top and side holes.

Comparison	Odds Ratio	95% CI	p value
Top/Side	1.02	0.80-1.23	0.914

Table 4.13. Odds Ratio of 24hr mortality of mosquitoes in the exit assay, comparison of top and side holes.

Comparison	Odds Ratio	95% CI	p value
Top/Side	1.01	0.79-1.22	0.914

4.4 Discussion

The implications of physical damage to a bed net for mosquito entry and subsequent survival is poorly described, with a particular dearth of literature for next-generation designs. Current WHO guidance for assessing the serviceability of a damaged net assumes holes on the top or side of the net are of equal importance, despite mounting evidence from video-based analysis that host-seeking *An. gambiae* activity is concentrated on the top of the net (WHO, 2013b; Parker et al. 2015; Sutcliffe et al. 2015; Gleave et al. 2022). Here, these findings indicate that hole location (top or side) is a strong predictor of *An. gambiae* bloodfeeding success, with mosquitoes predicted to be ten times more likely to bloodfeed if the hole is on the top of the PBO LLIN Olyset Plus compared to a similarly sized hole on the side. The percentage of mosquitoes bloodfed when a 15cm diameter circular hole was in the top of the PBO LLIN was 26.8% while the percentage when the hole was on the side was indistinguishable from zero. The ratio of bloodfeeding success between the top and the side was less extreme for the pyrethroid-only Olyset net at 4:1, with slightly fewer mosquitoes entering the top and slightly more entering through the side compared to the PBO-LLIN.

These findings indicate hole location is of great importance for the personal protection of an LLIN, with a top hole a high entry risk and an equivalently sized hole a low risk. This is consistent with previous video-based experiments with human-baited untreated nets conducted by Sutcliffe *et al.* (2017), which observed mosquitoes were more likely to encounter and pass through holes on the top compared to the sides. However, an important context of this disparity, reported in Chapter Three, is that side holes occur far more frequently than top holes (ratio of approximately 10:1 by total hole area). Thus, in practice side holes may still play an important role in providing access to host-seeking mosquitoes due

to the high frequency at which they occur. Nonetheless, these findings indicate that lack of consideration of hole location in the current WHO durability guidelines is not appropriate and that top holes should be weighted appropriately. Here, an appropriate risk-weighting would be 10:1 for top and side holes respectively however there is a need for future work to assess the reproducibility of these findings.

Despite the increased bioefficacy of Olyset Plus over Olyset Net in terms of 1hr knockdown and 24hr mortality, there was no statistical difference in mean bloodfeeding success between Olyset Plus and Olyset Net for any hole location. This observation indicates that improved bioefficacy of Olyset Plus did not improve the personal protective effect of the net against pyrethroid-resistant mosquitoes. This minimal association between bloodfeeding inhibition from bioefficacy is consistent with Randriamaherijaona *et al.* (2015) which observed no difference in the bloodfeeding success of pyrethroid-susceptible *An. gambiae* between holed pyrethroid and untreated nets. However, bioefficacy did have a profound impact on the survival of bloodfed mosquitoes, with 96.1% of all bloodfed mosquitoes dying. Consequently, while there was no difference in personal protection between the PBO-pyrethroid net and the pyrethroid-only net, the extremely high mortality of bloodfed mosquitoes with the addition of PBO would be expected to have important implications for preventing onwards transmission (Levitz *et al.* 2018; Unwin *et al.* 2022).

The benchtop assays broadly indicated trends in bioefficacy and bloodfeeding that were later reflected in behavioural assays. With 1hr knockdown and 24 mortality greatly elevated for Olyset Plus compared to Olyset Net under both benchtop and free-flying conditions. Furthermore, there was no difference in bloodfeeding success on the benchtop between Olyset Plus and Olyset Net, as was also later observed in free-flying assays. Neither Olyset Net

or Olyset Plus had any discernible insecticidal activity in the benchtop bloodfeeding assay, indicating this *An. gambiae* strain was able to feed directly through the gaps in the netting without consequence. However, it should be noted that bloodfeeding success in this benchtop assay was relatively low with only approximately a quarter of mosquito's blood-fed, though this may be due to the short three minute duration of the assay. This lack of insecticidal effect when feeding directly through either Olyset Plus or Olyset Net is consistent with previous benchtop assays using pyrethroid-resistant *An. gambiae* (Hauser et al. 2019).

4.4.1 Limitations

There are a number of limitations that should be considered when interpreting the results of this investigation. Firstly, the mosquitoes assessed in this study were released into the testing room in close proximity to the net whereas in a field setting they would have to actively enter the household structure from outside. Previous behavioural studies have observed that pyrethroid LLINs deter a greater proportion of *Anopheles* mosquitoes from entering compared to untreated nets however experimental hut trials in Cameroon observed similar deterrence rates for *An. funestus* between Olyset net and Olyset Plus (54.9% vs 49.0% respectively) (Menze et al. 2020). Another potential limitation is that outcomes were assessed for only a single hole size (15cm diameter) rather than across a range of plausible values. Consequently, it may be the case that the ratio of bloodfeeding success for top and side holes varies with hole size. A further potential limitation is that the specific mechanisms of pyrethroid resistance varies between strain, meaning bloodfeeding and bioefficacy outcomes observed here may not be universally applicable for all *An. gambiae* mosquitoes. However, in a concurrent study of *An. gambiae* behaviour around intact LLINs we observed no statistical differences in number of contacts or total contact time between two pyrethroid-resistant *An. gambiae* strains for both Olyset Net and PermaNet3.0 (Gleave *et al.* in submission). Additionally, it was observed that activity of pyrethroid resistant mosquitoes was relatively consistent throughout the testing window and confirmed that host-seeking activity occurs primarily on the top of the net for all *An. gambiae* strains tested. Additionally, while this study assessed only a side hole in the centre of the side panel there is evidence from video analysis using adhesive nets that mosquito activity is 10x higher in the upper 2/3rd of the side panel than the area below closest to the ground, though both areas have very low activity relative to the top panel (Sutcliffe et al. 2015). Consequently, hole encounter would be expected to

be even lower if the side hole had been further down on the net. Finally, post 24 hour mortality was not assessed in the current study, meaning delayed mortality effects or impacts on longevity could not be detected. However, as the mortality of bloodfed mosquitoes was extremely high (>95%) there would have been few live bloodfed mosquitoes to observe past 24 hours.

The impact of the shape on the net on mosquito bloodfeeding was not assessed in this experiment. While the net was kept as taught as possible to form a rectangular shape, in operational conditions it may drape down over the sleeping space in irregular shapes. Consequently, the flat roof assessed here may be somewhat artificial. In practice, a perfectly circular hole in the roof of the net may have a smaller cross section (as viewed from above) when allowed to droop down, presenting a smaller gap for mosquito entry.

4.4.2 Future work

Given the current assumption of the WHO physical assessment guidelines that all holes count equally in terms of assessing serviceability, future work should build on the current study to determine the appropriate weighting for holes on the top of the net relative to holes on the side. Future studies should aim to assess the ratio of bloodfeeding success of top to side holes for both other LLIN products and pyrethroid-resistant *An. gambiae* strains, in order to determine if the ratios observed here are consistent.

Future studies may wish to investigate the delayed mortality of bloodfed *An. gambiae* after encountering a damaged LLIN. Here, few bloodfed mosquitoes survived past 24 hours however this may not be the case with other LLIN products and requires assessment. Specifically, given the success of the next-generation LLIN Interceptor G2 (which contains the pyrethroid alpha-cypermethrin and the pyrrole slow-acting pyrrole insecticide chlorfenapyr) in field trials, there is a need to assess the bloodfeeding success and delayed mortality of pyrethroid-resistant *An. gambiae* mosquitoes with this net. Additionally, given only one hole size was assessed here, future studies may chose to investigated bloodfeeding and bioefficacy across different hole sizes.

Chapter Five: General Discussion

5.1 summary of key findings

The emergence of pyrethroid-resistance throughout sub-Saharan Africa has incentivised the development of novel bed net designs with chemistries that are intended to be effective against these mosquitoes. This study successfully met all of its aims and objectives in understanding how the efficacy of bed nets containing both a pyrethroid and PBO changes with operational use. Prior to this study, the change in bioefficacy of pyrethroid LLINs containing PBO against pyrethroid-resistant mosquitoes over time was not well described. Here I reported that despite being highly effective against such mosquitoes when new, the killing effect of Olyset Plus and PermaNet 3.0 declines with operational use, with approximately 50% surviving a three-minute exposure in benchtop assays after two years. The knockdown effect of Olyset Plus similarly declined over the same period, though that of PermaNet 3.0 remained high throughout. In Chapter Four, I report that the physical condition of these PBO-pyrethroid nets is similar to that of equivalent pyrethroid-only designs across time, confirming that the incorporation of PBO into these new designs did not adversely impact their physical integrity. Additionally, I report that the damage on these nets is concentrated on the side panels, with little damage on the top. In the final research chapter, I observed that free-flying pyrethroid-resistant *An. gambiae* were far more likely to enter and bloodfeed through a hole in the top of a net than in the side and that blood-fed mosquitoes had an extremely high risk of death when attempting to escape after bloodfeeding.

5.2 Implication for the evaluation and deployment of LLINs

The observation that pyrethroid nets supplemented with PBO were far more effective against pyrethroid-only designs against pyrethroid-resistant *An. gambiae* mosquitoes is consistent with a growing body of literature that PBO supplemented designs result in a 20-30% reduction in *Plasmodium* prevalence in children under the age of ten compared to standard nets (Staedke et al. 2020, Gleave et al. 2021). Additionally, the observation that the bioefficacy of PBO nets decreased with operational use is consistent with the narrowing gap between the protective effect of PBO and standard LLIN products observed over the same period in the concurrent trial of malaria outcomes (Staedke et al. 2020, Gleave et al. 2021).

Current WHO guidance to national malaria control programmes is to distribute LLINs at 3-5 year intervals. While there was only limited evidence of even conventional pyrethroid-only designs achieving this lifespan the evidence for LLIN designs supplemented with PBO is even less convincing. Throughout this study, the term 'PBO LLIN' has been used to describe pyrethroid nets supplemented with PBO, in line with wider documentations on the Uganda LLIN evaluation project. However, I argue here that the use of 'long lasting' here is inappropriate as it implies a property that has not been evidenced and may mislead decision makers to deploy them at 3-5 year intervals. The rapid reduction in PBO content in nets and the corresponding decline in bioefficacy after just two years (to less than 50% of their baseline values) indicates that, within the context of Uganda, they should be replaced at intervals shorter than three-five years. These findings are supported by a similar investigation in Kenya, observing that Olyset Plus PBO LLINs had lost 81% of PBO after two years (Gichuki et al. 2021). However, as the same study only assessed bioefficacy against pyrethroid-susceptible *An. gambiae*, their report that Olyset Plus 'passed' bioefficacy criteria at all timepoints is uninformative in an era of widespread of pyrethroid-resistance. Additionally, a

pre-print by Lukole et al. (in review) reports that after three years Olyset Plus nets sampled from Tanzania had lost 97% of their total PBO content compared to baseline. There is an urgent need for data from other settings to assess if this reduction in PBO content is consistently observed.

While there is currently no WHO requirement or guidance for testing against pyrethroid-resistant *An. gambiae*, this data is essential to informing decision makers. In the interim, subsequent studies should take the initiative to identify appropriate site-specific pyrethroid-resistant strains to test PBO-pyrethroid nets against. Peer-reviewers of future durability studies should be willing to make this a requirement for publication, as I myself have done in the past. An assessment of the bioefficacy of PBO-pyrethroid nets without a pyrethroid-resistant strain is simply not complete. Consequently, there is a clear and pressing need for a unified WHO guidance on assessing the bioefficacy of PBO-pyrethroid nets against pyrethroid-resistant *An. gambiae*.

A complication in assessing the performance of net designs with both a pyrethroid and PBO is the appropriate resistant strain to test against. While there is a strong argument that a site-specific strain is preferable as it provides insight into bioefficacy in that context, the counter argument is that this means studies in different settings cannot be directly compared as strains differ substantially in their resistance mechanisms. The alternative is that a small number of testing centres are identified by the WHO and net samples sent to these institutions for evaluation against a standardised pyrethroid-resistant strain.

The observation that building construction was a strong indicator of physical integrity has implications for LLIN distribution. That nets sampled from traditional thatched-roof houses were more likely to be damaged aligns with a concurrent study by Rugnao *et al* (2019) that

children living in such households were 15% more likely to test positive for *Plasmodium* infection (Rugnao et al. 2019). While LLINs are typically distributed nationally in 3-5 year cycles (with the important exception of programmes that distribute nets to pregnant women), there is an argument to be made for identifying regions and communities for which the operational lifespan of nets is lowest.

This study observed that WHO cone bioassays were a poor predictor of the performance of Olyset Plus against this pyrethroid-resistant strain. A possible explanation for this is the repellent properties of permethrin, which discourage contact, combined with the large surface area of insecticide-free surface inside the cone. These findings were consistent with early assessments of Olyset Net against a pyrethroid susceptible strain (Lindblade et al. 2005, Okumu et al. 2012). In this study, the WHO wireball greatly enhanced the observed bioefficacy of Olyset Plus samples assessed, indicating that mosquitoes tested were picking up a large dose of insecticide. The current WHO guidelines are to use the WHO tunnel test if a net performs poorly in the WHO cone bioassay, yet this is not ethically feasible in many institutions. Consequently, I argue here that the WHO wireball assay may be readily applied as an alternative to evaluate the bioefficacy of net samples in longitudinal durability assessments.

WHO Cone bioassays, the most commonly used laboratory method of assessing LLIN performance, was found to greatly underestimate the performance of the permethrin LLINs Olyset net and Olyset Plus against the pyrethroid-resistant *An. gambiae* strain. Given the repellent effect of permethrin (Cockcroft, Cosgrove and Wood 1998), I speculate that this disparity was due to the mosquitoes avoiding contact with the net in the WHO cone bioassay. These findings indicate that the large area of untreated surface in the cone assay (i.e. the

cone itself) makes it unsuitable for assessing the 1hr knockdown and 24 hr mortality of LLINs with repellent properties. This same point was raised in strong terms by Sutcliffe *et al.* (2005) in a letter to the editor of Tropical Medicine and International Health in response to early investigations of Olyset Nets performance in the field conducted by (Lindblade et al. 2005). This criticism would later form the basis of the requirement for the development of the WHO Tunnel Test and the guidelines specifying its use when sampled LLINs perform poorly in cone bioassays (<95 knockdown or <80% mortality against susceptible *An. gambiae*)(WHO, 2011; WHO, 2013). However, given that the WHO Tunnel test cannot be performed in many institutions due to animal welfare restrictions, a more accessible alternative is needed.

Here I demonstrate the benefits of the WHO wireball bioassay as an alternative to the WHO cone bioassay. The wireball provides the same insights as the cone into 1hr knockdown and 24hr mortality yet provides no insecticide-free surface for the mosquito to avoid contact. Consequently, the need for the logistically complex and ethically infeasible WHO tunnel test is reduced. While the tunnel test does provide some additional insight into mosquito repellence, the recent development of benchtop tools for assessing repellency, such as the 'baited-box assay' assess this directly without ethical concerns (Hughes et al. 2020). Given this, I recommend future investigations use the WHO wireball assay to assess the bioefficacy of LLIN products containing permethrin. However, before the WHO wireball can be used as a benchmarking tool, specific ambiguities in the existing guidelines must be addressed. At present there is no SOP for the WHO wireball, with only a single paragraph on the method in an early guidance document on assessing vector control tools (WHO, 2006). Subsequent studies vary in their use of a cube or sphere frame, resulting in large difference in internal volume. There is a need for a consensus SOP for the WHO wireball that clearly defines the apparatus and number of mosquitoes exposed in each assay.

5.3 Hole location and personal protection

This study is the first to explicitly measure bloodfeeding on human volunteers as a product of hole location. The finding that holes on the top of an LLIN result in a 4x-10x greater risk for bloodfeeding with *An. gambiae* compared to holes on the side is consistent with a large body of literature observing greater activity on the top of the net (Lynd and McCall 2013, Parker et al. 2015, Sutcliffe and Yin 2014, Sutcliffe and Colborn 2015, Sutcliffe, Ji and Yin 2017, Sutcliffe and Yin 2021). Furthermore, the model of population personal protection estimated that top holes had much larger implications than side holes, with the number of bites prevented only decreased by 2% when 50% of the net users having a 15cm hole in the side. When 50% of the population had an equivalent sized hole in the top personal protection decreased by 11%. Similar estimates of personal protection were made for both Olyset Net and Olyset Plus. These findings have important implications for assessing the serviceability of nets sampled from the field, indicating that the current assumption of the WHO guidelines that all holes are of equal importance regardless of where they are located should be reassessed. For these specific nets, the 15cm diameter hole puts them comfortably in the 'damaged' category of the pHI system, yet when the hole was in the side they still prevented >97% of bloodfeeding. However, these predictions are limited to personal protection, with the greater exit rate from top holes indicating that they may be more important for onwards transmission. As expected, no mosquitoes were able to bloodfed when there were no holes.

This investigation of damaged LLINs observed an interesting balance between two phenomena. Pyrethroid-resistant *An. gambiae* mosquitoes were ten times more likely to enter holes on the top of the PBO-LLIN Olyset Plus than on the side, yet total damage to the side was ten times greater by area than damage to the top. The key takeaway from these observations is that despite the higher entry risk for equally sized holes on the top of the net,

the sheer scale of damage to the side of the net means it is still of high importance. While holes on the top of individuals net should be weighted highly in assessing serviceability, a broader consideration of physical damage in LLIN design and distribution frequency should keep in mind that they are rare. Future studies of the physical integrity of LLINs would improve their insights by reporting the location of holes, perhaps using the 'functional areas' (top, upper 1/3rd, lower 2/3^{rds}) presented in this study. Furthermore, it should be noted that recording the location of holes has been included in the methodology for the LLIN durability assessment guidelines since 2013 but is not required for reporting as an outcome. Consequently, there may be a large resource of existing datasets on hole location for various LLIN products that is completely untapped. Additionally, to preserve the protective effect of LLINs in the field, it may be prudent for NMCPs to issue guidance that encourages net owners to prioritise repairing holes on the top of the net.

In this study there was no statistical difference in bloodfeeding inhibition between the pyrethroid-only Olyset Net and its PBO-pyrethroid equivalent Olyset Plus. This is consistent with experimental hut trials in Odisha state, India which observed very similar rates of bloodfeeding for Olyset Net and Olyset Plus, at 29.6% vs 30.6% of anophelines respectively (Gunasekaran et al. 2016). This observation of equivalent bloodfeeding inhibition against anophelines for Olyset Net and Olyset Plus is also corroborated by similarly designed experimental hut trials in Cameroon and Benin (Pennetier et al. 2013, Ngufor et al. 2022). Additionally, the broad observation that increased insecticidal effect has no additional benefit for personal protection is supported by a previous behavioural study by (Randriamaherijaona et al. 2015) across a range of hole sizes, observing that pyrethroid LLINs provided no additional benefit in bloodfeeding inhibition over untreated nets, across a range of hole sizes.

While the indirect protective effects of LLINs were not explicitly assessed in this study, bloodfeeding inhibition and bioefficacy outcomes would be expected to have implications for the general population in a practical setting. Even when Olyset Plus did not successfully prevent bloodfeeding, those mosquitoes that fed had a very high probability of dying as a result of the encounter (96.1%). Consequently, if the individual beneath such a net was infected, there would be a very low chance of onwards transmission. A previous study by Machani *et al.* (2019) observed that susceptible *An. gambiae* were able to better tolerate exposure to deltamethrin after a bloodmeal, with 24hr mortality falling from 83% to 35% with 60 minute exposures to 0.05% deltamethrin however the mosquitoes in that experiment were bloodfed eight hours prior to exposure rather than concurrently (Machani *et al.* 2019). Machani and colleagues observe that the expression of monooxygenase, β -esterase, and GSTs increased following the bloodmeal and suggest this makes the mosquitoes better able to tolerate insecticides. This increase in metabolic enzymes in response to bloodfeeding has also been observed in *An. funestus* (Spillings *et al.* 2008) and *An. arabiensis* (Oliver and Brooke 2014). However, these existing studies differ substantively from the current study as here the mosquito experiences concurrent insecticide and bloodmeal exposure and Olyset Plus is treated with PBO which inhibits the activity of metabolic enzymes. This highlights a knowledge gap onto the relationship between PBO and mosquito bloodfed status. Previous studies indicate that human blood is toxic to mosquitoes (Styer *et al.* 2007, Magalhaes *et al.* 2008), thus PBO may make it more susceptible to these effects. However, sides holes were observed to be more important for escape than for entry, allowing very few mosquitoes in but allowing a large minority to exit. Additionally, this study also observed that blood-fed *An. gambiae* were more likely to exit the net through a top hole than the side (60% vs 40% respectively). This is not readily explainable as a bloodfed mosquito was not following host

cues (due to the lack of host in the experiment) and the mosquitoes flight ability would be diminished by the weight of the bloodmeal (Kaufmann and Briegel 2004). However, given the importance of escaping the net for onwards transmission and the unexpectedly high mortality this is an area that requires further investigation. Current WHO guidelines for assessing the bioefficacy of LLINs test exclusively with non-bloodfed mosquitoes yet future studies should note that there may be additional insights to be gained from testing against bloodfed mosquitoes.

An important caveat of the findings of the current behavioural study are that the behavioural assays lasted only one hour rather than a whole night. As far fewer mosquitoes in these assays were incapacitated or killed by Olyset Net compared to Olyset Plus, it is possible these mosquitoes would have additional chances to infiltrate a net over a longer period of time. Additionally, the bioefficacy of these LLINs may be underestimated by this short assay time. The primary reason for keeping assays to one hour was to minimise discomfort to the volunteer however the lack of adverse effects reported here supports the arguments for longer assays in the future. A further caveat of the results of the current study is that that a difference in deterrence, the prevention of mosquitoes from entering a household, between LLIN products could not be observed by the design. However, experimental hut trials indicate deterrence from entering households is similar for Olyset Net and Olyset Plus thus is not expected to be a variable here (Pennetier et al. 2013, Gunasekaran et al. 2016, Ngufor et al. 2022). A further limitation on the interpretation of these results for personal protection is it does not account for biting which occurs during hours when an individual would not be expected to be indoors under their net. The extent of daytime feeding in sub-Saharan Africa is not well described. A recent study in Bangui, Central African Republic observed that 20-30% of bites occurred during the day (Sangbakembi-Ngounou et al. 2022), with *An. gambiae*, *An.*

coluzzii, *An. funestus*, and *An. pharoensis* all observed exhibit this behaviour. Daytime biting represents a significant challenge for conventional malaria control programmes as bed nets and IRS do not provide protection. Finally, there is a recent study that suggests the movement of mosquitoes in room-scale free-flying assays is influenced by airflow. Sutcliffe et al (2021) observed that while ~80% of activity occurred on the top of the net in warm still air conditions (27–30 °C), a high-speed cross-draft (speed unspecified) resulted in median activity on the top dropping to near zero, though this decline in activity was not statistically significant (p values unspecified).

5.4 Predictors of LLIN bioefficacy

This study indicates that the total PBO content of a PBO LLIN (for both Olyset Plus and PermaNet 3.0) was a strong predictor of insecticidal effect against pyrethroid-resistant *Anopheles gambiae* (s.s.). While the correlation is clear, the causal explanation for this link is not readily explainable here as this methodology measures the total content, both inside the fibres and on the surface of them, thus does not indicate how much of that chemistry is bioavailable to the mosquito. Here, the reduction in bioefficacy over time may indicate that the concentration of PBO is not being fully regenerated to baseline levels. Alternatively, or in addition, the reduction in total PBO content over time may have implications for the ratio of pyrethroid to PBO at the surface which may in turn impact bioefficacy against pyrethroid-resistant mosquitoes if it becomes suboptimal. The total content of pyrethroid was only minimally associated with bioefficacy against the pyrethroid-resistant strain. However, given that pyrethroid content stayed consistently high throughout the two year sampling period there was little variation to assess. The rapid loss of PBO, compared to the stability of the pyrethroids, indicates there must be some difference in the properties of these compounds that causes PBO to leech out more quickly over time. With this in mind, it should be noted that pyrethroid is a solid at room temperature and PBO is a liquid, which may have implications for how these compounds respond to handling and washing. Additionally, physical integrity was found to be a moderate indicator of PBO content, losing approximately a third of PBO after suffering the equivalent damage of two years of use (mean damage after two years = 125cm²). This trend in the relationship between physical damage and chemical content was observed to be very similar for Olyset Plus and PermaNet 3.0. However, the high variability in physical damage between individual nets (Figure 5.1), complicates the identification of trends in total damage across time when comparing LLIN products.

Additionally, it is important to note that these data cannot be assumed to apply beyond the context of Uganda and need to be assessed in other settings. With this in mind, the statistical modelling approach here is readily applicable to similar datasets that use standard WHO guidelines and uses software that is free-to-use, allowing future studies to address these same outputs.

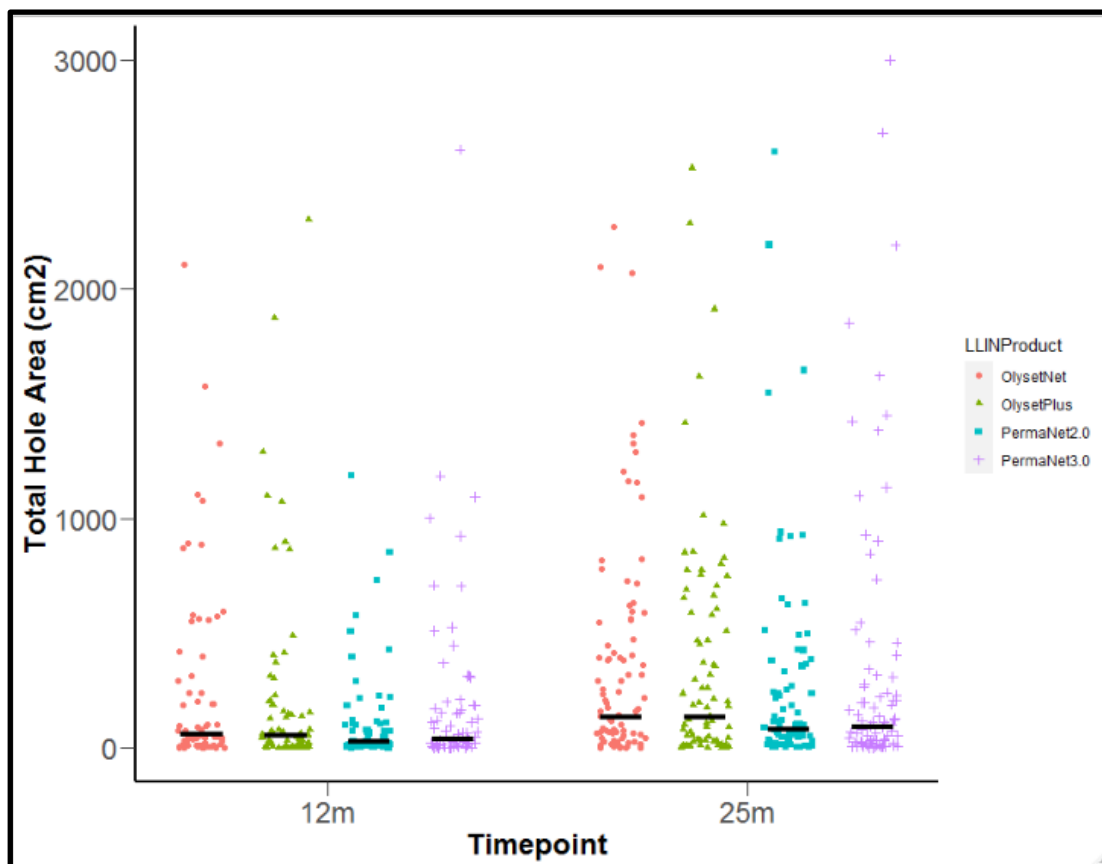


Figure 5.1 Total hole area (in cm²) for every net assessed. Note high variation between individual nets.

Benchtop bioefficacy and bloodfeeding assays were observed to be broadly indicative of outcomes in free-flying assays, not in terms of specific values but that they indicated broad trends between LLIN products. Specifically, Olyset Plus was observed to have much higher bioefficacy than Olyset Net in the wireball, which was reflected in the free-flying assays. However, KD and mortality in the wireball were substantially higher than in the free-flying assay (86% vs 38% and vs 66% vs 36.40% respectively). The disparity in outcomes between the wireball and free-flying assay may reflect their design, with the wireball forcing net contact and the free-flying assay allowing for avoidance. This is consistent with the disparity between the bioefficacy observed with the WHO wireball assay and the WHO cone bioassay, reported in Chapter 3. Additionally, there was no difference in bloodfeeding probability between the conventional pyrethroid net and the PBO-pyrethroid net in the benchtop bloodfeeding assay, which would also be reflected in free-flying assays. However, the relatively low bloodfeeding success in the untreated arm of the benchtop assay suggests a longer assay time is needed, with an argument that the length of the assay should be optimised to allow complete bloodfeeding on untreated netting. A notable difference in bioefficacy was observed between the benchtop wireball and bloodfeeding assays, with approximately 66% and 10% mortality respectively. The disparity between these assays despite using the same net sample for the same duration implies the mosquitoes are making less contact with the insecticide, which has strong parallels with the difference between the cone and wireball noted in Chapter Two.

5.5 Future work and next steps

In general, the key next step in evaluating the durability of PBO LLINs is to perform similar investigations in other settings. While rapid reductions in total PBO content and bioefficacy were observed here, these findings must be replicated across varying environmental and sociological contexts to demonstrate that the observations reported here are a property of the LLIN products themselves or specific to Uganda. Perhaps more importantly, there is a need to accumulate data on the longitudinal bioefficacy of Olyset Plus and PermaNet 3.0 against a variety of pyrethroid-resistant strains due to the variety of mutations and mechanisms between geographies. Additionally, I hope that the insights gained here from assessing hole location, household indicators, alternative bioassays designs, and novel statistical approaches encourage their inclusion in similar studies. Furthermore, the observation that the performance of Olyset Plus varies substantially depending on whether it is assessed in the WHO cone or wireball assay must be assessed on other strains (WHO, 2006). Future durability studies may also wish to investigate how the repellent effects on LLINs changes across time, with benchtop assays such as the video cone test or baited box assay allowing this outcome to be quantified without resorting to the ethically challenging WHO tunnel test.

The behavioural experiments performed here confirmed the hypothesis that *An. gambiae* s.s. mosquitoes were far more likely to enter holes on the top panel of an LLIN than holes on the side. The high entry risk for top holes provides a strong case for weighting holes by their location when assessing the condition of a net in the field. However, converging on the appropriate weighting values will require repeat experiments with different LLIN products and *An. gambiae* strains. Specifically, there is a need to assess entry into other 'next-gen' LLIN

products that are becoming widespread in distribution programmes in sub-Saharan Africa, such as PermaNet 3.0 and Interceptor G2.

Given the limitations of this behavioural work, namely that free-flying bloodfeeding assays lasted only one hour and assessed only one hole size, longer assays that evaluate bloodfeeding across the equivalent of a whole night are needed. There is also a need to assess the impact of hole size on bloodfeeding, to identify if the ratio of top to side entry here holds for smaller and larger holes. Additionally, due to recent, albeit weak, evidence that the concentration of mosquito activity on the top of the net is less pronounced when there is an airflow in the room, further work is needed to understand the impact of environmental conditions. Finally, there is a need to confirm if the findings reported here are replicated in a semi-field hut study in order to confirm that these phenomenon hold true in 'real world' conditions.

Chapter Six: References

- Abdel-Mohdy FA, Gaballa Fouda MM, Rehan MF, and Ali AS. (2009) Repellency of controlled-release treated-cotton fabrics based on permethrin and bioallethrin against mosquitoes. *The Journal of The Textile Institute*. 100(8):695-701.
- Anshebo, G. Y., P. M. Graves, S. C. Smith, A. B. Wills, M. Damte, T. Endeshaw, E. B. Shargie, T. Gebre, A. W. Mosher & A. E. Patterson (2014) Estimation of insecticide persistence, biological activity and mosquito resistance to PermaNet® 2 long-lasting insecticidal nets over three to 32 months of use in Ethiopia. *Malaria Journal*, 13, 1-14.
- Baba, E., P. Hamade, H. Kivumbi, M. Marasciulo, K. Maxwell, D. Moroso, A. Roca-Feltrer, A. Sanogo, J. S. Johansson & J. Tibenderana (2020) Effectiveness of seasonal malaria chemoprevention at scale in west and central Africa: an observational study. *The Lancet*, 396, 1829-1840.
- Bardají, A., B. Sigauque, L. Bruni, C. Romagosa, S. Sanz, S. Mabunda, I. Mandomando, J. Aponte, E. Sevene & P. L. Alonso (2008) Clinical malaria in African pregnant women. *Malaria Journal*, 7, 1-7.
- Bartoloni, A. & L. Zammarchi (2012) Clinical aspects of uncomplicated and severe malaria. *Mediterranean journal of hematology and infectious diseases*, 4.
- Batisso, E., T. Habte, G. Tesfaye, D. Getachew, A. Tekalegne, A. Kilian, B. Mpeka & C. Lynch (2012) A stitch in time: a cross-sectional survey looking at long lasting insecticide-treated bed net ownership, utilization and attrition in SNNPR, Ethiopia. *Malaria journal*, 11, 1-7.
- Bauserman, M., A. L. Conroy, K. North, J. Patterson, C. Bose & S. Meshnick. 2019. An overview of malaria in pregnancy. In *Seminars in perinatology*, 282-290. Elsevier.
- Bertozi-Villa, A., C. A. Bever, H. Koenker, D. J. Weiss, C. Vargas-Ruiz, A. K. Nandi, H. S. Gibson, J. Harris, K. E. Battle & S. F. Rumisha (2021) Maps and metrics of insecticide-treated net access, use, and nets-per-capita in Africa from 2000-2020. *Nature communications*, 12, 1-12.
- Bhatt, S., D. Weiss, E. Cameron, D. Bisanzio, B. Mappin, U. Dalrymple, K. Battle, C. Moyes, A. Henry & P. Eckhoff (2015) The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature*, 526, 207-211.
- Bousema, T., L. Okell, I. Felger & C. Drakeley (2014) Asymptomatic malaria infections: detectability, transmissibility and public health relevance. *Nature Reviews Microbiology*, 12, 833-840.
- Briët, O. J., M. A. Penny, D. Hardy, T. S. Awolola, W. Van Bortel, V. Corbel, R. K. Dabiré, J. Etang, B. G. Koudou & P. K. Tangu (2013) Effects of pyrethroid resistance on the cost effectiveness of a mass distribution of long-lasting insecticidal nets: a modelling study. *Malaria journal*, 12, 1-12.
- Chen, I., S. E. Clarke, R. Gosling, B. Hamainza, G. Killeen, A. Magill, W. O'Meara, R. N. Price & E. M. Riley (2016) "Asymptomatic" malaria: a chronic and debilitating infection that should be treated. *PLoS medicine*, 13, e1001942.

- Childs, L. M. & O. F. Prosper (2020) The impact of within-vector parasite development on the extrinsic incubation period. *Royal Society open science*, 7, 192173.
- Churcher, T. S., N. Lissenden, J. T. Griffin, E. Worrall & H. Ranson (2016) The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *Elife*, 5, e16090.
- Clarke, S. E., C. Bøgh, R. C. Brown, M. Pinder, G. E. Walraven & S. W. Lindsay (2001) Do untreated bednets protect against malaria? *Transactions of the royal society of tropical medicine and hygiene*, 95, 457-462.
- Cockcroft, A., J. Cosgrove & R. Wood (1998) Comparative repellency of commercial formulations of deet, permethrin and citronellal against the mosquito *Aedes aegypti*, using a collagen membrane technique compared with human arm tests. *Medical and veterinary entomology*, 12, 289-294.
- Coetzee, M. 2004. Distribution of the African malaria vectors of the *Anopheles gambiae* complex. 103-104.
- Curtis, C., J. Myamba & T. Wilkes (1996) Comparison of different insecticides and fabrics for anti-mosquito bednets and curtains. *Medical and veterinary entomology*, 10, 1-11.
- Dimala, C.A., Kika, B.T., Kadia, B.M. and Blencowe, H. (2018). Current challenges and proposed solutions to the effective implementation of the RTS, S/AS01 Malaria Vaccine Program in sub-Saharan Africa: A systematic review. *PloS one*, 13(12), p.e0209744.
- Dekker, T., B. Steib, R. Cardé & M. Geier (2002) L-lactic acid: a human-signifying host cue for the anthropophilic mosquito *Anopheles gambiae*. *Medical and veterinary entomology*, 16, 91-98.
- Edi, C. V., L. Djogbenou, A. M. Jenkins, K. Regna, M. A. Muskavitch, R. Poupardin, C. M. Jones, J. Essandoh, G. K. Ketoh, M. J. Paine, Koudou B., Donnelly M.J., Ranson H., Weetman, D. (2014) CYP6 P450 enzymes and ACE-1 duplication produce extreme and multiple insecticide resistance in the malaria mosquito *Anopheles gambiae*. *PLoS genetics*, 10, e1004236.
- Galactionova K, Tediosi F, De Savigny D, Smith T, Tanner M. Effective coverage and systems effectiveness for malaria case management in sub-Saharan African countries. (2015) *PloS one*. 10(5):e0127818.
- Ensley, S. M. 2018. Pyrethrins and pyrethroids. In *Veterinary toxicology*, 515-520. Elsevier.
- Gelabert, P., I. Olalde, T. De-Dios, S. Civit & C. Lalueza-Fox (2017) Malaria was a weak selective force in ancient Europeans. *Scientific reports*, 7, 1-10.
- Gichuki, P. M., L. Kamau, K. Njagi, S. Karoki, N. Muigai, D. Matoke-Muhia, N. Bayoh, E. Mathenge & R. S. Yadav (2021) Bioefficacy and durability of Olyset® Plus, a permethrin and piperonyl butoxide-treated insecticidal net in a 3-year long trial in Kenya. *Infectious diseases of poverty*, 10, 1-11.
- Gleave, K., N. Lissenden, M. Chaplin, L. Choi & H. Ranson (2021) Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. *Cochrane Database of Systematic Reviews*.
- Gleave, K., F. Mechan & L. Reimer (2022) The effects of temephos, permethrin and malathion selection on the fitness and fecundity of *Aedes aegypti*. *Medical and veterinary entomology*, 36, 56-65.

- Gnanguenon, V., R. Azondekon, F. Oke-Agbo, R. Beach & M. Akogbeto (2014) Durability assessment results suggest a serviceable life of two, rather than three, years for the current long-lasting insecticidal (mosquito) net (LLIN) intervention in Benin. *BMC infectious diseases*, 14, 1-10.
- Govella, N. J. & H. Ferguson (2012) Why use of interventions targeting outdoor biting mosquitoes will be necessary to achieve malaria elimination. *Frontiers in physiology*, 3, 199.
- Guglielmo, F., H. Ranson, N. f. Sagnon & C. Jones (2021) The issue is not 'compliance': exploring exposure to malaria vector bites through social dynamics in Burkina Faso. *Anthropology & medicine*, 28, 508-525.
- Gunasekaran, K., S. S. Sahu, T. Vijayakumar, S. Subramanian, R. S. Yadav, O. Pigeon & P. Jambulingam (2016) An experimental hut evaluation of Olyset Plus, a long-lasting insecticidal net treated with a mixture of permethrin and piperonyl butoxide, against *Anopheles fluviatilis* in Odisha State, India. *Malaria Journal*, 15, 1-10.
- Haji, K. A., B. O. Khatib, E. Obi, K. Dimoso, H. Koenker, S. Babalola, G. Greer, N. Serbantez, F. Abbas & A. Ali (2020) Monitoring the durability of the long-lasting insecticidal nets Olyset® and PermaNet® 2.0 in similar use environments in Zanzibar. *Malaria journal*, 19, 1-17.
- Hancock PA, Hendriks CJ, Tangena JA, Gibson H, Hemingway J, Coleman M, Gething PW, Cameron E, Bhatt S, Moyes CL. (2020) Mapping trends in insecticide resistance phenotypes in African malaria vectors. *PLoS biology*. 25;18(6):e3000633.
- Hauser, G., K. Thiévent & J. C. Koella (2019) The ability of *Anopheles gambiae* mosquitoes to bite through a permethrin-treated net and the consequences for their fitness. *Scientific Reports*, 9, 1-8.
- Hawkes, F., B. O. Manin, S. H. Ng, S. J. Torr, C. Drakeley, T. H. Chua & H. M. Ferguson (2017) Evaluation of electric nets as means to sample mosquito vectors host-seeking on humans and primates. *Parasit Vectors*, 10, 338.
- Hodgson E, Levi PE. (1999) Interactions of piperonyl butoxide with cytochrome P450. (pp. 41-II). *Academic Press*.
- Hogan, A. B., P. Winskill & A. C. Ghani (2020) Estimated impact of RTS, S/AS01 malaria vaccine allocation strategies in sub-Saharan Africa: A modelling study. *PLoS medicine*, 17, e1003377.
- Hougard, J.-M., S. Duchon, F. Darriet, M. Zaim, C. Rogier & P. Guillet (2003) Comparative performances, under laboratory conditions, of seven pyrethroid insecticides used for impregnation of mosquito nets. *Bulletin of the World Health Organization*, 81, 324-333.
- Howarth, J. (1988) The global distribution of malaria in the world and the present control effort. Wernsdorfer WH, McGregor I, eds. *Malaria: Principles and Practice of Malariology*.
- Howick, V. M., A. J. Russell, T. Andrews, H. Heaton, A. J. Reid, K. Natarajan, H. Butungi, T. Metcalf, L. H. Verzier & J. C. Rayner (2019) The Malaria Cell Atlas: Single parasite transcriptomes across the complete Plasmodium life cycle. *Science*, 365, eaaw2619.

- Hughes, A., G. M. Foster, A. Guy, A. Matope, M. Abe, D. Towers & P. J. McCall (2020) Quantifying late-stage host-seeking behaviour of *Anopheles gambiae* at the insecticidal net interface using a baited-box bioassay. *Malaria Journal*, 19, 1-11.
- Kaufmann, C. & H. Briegel (2004) Flight performance of the malaria vectors *Anopheles gambiae* and *Anopheles atroparvus*. *Journal of vector ecology*, 29, 140-153.
- Keiding, N., K. L. Albertsen, H. C. Rytgaard & A. L. Sørensen (2019) Prevalent cohort studies and unobserved heterogeneity. *Lifetime Data Analysis*, 25, 712-738.
- Killeen, G. F. & H. Ranson (2018) Insecticide-resistant malaria vectors must be tackled. *The Lancet*, 391, 1551-1552.
- Koenker, H., A. Kilian, C. Z. de Beyl, E. O. Onyefunafua, R. A. Selby, T. Abeku, M. Fotheringham & M. Lynch (2014) What happens to lost nets: a multi-country analysis of reasons for LLIN attrition using 14 household surveys in four countries. *Malaria journal*, 13, 1-10.
- Kolaczinski, J. & C. Curtis (2004) Chronic illness as a result of low-level exposure to synthetic pyrethroid insecticides: a review of the debate. *Food and chemical toxicology*, 42, 697-706.
- Kreznoski, P. J., D. R. Bangsberg & A. C. Tsai (2018) Quantifying bias in measuring insecticide-treated bednet use: meta-analysis of self-reported vs objectively measured adherence. *Journal of global health*, 8.
- Larsen, D. A., P. Hutchinson, A. Bennett, J. Yukich, P. Anglewicz, J. Keating & T. P. Eisele (2014) Community coverage with insecticide-treated mosquito nets and observed associations with all-cause child mortality and malaria parasite infections. *The American journal of tropical medicine and hygiene*, 91, 950.
- Levitz, L., M. Janko, K. Mwandagalirwa, K. L. Thwai, J. L. Likwela, A. K. Tshetu, M. Emch & S. R. Meshnick (2018) Effect of individual and community-level bed net usage on malaria prevalence among under-fives in the Democratic Republic of Congo. *Malaria journal*, 17, 1-8.
- Lindblade, K. A., E. Dotson, W. A. Hawley, N. Bayoh, J. Williamson, D. Mount, G. Olang, J. Vulule, L. Slutsker & J. Gimnig (2005) Evaluation of long-lasting insecticidal nets after 2 years of household use. *Tropical Medicine & International Health*, 10, 1141-1150.
- Lindblade, K. A., L. Steinhardt, A. Samuels, S. P. Kachur & L. Slutsker (2013) The silent threat: asymptomatic parasitemia and malaria transmission. *Expert review of anti-infective therapy*, 11, 623-639.
- Lindsay, S., J. Adiamah, J. Miller & J. Armstrong (1991) Pyrethroid-treated bednet effects on mosquitoes of the *Anopheles gambiae* complex in The Gambia. *Medical and veterinary entomology*, 5, 477-483.
- Lorenz, L. M., J. Bradley, J. Yukich, D. J. Massue, Z. Mageni Mboma, O. Pigeon, J. Moore, A. Kilian, J. Lines & W. Kisinza (2020) Comparative functional survival and equivalent annual cost of 3 long-lasting insecticidal net (LLIN) products in Tanzania: a randomised trial with 3-year follow up. *PLoS medicine*, 17, e1003248.
- Lorenz, L. M., H. J. Overgaard, D. J. Massue, Z. D. Mageni, J. Bradley, J. D. Moore, R. Mandike, K. Kramer, W. Kisinza & S. J. Moore (2014) Investigating mosquito net durability for malaria control in Tanzania-attrition, bioefficacy, chemistry,

- degradation and insecticide resistance (ABCDR): study protocol. *BMC public health*, 14, 1-11.
- Lucas, E. R., A. Miles, N. J. Harding, C. S. Clarkson, M. K. Lawniczak, D. P. Kwiatkowski, D. Weetman, M. J. Donnelly & A. G. Consortium (2019) Whole-genome sequencing reveals high complexity of copy number variation at insecticide resistance loci in malaria mosquitoes. *Genome research*, 29, 1250-1261.
- Lynd, A. & P. J. McCall (2013) Clustering of host-seeking activity of *Anopheles gambiae* mosquitoes at the top surface of a human-baited bed net. *Malaria journal*, 12, 1-8.
- Machani, M. G., E. Ochomo, D. Zhong, G. Zhou, X. Wang, A. K. Githeko, G. Yan & Y. A. Afrane (2020) Phenotypic, genotypic and biochemical changes during pyrethroid resistance selection in *Anopheles gambiae* mosquitoes. *Scientific reports*, 10, 1-8.
- Magalhaes, T., D. Brackney, J. Beier & B. Foy (2008) Silencing an *Anopheles gambiae* catalase and sulfhydryl oxidase increases mosquito mortality after a blood meal. *Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America*, 68, 134-143.
- Malima, R. C., S. M. Magesa, P. K. Tungu, V. Mwingira, F. S. Magogo, W. Sudi, F. W. Mosha, C. F. Curtis, C. Maxwell & M. Rowland (2008) An experimental hut evaluation of Olyset® nets against anopheline mosquitoes after seven years use in Tanzanian villages. *Malaria journal*, 7, 1-8.
- Massue, D. J., S. J. Moore, Z. D. Mageni, J. D. Moore, J. Bradley, O. Pigeon, E. J. Maziba, R. Mandike, K. Kramer & W. N. Kisinza (2016) Durability of Olyset campaign nets distributed between 2009 and 2011 in eight districts of Tanzania. *Malaria journal*, 15, 1-11.
- McBride, C. S. (2016) Genes and odors underlying the recent evolution of mosquito preference for humans. *Current Biology*, 26, R41-R46.
- McMeniman, C. J., R. A. Corfas, B. J. Matthews, S. A. Ritchie & L. B. Vosshall (2014) Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. *Cell*, 156, 1060-1071.
- Menze, B. D., M. F. Kouamo, M. J. Wondji, W. Tchapga, M. Tchoupo, M. O. Kusimo, C. S. Mouhamadou, J. M. Riveron & C. S. Wondji (2020) An experimental hut evaluation of PBO-based and pyrethroid-only nets against the malaria vector *Anopheles funestus* reveals a loss of bed nets efficacy associated with GSTe2 metabolic resistance. *Genes*, 11, 143.
- Meuwissen, J. H. & T. Ponnudurai (1988) Biology and biochemistry of sexual and sporogonic stages of *Plasmodium falciparum*: a review. *Biology of the Cell*, 64, 245-249.
- Meza, F. C., K. S. Kreppel, D. F. Maliti, A. T. Mlwale, N. Mirzai, G. F. Killeen, H. M. Ferguson & N. J. Govella (2019) Mosquito electrocuting traps for directly measuring biting rates and host-preferences of *Anopheles arabiensis* and *Anopheles funestus* outdoors. *Malaria journal*, 18, 1-11.
- Minakawa, N., J. O. Kongere, G. O. Sonye, P. A. Lutiali, B. Awuor, H. Kawada, R. Isozumi & K. Futami (2021) Long-Lasting Insecticidal Nets Incorporating Piperonyl Butoxide Reduce the Risk of Malaria in Children in Western Kenya: A Cluster Randomized Controlled Trial. *The American Journal of Tropical Medicine and Hygiene*, 105, 461.

- Mitchell, S. N., D. J. Rigden, A. J. Dowd, F. Lu, C. S. Wilding, D. Weetman, S. Dadzie, A. M. Jenkins, K. Regna & P. Boko (2014) Metabolic and target-site mechanisms combine to confer strong DDT resistance in *Anopheles gambiae*. *PloS one*, 9, e92662.
- Moyes, C. L., D. K. Athinya, T. Seethaler, K. E. Battle, M. Sinka, M. P. Hadi, J. Hemingway, M. Coleman & P. A. Hancock (2020) Evaluating insecticide resistance across African districts to aid malaria control decisions. *Proceedings of the National Academy of Sciences*, 117, 22042-22050.
- Mugenzi, L. M., B. D. Menze, M. Tchouakui, M. J. Wondji, H. Irving, M. Tchoupo, J. Hearn, G. D. Weedall, J. M. Riveron & C. S. Wondji (2019) Cis-regulatory CYP6P9b P450 variants associated with loss of insecticide-treated bed net efficacy against *Anopheles funestus*. *Nature communications*, 10, 1-11.
- Musiba RM, Tarimo BB, Monroe A, Msaky D, Ngowo H, Mihayo K, Limwagu A, Chilla GT, Shubis GK, Ibrahim A, Greer G. (2022) Outdoor biting and pyrethroid resistance as potential drivers of persistent malaria transmission in Zanzibar. *Malaria Journal*. 21(1):1-4.
- Neghina, R., I. Iacobiciu, A. M. Neghina & I. Marincu (2010) Malaria, a journey in time: in search of the lost myths and forgotten stories. *The American journal of the medical sciences*, 340, 492-498.
- Ngufor, C., A. Agbevo, J. Fagbohoun, A. Fongnikin & M. Rowland (2020) Efficacy of Royal Guard, a new alpha-cypermethrin and pyriproxyfen treated mosquito net, against pyrethroid-resistant malaria vectors. *Scientific reports*, 10, 1-15.
- Ngufor, C., J. Fagbohoun, A. Agbevo, H. Ismail, J. D. Challenger, T. S. Churcher & M. Rowland (2022) Comparative efficacy of two pyrethroid-piperonyl butoxide nets (Olyset Plus and PermaNet 3.0) against pyrethroid resistant malaria vectors: a non-inferiority assessment. *Malaria journal*, 21, 1-13.
- Ohm, J. R., F. Baldini, P. Barreaux, T. Lefevre, P. A. Lynch, E. Suh, S. A. Whitehead & M. B. Thomas (2018) Rethinking the extrinsic incubation period of malaria parasites. *Parasites & vectors*, 11, 1-9.
- Okumu, F. O., B. Chipwaza, E. P. Madumla, E. Mbeyela, G. Lingamba, J. Moore, A. J. Ntamatungro, D. R. Kavishe & S. J. Moore (2012) Implications of bio-efficacy and persistence of insecticides when indoor residual spraying and long-lasting insecticide nets are combined for malaria prevention. *Malaria journal*, 11, 1-13.
- Oliver, S. V. & B. D. Brooke (2014) The effect of multiple blood-feeding on the longevity and insecticide resistant phenotype in the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). *Parasites & vectors*, 7, 1-12.
- Paaijmans, K. P., S. Blanford, B. H. Chan & M. B. Thomas (2012) Warmer temperatures reduce the vectorial capacity of malaria mosquitoes. *Biology letters*, 8, 465-468.
- Parker, J. E., N. Angarita-Jaimes, M. Abe, C. E. Towers, D. Towers & P. J. McCall (2015) Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. *Scientific reports*, 5, 1-14.
- Pennetier, C., A. Bouraima, F. Chandre, M. Piameu, J. Etang, M. Rossignol, I. Sidick, B. Zogo, M.-N. Lacroix & R. Yadav (2013) Efficacy of Olyset® Plus, a new long-lasting insecticidal net incorporating permethrin and piperonyl-butoxide against multi-resistant malaria vectors. *Plos one*, 8, e75134.

- Price, R. N., R. J. Commons, K. E. Battle, K. Thriemer & K. Mendis (2020) Plasmodium vivax in the Era of the Shrinking P. falciparum Map. *Trends in parasitology*, 36, 560-570.
- Protopopoff, N., J. F. Mosha, E. Lukole, J. D. Charlwood, A. Wright, C. D. Mwalimu, A. Manjurano, F. W. Mosha, W. Kisinza & I. Kleinschmidt (2018) Effectiveness of a long-lasting piperonyl butoxide-treated insecticidal net and indoor residual spray interventions, separately and together, against malaria transmitted by pyrethroid-resistant mosquitoes: a cluster, randomised controlled, two-by-two factorial design trial. *The Lancet*, 391, 1577-1588.
- Pryce, J., M. Richardson & C. Lengeler (2018) Insecticide-treated nets for preventing malaria. *Cochrane Database of Systematic Reviews*.
- Raji, J. I. & M. DeGennaro (2017) Genetic analysis of mosquito detection of humans. *Current opinion in insect science*, 20, 34-38.
- Randriamaherijaona, S., O. J. Briët, S. Boyer, A. Bouraima, R. N'Guessan, C. Rogier & V. Corbel (2015) Do holes in long-lasting insecticidal nets compromise their efficacy against pyrethroid resistant Anopheles gambiae and Culex quinquefasciatus? Results from a release–recapture study in experimental huts. *Malaria journal*, 14, 1-22.
- Ranson, H. & N. Lissenden (2016) Insecticide resistance in African Anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends in parasitology*, 32, 187-196.
- Reimer, L., E. Fondjo, S. Patchoké, B. Diallo, Y. Lee, A. Ng, H. M. Ndjemai, J. Atangana, S. F. Traore & G. Lanzaro (2014) Relationship between kdr mutation and resistance to pyrethroid and DDT insecticides in natural populations of Anopheles gambiae. *Journal of medical entomology*, 45, 260-266.
- Rosenthal PJ. (2022) Malaria in 2022: Challenges and Progress. *The American Journal of Tropical Medicine and Hygiene*;106(6):1565.
- Ross, R. (1898) The role of the mosquito in the evolution of the malarial parasite. *The Lancet*, 152, 488-490.
- Rugnao, S., S. Gonahasa, C. Maiteki-Sebuguzi, J. Opigo, A. Yeka, A. Katureebe, M. Kyohere, A. Lynd, J. Hemingway & M. J. Donnelly (2019) LLIN Evaluation in Uganda Project (LLINEUP): factors associated with childhood parasitaemia and anaemia 3 years after a national long-lasting insecticidal net distribution campaign: a cross-sectional survey. *Malaria journal*, 18, 1-12.
- Russell, T. L., N. J. Govella, S. Azizi, C. J. Drakeley, S. P. Kachur & G. F. Killeen (2011) Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. *Malaria journal*, 10, 1-10.
- Sangbakembi-Ngounou, C., C. Costantini, N. M. Longo-Pendy, C. Ngoagouni, O. Akone-Ella, N. Rahola, S. Cornelie, P. Kengne, E. R. Nakouné & N. P. Kommas (2022) Diurnal biting of malaria mosquitoes in the Central African Republic indicates residual transmission may be “out of control”. *Proceedings of the National Academy of Sciences*, 119, e21104282119.
- Shah, M. P., J. Hwang, L. Choi, K. A. Lindblade, S. P. Kachur & M. Desai (2021) Mass drug administration for malaria. *Cochrane Database of Systematic Reviews*, 9, CD008846.

- Silva, A. P. B., J. M. M. Santos & A. J. Martins (2014) Mutations in the voltage-gated sodium channel gene of anophelines and their association with resistance to pyrethroids—a review. *Parasites & vectors*, 7, 1-14.
- Sinka, M., S. Pironon, N. Massey, J. Longbottom, J. Hemingway, C. Moyes & K. Willis (2020) A new malaria vector in Africa: Predicting the expansion range of *Anopheles stephensi* and identifying the urban populations at risk. *Proceedings of the National Academy of Sciences*, 117, 24900-24908.
- Sinka, M. E., M. J. Bangs, S. Manguin, M. Coetzee, C. M. Mbogo, J. Hemingway, A. P. Patil, W. H. Temperley, P. W. Gething & C. W. Kabaria (2010) The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. *Parasites & vectors*, 3, 1-34.
- Smith, D. L., A. K. Musiime, K. Maxwell, S. W. Lindsay & S. Kiware (2021) A new test of a theory about old mosquitoes. *Trends in Parasitology*, 37, 185-194.
- Solomon, T., E. Loha, W. Deressa, M. Balkew, T. Gari, H. J. Overgaard & B. Lindtjørn (2018) Bed nets used to protect against malaria do not last long in a semi-arid area of Ethiopia: a cohort study. *Malaria journal*, 17, 1-14.
- Sougoufara, S., E. C. Ottih & F. Tripet (2020) The need for new vector control approaches targeting outdoor biting Anopheline malaria vector communities. *Parasites & Vectors*, 13, 1-15.
- Spillings, B. L., M. Coetzee, L. L. Koekemoer & B. D. Brooke (2008) The effect of a single blood meal on the phenotypic expression of insecticide resistance in the major malaria vector *Anopheles funestus*. *Malaria journal*, 7, 1-9.
- Spurlock, F. & M. Lee. 2008. Synthetic pyrethroid use patterns, properties, and environmental effects. ACS Publications.
- Staedke, S. G., S. Gonahasa, G. Dorsey, M. R. Kamya, C. Maiteki-Sebuguzi, A. Lynd, A. Katureebe, M. Kyohere, P. Mutungi & S. P. Kigozi (2020) Effect of long-lasting insecticidal nets with and without piperonyl butoxide on malaria indicators in Uganda (LLINEUP): a pragmatic, cluster-randomised trial embedded in a national LLIN distribution campaign. *The Lancet*, 395, 1292-1303.
- Stebbins, R. C., M. Emch & S. R. Meshnick (2018) The effectiveness of community bed net use on malaria parasitemia among children less than 5 years old in Liberia. *The American journal of tropical medicine and hygiene*, 98, 660.
- Sutcliffe, J. & K. L. Colborn (2015) Video studies of passage by *Anopheles gambiae* mosquitoes through holes in a simulated bed net: effects of hole size, hole orientation and net environment. *Malaria Journal*, 14, 1-13.
- Sutcliffe, J. & K. L. Colborn (2015) Video studies of passage by *Anopheles gambiae* mosquitoes through holes in a simulated bed net: effects of hole size, hole orientation and net environment. *Malaria Journal*, 14, 1-13.
- Sutcliffe, J., X. Ji & S. Yin (2017) How many holes is too many? A prototype tool for estimating mosquito entry risk into damaged bed nets. *Malaria journal*, 16, 1-21.
- Sutcliffe, J. F. & S. Yin (2014) Behavioural responses of females of two anopheline mosquito species to human-occupied, insecticide-treated and untreated bed nets. *Malaria journal*, 13, 1-19.

- Sutcliffe, J. F. & S. Yin (2021) Effects of indoor air movement and ambient temperature on mosquito (*Anopheles gambiae*) behaviour around bed nets: implications for malaria prevention initiatives. *Malaria journal*, 20, 1-14.
- Stopard, I. J., T. S. Churcher & B. Lambert (2021) Estimating the extrinsic incubation period of malaria using a mechanistic model of sporogony. *PLoS computational biology*, 17, e1008658.
- Strode, C., S. Donegan, P. Garner, A. A. Enayati & J. Hemingway (2014) The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African anopheline mosquitoes: systematic review and meta-analysis. *PLoS medicine*, 11, e1001619.
- Styer, L. M., S. L. Minnick, A. K. Sun & T. W. Scott (2007) Mortality and reproductive dynamics of *Aedes aegypti* (Diptera: Culicidae) fed human blood. *Vector-borne and zoonotic diseases*, 7, 86-98.
- Sypniewska, P., J. F. Duda, I. Locatelli, C. R. Althaus, F. Althaus & B. Genton (2017) Clinical and laboratory predictors of death in African children with features of severe malaria: a systematic review and meta-analysis. *BMC medicine*, 15, 1-17.
- Tang, W., D. Wang, J. Wang, Z. Wu, L. Li, M. Huang, S. Xu & D. Yan (2018) Pyrethroid pesticide residues in the global environment: an overview. *Chemosphere*, 191, 990-1007.
- Tangena, J.-A. A., C. M. Hendriks, M. Devine, M. Tamaro, A. E. Trett, I. Williams, A. J. DePina, A. Sisay, R. Herizo & H. T. Kafy (2020) Indoor residual spraying for malaria control in sub-Saharan Africa 1997 to 2017: an adjusted retrospective analysis. *Malaria journal*, 19, 1-15.
- Tchouakui, M., Riveron, J.M., Djonabaye, D., Tchapga, W., Irving, H., Soh Takam, P., Njiokou, F. and Wondji, C.S., (2018). Fitness costs of the glutathione S-transferase epsilon 2 (L119F-GSTe2) mediated metabolic resistance to insecticides in the major African malaria vector *Anopheles funestus*. *Genes*, 9(12), 645.
- Tchouakui, M., J. Riveron, Miranda, L. M. Mugenzi, D. Djonabaye, M. J. Wondji, M. Tchoupo, W. Tchapga, F. Njiokou & C. S. Wondji (2020) Cytochrome P450 metabolic resistance (CYP6P9a) to pyrethroids imposes a fitness cost in the major African malaria vector *Anopheles funestus*. *Heredity*, 124, 621-632.
- Tchouakui, M., L. M. Mugenzi, M. J. Wondji, M. Tchoupo, F. Njiokou & C. S. Wondji (2021) Combined over-expression of two cytochrome P450 genes exacerbates the fitness cost of pyrethroid resistance in the major African malaria vector *Anopheles funestus*. *Pesticide biochemistry and physiology*, 173, 104772.
- Tinto, H., W. Otieno, S. Gesase, H. Sorgho, L. Otieno, E. Liheluka, I. Valéa, V. Sing'oei, A. Malabeja & D. Valia (2019) Long-term incidence of severe malaria following RTS, S/AS01 vaccination in children and infants in Africa: an open-label 3-year extension study of a phase 3 randomised controlled trial. *The Lancet Infectious Diseases*, 19, 821-832.
- Toe KH, Müller P, Badolo A, Traore A, Sagnon N, Dabiré RK, Ranson H. (2018) Do bednets including piperonyl butoxide offer additional protection against populations of

- Anopheles gambiae sl. that are highly resistant to pyrethroids? An experimental hut evaluation in Burkina Faso. *Medical and veterinary entomology*.;32(4):407-16.
- Toé, K. H., F. Mehan, J.-A. A. Tangena, M. Morris, J. Solino, E. F. Tchicaya, A. Traoré, H. Ismail, J. Maas & N. Lissenden (2019) Assessing the impact of the addition of pyriproxyfen on the durability of permethrin-treated bed nets in Burkina Faso: a compound-randomized controlled trial. *Malaria journal*, 18, 1-16.
- Tuteja, R. (2007) Malaria– an overview. *The FEBS journal*, 274, 4670-4679.
- Unwin, H.J.T., Smith, E.S., Churcher, T.S. and Ghani, A.C., 2022. Quantifying the direct and indirect protection provided by insecticide treated bed nets against malaria. *medRxiv*.
- van Eekelen, R., P. M. Bossuyt, M. van Wely & N. van Geloven (2021) Reducing survivorship bias due to heterogeneity when comparing treated and controls with a different start of follow up. *arXiv preprint arXiv:2105.07685*.
- Vanden Eng, J. L., D. P. Mathanga, K. Landman, D. Mwandama, A. A. Minta, M. Shah, J. Sutcliffe, J. Chisaka, K. A. Lindblade & L. Steinhardt (2017) Assessing bed net damage: comparisons of three measurement methods for estimating the size, shape, and distribution of holes on bed nets. *Malaria journal*, 16, 1-13.
- Venugopal, K., F. Hentzschel, G. Valkiūnas & M. Marti (2020). Plasmodium asexual growth and sexual development in the haematopoietic niche of the host. *Nature Reviews Microbiology*, 18, 177-189.
- Webster, J.P., Molyneux, D.H., Hotez, P.J. and Fenwick, A. (2014). The contribution of mass drug administration to global health: past, present and future. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 369(1645), p.20130434.
- Webster, B., E. S. Lacey & R. T. Cardé (2015). Waiting with bated breath: opportunistic orientation to human odor in the malaria mosquito, *Anopheles gambiae*, is modulated by minute changes in carbon dioxide concentration. *Journal of chemical ecology*, 41, 59-66.
- Wheldrake, A., Guillemois, E., Arouni, H., Chetty, V. and Russell, S.J., (2021). The causes of holes and loss of physical integrity in long-lasting insecticidal nets. *Malaria Journal*, 20(1), pp.1-10.
- Wiebe, A., J. Longbottom, K. Gleave, F. M. Shearer, M. E. Sinka, N. C. Massey, E. Cameron, S. Bhatt, P. W. Gething & J. Hemingway (2017) Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance. *Malaria journal*, 16, 1-10.
- World Health Organisation (2011). Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions. World Health Organization.
- World Health Organisation (2012). Policy recommendation: seasonal malaria chemoprevention (SMC) for Plasmodium falciparum malaria control in highly seasonal transmission areas of the Sahel sub-region in Africa. *World Health Organization, Geneva*.
- World Health Organisation. (2013a). Guidelines for laboratory and field-testing of long-lasting insecticidal nets. World Health Organization.
- World Health Organisation. (2013b). WHO guidance note for estimating the longevity of long-lasting insecticidal nets in malaria control. World Health Organization.

- World Health Organisation. (2017a). Conditions for deployment of mosquito nets treated with a pyrethroid and piperonyl butoxide: recommendations. World Health Organization.
- World Health Organisation. (2017b). Report of the twentieth WHOPES working group meeting, WHO/HQ, Geneva, 20–24 March 2017. World Health Organization.
- World Health Organisation. (2018) WHO recommended insecticides for indoor residual spraying against malaria vectors. Geneva, World Health Organization, 2018.
- World Health Organisation. (2020) World malaria report 2020: 20 years of global progress and challenges.
- World Health Organisation. (2021a). Interim recommendations for use of the AZD1222 (ChAdOx1-S [recombinant]) vaccine against COVID19 developed by Oxford University and AstraZeneca: interim guidance, 10 February 2021. World Health Organization.
- World Health Organisation. (2021b). World malaria report 2021.
- Zaim, M., A. Aitio & N. Nakashima (2000). Safety of pyrethroid-treated mosquito nets. *Medical and veterinary entomology*, 14, 1-5.
- Zwiebel, L. & W. Takken (2004). Olfactory regulation of mosquito–host interactions. *Insect biochemistry and molecular biology*, 34, 645-652.

Appendix I: Comparison of WHO Cone and WHO Wireball:

Supplementary Table 1. Comparison of bioefficacy outcomes with pyrethroid resistance *An. gambiae* ('Busia') after three minute exposure to Olyset Net in WHO Cone and WHO Wireball.

Olyset Net	Timepoint	WHO Cone	WHO Wireball
1hr Knockdown	Baseline	3.30 (95% CI: 0-7.09)	11.79 (95% CI: 4.18-19.42)
	12m	3.84 (95% CI: 0-12.74)	32.05 (95% CI: 4.79-59.30)
	25m	8.35 (95% CI: 0-22.69)	22.56 (95% CI: 11.40-33.96)
24hr Mortality	Baseline	5.94 (95% CI: 3.19-8.69)	7.14 (95% CI: 2.39-11.88)
	12m	3.60 (95% CI: 0.18-7.02)	24.60 (95% CI: 5.35-43.86)
	25m	2.60 (95% CI: 0.53-4.67)	9.29 (95% CI: 4.94-14.64)

Supplementary Table 2. Comparison of bioefficacy outcomes with pyrethroid resistance *An. gambiae* ('Busia') after three minute exposure to Olyset Net in WHO Cone and WHO Wireball.

Olyset Plus	Timepoint	WHO Cone	WHO Wireball
1hr Knockdown	Baseline	32.58 (95% CI: 15.57-49.59)	98.83 (95% CI: 94.43-100)
	12m	18.24 (95% CI: 0.97-35.51)	73.92 (95% CI: 54.88-92.97)
	25m	3.54 (95% CI: 0.70-10.54)	45.72 (95% CI: 22.84-68.61)
24hr Mortality	Baseline	12.19 (95% CI: 5.45-17.01)	87.72 (95% CI: 77.68-97.76)
	12m	5.67 (95% CI: 3.69-7.64)	24.60 (95% CI: 5.35-43.86)
	25m	3.34 (95% CI: 0-8.71)	25.92 (95% CI: 11.92-39.92)

Appendix II: Ethical approval documentation

Dear Mr Mechan,

Re. Research Protocol (21-065) 'Impact of hole location on entry rate of Anopheles mosquitoes into host-baited bed nets: comparison of damage on the top and sides of the net'

LSTM Research Ethics Committee Full Approval

Document Title	Version Number	Version Date
Protocol	V1.0	16/08/2021

Thank you for your letter of 14 October 2021 responding to the action points raised by the Committee. I can confirm that the protocol now has formal ethical approval from the LSTM Research Ethics Committee.

The approval is for the duration of your study and will therefore expire on 5 November 2022. You must submit a protocol amendment to extend approval beyond this date.

Approval is conditional upon submission of protocol amendments, notices and annual reports for REC Chair review via lstmrec@lstmed.ac.uk.

As your study is Sponsored by LSTM, please carefully read 'LSTM Sponsor Approval in Full' letter to ensure that you abide by the conditions of your approval.

Yours sincerely



Professor Graham Devereux
Chair
LSTM Research Ethics Committee

Researching and educating to save lives A Company Limited by Guarantee. Registered Number 83405, England and Wales. Registered Charity Number 222655.

RECTEM010 v3.0

Release date: 17/11/2020 Issued by: RGEO



FOR OFFICE USE ONLY	Application Number	Date considered

GOVERNANCE & ETHICS APPLICATION FORM

Please refer closely to the [Guidance Notes](#) when completing this form.

Please ensure that this form is fully completed including all attachments, so that the study can be properly reviewed by the Research Ethics Committee. If any documentation is missing, proposals will not be submitted for review.

Please confirm that this application is for:	YES	NO	N.B. Usually, LSTM will be Sponsor when an LSTM PI is the grant recipient
LSTM Sponsorship Approval	X		
LSTM REC Approval	X		

*If the Sponsor is not LSTM, provide documents which confirm Sponsorship

STUDY OVERVIEW

Main Applicant and Research Team				
List LSTM research team members and collaborators from partner institutions.				
Name	Organisation	Qualifications	Role in Study	Country
Frank Mechan	LSTM	MRes (PhD student)	Researcher	UK
Dr Lisa Reimer	LSTM	PhD	Supervisor	UK
Prof Philip McCall	LSTM	PhD	Supervisor	UK
Jonathan Thornton	LSTM	MSc	Technical	UK
Project Title:	Impact of hole location on entry rate of <i>Anopheles</i> mosquitoes into host-baited bed nets: comparison of damage on the top and sides of the net			
Applicant Full Name: (including title)	Frank Mechan			
Email address:	frank.mechan@lstmed.ac.uk			
Postal Address (if not LSTM):				
Telephone number:	07576266655			
Administrative Contact Name: (if applicable)				
Administrative Contact Email:				

REC Submission Fee			
Please go to REC payment information for fee structure and pre-prepared ISF Forms			
Is the proposed work already funded?	Yes		
	Formal Peer Review	Informal Peer Review	No Peer Review
How has the study been peer reviewed? Tick the appropriate box			X
Total budget of proposal	£ 500	Name of Funder	Medical Research Council, Doctoral Training Partnership studentship PhD bench fees used to cover costs of proposed project and REC submission fee

--	--	--	--	--

Project Details					
Proposed start date	01/10/2021	Proposed end date	30/03/2022	Total number of participants (refer to A.8)	8

Type of Research		
What type of research project is it?	Yes	No
Clinical Trial		
1. Involve a novel investigational drug or device		X
2. Involve a licensed drug or device		X
Human Tissue Study		
3. Involve the collection of samples of human blood, bodily secretions or tissue		X
4. Involve collection and/ or storage of human tissue samples on LSTM premises		X
5. Involve use of human tissue stored in a tissue bank or previously collected from consenting individuals (i.e. in a separate research study)		X
6. Human tissue collected in any other context		X
Human Contact Study		
7. Involve any form of quantitative or qualitative methods	X	
8. Vector studies involving human participants	X	
9. Involve exposing humans to an existing or modified non-medicinal intervention, training or process, including a new system		X
Data Study		
10. Data collected during a separate research study, conducted in the past (does require consent)		X
11. Data from patient records or public health surveillance (does not require consent)		X
12. Other, please specify:		
Is this study part of a Research degree? (PhD/ MPhil/ MD) If so, who is your supervisor?		
<i>Supervisor name:</i> Dr Lisa Reimer		

Sponsorship and In-Country/Other Ethical Approval				
If LSTM is not Sponsor, documents confirming Sponsorship must be enclosed				
	Sponsoring Institution	Received	Pending	Not Applicable
1	LSTM		X	
Please list the country(ies) where the research will be carried out and the status of in-country ethical approval <i>Add additional lines as necessary</i>				
	Country	Received	Pending	Not Required
1	United Kingdom		X	
2				

3				
Have you submitted this proposal to any other Research Ethics Committees not named above? If yes, please state name of institution:				
1				

All correspondence from partner institutions must reference the same study title as indicated on this application.

Please note if you have marked 'Not Required' for any country above, written evidence must be provided to confirm that in-country ethical approval is not required. This evidence should be appended to the application.

Acceptable evidence includes:

- a letter from the national Ministry of Health or other relevant regulatory authority
- a letter from an authorised signatory at a local partner

A letter from a co-investigator or other researcher at a local partner institution is not sufficient evidence.

SECTION A Study Details

A.1	GLOSSARY OF TERMS Please provide a list of specialist or scientific acronyms used in the application, with their full name and any relevant explanation that would be helpful to committee members that may not be an expert in your area of work. Please limit this list to 10 acronyms.
<p>LLIN: Long-lasting insecticidal net PBO: Piperonyl butoxide</p>	
A.2	LAY SUMMARY: Please use simple language which is understandable to a non-scientific/non-academic audience. This section must not exceed 500 words.

Overall Aim

Long lasting insecticidal nets (LLINs) protect people from the bites of malaria transmitting mosquitoes as they sleep. LLINs provide both personal protection to the person underneath them (by forming a physical and chemical barrier) and community protection to other nearby households (by killing mosquitoes that may have gone on to bite others at a later time). However, LLINs get damaged with use, resulting in holes that mosquitoes can potentially enter. Recent studies have indicated that mosquito activity is heavily focused on the top of the net, here we hypothesise that holes on the top of the net pose the greatest risk for allowing entry to the mosquito.

The aim of this study is to investigate if the location of a hole on a net (top or side) impacts the probability a host-seeking mosquito will successfully enter through the hole and survive the encounter.

This study will address the above questions for both conventional (insecticide-only) bed nets and for 'next generation' nets that contain an additional compound to be used against insecticide-resistant mosquitoes.

Methods in Brief

In our study, a human volunteer will lie beneath a bed net in a climate-controlled room (located in a purpose built room in LSTM Accelerator building).

Two types of net will be assessed:

- (1) A standard insecticide-only net
- (2) An equivalent insecticide net that also contains the synergist piperonyl butoxide

Each net will be in one of three states of physical integrity:

- (a) Completely intact
- (b) One 15cm diameter circular hole in centre of top panel
- (c) One 15cm diameter circular hole in the centre of a side panel

All combinations of net type and physical integrity in the table below will be assessed.

	NET TYPE	
DAMAGE STATUS	Insecticide only	Insecticide + PBO
Completely intact	6 observations	6 observations
Hole in side	6 observations	6 observations
Hole in top	6 observations	6 observations

In each observation, a volunteer will be asked to lie under a net. Once the volunteer is in place, the lights will be turned off and twenty (20) mosquitoes released into the room. For the duration of the assay, these mosquitoes will be free to move around the room and approach the net. After 60 minutes, the lights will be turned back on and all mosquitoes in the room collected by an aspiration tube into one of four cups labelled as follows:

- (1) Found inside net and bloodfed
- (2) Found inside net and not bloodfed
- (3) Found outside net and bloodfed
- (4) Found outside net and not bloodfed

Once all mosquitoes have been collected, the volunteer will be asked to step out from under the net.

The number of mosquitoes in an incapacitated state in each cup will be counted one hour after collection. The number of mosquitoes dead in each cup will be counted 24 hours after collection.

A.3	ETHICAL ISSUES: Please list any anticipated ethical issues and briefly state how you will address them. All projects will have ethical issues, which may relate to informed consent, potential conflicts of interest, handling confidential data etc.
------------	--

In the course of this study, participants may be bitten by mosquitoes. Typically, mosquito bites result in minor discomfort and itching. In rare cases, specific individuals may exhibit a more pronounced response, including swelling at the site of the bite.

In this study, only individuals that have previously worked in an insectary and are approved by LSTM to arm-feed mosquitoes will be considered for participation (as such individuals are routinely bitten in the course of their work thus will be aware of their body's response to a bite). Additionally, participants may suffer minor discomfort due to the hot and humid environment of the room. However, this too is mitigated by only including individuals familiar with working in insectary conditions and limiting the time inside to 60 minutes.

Care will be taken to avoid any potential participants feeling pressured to volunteer, which will be made clear in the consent form. Potential participants will be made fully aware that being bitten by a mosquito during the course of the study is likely and be made aware of the potential side effects associated with mosquito bites.

A.4	JUSTIFICATION FOR THE RESEARCH: Give a brief explanation of the importance of the research to be conducted. What needs will it address and how will it build on previous research? (<i>max. 300 words</i>)
------------	---

Long-lasting insecticidal nets (LLINs) provide protection from the bites of mosquitoes as their occupant sleeps, reducing their exposure to malaria-causing parasites^[1]. These nets provide physical protection through tightly woven fabric and chemical protection through insecticide on the surface. However, over time LLINs become physically damaged through routine use and washing^[2]. The extent to which these holes provide access to mosquitoes is not well understood.

Current WHO guidance for assessing physical damage involves calculating the total area of holes, then categorising according to a simple metric (where $\geq 643\text{cm}^2$ = 'needs replaced')^[3]. In this metric, all holes are counted equally regardless of where they occur on the net. Despite this, there is growing evidence that mosquito activity around an LLIN is heavily focused (>80%) on the top above the sleeper^[4]. Consequently, these holes on the top surface may be more important for allowing passage to mosquitoes however there is a lack of behavioral studies that address this question.

This knowledge gap is further compounded by the widespread rise of insecticide resistance in mosquitoes, allowing them to tolerate exposure to LLINs^[5]. As mosquitoes become better able to withstand contact with LLINs, there is concern that they will be more likely to navigate through holes and bite the occupant. In response, so called 'next generation'

LLINs containing the synergist piperonyl butoxide have been developed to restore susceptibility. However, the protective efficacy of this new class of nets once damaged is completely unaddressed.

References

1. Churcher, T.S., Lissenden, N., Griffin, J.T., Worrall, E. and Ranson, H., 2016. The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *Elife*, 5, p.e16090.
2. WHO. Methods for maintaining coverage with long-lasting insecticidal nets (LLINs). Vector Control Technical Expert Group, Report to MPAC September 2013. Geneva: World Health Organization, 2013.
3. WHO. Estimating functional survival of long-lasting insecticidal nets from field data. Vector Control Technical Expert Group, Report to MPAC September 2013. Geneva: World Health Organization, 2013.
4. Parker JE, Angarita-Jaimes N, Abe M, Towers CE, Towers D, McCall PJ. Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. *Scientific reports*. 2015 Sep 1;5(1):1-4.
5. Ranson H, Lissenden N. Insecticide resistance in African *Anopheles* mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends in parasitology*. 2016 Mar 1;32(3):187-96.

A.5	OBJECTIVES: List the major objectives of the study. These must be achievable by the proposed design and methods. Please list the key outcome measure for each objective. (<i>max. 300 words</i>)	
	Objective 1: Compare the blood-feeding rate pyrethroid-resistant <i>An. gambiae</i> mosquitoes when exposed to each net type/damage status combination	Outcome The proportion of mosquitoes blood fed
	Objective 2: Compare the proportion of pyrethroid-resistant <i>An. gambiae</i> mosquitoes alive after 24 hours in each net type/damage status combination.	Outcome The proportion of mosquitoes alive after 24 hours.

A.6 Please	<p>METHODOLOGY: Please describe the methods for each objective (if different) and justify the rationale behind the chosen methodology. Please use simple language which is clear to a non-scientific/non-academic audience. Please keep this section concise and ensure that specialist terms are explained. APPLICATION FORM</p> <p>Where possible please use diagrams to summarise. INTERNAL LSTM APPLICANTS ONLY (<i>max. 1,500 words, Clinical Trials: 2,000-words</i>)</p>
---------------	--

GOVERNANCE & ETHICS APPLICATION FORM**INTERNAL LSTM APPLICANTS ONLY**

The following covers both Objective 1 & 2

Study design

The aim of this study is to investigate the impact of physical damage to a bed net on the probability a host-seeking *Anopheles* mosquito will successfully enter to bite a person inside and survive the encounter. To achieve this, the circumstances of a mosquito approaching a sleeper under a damaged bed net will be reconstructed. In each arm of the study, human volunteers will lie beneath a bed net with damage on different parts of the net. The effectiveness of these nets in preventing blood-feeding, and killing the mosquito in each arm will be quantified. A human participant is necessary inside the net to provide the chemical cues that attract mosquitoes to approach.

In total, two different designs of bed net will be used: pyrethroid insecticide only ('*Olyset*') and pyrethroid insecticide + piperonyl butoxide ('*Olyset Plus*'). For each of these two net designs, three different damage patterns will be assessed (for a total of six combinations).

Volunteers will be asked to return for multiple testing days. Specifically, volunteers will be asked to participate on three occasions: one session for each of the three damage patterns (top hole, side hole, fully intact) for a given net design. However, if a volunteer is willing to participate in more than three sessions, the additional sessions will be included in a different arm.

Volunteer Recruitment

Multiple participants are needed as people vary in their attractiveness to mosquitoes. We will invite potential participants to take part in three 60 minute sessions (each taking place on a different day). Each sessions will consist of sleeping under a given net type in one of three states of physical condition (fully intact, hole in side, hole in top). However, there is no obligation for a participant to take part in more than one session.

Participants are not precluded from taking part in more than one arm of the study if they volunteer to do more than three sessions.

We intend to recruit twelve (12) participants.

Experimental set up

The volunteer will lie on a single bed in the centre of a climate-controlled room (Temperature: 27°C±3°C, Humidity: 75%±5%). The bedding will be a simple sheet covered mattress and pillow with no quilt or blanket. We will ask the volunteer to wear a short-sleeved t-shirt and to avoid fragrances/perfume on the day they are participating. We will ask the volunteer to try to avoid responding to the mosquito if it enters the net, and to keep movement to a minimum in general. The bed will be surrounded by a plastic frame (length: 180cm, width: 170cm, height: 150cm), over which the bed net will be secured.

Damage to nets will be cut into the centre of the relevant net panel using scissors. A circular hole measuring 15cm in diameter will be cut into either the centre of the top panel or the centre of a side panel. Only one side panel of a net will have a hole at one time, with the side panel varying randomly each session.

Once the volunteer is in place on the bed and the net is secured in place, the researcher will leave the room and turn off the lights. The researcher will then release twenty mosquitoes into the room from outside (using a simple but well-established string and cup mechanism). After one hour the researcher will re-enter the room and collect all mosquitoes in the room into a plastic cup using a mechanical aspirator, while the participant will be asked to collect any inside the net before they leave.

Outcomes

The number of mosquitoes blood-fed counted immediately after the session and the number of mosquitoes dead counted after 24 hours.

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

Mosquito source and characteristics

Mosquitos used are *Anopheles gambiae* species (strain 'Busia'), from a colony currently maintained at LSTM. This colony was established in 2018 from collections in Busia, Uganda and have been since characterised to possess moderate pyrethroid-resistance (i.e. can survive a three-minute cone bioassay exposure to a standard pyrethroid-only net).

GOVERNANCE & ETHICS APPLICATION FORMINTERNAL LSTM APPLICANTS ONLY

Mosquitoes used in the experiment will be 3-5 days old females (as is conventional in WHO bioassays) and will not have fed on human blood prior to the experiment.

A.9 ELIGIBILITY CRITERIA		
Inclusion Criteria	Exclusion Criteria	Reason for Exclusion
Current/Previous work in an insectary including mosquito arm-feeding.	History of adverse reactions to mosquito bites Recently travel to countries where mosquito-borne diseases are endemic.	Potential for allergic reaction due to being bitten in study Extremely low probability that mosquitoes may pass a vectorborne infection from a participant onto other participants or researchers.

A.7 PROCEDURES Please detail any clinical, social science or other research procedures to which participants will be subjected.			
Procedure	To be carried out by:	Organisation	
1. Participant asked to lie down under bed net in testing room for 60 minutes	Frank Mechan/Jonathan Thornton	LSTM	
2. Participant asked to allow mosquitoes to bite their arms or legs	Frank Mechan/Jonathan Thornton	LSTM	
3.			
4.			
<i>Add rows if necessary</i>			

A.8	PARTICIPANTS: How many participants will be recruited? If you are unable to give precise figures, please give estimates. Please state the age of legal majority in the country of research.	Age of legal majority in country(ies)	<u>18</u> yrs.
-----	--	--	----------------

A.10	RECRUITMENT AND INFORMED CONSENT: Please describe how you will recruit and consent each group of study participants. Please use diagrams where possible. You must include details of: i Identification of potential participants ii Information given to potential participants iii How, where and by whom will the first approach be made? iv How will consent be recorded? Please give details of how you will obtain informed consent/assent/proxy consent.		
------	---	--	--

GOVERNANCE & ETHICS APPLICATION FORM**INTERNAL LSTM APPLICANTS ONLY**

AGE/SEX	Neonates (<28 days)	Infants (1-12 months)	Young children (1-4 years)	Older children (5-9 years)	Early adolescents (10-14 years)	Older adolescents (15 years – age of majority)	Adults (≥ age of majority)
Males							6
Females							6
Where participants will not be individual participants, state number of households:						households	

- i) Only individuals who have been approved to work in a mosquito insectary perform arm-feeding (where an individual allows mosquitoes to bite their arm to supply blood nutrients needed for reproduction) will be eligible for participation.
- ii) Participants will be provided with a full description of the study aims, methodology, and potential risks.
- iii) I will approach potential participants via a group email to insectary users .
- iv) I will ask participants to sign a consent form (attached)

A.11	COMPENSATION: Please outline any reimbursements or compensation (financial or otherwise) that will be offered to potential participants or individuals as part of their participation in this research.
<p>No compensation will be given as there is no precedent for compensation in mosquito behaviour experiments at LSTM and would not wish this to be a motivating reason for a participant to take part.</p>	

A.12	DISSEMINATION: outline plans of results. For pilot studies , will the results inform future studies?
<p>Results will be published in reputable journals and shared with colleagues at both internal and external conferences. Results will also be shared directly with all participants to show them how the data was used.</p>	

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

SECTION B Ethical Issues and Consequences

Consider how you will protect the health, dignity and well-being of participants, staff and members of the public. Please also show awareness of impact on health services.

B.1	ADVERSE EFFECTS, DISCOMFORT OR RISKS: Outline the potential adverse effects, discomfort or risks that may result from the study for participants, investigators and members of the public and how you will minimise them.	
B.1.1 Participants	Insect bites resulting in itching, allergic reactions to bite	<p>There is a moderate-high chance that the volunteer will be bitten due to the hole in the net and the mosquito’s moderate ability to survive exposure to the insecticide.</p> <p>The relatively low numbers of mosquitoes released in a single sitting (n = 20) limits the number of bites the volunteer will receive.</p> <p>Bites can be treated with a topical antihistamine cream (‘Anthisan’) if the volunteer wishes. Additionally, we will offer a commercial oral antihistamine (10mg cetirizine hydrochloride).</p> <p>A small, untreated piece of netting will be provided for the participant to cover their head if they wish.</p>

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

	<p>Discomfort from room temperature (~27°C) and humidity (~70%)</p> <p>Discomfort or irritation caused by contact with insecticide treated netting</p> <p>Transmission of Covid-19</p>	<p>Discomfort in the room’s tropical climate is minimised by the volunteer spending only 60 mins per test in the room. (maximum).</p> <p>All of the LLINs used have received WHO prequalification: certified safe and recommended for use worldwide.</p> <p>Irritation resulting from LLIN contact is extremely rare and tends to be localised to the skin surfaces where contact was made, and temporary in character. Nonetheless, adverse events will be monitored throughout the study.</p> <p>Researchers will wear a face covering covering and disposable gloves at all times and participants will only remove their mask while alone in the testing room.</p> <p>There will be an interval of at least minimum of 30 minutes between each experimental run to allow the air in the testing room to be fully replaced.</p> <p>Bedding will be changed after every experimental run and the plastic sheeting below wiped down with antiviral spray. A separate pillow cover and sheets will be used for each participant.</p>
<p>B.1.2 Investigators</p>	<p>Insect bites.</p> <p>Irritation when handling LLINs</p>	<p>The likelihood of mosquito bites to investigators is minimised by adhering to standard good practices.</p> <p>Any bites can be treated with a topical antihistamine cream (‘Anthisan’).</p> <p>Study staff will wear gloves and appropriate clothing when handling nets.</p> <p>All materials used during the study will be disposed of correctly.</p>

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

B.1.3 Members of the public	Potential risks, adverse effects, discomfort or risks NA	Steps to be taken to minimise adverse effects, discomfort and risks NA
B.2	VULNERABLE GROUPS: Please identify vulnerable groups that will be included in this study. How will you minimise any harm to each group identified?	
	Include any potential safeguarding issues that may arise during your research; how will you protect vulnerable adults and children? Are there any common practices or traditions that could cause harm? How will you ensure you protect staff and students who work in isolated areas?	
<p>No individuals from vulnerable groups will be recruited into the study.</p> <p>Only trained insectary workers will be recruited into the study.</p> <p>Experiment will be monitored while in progress to provide assistance if necessary.</p>		
B.3	SAFEGUARDING LEAD: Who will have lead responsibility for any vulnerable child or adult safeguarding issues identified during the project? Consider how safeguarding incidents will be recorded and reported.	
<p>No vulnerable adults or children will be recruited into the study.</p> <p>Dr Lisa Reimer will have lead responsibility for safeguarding issues, participants will be provided with the contact number of said safeguarding lead and LSTM's designated Safeguarding Officer to report abuse, harassment, or neglect by a study team member.</p>		

GOVERNANCE & ETHICS APPLICATION FORMINTERNAL LSTM APPLICANTS ONLY

B.4	CONSEQUENCES FOR LOCAL HEALTH SERVICES	
<p>What demands will this research place on local health services?</p> <p>In the event of a serious adverse reaction (i.e. a allergic response more severe than minor redness and itching at the site of a bite), the participant will be directed to the local Accident and Emergency department:</p> <p>Liverpool Royal University Hospital, Prescot St, Liverpool L7 8XP</p>	<p>Any participant who experiences a serious adverse reaction will be discontinued from taking part in the study. A replacement participant will be sought.</p>	
B.5	MONITORING AND OVERSIGHT: Please give details of the proposed arrangements for independent monitoring and oversight of the trial and how any data and safety monitoring function will be carried out.	
<p><i>If this is not a clinical trial, go to Section C</i></p>		
B.6	RECORDING AND REPORTING SAEs: Provide details of how you propose to manage the recording and reporting of serious adverse events.	
<p>Serious adverse events will be recorded and reporting as per LSTM REC guidelines.</p>		

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

SECTION C Statistics, Data and Sample Management

C.1	<p>SAMPLE SIZE: Please justify your choice of sample size (as described in A.9). Please ensure that the sample size calculation is based on the primary outcome measure as detailed in A.5.</p> <p>Applicants are encouraged to include screen shots of calculations when performed using software.</p> <p>Note that screen shots are not sufficient in themselves and need to be accompanied by justification of the values used in the calculations.</p>
------------	---

GOVERNANCE & ETHICS APPLICATION FORMINTERNAL LSTM APPLICANTS ONLY

The sample size calculation for the primary outcome (proportion alive) is based on a previous behavioural study by Randriamaherijaona *et al.* (2015) that investigated mosquito passage through holes in LLINs. The comprehensive reporting of explanatory power associated with each variable tested (LLIN type, mosquito resistance status, hole area) allows well informed power analysis for the proposed study.

While their study investigated only conventional pyrethroid nets (i.e. did not include PBO-LLINs), the effect size of LLIN type (insecticide vs non-insecticide) and mosquito resistance status (resistant vs susceptible) are informative for the proposed study. They report that net type and resistance status together explain a total of 34% of variation in the data, rising to 92% with the inclusion of hole size (in cm²). However, as their study included holes sizes much larger than the proposed current study, we have chosen to be conservative in our estimates by using the lower value of **34% in our sample size calculations**.

Here we use the R package 'pwr' to calculate the necessary sample size.

Using the approaches developed by Cohen (1998) this method takes an assumed effective size (here **0.34**) and calculates the total number of samples needed to differentiate between treatment groups (assuming a balanced experimental design).

The number of treatment groups is specified here by the number of 'degrees of freedom' (effectively the number of parameters in the model that can vary). Here the total number of degrees of freedom in the model is four: **1** (for net type) + **2** (for hole location) + **1** for the model intercept = **4**

As is convention, we aim for a type 1 error probability (significance level) of **0.05** and a type 2 error probability of **0.2** (equivalent to 80% power).

Effect size: 0.34

Degrees of freedom: 4

Type I error prob: 0.05

Type II error prob: 0.20

```
> pwr.f2.test(u = 4,
+           f2 = 0.34,
+           sig.level = 0.05,
+           power = 0.80)

Multiple regression power calculation

      u = 4
      v = 35.14392
      f2 = 0.34
sig.level = 0.05
  power = 0.8
```

Total samples needed (fully balanced experimental design) = **36**

Allowing six observations of all pairwise net type and hole location combinations.

References:

Randriamaherijaona, S., Briët, O.J., Boyer, S., Bouraima, A., N'Guessan, R., Rogier, C. and Corbel, V., 2015. Do holes in long-lasting insecticidal nets compromise their efficacy against pyrethroid resistant *Anopheles gambiae* and *Culex quinquefasciatus*? Results from a release–recapture study in experimental huts. *Malaria journal*, 14(1), pp.1-22.

Cohen, J. (1988). *Statistical power analysis for the behavioral sciences* (2nd ed.). Hillsdale, NJ: Lawrence Erlbaum.

GOVERNANCE & ETHICS APPLICATION FORMINTERNAL LSTM APPLICANTS ONLY

C.2	MAJOR METHODS OF ANALYSIS: What are the major methods you intend to use to analyse the data? These should be clearly linked to the outcome measures listed in section A.5.
<p>Associations between outcomes and variables of interest will be quantified using Generalized Linear Mixed Models (GLMMs). To account for unexplained variation between individual volunteers, a unique ID will be assigned to each participant and included in the models as a random effect.</p>	

C.3	<p>MANAGEMENT OF SAMPLES & DATA: For each type of data/sample to be collected, please describe the procedures in place during;</p> <ul style="list-style-type: none"> i Collection and Processing ii Analysis iii Storage and Transportation iv Surplus material (human tissue) <p>Consider how data quality will be assured, and how participant privacy and confidentiality will be maintained.</p>
<p>Mosquito samples Mosquitoes collected during the study will be kept within paper cups for 24 hours after each assay. All assessments of mosquito condition (blood-fed status, incapacitation, mortality) are assessed visually by an experienced researcher. At the end of the 24 hr period, mosquitos will be starved for 24 hours before disposal in autoclave waste.</p> <p>Data handling Paper data-entry sheets will be immediately destroyed once scanned into digital format. Data entry will be performed on Microsoft Excel and stored on a password locked computer and only de-identified data will be used for data analysis. Electronic data will be stored for five years on an LSTM server.</p> <p>Only named investigators will have access to the data prior to publication. The final databases will be publicly available once study findings have been published. However, no identifying information on study participants will be included.</p>	

GOVERNANCE & ETHICS APPLICATION FORMINTERNAL LSTM APPLICANTS ONLY

D.1	TRAINING		
Please indicate the basis on which the persons identified in A.7 are considered competent to carry out these procedures. List any staff training required prior to commencement of the study.			
<p><i>According to ICH GCP (International Conference on Harmonisation - Good Clinical Practice), all clinical research staff should have a minimum of Protocol training, plus GCP training, or Good Research Practice for Social Science research. Research team members must also have training on Informed Consent where appropriate. These mandatory training requirements should be in place at the time the study commences.</i></p>			
Staff Member	Title	Experience/Competencies	Training Required
Frank Mechan	Mr.	Trained in mosquito handling, bioassays, behavioural analyses	Practice recovering mosquitoes from the testing room prior to study commencement
Lisa Reimer	Dr.	Trained in mosquito handling, bioassays, behavioural analyses	none
Philip McCall	Prof.	Trained in mosquito handling, bioassays, behavioural analyses	none
Jonathan Thornton	Mr.	Trained in mosquito handling and bioassays	none

GOVERNANCE & ETHICS APPLICATION FORMINTERNAL LSTM APPLICANTS ONLY**SUBMITTED DOCUMENTS**

The following document list must be completed, itemising each document.

Please refer to LSTM [Version Control A Good Practice Guide](#).

Documents may include:

- Participant Information Sheets
- Consent Forms
- Case Report Forms
- Social Science Data Collection Tools (Interview Guides, Questionnaires etc.)
- Translator Agreement
- Research Protocol

Title	Version No.	Date
<i>Participant Information Sheet and consent form</i>	1.0	13/08/21

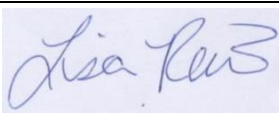
Please collate documents into 4 application packs, and 1 combined PDF as per instructions on our

[SharePoint page](#) The signed Declaration Page should be included in both paper packs and PDF.

The collated paper applications should be sent to Lindsay Troughton, Secretary, Research Ethics Committee, Liverpool

School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA

Plus, combined PDF application via e-mail: lstmrec@lstmed.ac.uk

If proposal is for work relating to a PhD:	
Supervisor	Lisa Reimer
Department	Vector
<p>By signing below, I confirm that:</p> <ul style="list-style-type: none"> • The application is clearly written and can be understood by a lay person • The objectives can be met by the proposed methodology • Participants will be identified, recruited and consented in accordance with ethical guidelines • The participant information sheets, and consent/assent forms are appropriate for the target audience 	
Supervisor Signature	

GOVERNANCE & ETHICS APPLICATION FORMINTERNAL LSTM APPLICANTS ONLY**DECLARATION: TO BE SIGNED BY MAIN APPLICANT**

Applicants must initial each declaration or 'N/A' in the right-hand column if not applicable	Initial (by hand)	N/A
i) I confirm that the details of this proposal are a true representation of the research to be undertaken.	FM	
ii) I agree to abide by the ethical principles underlying the Declaration of Helsinki .	FM	
iii) I agree to abide by LSTM Code of Conduct and LSTM Safeguarding Policy .	FM	
iv) I confirm that I and all staff who are involved in the research and/or in obtaining consent from participants will receive formal training in Good Clinical Practice/Good Research Practice before the research project commences.	FM	
v) I undertake to seek In-Country Ethical Approval in the country(ies) where the research is to be carried out and abide by local regulations, including those on data and human tissue.	FM	
vi) If protocol amendments are required as the research progresses, I will submit these to the Liverpool School of Tropical Medicine Research Ethics Committee and in-country authorities for approval.	FM	
vii) I will ensure that the research does not deviate from the protocol described. In the event that a protocol deviation does occur, I will submit these to the Liverpool School of Tropical Medicine Research Ethics Committee and in-country authorities for approval.	FM	
viii) I will provide the Research Ethics Committee with an annual report, due each year on the original approval date, and an end of study report once all activities are completed.	FM	
ix) I understand that all conditions apply to any co-applicants, researchers and other staff involved in the study, and that it is my responsibility to ensure that they abide by them.	FM	
For studies using 'human tissue' x) I confirm I will abide by LSTM's Policies and Standard Operating Procedures relating to activities involving human tissue. * Human tissue (relevant material) is defined as any material that has come from a human body and consists of, or includes, human cells.		NA

Please do not staple, paperclip only

Liverpool School of Tropical Medicine



GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

Where application form has been completed by junior researcher on behalf of the PI xi) As PI, I have reviewed this application and am satisfied that it is at an acceptable standard.		LR	
Signed:	<i>Frank Mechar</i>	Date:	16/08/2021

From time to time the Committee uses past ethics applications for training purposes or to give examples to new applicants. In all cases the applications are anonymised.

If you **DO NOT** consent to your application being used for these purposes, please tick the box.

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

**VOLUNTEER INFORMATION SHEET
& CONSENT FORM**

Study Title: Impact of hole location on entry rate of *Anopheles* mosquitoes into host-baited bed nets:
comparison of damage on the top and sides of the net

IRB: 21-065

Sponsor: LSTM

Participant identification number:

What is this study?

Long lasting Insecticidal Nets (LLINs) are the cornerstone of malaria control strategy, protecting individuals from the bites of *Anopheles* mosquitoes as they sleep. However, LLINs become damaged by routine use over time. The impact of this damage on the protective effect of the LLIN is not well understood. Given recent studies indicating that mosquito activity is heavily focussed on the top of the net, this study will investigate if the location of the hole of the net (top or side) impacts the probability a mosquito will successfully enter the net and survive the encounter.

You are invited to take part in a research study to gather information that may be useful in developing new LLIN designs that are more resilient to damage. Before you commit to taking part, please read the following information. Please feel free to ask any questions you may have. This study will be undertaken here at the Liverpool School of Tropical Medicine (LSTM)

Why are volunteers needed?

Malaria transmitting mosquitoes' need human blood into order to successfully reproduce and will typically approach humans as they sleep to obtain a bloodmeal. Mosquitoes identify humans by cues from the human body including CO², body heat, and volatile chemicals on the skin. Consequently, a human participant is needed underneath the nets assessed in this study to entice the mosquito to approach and attempt to enter it. People vary in their attractiveness to mosquitoes, and by using several different people we can ensure that the results gained are more reliable.

You have been invited to take part due to your experience working in insectaries and arm-feeding mosquitoes. As some people have a pronounced inflammatory response to being bitten by mosquitoes, these criteria minimise the chance you will have an adverse reaction to mosquito bites.

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

What do volunteers have to do?

If you chose to take part; you will be asked to come to our testing room in the LSTM Accelerator building where we have recreated the conditions of a sleeping space in tropical conditions protected by a holed LLIN. You will be asked to lie down inside the LLIN while we release mosquitoes into the room. As the LLIN will have a hole in it there is a high chance that some mosquitoes will bite you. Volunteers will be offered a piece of untreated netting to protect their head and shoulders if they choose. We ask that you lie down, in the dark, for 60 minutes. The room will be warm (about 27°C) and humid (about 80%). A researcher from the study will be monitoring the experiment (from outside the room) and will provide assistance as required. After the 60 minutes have elapsed, the volunteer will be asked to collect any mosquitoes from inside the net using a mechanical aspirator.

Volunteers may listen to music or sleep while lying (as motionless as is comfortable) on the bed. We ask volunteers to wear light clothing (such as a short sleeved t-shirt) and to refrain from wearing perfume, aftershave or any other strong scent. Volunteers will be invited to take part in three sessions (always on different days), though there is no obligation to take part in more than one session.

Is it dangerous?

As the purpose of the study is to investigate mosquitoes entering damaged nets, there is a high chance volunteers will be bitten in the course of their participation. However, as eligibility is conditional on experience arm-feeding the probability of an adverse reaction to these bites is low. Additionally, the mosquitoes used will come from our laboratory colonies and will not be able to transmit any infections. The treated bednets are made from insecticide-treated materials that have been declared safe by the WHO. However, in the very unlikely event that you experience any problems either during or after the experiments, please tell us immediately (frank.mechan@lstmed.ac.uk /07576266655).

A number of measure will be put in place to minimise the risk of covid-19 when conducting the experiment. Research staff will wear a mask and gloves at all time. Additionally, separate bedding will be used for each participant and the air air in the testing room fully recycled between each testing period.

Will my taking part in this study be confidential?

Volunteers will not be named, though we will acknowledge participation appropriately in any publications or reports resulting from this study as is common practice. You will be given an anonymous ID number for data analysis.

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

What if I don't want to do this?

You are under no pressure or obligation to participate in this study and if you decide to take part you may end your involvement at any time without explanation.

Safeguarding

The study team and data collectors are expected to behave ethically and responsibly at all times and follow the LSTM code of conduct. This means that they must not ask you for any financial, physical or sexual favours in return for taking part in this research. If you experience any abuse, harassment or neglect by a study team member you can contact the study Safeguarding Lead – Lisa Reimer on +44 (0)151 705

3107/lisa.reimer@lstmed.ac.uk. You may call this number at any time. You may also raise a safeguarding concern directly with LSTM Designated Safeguarding Officer Philippa Tubb on +44 (0)151 705 3744/safeguarding@lstmed.ac.uk. LSTM's safeguarding commitment is described on [LSTM Safeguarding webpage](#).

Thank you for considering participation. Please ask any questions you wish.

If you understand what you are being asked and are willing to volunteer, please read and sign here

DECLARATION

I am volunteering to participate in this project evaluating the impact of damage to long-lasting insecticidal nets and I understand that I can withdraw from the study at any time, without explanation.

Name

Signature

Date

For information, or in the event of any problems, please contact:

Name: Frank Mechan Email: frank.mechan@lstmed.ac.uk Phone: 07576266655

Name: Lisa Reimer Email: lisa.reimer@lstmed.ac.uk Phone: 0151 705 3107

Research Ethics Committee Chair:

Graham.Deveruex@lstmed.ac.uk 0151 702 9551

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

Group email to be sent to vector insectary users

Hello,

I'm Frank Mechan, a PhD student here in the vector department at LSTM.

I am hoping to start a study soon on the behaviour of *Anopheles* mosquitoes around damaged nets. This study aims to help us better understand how mosquitoes get into and out of nets with holes when seeking humans to bite. The goal is that this data will contribute towards the development of bed nets that are more resilient to damage and provide protection for longer in the field.

To do this I will need volunteers to lie down under a net in a testing room for one hour while mosquitoes are released. As these nets will have holes in them, there is a chance that volunteers may be bitten by mosquitoes during this time.

If you are interested in learning more about the study please get in touch.

Kind
regards,
Frank

Hello again [participant]

Thank you for your interest in our study.

For more information, please read the Participant Information Sheet and Consent Form attached.

If you have read the information and wish to participate, please return the consent form signed. Feel free to ask any questions you may have.

Kind
regards,
Frank

GOVERNANCE & ETHICS APPLICATION FORMINTERNAL LSTM APPLICANTS ONLY**Protocol**

Impact of hole location on entry rate of *Anopheles* mosquitoes into host-baited bed nets: comparison of damage on the top and sides of the net

(V1 – Frank Mechan 16/8/21)

Study design

To investigate if the location of damage on a bed net impacts the entry rate and survival of host-seeking *Anopheles* mosquitoes approaching the net. To achieve this, the conditions of a sleeping space occupied by a participant and protected by a bed net will be recreated in a climate controlled testing room. A total of ten (10) volunteers will be included, who may participate repeatedly in any of the three arms of the study. The arms of the study are as follows; each a different type of bed net: **(1)** a fully intact net, **(2)** a single 15cm diameter circular hole in the centre of the top, or **(3)** a single 15cm diameter circular hole in the centre of the side. Damage to nets will be cut into the centre of the appropriate net panel with scissors. In each test, twenty (20) female, lab reared, pyrethroid-resistant *An. gambiae* mosquitoes will be released into the room and recollected after one hour. The outcomes of the trial are **(a)** the proportion of all mosquitoes blood-fed and **(b)** the proportion of mosquitoes dead after 24 hours.

The experiment will be repeated for each of three types of bed net, **(1)** an insecticide-free control net, **(2)** a pyrethroid insecticidal net (brand name 'Olyset'), **(3)** an insecticidal net containing both a pyrethroid and piperonyl butoxide (brand name 'Olyset Plus').

In each assay, The volunteer will lie on a single bed in the centre of a climate-controlled room (Temperature: 27°C±3°C, Humidity: 75%±5%). The bedding will be a simple sheet covered mattress and pillow with no quilt or blanket. We will ask the volunteer to wear a short-sleeved t-shirt and to avoid fragrances/perfume on the day they are participating. We will ask the volunteer to try to avoid responding to the mosquito if it enters the net, and to keep movement to a minimum in general. The bed will be surrounded by a plastic frame (length: 180cm, width: 170cm, height: 150cm), over which the bed net will be secured.

Once the volunteer is in place on the bed and the net is secured in place, the researcher will leave the room and turn off the lights. The researcher will then release twenty mosquitoes

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

into the room from outside (using a simple but well-established string and cup mechanism). After one hour the researcher will re-enter the room and collect any mosquitoes in the room into a plastic cup using a mechanical aspirator, while the participant will be asked to collect any inside the net before they leave.

Mosquito source and characteristics

Mosquitos used are *Anopheles gambiae* species (strain 'Busia'), from a colony currently maintained at LSTM. This colony was established in 2018 from collections in Busia, Uganda and have been since characterised to possess moderate pyrethroid-resistance (can survive a three-minute exposure to a standard pyrethroid-only net). Mosquitoes used in the experiment will be 3-5 days old females (as is conventional in WHO bioassays) and will not have fed on human blood prior to the experiment.

Study population and selection criteria

All participants will require full informed consent and meet the following criteria; male or female aged 18-60 (inclusive), currently working at LSTM, must be trained and approved for insectary work, and must be approved for mosquito arm-feeding.

Subjects will be excluded from the study based on the following criteria; history of adverse reactions to insect bites.

Recruitment method

Participants will be identified through LSTMs skill database and invited by emailed.

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

Data collection

The study outcomes, mosquito blood-fed status and mosquito mortality, will be assessed visually by an experienced researcher. Data will be stored initially in paper format in standard mosquito testing input sheets then later inputted into digital format in a spreadsheet. Paper copies will be kept on record.

Adverse reactions

Participants may experience minor redness and itching as a result of mosquito bites. There is no expectation of adverse reactions due to all participants having arm-fed recently (thus being aware of their bodies response to bites). All participants will be given the contact information of the investigators to report any adverse reactions.

Reasons for withdrawal

A participant will be discontinued from taking part for the following reasons:

- Withdrawal of consent
- Adverse reaction to mosquito bite (at the discretion of the investigators).

All participants are free to withdraw from the study at any time for any reason.

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

Methods

The following procedures will be performed prior to each flight assay

1. Mosquito rearing in insectary (with lighting set to turn off at 10am-10pm so mosquitoes perform nighttime behaviours during working day).
2. Transfer 20 females into cup.
3. Mosquito starved (no sugar given) for 24 hours in climate-controlled room adjacent to testing room.
4. Testing room cleaned and air allowed to completely replace.
5. Sleeping space prepared for next participant (fresh sheet/pillow).
6. Appropriate net type set up around space.
7. Cup containing assay-ready mosquitoes set up in position for release

The following procedures will be performed during each flight assay

1. Participant directed into sleeping space and net secured over them.
2. Investigator leaves room and lights turned off
3. Mosquitoes released
4. 60 minutes elapse
5. Lights turned on and investigator re-enters room
6. All mosquitoes outside net collected using mechanical aspirator ○ Collected into one of two cups:
 - (A) outside blood-fed
 - (B) outside not blood-fed
7. All mosquitoes inside net collected using mechanical aspirator ○ Collected into one of two cups:
 - (A) inside blood-fed
 - (B) inside not blood-fed
8. Participant directed to step out of net and leave room

The following procedures will be performed after each flight assay

1. Bedding is removed.
2. After one hour has elapsed the number of mosquitoes 'knocked down' is counted.
3. After 24 hours has elapsed the number of mosquitoes dead is counted.

GOVERNANCE & ETHICS APPLICATION FORM

INTERNAL LSTM APPLICANTS ONLY

Statistical analysis plan

The primary endpoints for this study are:

(1A) proportion of mosquito's blood fed in each damage category for a pyrethroid-only net

(1B) proportion of mosquito's blood fed in each damage category for a pyrethroid-PBO net

(2A) proportion of mosquito's dead in each damage category for a pyrethroid-only net

(2B) proportion of mosquito's dead in each damage category for a pyrethroid-PBO net

Data analysis will be conducted using R (version 3.6.0). Associations between outcomes and variables of interest will be quantified using Generalized Linear Mixed Models (GLMMs) using the 'lme4' package (version 1.1-21). To account for unexplained variation between individual volunteers, a unique ID will be assigned to each participant and included in the models as a random effect.

Data handling

Only named investigators will have access to the data. Hard copies will be retained for the duration for the study and kept locked in a cabinet. Data entry will be performed on Microsoft Excel and stored on a password locked computer. Only de-identified data will be used for data analysis. All hard copy data documents will be shredded within five years of the conclusion of the study.

Protocol deviation

Any deviation from the protocol will be submitted to the REC per reporting guidelines.

Conflicts of interest

No conflicts of interest reported.

Please do not staple, paperclip only

Liverpool School of Tropical Medicine



GOVERNANCE & ETHICS APPLICATION FORM

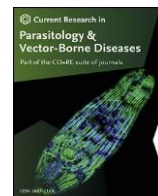
INTERNAL LSTM APPLICANTS ONLY

Appendix III: Peer-reviewed publication of Chapters Two & Three

Current Research in Parasitology & Vector-Borne Diseases 2 (2022) 10009 2



Contents lists available on ScienceDirect
Current Research in Parasitology & Vector-Borne Diseases
journal homepage: www.editorialmanager.com/crvb



LLIN evaluation in Uganda project (LLINEUP): The fabric integrity, chemical content and bioefficacy of long-lasting insecticidal nets treated with and without piperonyl butoxide across two years of operational use in Uganda



Frank Mehan^{a,*}, Agaba Katureebe^{b,1}, Violet Tuhaise^b, Martin Mugote^b, Ambrose Oruni^a, Ismail Onyige^b, Kawesa Bumali^b, Jonathan Thornton^a, Kilama Maxwell^c, Mary Kyohere^c, Moses R. Kanya^{b,d}, Peter Mutungi^b, Simon P. Kigozi^b, Adoke Yeka^b, Jimmy Opigo^e, Catherine Maiteki-Sebuguzi^b, Samuel Gonahasa^b, Janet Hemingway^a, Grant Dorsey^f, Lisa J. Reimer^a, Sarah G. Staedke^{b,g}, Martin J. Donnelly^a, Amy Lynd^{a,**}

^aDepartment of Vector Biology, Liverpool School of Tropical Medicine, Liverpool, UK ^bInfectious Diseases Research Collaboration, Uganda ^c

Department of Medicine, Makerere University, Kampala, Uganda ^dMakerere University - Johns Hopkins University (MUJHU) Research Collaboration, Kampala, Uganda ^eNational Malaria Control Division, Ministry of Health, Kampala, Uganda ^fDepartment of Medicine, University of California, San Francisco, USA

^gDepartment of Clinical Research, London School of Hygiene & Tropical Medicine, London, UK

ARTICLE INFO

Keywords:

LLIN
Malaria
Durability
Insecticide
Bioefficacy
Piperonyl butoxide

ABSTRACT

Long-lasting insecticidal nets (LLINs) supplemented with the synergist piperonyl butoxide have been developed in response to growing pyrethroid resistance; however, their durability in the field remains poorly described. A pragmatic cluster-randomised trial was embedded into Uganda's 2017–2018 LLIN distribution to compare the durability of LLINs with and without PBO. A total of 104 clusters (health sub-districts) were included with each receiving one of four LLIN products, two with pyrethroid + PBO (Olyset Plus and PermaNet 3.0) and two pyrethroid-only (Olyset Net and PermaNet 2.0). Nets were sampled at baseline, 12 and 25 months postdistribution to assess physical condition, chemical content, and bioefficacy. Physical condition was quantified using proportionate Hole Index and chemical content measured using high-performance liquid chromatography. Bioefficacy was assessed with three-minute World Health Organisation (WHO) Cone and Wireball assays using pyrethroid-resistant *Anopheles gambiae*, with 1-h knockdown and 24-h mortality recorded. There was no difference in physical durability between LLIN products assessed ($P = 0.644$). The pyrethroid content of all products remained relatively stable across time-points but PBO content declined by 55% ($P < 0.001$) and 58% ($P < 0.001$) for Olyset Plus and PermaNet 3.0 respectively. Both PBO LLINs were highly effective against pyrethroid-resistant mosquitoes when new, knocking down all mosquitoes. However, bioefficacy declined over time with Olyset Plus knocking down 45.72% (95% CI: 22.84–68.62%, $P = 0.021$) and Permanent 3.0 knocking down 78.57% (95% CI: 63.57–93.58%, $P < 0.001$) after 25 months. Here we demonstrate that both Olyset Plus and PermaNet 3.0 are as durable as their pyrethroid-only equivalents and had superior bioefficacy against pyrethroid-resistant *An. gambiae*. However, the superiority of PBO-LLINs decreased with operational use, correlating with a reduction in total PBO content. This decline in bioefficacy after just two years is concerning and there is an urgent need to assess the durability of PBO LLINs in other settings.

* Corresponding author.

** Corresponding author.

E-mail addresses: frank.mechan@lstmed.ac.uk (F. Mechan),
amy.lynd@lstmed.ac.uk (A. Lynd). 1 Equal contributors.

<https://doi.org/10.1016/j.crpvbd.2022.100092>

Received 1 April 2022; Received in revised form 6 May 2022; Accepted 18 May 2022

2667-114X/© 2022 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Introduction

Long-lasting insecticidal nets (LLINs) are the cornerstone of global malaria control strategies, forming a physical and chemical barrier against the bites of *Anopheles* mosquitoes (Bhatt et al., 2015; Churcher et al., 2016; Pryce et al., 2018). Progress in reducing malaria burden in sub-Saharan Africa achieved in the first decade of the 21st century has been attributed, in large part, to mass distribution of LLINs (Bhatt et al., 2015). LLINs are intended to maintain an effective level of protection for at least three years, with the expectation that distributions will take place at two-to-three-year intervals (WHO, 2013a, 2016). However, recent studies suggest that the lifespan of LLINs may be less than three years (Gnanguenon et al., 2014; Toe et al., 2019; Lorenz et al., 2020). To ensure the continued success of malaria control efforts, National Malaria Control Programmes (NMCPs) must identify LLIN products that demonstrate durability within the socio-economic and environmental context of their country.

The WorldHealth Organisation (WHO) currently recommends the use of pyrethroid and pyrrole insecticides on LLINs (WHO, 2017b); however, the effectiveness of LLINs is threatened by widespread pyrethroid resistance (Ranson & Lissenden, 2016; Churcher et al., 2016; Hemingway et al., 2016). The development of target site alterations and metabolic resistance enables mosquitoes to better tolerate insecticide exposure, increasing the probability they will obtain a blood meal and survive the encounter (Irish et al., 2008; Asidi et al., 2012; Strode et al., 2014). While there is evidence that pyrethroid LLINs retain some protective effect against resistant mosquito populations (Alout et al., 2016; Viana et al., 2016), the threat of resistance has incentivised the development of new classes of LLIN. Due to the limited alternatives to pyrethroids, initial efforts to maintain the impact of LLINs have focused on secondary compounds that restore the susceptibility of pyrethroid-resistant mosquitoes. Piperonyl butoxide (PBO) is a synergist that inhibits the cytochrome P450 enzymes within the mosquito which detoxify insecticides (Darriet & Chandre, 2011). In 2017, the WHO provided an interim endorsement of use of pyrethroid LLINs containing PBO in areas of moderate pyrethroid resistance (WHO,

2017a) and a 2021 Cochrane review concluded that PBO-LLINs were associated with a reduction in parasite prevalence in areas of moderate-high pyrethroid resistance compared to pyrethroid-only nets (Gleave et al., 2021). However, the same review emphasised that evidence of the durability of these PBO-LLINs under operational conditions is lacking.

LLINs are known to lose insecticide content during routine use (WHO, 2013b). As nets are handled and washed, the insecticide at the surface is depleted then gradually regenerated by a reservoir within the fibres (Gimnig et al., 2005). Pyrethroid LLINs are designed with sufficient insecticide reserves to continue regenerating for at least three years, with the expectation they will be replaced before this time (WHO, 2013a). Currently, WHO LLIN durability guidelines quantify performance against objective bioefficacy benchmarks to assess if a three-year operational lifespan is achieved (WHO, 2011, 2013a), yet there is emerging evidence to suggest that bioefficacy varies substantially between products and may fall below defined efficacy thresholds within three years (Gnanguenon et al., 2014; Toe et al., 2019; Lorenz et al., 2020).

Table 1

In Uganda, the country with the highest malaria burden in East Africa, progress in controlling transmission has faltered (Lynd et al., 2019). The declining efficacy of conventional control strategies coincides with emerging evidence of both high levels of knockdown resistance (kdr) and metabolic resistance in mosquito populations throughout the country (Lynd et al., 2019; Njoroge et al., 2021). As part of a commitment to achieve universal coverage of LLINs, the Ugandan Ministry of Health initiated a mass distribution of LLINs and PBO LLINs in 2017. A randomised control trial was embedded within this distribution programme to evaluate the impact of LLINs with and without PBO (Staedke et al., 2019). From this, it was demonstrated that PBO-LLINs reduce parasite prevalence in children aged 2–10 years-old and vector density more effectively than conventional LLINs for at least 25 months (Staedke et al., 2020; Gleave et al., 2021). The present study was conducted as part of the same trial to evaluate the durability of the PBO-LLINs. Here the physical integrity, chemical integrity,

and bioefficacy of two PBO-LLIN products are assessed in comparison with their pyrethroid-only equivalents at

Specifications of LLIN products assessed in study. The target dose was defined as the amount of chemical per kg of fabric.

Product name	Manufacturer	Fabric type	Active ingredient target dose (w/manufacturing tolerance)
Olyset Net	Sumitomo Chemical Ltd.	Polyethylene (150 denier)	Permethrin: 20 5.0 g/kg
Olyset Plus	Sumitomo Chemical Ltd.	Polyethylene (150 denier)	Permethrin: 20 5.0 g/kg
PermaNet 2.0	Vestergaard Frandsen	Polyester (100 denier)	PBO: 10 2.5 g/kg
PermaNet 3.0	Vestergaard Frandsen	roof: Polyethylene (100 denier); sides: Polyester (75 denier)	Deltamethrin: 1.4 0.35 g/kg Deltamethrin: 4.0 1.0 g/kg (roof); 2.8 0.525 g/kg (sides)
			PBO: 25 2.5 g/kg (roof)

12 and 25 months post-distribution.

Materials and methods

Study site

The trial protocol for this study has been published (Staedke et al., 2019). A total of 104 clusters (health sub-districts, HSDs) in eastern and western Uganda were randomly assigned to receive one of four LLIN products, including two LLINs with PBO (PermaNet 3.0 and Olyset Plus) and two LLINs without PBO (PermaNet 2.0 and Olyset Net).

Cross-sectional community surveys were carried out in 50 households per cluster (5200 households per survey) to confirm presence of the expected LLIN product from the distribution and entomological surveillance undertaken in 10 households per cluster. Efficacy data from this study have been published previously (Staedke et al., 2020). In the present study, we quantify the chemical and physical integrity of 400 LLINs, 97–100 nets of each type (Supplementary Table S1), withdrawn from households after 12 months and 25 months (total of 800 nets). These nets were assessed alongside unused nets of the same LLIN products.

LLIN description

Four LLIN products were distributed and assessed in this study: Olyset Net treated with permethrin; PermaNet 2.0 treated with deltamethrin; Olyset Plus treated with permethrin and PBO; and PermaNet 3.0 treated with deltamethrin and incorporating PBO on the top surface of the net only. All nets were 180 cm long 170 cm wide 170 cm high; the chemical and fabric specifications of each LLIN product are shown in Table 1.

Field collections

Net sampling was performed at baseline, 12 months, and 25 months post-distribution. At baseline, a total of 20 nets were retained (5 of each

LLIN product) from the LLINs that were to be distributed during the campaign to be used as baseline samples. Post-distribution, at 12 and 25 months, 100 LLINs of each type were collected from houses enrolled in the community survey (across the 104 clusters). This sample size was a pragmatic decision based on available human capacity and estimated processing time, and on availability of replacement nets.

Nets were sampled and exchanged for a new net of the same type. Nets were identified as part of the study by a unique ID number (net ID) attached to each net. If no study net was found at the selected household or the net was an unexpected type, then the next household on the reserve list was sampled instead. No more than one net per household was sampled. Information on the construction of the dwelling was recorded, with the household categorised as ‘improved’ if it had both brick walls and an iron roof. Otherwise, the dwelling was categorised as

‘traditional’.

On collection, sampled nets were labelled and placed individually in zip-lock bags. All sampled nets were transported to the project office in Bugembe, Jinja, Uganda, for physical assessment and processing. After physical measurements were recorded, seven 30 30 cm pieces were cut from each net (one from centre of each side panel and three from the top) and samples sent to

the Liverpool School of Tropical Medicine (Liverpool, UK) for chemical and bioefficacy assessment.

Physical integrity

To assess the physical integrity of the net fabric, nets were placed over a metal frame measuring W160 L180 H170 cm and any holes > 0.5 cm recorded (Lorenz et al., 2014). The size of a hole was defined by its length (the longest dimension) and width (measurement perpendicular to length measurement). Holes smaller than 0.5 cm (in length or width) and holes that had been repaired were noted but not included in the final dataset. Hole size was calculated using the formula for an ellipse (area $\frac{1}{4}\pi$ length width). The total area of damage on a net was summed and used to categorise the net within the WHO proportionate Hole Index (pHI) categories: 'good' (0–64 cm²), 'damaged' (65–642 cm²); or 'too torn' (643 cm²) (WHO, 2013b). Additionally, the proportion of nets of each LLIN product with at least one hole was calculated for each time-point.

Following physical integrity testing, two 30 × 30 cm square net pieces were sampled from the top of each LLIN for bioefficacy and chemical assessment. The rationale for using pieces cut from the top for chemical and bioefficacy testing was to allow fair comparison with PermaNet 3.0 which has PBO on the roof only, as well as literature indicating that *Anopheles gambiae* (s.l.) activity around an occupied bednet is focussed primarily on the top surface (Lynd & McCall, 2013; Sutcliffe & Yin, 2014, 2021). The samples were wrapped in aluminium foil and stored at room temperature prior to use in WHO cone bioassays. Samples were subsequently stored at 4 °C until chemical content and bioefficacy was assessed.

Chemical integrity

To quantify the content of active ingredients, chemical analysis was performed using high-performance liquid chromatography (HPLC) after extraction in 10% 1-propanol in heptane. A total of 30 nets of each LLIN type were analysed at each time-point, with two samples taken from each net.

The HPLC analysis was performed on an Agilent 1100 Series machine (Agilent, California, USA) at a

wavelength of 226 nm, using a modification of the methods published by Ngufor et al. (2022). Quantities of permethrin, deltamethrin and piperonyl butoxide were calculated by comparison to standard curves of each compound

(PESTANAL[®], analytical standard, Sigma-Aldrich, Missouri, USA) and corrected against internal standard dicyclohexyl phthalate (DCP). HPLC data were analysed using OpenLAB software v2.1 (Agilent, California, USA).

WHO cone bioassays

To assess bioefficacy, WHO cone bioassays were performed using the protocol outlined in the WHO durability monitoring guidelines (WHO, 2011, 2013a).

Bioefficacy testing was performed on the same nets assessed for chemical content. The two pieces from each net were each tested in duplicate, thus a total of four cone exposures were performed per net. Cone bioassay design followed the WHO protocol, with the testing board angled at 45° (WHO, 2011; Owusu & Müller, 2016). Ambient conditions in the testing room were targeted to a temperature of 27 ± 2 °C and a relative humidity of 80 ± 10%. All mosquitoes used were 3–5-day-old unfed females, reared in temperature and humidity-controlled insectaries. Each exposure lasted 3 minutes, with 7 mosquitoes per cone. Thus, 24 mosquitoes were used in each cone exposure assay per net piece for each mosquito strain.

Two different mosquito strains were used in the cone bioassays: 'Kisumu' and 'Busia'. 'Kisumu' is a pyrethroid-susceptible strain of *An. gambiae* collected in 1975 from what is now Kisumu County (formerly Kisumu District), in western Kenya. 'Busia' is a strain established in November 2018 from mosquitoes collected in Busia, eastern Uganda, by Ambrose Oruni. This strain has been previously characterised as possessing resistance to pyrethroids through both target site alterations (Vgsc-1014S) and metabolic resistance mechanisms (Cyp4j5, Cyp6aa1 and Coeae1d) (Lynd et al., 2019; Njoroge et al., 2021). WHO tube assays with standard discriminating doses indicate 'Busia' is more resistant to permethrin than deltamethrin (Supplementary Fig. S1).

WHO bioefficacy criteria are defined as the proportion of nets that achieve either 80% mortality or 95% knockdown against pyrethroidsusceptible *An. gambiae* (s.s.) mosquitoes. An LLIN product was considered to have passed if 80% of nets met these criteria at all time-points up to 24 months. Chemical and physical integrity data are not included in bioefficacy criteria.

WHO wireball assays

Given previous literature indicating that WHO cone bioassays are insufficient to assess the bioefficacy of LLIN products containing insecticides with high contact irritancy (WHO, 2006, 2011; Okumu et al., 2012), such as permethrin, supplemental WHO wireball assays were performed on the same samples used in the WHO cone bioassays. The purpose of this secondary testing was to assess bioefficacy under conditions where there were no surfaces on which the mosquito could rest to avoid contact (such as the cone itself in the WHO cone assay). While the WHO Tunnel test is recommended as a secondary assay for assessing nets with high contact irritancy, the present study could not undertake this technique due to the ethical issues surrounding the use of small mammals as bait.

In the WHO wireball method, the net to be tested is affixed around a wire cube measuring 15 × 15 × 15 cm (WHO, 2006). As in the cone bioassay, seven 3–5-day-old females were released into the wireball for three minutes then assessed for 1 h knockdown and 24 h mortality.

Data analysis

Data analyses were conducted using R (version 3.6.0), all graphs were produced using the ggplot2 package (version 3.2.1). Associations between outcomes and variables of interest were quantified using generalized linear mixed models (GLMMs) using the lme4 package (version 1.1-21). To account for unexplained variation between separate pieces from individual nets and between clusters, the net ID (a unique identifier for each net distributed) and HSD number were each included in the models as a random effect. The model selection process used stepwise regression, working backwards from a maximally complex model to produce the most parsimonious fit. Variables that did not

significantly increase explanatory power, as indicated by log-likelihood ratio tests (LRTs) (lmerTest package, version 0.9-37), were excluded from the final model. All possible interactions between variables were considered in the model selection process; for succinctness, only significant interactions are presented. The P-values reported are the output of these LRTs. Pairwise comparisons between levels within a categorical variable were performed using least square means with the lsmeans package (version 2.30-0).

To quantify the relationship between chemical integrity and bioefficacy, the HPLC outputs for each net were combined with their corresponding WHO cone assay or WHO wireball assay mortality data (for PermaNet 3.0 and Olyset Plus, respectively). A GLMM was then fit separately to the PermaNet 3.0 and Olyset Plus data, with pyrethroid content and PBO content each fit as a fixed effect. Model selection and Pvalue reporting was performed as above. The 3D plots were produced using the plot3D package (version 1.4).

Results

Physical integrity

Proportion of nets in each pHl category

At 12 months post-distribution, the proportion of nets classified as 'too torn' on the pHl scale was 0.066 (Fig. 1A), with this proportion approximately doubling after 25 months (Fig. 1B) to 0.125 (OR: 2.017, 95% CI: 1.268–3.208, $P < 0.001$; Supplementary Table S2). There was no significant difference in the proportion of nets that were 'too torn' between LLIN products ($P = 0.644$).

When categorised by the type of housing they were collected from, it was observed that nets from traditional housing were more likely to be in poor physical condition than those from improved housing (OR: 3.350, 95% CI: 1.865–6.016, $P = 0.003$; Supplementary Table S2). After 25 months in operational use, the proportion of nets from traditional housing categorised as 'too torn' was 0.297 compared to 0.112 for improved housing (Supplementary Fig. S2).

Proportion of nets with at least one hole

The proportion of nets of each type with at least one hole at 12- and 25 months post-distribution is shown in Fig. 1C. The overall proportion

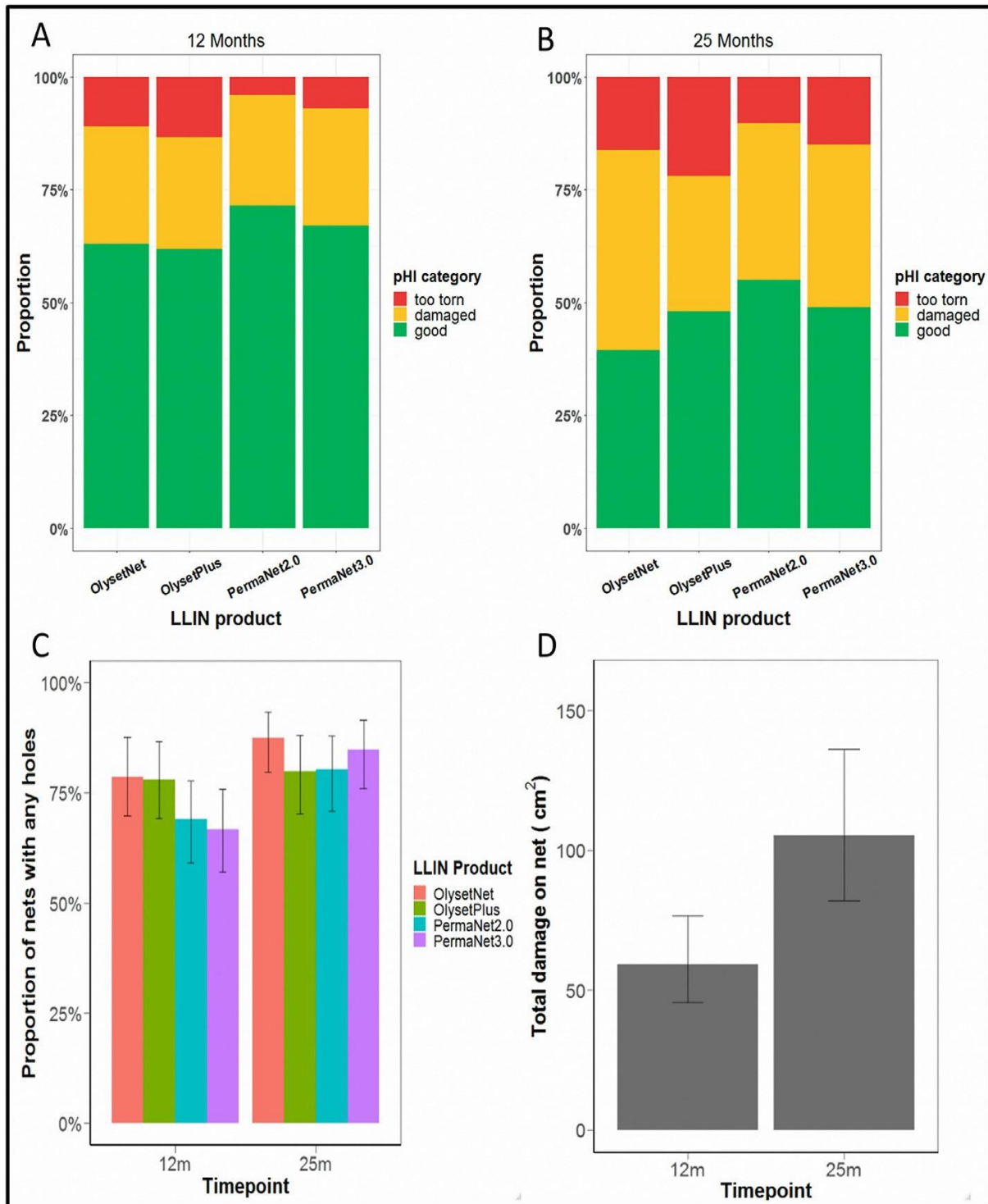


Fig. 1. Physical integrity outcomes at 12 and 25 months post-distribution. A Percentage of collected nets in each pH category ('too torn', 'damaged', 'good') at 12 months. B Percentage of collected nets in each pH category at 25 months. C Percentage of nets with at least one hole. D Mean total surface area of damage per net at 12 and 25 months post-distribution across all LLIN products.

of nets with at least one hole after 12 months in operational conditions was 0.727, increasing to 0.829 after 25 months (OR: 1.821, 95% CI: 1.289–2.571, $P < 0.001$). There was no difference in the proportion of nets with at least one hole between the four LLIN products tested at any time-point ($P \geq 0.306$).

Total surface area of holes

There was no difference in total hole area between any of the four LLIN products tested ($P \geq 0.270$). However, across all net types there was an overall increase in holed area from 12 months post-distribution to 25 months post-distribution ($P \geq 0.0005$; [Fig. 1D](#)), which approximately doubled from 59.33 cm² (95% CI: 45.08

83.43–136.86).

Chemical integrity

At baseline, all net samples tested met or exceeded the minimum target dose of active ingredients per their respective manufacturer specifications ([Table 2](#)).

Deltamethrin

The deltamethrin content of PermaNet 3.0 was lower at each subsequent time-point ($P < 0.001$; [Fig. 2A](#)). In the period from baseline to 25 months, mean deltamethrin content of PermaNet 3.0 nets declined from 4.98 g/kg (95% CI: 4.08–6.01) to 3.48 g/kg (95% CI: 3.19–3.78). Despite this, the deltamethrin content of all PermaNet 3.0 nets collected at 25 months remained within the range of the target dose (3.0–5.0 g/kg). For PermaNet 2.0, mean deltamethrin content after 25 months was not statistically different from baseline ($P \geq 0.071$).

Permethrin

The permethrin content of Olyset Plus varied across the sampled timepoints ($P < 0.001$; [Fig. 2B](#)) however pairwise comparison indicated no overall difference between baseline and the final time-point at 25 months ($P \geq 0.591$). Mean permethrin content in Olyset Plus at baseline was 16.08 (95% CI: 13.70–18.62), declining

to 14.54 (95% CI: 13.64–15.35) after 12 months, then increasing to 17.39 (95% CI: 16.53–18.22) after 25 months. A similar pattern was observed for Olyset Net, with permethrin content varying across time-points overall ($P < 0.001$), yet pairwise comparison indicating no overall difference between baseline and the 25-month time-point ($P \geq 0.327$).

PBO

The PBO content of PermaNet 3.0 declined across the sampled timepoints ($P < 0.001$; [Fig. 2C](#)). PBO content for PermaNet 3.0 at baseline was 26.81 g/kg (95% CI: 22.80–31.07) before declining sharply to 15.28 g/kg (95% CI: 13.74–16.71) after 12 months ($P \geq 0.001$), then falling further to 11.03 g/kg (95% CI: 9.35–12.67) after 25 months ($P \geq 0.001$).

A similar downwards trend in PBO was observed for Olyset Plus across time-points ($P < 0.001$). At baseline mean PBO content was

8.17 g/kg (95% CI: 6.51–9.82) before declining to 5.03 g/kg (95% CI: 4.37–5.74) after 12 months ($P \geq 0.002$). From 12 months to 25 months

Table 2

post-distribution, PBO content further fell to 3.66 g/kg (95% CI:

2.97–4.28, $P \geq 0.013$).

Bioefficacy

Cone bioassay: pyrethroid-susceptible *An. gambiae*

All LLINs were effective per WHO definition against the pyrethroid-susceptible 'Kisumu' strain (defined as achieving either 95% knockdown or 80% mortality), both when new and 12 months post-distribution. Overall mean cone mortality was 96.93% (95% CI: 95.77–98.10%) at baseline. Adjusted cone mortality was statistically indistinguishable between LLIN products ($P \geq 0.522$) and did not vary significantly between time-points ($P \geq 0.589$).

Cone bioassay: pyrethroid-resistant *An. gambiae*

Bioefficacy against the pyrethroid-resistant strain in cone assays varied between PBO-LLINs. Knockdown for PermaNet 3.0 remained very high throughout, achieving 99.7% (95% CI: 97.26–99.65; Fig. 3A) at baseline and remaining stable to 12 months ($P = 0.441$), though declining to 78.57% (95% CI: 63.57–93.58%, $P < 0.001$) after 25 months. PermaNet 3.0 was fully lethal against the pyrethroid-resistant strain when new, but mortality declined with operational use, falling by 26.8% (95% CI: 16.28–37.33%) for each year in the field ($P < 0.001$; Fig. 3B). In comparison, both mortality and knockdown with PermaNet 2.0 against the pyrethroid-resistant strain was very low at all time-points (3% and 6% respectively).

Knockdown with Olyset Plus was 46.98% (95% CI: 18.55–79.13%) when new but fell considerably to 3.54% (95% CI: 0.7–10.54%) after two years ($P = 0.005$). Mortality with Olyset Plus in cone assays was low throughout, killing 12.19% (95% CI: 5.45–17.01%) at baseline and 3.34% (95% CI: 0–8.71%) after two years but with no significant difference between time-points ($P = 0.226$; Fig. 3B). Knockdown and mortality with Olyset Net was low at all time-points (9% and 6% respectively).

Wireball assay: pyrethroid-resistant *An. gambiae*

Due to the unexpectedly low bioefficacy of Olyset Plus in the WHO cone assay, the same net samples were assessed in WHO wireball assays. Olyset Net was also assessed in wireball assays for comparison.

In the wireball assay, Olyset Plus knocked down 98.93% (95% CI: 94.43–100%; Fig. 4A) of pyrethroid-resistant mosquitoes at baseline. After 12 months knockdown had not significantly reduced (73.92%, 95% CI: 54.88–92.97%, $P = 0.376$); however, there was an overall decline to 45.72% (95% CI: 22.84–68.62, $P = 0.021$) after 25 months. Mortality for Olyset Plus against the pyrethroid-resistant strain in wireball assays at baseline was similarly improved compared to the cone assay, killing 87.72% at baseline (95% CI: 77.68–97.76%; Fig. 4B). However, after 12 months mortality has declined to 44.15% (95% CI: 29.32–58.98%,

$P = 0.002$) though the subsequent decline to 25.92% (95% CI: 11.92–39.93%) at 25 months was not statistically significant ($P = 0.216$).

The bioefficacy of Olyset Net in the wireball assay was low at all sampled time-points, with overall mean knockdown and mortality 22% and 13.5% respectively.

Mean chemical content (in g/kg) for each active ingredient in each LLIN product at baseline, 12 months, and 25 months post-distribution. Values in parentheses indicate 95% confidence interval

Active ingredient	LLIN product	Time-point		
		Baseline	12 months	25 months
Deltamethrin	PermaNet 2.0	1.3 (0.8–1.9)	1.1 (0.9–1.3)	0.7 (0.5–0.9)
	PermaNet 3.0	5.0 (4.1–5.9)	4.2 (4.0–4.5)	3.5 (3.2–3.8)
Permethrin	Olyset Net	19.5 (19.9–21.1)	17.0 (16.4–17.6)	18.2 (17.6–18.7)
	Olyset Plus	16.1 (13.6–18.5)	14.5 (13.7–15.4)	17.4 (16.5–18.3)
PBO	PermaNet 3.0	26.8 (22.9–30.7)	15.3 (13.7–16.9)	11.0 (9.4–12.7)
	Olyset Plus	8.2 (6.7–9.8)	5.0 (4.4–5.7)	3.7 (3.0–4.3)

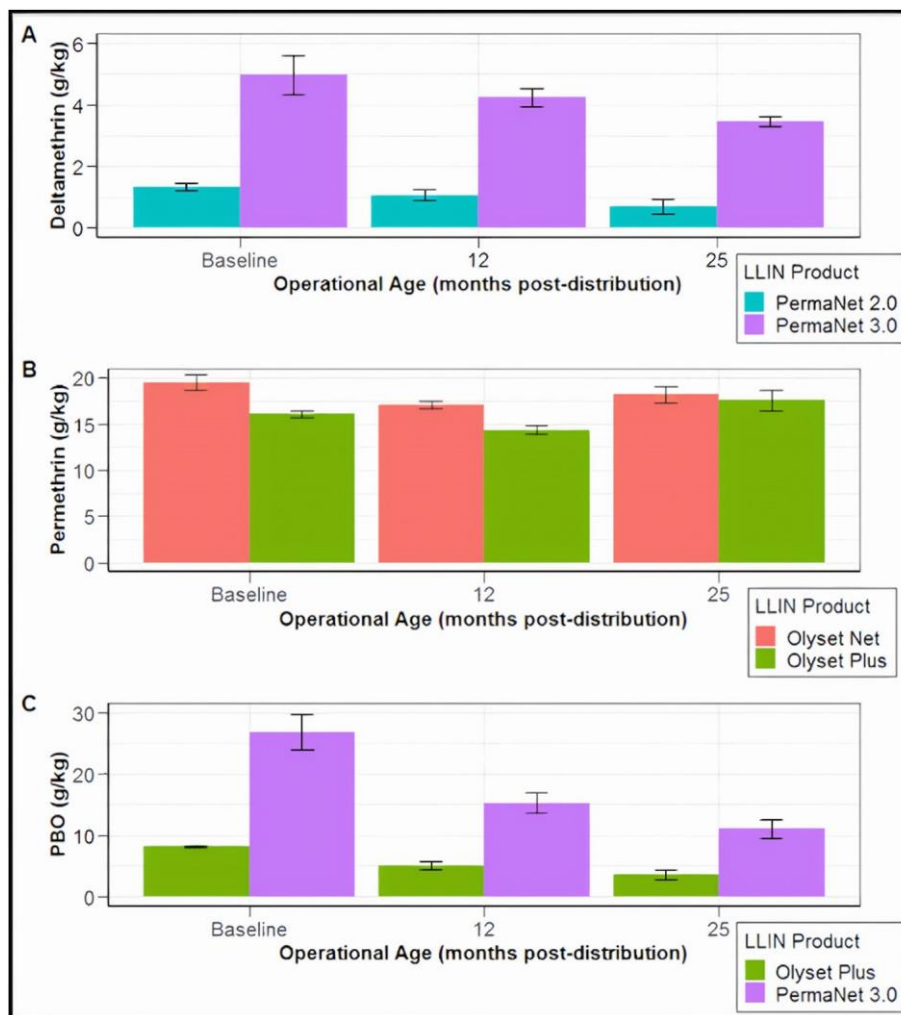


Fig. 2. Mean concentration of deltamethrin (A), permethrin (B) and PBO (C) detected in net samples at each sampled time-point (measured using HPLC). Error bars indicate 95% confidence intervals.

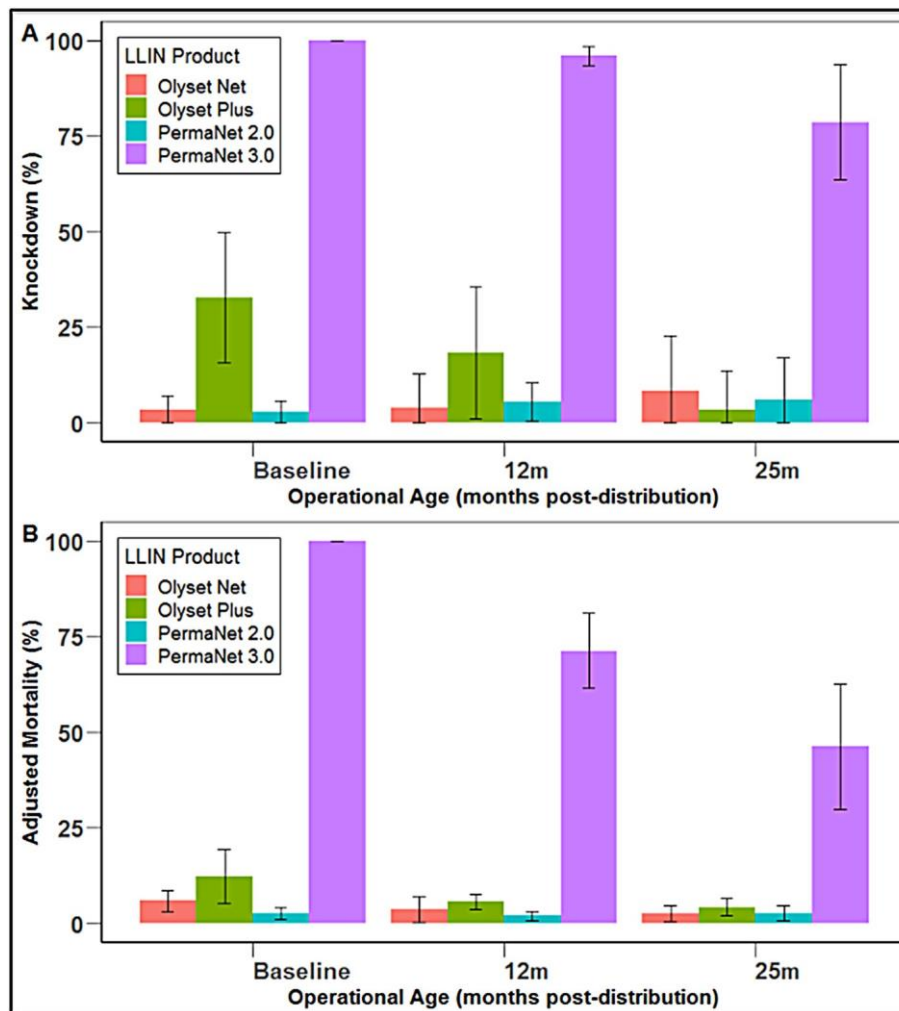
Relationship between chemical integrity and bioefficacy

The relationship between chemical integrity and predicted mortality for the pyrethroid-resistant *An. gambiae* (s.s.) 'Busia' line is shown in Fig. 5. For PermaNet 3.0 in the WHO cone bioassay, mortality was dependent on both total deltamethrin content and total PBO content, as indicated by a significant interaction between the two variables ($P < 0.001$; Fig. 5A). Modelling indicated there is a non-linear association between PBO content and mortality, with mortality falling more sharply with each consecutive g/kg of PBO that is lost (Fig. 5C). When the deltamethrin value was fixed at the mean of the data (4.42 g/kg), a reduction in PBO from 25 g/kg to 15 g/kg resulted in predicted mortality falling from 98% to 90%. Furthermore, a reduction in PBO content from 15 g/kg to 5 g/kg

resulted in a decline in predicted mortality from 90% to 57%. Consequently, the model predicted that to achieve 80% mortality against this pyrethroid-resistant mosquito strain a minimum of 11 g/kg PBO was needed.

For Olyset Plus in the WHO wireball bioassay, mortality had no statistical relationship with total permethrin content ($P = 0.583$) and was instead directly correlated with total PBO content ($P < 0.001$; Fig. 5B). Modelling indicated there was a linear association between PBO content and predicted mortality, with mortality falling by 11.12% for each g/kg PBO that is lost (Fig. 5D). The model predicted that to achieve 80% mortality against this strain, a minimum of 7.7 g/kg PBO was needed.

Discussion



Physical integrity

There was no difference in physical integrity outcomes between any of the four LLINs tested after 25 months in operational conditions. Thus, PBO-LLINs nets were as physically durable as their pyrethroid-only equivalents. Furthermore, it was observed that nets sampled from 'traditional' thatched-roof housing were almost three times more likely to be in the most severely damaged category than nets from 'improved' iron-roofed housing. While this disparity may be associated with the housing structure itself (such as the presence of straw), housing type may in fact be an indicator of other household variables such as the construction of the bed frame, the presence of animals indoors, or the type of cooking

material used in the household (Gnanguenon et al., 2014). More generally, these household variables are expected to be indicative of overall socioeconomic status which may impact an individual's day-to-day behaviour and use of their net. Nonetheless, there may be an argument to distribute nets more frequently than three years in regions where traditional housing remains common. It should be noted that the net attrition rate was high, with adequate coverage of LLINs (one LLIN for every two residents) decreasing from 71% at baseline to 35% after 25 months (Maiteki-Sebuguzi et al., unpublished data), indicating that LLIN attrition after distribution is an issue. If, as might be expected, individuals chose to discard damaged nets at a

Fig. 3. Mean knockdown (A) and adjusted mortality (B) in WHO cone bioassays with pyrethroid-resistant *An. gambiae* (s.s.) strain 'Busia' for each LLIN product tested at baseline, 12 months, and 25 months in the field.

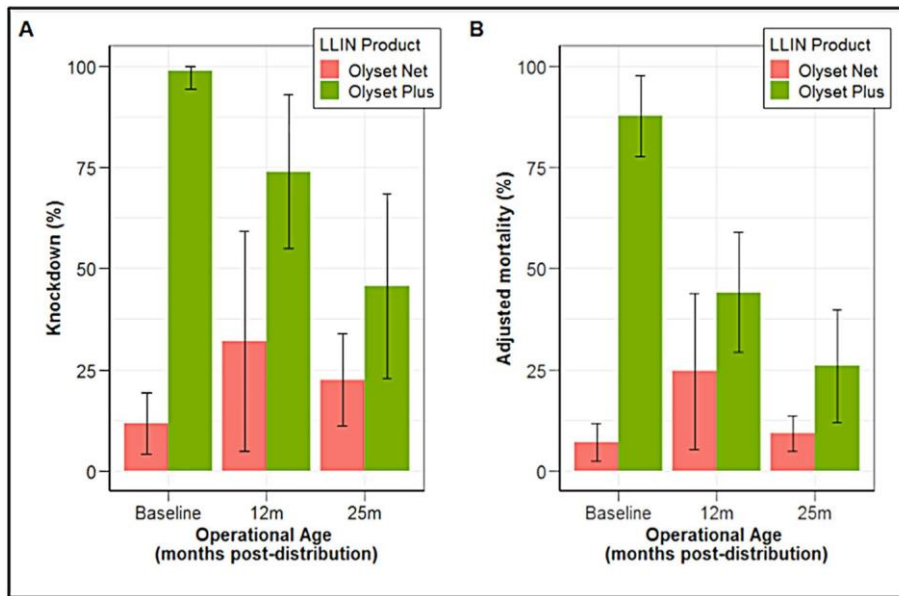


Fig. 4. Mean knockdown (A) and adjusted mortality (B) in WHO wireball assays with pyrethroid-resistant *An. gambiae* strain 'Busia' for Olyset Net and Olyset Plus at baseline, 12 months, and 25 months in the field.

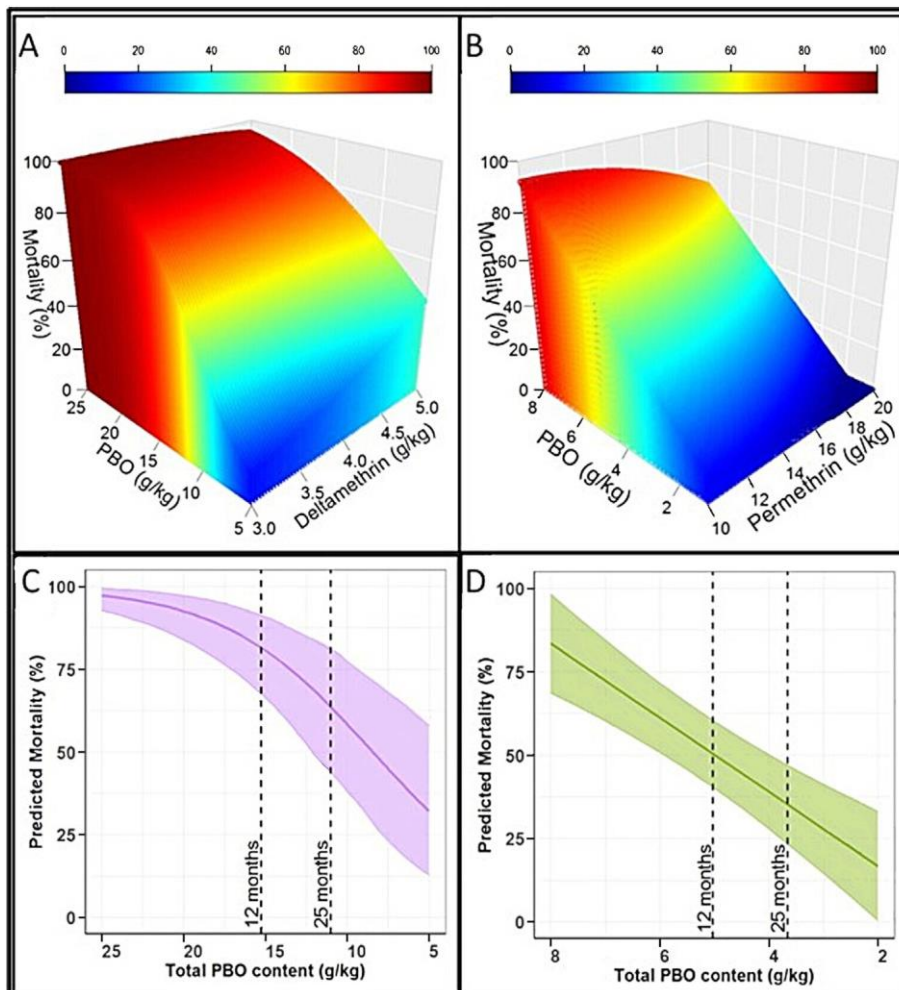


Fig. 5. Relationship between total chemical content and bioefficacy against pyrethroid-resistant *An. gambiae* (s.s.). A PermaNet 3.0 in WHO cone bioassays. B Olyset Plus in WHO wireball bioassays. C PermaNet 3.0 in WHO cone with deltamethrin value fixed at mean (4.42 g/kg). D Olyset Plus in WHO wireball with permethrin

value fixed at mean (15.45 g/kg).

higher rate than nets in good condition, then the physical damage observed in the present study may be an underestimate.

The current physical integrity outputs outlined in the WHO durability guidelines cannot be directly interpreted in terms of personal and community protection from mosquito bites. There is a need to better understand the impact of declining physical integrity on both mosquito blood-feeding inhibition and mortality. There is empirical evidence that damage to pyrethroid LLINs reduces personal protection from bites, but that mortality is independent from holed surface area and instead dependent on resistance status (Randriamaherijaona et al., 2015). Consequently, damaged LLINs would be expected to retain community effect against mosquito populations that are susceptible to their chemistry. Despite this, the median retention time of LLINs is well below three years in many settings (1.64 years across sub-Saharan Africa and 1.66 years for Uganda) (Bertozzi-Villa et al., 2021). Given evidence that perception of physical integrity is the primary consideration in retention (Koenker et al., 2014), developing more durable LLIN products may have epidemiological impacts beyond what would be indicated by studies of mosquito behaviour, due to improved retention.

In the current WHO durability guidelines, the location of holes on the net surface is not factored into categorisation of net condition by proportionate Hole Index. Recent behavioural experiments demonstrate that *An. gambiae* host-seeking activity occurs primarily on the top surface of the LLIN (Lynd & McCall, 2013; Sutcliffe & Yin, 2014, 2021; Parker et al., 2015; Sutcliffe et al., 2017). This highlights an important knowledge gap in the relationship between hole location on a net and the probability of mosquito entry and net effectiveness.

Chemical integrity

The pyrethroid content of the LLINs assessed was relatively stable across the two years of the study, with the exception of PermaNet 3.0 which declined by ~30% (yet was still within the manufacturer's target range). The stability of pyrethroids over two years observed here is consistent with studies from a range of settings (Lorenz et al., 2014, 2020; Toe et al., 2019). In contrast, the PBO content of both PBO-LLINs declined more rapidly over the same time period, with under half of the initial content remaining after 25 months. Nonetheless, despite this decline in PBO content, the concurrent trial of epidemiological outcomes in the study site demonstrated that PBO-LLINs maintained superior protection over their conventional equivalents up to 25 months (Staedke et al., 2020; Gleave et al., 2021).

While a strong correlation between total PBO content and bioefficacy was observed for both PBO-LLINs, this relationship may not be causal and total chemical content quantified by HPLC may not be representative of the concentration at the surface bioavailable to mosquitoes. There is currently a lack of tools for quantifying the concentration important for future studies seeking to link chemical composition to bioefficacy.

Bioefficacy

Both Olyset Plus and PermaNet 3.0 tested demonstrated superior bioefficacy against the pyrethroid-resistant strain than their pyrethroid-only equivalents. This observation is consistent with the previously reported finding that these nets reduced childhood parasitaemia in the study area where these nets were collected (Staedke et al., 2020). However, while both PBO-LLINs tested were highly effective against the pyrethroid-resistant strain at baseline, their bioefficacy diminished with

operational use (with the mortality associated with Olyset Plus and PermaNet 3.0 decreasing to 26% and 46%, respectively, after two years). The diminishing differential in bioefficacy between PBO-LLINs and their pyrethroid-only equivalents is also consistent with the observation that differential impact on childhood parasitaemia narrowed over the same time. The steep reduction in bioefficacy with both PBO-LLINs against a study site-specific pyrethroid-resistant strain is greatly concerning. These nets were distributed with the expectation they will be replaced after three years, yet these findings indicate that they have greatly diminished killing effect after the first two years. While the bioefficacy values themselves are specific to the 'Busia' strain, there is an urgent need to investigate if this downwards trend is observed in other settings. Given these findings, there is an argument that, within the Ugandan context, LLINs should be distributed on a two-rather than three-year cycle to maintain efficacy.

The low knockdown and mortality observed with Olyset Plus in the WHO cone bioassay was in strong contrast with the high bioefficacy observed with the same nets in the WHO wireball bioassay. This difference in outcomes between methodologies may be associated with the excitorepellency of permethrin, manifesting as reduced contact with the net surface. As the wireball method surrounds the mosquito on all sides with netting, there is no insecticide-free surface to rest on and a greater insecticidal effect is observed. Consequently, future investigations with excito-repellent LLINs may wish to also include an assay that prevents avoidance from the net, such as the WHO wire-ball assay (WHO, 2006). The WHO tunnel test would also address excito-repellency; however, in practice the aforementioned ethical issues prevent many institutes from performing it.

Conclusions

This LLIN durability study was conducted alongside a trial into the epidemiological effectiveness of PBO-LLINs in protecting against the bites of *Anopheles* mosquitoes in Uganda, where there is widespread pyrethroid resistance. Here, we demonstrate that both Olyset Plus and PermaNet 3.0 were as physically durable as their conventional equivalents and had superior bioefficacy against pyrethroid-resistant *An. gambiae* (s.s.) mosquitoes from the trial site. However, the superiority of PBO-LLINs over conventional LLINs in bioassays narrowed with the operational life of the net, correlating with a decline in PBO content. Additionally, we observed that nets collected from traditional thatched-roof housing were far more likely to be severely damaged than nets from improved iron-roofed housing. The diminished bioefficacy of PBO-LLINs against pyrethroid-resistant mosquitoes after just two years of operational use is of great concern and there is an urgent need to assess the durability of these LLIN products in other settings. Given these findings, we suggest that control programmes should consider distributing PBO-LLINs at more frequent intervals than three years and prioritise regions where traditional housing is common. Additionally, the contrasting performance of the same Olyset Plus nets in the WHO cone assay and the WHO wireball bioassay highlights that LLIN products with excitorepellent properties should be assessed with approaches that minimise avoidance from the net surface.

Funding

This project was funded primarily by the Against Malaria Foundation, with additional funding from the Department for International Development, the Innovative Vector Control Consortium, the Bill and Melinda Gates Foundation. The content of the manuscript is solely the responsibility of the authors.

Ethical approval

The trial was approved by the Uganda National Council for Science and Technology (UNCST Ref. HS 2176), Makerere University School of Medicine Research & Ethics Committee (SOMREC 2016-133), London School of Hygiene & Tropical Medicine Ethics Committee (LSHTM Ref. 12019), and the Liverpool School of Tropical Medicine (LSTM Ref. 16-072), which is the sponsoring institute.

CRediT author statement

Frank Mehan: Investigation, methodology, formal analysis, investigation, resources, data curation, visualization, writing - original draft. Agaba Katureebe: Investigation, methodology, supervision, project administration, data curation, resources, writing - original draft. Violet Tuhaise: Data curation, investigation, resources, supervision, writing review & editing. Martin Mugote: Investigation, resources, writing review & editing. Ambrose Oruni: Investigation, resources, writing review & editing. Ismail Onyige: Investigation, resources, writing - review & editing. Kawesa Bumali: Investigation, resources, writing - review & editing. Jonathan Thornton: Investigation, resources, methodology, writing - review & editing. Kilama Maxwell: Investigation, methodology, writing - review & editing. Mary Kyohere: Investigation, methodology, supervision, project administration, data curation, resources, writing - review & editing. Moses R. Kamya: Project administration, methodology, investigation, data curation, writing review & editing. Peter Mutungi: Data curation, investigation, resources, writing - review & editing. Simon P. Kigozi: Methodology, formal analysis, data curation, writing - review & editing. Adoke Yeka: Methodology, investigation, resources, data curation, project administration, writing - review & editing. Jimmy Opigo: Conceptualization, methodology, resources, writing - review & editing. Catherine MaitekiSebuguzi: Project administration, resources, data curation, writing review & editing. Samuel Gonahasa: Conceptualization, supervision, methodology, project administration, writing - review & editing. Janet Hemingway: Conceptualization, supervision, methodology, writing review & editing. Grant Dorsey: Conceptualization, formal analysis, methodology, writing - review & editing. Lisa J. Reimer: Supervision, methodology, writing - review & editing. Sarah G. Staedke: Conceptualization, supervision, methodology, project administration, writing review & editing. Martin J. Donnelly: Conceptualization, supervision, methodology, writing - original draft. Amy Lynd: Conceptualization, supervision, investigation, writing - original draft, methodology, formal analysis, investigation, resources, data curation, visualization. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crpvbd.2022.100092>.

References

- Alout, H., Dabire, R.K., Djogbenou, L.S., Abate, L., Corbel, V., Chandre, F., Cohuet, A., 2016. Interactive cost of Plasmodium infection and insecticide resistance in the malaria vector *Anopheles gambiae*. *Sci. Rep.* 6, 29755.
- Asidi, A., N'Guessan, R., Akogbeto, M., Curtis, C., Rowland, M., 2012. Loss of household protection from use of insecticide-treated nets against pyrethroid-resistant mosquitoes. *Benin. J. Emerg. Infect. Dis.* 18, 1101.
- Bertozzi-Villa, A., Bever, C.A., Koenker, H., Weiss, D.J., Vargas-Ruiz, C., Nandi, A.K., et al., 2021. Maps and metrics of insecticide-treated net access, use, and nets-percapita in Africa from 2000-2020. *Nat. Commun.* 12, 3589.
- Bhatt, S., Weiss, D., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., et al., 2015. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature* 526, 207-211.
- Churcher, T.S., Lissenden, N., Griffin, J.T., Worrall, E., Ranson, H., 2016. The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *eLife* 5, e16090.

- Darriet, F., Chandre, F., 2011. Combining piperonyl butoxide and dinotefuran restores the efficacy of deltamethrin mosquito nets against resistant *Anopheles gambiae* (Diptera: Culicidae). *J. Med. Entomol.* 48, 952–955.
- Gimnig, J.E., Lindblade, K.A., Mount, D.L., Atieli, F.K., Crawford, S., Wolkon, A., et al., 2005. Laboratory wash resistance of long-lasting insecticidal nets. *Trop. Med. Health* 10, 1022–1029.
- Gleave, K., Lissenden, N., Chaplin, M., Choi, L., Ranson, H., 2021. Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. *Cochrane Database Syst. Rev.* 5, CD012776.
- Gnanguenon, V., Azondekon, R., Oke-Agbo, F., Beach, R., Akogbeto, M., 2014. Durability assessment results suggest a serviceable life of two, rather than three, years for the current long-lasting insecticidal (mosquito) net (LLIN) intervention in Benin. *BMC Infect. Dis.* 14, 69.
- Hemingway, J., Ranson, H., Magill, A., Kolaczinski, J., Fornadel, C., Gimnig, J., et al., 2016. Averting a malaria disaster: will insecticide resistance derail malaria control? *Lancet* 387, 1785–1788.
- Irish, S., N'guessan, R., Boko, P., Metonnon, C., Odjo, A., Akogbeto, M., Rowland, M., 2008. Loss of protection with insecticide-treated nets against pyrethroid-resistant *Culex quinquefasciatus* mosquitoes once nets become holed: an experimental hut study. *Parasit. Vectors* 1, 17.
- Koenker, H., Kilian, A., de Beyl, C.Z., Onyefunafoa, E.O., Selby, R.A., Abeku, T., et al., 2014. What happens to lost nets: a multi-country analysis of reasons for LLIN attrition using 14 household surveys in four countries. *Malar. J.* 13, 464.
- Lorenz, L.M., Bradley, J., Yukich, J., Massue, D.J., Mageni, Z., Pigeon, O., et al., 2020. Comparative functional survival and equivalent annual cost of 3 long-lasting insecticidal net (LLIN) products in Tanzania: a randomised trial with 3-year follow up. *PLoS Med.* 17, e1003248.
- Lorenz, L.M., Overgaard, H.J., Massue, D.J., Mageni, Z.D., Bradley, J., Moore, J.D., et al., 2014. Investigating mosquito net durability for malaria control in Tanzania-attrition, bioefficacy, chemistry, degradation and insecticide resistance (ABCDR): study protocol. *BMC Publ. Health* 14, 69.
- Lynd, A., Gonahasa, S., Staedke, S.G., Oruni, A., Maiteki-Sebuguzi, C., Dorsey, G., Opigo, J., et al., 2019. LLIN Evaluation in Uganda Project (LLINEUP): a cross-sectional survey of species diversity and insecticide resistance in 48 districts of Uganda. *Parasit. Vectors* 12, 94.
- Lynd, A., McCall, P.J., 2013. Clustering of host-seeking activity of *Anopheles gambiae* mosquitoes at the top surface of a human-baited bed net. *Malar. J.* 12, 267.
- Ngufor, C., Fagbohoun, J., Agbevo, A., Ismail, H., Challenger, J.D., Churcher, T.S., Rowland, M., 2022. Comparative efficacy of two pyrethroid-piperonyl butoxide nets (Olyset Plus and PermaNet 3.0) against pyrethroid resistant malaria vectors: a noninferiority assessment. *Malar. J.* 21, 20.
- Njoroge, H., Van't Hof, A., Oruni, A., Pipini, D., Nagi, S.C., Lynd, A., 2021. Identification of a rapidly-spreading triple mutant for high-level metabolic insecticide resistance in *Anopheles gambiae* provides a real-time molecular diagnostic for anti-malarial Intervention deployment. *bioRxiv*. <https://www.biorxiv.org/content/10.1101/2021.02.11.429702v2>.
- Okumu, F.O., Chipwaza, B., Madumla, E.P., Mbeyela, E., Lingamba, G., Moore, J., 2012. Implications of bio-efficacy and persistence of insecticides when indoor residual spraying and long-lasting insecticide nets are combined for malaria prevention. *Malar. J.* 11, 378.
- Owusu, H.F., Müller, P., 2016. How important is the angle of tilt in the WHO cone bioassay? *Malar. J.* 15, 1.
- Parker, J.E., Angarita-Jaimes, N., Abe, M., Towers, C.E., Towers, D., McCall, P.J., 2015. Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. *Sci. Rep.* 5, 13392.
- Pryce, J., Richardson, M., Lengeler, C., 2018. Insecticide-treated nets for preventing malaria. *Cochrane Database Syst. Rev.* 11, CD000363.
- Randriamaherijaona, S., Briët, O.L., Boyer, S., Bouraima, A., N'Guessan, R., Rogier, C., Corbel, V., 2015. Do holes in long-lasting insecticidal nets compromise their efficacy against pyrethroid resistant *Anopheles gambiae* and *Culex quinquefasciatus*? Results from a release-recapture study in experimental huts. *Malar. J.* 14, 332.
- Ranson, H., Lissenden, N., 2016. Insecticide resistance in African *Anopheles* mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends Parasitol.* 32, 187–196.
- Staedke, S.G., Gonahasa, S., Dorsey, G., Kanya, M.R., Maiteki-Sebuguzi, C., Lynd, A., et al., 2020. Effect of long-lasting insecticidal nets with and without piperonyl butoxide on malaria indicators in Uganda (LLINEUP): a pragmatic, cluster-randomised trial embedded in a national LLIN distribution campaign. *Lancet* 395, 1292–1303.
- Staedke, S.G., Kanya, M.R., Dorsey, G., Maiteki-Sebuguzi, C., Gonahasa, S., Yeka, A., et al., 2019. LLIN Evaluation in Uganda Project (LLINEUP) - impact of long-lasting insecticidal nets with, and without, piperonyl butoxide on malaria indicators in Uganda: study protocol for a cluster-randomised trial. *Trials* 20, 321.
- Strode, C., Donegan, S., Garner, P., Enayati, A.A., Hemingway, J., 2014. The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African anopheline mosquitoes: systematic review and meta-analysis. *PLoS Med.* 11, e1001619.
- Sutcliffe, J.F., Ji, X., Yin, S., 2017. How many holes is too many? A prototype tool for estimating mosquito entry risk into damaged bed nets. *Malar. J.* 16, 304.
- Sutcliffe, J.F., Yin, S., 2014. Behavioural responses of females of two anopheline mosquito species to human-occupied, insecticide-treated and untreated bed nets. *Malar. J.* 13, 294.
- Sutcliffe, J.F., Yin, S., 2021. Effects of indoor air movement and ambient temperature on mosquito (*Anopheles gambiae*) behaviour around bed nets: implications for malaria prevention initiatives. *Malar. J.* 20, 427.
- Toe, K.H., Mechan, F., Tangena, J.-A.A., Morris, M., Solino, J., Tchicaya, E.F., et al., 2019. Assessing the impact of the addition of pyriproxyfen on the durability of permethrin-treated bed nets in Burkina Faso: a compound-randomized controlled trial. *Malar. J.* 18, 383.
- Viana, M., Hughes, A., Matthiopoulos, J., Ranson, H., Ferguson, H.M., 2016. Delayed mortality effects cut the malaria transmission potential of insecticide-resistant mosquitoes. *Proc. Natl. Acad. Sci. U.S.A.* 113, 8975–8980.
- WHO, 2006. Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/69296>.
- WHO, 2011. Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/44610>.
- WHO, 2013a. Guidelines for laboratory and field-testing of long-lasting insecticidal nets. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/80270>.
- WHO, 2013b. WHO guidance note for estimating the longevity of long-lasting insecticidal nets in malaria control. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/338345?show%full>.
- WHO, 2016. Estimating functional survival of long-lasting insecticidal nets from field data. Vector Control Technical Expert Group Report to MPAC, September 2013. World Health Organization, Geneva. https://files.givewell.org/files/DWDA%2009/Interventions/Nets/LLIN_Equivalent_Coverage_Years/WHO_Vector_Control_Technical_Expert_Group_Report_to_MPAC_2013.pdf.
- WHO, 2017a. Conditions for deployment of mosquito nets treated with a pyrethroid and piperonyl butoxide: recommendations. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/258939>.
- WHO, 2017b. Report of the twentieth WHOPES working group meeting. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/258921>.

Appendix IV: Additional relevant works published during this PhD studentship

Assessing the impact of the addition of pyriproxyfen on the durability of permethrin-treated bed nets in Burkina Faso: a compound-randomized controlled trial

Toé KH, **Mechan F**, Tangena JA, Morris M, Solino J, Tchicaya EF, Traoré A, Ismail H, Maas J, Lissenden N, Pinder M. *Malaria journal*. 2019 Dec;18(1):1-6.DOI: [10.1186/s12936-019-3018-1](https://doi.org/10.1186/s12936-019-3018-1)

The effects of temephos, permethrin and malathion selection on the fitness and fecundity of *Aedes aegypti*

Gleave K, **Mechan F**, Reimer LJ.. *Medical and veterinary entomology*. 2022 Mar;36(1):56-65.DOI: [10.1111/mve.12551](https://doi.org/10.1111/mve.12551)

The seasonal dynamics and biting behavior of potential *Anopheles* vectors of *Plasmodium knowlesi* in Palawan, Philippines

Malijan RP, **Mechan F**, Braganza JC, Valle KM, Salazar FV, Torno MM, Aure WE, Bacay BA, Espino FE, Torr SJ, Fornace KM. *Parasites & vectors*. 2021 Dec;14(1):1-6.
DOI: [10.1186/s13071-021-04853-9](https://doi.org/10.1186/s13071-021-04853-9)

RESEARCH

Open Access



Assessing the impact of the addition of pyriproxyfen on the durability of permethrin-treated bed nets in Burkina Faso: a compound-randomized controlled trial

Kobié H. Toé^{1†}, Frank Mechan^{2†}, Julie-Anne A. Tangena^{2†}, Marion Morris², Joanna Solino², Emile F. S. Tchicaya³, Alphonse Traoré¹, Hanafy Ismail², James Maas², Natalie Lissenden², Margaret Pinder^{4,5}, Steve W. Lindsay⁴, Alfred B. Tiono¹, Hilary Ranson^{2*}  and N'Falé Sagnon¹

Abstract

Background: Long-lasting insecticidal nets (LLINs) treated with pyrethroids are the foundation of malaria control in sub-Saharan Africa. Rising pyrethroid resistance in vectors, however, has driven the development of alternative net formulations. Here the durability of polyethylene nets with a novel combination of a pyrethroid, permethrin, and the insect juvenile hormone mimic, pyriproxyfen (PPF), compared to a standard permethrin LLIN, was assessed in rural Burkina Faso.

Methods: A compound-randomized controlled trial was completed in two villages. In one village 326 of the PPF-permethrin nets (Olyset Duo) and 327 standard LLINs (Olyset) were distributed to assess bioefficacy. In a second village, 170 PPF-permethrin nets and 376 LLINs were distributed to assess survivorship. Nets were followed at 6-monthly intervals for 3 years. Bioefficacy was assessed by exposing permethrin-susceptible and resistant *Anopheles gambiae* sensu lato mosquito strains to standard World Health Organization (WHO) cone and tunnel tests with impacts on fertility measured in the resistant strain. Insecticide content was measured using high-performance liquid chromatography. LLIN survivorship was recorded with a questionnaire and assessed by comparing the physical integrity using the proportionate hole index (pHI).

Results: The PPF-permethrin net met WHO bioefficacy criteria ($\geq 80\%$ mortality or $\geq 95\%$ knockdown) for the first 18 months, compared to 6 months for the standard LLIN. Mean mosquito mortality for PPF-permethrin nets, across all time points, was 8.6% (CI 2.6–14.6%) higher than the standard LLIN. Fertility rates were reduced after PPF-permethrin net exposure at 1-month post distribution, but not later. Permethrin content of both types of nets remained within the target range of 20 g/kg \pm 25% for 242/248 nets tested. The pyriproxyfen content of PPF-permethrin nets declined by 54%, from 10.4 g/kg (CI 10.2–10.6) to 4.7 g/kg (CI 3.5–6.0, $p < 0.001$) over 36 months. Net survivorship was poor, with only 13% of PPF-permethrin nets and 12% of LLINs still present in the original household after 36 months. There was no difference in the fabric integrity or survivorship between the two net types.

Conclusion: The PPF-permethrin net, Olyset Duo, met or exceeded the performance of the WHO-recommended standard LLIN (Olyset) in the current study but both net types failed the 3-year WHO bioefficacy criteria.

*Correspondence: Hilary.Ranson@lstm.ac.uk

[†]Kobié H. Toé, Frank Mechan, and Julie-Anne A. Tangena contributed equally to this work

²Vector Biology Department, Liverpool School of Tropical Medicine, Liverpool, UK

Full list of author information is available at the end of the article



© The Author(s) 2019. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated.

Keywords: *Anopheles gambiae*, Burkina Faso, Long-lasting insecticidal nets, Malaria control, Net durability, Olyset, Olyset Duo, Permethrin, Pyriproxyfen

Background

The massive deployment of long-lasting insecticidal nets (LLINs) across sub-Saharan Africa has been a major factor in the rapid decline of malaria cases in the first 15 years of this century [1]. In Burkina Faso, one of the countries with the highest malaria burden in Africa, a total of 29 million nets have been distributed during three rounds of national LLIN distributions in 2010, 2013 and 2016. Since the start of the net distribution programme malaria-related mortality has declined, yet the drop in number of malaria cases has stalled, with 7.7 million cases in 2015 and 7.9 million cases in 2017 [2]. In rural Burkina Faso, approximately 61% of the population are infected with malaria parasites at any one time [3]. Most LLINs distributed in Burkina Faso to date have been pyrethroid-only nets, with a small number of piperonyl butoxide (PBO) nets distributed in 2010 and 2013 [4]. Resistance to pyrethroid insecticides is widespread in African malaria vectors [5] and has reached exceptionally high levels in Burkina Faso [6]. In the Cascades region of Burkina Faso, the site of the current trial, pyrethroid-only LLINs are no longer effective at killing the local mosquito populations [7].

Efforts to maintain the efficacy of bed nets in areas of pyrethroid resistance have driven the development of alternative net types containing either insecticide synergists, multiple insecticides or insecticides plus insect growth regulators [8]. Data on the efficacy and durability of these 'next generation' nets are essential for future control and elimination of malaria. This study compared the bioefficacy and survivorship of novel manufactured nets containing both pyrethroid insecticide and the insect growth regulator pyriproxyfen (PPF) (Olyset Duo nets) with comparator nets containing the pyrethroid alone (Olyset nets). PPF is a World Health Organization (WHO) approved larvicide and is highly effective at inhibiting the emergence of mosquito larvae, although the practical limitations of delivering PPF to the aquatic habitats of anopheline mosquitoes at scale has limited its use in malaria control to date. PPF also interferes with the reproductive output of *Anopheles* spp. and can reduce the longevity of adult mosquitoes [9–11]. Encouraging results from experimental hut studies of Olyset Duo [12–14] led to the first clinical trial of a dual active bed net. A cluster randomized control field trial in an area of intense seasonal malaria transmission and high pyrethroid resistance in the local vector population, found that the PPF-permethrin net was protective

and reduced the clinical incidence of malaria in children by 12% compared to standard pyrethroid-only LLINs [7].

For any candidate LLIN to be recommended by WHO, it requires the demonstration that it is effective under operational conditions for at least 3 years. Following the WHO Pesticide Evaluation Scheme (WHOPES) guidelines, candidate LLINs are assessed on their bioefficacy, insecticide content, and survivorship [15–17]. The current study was undertaken to assess the durability of the PPF-permethrin Olyset Duo net, in the field compared to a standard, WHO approved, permethrin-only net (Olyset). The primary objective was to determine whether the bioefficacy of the PPF-permethrin net was superior to a standard permethrin LLIN over 36 months with secondary and tertiary objectives to compare the physical integrity, net survivorship and insecticide content over the course of the study. This is the first durability study evaluating the performance of 'next generation' nets under operational settings.

Methods

Study design and study area

A detailed description of the study protocol, which follows WHO guidelines for measuring the durability of LLINs, has been reported previously [18]. In this cluster-randomized controlled trial, the durability of a PPF-permethrin net (Olyset[®] Duo, 2% permethrin 1% pyriproxyfen, Sumitomo Chemical Ltd) was compared with a conventional pyrethroid LLIN (Olyset[®], 2% permethrin, Sumitomo Chemical Ltd) incorporated into high-density polyethylene fibres. Nets were 'Extra Family' size, rectangular nets (180 cm wide, 90 cm long, 150 cm high, 150 denier) manufactured with an enhanced knitting pattern that was introduced in 2013.

This study enrolled residents of two rural villages, Dalamba (10° 31' 6.07" N, 4° 18' 48.01" E) and Sanako (10° 36' 17.56" N, 4° 22' 22.17" E), in Sidéradougou health centre area in Mangodara District, the Cascades region of Burkina Faso. Both villages are approximately 6 km from the study sites of a large clinical trial of these nets [7]. Dalamba comprised of 156 households in 108 compounds and neighbouring Sanako had 111 households in 71 compounds. Compounds are small group of houses typically enclosed by a wall. The houses in the villages are made with mud or cement walls with thatched or metal roofs. At the beginning of this trial, informed consent was provided by the household heads for each household

enrolled (Additional file 1). Only one compound head (from Dalamba) refused to participate.

Clustering occurred at compound level with each compound randomly allocated either PPF-permethrin nets or permethrin-only LLINs. A total of 653 nets (326 PPF-permethrin and 327 LLINs) were distributed in Dalamba and 546 nets (170 PPF-permethrin and 376 LLINs) in Sanako from July to August 2014. The distribution of nets was not equal in Sanako as the number of nets was allocated according to the number of people in the compound. The householder and research team were blinded to the net type [18]. During the enrolment and informed consent procedures, householders were encouraged not to exchange the nets. In a separate program, a government-led national LLIN campaign distributed PermaNet 2.0 to all households in the study villages in July 2016, 2 years into the study described here.

In both villages, seven rounds of net sampling and surveys were performed over 36 months from September 2014 until September 2017. Nets were sampled 1 month after distribution and subsequently at 6, 12, 18, 24, 30 and 36 months. In Dalamba, nets were destructively sampled for bioefficacy testing and replacement nets provided. In Sanako, nets were left in situ for assessment of physical integrity and survivorship over the complete length of the trial. An outline of the study design is provided in Additional file 2.

Bioefficacy (Dalamba village)

Bed nets were sampled from Dalamba according to a pre-defined random sampling schedule [18]. The net number, household identification number, and name of the household head were used by the field team to identify nets for sampling. A total of 48 nets, designed to represent 24 of each net type, were targeted for each collection round but, due to high attrition rates, the total number of nets available to be sampled was consistently lower (Table 1). Three panels of 25 cm² from

each net, sampled from three different sides of the net were used in cone bioassays and tunnel bioassays at the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) insectaries in Banfora and Ouagadougou. A further four panels (five in round 1) of 30 cm² were sampled from each net and sent to Liverpool School of Tropical Medicine (LSTM) for additional insecticide content analysis, cone bioassays and fertility analysis.

Cone tests

Cone tests were performed at the Banfora and Ouagadougou insectaries by CNRFP using batches of five, three to 5-day old unfed *Anopheles gambiae* from the permethrin susceptible Kisumu strain. Ambient conditions in the testing room ranged from 27 ± 2 °C and 80 ± 10% relative humidity. Mosquitoes were exposed to the three 25 cm² panels sampled from each net in triplicates for 3 min. Each net panel was tested four times so that 20 mosquitoes were exposed to each panel, 60 to each net. Mosquitoes were provided a sugar meal post exposure. Knockdown was recorded after 60 min and mortality after 24 h. Four panels 30 cm² from the same nets were sent to LSTM and further cone bioassays performed on three of these panels. For cone bioassays at LSTM both Kisumu and Tiassalé 13 mosquito strains were used; Tiassalé 13 (hereafter Tiassalé) is a pyrethroid resistant strain of *An. gambiae* sensu lato originally from Côte d'Ivoire [19] in which pyrethroid resistance is conferred by a combination of target site and metabolic (cytochrome P450) mechanisms. Tiassalé mosquitoes surviving the cone bioassays were retained for blood feeding and assessed for fecundity as described below.

Table 1 Number of nets from Dalamba on which tests were performed in each sampling round

Time after deployment (months)	Chemical content LSTM		Kisumu cone bioassay CNRFP		Tunnel tests CNRFP		Kisumu cone bioassay LSTM		Tiassalé cone bioassay LSTM	
	PPF-permethrin	LLIN	PPF-permethrin	LLIN	PPF-permethrin	LLIN	PPF-permethrin	LLIN	PPF-permethrin	LLIN
1	24	24	24	24	0	0	24	24	24	24
6	22	21	24	21	0	2	22	21	22	21
12	21	23	22	22	1	5	21	23	21	23
18	17	14	17	14	4	7	3	1	17	14
24	15	19	14	20	16	19	14	16	14	16
30	12	12	17	16	12	12	9	3	8	2
36	12	12	20	12	16	10	0	0	0	0

Tunnel tests

Tunnel tests were performed at the CNRFP insectary in Ouagadougou on nets that did not reach the target of $\geq 95\%$ 1 h knockdown or $\geq 80\%$ 24 h mortality after exposure of susceptible mosquitoes in a cone test. Tunnel tests were performed according to WHO protocols [15] using a guinea pig as a host. 100 five to eight-day old unfed female Kisumu mosquitoes were introduced into the tunnel at 18.00 h and the test terminated at 09.00 h the following day. The number of blood fed and dead mosquitoes were counted. A control, using untreated netting, was run for each round. The numbers of nets tested in tunnel assays are shown in Table 1.

Reduction in offspring

Nets in Rounds 1–5 were tested for their effect on mosquito reproductive output. At 24 h post exposure, Tiassalé mosquitoes surviving the cone bioassay were offered a human blood meal on an artificial blood feeder (Hemotek, UK) in the dark for 45 min. Mosquitoes exposed to the same net were pooled before blood feeding (maximum of 60 mosquitoes per cup). Unfed mosquitoes were removed, and blood-fed females maintained with access to a sugar solution for a further 3 days. Mosquitoes were then transferred into individual oviposition tubes (flat bottom 30 ml cell culture tubes, Fisher) containing moist cotton wool, covered in filter paper. Tubes were covered with netting and cotton wool soaked in deionized water placed on the netting. Mosquitoes were left for a further 3 days to oviposit. Any filter papers containing eggs were removed and the number of eggs counted under a dissection microscope before floating in approximately 50 ml of water in plastic pots. TetraMin fish food (Tetra, Germany) was added as food for any larvae that hatched. The number of eggs hatched after 2 to 3 days was recorded. Oviposition rate (proportion of survived blood-fed mosquitoes that laid eggs), fecundity (number of eggs laid per survived blood-fed female), hatch rate (proportion of eggs that hatched) and fertility (number of hatched eggs per survived blood-fed female) were compared between mosquitoes exposed to untreated control nets, and mosquitoes exposed to either LLIN or PPF-permethrin nets.

Chemical analysis

High-performance liquid chromatography (HPLC) was used to measure the insecticide content of the PPF-permethrin nets and LLINs. Briefly, a representative sample with an area of 48 cm² (approximately 0.2 g net fibre) was cut in triplicates from the four panels of each net (total of 12 samples per net). Net samples were boiled at 85 °C for 45 min with 5 ml solution of 4% 1-propanol in

heptane containing 100 µg/ml of internal standard dicyclohexyl phthalate (DCP). 1 ml of the insecticide extract was transferred to a clean glass tube, evaporated to dryness at 40 °C, reconstituted in one ml of acetonitrile and centrifuged at 20,000×g for 15 min. HPLC analysis was performed by the injection of 10-µl aliquots of the samples on a reverse-phase Hypersil GOLD C18 column (75 Å, 250 × 4.6 mm, 5-µm particle size; Thermo Scientific) at room temperature. A mobile phase of 70% acetonitrile in water was used at a flow rate of 1 ml min⁻¹ to separate permethrin, pyriproxyfen and DCP. Chromatographic peaks of the insecticides and DCP were detected at a wavelength of 232 nm with the Ultimate 3000 UV detector and analysed with Dionex Chromeleon™ 6.8 Chromatography Data System software. Quantities of pyriproxyfen and permethrin were calculated from standard curves established by known concentrations of the insecticide authenticated standards and corrected by internal standard readings in each sample relative to control. Final insecticide content in gram per kilogram (g/kg) net material was estimated using the following equation:

$$I = \left(\frac{x}{a}\right) \times \left(\frac{0.005}{m}\right) \times C$$

where I is the insecticide content in g/kg, x is the insecticide peak area at 232 nm obtained from HPLC, a is the slope of insecticide standard curve, m is the mosquito net sample mass in gram and C is the internal standard correction factor calculated from dividing the peak area of 100 µg/ml DCP by the DCP peak area obtained for the unknown. The method accuracy coefficient of variation was lower than 10% for both permethrin and pyriproxyfen extracted from new nets.

Net survivorship (Sanako village)

In Sanako village, net survivorship was recorded during the seven rounds of data collection. Functional survivorship is defined as the proportion of nets still in households in serviceable condition and is calculated by measuring the total number of nets in 'good' + 'acceptable' condition (see below) hung over a bed × 100/total number of each net type distributed to surveyed households, excluding the number lost by attrition. At every compound, information on the presence of the distributed nets and their physical condition was recorded and the head of the household or an adult person from the family was asked a set of questions on the use of bed nets in the household (Additional file 3). Distributed nets were identified by a code written with indelible markers on the label of the net. Nets found at a different household than the one where they were originally distributed were excluded from the study.

Fabric integrity (Sanako village)

Fabric integrity was measured using the results from the questionnaire on the physical condition of the nets. The number of holes, their size and their location on the net was noted. These data were used to calculate the proportion of torn nets, proportion of nets with any holes and the proportionate Hole Index (pHI) as defined by the WHO [16] ($pHI = (1 \times \text{number of size-1 holes}) + (23 \times \text{number of size-2 holes}) + (196 \times \text{number of size-3 holes}) + (576 \times \text{number of size-4 holes})$). The $pHI \leq 64$ is defined as 'good', $pHI 65-642$ as 'acceptable' and $pHI \geq 643$ as 'torn'.

Data analysis

Data analyses were conducted using the R programming language (version 3.6.0) and plots produced with the ggplot2 package (version 3.2.1). Generalized Linear Mixed Models (GLMMs) (lme4 version 1.1-21) were used to identify factors significantly associated with bioefficacy, chemical analysis, fabric integrity and attrition. To account for unexplained variation between individual nets, the net ID was included in all models as a random effect. The model selection process used stepwise regression with the maximally complex model with all fixed variables and all two-way interactions fit. The contribution of each variable to the explanatory power of the model was evaluated using log-likelihood ratio tests (LRTs). The final model consisted of all explanatory variables which had statistically significant LRTs.

Mosquito mortality

Cone test and tunnel test mortality results were adjusted with Abbott's formula [20], and net types compared over time using GLMMs with a gaussian distribution.

Reduction in offspring

The impact of exposure to the different net types on fecundity and fertility was analysed using GLMM.

Chemical analysis

The mean concentration, and 95% confidence intervals, of permethrin and pyriproxyfen was calculated and the chemical content contained in the two net types over time was compared using GLMMs with a gaussian distribution.

Fabric integrity

The physical integrity of the two net types was compared using: (1) the proportion of torn nets (nets in

poor condition), (2) the proportion of nets with holes and (3) the proportionate hole index (pHI) [15, 16]. The following formula were used:

Proportion of torn nets = (Total number of each type of net where the nets are not long enough to be tucked under the mattress, or are torn or badly damaged, or have more than 5 holes (finger width, diameter approx. 2 cm)) / (Total number of each net type found and assessed in surveyed households).

Proportion of nets with any holes = (Total number of nets with any hole / Total number of each net type found and assessed in surveyed households).

Net survival

Survivorship of nets was compared using GLMM with a binomial logit link function. The response variable of this model was a binary variable in which a net is either: (1) in functional use at the allocated location or (0) not in functional use and/or not at the allocated location.

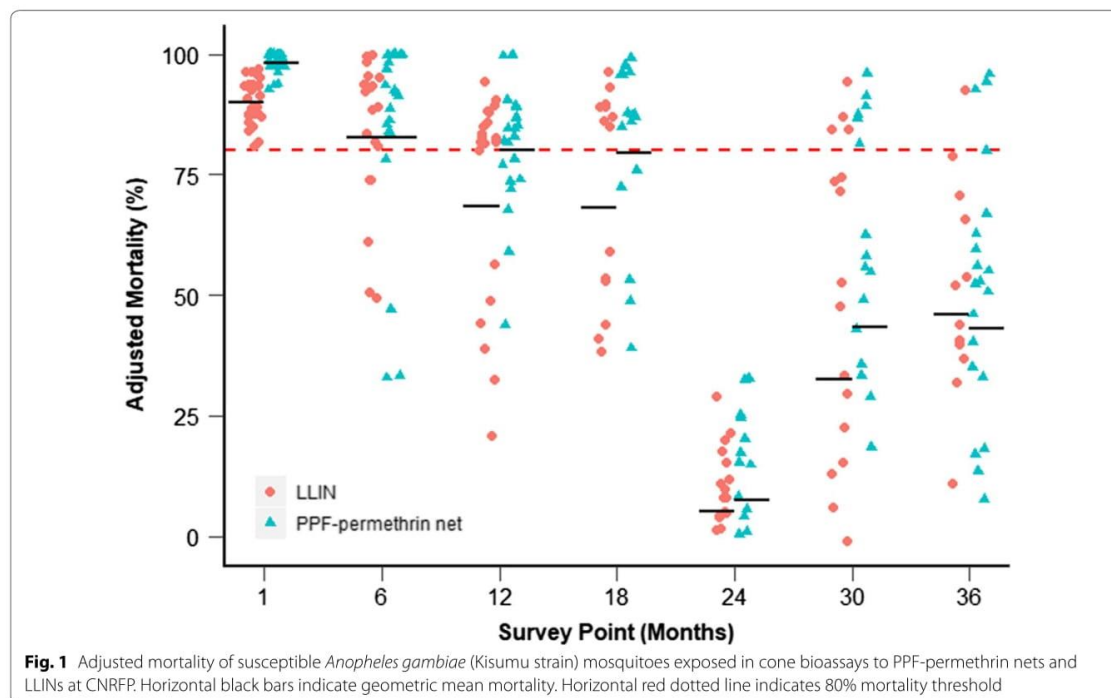
Ethics

The trial was approved by the Ethics Committee for Research in Health, Ministry of Scientific Research and Innovation, Burkina Faso (2014-0-0250) and the School of Biological Sciences ethics committee, Durham University, UK (SBBS/EC/PV120914).

Results**Bioefficacy of nets****Cone and tunnel tests for pyrethroid susceptible mosquitoes**

A total of 138 PPF-permethrin nets and 128 LLINs were tested with WHO cone bioassays on pyrethroid susceptible *An. gambiae* sensu stricto (s.s.) (Kisumu strain) in Burkina Faso at CNRFP (Fig. 1). Additionally, a proportion of these nets (84 PPF-permethrin nets and 85 LLINs) were also tested on the Kisumu strain at LSTM for validation of results (Additional file 4).

Mean mortality for the permethrin-only LLIN met the 80% threshold at baseline and 6 months but fell below this threshold from 12 months onwards; the threshold mortality for the PPF-permethrin nets was reached for the first 18 months. Dramatically reduced levels of mortality were observed for both net types at the 24-month sampling point (PPF-permethrin 10.0% and LLIN 6.9%). Mortality, however, increased unexpectedly again at 30 and 36 months. A similar pattern in efficacy was observed for the susceptible Kisumu strain at LSTM (Additional file 4), with mortality for both net types at its lowest at 24 months and increasing again at 30 months (the final nets from 36 months were not evaluated at LSTM). Knockdown data showed trends similar to the mortality data, with very low knock down rates at 24 months and higher rates in subsequent months (Additional file 5).



The best fit model determined that the mean mortality for PPF-permethrin nets over time was 8.6% (CI 2.6–14.6%) higher than for the LLINs ($\chi^2 = 11.244$, $df = 1$, $p < 0.006$). Comparison with the null model indicated that the model explained approximately 58.6% of total deviance in the cone mortality data.

Tunnel tests (Fig. 2), performed on the 104 (49 PPF-Permethrin and 55 LLINs) nets which did not meet the WHO criteria of $\geq 95\%$ knockdown and/or $\geq 80\%$ mortality after cone tests, showed that 19 nets passed the tunnel test criteria for mortality and/or blood feeding, of which 11 were PPF-permethrin nets and 9 LLINs (Table 2).

Combining the results from the cone bioassays and the tunnel tests, the PPF-permethrin net met the WHOPES bioefficacy criteria for 12 months longer than the standard permethrin-only LLIN (18 months compared to 6). At the final time point of 36 months post distribution, just 67% of PPF-permethrin nets (8/12) and 58% of standard LLINs (7/12) met the WHOPES bioefficacy criteria (Table 2).

Cone tests for pyrethroid resistant mosquitoes

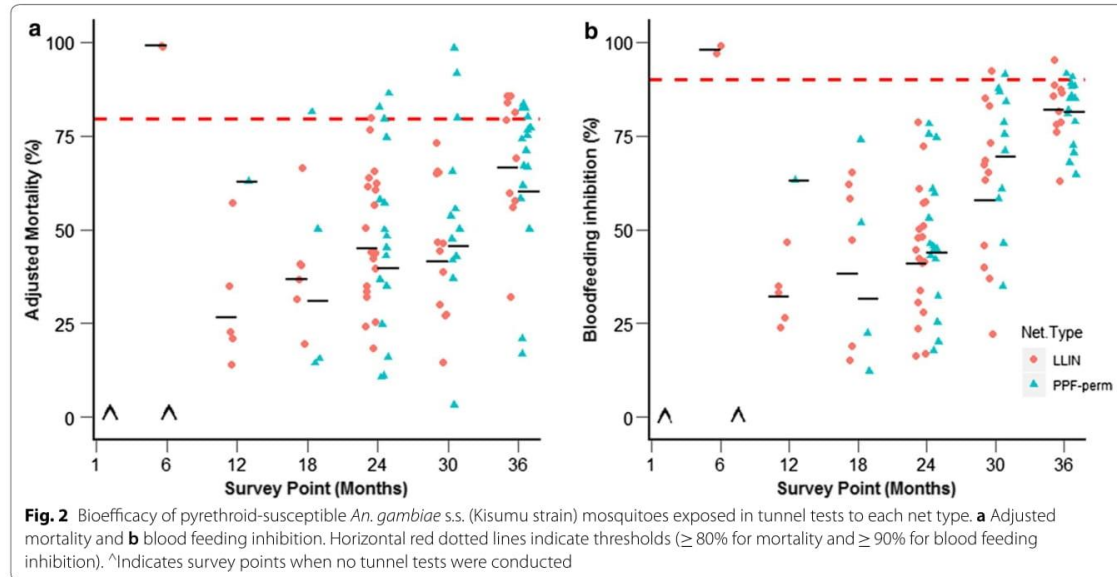
Pyrethroid-resistant *An. gambiae* s.s. (Tiassalé strain) were used to test the bioefficacy of 106 PPF-permethrin nets and 100 LLINs at LSTM using WHO cone

bioassays. Mosquito mortality over all time points was 14.6% (CI 9.7–19.5%) higher for PPF-permethrin nets than LLINs ($\chi^2 = 43.55$, $df = 2$, $p < 0.001$, Fig. 3). Mortality, however, was markedly more variable when mosquitoes were exposed to PPF-permethrin nets than LLINs. At 18 months and after, there was no difference in mortality between net types (18 months: $p = 0.42$, 24 months: $p = 0.74$, 30 months: $p = 0.72$). Comparison with the null model indicated that the model explained approximately 58.6% of total deviance in the data. Knockdown data are shown in Additional file 6.

Reduction in offspring

Pyrethroid-resistant mosquitoes that survived exposure in cone bioassays, were offered a blood meal and then retained for oviposition assays. Of 10,793 pyrethroid-resistant mosquitoes exposed to either net type, 56.3% (6078) survived 24 h after blood feeding and 3018 (49.7%) of the survivors laid eggs. Oviposition rates in mosquitoes exposed to untreated nets were variable, ranging from 0.43 to 0.96 in the different survey periods (Additional file 7). No comparisons were made for nets 18 months post-distribution as only one successful control exposure was done.

One-month post-distribution fertility was 80% lower for mosquitoes exposed to PPF-permethrin nets



compared to mosquitoes exposed to the control nets ($p=0.0001$), while there was no difference between LLINs and controls ($p=0.439$, Fig. 4). The reduction in fertility in PPF-permethrin nets was due to 40% fewer mosquitoes laying eggs (oviposition $p=0.01$), which resulted in 47% fewer eggs laid (fecundity $p=0.007$). Additionally, the eggs had a 60% lower hatch rate ($p<0.001$) than eggs laid by control mosquitoes (Additional file 7). There was no significant impact on fertility of the PPF-permethrin nets beyond 1 month (Fig. 4 and Additional file 7).

Chemical analysis

The insecticide content of 123 PPF-permethrin nets and 125 LLINs was measured. The permethrin content of both net types declined over 18 months and then rose from 24 months onwards ($\chi^2=505.33$, $df=1$, $p<0.001$, Fig. 5). Permethrin content of PPF-permethrin nets fell from 19.1 g/kg (95% CI 18.5–19.6) to 10.20 g/kg (95% CI 8.7–11.8) at 18 months and rose back to 15.4 g/kg (95% CI 13.1–17.6) at 36 months. Permethrin content of the LLINs fell from 18.9 g/kg (95% CI 18.6–19.4) to 14.3 g/kg (95% CI 13.3–15.2) and rose back to 18.6 g/kg (95% CI 17.2–19.9) at 36 months. At the end of the study, at

Table 2 Summary of cone and tunnel test results

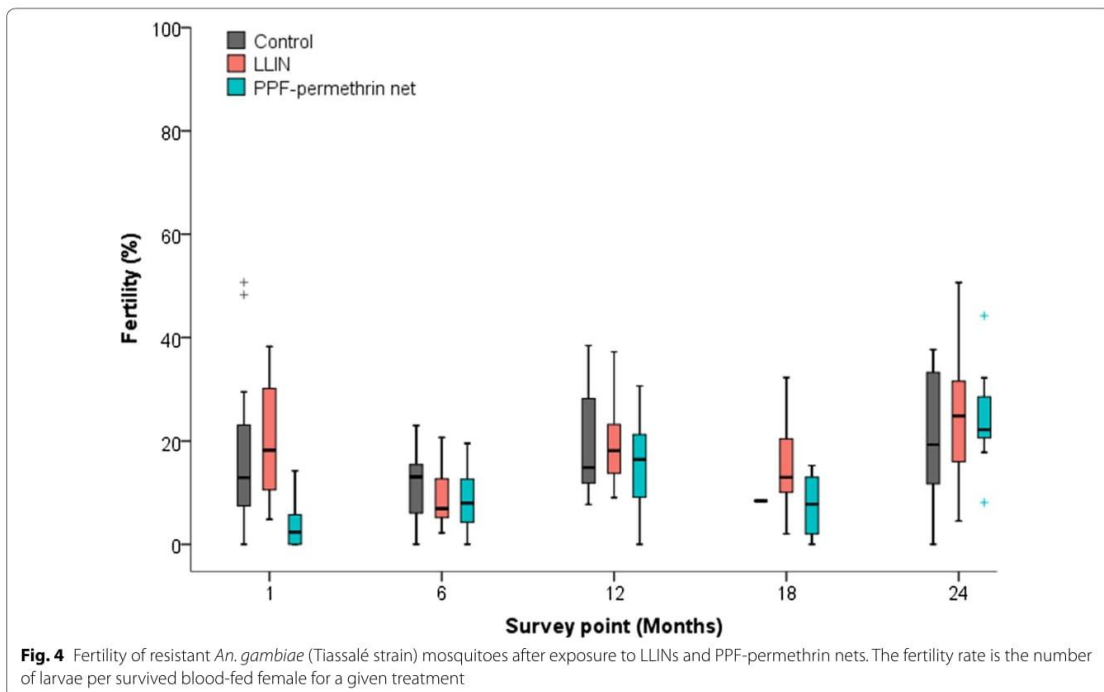
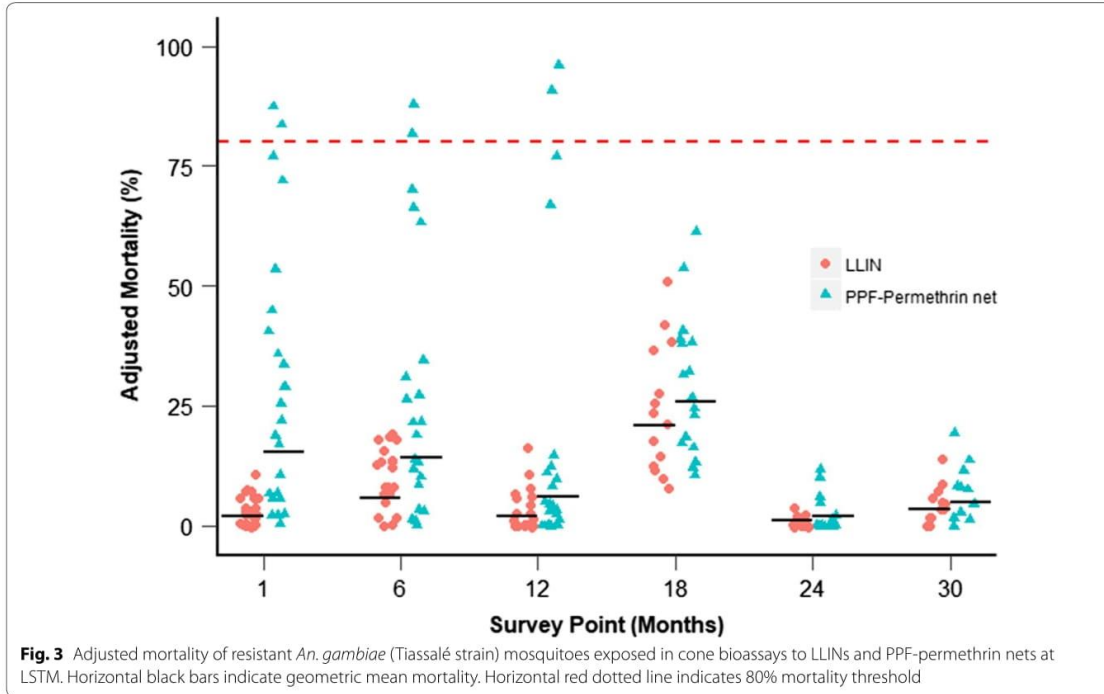
	Net type	Time after net distribution (months)						
		1	6	12	18	24	30	36
Cone test	PPF-permethrin	100% (24/24)	100% (23/23)	95.4% (21/22)	76.47% (13/17)	0% (0/14)	37.5% (6/16)	20.0%(4/20)
	LLIN	100% (24/24)	90.0% (18/20)	72.7% (16/22)	57.14% (8/14)	0% (0/20)	25.0% (4/16)	16.6% (2/12)
Tunnel test	PPF-permethrin	N/A	N/A	0% (0/1)	25% (1/4)	21.4% (3/14)	30.0% (3/10)	25% (4/16)
	LLIN	N/A	100% (2/2)	0% (0/5)	0% (0/6)	5.55% (1/18)	0% (1/12)	50% (5/10)
Overall ^a	PPF-permethrin	<i>100%</i> (24/24)	<i>100%</i> (23/23)	<i>95.4%</i> (21/22)	<i>82.35%</i> (14/17)	21.4% (3/14)	56.25% (9/16)	66.66% (8/12)
	LLIN	<i>100%</i> (24/24)	<i>100%</i> (20/20)	72.7% (16/22)	64.3% (8/14)	5% (1/20)	31.25% (5/16)	58.33% (7/12)

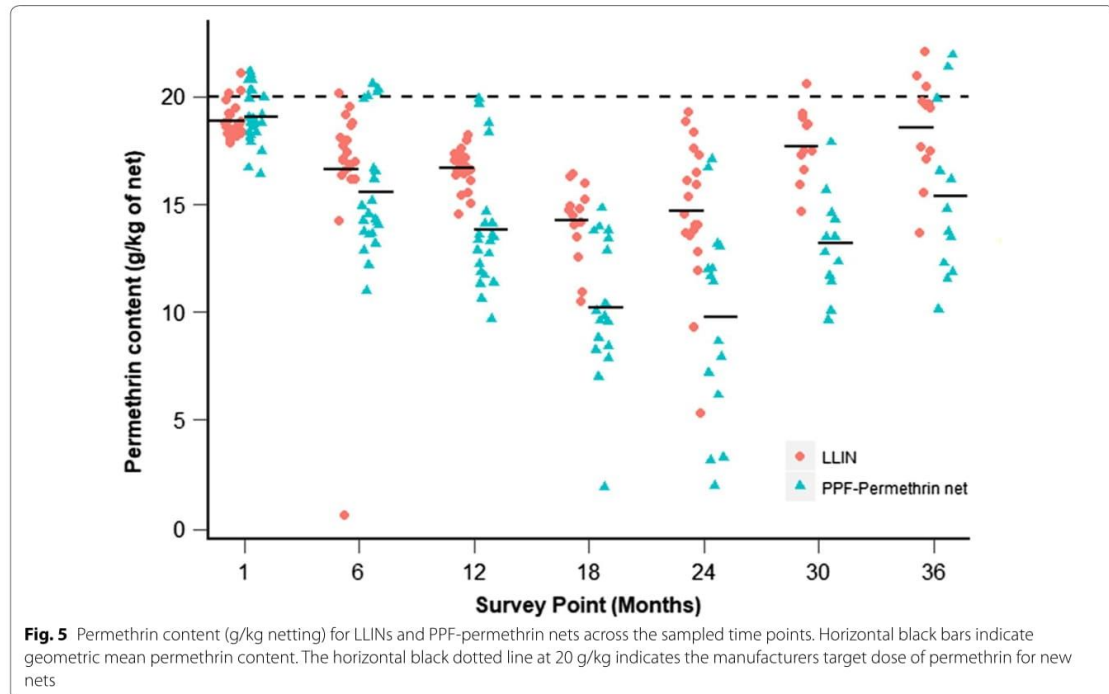
WHO cut off criteria for cone bioassay is $\geq 95\%$ knock down and/or $\geq 80\%$ mortality. For tunnel tests cut off criteria is $\geq 80\%$ mortality and/or $\geq 90\%$ blood feeding inhibition

Italic indicates pass, bolditalic indicates fail

N/A No nets tested

^a Nets meet overall WHO criteria for a given timepoint if 80% of nets pass either cone or tunnel tests





36 months, the permethrin content of LLINs was similar to nets collected immediately after distribution ($p = 0.99$).

The permethrin content of the two net types differed across the sampled time points (Fig. 5), with the permethrin content in PPF-permethrin nets 14% (2.59 g/kg, 95% CI 1.86–3.31) lower than that of LLINs ($\chi^2 = 44.067$, $df = 1$, $p < 0.001$). Comparison with the null model indicated that the best-fit permethrin model explained approximately 42.5% of total deviance in the data.

The pyriproxyfen content of PPF-permethrin nets declined for the first 24 months of the study but again, a small increase in the concentration of PPF was observed for nets surveyed in year three (Fig. 6). From immediately post distribution to 36 months, the mean pyriproxyfen concentration in permethrin-PPF nets had declined by 54% from 10.4 g/kg (CI 10.2–10.6) to 4.7 g/kg (CI 3.5–6.0, GLMM, $p < 0.001$).

Fabric integrity

The proportion of torn nets was low throughout the study for both net types, ranging from 0% at 1 month to its peak of 11% at 18 months post-distribution. There was no difference in the proportion of torn nets between net types ($p = 0.089$). However, the odds of a net having at least one hole was 47% higher overall for PPF-permethrin

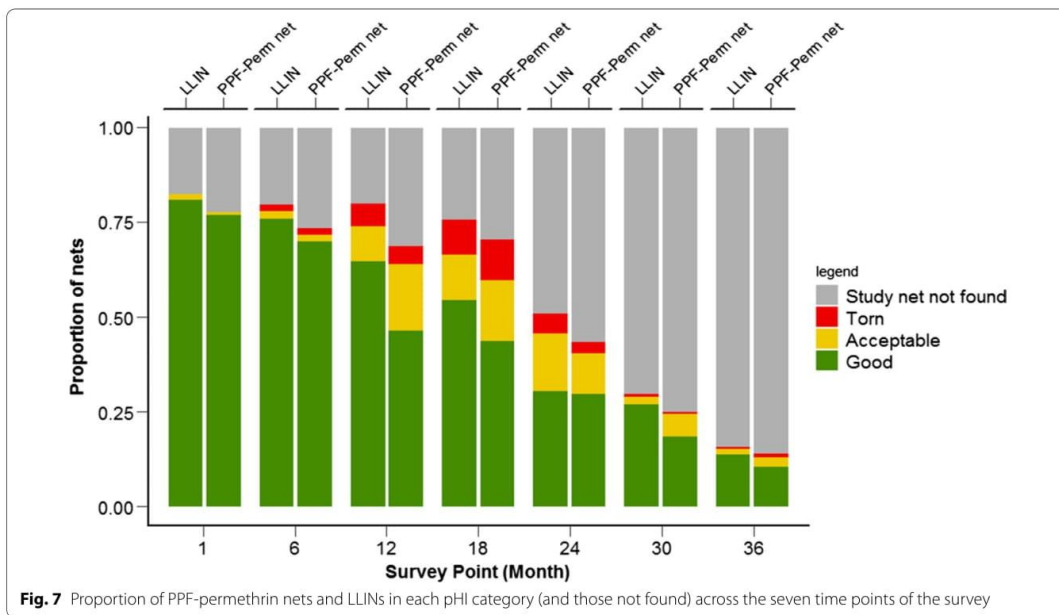
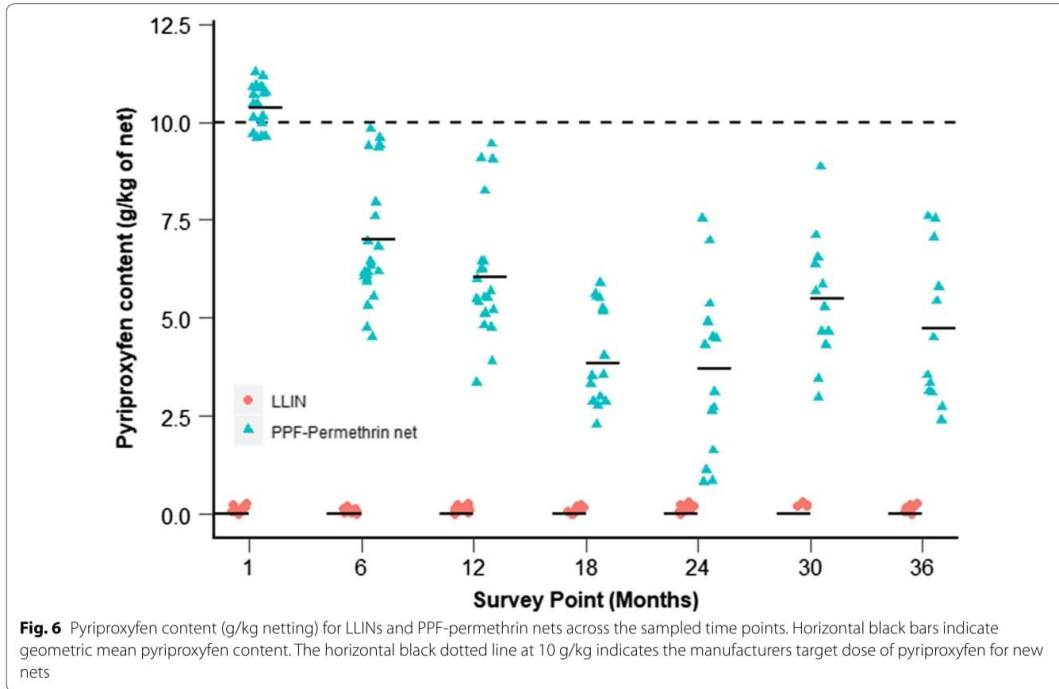
nets LLINs ($\chi^2 = 15.77$, $df = 1$, $p < 0.001$). The proportion of project nets in 'good' condition decreased during the study, falling from 77.1% (131/170) at 1 month to 10.6% (18/169) at 36 months for PPF-permethrin nets and 81.1% (304/375) to 13.9% (52/374) for LLINs. This is largely driven by net attrition with the majority of nets lost from households after 24 months (Fig. 7).

At 36 months, when lost/discarded nets are excluded, nets in 'good' condition accounted for 75% (18/24) and 88.1% (52/59) of remaining PPF-permethrin nets and LLINs respectively.

Net survival

There was a decline in functional survivorship during the study, with only 13.1% of PPF-permethrin nets and 12.4% of LLINs found hanging in the compound they were originally distributed to, in acceptable or good condition after 3 years. There was no difference between the survivorship of PPF-permethrin nets and LLINs at any point in the study ($\chi^2 = 0.126$, $df = 1$, $p = 0.72$). Comparison with the null model indicated that the best fit model explained approximately 36.4% of total deviance in the data.

The overall change in functional survivorship across the study was non-linear with a distinct second peak at 12 months post-distribution (Fig. 8). The model



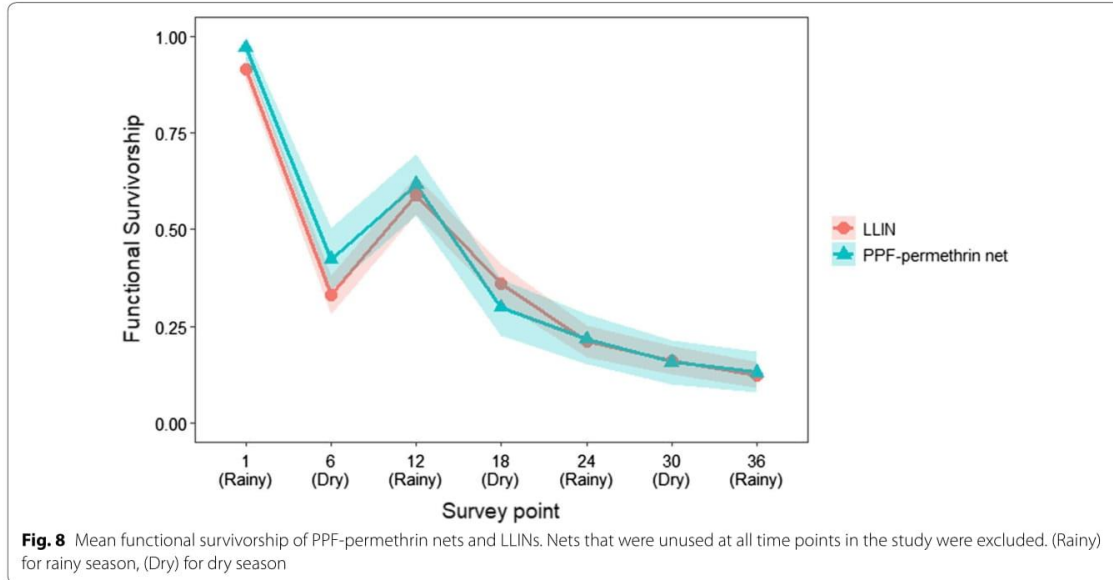


Table 3 Odds Ratios and 95% confidence intervals for impact of modelled variables on net survivorship

Variable	Odds ratio	95% confidence intervals	p-value
Net type			
(LLIN)	1.00		
PPF-permethrin net	1.07	0.73–1.56	0.72
Survey No.			
1	0.74	0.68–0.80	< 0.001*
Season			
(Dry)	1.00		
Rainy	24.50	15.79–38.02	< 0.001*
Survey No.: season (rainy)	0.55	0.52–0.61	< 0.001*

* Significant impact on functional survivorship, p < 0.05

attributed this non-linearity to seasonal variation, with the odds that a net was in use at its designated location increasing during the rainy season (Table 3). The positive effect of the rainy season on survivorship declined in magnitude as the study progressed.

Sampling bias

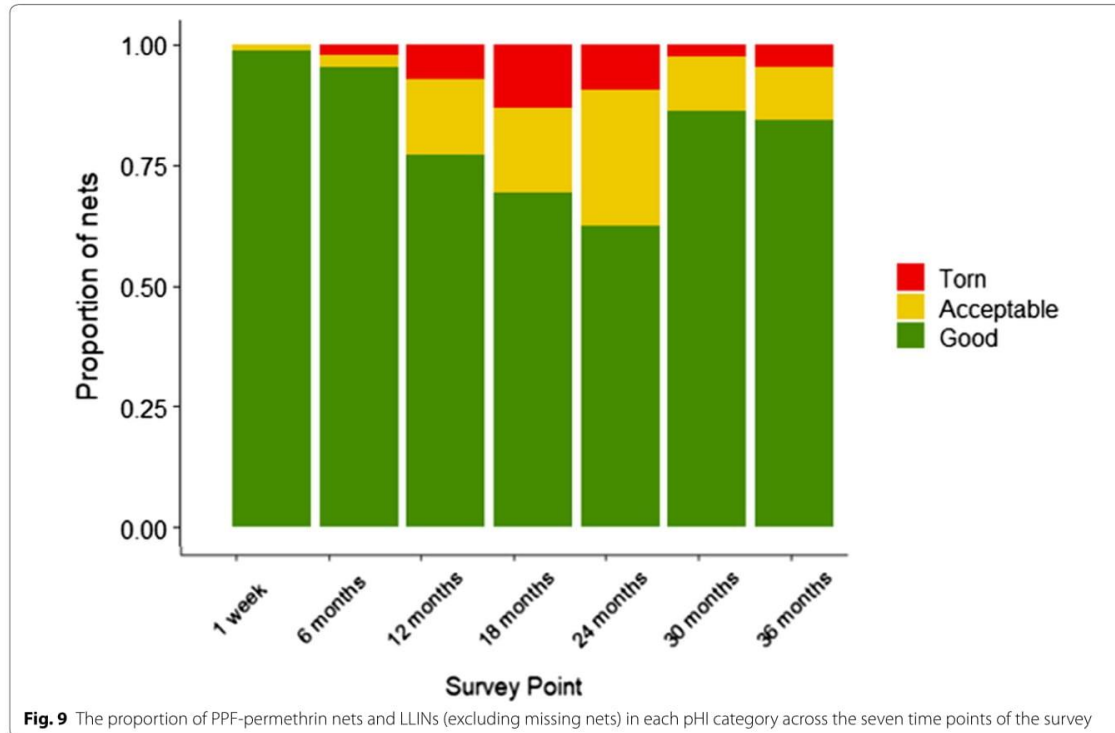
The unexpected changes in the chemical content and associated bioefficacy observed in this study are indicative of a sampling bias in the quality of nets collected at each timepoint. As measurement can only be performed on nets that remain to be sampled, those nets that are destroyed or lost are censored from sampling. If nets in

poor physical condition are disproportionately more likely to be discarded over time than nets in good condition, random samples of the remaining nets will be biased towards higher quality nets. This bias towards nets in better conditions in later time points can be directly observed when considering the physical integrity of remaining nets at each time point (Fig. 9).

Discussion

Bioefficacy of nets

This study was the first to measure the durability of dual active ‘next generation’ nets under operational conditions according to the WHOPEP criteria on bioefficacy, insecticide content, and survivorship. Both net types met the bioefficacy criteria for 6 months post distribution, however, the standard Olyset LLIN failed thereafter. The candidate PPF-permethrin net, Olyset Duo, continued to meet the threshold for a further 12 months than the LLIN, remaining effective until 18 months post-distribution. Typically, nets are only recommended by the WHO provided they meet the defined bioefficacy criteria for 3 years however older LLIN products on the market may have instead been evaluated under less-stringent guidelines that are no longer in use. Importantly, the standard LLIN used in this comparison, did not fulfil the criteria needed for approval; a similar observation was made in a recent study in Tanzania [21]. Contrary to these results, a retrospective study in Tanzania carried out in 2013 found that more than 96% of Olyset nets in serviceable condition passed the WHO cone/tunnel tests cut off criteria



36 months post distribution [22]. Similarly, a study in western Kenya from 2015 measured 100% mortality up to 3 years post-distribution for Olyset nets [23]. Both East African surveys were based on one sampling point, and, as the results in this study show, it is difficult to interpret net durability data in the absence of intermediate data on net usage over time. Olyset, the standard LLIN used as the comparator in this study, has been distributed to millions of people in sub-Saharan Africa, so the underperformance of this net is a cause of great concern. At a time when malaria prevalence is rising in several sub-Saharan African countries [2] it is important that the bioefficacy of Olyset and other types of nets are monitored when deployed to ensure that they provide protection for at least 3 years.

Tests with pyrethroid-resistant mosquitoes showed that the PPF-permethrin net provided greater bioefficacy for the first 18 months than did standard LLINs but declined to very low levels in both nets thereafter. The increased mortality found with Olyset Duo nets could be attributed to the higher permethrin bleed rate compared to Olyset, as has been previously proposed [12, 13]. Although the results of the chemical analysis found that the mean permethrin concentration was lower in PPF-treated nets than LLINs, the concentration of permethrin

detected in net fibres is not necessarily representative of the bioavailability of permethrin to a mosquito on the surface. Alternatively, or in addition, as both pyrethroids and pyriproxyfen have been shown to be metabolized by the same mosquito cytochrome P450s, this increased mortality observed in the PPF-permethrin combination may be indicative of a saturation effect whereby mosquitoes are unable to simultaneously detoxify the two active ingredients on the net [24]. These findings are supported by those from experimental hut trials in Benin and Côte d'Ivoire, where *Anopheles* are highly resistant to pyrethroids, also found higher levels of mortality with PPF-permethrin nets than LLINs [12, 13].

The sterilizing effect of the PPF-permethrin nets was short lived. One-month post distribution the fertility of mosquitoes exposed to PPF-permethrin nets was 80% lower than mosquitoes exposed to either untreated or standard LLINs but no significant reduction in fertility was observed at any subsequent time point. This contrasts with entomological data collected during a clinical trial [7] which found evidence of a strong sterilizing effect of PPF-permethrin nets 1-year post distribution (Grisales, manuscript in preparation). Both laboratory cone bioassays and experimental hut studies on PPF-permethrin nets found that net washing reduced the sterilizing

effect of PPF but the impact of washing was much greater in the cone bioassays [14]; the authors attribute this to the short duration of the cone bioassay which may not allow mosquitoes to pick up sufficient PPF to cause complete sterilization. However, this explanation is not supported by video tracking studies which show that the actual time mosquitoes spend in contact with a LLIN is very low, and typically less than 3 min [25]. Nevertheless, it would be informative to perform tunnel tests, or similar tests with longer exposure times on the pyrethroid resistant strain to assess the duration of the sterilizing effect of the PPF in the Olyset Duo nets under controlled conditions.

Net survivorship

The secondary objective of this study was to determine the physical integrity of the two net types for the first 36 months after distribution. There was little evidence of a difference in the physical durability of the two nets. A higher proportion of the PPF-permethrin nets had at least one hole but neither the proportion of torn nets nor the proportional hole index differed between the two net types. The attrition rate observed for both net types was very high with only 12% of all PPF-permethrin nets and 13% of standard permethrin only LLINs in good or acceptable condition *and* present in the household to which they were originally assigned 36 months post-distribution. The similar rates of survivorship between the two net types indicates that the high attrition is associated with the environmental or sociological characteristics of the setting rather than any differential preference or physical durability of either net type. Unfortunately, although the survey data did ask follow-up questions if the net was not found in the expected location, the large amount of missing data and/or ambiguous answers precluded us from accurately assigning causes for net attrition. A meta-analysis of data from 14 different national net distribution campaigns found that a similar proportion of nets that had left the household had been given away, rather than destroyed [26]. The national distribution of PermaNet 2.0 LLINs to the study site approximately 24 months into the trial may have contributed to the accelerated attrition of study nets. When individuals received these new government distributed nets, they may have then had less incentive to utilize, maintain and/or retain their existing study net, as has been described previously in qualitative surveys conducted on LLIN users in Senegal [27].

This study explicitly quantifies the impact of seasonal changes on the functional survivorship of the candidate LLIN. The arrival of the rainy season had a strong positive effect on the probability that a net would be hanging up in a sleeping place (taken to be an indicator of use).

It is expected that an individual's motivation to use their net would be increased by the higher biting densities of mosquitoes associated with the rainy season. Additionally, this could also be interpreted as the dry season having a strong negative effect on the probability that a net being used, due to the discomfort of sleeping under a net during hot nights. This fluctuation in functional survivorship over time highlights that net attrition is not necessarily permanent and nets may return to functional use at a later time point. Future evaluations of novel LLINs should consider the impact of seasonal variation on net utilization when attempting to model survivorship over time.

Survivorship bias

Observations of the bioefficacy and chemical analysis data highlight a seemingly counterintuitive finding. The performance of the nets decreases for the first 2 years of use and then increases. As the sampling process is destructive, the same nets are not sampled at each time point. Nevertheless, it is initially difficult to explain why insecticide content and bioefficacy could increase over time. It is possible that there is a survivorship bias in the sampling methodology. If nets in poor physical condition are disproportionately more likely to be discarded over time than nets in good condition, random samples of the remaining nets will be biased towards nets in good condition. This is consistent with a survivorship bias where an unrepresentative subgroup of nets disproportionately persisted to the latter stages of the trial, accounting for an increasingly large proportion of the remaining nets. This bias towards nets in better conditions in later time points can be directly observed when considering the physical integrity of remaining nets at each time point. This illustrates that the proportion of nets in 'torn' condition is not a reliable metric of durability in itself as it may be biased by non-random discarding of nets as the study progresses.

The apparent increase in net quality at 30- and 36-months post-distribution (as evidenced by chemical analysis and bioassays on the susceptible strain) could also be accounted for by users not utilizing their assigned nets immediately and these nets then being introduced at later time points. Even 1 month after net distribution, only three quarters of the nets were in use in the expected households. It is possible that some of the survey nets were only utilized after other nets in the household became too torn, or as household number increased. This could potentially explain the phenomenon in the chemical and biological efficacy data where performance of both net types was lowest at 24 months and then increased again at 30 and 36 months. Unfortunately, it was not possible to reliably ascertain whether

nets were detected in use for the first-time mid-way through the study.

Potential improvements to study protocol

The study protocol was based on WHO recommendations for evaluating LLINs in the field [15, 16], but for combination LLINs, like PPF-pyrethroid nets, there are a number of ways in which the protocols could be improved.

1. Cone bioassays may not be the most appropriate method for assessing bioefficacy for all active ingredients, particularly those with repellent or contact irritant properties where contact with the net may be less than the targeted 3 min. Poor results from cone bioassays on Olyset nets have been reported by us and others previously [28, 29]. Nevertheless, in the current study, nets that failed cone tests generally also performed poorly in tunnel tests, suggesting that the duration of contact was not responsible for the low mortality rates.
2. Bioassays to assess the impact of the nets on mosquito fertility were extremely time consuming to complete. The results were also confounded by low levels of oviposition in the controls (typically less than 50%). This has also confounded a previous study [13]. An alternative and less labour-intensive method to assess the impact of pyriproxyfen exposure is to examine the morphology of the ovaries. However, further work is required to determine the correlation between these two measurements.
3. In general, to assess the bioefficacy of dual active ingredient nets, it is necessary to use a strain with resistance to one of the two insecticides. It is important that the same strain is used throughout, and that resistance is maintained at a stable level throughout the study. This is challenging when studies last for 3 years. An alternative option might be to test all of the net samples from the different collection rounds at the end of the study but further information about the stability of the active ingredients under different storage conditions is needed from the manufacturers.
4. The randomization of nets to village compounds, and the differing compound size, meant that an unequal number of the two net types were distributed. This, coupled with the high attrition rate, meant that there were insufficient nets for the stated sample sizes in later sampling rounds. The original sample size calculations [18] assumed a 10% loss rate every year but in reality, the attrition rate was much higher.
5. The WHO proportionate Hole Index (pHI) was used to measure the number of holes on the net. This index does not account for the location of the holes

yet several studies [30, 31] have shown that host-seeking *Anopheles* mosquitoes are more active at the top of the nets compared to the sides, suggesting that holes on the top might give easy access into the nets. Consideration should be given to amending the pHI to weight holes depending on their location.

6. More succinct surveys, asking fewer questions would improve the quality of the data on survivorship and net attrition. The lengthy data on household income, level of education etc. (Additional file 3) were not needed for the stipulated analysis. Furthermore, the head of the household, typically male, was interviewed for the surveys whereas several of the questions, such as household composition, bed net usage and net washing patterns would have been more appropriately addressed by the female household head.
7. The data strongly suggests that nets sampled at the end of the study had not all been in use for the full 3 years making it difficult to draw firm conclusions about the longevity of individual nets. Furthermore, a high proportion of the nets moved between houses in the study. Amending the data collection tools to track the location of each of the distributed nets, regardless of whether they were located in the originally assigned household, would enable more precise measurements of net survivorship.

Finally, the current study involved 6 monthly assessments, enabling us to detect seasonal variations in net usage. Other durability studies typically survey the nets on an annual basis and, whilst this is probably a more pragmatic approach for future studies given the workload involved, it is important to note that the time of year at which surveys are performed could dramatically impact the proportion of nets hanging over a sleeping space due to the variation in seasonality observed. Regardless of the frequency of the surveys, comparison between studies would be facilitated by ensuring consistent methods of data collection and reporting between studies.

Conclusion

This is the first report of a full 3-year durability study on a combination or 'next generation' net. Although a clinical trial of the PPF-Permethrin Olyset® Duo net showed this net provides better protection from malaria than standard pyrethroid only nets, it is unlikely that, based on the results from the current study, this particular product would meet the WHO's definition of a 'long-lasting insecticidal net'. It is of concern that there are now many new types of nets in operational use, including PBO-pyrethroid nets and, from 2019, dual active ingredient nets, for which no data on their durability and longitudinal

performance in the field is available. Furthermore, the poor performance of a conventional LLIN in this study highlights that all net types must be continuously monitored for their durability, including the ones that are currently used as standard LLINs.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12936-019-3018-1>.

Additional file 1. Information sheet and consent form.

Additional file 2. Study profile.

Additional file 3. Questionnaire administered to heads of households.

Additional file 4. Adjusted mortality of susceptible *An. gambiae* (Kisumu strain) mosquitoes exposed in cone bioassays to PPF-permethrin nets and LLINs at LSTM.

Additional file 5. Adjusted knockdown of susceptible *An. gambiae* (Kisumu strain) mosquitoes exposed in cone bioassays to PPF-permethrin nets and LLINs.

Additional file 6. Adjusted knockdown of resistant *An. gambiae* (Tiassalé strain) mosquitoes exposed in cone bioassays to LLINs and PPF-permethrin nets at LSTM.

Additional file 7. Fecundity and fertility study result for resistant *An. gambiae* (Tiassalé 13 strain) mosquitoes exposed in cone bioassays to PPF-permethrin nets and LLINs.

Abbreviations

CNRFP: Centre National de Recherche et de Formation sur le Paludisme; DCP: dicyclohexyl phthalate; GLMM: Generalized Linear Mixed Model; LLINs: long-lasting insecticidal nets; LRTs: log-likelihood ratio tests; LSTM: Liverpool School of Tropical Medicine; PBO: pyriproxyfen; PHI: proportionate hole index; WHO: World Health Organization.

Acknowledgements

We would like to thank the population of Dalambo and Sanako village for their participation in this study. We thank Chris Williams, Kyle Walker, Ben Rogerson and John Morgan for technical support at LSTM and Moussa Guelbéogo for technical support at CNRFP. We also gratefully acknowledge Federica Guglielmo and Emma Sayer's help in analysing the survey data.

Authors' contributions

HR, MP and SWL designed the study. KHT and EFST led and conducted the majority of the field work with support from AT and supervision from NS and ABT. MM, JS and NL conducted and analysed the bioassays at LSTM. HI performed and analysed the HPLC data. J-AT and FM, with support from JM analysed the data. HR, FM, J-AT drafted the paper with input from SWL. All authors read and approved the draft. All authors read and approved the final manuscript.

Funding

This study was funded, in part, by the Innovative Vector Control Consortium (IVCC) and Sumitomo Chemicals Ltd provided the nets free of charge. The data were shared with the funders in advance of publication but they had no influence on the design of the study, the collection, analysis or interpretation of the data, or the writing of the manuscript.

Availability of data and resources

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The trial was approved by the Ethics Committee for Research in Health, Ministry of Scientific Research and Innovation, Burkina Faso (2014-0-0250) and the School of Biological Sciences ethics committee, Durham University, UK (SBBS/

EC/PV120914). The authors declare no competing interests. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Centre National de Recherche et de Formation sur le Paludisme, Ouagadougou, Burkina Faso. ² Vector Biology Department, Liverpool School of Tropical Medicine, Liverpool, UK. ³ Swiss Centre for Scientific Research in Côte d'Ivoire, Abidjan, Côte d'Ivoire. ⁴ Department of Biosciences, Durham University, Durham, UK. ⁵ Medical Research Council Unit, The Gambia at the London School of Hygiene and Tropical Medicine, Banjul, The Gambia.

Received: 11 September 2019 Accepted: 20 November 2019

Published online: 02 December 2019

References

- Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature*. 2015;526:207–11.
- WHO. World malaria report 2018. Geneva: World Health Organization; 2018.
- Pombi M, Calzetta M, Guelbeogo WM, Manica M, Perugini E, Pichler V, et al. Unexpectedly high Plasmodium sporozoite rate associated with low human blood index in *Anopheles coluzzii* from a LLIN-protected village in Burkina Faso. *Sci Rep*. 2018;8:12806.
- Tesfazghi K, Traore A, Ranson H, N'Fale S, Hill J, Worrall E. Challenges and opportunities associated with the introduction of next-generation long-lasting insecticidal nets for malaria control: a case study from Burkina Faso. *Implement Sci*. 2016;11:103.
- Ranson H, Lissenden N. Insecticide resistance in African Anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends Parasitol*. 2016;32:187–96.
- Toé KH, Jones CM, N'fale S, Ismail HM, Dabiré RK, Ranson H. Increased pyrethroid resistance in malaria vectors and decreased bed net effectiveness Burkina Faso. *Emerg Infect Dis*. 2014;20:1691–6.
- Tiono AB, Ouédraogo A, Ouattara D, Bougouma EC, Coulibaly S, Diarra A, et al. Efficacy of Olyset Duo, a bednet containing pyriproxyfen and permethrin, versus a permethrin-only net against clinical malaria in an area with highly pyrethroid-resistant vectors in rural Burkina Faso: a cluster-randomised controlled trial. *Lancet*. 2018;392:569–80.
- WHO. List of WHO prequalified vector control products date. Geneva: World Health Organization. <https://www.who.int/pq-vector-control/prequalified-lists/LOPrequalifiedProducts20190321.pdf?ua=1>. Accessed 21 Mar 2019.
- Ohashi K, Nakada K, Ishiwatari T, Miyaguchi J, Shono Y, Lucas JR, et al. Efficacy of pyriproxyfen-treated nets in sterilizing and shortening the longevity of *Anopheles gambiae* (Diptera: culicidae). *J Med Entomol*. 2012;49:1052–8.
- Harris C, Lwetoijera DW, Dongus S, Matowo NS, Lorenz LM, Devine GJ, et al. Sterilising effects of pyriproxyfen on *Anopheles arabiensis* and its potential use in malaria control. *Parasit Vectors*. 2013;6:144.
- Jaffer A, Protopopoff N, Moshia FW, Malone D, Rowland MW, Oxborough RM. Evaluating the sterilizing effect of pyriproxyfen treated mosquito nets against *Anopheles gambiae* at different blood-feeding intervals. *Acta Trop*. 2015;150:131–5.
- Koffi AA, Ahoua Alou LP, Djenontin A, Kabran JPK, Dosso Y, Kone A, et al. Efficacy of Olyset® Duo, a permethrin and pyriproxyfen mixture net against wild pyrethroid-resistant *Anopheles gambiae* s.s. from Côte d'Ivoire: An experimental hut trial. *Parasite*. 2015;22:28.
- Ngufor C, N'Guessan R, Fagbohoun J, Odjo A, Malone D, Akogbeton M, et al. Olyset Duo® (a pyriproxyfen and permethrin mixture net): an experimental hut trial against pyrethroid resistant *Anopheles gambiae* and *Culex quinquefasciatus* in southern Benin. *PLoS ONE*. 2014;9:e93603.

14. Nguifor C, N'Guessan R, Fagbohoun J, Todjinou D, Odjo A, Malone D, et al. Efficacy of the Olyset Duo net against insecticide-resistant mosquito vectors of malaria. *Sci Transl Med*. 2016;8:356ra121.
15. WHO. Guidelines for laboratory and field-testing of long-lasting insecticidal nets. WHO/HTM/NTD/WHOPE/20131. Geneva: World Health Organization. 2013:93. <https://apps.who.int/iris/handle/10665/80270>. Accessed 4 Sept 2019.
16. WHO. Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions. Control of Neglected Tropical Diseases, WHO Pesticide Evaluation Scheme, and Global Malaria Programme Vector Control Unit 2011. https://apps.who.int/iris/bitstream/handle/10665/44610/9789241501705_eng.pdf. Accessed 4 Sept 2019.
17. WHO. Guidance note for estimating the longevity of long-lasting insecticidal nets in malaria control. Geneva: World Health Organization; 2013.
18. Sagnon NF, Pinder M, Tchicaya EFS, Tiono AB, Faragher B, Ranson H, et al. To assess whether addition of pyriproxyfen to long-lasting insecticidal mosquito nets increases their durability compared to standard long-lasting insecticidal mosquito nets: study protocol for a randomised controlled trial. *Trials*. 2015;16:195.
19. Edi CV, Djogbenou L, Jenkins AM, Regna K, Muskavitch MAT, Poupardin R, et al. CYP6 P450 enzymes and ACE-1 duplication produce extreme and multiple insecticide resistance in the malaria mosquito *Anopheles gambiae*. *PLoS Genet*. 2014;10:e1004236.
20. Abbott WS. A method of computing the effectiveness of an insecticide. 1925. *J Am Mosq Control Assoc*. 1987;3:302–3.
21. Lorenz LM, Bradley J, Yukich J, Massue DJ, Mboma M, Pigeon O, et al. Comparative functional survival and equivalent annual cost of three long lasting insecticidal net (LLIN) products in Tanzania. *Medrxiv (preprint)* 2019. <https://www.medrxiv.org/content/10.1101/19002212v1>. Accessed 23 Oct 2019.
22. Massue DJ, Moore SJ, Mageni ZD, Moore JD, Bradley J, Pigeon O, et al. Durability of Olyset campaign nets distributed between 2009 and 2011 in eight districts of Tanzania. *Malar J*. 2016;15:176.
23. Wanjala CL, Zhou G, Mbugi J, Simbauni J, Afrane YA, Ototo E, et al. Insecticidal decay effects of long-lasting insecticide nets and indoor residual spraying on *Anopheles gambiae* and *Anopheles arabiensis* in Western Kenya. *Parasit Vectors*. 2015;8:588.
24. Yunta C, Grisales N, Nász S, Hemmings K, Pignatelli P, Voice M, et al. Pyriproxyfen is metabolized by P450 s associated with pyrethroid resistance in *Anopheles gambiae*. *Insect Biochem Mol Biol*. 2016;78:50–7.
25. Parker JEA, Angarita-Jaimes N, Abe M, Towers CE, Towers D, McCall PJ. Infrared video tracking of *Anopheles gambiae* at insecticide-treated bed nets reveals rapid decisive impact after brief localised net contact. *Sci Rep*. 2015;16:270.
26. Koenker H, Kilian A, De Beyl CZ, Onyefunfoa EO, Selby RA, Abeku T, et al. What happens to lost nets: a multi-country analysis of reasons for LLIN attrition using 14 household surveys in four countries. *Malar J*. 2014;13:464.
27. Loll DK, Berthe S, Faye SL, Wone I, Koenker H, Arnold B, et al. User-determined end of net life in Senegal: a qualitative assessment of decision-making related to the retirement of expired nets. *Malar J*. 2013;12:337.
28. Lenhart A, Orelus N, Maskill R, Alexander N, Streit T, McCall PJ. Insecticide-treated bednets to control dengue vectors: preliminary evidence from a controlled trial in Haiti. *Trop Med Int Health*. 2008;13:56–67.
29. Bagi J, Grisales N, Corkill R, Morgan JC, N'Falé S, Brogdon WG, et al. When a discriminating dose assay is not enough: measuring the intensity of insecticide resistance in malaria vectors. *Malar J*. 2015;14:210.
30. Lynd A, McCall PJ. Clustering of host-seeking activity of *Anopheles gambiae* mosquitoes at the top surface of a human-baited bed net. *Malar J*. 2013;12:267.
31. Sutcliffe J, Ji X, Yin S. How many holes is too many? A prototype tool for estimating mosquito entry risk into damaged bed nets. *Malar J*. 2017;16:304.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more [biomedcentral.com/submissions](https://www.biomedcentral.com/submissions)



The effects of temephos, permethrin and malathion selection on the fitness and fecundity of *Aedes aegypti*

K. GLEAVE , F. MECHAN  and L. J. REIMER 

Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, U.K.

Abstract. The recent scale-up of insecticide use has led to the rapid spread of insecticide resistance (IR) in mosquito populations across the world. Previous work has suggested that IR mechanisms could influence mosquito life-history traits, leading to alterations in fitness and key physiological functions. This study investigates to what extent mosquito fitness may be affected in a colony of *Aedes aegypti* after selection with temephos, permethrin or malathion insecticides. We measured immature development, sex ratio, adult longevity, energetic reserves under different rearing conditions and time points, ingested bloodmeal volume, mosquito size, male and female reproductive fitness and flight capability in the unexposed offspring of the three selected strains and unselected strain. We found that insecticide selection does have an impact on mosquito fitness traits in both male and female mosquitoes, with our temephos-exposed strain showing the highest immature development rates, improved adult survival, larger females under crowded rearing and increased sperm number in males. In contrast, this strain showed the poorest reproductive success, demonstrating that insecticide selection leads to trade-offs in life-history traits, which have the potential to either enhance or limit disease transmission potential.

Key words. Energetic resources, flight, insecticide resistance, larvicide, life-history parameters, mosquito.

Introduction

Insecticide resistance (IR) in disease vectors is at a crucial tipping point. The recent scale-up of insecticide-based vector control has protected hundreds of millions of people from disease exposure (Bhatt *et al.*, 2016), but has also resulted in the emergence and rapid spread of IR mechanisms across the world (Vontas *et al.*, 2012; Ranson & Lissenden 2016; WHO 2018). Within the major arbovirus vector *Aedes aegypti*, resistance has evolved to the four insecticide classes most commonly used for public health (Ranson *et al.*, 2010; Moyes *et al.*, 2017), with resistance to both larval and adult insecticides well documented in field populations (Montella *et al.*, 2007). This has led to a reduction in the efficacy of current insecticide-based control strategies (Moyes *et al.*, 2017). However, IR is energetically costly and can reduce mosquito fitness in the absence of insecticides, with effects ranging from minimal to highly damaging (Martins *et al.*, 2012; Brito *et al.*, 2013; Belinato & Martins 2016).

Resistance mechanisms cause significant changes to key physiological functions in the vector, such as depleting energy resources (Diniz *et al.*, 2015), affecting development time (Martins *et al.*, 2012; Rahim *et al.*, 2017; Ramos *et al.*, 2018) or altering immune functions (Vontas *et al.* 2005), which can lead to changes in disease transmission. Metabolic resistance, caused by elevated enzyme activity, can be energetically costly with resources diverted for sequestration, metabolism and detoxification of insecticides (Saingamsook *et al.*, 2019). Previous studies have shown that metabolic resistance to temephos is associated with a reduction in egg batch size (Martins *et al.*, 2012; Diniz *et al.*, 2015; Viana-Medeiros *et al.*, 2017). Removing insecticide pressures from an environment results in lower frequencies of resistant alleles in mosquito populations, suggesting there is a fitness cost to maintaining these alleles in the absence of insecticide (Coustau *et al.*, 2000; David *et al.*, 2018).

Lipids and glycogen are important energy resources used for processes such as flight, vitellogenesis and immune responses

Correspondence: Dr Lisa J. Reimer, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, U.K. Tel.: 0151 705 3107; E-mail: lisa.reimer@lstmed.ac.uk

56 © 2021 The Authors. *Medical and Veterinary Entomology* published by John Wiley & Sons Ltd on behalf of Royal Entomological Society. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

(Steele, 1981). Glycogen stores are released from within cells and provide a source of energy for immediate flight, whereas ingested carbohydrates are converted to lipids that are directly involved in oogenesis, moulting and sustained flight (Beenackers *et al.*, 1981). Resource-based trade-offs have been previously observed in insecticide-resistant mosquito populations, with the over-production of detoxifying enzymes requiring an extensive investment of resources. This can lead to depleted lipid stores, likely because lipids play a vital role in amino acid synthesis, thus leading to a knock-on negative impact on life-history traits, which rely on stored energy reserves (Rivero *et al.*, 2010). If the availability of these resources is altered at either the larval or adult stage then development, reproduction and movement will be affected.

Research into mosquito behaviour, fitness and fecundity tends to focus on measurements of females and their offspring. However, the physiological and behavioural traits observed in females post-mating (egg development, oviposition rates and host-seeking behaviours) are partially attributed to the receipt of male seminal fluid proteins and sperm (Hiss & Fuchs, 1972; Downe, 1975; Adlakha & Pillai, 1976; Klowden, 1993; Villarreal *et al.*, 2018). Both positive and negative associations between resistance and male reproductive success have been demonstrated, with Arnaud *et al.* (2005) reporting that insecticide-resistant beetles have improved reproductive success and are superior sperm competitors, whereas, in resistant mosquitoes, Belinato *et al.* (2012) saw a reduced frequency of female insemination.

While many studies have reported negative effects of IR on fitness and fecundity, a few studies have documented positive effects. Chan & Zairi (2013) demonstrated that permethrin-resistant *Aedes albopictus* survived longer when starved and produced larger females under crowded rearing densities than their susceptible counterparts. If resistant female mosquitoes show increased longevity, they are more likely to survive through a pathogen's extrinsic incubation period, increasing transmission potential (Kramer & Ebel, 2003).

Numerous limitations from previous studies likely contribute to poor concordance in study outcomes. Often only one or two fitness-related phenotypes were measured, despite the interdependency between longevity, male and female fecundity and energy resources. Furthermore, there are very few comparable pairs of resistant and susceptible strains, which only differ in resistance phenotype.

Our study aimed to investigate the fitness costs associated with IR by measuring energetic reserves, development, longevity, reproduction and flight in four strains of *A. aegypti* with different histories of insecticide exposure.

Materials and Methods

Establishment and maintenance of four A. aegypti strains

An *A. aegypti* colony from Recife, Brazil, was used to create four strains via exposure over 10 generations to either the larval organophosphate temephos (REC-R), adult pyrethroid permethrin (REC-P), adult organophosphate malathion (REC-M), or no insecticide exposure (REC-U) (Thornton *et al.*, 2020).

All four strains were established and maintained under standard controlled conditions ($27^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and 80% relative humidity, 12:12 light/dark cycle) in an insectary at the Liverpool School of Tropical Medicine. Eggs were obtained by feeding mated adult females on human blood using a Hemotek feeder (Hemotek Ltd, Blackburn, U.K.). To standardize rearing conditions, 200 first instar larvae were counted and placed in plastic larval rearing trays ($23.5 \times 34.5 \times 7.5$ cm) containing 1 L of deionized (DI) water and one Brewer's yeast tablet (500 mg). To mimic high larval density rearing, 500 first instar larvae were counted and placed in rearing trays with 1 L of DI water and 1 yeast tablet. For each strain, four larval trays at each density were reared to use for testing and larvae were fed with one yeast tablet every other day. Adults were maintained on 10% sugar solution.

Resistance profiles. Resistance ratios after 1 year of selection, using lethal concentration (LC) 50 and LC95, were previously examined and compared to a fully susceptible New Orleans colony (Thornton *et al.*, 2020). For permethrin, REC-P was five times more resistant than REC-U, REC-M and REC-R. For malathion, REC-R and REC-M were slightly more resistant ($\sim 2\times$) than REC-U or REC-P. With temephos, REC-R, REC-M and REC-P were more resistant ($>2\times$) than REC-U (Table S1).

This study investigated the impact of insecticide selection regimes on four main physiological aspects of mosquito fitness: life-history traits, energy reserves, reproductive fitness and flight capability. The effect of different larval rearing densities and mosquito age were also considered. Figure 1 shows the study design and experimental pathway for each cohort of mosquitoes.

Mosquito life traits

Immature development time. Mosquitoes from each of the four strains, at both rearing densities (standard rearing trays: REC-R $n = 3$, REC-U $n = 3$, REC-M $n = 2$, REC-P $n = 3$; crowded rearing trays: REC-R $n = 2$, REC-U $n = 2$, REC-M $n = 2$, REC-P $n = 1$), were separated by sex upon pupation into individual male and female holding containers. The number pupating per day was recorded. Mosquito eclosion was recorded for each sex and strain, and adults were retained in separate containers prior to assays.

Longevity

Longevity was recorded for mosquitoes from each strain, at the standard rearing density of 200 larvae/tray. Four cups of females and four cups of males each containing 20 adults were maintained on 10% sugar solution and monitored until all mosquitoes had naturally died. Due to different eclosion dates, each strain had a staggered start date, with the longest experiment lasting for a total of 60 days. The temperature and humidity of the insectary remained constant ($27^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and 80% relative humidity) and cup placement rotated daily to ensure standardized conditions. Death was recorded daily.

Objective	Cohort	Outcome	Measured endpoints	Target sample size per strain
Life traits	Standard density	Immature development	Number successfully pupated and time to pupation and sex ratio	3 trays, $n = 200$
			Number successfully eclosed and time to eclosion and sex ratio	
		Adult longevity	Day of death	80 females, 80 males
	Crowded density	Immature development	Time to pupation and sex ratio	2 trays, $n = 500$
Time to eclosion and sex ratio				
Energy reserves	Standard density	Bloodmeal volume	Haemoglobin content *	10 females
			Reserves (day 2)	Lipid content (ug/mL) *
		Glycogen content (ug/mL) *		
		Reserves (day 8)	Lipid content (ug/mL) *	16 females
	Glycogen content (ug/mL) *			
	Crowded density	Reserves (day 2)	Lipid content (ug/mL) *	16 females
			Glycogen content (ug/mL) *	
		Reserves (day 8)	Lipid content (ug/mL) *	16 females
Glycogen content (ug/mL) *				
Reproductive fitness	Male	Fertility	Total sperm count per male *	15 males
			Sperm number per mm of wing length	
		Individual mating success	Number of females inseminated per male	22 males
	Cross mating success	Number of females inseminated per male	10 males	
	Female	Female fecundity	Total egg number per female fed to repletion	20 females
Total L1 per female fed to repletion				
Flight capability	Female	Flight distance	Total distance (m)	33 females
			Average speed (m/s)	
		Flight bursts	Number of bursts over test period	

Fig 1. Study objectives, measured endpoints and target sample sizes. *Wing length measurements were taken for each of the mosquitoes in this assay. The sample size calculation for each primary outcome was based on a pilot study. Statistical modelling of the relationship between measured endpoint and strain indicated that differences between strains explained approximately 10% of variation in the data. Thus, on the assumption of an effect size of 0.1, the R package 'pwr' was used to calculate the minimum sample size under the following assumptions: degrees of freedom for numerator: 5; type I error prob: 0.05; type II error prob: 0.20; effect size: 0.1.

Quantification of energy resources

Bloodmeal volume. Bloodmeal volume was evaluated by quantifying haemoglobin amount (Briegel *et al.*, 1979), using Drabkin's reagent method. Midguts of blood-fed female mosquitoes were dissected 1 h post bloodmeal and the carcass was stored at -20°C for subsequent wing measurements. Individual midguts were placed into 1.5-mL Eppendorf tubes

containing 500 μL Drabkin's reagent and one metal ball bearing on ice. Samples were agitated in a tissue lyser for 1 min at 15 Hz and another 500 μL Drabkin's reagent was added. Samples were centrifuged at 12770 g for 15 min, before 200 μL of each sample was loaded onto a flat bottomed 96-well plate and read at 540 nm using Gen5 Epoch plate reader. Triplicate readings were recorded for each sample and an average was taken.

Wing length. Wing length was used as an estimate for body size. The right-wing from each female was removed from the thorax and an image was taken using a GXCAM ECLIPSE Wi-Fi microscope camera attached to a GX Stereo microscope. The length of the wing from the axial vein to the distal end of the R1 vein (not including the hairs on the edges of wings) was measured using GXCAM software (GXCAM Ver6.7).

Lipid and glycogen. We determined the lipid and glycogen content of mosquitoes using a standard protocol (*Methods in Anopheles Research*, 2015) with vanillin and anthrone reagents. Mosquitoes from all four strains, at both rearing densities, were split into two separate cohorts to allow energy analysis at two different time points; reserves measured at two days post-emergence (DPE) and reserves measured at eight DPE.

Reproductive fitness

Sperm number

Male and female mosquitoes were separated upon pupation and allowed to emerge in separate holding containers. Fifteen 1-day-old males were removed and individually knocked down on ice before dissection of the testes and seminal vesicles into 50 μ L of phosphate-buffered saline (PBS). Samples were torn gently with dissecting pins and pins washed with 150 μ L of PBS to obtain a final stock volume of 200 μ L. Samples were mixed and 10 μ L transferred into multi-well slides (20 individual wells per mosquito). Slides were air-dried, fixed with 70% ethanol and stained with Giemsa dye. Mosquito sperm heads were counted under $\times 40$ magnification. One wing from each male was measured using the method described earlier.

Individual mating success

To determine individual mating success, 22 virgin male mosquitoes of each strain were housed individually in holding cups with three virgin females of the same strain. Males were given four days to mate. On the fourth day, female mosquitoes were knocked down briefly on ice and all three spermatheca were scanned for spermatozoa. Mosquitoes were recorded as either 'positive' or 'negative' for insemination.

Cross mating success. Following the results of strain-specific differences in mating success, REC-M and REC-R strains were further evaluated through a cross mating experiment to determine whether mating success was a male or female trait. The same method was repeated, with 10 virgin males individually housed with three virgin females from either the same strain or the alternate strain, resulting in four different crosses.

© 2021 The Authors. *Medical and Veterinary Entomology* published by John Wiley & Sons Ltd on behalf of Royal Entomological Society, *Medical and Veterinary Entomology*, 36, 56–65

Female fecundity

Three mosquito rearing cages (28.5 \times 29.5 \times 28 cm) for REC-R, REC-U and REC-M, and two rearing cages for REC-P, were prepared with 30 female and 30 male mosquitoes introduced at the same time. Females were given four days to mate and then offered a human bloodmeal using a Hemotek membrane feeding system. All non-fed females were removed from the cage, and an oviposition pot containing damp cottonwool and filter paper was placed into the cage three days later, left overnight and then removed the following day. Multiple parameters were recorded: number of females fed to repletion, number of eggs laid and L1 hatch rate.

Quantification of flight ability

To investigate the effects of IR on mosquito flight ability, we used a tethered insect flight mill (provided by Dr. Jason Lim of Rothamsted Research), housed under standard insectary conditions. Due to low numbers of REC-M at the time of this assay, we only compared females from three strains: REC-R ($n = 33$), REC-U ($n = 66$) and REC-P ($n = 33$). REC-U females were flown at the same time as either REC-R or REC-P females to serve as a comparator.

Then, 2–5-day-old, non-blood-fed, virgin mosquitoes were knocked down briefly on ice before attachment to the tethered flight mill as follows. The rotor arm of the flight mill (radius 4 cm) was dipped into non-solvent glue and held gently onto the upper thorax of the mosquito, avoiding the wings. Mosquitoes on the rotor arm were then placed into one of the eight tethered flight mills, held in place between two opposing magnets to minimize friction, and briefly observed to check flight capability (Fig. S1). After a 30-minute recovery period, mosquitoes could fly freely for one h. The distance covered every five second (to the nearest 10 cm) was recorded using the flight mill software (Flight Mill Version 2).

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics (Version 24) or in RStudio (R version 3.6.0). To evaluate differences between strains in number of mosquitoes successfully pupating and eclosing, t tests were performed in SPSS, with differences in sex ratio for both pupae and adults analysed using chi-square test. Differences in the longevity of female and male mosquitoes from each strain were investigated using Kaplan–Meier survival curves and compared using Logrank (Mantel–Cox).

To determine if bloodmeal volume, wing length or energy content differed between strains, we used generalized linear mixed models (GLMMs) using the 'lme4' package in R. GLMMs for energy resources were fit with a Gaussian distribution. To account for variation in body size between individual mosquitoes, wing length was included in the GLMM as a random effect. Stepwise regression was used for model selection. All explanatory variables and two-way interactions were fit, and their significance was tested using log-likelihood ratio

tests by comparison to a null model with only an intercept. Pairwise comparisons between categories were conducted using Tukey range tests ('lsmeans' package Version 2.30-0), with the *p* value significance threshold adjusted using the Bonferroni correction method. To investigate male fecundity, we analysed sperm number per mm of wing length for each strain. For individual mating and cross mating, we investigated the associations between the proportion of females successfully inseminated and strain using GLMMs fit with a binomial distribution, following the same method as previously described. Statistical significance of female fecundity was investigated using *t* tests.

Flight ability parameters (average speed, maximum speed, number of flight bursts and flight burst length) were analysed using RStudio prior to further analysis using SPSS. Individuals, which flew less than 50 m, were not included in analysis to rule out the possibility that attachment to the flight mill may have compromised their flight. Then, *t* tests were carried out using SPSS.

Results

Mosquito life traits

Immature development time. At standard rearing density, REC-R and REC-U had the highest pupation and eclosion rates,

and at the crowded rearing density, REC-R had the highest pupation and eclosion rate (Table 1). Female-to-male ratios also differed between strains for both pupae and adult mosquitoes (Table 1). For all strains, the time to 50% pupation and eclosion was slower in the higher density trays.

Longevity. With a mean female survival of 28.07 days [95% confidence intervals (95% CI) 25.23–30.91], REC-R had greater longevity than REC-U (20.49 days, 95% CI 18.74–22.25, *p* < 0.001), REC-M (22.68 days, 95% CI 20.99–24.37, *p* < 0.001) and REC-P (21.45 days, 95% CI 20.24–22.67, *p* < 0.001).

With a mean male survival of 35.13 days (95% CI 32.52–37.73), REC-R had greater longevity than REC-U (25.86 days, 95% CI 22.81–28.91, *p* < 0.001) and REC-M (27.09 days, 95% CI 24.67–29.52, *p* < 0.001). REC-P had a mean survival of 36.80 days (95% CI 34.51–39.09), also surviving significantly longer than REC-U (*p* < 0.001) and REC-M (*p* < 0.001) (Fig. 2).

Energy resources. To determine whether energetic resources differed between strains, we first explored adult body size, followed by the relationship between body size and blood volume consumed.

At the standard rearing density REC-R, REC-U and REC-P female mosquitoes were all significantly larger than REC-M

Table 1. Mosquito pupation, eclosion and sex ratios by strain and rearing density.

Density	Strain	Mean number pupated and time to 50% pupation			Pupae sex ratio (F:M)	Mean number eclosed and time to 50% eclosed			Adult sex ratio (F:M)
		Female	Male	% Pupated		Female	Male	% Eclosed	
200 larvae/tray	REC-R	96.0 (SD ± 2.4) 4 days	110.3 (SD ± 6.3) 3 days	100.0	1:1.15	80.3 (SD ± 9.2) 7 days	98.7 (SD ± 1.7) 5 days	89.5	1:1.23
	REC-U	92.0 (SD ± 7.8) 4 days	115.0 (SD ± 0) 3 days	100.0	1:1.23	87.0 (SD ± 7.9) 7 days	98.7 (SD ± 2.9) 5 days	92.8	1:1.13
	REC-M	75.5 (SD ± 13.5) 4 days	75.5 (SD ± 11.5) 2 days	75.5*	1:1	54.0 (SD ± 10) 5 days	54.5 (SD ± 2.5) 5 days	54.25*	1:1
	REC-P	76.7 (SD ± 10.2) 3 days	83.3 (SD ± 18.4) 2 days	80.0*	1:1.09	59.7 (SD ± 8.3) 6 days	63 (SD ± 13.4) 5 days	61.0*	1:1.07
500 larvae/tray	REC-R	213.0 (SD ± 6.0) 8 days	256.5 (SD ± 2.5) 4 days	93.9*	1:1.20	155.0 (SD ± 4) 10 days	217.4 (SD ± 1.5) 6 days	74.5*	1:1.40
	REC-U	118.5 (SD ± 6.5) 6 days	149.5 (SD ± 3.5) 4 days	53.6	1:1.26	88.5 (SD ± 1.5) 8 days	117 (SD ± 5) 6 days	41.1	1:1.32
	REC-M	111.5 (SD ± 2.5) 5 days	195.0 (SD ± 19.0) 3 days	61.3	1:1.75	79.5 (SD ± 0.5) 8 days	145.5 (SD ± 19.5) 6 days	45.0	1:1.83
	REC-P	217.0 (SD ± 0) 6 days	260.0 (SD ± 0) 4 days	47.6	1:1.19	160 (SD ± 0) 8 days	214 (SD ± 0) 7 days	37.4	1:1.34

*Significant difference when compared to REC-U (*p* < 0.05).

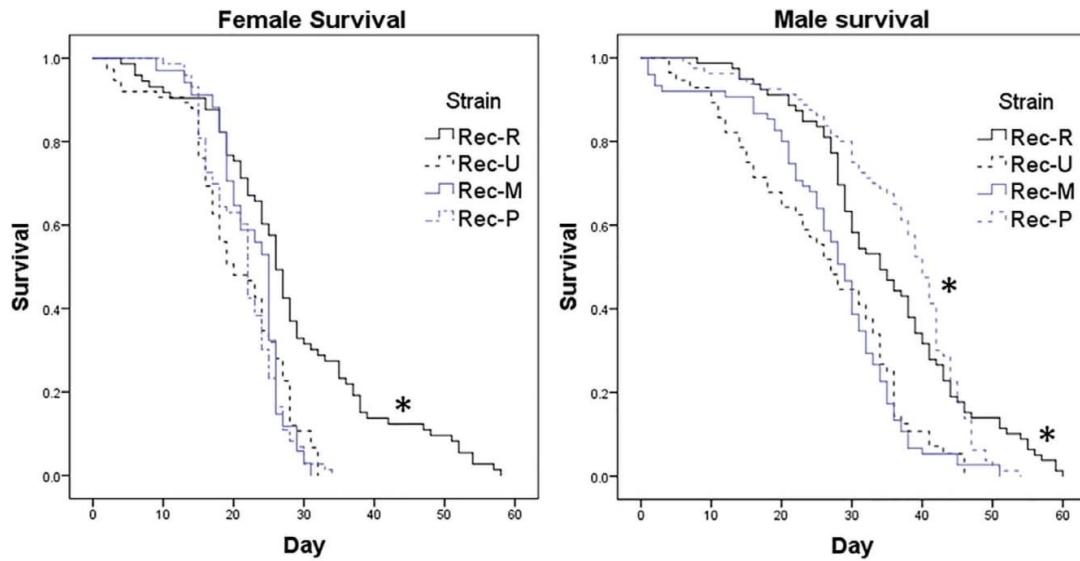


Fig 2. (A) Kaplan–Meier survival curves of REC-R ($n = 71$), REC-U ($n = 73$), REC-M ($n = 34$) and REC-P ($n = 76$) female mosquitoes and (B) Kaplan–Meier survival curves of REC-R ($n = 77$), REC-U ($n = 54$), REC-M ($n = 74$) and REC-P ($n = 77$) male mosquitoes. * $p < 0.05$.

(Fig. 3) (Table S2 and Fig. S2). At the crowded rearing density, there was a significant difference in size between all strains of mosquito.

There was a positive correlation ($R^2 = 0.27$) between bloodmeal volume and wing length ($\chi^2 = 15.599$, $df = 1$, $p < 0.001$), with no difference in this relationship between strains ($\chi^2 = 1.111$, $df = 3$, $p = 0.57$).

Lipid. The fixed effects of ‘strain’, ‘density’ and ‘age’ each contributed significantly to the explanatory power of the best fit model of lipid content (Table S3).

There was a significant interaction between ‘strain’ and ‘density’ ($\chi^2 = 34.138$, $df = 3$, $p < 0.001$). When reared at standard density there were no differences between any combinations of strains, however, at high-density lipid content for both REC-R and REC-U was significantly higher than REC-P [REC-P – REC-R ($p = < 0.001$, 95% CI -49.24 to -16.42), REC-P – REC-U ($p = 0.008$, 95% CI -51.27 to 12.347); Table S4].

The best fit model for lipid content also reported a significant interaction between ‘strain’ and ‘age’ ($\chi^2 = 50.503$, $df = 3$, $p < 0.001$; Fig. S3). At two DPE lipid content for REC-R was significantly higher than REC-M and REC-P [REC-M – REC-R ($p = < 0.001$, 95% CI -55.78 to -21.79), REC-P – REC-R ($p = < 0.001$, 95% CI -57.01 to -25.07)]. All other pairwise comparisons at two DPE were not significantly different. At eight DPE, REC-M lipids were significantly higher than REC-P with no difference between all other pairwise comparisons [REC-M – REC-P ($p = < 0.001$, 95% CI 17.73 – 54.70); Table S5].

Glycogen. The fixed effects of ‘strain’, ‘density’ and ‘age’ each contributed significantly to the explanatory power of the best fit model for glycogen content (Table S6).

There was a significant interaction between ‘strain’ and ‘density’, indicating that the relationship between strain and glycogen content was dependent on density at the larval stage ($\chi^2 = 22.241$, $df = 3$, $p < 0.001$). Pairwise comparisons showed that at standard density the mean glycogen content for REC-R was higher than both REC-P and REC-U, all other combinations were not significantly different [REC-R – REC-P ($p = 0.003$, 95% CI 7.35 – 25.85), REC-R – REC-U ($p = < 0.001$, 95% CI 8.83 – 26.71); Table S7]. However, when reared at high density there was no difference in glycogen contents between any combinations of strains.

The interaction between ‘strain’ and ‘age’ also contributed to the model of glycogen content, indicating that the relationship between strain and glycogen content varied depending on the DPE ($\chi^2 = 24.985$, $df = 3$, $p < 0.001$). At two DPE, glycogen content for REC-R was significantly higher than REC-M, REC-P and REC-U, with no significant difference between any combination of these other strains [REC-M – REC-R ($p = 0.005$, 95% CI -26.74 to -7.02), REC-P – REC-R ($p = < 0.001$, 95% CI -29.25 to -10.47), REC-R – REC-U ($p = < 0.001$, 95% CI 12.56 – 31.73); Table S8 and Fig. S3]. At eight DPE, there was no difference between any combinations of strains.

Reproductive fitness

Sperm number. REC-R contained a significantly higher number of sperm per mm of wing length than all other strains

Table 2. Mean sperm number, wing length and sperm number per mm of wing length for each of the four strains.

Strain	N	Sperm number (95% CI)	Wing length (mm) (95% CI)	Sperm number/mm wing length (95% CI)
REC-R	14	3806.14 (2222.24–5390.05)	2.60 (2.55–2.66)	1475.22* (851.17–2099.28)
REC-U	15	1779.07 (1033.09–2525.04)	2.62 (2.57–2.68)	681 (394.14–969.01)
REC-M	15	1318.27 (629.16–2007.37)	2.57 (2.53–2.61)	511.20 (244.88–777.53)
REC-P	14	1719.86 (1182.61–2257.10)	2.61 (2.56–2.65)	657.12 (448.64–865.60)

*Significant difference compared to all other strains $p < 0.05$.

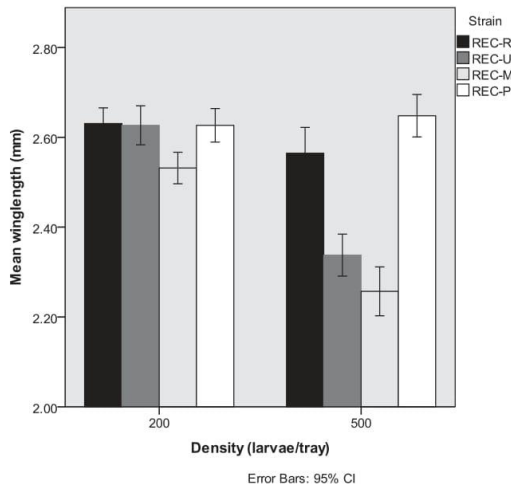


Fig 3. Wing length of four strains of *Aedes aegypti*, reared at standard 200/tray (REC-R $n = 36$, REC-U $n = 38$, REC-M $n = 35$, REC-P $n = 32$) and crowded 500/tray (REC-R $n = 32$, REC-U $n = 32$, REC-M $n = 35$, REC-P $n = 32$) larval densities. Different letters indicate statistically significant differences between strains ($p < 0.05$) per density, with 95% confidence intervals.

[Correction added on 19 November 2021, after first online publication: Figure 3 has been replaced with correct figure.]

[REC-U $t(27) = 2.5487$, $p = 0.017$; REC-M $t(27) = 3.1404$, $p = 0.004$; REC-P $t(26) = 2.6862$, $p = 0.012$] (Table 2).

Individual mating success. Binomial regression analysis showed that overall strain was a statistically significant factor for individual mating success over the 3-day period ($\chi^2 = 14.675$, $df = 3$, $p = 0.002$).

A significant difference in mating success was observed between REC-M and REC-R ($p = 0.002$, 95% CI 0.188) (Fig. 4 and Table S9). All other pairwise comparisons were not significantly different.

Cross mating. Mating success was explored further through cross mating of the poorest performing strain (REC-R) and the highest performing strain (REC-M). Results show that mating success is a male trait and again that strain is a significant factor

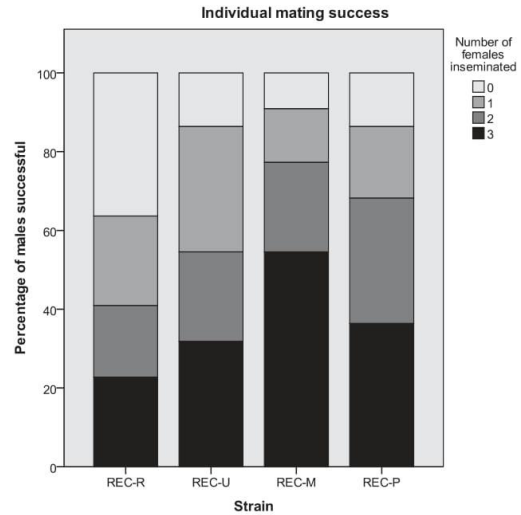


Fig 4. Individual mating success of one male mosquito ($n = 22$ per strain) with three female mosquitoes ($n = 66$ per strain).

($\chi^2 = 15.372$, $df = 3$, $p = 0.002$). REC-M males were more successful at inseminating both REC-M females ($p = 0.033$, 95% CI 11.976) and REC-R females ($p = 0.066$, 95% CI 6.345), than REC-R males were (Table S10).

Female fecundity. REC-U females produced a larger mean egg batch per female (35.02 eggs/female) than REC-R (18.03 eggs/female) and REC-M (22.60 eggs/female); however, neither comparison was statistically significant (REC-R $p = 0.122$, 95% CI -40.137 to 6.964; REC-M $p = 0.289$, 95% CI -40.176 to 15.642; Table 3). REC-U also had a higher larval hatch rate per female (26.6 larvae/female) than REC-R (13.2 larvae/female), REC-M (9.9 larvae/female) and REC-P (16.1 larvae/female); however, no comparisons were significantly different (REC-R $p = 0.205$, 95% CI -847.97 to 249.97; REC-M $p = 0.143$, 95% CI -952.18 to 198.84; REC-P $p = 0.353$, 95% CI -1147.32 to 559.65).

Quantification of flight ability

A total of 99 mosquitoes were flown on the tethered insect flight mill. REC-P flew a longer distance within an hour than

Table 3. Fecundity of females fed to repletion.

Strain	<i>N</i>	Mean eggs	Mean L1	% Hatch
REC-R	63	18.03	13.2	73.0
REC-U	65	35.02	26.6	75.8
REC-M	60	22.6	9.9	44.0
REC-P	35	41.9	16.1	38.4

REC-R; however, neither strain was statistically significant compared to REC-U (Table 4) [REC-P $t(69) = 0.2792$, $p = 0.7809$; REC-R $t(71) = 0.8975$, $p = 0.3725$]. REC-P also showed more sustained flight when compared to REC-U, with less than half of the number of flight bursts of REC-R [REC-P $t(69) = 1.2982$, $p = 0.1985$; REC-R $t(71) = 0.5759$, $p = 0.5665$]; however, this was not statistically significant.

These results show that insecticide selection does have an impact on the life-history traits of both female and male mosquitoes. Compared to all other strains, REC-R had the highest pupation and eclosion rates at both rearing densities, female and male adults survived longer, females were larger at the crowded rearing density and males produced more sperm per mm of wing length. However, REC-R males and females had the poorest reproductive fitness with males inseminating the fewest females and females laying the fewest eggs. In comparison, REC-M had the smallest females at both rearing densities, but the highest individual female insemination success rate.

Discussion

Throughout this study, the temephos exposed REC-R strain has shown the most noticeable differences in fitness and fecundity when compared to the other exposed and unexposed. With higher pupation numbers at both rearing densities, males and females surviving longer, increased energy resources under certain conditions and highest sperm number, our results suggest a fitness advantage due to sustained temephos selection pressure. However, despite the increased sperm number seen in REC-R, there appears to be a net fecundity cost due to poor male mating success and lower mean egg numbers.

One possible explanation for why REC-R males had the highest sperm count but lowest insemination success is that this strain produces a larger ejaculate but at less frequent intervals. This result is mirrored in work by Belinato *et al.* (2012) who saw that mating efficacy was inversely proportional to temephos resistance ratio, and in work by Diniz *et al.* (2015) who showed that resistance status impacts male mating success. Body size is a well-documented factor in male mating success, with previous studies (Ponlawat & Harrington, 2007, 2009) reporting that *A. aegypti* body size was correlated with sperm number. However, our study confirmed that the significant differences in sperm number between strains were not attributable to differences in body size.

Our results on female fecundity are again similar to Belinato *et al.* (2012), who showed females from a highly resistant

temephos field strain laid fewer eggs than the susceptible counterpart. One limitation of our study is we were unable to measure fecundity throughout the female's lifespan due to an unavoidable change in blood source after the first gonotrophic cycle.

While reduced fecundity in resistant strains could lead to lower mosquito densities, adult female longevity is a crucial factor in the vectorial capacity of wild mosquito populations. REC-R female and male mosquitoes survived for significantly longer than other strains in this study; however, previous work using a different *A. albopictus* reported that temephos resistant field strains had a shorter lifespan than their susceptible counterpart (Rahim *et al.*, 2017). There are important differences between our study design and the one followed by Rahim *et al.* (2017), most notably, we tested laboratory mosquitoes with an extended history of insecticide pressure, in contrast to a progeny originating from only one round of larval temephos exposure. We also did not offer a bloodmeal to females during the longevity assay and instead provided continued access to sucrose solution.

Results from energy content analysis show that teneral energy reserves do not explain the stark differences in fitness traits for REC-R. There was no significant difference in lipid or glycogen content observed between strains, instead differences were only observed between the two larval rearing densities and mosquito age. Energy content cannot, therefore, explain reductions in egg batch size, improved immature development or increased longevity. With lipids and glycogen being important for use in flight, we were not surprised to observe no difference in flight duration or flight burst number between strains.

It is important to note that while the strains used all originated from the same parental colony, these fitness experiments were carried out under laboratory-controlled conditions. The Recife colony used for selection had a background of previous temephos exposure and each strain underwent differential selection with exposure to insecticides using concentrations at 50% lethal dose (LD) over a period of 12 months. The physiological costs of resistance are often underestimated within a laboratory setting due to a lack of stress factors that are experienced in the field. In this study, however, we took the stress of larval crowding into consideration when assessing life-history traits.

Interestingly, our data suggest that continued selection to the organophosphate temephos at larval stages leads to shorter developmental time and increased longevity but reduced fecundity in the unexposed offspring. However, switching to selection with the organophosphate malathion in adult stage leads to better reproductive fitness but at the cost of longevity. With spermatogenesis thought to peak at the pupal stage, one explanation is that exposure during larval development can only lead to resource allocation that benefits longevity rather than reproduction. Conversely, improved fecundity in strains historically exposed during the adult life stage suggests that resources are diverted to offspring production rather than adult survival. These results have worrying implications for vector control programmes that target larval stages with insecticides, as longevity of the vector population is a key determinant of disease transmission potential.

Table 4. Mean flight distance and number of flight bursts over 1 h.

Strain	<i>N</i>	Distance (m) (95% CI)	Ratio*	Number flight bursts (95% CI)	Ratio*
REC-R	23	751.93 (387.39–1116.47)	0.80	21.22 (12.63–29.80)	1.20
REC-P	21	1012.57 (508.92–1519.22)	1.07	9.81 (2.87–16.75)	0.55
REC-U	50	944.64 (701.27–1188.01)	–	17.70 (10.32–25.08)	–

*Ratio compared to REC-U mosquitoes flown at the same time.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The set-up of the tethered insect flight mill used to assess the flight capability of mosquitoes. Mosquitoes fly around a radius measuring 4 cm, causing the light encoder to periodically break a laser beam, which measures distance. One full rotation of the flight mill rotor arm = 25.13 cm. Image taken from (Somerville *et al.*, 2019).

Fig. S2. Bloodmeal volume relationship. Relationship between wing length and bloodmeal volume is not statistically distinguishable between strains. Shaded areas show upper and lower CIs for the line of best fit as predicted by the model. CIs overlap at all points in range, so all strains follow the same linear relationship.

Fig. S3. Predicted mean energy content for each *Aedes aegypti* strain reared at two different larval densities; lipid content at two days post-emergence (DPE) (A), lipid content at eight DPE (B), glycogen content at two DPE (C) and glycogen content at eight DPE (D).

Table S1. Lethal concentrations and resistance ratios of Recife strains for three insecticides (i.e. permethrin, malathion and temephos). Taken from Thornton *et al.* (2020).

Table S2. Mean wing length comparisons of four strains of *Aedes aegypti* reared at two different larval densities.

Table S3. GLMM lipid model statistics.

Table S4. The effects of strain and density on lipid content.

Table S5. The effects of strain and age on lipid content.

Table S6. GLMM glycogen model statistics.

Table S7. The effects of strain and density on glycogen content.

Table S8. The effects of strain and age on glycogen content.

Table S9. Differences in individual mating success between all four strains of *Aedes aegypti*.

Table S10. Cross mating success between REC-M and REC-R males when given the opportunity to mate with REC-M and REC-R females.

Acknowledgements

We thank Jonathon Thornton, Liverpool School of Tropical Medicine, for his continued assistance in mosquito husbandry

and Alastair G. T. Somerville for his assistance with tethered insect flight mill experiments. LJR is on the editorial board of Medical and Veterinary Entomology; however, they played no role in the assessment of the manuscript. This work was supported by Wellcome Trust (202137) to LJR.

All other authors declare no conflict of interest.

Author contributions

LJR and KG conceived and designed the study, KG collected the data, KG and FM analysed the data, KG wrote the first draft of the manuscript. All authors contributed to the final draft of the manuscript.

Date availability statement

The data that support the findings of this study are openly available in Open Science Framework at <https://osf.io/crsmu/> (DOI 10.17605/OSF.IO/CRSMU)

References

- Adlakha, V. & Pillai, M.K.K. (1976) Role of male accessory gland substance in the regulation of blood intake by mosquitoes. *Journal of Insect Physiology*, **22**, 1441–1442. [https://doi.org/10.1016/0022-1910\(76\)90206-7](https://doi.org/10.1016/0022-1910(76)90206-7).
- Arnaud, L., Haubruge, E. & Gage, M.J.G. (2005) The malathion-specific resistance gene confers a sperm competition advantage in *Tribolium castaneum*. *Functional Ecology*, **19**, 1032–1039. <https://doi.org/10.1111/j.1365-2435.2005.01055.x>.
- Beenackers, A.M.T., Horst, D.J.V.D. & Marrewijk, W.J.A.V. (1981) Role of lipids in energy metabolism. *Energy Metabolism in Insects* (ed. by R.G.H. Downer), pp. 53–100. Springer: New York.
- Belinato, T.A. & Martins, A.J. (2016) Insecticide resistance and fitness cost. *InTechOpen*, **32**, 243–261. <https://doi.org/10.5772/61826>.
- Belinato, T.A., Martins, A.J. & Valle, D. (2012) Fitness evaluation of two Brazilian *Aedes aegypti* field populations with distinct levels of resistance to the organophosphate temephos. *Memorias Do Instituto Oswaldo Cruz*, **107**, 916–922. <https://doi.org/10.1590/S0074-02762012000700013>.
- Bhatt, S., Weiss, D.J., Cameron, E., Bisanzio, D., Mappin, B. & Dalrymple, U. (2016) The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature*, **526**, 207–211. <https://doi.org/10.1038/nature15535>.
- Briegleb, H., Lea, A.O. & Klowden, M.J. (1979) Hemoglobinometry as a method for measuring blood meal sizes of mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*, **15**, 235–238. <https://doi.org/10.1093/jmedent/15.3.235>.

- Brito, L.P., Linss, J.G.B., Lima-Camara, T.N. *et al.* (2013) Assessing the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost. *PLoS One*, **8**, e60878. <https://doi.org/10.1371/journal.pone.0060878>.
- Chan, H.H. & Zairi, J. (2013) Permethrin resistance in *Aedes albopictus* (Diptera: Culicidae) and associated fitness costs. *Journal of Medical Entomology*, **50**, 362–370. <https://doi.org/10.1603/ME12182>.
- Coustau, C., Chevillon, C. & Ffrench-Constant, R. (2000) Resistance to xenobiotics and parasites: Can we count the cost? *Trends in Ecology and Evolution*, **15**, 378–383. [https://doi.org/10.1016/S0169-5347\(00\)01929-7](https://doi.org/10.1016/S0169-5347(00)01929-7).
- David, M.R., Garcia, G.A., Valle, D. & Maciel-De-Freitas, R. (2018) Insecticide resistance and fitness: The case of four *Aedes aegypti* populations from different Brazilian Regions. *BioMed Research International*, **2018**, 1–12. <https://doi.org/10.1155/2018/6257860>.
- Diniz, D.F.A., Melo-Santos, M.A.V.D., Santos, E.M.D.M. *et al.* (2015) Fitness cost in field and laboratory *Aedes aegypti* populations associated with resistance to the insecticide temephos. *Parasites and Vectors*, **8**, 1–15. <https://doi.org/10.1186/s13071-015-1276-5>.
- Downe, A.E.R. (1975) Internal regulation of rate of digestion of blood meals in the mosquito, *Aedes aegypti*. *Journal of Insect Physiology*, **21**, 1835–1839. [https://doi.org/10.1016/0022-1910\(75\)90250-4](https://doi.org/10.1016/0022-1910(75)90250-4).
- Hiss, E.A. & Fuchs, M.S. (1972) The effect of matrone on oviposition in the mosquito, *Aedes aegypti*. *Journal of Insect Physiology*, **18**, 2217–2227. [https://doi.org/10.1016/0022-1910\(72\)90250-8](https://doi.org/10.1016/0022-1910(72)90250-8).
- Klowden, M.J. (1993) Mating and nutritional state affect the reproduction of *Aedes albopictus* mosquitoes. *Reproduction*, **9**, 169–173.
- Kramer, L.D. & Ebel, G.D. (2003) Dynamics of flavivirus infection in mosquitoes. *Advances in Virus Research*, Amsterdam, Netherlands: Elsevier, pp. 187–232.
- Martins, A.J., Ribeiro, C.D.e.M., Bellinato, D.F., Peixoto, A.A., Valle, D. & Lima, J.B.P. (2012) Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected *Aedes aegypti* populations. *PLoS One*, **7**, 1–9. <https://doi.org/10.1371/journal.pone.0031889>.
- Methods in Anopheles Research*. 3rd edn. (2015). <http://www.mr4.org>.
- Montella, I.R., Martins, A.J., Viana-Medeiros, P.F., Lima, J.B.P., Braga, I.A. & Valle, D. (2007) Insecticide resistance mechanisms of Brazilian *Aedes aegypti* populations from 2001 to 2004. *American Journal of Tropical Medicine and Hygiene*, **77**, 467–477. <https://doi.org/10.4269/ajtmh.2007.77.467>.
- Moyes, C.L., Vontas, J., Martins, A.J. *et al.* (2017) Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. *PLoS Neglected Tropical Diseases*, **11**, 1–20. <https://doi.org/10.1371/journal.pntd.0005625>.
- Ponlawat, A. & Harrington, L.C. (2007) Age and body size influence male sperm capacity of the dengue vector *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology*, **44**, 422–426. [https://doi.org/10.1603/0022-2585\(2007\)44\[422:aabsim\]2.0.co;2](https://doi.org/10.1603/0022-2585(2007)44[422:aabsim]2.0.co;2).
- Ponlawat, A. & Harrington, L.C. (2009) Factors associated with male mating success of the dengue vector mosquito, *Aedes aegypti*. *The American Journal of Tropical Medicine and Hygiene*, **80**, 395–400.
- Rahim, J., Ahmad, A.H. & Maimusa, A.H. (2017) Effects of temephos resistance on life history traits of *Aedes albopictus* (Skuse) (Diptera: Culicidae), a vector of arboviruses. *Revista Brasileira de Entomologia*, **61**, 312–317. <https://doi.org/10.1016/j.rbe.2017.08.001>.
- Ramos, R.S., Araújo, V.C.R.D., Pereira, R.R. *et al.* (2018) Investigation of the lethal and behavioral effects of commercial insecticides on the parasitoid wasp *Copidosoma truncatellum*. *Chemosphere*, **191**, 770–778. <https://doi.org/10.1016/j.chemosphere.2017.10.113>.
- Ranson, H., Burhani, J., Lumjuan, N. & Black, W.C. IV (eds.) (2010) Insecticide resistance in dengue vectors. *Tropika*, **1**, 307–316.
- Ranson, H. & Lissenden, N. (2016) Insecticide resistance in African anopheles mosquitoes: A worsening situation that needs urgent action to maintain malaria control. *Trends in Parasitology*, **32**, 187–196. <https://doi.org/10.1016/j.pt.2015.11.010>.
- Rivero, A., Vézilier, J., Weill, M., Read, A.F. & Gandon, S. (2010) Insecticide control of vector-borne diseases: When is insecticide resistance a problem? *PLoS Pathogens*, **6**, 5–6. <https://doi.org/10.1371/journal.ppat.1001000>.
- Saingamsook, J., Yanola, J., Lumjuan, N., Walton, C. & Somboon, P. (2019) Investigation of relative development and reproductive fitness cost in three insecticide-resistant strains of *Aedes aegypti* from Thailand. *Insects*, **10**, 1–16. <https://doi.org/10.3390/insects10090265>.
- Somerville, A.G.T., Gleave, K., Jones, C.M. & Reimer, L.J. (2019) The consequences of *Brugia malayi* infection on the flight and energy resources of *Aedes aegypti* mosquitoes. *Sci Rep*, **9**, 18449. <https://doi.org/10.1038/s41598-019-54819-2>.
- Steele, J.E. (1981) The role of carbohydrate metabolism in physiological function. *Energy Metabolism in Insects*. Plenum, New York.
- Thornton, J., Gomes, B., Ayres, C. & Reimer, L. (2020) *Insecticide resistance selection and reversal in two strains of Aedes aegypti*. Wellcome Open Research.
- Viana-Medeiros, P.F., Bellinato, D.F., Martins, A.J. & Valle, D. (2017) Insecticide resistance, associated mechanisms and fitness aspects in two Brazilian *Stegomyia aegypti* (= *Aedes aegypti*) populations. *Medical and Veterinary Entomology*, **31**, 340–350. <https://doi.org/10.1111/mve.12241>.
- Villarreal, S.M., Pitcher, S., Helinski, M.E.H., Johnson, L., Wolfner, M.F. & Harrington, L.C. (2018) Male contributions during mating increase female survival in the disease vector mosquito *Aedes aegypti*. *Journal of Insect Physiology*, **108**, 1–9. <https://doi.org/10.1016/j.jinsphys.2018.05.001>.
- Vontas, J., Blass, C., Koutsos, A.C. *et al.* (2005) Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. *Insect Molecular Biology*, **14**, 509–521. <https://doi.org/10.1111/j.1365-2583.2005.00582.x>.
- Vontas, J., Kioulos, E., Pavlidi, N., Morou, E., Della Torre, A. & Ranson, H. (2012) Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. *Pesticide Biochemistry and Physiology*, **104**, 126–131. <https://doi.org/10.1016/j.pestbp.2012.05.008>.
- WHO (2018) *Global report on insecticide resistance in malaria vectors*, Geneva: World Health Organisation, pp. 2010–2016.

Accepted 14 October 2021


First published online 9 November 2021

RESEARCH

Open Access



The seasonal dynamics and biting behavior of potential *Anopheles* vectors of *Plasmodium knowlesi* in Palawan, Philippines

Richard Paul B. Malijan¹, Frank Mechan², Jessie C. Braganza Jr¹, Kristelle Mae R. Valle¹, Ferdinand V. Salazar¹, Majhalia M. Torno^{1,3}, Wilfredo E. Aure¹, Brian A. Bacay¹, Fe Esperanza Espino⁴, Stephen J. Torr², Kimberly M. Fornace⁵, Chris Drakeley⁵ and Heather M. Ferguson^{6*} 

Abstract

Background: A small number of human cases of the zoonotic malaria *Plasmodium knowlesi* have been reported in Palawan Island, the Philippines. Identification of potential vector species and their bionomics is crucial for understanding human exposure risk in this setting. Here, we combined longitudinal surveillance with a trap-evaluation study to address knowledge gaps about the ecology and potential for zoonotic spillover of this macaque malaria in Palawan Island.

Methods: The abundance, diversity and biting behavior of human-biting *Anopheles* mosquitoes were assessed through monthly outdoor human landing catches (HLC) in three ecotypes representing different land use (forest edge, forest and agricultural area) across 8 months. Additionally, the host preference and biting activity of potential *Anopheles* vectors were assessed through comparison of their abundance and capture time in traps baited with humans (HLC, human-baited electrocuting net—HEN) or macaques (monkey-baited trap—MBT, monkey-baited electrocuting net—MEN). All female *Anopheles* mosquitoes were tested for the presence of *Plasmodium* parasites by PCR.

Results: Previously incriminated vectors *Anopheles balabacensis* and *An. flavirostris* accounted for > 95% of anophelines caught in longitudinal surveillance. However, human biting densities were relatively low (*An. balabacensis*: 0.34–1.20 per night, *An. flavirostris*: 0–2 bites per night). Biting densities of *An. balabacensis* were highest in the forest edge, while *An. flavirostris* was most abundant in the agricultural area. The abundance of *An. balabacensis* and *An. flavirostris* was significantly higher in HLC than in MBT. None of the 357 female *Anopheles* mosquitoes tested for *Plasmodium* infection were positive.

Conclusions: The relatively low density and lack of malaria infection in *Anopheles* mosquitoes sampled here indicates that exposure to *P. knowlesi* in this setting is considerably lower than in neighboring countries (i.e. Malaysia), where it is now the primary cause of malaria in humans. Although anophelines had lower abundance in MBTs than in HLCs, *An. balabacensis* and *An. flavirostris* were caught by both methods, suggesting they could act as bridge vectors between humans and macaques. These species bite primarily outdoors during the early evening, confirming that insecticide-treated nets are unlikely to provide protection against *P. knowlesi* vectors.

Keywords: *Anopheles balabacensis*, *Anopheles flavirostris*, *Plasmodium knowlesi*, Vector behavior, Philippines

*Correspondence: Heather.Ferguson@glasgow.ac.uk

⁶ Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow G12 8QQ, UK

Full list of author information is available at the end of the article



© The Author(s) 2021. This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

Background

The Philippines has established a subnational/territorial malaria elimination strategy, through which zero indigenous cases were reported in 78 out of 81 provinces in 2019 [1–3]. The primary malaria species of public health importance in the Philippines are *Plasmodium falciparum* and *P. vivax* which respectively comprise ~88% and 9% of the total indigenous malaria cases [1]. Malaria transmission in the country is now confined to a few provinces including Palawan [1–3]. Concern has been raised that the emergence of the zoonotic malaria parasite *P. knowlesi* as a public health problem in several Southeast Asian countries may threaten regional elimination [4, 5]. Human cases of *P. knowlesi* infection in Palawan, Philippines were first confirmed in 2008, based on molecular detection from blood slides that had been previously diagnosed by microscopy as *P. malariae* [6]. Recent serological work indicates that *P. knowlesi* transmission in Palawan is ongoing, with community sampling reporting that 1.1% of individuals tested positive for the *P. knowlesi*-specific PkSERA3 ag1 antibody response [7]. In response to the emerging threat of *P. knowlesi*, an international collaboration was established in 2012 to investigate the risk factors for human infections and identify populations at risk. The MONKEYBAR project focused investigation on two known areas of transmission: Sabah in Malaysian Borneo and Palawan Island in the Philippines [7, 8]. Although human infections of *P. knowlesi* have been reported in both settings [5, 7, 9], cases have been sporadic in Palawan [6, 10, 11] whilst *P. knowlesi* is now the leading cause of human malaria in Sabah [5, 12, 13].

The primary reservoirs of *P. knowlesi* are the long-tailed (*Macaca fascicularis*) and pig-tailed (*M. nemestrina*) macaques that are widely distributed throughout Southeast Asia [14, 15]. Long-tailed macaques are the only monkey species in the Philippines, and are widely distributed throughout the country including Palawan [16]. While long-tailed macaques have been confirmed as reservoirs of *P. knowlesi* in Palawan [17], there is limited understanding of the ecology of *P. knowlesi* transmission and potential for human spillover in this setting. Of particular concern is whether human *P. knowlesi* cases will continue to be sporadic and rare in Palawan, or will transition into substantial spillover into human populations as has occurred in the neighboring area of Sabah, Malaysian Borneo; which is < 100 km across the sea from Palawan. Variation in epidemiological potential may be related to differences in vector species and their interactions with human and macaque host species. Understanding the local ecology of transmission is vital to identify both spillover potential and control strategies [14].

Competent *Anopheles* species that feed on both human and monkey hosts could act as *P. knowlesi* bridge vectors [15, 18]. Mosquitoes in the *Anopheles leucosphyrus* group have been implicated as *P. knowlesi* vectors and capable of cross-species transfer between macaques to humans [12, 18–21]. Primary vector species vary geographically [22–25], with *An. balabacensis* and *An. donaldi* being the most important in Sabah [26, 27]. In the Philippines, there has been relatively limited investigation of *Anopheles* vectors of simian malaria. Early work (1970s) indicated that *An. balabacensis* is the most likely vector of simian malaria on Palawan [28]; however, there has been no recent confirmation of the role of this vector within the period of *P. knowlesi* emergence in humans.

Investigating the ecology and behavior of potential vectors of *P. knowlesi*, and incrimination of the vector species responsible for cross-species transmission are essential to identify human populations at risk and develop appropriate control strategies [29]. The gold standard method for directly measuring human exposure to malaria vectors is the human landing catch (HLC) [30, 31]. However, this approach raises ethical concerns by exposing people to mosquitoes that might be infected with mosquito-borne diseases; many of which have no or limited prophylactic and treatment options. Previously, monkey-baited traps (MBT) have been used as the reference method for estimating mosquito biting rates on monkeys; however estimates from this approach are not directly comparable to HLC due to differences in procedures, and it raises animal welfare concerns [15, 19, 25]. The development of alternative mosquito trapping methods that can provide more standardized comparisons of mosquito attraction to humans and macaques, without risking host exposure to infection, would be of great value.

Electrocuting traps may offer a solution to some issues associated with traditional mosquito trapping methods [36] by using host odor to attract and sample mosquitoes [37, 38]. Such traps were originally used to sample tsetse flies in Africa and similar traps using host odor have been evaluated for mosquitoes [32, 33]. One type of electrocuting trap, the electric net (E-net), was recently evaluated for sampling mosquito vectors of *P. knowlesi* in Sabah, Malaysia [19]. Here, E-nets baited with humans and macaques generally had poorer performance than HLCs, but higher than MBTs. The potential for wider application of E-nets as a general surveillance tool for zoonotic malaria vectors has yet to be demonstrated.

Here we combined longitudinal surveillance of potential *P. knowlesi* vectors in Palawan Island with a trap-evaluation study to identify potential vector species and investigate how their ecology and infection varied between ecotypes. The aims of the longitudinal study were to characterize the abundance, diversity, seasonal

dynamics, biting behavior and *Plasmodium* spp. infection rates of potential human-biting vectors in three different habitats: forest, forest edge and agricultural. This study also aimed to assess different trapping methods for sampling human- and macaque-biting vectors in order to identify species that could act as bridge vectors.

Methods

Study site

Two separate field experiments were conducted in Barangay (Brgy.) Bacungan, Puerto Princesa City, Palawan in 2015: (1) a longitudinal study of human-biting mosquitoes and (2) a comparison of human- and monkey-baited traps (Fig. 1). Barangay Bacungan is an area with intact secondary forest and some remaining primary forest. This study site was selected based on the locations of previously reported human *P. knowlesi* cases and was the site of integrated entomology, primatology and social studies within a wider research program on risk factors for *P. knowlesi* [6, 7, 9].

The presence of *An. balabacensis*, a vector of *P. knowlesi* [27, 28, 34], was confirmed from pilot mosquito collections as well as sightings of long-tailed macaques (*M. fascicularis*). The relative accessibility and safety of the area year-round were also considered in the selection of the sites. Experiments were conducted between May

and December 2015, coinciding with the northeast monsoon season of high rainfall.

Trapping techniques

Four trapping techniques were used in this study to characterize the behavior of potential *Anopheles* vectors in Palawan.

Human landing catch (HLC)

Human landing catches were performed outdoors from 18:00 to 06:00. All trained HLC collectors were male residents in the study site, aged between 20 and 40 years. For the longitudinal study, two collectors performed the HLC in a pair, wherein one individual exposed their bare legs while seated (Fig. 2a) and the second used a manual aspirator to collect any mosquitoes that landed on the other's legs. Aspirated mosquitoes were transferred into separate collection cups labeled with the sampling station and hour of collection. At midnight, the collectors swapped roles so that each individual performed as both collector and bait over the course of the night. This protocol was modified slightly for the trap evaluation study, where only one person carried out each HLC, acting as both bait and collector.

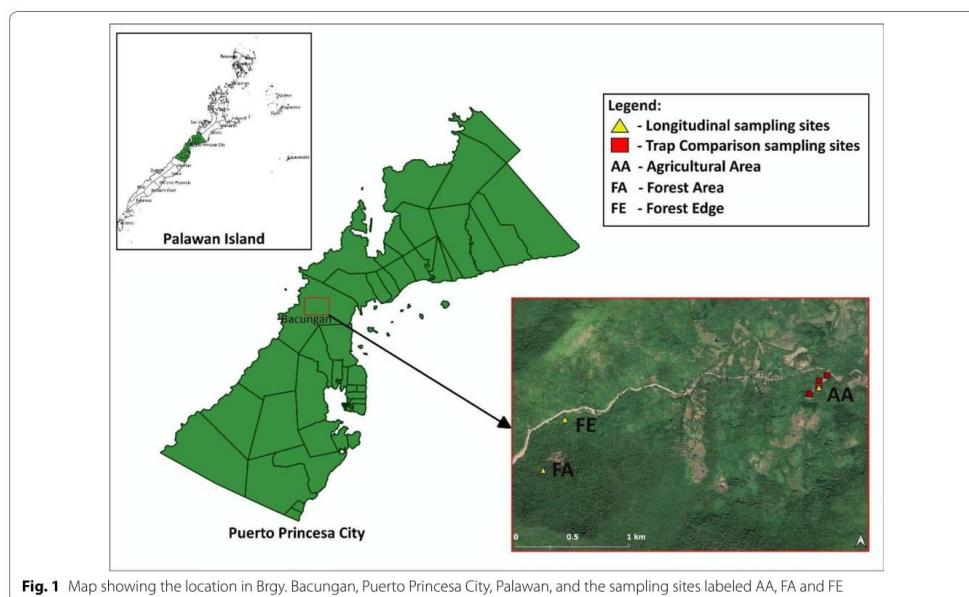


Fig. 1 Map showing the location in Brgy. Bacungan, Puerto Princesa City, Palawan, and the sampling sites labeled AA, FA and FE



Monkey-baited trap (MBT)

In previous studies, monkey-baited traps have been used as the reference method to sample mosquitoes attracted to macaques [15, 19, 25]. In this study, one adult female long-tailed macaque (*M. fascicularis*) was placed inside a steel cage measuring 2.0 × 2.0 × 2.0 m and fitted with wire mesh to prevent entry of mosquitoes. The origin of macaques used in this study and their holding conditions are described in Additional file 1.

During mosquito trapping, a large untreated mosquito net (3.9 m × 3.0 m × 3.3 m) was suspended around the cage with the door flap open (Fig. 2b). Mosquitoes attracted to the macaque entered the outer net but the wire mesh of the monkey cage prevented them from feeding on the macaque. This internal protective net was not used in most previous work with MBTs [15, 25, 34], but was incorporated into MBT design here and in another recent study by Hawkes et al. [19] in Sabah, Malaysia, as a requirement of the ethics approval granted to work with primates. Mosquitoes resting between the cage and outer net were collected every hour from 18:00 to 06:00 using a CDC backpack aspirator (Fig. 2c).

Electrocuting nets (HEN and MEN)

Electrocuting net traps work by piping the scent from a single host, housed in an enclosed tarpaulin tent, out to a collection point that is covered with an electrified surface. Two versions of this trap were used in this study: a human-baited electrocuting net (HEN) and a monkey-baited electrocuting net (MEN). In the HEN, the tent contained a human male (same volunteers as in the HLC collections) while in the MEN the tent contained a female long-tailed macaque (same macaques as participated in MBT). The host's scent was pumped from the tent to an electrified grid (Fig. 2d) via a 6-m PVC pipe using a co-axial fan (120 × 120 × 25 mm, 3100 RPM speed, air volume 3.229 m³/min). The electrified grid, measuring 1 m tall by 0.5 m wide, consisted of vertically-arranged copper wires (0.2 mm thick) spaced 5 mm apart. Alternate wires in each bank were charged by a transformer with a DC input of 12 V (3 amps) and an output of ~50 kV pulsing at ~70 Hz. Mosquitoes stunned by the electrified grid were collected in pans with water and liquid soap (to break the surface tension of the water and allow the mosquitoes to sink before escape), each pan extending 44 cm from each side of the electrified grid. The electrocuting net trap was used to collect mosquitoes from 18:00

to 06:00, with mosquito specimens collected once from the pan at the end of the night and transferred into a collection cup containing 70% ethanol. The electrified grids were also inspected for any mosquitoes attached to the wires.

Longitudinal study of human-biting mosquitoes

This study investigated the abundance and biting activity of *Anopheles* mosquitoes at three sites on Palawan Island between May and December 2015. Each collection site was broadly representative of an ecotype in Brgy. Bacungan: agricultural area (9° 53.320' N, 118° 39.076' E), forest edge (9° 53.167' N, 118° 37.850' E) and forest area (9° 52.921' N, 118° 37.744' E). There was a minimum distance between sites of 1 km (Fig. 1). A GPS device (Garmin 62SC) was used to ensure that collections were conducted repeatedly in the same spot.

The agricultural area was cleared land used for small-scale, low-input farming of mixed crops of fruit-bearing trees (mango, cashew, jackfruit) and upland rice. The forest edge area was located at the margin between secondary forest and a cleared agricultural area, characterized by a mixture of small trees and shrubs and surrounded by bamboo clusters. The forest area (secondary forest) was characterized by having more than 10% canopy cover, presence of tree species with a minimum height of 5 m, and no or low anthropogenic disturbances.

Monthly mosquito collections were carried out simultaneously at the three sites for three consecutive nights between May and December 2015. On each night, HLC collections were conducted hourly between 18:00 and 06:00. Simultaneous collections were made at each site on each night of collection by three separate teams. These teams rotated between sampling sites each night.

Comparison of human- and monkey-baited traps

This study was designed to compare mosquito collection techniques that use human and macaque hosts. The aim was to characterize the host preference of *Anopheles* species including potential *P. knowlesi* vectors by contrasting their relative abundance in traps baited with humans versus macaques. Outdoor collections of human- and monkey-biting mosquitoes were conducted using HLC, MBT, HEN and MEN at four collection stations: I (118° 39.076' E, 9° 53.353' N), II (118° 39.116' E, 9° 53.368' N), III (118° 39.074' E, 9° 53.320' N) and IV (118° 39.031' E, 9° 53.290' N).

To minimize the influence of environmental factors, all collection sites were located within the same agricultural area, with each sampling station spaced approximately 100 m apart in a Latin square design. Collections using each of the four trapping methods were conducted simultaneously from 18:00 to 06:00, with one trap at each of

the four collection stations. Traps were rotated between stations each night to give a complete replicate every four collection nights. These 4-day replicates were carried out over 40 non-continuous nights between May to July 2015, providing a total of 10 full replicates of each trap in each collection station.

Mosquito processing and identification

Mosquitoes captured within the same one-hour period were stored together in a holding cup and labeled by hour, collection site and trap type used. A field supervisor visited the teams hourly to gather and replace the collection cup. Immediately upon collection, mosquitoes were killed using ethyl acetate then placed in a cell culture plate (12-well; 12.5 × 8.5 × 2 cm) which was subdivided by time of collection.

All collected mosquitoes were taken to a field laboratory the day after the collection night for morphological identification. All mosquitoes (male and female) were identified to genus level based on morphology. Female *Anopheles* mosquitoes were identified further to species level using illustrated keys [40], while non-anopheline mosquitoes (male and female) were segregated by genus level and later identified to species level in the field laboratory [35]. After identification, all mosquito samples were placed in 1.5 ml microcentrifuge tubes lined with filter paper and silica gel. For samples collected using E-nets, mosquitoes were placed in 1.5 ml microcentrifuge tubes with 70% ethanol instead of filter paper and silica gel as stunned mosquitoes had already been soaked in a water pan.

Each microcentrifuge tube was labeled with a unique collection number corresponding to the date of collection, the time of collection, the collection station, trap type and initial species identification. Validation of *Anopheles* morphological identification was conducted by entomologists at the Research Institute for Tropical Medicine (RITM), Muntinlupa City, Metro Manila. All samples were stored in an incubator (Thermo Fisher Scientific) at 37 °C prior to processing for molecular analysis.

Molecular detection of *Plasmodium* in mosquitoes

All female *Anopheles* mosquitoes collected during the study were screened for malaria parasites. The head and thorax of dried female *Anopheles* specimens were separated from the rest of their body and placed individually in separate microcentrifuge tubes. For HEN and MEN collections, the ethanol used for mosquito preservation was allowed to evaporate completely by placing sample tubes in an AccuBlock dry bath (Labnet, USA) set at 70 °C, with whole mosquito specimens used for DNA extraction. Genomic DNA was extracted from the head

and thorax of each mosquito using the DNeasy tissue kit (Qiagen, Germany) according to the manufacturer's protocol. Eluted DNA from the same mosquito species collected from the same trap was pooled in a separate microcentrifuge tube (maximum of 10 eluted DNA per pool) and kept in a freezer at -20°C until required.

Detection of malaria parasites from the pooled specimens was conducted using a nested PCR assay using primers based on the *Plasmodium* small subunit ribosomal RNA (SSU rRNA). Primers and protocols used for *Plasmodium* detection were as described by Singh et al. [36]. For *Plasmodium*-positive pools, the first nested PCR assay was performed again for each sample from the pool. A second nested PCR assay was performed on the *Plasmodium*-positive samples to determine the species using nine species-specific primers (Additional file 2).

Nested PCR assays were performed with 25 μl final volume consisting of 5.0 μl of 5X PCR buffer (Promega), 0.5 μl of dNTP (10 mM) mixture (Promega), 3.0 μl of 25 mM MgCl_2 (Promega), 1.0 μl each of 10 μM forward and reverse primers, 0.3 μl of Taq DNA polymerase (5 U/ μl), 2.0 μl of the DNA template and sterile dH_2O up to 25 μl final volume.

The PCR conditions used were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min. The annealing temperature was set based on the optimum temperature of the primers (Additional file 1). After completion of the first PCR, 2.0 μl of the first PCR product was used as a template in the second PCR.

Statistical analysis

Statistical analysis was conducted using the R programming language (version 3.2.3). Generalized linear mixed models (GLMM) were constructed to analyze the variables of interest (nightly or hourly mosquito abundance) using key explanatory variables of collection site, hour and month (for longitudinal study) or trapping method and host bait (for trap comparison study). Graphs were produced using ggplot2 (version 2.2.1). All confidence intervals were estimated with bootstrap resampling of 10,000 samples using the 'boot' package (version 1.3-19).

Stepwise regression was used for model selection. All fixed explanatory variables and two-way interactions were fit and their significance tested using log-likelihood ratio tests (LRTs). The distribution fit to each model was chosen by considering the nature and dispersion of the data. To investigate significant associations between factor levels, post hoc comparisons were performed using Tukey tests.

Separate models of nightly and hourly abundance were fit to the data for each known *Anopheles* vector species

(*An. balabacensis* and *An. flavirostris*) to investigate spatial and seasonal variation in their biting density between different ecotypes. The response variable for nightly models was the number of females collected per night, while the response variable for each hourly model was the number of females collected in each hourly period (18:00–19:00 to 05:00–06:00). Due to overdispersion in the mosquito count data, a negative binomial distribution was determined to be the best fit for all nightly and hourly models.

To investigate spatial variation in abundance, the site of collection was fit to the nightly and hourly models as a fixed factorial effect, with the unique mosquito collector identification (ID) and date of collection as random effects. Seasonal variation in nightly biting density was investigated with month of collection fit as a fixed continuous effect and as a quadratic variable to allow peaks in monthly biting density to be detected. To investigate hourly variations in biting density, the time at which the mosquito was collected was fit to the hourly model as a fixed continuous effect and as a quadratic variable with month of collection as a random effect.

To investigate variation in the biting density between collections with different human-baited and monkey-baited techniques, a model of nightly abundance was fit for each potential *Anopheles* vector species. The response variable for each model was the number of *Anopheles* females collected per night. Variations in abundance between traps were investigated with trap type fit to the model as a fixed factorial effect, while the collection station, date of collection and collector ID were included as random effects. A Poisson distribution was determined to be the best fit for all nightly models in this trap evaluation.

Mosquito diversity

The species diversity indices were calculated for each trap type based on the *Anopheles* mosquito species collected. Species richness (R) is the total number of species collected by each trap type, accompanied by the Gini-Simpson diversity index (1-D), where

$$1 - D = 1 - \frac{\sum n_i(n_i - 1)}{N(N - 1)},$$

the 95% confidence limit of which is

$$\pm 2 \sqrt{\frac{\sum \left(\frac{n_i}{N}\right)^2 - \left(\sum \left(\frac{n_i}{N}\right)\right)^2}{N(N - 1)}},$$

where n_i is the abundance of species i , and N is the total number of individuals in a sample.

Results

Longitudinal study of human-biting mosquitoes

In total, 4857 mosquitoes were collected across all sites over the 8 months of longitudinal sampling (Additional file 3: Table S1). Other *Anopheles* species were found in very low numbers, and only in the agricultural area and forest edge. A total of 124 female *Anopheles* mosquitoes belonging to nine species were obtained, of which *An. balabacensis* and *An. flavirostris* dominated (93.5% of all *Anopheles* females; Additional file 3: Table S2).

Anopheles balabacensis and *An. flavirostris* abundance across months

On account of their known role in transmission of malaria in the Philippines, further analysis was restricted to *An. balabacensis* and *An. flavirostris*. In general, the abundance of *An. balabacensis* was low (mean 0.34 to 1.20 per collection night). The best-fit model indicated that *An. balabacensis* density varied between sites ($\chi^2=7.92$, $df=1$, $p<0.001$), with biting density highest in the forest edge and lowest in the forest area (Additional file 4: Table S1). Comparison with the null model indicated that the best-fit model explained approximately 88.7% of the total deviance in the data.

Anopheles balabacensis were collected in all months in the agricultural area and forest edge sites but were only observed in the forest area from May to July. There was no significant interaction between the month of collection and collection site ($\chi^2=4.354$, $df=2$, $p=0.339$), indicating a similar temporal pattern of abundance in all sites (Additional file 4: Table S1). The best-fit model indicated that abundance of *An. balabacensis* varied between months ($\chi^2=10.68$, $df=1$, $p=0.01$), with the highest biting density occurring in May, followed by a decline until December (Fig. 3).

The nightly density of *An. flavirostris* was also relatively low across the study area (mean 0 to 2 per collection night) with numbers ranging from 0 to 10 per night in the agricultural and forest edge sites, and none being collected in the forest site. The best fit model predicted that the abundance of *An. flavirostris* was higher in the agricultural area than the forest edge (Additional file 4: Table S2). Comparison with the null model indicated that the model explained approximately 78.4% of the total deviance in the data.

Anopheles flavirostris was collected in the agricultural area across all months of collection; however, none were collected in the forest edge in July, August or December. There was no significant interaction between the month of collection and collection site ($\chi^2=0.43$, $df=1$, $p=0.74$), indicating similar seasonal patterns of *An. flavirostris* in the agricultural and forest edge areas

(Additional file 4: Table S2). A quadratic association was observed between abundance of *An. flavirostris* and month of collection ($\chi^2=15.248$, $df=2$, $p<0.001$), characterized by peaks in abundance occurring in May and December (Fig. 4).

Hourly biting activity of *An. balabacensis* and *An. flavirostris*

Host-seeking *An. balabacensis* were collected throughout the sampling night (18:00–06:00) in both the agricultural and forest edge sites; however, no specimens were collected in the forest area before 19:00 or after 01:00. The number of *An. balabacensis* collected varied significantly throughout the night ($\chi^2=34.93$, $df=5$, $p<0.001$; Additional file 4: Table S3), with the model not detecting a difference in biting behavior between sites. Comparison with the null model indicated that the best-fit model explained approximately 92.6% of the total deviance in data.

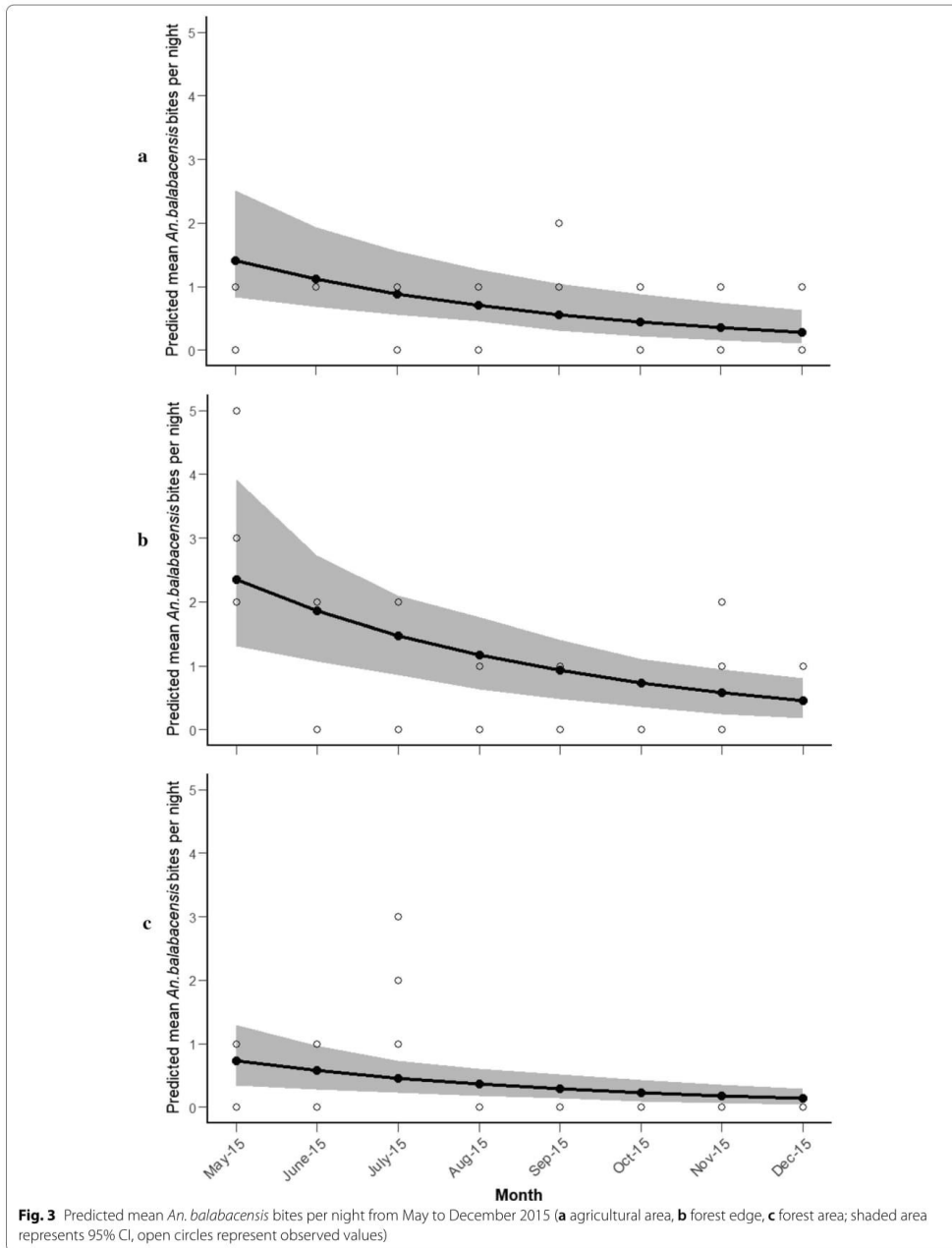
The number of *An. balabacensis* collected varied significantly over the course of a night ($\chi^2=34.93$, $df=5$, $p<0.001$), with the model predicting a single peak in abundance occurring between 21:00–22:00 followed by a gradual decline until dawn (Fig. 5). Approximately 60% of *An. balabacensis* bites occurred before 22:00 across all sites.

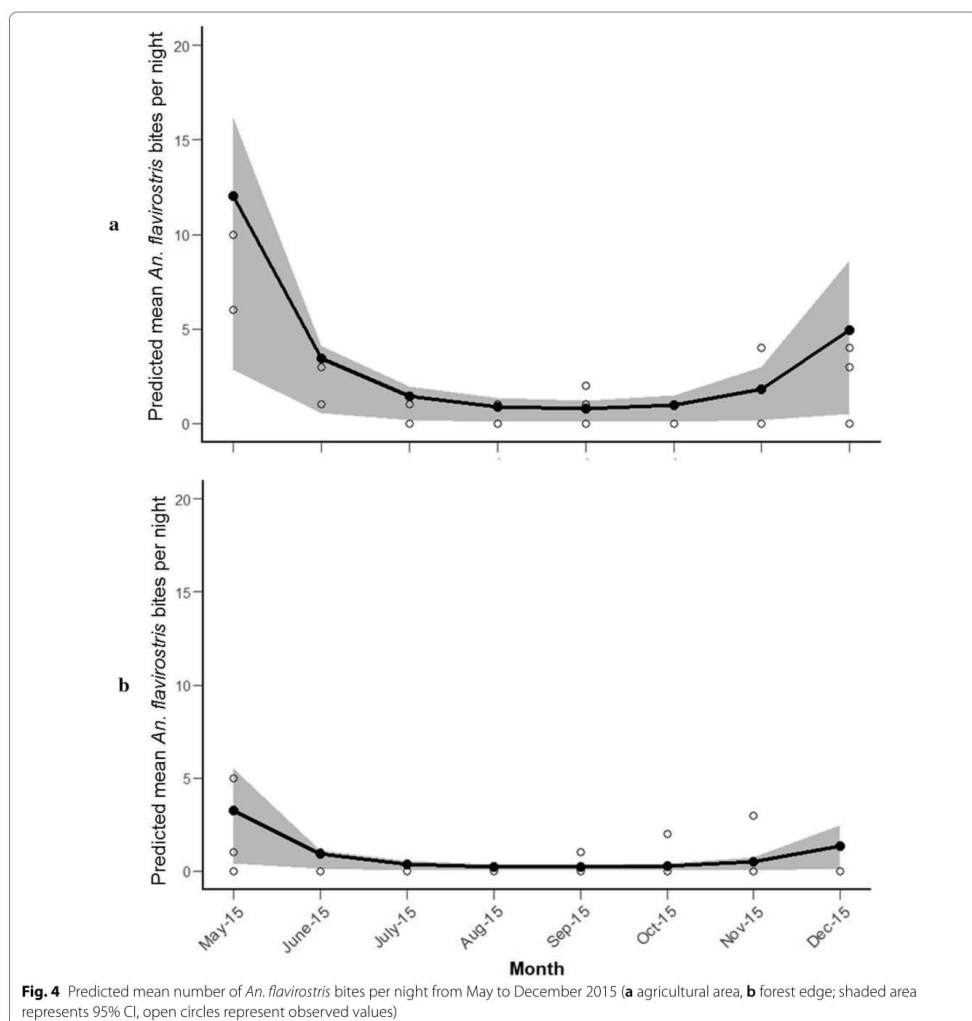
Anopheles flavirostris was collected throughout the night in both the agricultural area and forest edge. The number of *An. flavirostris* collected varied significantly throughout the night ($\chi^2=19.174$, $df=2$, $p<0.001$; Additional file 4: Table S4). Comparison with the null model indicated that the best fit model explained approximately 87.5% of the total deviance in data. There was no significant interaction between *An. flavirostris* biting time and sample site ($\chi^2=2.30$, $df=1$, $p=0.112$) indicating the same hourly biting pattern in the agricultural area and forest edge. The model estimated peak abundance of *An. flavirostris* from 23:00 to 00:00 (Fig. 6), and only 33.86% of *An. flavirostris* bites occurring before 22:00.

Comparison of human- and monkey-baited collections of *Anopheles* mosquitoes

Species composition of *Anopheles* mosquitoes collected in different traps

A total of 6591 mosquitoes were collected in all traps across 40 nights of outdoor collection, of which 3942 (59.81%) were females and subsequently identified to species level (Additional file 5: Table S1). Restricting analysis to the *Anopheles* genus, the majority of females were collected in MBT and the lowest numbers in HEN and MEN (Additional file 5: Table S2). The MBT collected the highest number of *Anopheles* species, with nine, while HEN, MEN and HLC caught eight, seven and four, respectively.





The Gini-Simpson diversity index indicated that anopheline diversity was highest in the MEN trap and lowest in HLC collections.

Nightly abundance of Anopheles mosquitoes in each trap type

Statistical analysis of trap performance was conducted only for *An. balabacensis*, *An. flavirostris*, *An. dispar*

and *An. greeni*, as the abundance of all other anopheline species was too low for robust analysis. For each of these species, statistical comparisons were made only between traps that collected at least one specimen. *Anopheles balabacensis* was collected in HLC and MBT at densities ranging from zero to three individuals per night, with none collected in the E-net traps. The mean abundance of *An. balabacensis* was approximately five times higher in HLC than in MBT collections

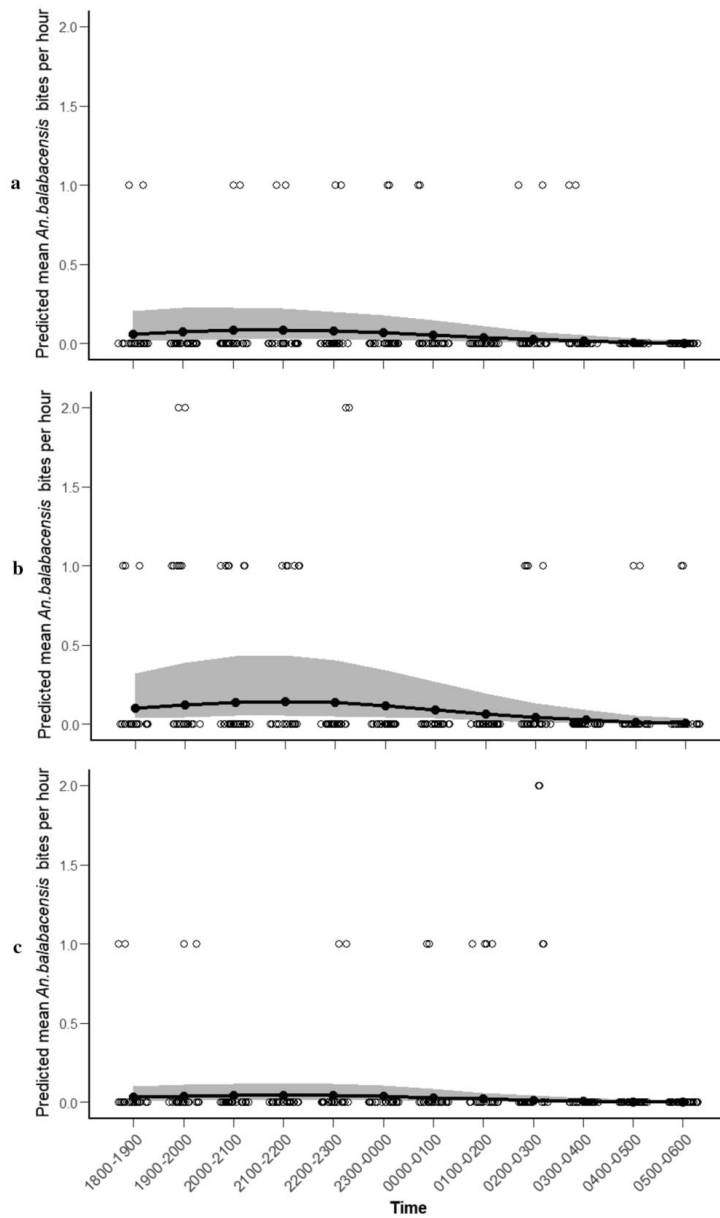
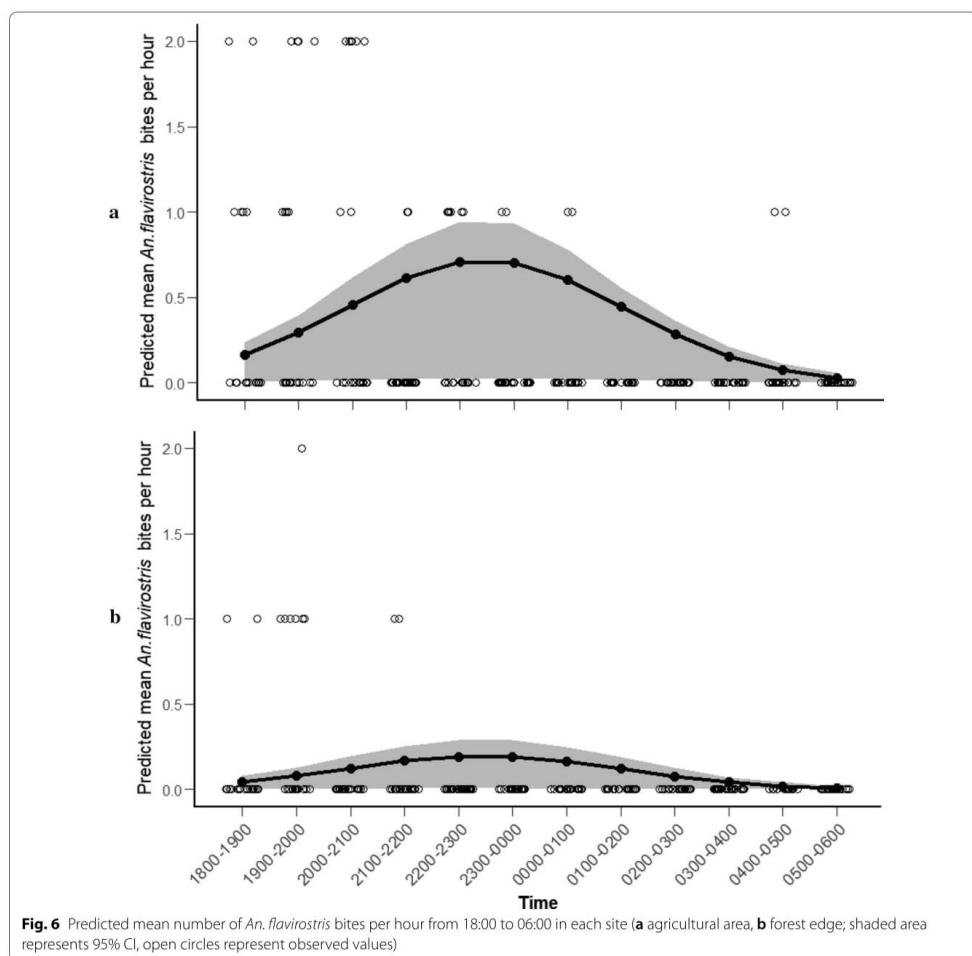


Fig. 5 Predicted mean number of *An. balabacensis* bites per hour from 18:00 to 06:00 in each site (**a** agricultural area, **b** forest edge, **c** forest site; shaded area represents 95% CI, open circles represent observed values)

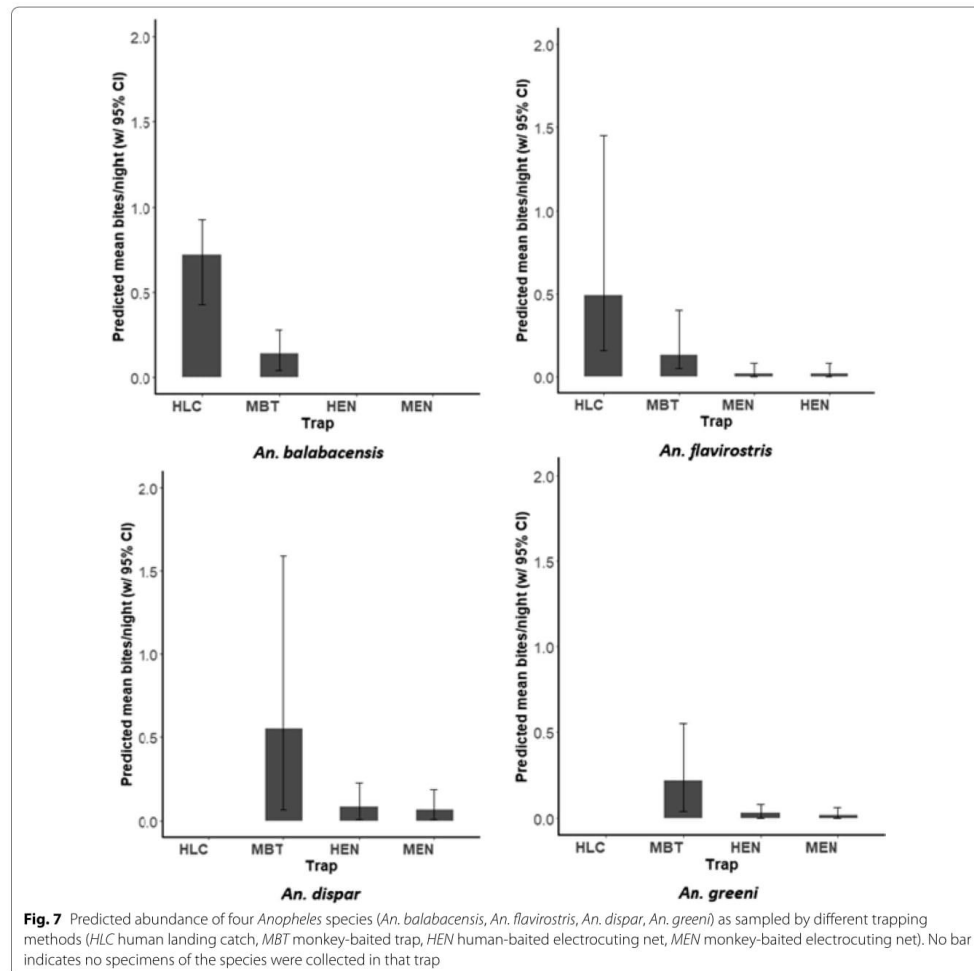


($\chi^2 = 11.66$, $df = 1$, $p = 0.001$, Fig. 7, Additional file 4: Table S5).

Anopheles flavirostris was collected in all four trap types. Biting densities of *An. flavirostris* ranged from zero to four bites per night and varied significantly between traps ($\chi^2 = 36.93$, $df = 3$, $p = 0.001$, Fig. 7). The HLC collected approximately 3.3 times more *An. flavirostris* than the MBT, with no difference between abundance in MBT,

HEN and MEN ($p > 0.05$ in all cases; Additional file 5: Table S6).

Anopheles dispar and *An. greeni* were not collected by HLC. Biting densities of *An. dispar* varied significantly between trap types ($\chi^2 = 34.56$, $df = 2$, $p = 0.001$, Fig. 7), with abundance in MBT being 7.8 times higher than in HEN and MEN (Additional file 4: Table S7). *Anopheles greeni* densities varied significantly between trap



types (MBT, HEN, MEN), with MBT yielding approximately 7.3 times more *An. greeni* than HEN or MEN ($\chi^2=26.73$, $df=2$, $p=0.001$; Additional file 4: Table S8, Fig. 7). However, there was no significant difference between collections with HEN and MEN ($p=0.89$).

Molecular detection of *Plasmodium* in *Anopheles* mosquitoes

All female *Anopheles* mosquitoes ($n=357$) collected during the study were tested for the presence of malaria parasites. A total of 120 pooled samples

underwent a first round of nested PCR and all were all negative for *Plasmodium* parasites.

Discussion

To better understand the low incidence of *P. knowlesi* in Palawan despite its close proximity to a major focus of infection in nearby Sabah, Malaysian Borneo, here we characterized the ecology and biting behavior of potential *Anopheles* vectors across three ecotypes representative of land use. Two known malaria vectors, *An. balabacensis* (the vector of *P. knowlesi* in Sabah) and *An. flavirostris*,

were detected in longitudinal surveillance in Palawan, representing 44% and 49% of all anophelines, respectively. However, mean nightly human-biting densities were low, ranging from 0.34 to 1.20 for *An. balabacensis* and 0 to 2 for *An. flavirostris*. A substantial proportion of *Anopheles* bites occurred before 10 pm, a time when residents in Palawan would typically be active and unprotected by insecticide-treated nets. No *Plasmodium*-infected mosquitoes were found, though the small number collected meant that detection power was limited. Sampling with human- and macaque-baited traps indicated that both vector species are attracted to each host type, and could thus serve as bridge vectors for *P. knowlesi*. In summary, while potential vectors of *P. knowlesi* are present in Palawan, their comparatively low densities and infection rates indicate that human exposure to *P. knowlesi* is considerably lower in this setting than in nearby Sabah, where this parasite is the primary cause of malaria in humans.

The outdoor biting densities of potential *P. knowlesi* vectors *Anopheles* in Palawan were approximately seven times lower than that found in recent studies in northern Sabah (e.g. *An. balabacensis* ranging from 1.81 to 7.84 bites per night) [19, 27]. More recent, broader sampling across Sabah state revealed substantial geospatial variation in *An. balabacensis* biting densities, confirming that *An. balabacensis* abundance is highly heterogeneous even across even short distances [26]. The relative abundance of *An. balabacensis* observed in the current study (44% of all anophelines) falls in the middle range of what has been previously reported in other settings in Malaysian Borneo (e.g. from 15% [26], 40% [19] and 95% [27]).

No *Anopheles* specimen collected in this study tested positive for *Plasmodium*, thus definitive incrimination of the contemporary *P. knowlesi* vector in Palawan was not possible. For comparison, in Sabah the *Plasmodium* infection rates (all species) in *An. balabacensis* ranged from 1.45–3% [26, 27, 37–39], with *P. knowlesi*-specific rates ranging from 0–3% [26, 27, 37–39]. Thus even in areas of high *P. knowlesi* transmission to humans, infection rates in vectors are relatively low. Failure to detect *P. knowlesi* in vectors collected here should not be interpreted as evidence of an absence of transmission. The relatively small number ($n=357$) of *Anopheles* collected may have insufficient to detect infection, especially if transmission was occurring at low levels. A much larger sample may be required to accurately estimate the prevalence of *P. knowlesi* infection in *Anopheles* populations in Palawan. Although not confirmed in this study, we hypothesize that *An. balabacensis* remains the most likely *P. knowlesi* vector in Palawan based on previous work [28].

The biting density of *An. balabacensis* and *An. flavirostris* varied between collection sites. *Anopheles*

balabacensis was more abundant in the forest edge than forest site, with density in the agricultural site being statistically indistinguishable from either forest site. Previous focal sampling in northern Sabah showed that *An. balabacensis* was also more abundant at forest edges than in human settlements [21], and in farm and forest than in peri-domestic habitats [26]. Thus, our findings are consistent in highlighting the suitability of forest edge habitats for *An. balabacensis*. In contrast, *An. flavirostris* density was significantly higher in the agricultural than the forest edge habitat, with no individuals collected in the forest site in contrast to previous studies in Palawan [40, 41]. The absence of *An. flavirostris* in our forest site may be due to site-specific effects, with broader sampling over a range of forest sites required to confirm habitat associations.

There was evidence of seasonality in both *An. balabacensis* and *An. flavirostris* populations in Palawan, although the pattern varied somewhat between vector species. The abundance of *An. balabacensis* was highest in May, followed by a gradual decrease through the remaining months of surveillance until December. In contrast, longitudinal sampling in Sabah [27] revealed month-to-month variation in *An. balabacensis* but no consistent seasonal trend between sites. Seasonality in *An. flavirostris* was characterized by peaks in biting density in May and December, with a decline in density during the intermediate months. As vectors were only sampled from May to December here, it is possible that the annual peak in *An. balabacensis* or *An. flavirostris* lies outside the sampling period investigated. However this is unlikely as rainfall and malaria transmission are strongly seasonal in Palawan, with the peak period of rains and malaria transmission (June–August/September) falling within the sampling period [42]. Notably, the peaks in *An. balabacensis* (May) and *An. flavirostris* (May and December) occurred outside the main period of rains in Puerto Princesa City.

The trap evaluation study revealed substantial differences in *Anopheles* species composition between trapping methods. *Anopheles balabacensis* and *An. flavirostris* were most abundant in HLC whereas *An. dispar* and *An. greeni* were dominant in MBTs. The *P. knowlesi* vector *An. balabacensis* was five times more abundant in HLCs than in MBTs. This difference may reflect a preference for humans over macaques for *An. balabacensis*; however, results from HLC and MBT may not be directly comparable due to non-host-related differences in trapping methods. Nevertheless, these results are consistent with a similar study in Sabah, where *An. balabacensis* was collected more frequently with HLC than with MBT [19]. To our knowledge, this is the first direct comparison of *An. flavirostris*

host-seeking on human and macaque hosts. Similar to *An. balabacensis*, *An. flavirostris* was more abundant (~3.3 times) in HLC than MBT collections. *Anopheles flavirostris* has been previously described as zoophilic based on comparisons between human- and water buffalo-baited collections [41, 43, 44]. The other two *Anopheles* species that were common in MBTs, *An. dispar* and *An. greeni*, are indigenous to the Philippines. There is no definitive evidence that these species are involved in human malaria transmission [30]; but their apparent preference for macaques over humans here indicates that they have potential to act as vectors for simian malaria.

In comparison to the HLC and MBT methods, the E-nets used in this study (HEN and MEN) performed relatively poorly for anopheline surveillance. Whilst almost all mosquitoes in HLCs were *An. balabacensis* and *An. flavirostris*, HEN collected no *An. balabacensis* and only one *An. flavirostris*. Similarly, the MEN collected fewer anophelines than the MBT, although numbers were sufficient to give an adequate representation of species diversity. The poor sampling efficiency of the E-nets and lack of difference in species composition between HEN and MEN compared to that between HLC and MBT collections is consistent with previous evaluations in Sabah [19]. The E-net traps' poorer performance relative to the HLC and MBT may be due to the design of the current prototype, where host odors are pumped from the tent along the length of PVC pipe to the electrified grid. The long (6 m) pipe or relatively fast movement of air may reduce or dilute the quantity or quality of the odor cues needed by mosquitoes to identify and locate their preferred host species. In summary, these findings indicate that the E-net traps used here do not provide an appropriate representation of the standard HLC and MBT methods.

Current malaria control strategies in Palawan rely on the use of LLINs and IRS [1]. As these interventions primarily target indoor biting mosquitoes, they are likely insufficient for protection against the outdoor, early-biting vectors of *P. knowlesi*. For example, in outdoor collections here, 60.37% of biting by *An. balabacensis* and 33.68% by *An. flavirostris* occurred between 18:00 and 22:00; a period in the evening when many people in rural communities in Palawan would still be outdoors. These findings are consistent with previous investigations in Sabah, where a large proportion of outdoor *An. balabacensis* bites occurred outdoors in the early evening, with almost no evidence of indoor biting [20, 37]. Clearly, additional vector control strategies that can protect people outside of homes are needed to reduce the risk of *P. knowlesi* exposure in Palawan and other settings where it is emerging.

Conclusions

The monkey malaria *P. knowlesi* is now the primary cause of human malaria in Malaysian Borneo; however only sporadic human cases have been reported in the nearby island of Palawan. By investigating the ecology and behavior of potential *P. knowlesi* vectors in Palawan, this study indicates that this disparity may be due to the relatively lower density and infection rates in mosquitoes even though known vector species are present. The reason for lower vector densities in this setting is unknown, but may relate to differences in land use and fragmentation between Palawan and northern Sabah.

While the risk of *P. knowlesi* spillover to humans in Palawan is low at present, it could increase with land use or other socioecological changes. To mitigate against the risk of *P. knowlesi* and other malaria species transmitted by exophilic vectors, control strategies in Palawan may need to be expanded to incorporate methods that protect people when they are outdoors.

Abbreviations

GLMM: Generalized linear mixed models; HEN: Human-baited electrocuting net; HLC: Human landing catch; IRS: Indoor residual spraying; LLIN: Long-lasting insecticidal net; MBT: Monkey-baited trap; MEN: Monkey-baited electrocuting net; PCR: Polymerase chain reaction; RITM: Research Institute for Tropical Medicine.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13071-021-04853-9>.

Additional file 1. Ethical considerations for the use of non-human primates.

Additional file 2. Detail of PCR primers used for detection of malaria parasite species in *Anopheles* mosquito specimens.

Additional file 3: Table S1. Summary of total number of mosquitoes caught in the different collection sites in the longitudinal study. **Table S2.** Female *Anopheles* species collected in each sampling site in Puerto Princesa City, Palawan from May to December 2015.

Additional file 4: Table S1. Summary of modeled coefficients for the nightly abundance of *An. Balabacensis* collected in each site in the longitudinal study from May to December 2015 (a – statistical difference relative to zero, b – statistical difference relative to reference category, * – indicates statistically significant difference). **Table S2.** Summary of modeled coefficients for the nightly abundance of *An. flavirostris* collected in the longitudinal study from May to December 2015 (a – statistical difference relative to zero, b – statistical difference relative to reference category, * – indicates statistically significant difference). **Table S3.** Summary of modeled coefficients for the hourly abundance of *An. balabacensis* collected in longitudinal study from May to December 2015 (a – statistical difference relative to zero, b – statistical difference relative to reference category, * – indicates statistically significant difference).

Table S4. Summary of modeled coefficients for the hourly abundance of *An. flavirostris* collected in longitudinal study from May to December 2015 (a – statistical difference relative to zero, b – statistical difference relative to reference category, * – indicates statistically significant difference). **Table S5.** Summary of modeled coefficients for the nightly abundance of *An. balabacensis* collected in each trap (HLC – human landing catch, MBT – monkey-baited trap, HEN – human-baited electrocuting net, MEN – monkey-baited electrocuting net, a – statistical difference relative to zero,

b – statistical difference relative to reference category, * – indicates statistically significant difference). **Table S6.** Summary of modeled coefficients for the nightly abundance of *An. flavirostris* collected in each trap (HLC – human landing catch, MBT – monkey-baited trap, HEN – human-baited electrocuting net, MEN – monkey-baited electrocuting net, a – statistical difference relative to zero, b – statistical difference relative to reference category, * – indicates statistically significant difference). **Table S7.** Summary of modeled coefficients for the nightly abundance of *An. dispar* collected in each trap (HLC – human landing catch, MBT – monkey-baited trap, HEN – human-baited electrocuting net, MEN – monkey-baited electrocuting net, a – statistical difference relative to zero, b – statistical difference relative to reference category, * – indicates statistically significant difference). **Table S8.** Summary of modeled coefficients for the nightly abundance of *An. greeni* collected in each trap (HLC – human landing catch, MBT – monkey-baited trap, HEN – human-baited electrocuting net, MEN – monkey-baited electrocuting net, a – statistical difference relative to zero, b – statistical difference relative to reference category, * – indicates statistically significant difference).

Additional file 5: Table S1. Summary of total number of mosquitoes caught using the different trapping techniques in the trap comparison study. **Table S2.** Total number of female *Anopheles* mosquitoes collected in each trap and associated diversity indices (HLC – human landing catch, MBT – monkey-baited trap, HEN – human-baited electrocuting net, MEN – monkey-baited electrocuting net).

Acknowledgements

The authors would like to thank RITM IACUC and Dr. Glenn Rebono for providing guidance on health, safety and welfare aspects of working with non-human primates, J. Butigan for support with animal handling, and the DENR-BMB through the PWRCC for donating macaque for use in this study. We thank Judy Dorothy Madarcos, Elizabeth Downe and the MONKEYBAR field team in Palawan for support throughout the study. We thank the community of Brgy. Bacungan for their cooperation and interest during this research.

Authors' contributions

FVS, CD, MMT, WEA, FEE, SJT, KMF and HMF conceived the study. RPBM, MMT, JCBJ, WEA, KMRV, KMF, SJT, HMF and FVS designed the experiments. RPBM, JCBJ, KMRV, WEA and MMT implemented the experiments and identified the samples. BAB and RPBM carried out molecular analysis. FM and HMF carried out statistical analysis. RPBM, FM, HMF and FVS drafted the manuscript. All authors read and approved the final manuscript.

Funding

This research was funded by the Medical Research Council, Natural Environment Research Council, Economic and Social Research Council, and Biotechnology and Biological Sciences Research Council through the Environmental and Social Ecology of Human Infectious Diseases Initiative, grant no. G1100796.

Availability of data and materials

Summary data supporting the conclusions of this article are included within the article and its additional files. Raw data can be made available through request to the Director of RITM, Philippines.

Declarations

Ethics approval and consent to participate

This study was approved by the RITM Institutional Review Board (IRB Protocol No. 2012-28) and the London School of Hygiene and Tropical Medicine Research Ethics Committee (LSHTM ethics ref. 6302), the Palawan Council for Sustainable Development (PCSD) (Wildlife Gratuitous Permit No. 2014-02 and Sustainable Environmental Plan Clearance No. RES-103013-007), RITM Institutional Animal Care and Use Committee (IACUC) (Protocol Number: 2012-05) and LSHTM Animal Welfare and Ethical Review Body (AWERB) (AWERB Ref: 2012/8N revised). Consent was obtained from the village leader of Brgy. Bacungan before data collection commenced.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Medical Entomology, Research Institute for Tropical Medicine, Alabang, 1781 Muntinlupa City, Metro Manila, Philippines. ²Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool L3 5Q4, UK. ³Present Address: Taxonomy & Pesticide Efficacy Branch, Vector Biology & Control Division, Environment Health Institute, National Environment Agency, Ministry of Sustainability and the Environment, 11 Biopolis Way, Singapore 138667, Singapore. ⁴Department of Parasitology, Research Institute for Tropical Medicine, Alabang, 1781 Muntinlupa City, Ma, Metro Manila, Philippines. ⁵Faculty of Infectious and Tropical Diseases, London School of Tropical Medicine and Hygiene, London WC1E 7HT, UK. ⁶Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow G12 8QQ, UK.

Received: 25 May 2021 Accepted: 17 June 2021

Published online: 07 July 2021

References

- Organization WH. World malaria report 2020: 20 years of global progress and challenges. Geneva: Organization WH; 2020.
- Philippines DoH-Rot. Shell, DOH aim to wipe out malaria in PH by 2030; 2020. <https://doh.gov.ph/press-release/Phl-Malaria-Free-by-2030-DOH>.
- D N. Only 4 provinces have cases of malaria. <https://mb.com.ph/2020/02/15/only-4-ph-provinces-have-cases-of-malaria/>. Accessed 13 Jan 2021.
- Brock PM, Fornace KM, Parmiter M, Cox J, Drakeley CJ, Ferguson HM, et al. *Plasmodium knowlesi* transmission: integrating quantitative approaches from epidemiology and ecology to understand malaria as a zoonosis. *Parasitology*. 2016;143(4):389–400.
- Naik DG. mediated zoonotic malaria: a challenge for elimination. *Trop Parasitol*. 2020;10(1):3–6.
- Luchavez J, Espino F, Curameng P, Espina R, Bell D, Chiodini P, et al. Human Infections with *Plasmodium knowlesi*, the Philippines. *Emerg Infect Dis*. 2008;14(5):811–3.
- Fornace KM, Herman LS, Abidin TR, Chua TH, Daim S, Lorenzo PJ, et al. Exposure and infection to *Plasmodium knowlesi* in case study communities in Northern Sabah, Malaysia and Palawan, The Philippines. *PLoS Negl Trop Dis*. 2018;12(6):e0006432.
- Fornace KM, Drakeley CJ, William T, Espino F, Cox J. Mapping infectious disease landscapes: unmanned aerial vehicles and epidemiology. *Trends Parasitol*. 2014;30(11):514–9.
- Zaw MT, Lin Z. Human *Plasmodium knowlesi* infections in South-East Asian countries. *J Microbiol Immunol Infect*. 2019;52(5):679–84.
- Takaya S, Kutsuna S, Suzuki T, Komaki-Yasuda K, Kano S, Ohmagari N. Case report: *Plasmodium knowlesi* infection with Rhabdomyolysis in a Japanese traveler to Palawan, the Philippines. *Am J Trop Med Hyg*. 2018;99(4):967–9.
- De Canale E, Sgarabotto D, Marini G, Menegotto N, Masiero S, Akkouche W, et al. *Plasmodium knowlesi* malaria in a traveller returning from the Philippines to Italy, 2016. *N Microbiol*. 2017;40:291–4.
- Ang JXD, Kadir KA, Mohamad DSA, Matusop A, Divis PCS, Yaman K, et al. New vectors in northern Sarawak, Malaysian Borneo, for the zoonotic malaria parasite, *Plasmodium knowlesi*. *Parasit Vectors*. 2020;13(1):472.
- Cooper DJ, Rajahram GS, William T, Jelip J, Mohammad R, Benedict J, et al. *Plasmodium knowlesi* Malaria in Sabah, Malaysia, 2015–2017: ongoing Increase in Incidence Despite Near-elimination of the Human-only *Plasmodium* Species. *Clin Infect Dis*. 2020;70(3):361–7.
- Lee KS, Divis PC, Zakaria SK, Matusop A, Julin RA, Conway DJ, et al. *Plasmodium knowlesi*: reservoir hosts and tracking the emergence in humans and macaques. *PLoS Pathog*. 2011;7(4):e1002015.
- Vythilingam I, Noorazian YM, Huat TC, Jiram AI, Yusri YM, Azahari AH, et al. *Plasmodium knowlesi* in humans, macaques and mosquitoes in peninsular Malaysia. *Parasit Vectors*. 2008;1(1):26.

16. Ong P, Richardson M. *Macaca fascicularis*. The IUCN red list of threatened species; 2008.
17. Gamalo LE, Dimalibot J, Kadir KA, Singh B, Paller VG. *Plasmodium knowlesi* and other malaria parasites in long-tailed macaques from the Philippines. *Malar J*. 2019;18(1):147.
18. Vythilingam I, Hii J, Manguin S. Simian malaria parasites: special emphasis on *Plasmodium knowlesi* and their *Anopheles* vectors in Southeast Asia. In: *Anopheles mosquitoes—new insights into malaria vectors*. Rijeka: Intech Open; 2013. p. 487–510.
19. Hawkes F, Manin BO, Ng SH, Torr SJ, Drakeley C, Chua TH, et al. Evaluation of electric nets as means to sample mosquito vectors host-seeking on humans and primates. *Parasit Vectors*. 2017;10(1):338.
20. Vythilingam I. *Plasmodium knowlesi* in humans: a review on the role of its vectors in Malaysia. *Trop Biomed*. 2010;27(1):1–12.
21. Hawkes FM, Manin BO, Cooper A, Daim S, Homathevi R, Jelip J, et al. Vector compositions change across forested to deforested ecotones in emerging areas of zoonotic malaria transmission in Malaysia. *Sci Rep*. 2019;9(1):13312.
22. Khim N, Siv S, Kim S, Mueller T, Fleischmann E, Singh B, et al. *Plasmodium knowlesi* infection in humans, Cambodia, 2007–2010. *Emerg Infect Dis*. 2011;17(10):1900–2.
23. Marchand RP, Culleton R, Maeno Y, Quang NT, Nakazawa S. Co-infections of *Plasmodium knowlesi*, *P. falciparum*, and *P. vivax* among humans and *Anopheles dirus* mosquitoes, Southern Vietnam. *Emerg Infect Dis*. 2011;17(7):1232–9.
24. Nakazawa S, Marchand RP, Quang NT, Culleton R, Manh ND, Maeno Y. *Anopheles dirus* co-infection with human and monkey malaria parasites in Vietnam. *Int J Parasitol*. 2009;39(14):1533–7.
25. Jiram AI, Vythilingam I, NoorAzian YM, Yusof YM, Azahari AH, Fong MY. Entomologic investigation of *Plasmodium knowlesi* vectors in Kuala Lipis, Pahang, Malaysia. *Malar J*. 2012;11:213.
26. Brown R, Chua TH, Fornace K, Drakeley C, Vythilingam I, Ferguson HM. Human exposure to zoonotic malaria vectors in village, farm and forest habitats in Sabah, Malaysian Borneo. *PLoS Negl Trop Dis*. 2020;14(9):e0008617.
27. Wong ML, Chua TH, Leong CS, Khaw LT, Fornace K, Wan-Sulaiman WY, et al. Seasonal and spatial dynamics of the primary vector of *Plasmodium knowlesi* within a major transmission focus in Sabah, Malaysia. *PLoS Negl Trop Dis*. 2015;9(10):e0004135.
28. Tsukamoto M, Miyata A. Surveys on simian malaria parasites and their vector. *Trop Med*. 1975;20(1):39–50.
29. Moyes CL, Shearer FM, Huang Z, Wiebe A, Gibson HS, Nijman V, et al. Predicting the geographical distributions of the macaque hosts and mosquito vectors of *Plasmodium knowlesi* malaria in forested and non-forested areas. *Parasit Vectors*. 2016;9:242.
30. Curtis C. *Mosquito ecology: field sampling methods* (2nd edn); edited by MW Service, Elsevier Applied Science, 1992. £ 200.00 (xiii+ 998 pages) ISBN 1 85166 798 9. Elsevier Current Trends; 1993.
31. Organization WH. *Manual on practical entomology in malaria*. Geneva: World Health Organization; 1995.
32. Torr SJ, Della Torre A, Calzetta M, Costantini C, Vale GA. Towards a fuller understanding of mosquito behaviour: use of electrocuting grids to compare the odour-orientated responses of *Anopheles arabiensis* and *An. quadriannulatus* in the field. *Med Vet Entomol*. 2008;22(2):93–108.
33. Torr SJ, Vale GA. Know your foe: lessons from the analysis of tsetse fly behaviour. *Trends Parasitol*. 2015;31(3):95–9.
34. Tan CH, Vythilingam I, Matusop A, Chan ST, Singh B. Bionomics of *Anopheles latens* in Kapit, Sarawak, Malaysian Borneo in relation to the transmission of zoonotic simian malaria parasite *Plasmodium knowlesi*. *Malar J*. 2008;7:52.
35. Rattanarithkul R, Harbach RE, Harrison BA, Panthusiri P, Coleman RE, Richardson JH. Illustrated keys to the mosquitoes of Thailand VI Tribe Aedini. *Southeast Asian J Trop Med Public Health*. 2010;41(Suppl 1):1–225.
36. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *Am J Trop Med Hyg*. 1999;60(4):687–92.
37. Chua TH, Manin BO, Vythilingam I, Fornace K, Drakeley CJ. Effect of different habitat types on abundance and biting times of *Anopheles balabacensis* Baisas (Diptera: Culicidae) in Kudat district of Sabah, Malaysia. *Parasit Vectors*. 2019;12(1):364.
38. Manin BO, Ferguson HM, Vythilingam I, Fornace K, William T, Torr SJ, et al. Investigating the contribution of peri-domestic transmission to risk of zoonotic malaria infection in humans. *PLoS Negl Trop Dis*. 2016;10(10):e0005064.
39. Chua TH, Manin BO, Daim S, Vythilingam I, Drakeley C. Phylogenetic analysis of simian *Plasmodium* spp. infecting *Anopheles balabacensis* Baisas in Sabah, Malaysia. *PLoS Negl Trop Dis*. 2017;11(10):e0005991.
40. Oberst RB, Schultz GW, Laughlin LW, Sy NE, Santos MN, Casimiro C. Epidemiological study of malaria in Palawan. *Philippine J Microbiol Infect Dis*. 1988;17:41–8.
41. Schultz GW. Biting activity of mosquitos (Diptera: Culicidae) at a malarious site in Palawan, Republic of The Philippines. *Southeast Asian J Trop Med Public Health*. 1992;23(3):464–9.
42. Reyes RA, Fornace KM, Macalinao MLM, Boncayao BL, De La Fuente ES, Sabanal HM, et al. Enhanced health facility surveys to support malaria control and elimination across different transmission settings in the Philippines. *Am J Trop Med Hyg*. 2021;104:968–78.
43. Schultz GW. A survey of the mosquitos (Diptera: Culicidae) of Napsan, Palawan, Republic of the Philippines. *Southeast Asian J Trop Med Public Health*. 1993;24(2):376–83.
44. Torres EP, Salazar NP, Belizario VY, Saul A. Vector abundance and behaviour in an area of low malaria endemicity in Bataan, the Philippines. *Acta Trop*. 1997;63(4):209–20.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions



