Evolution and evaluation of long-lasting treated nets: from long-lasting insecticide treatment kits to dual active ingredient LLINs to control resistant mosquitoes.

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Thesis submitted to University of London in fulfillment of the requirements for the Doctor of Philosophy

2024

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Declaration:

I, **Patrick Kija Tungu**, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

I confirm that no funding was received from any funding body.

Abstract

Long-lasting insecticidal nets (LLINs) in which wash-resistant formulation of insecticide is coated or incorporated into the netting of synthetic polymer fibres during manufacture are the most widely used method for malaria prevention in Africa and Asia. By 2021, 38% of the population at risk of malaria in Africa were sleeping under an ITN, compared to <2% in 2002. This coverage has made a major contribution to the halving of the global malaria burden over the last two decades.

With the LLINs' success, come new technical and logistical challenges that compromised their effectiveness. Major challenges facing LLIN intervention include inadequate LLIN coverage, inappropriateness in humanitarian emergencies and the evolution and spread of insecticide resistance.

The work in this thesis was designed to identify and contribute to the solution of these problems. This required testing and evaluation in laboratory, experimental hut and field trials of several new LLIN products for purposes of product validation for WHO. It required improvement of WHO testing and evaluation guidelines to which we contributed, investigation of new long-lasting treatment kits to treat other types of materials to solve problems in niche situations and to help reduce gaps in LLIN coverage. To combat the problem of resistance required evaluation of alternative insecticides and synergists to curb the limitations of standard LLINs for control of resistant vector. This necessitated studying the added efficacy of alternative / combination insecticides alongside standard pyrethroid-only nets to control resistant vector populations, and meta-analysis of evaluations before and after the evolution of resistance.

Along this journey the studies in this thesis have helped advance LLIN product quality, contributed to WHO guideline revision, helped solve outstanding problems of malaria vector control, and have investigated the durability of different synthetic polymers and alternative novel insecticides; and by so doing have helped advance malaria prevention.

Dedicated to my family.

Acknowledgements

I would like to thank the following:

Mark Rowland, my PhD supervisor, for his keen and ready support both of my PhD and other work. His advice and guidance were unstinting, without them this work would not have begun, progressed or been completed.

Dr Stephen Magesa, my co-supervisor for his advice, assistance and for his push and encouragement.

Caroline Maxwell for her generous spirit of giving up her time and provided me with emotional support and organizing a place for my stay in the UK during my initial stages of my PhD the most wonderful wedding whilst I was caught up in work and thesis writing.

Centre Manager, administration and other Staff at the National Institute for Medical Research – Amani Research Centre for their technical, logistic and personal support, and for allowing me the study leave and to conduct research at its labs and field sites.

I extend special thanks to Wema Sudi, Joseph Myamba, Frank Magogo, Josephine Nyongole, Abdallah Telaki, Thobias Sikanyika, Benard Batengana, Elisante Michael, Emanuel Festo, and Charles Masenga for technical support to the laboratory and huts trials.

I am grateful to the volunteers who slept under nets in the huts and to community leaders of Zeneti and Mabogini village for their support.

Innovative Vector Control Consortium, WHOPES, BASF, Syngenta and Bayer for providing the funding for the field research.

My parents, **Lameck** and **Salome**, for their care and emotional support.

My Wife, **Agnes Tungu**, for her patience, tolerance, advice, encouragement, support and cuisine.

My children **Daniel** and **Uriel** for giving up their precious time allowing me to complete my thesis.

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Glossary

Abbreviation	Meaning
Ace-1^R	Insensitive Acetyl cholinesterase gene
ACT	Artemisinin-based Combination Treatment
NMCPs	National Malaria Control Programs
CCE	Carboxyl Choline Esterases
CDC	Centres for Disease Control
CFP	Chlorfenapyr
CS	Microencapsulated formulation
CYP	Cytochrome P450
DDT	Dicloro-diphenyl-tricloroethane
DNA	De-oxyribonucleic acid
EC	Emulsifiable Concentrate formulation
EIR	Entomologic Inoculation Rate
GABA	γ-amino-butyric acid
GPIRM	Global Programme for Insecticide Resistance Management
GST	Glutathione S-transferase
HDPE	High density polyethylene
IGR	Insect Growth Regulator
IRM	Insecticide Resistance Management
IRS	Indoor Residual insecticide Spraying
ITN	Insecticide Treated Nets
IVCC	Innovative Vector Control Consortium
kdr	Knock-Down Resistance

LLIN	Long Lasting Insecticide Treated Nets
LN	Long Lasting Insecticide Treated Nets
LSHTM	London School of Hygiene and Tropical Medicine
MFOs	Mixed Functional Oxidases
NIMR	National Institute for Medical Research
OP	Organophosphate
PBO	Piperonyl Butoxide
PCR	Polymerase Chain Reaction
RBM	Roll Back Malaria
RCT	Randomized Control Trial
VGSC	Voltage Gated Sodium Channel
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme
WHOPQT	World Health Organization Prequalification Team

PART ONE

Part One of the thesis provides an introduction, a literature review, the objectives of this thesis, and an explanation why appendices that describe basic methods and technical information in greater depth were necessary.

Prologue:

Part 1 contains a literature review of current knowledge of malaria vector control, the threats and impact of insecticide resistance, and the recommended strategies for improving control of resistant vectors and managing resistance. The objectives of the thesis are introduced.

Due to the length of the thesis, it was decided that the section that described the methods of vector control in greater depth should be placed in the appendices (appendix 1). This does not detract from the thesis itself because a thesis by papers contains a mix of self-contained chapters already published in which methods are already adequately described, and chapters that are destined to become papers in due course. Any shortcomings in the methods described in individual chapters (published and unpublished papers) are covered in greater depth in appendix 1.

The Literature Review is presented in Chapter 1 and the extended methods are described Appendix 1.

The other appendix (Interceptor Phase 1 & 2; appendix 2) was led by a senior author (R Malima) in which the second author (P Tungu) supervised the field team and ran the analysis of the paper. Appendix 2 gives background insight to Part 2 (chapters 2, 3 and 4) and is recommended to be read before reading Part 2.

Chapter 1: Literature review

This chapter is a review of current knowledge of malaria vector control, the threats and impact of insecticide resistance and the recommended strategies for improving vector control and managing resistance. It also discusses the justification and objectives of the thesis.

Introduction and Literature Review

1.1.Long-lasting insecticidal nets

Insecticide-treated mosquito nets (ITNs) are an effective and cost-effective measure to reduce malaria morbidity and mortality [1, 2]. The results of six major cluster randomized, controlled trials, in different areas of Africa, have indicated that the use of such nets can lead to significant reductions in mortality among young children [3-10]. The use of ITNs has resulted in substantial reduction in malaria morbidity and mortality correlated with a reduction in vector biting rates and parasite inoculation rates [11-15]. Much emphasis is now placed on insecticidal bed nets for control of malaria transmission [16-25]. Treated nets not only provide a considerable degree of personal protection to individual net users, but, if used by whole community, can reduce the infective vector population [26, 27]. If the coverage is good, they also provide community protection by significantly reducing the vector populations.

Unfortunately, it was found that most standard conventionally treated ITN lose much of their insecticidal activity after just a few washes with soap or detergent [16, 18, 19, 22, 26, 28-32]. Conventional ITNs needed to be re-treated with insecticide at least once a year, or after two or three washes, or else their effective protection in the long-term may be compromised [33]. Although nets that have lost most of their insecticide after repeated washing can be successfully re-treated, the observed frequencies of such re-treatment, when the nets are in routine use in the field, have been found to be very low [26, 33-45]. There were several reasons for this. Firstly, there are weaknesses in the logistics for re-treatment systems, secondly many African families are too poor to buy insecticide kits for re-treatment and thirdly, many of them may not want to retreat their nets or do not think it is necessary.

To overcome this constraint, some manufacturers developed long-lasting insecticidal nets (LLINs), which are ITNs that have increased wash resistance and do not need to be re-treated with insecticide after wash to stay effective. LLINs are presented as ready-to-use industrially pre-treated mosquito nets that have been given a special insecticide treatment that is more durable and resistant to washing than the conventional dipping method. In LLINs, insecticides remain

present, either incorporated into, or coated around, the fiber at toxic concentrations for malaria vectors even after multiple washes. Biological activity then lasts for the lifetime of the net with no need for repeated re-treatment [18, 47-51]. Ideally, to fit the WHO [52] definition of an LLIN, the insecticidal activity should last as long as the nets expected lifespan (3-5 years). Long-lasting insecticidal nets (LLINs) are presently the most technologically advanced form and sustainable intervention for vector control. LLINs have become a valuable tool for vector control against malaria and other mosquito-borne diseases [53-55].

The 58th World Health Assembly set targets, namely that 80% LLIN coverage to those at risk of or suffering from malaria benefits from major preventive and curative interventions by the end of 2015 [56].

The proportion of the population with access to ITNs has increased markedly in sub-Saharan Africa over the twenty years since 2000. Based on data from household surveys, between 2000 and 2015, the proportion of the population sleeping under an ITN has increased markedly in sub-Saharan Africa, from less than 2% in 2000 to an estimated 55% in 2015 and the proportion of children under five sleeping under an ITN in sub-Saharan Africa increased from less than 2 per cent to an estimated 68 per cent [57]. Although there is an increasing number of LLINs that have been delivered to Malaria endemic sub-Saharan African countries, still 1 in 4 children in sub-Saharan Africa still lives in a household with no ITN and no protection provided by indoor residual spraying [57]; thus, there is still demand for LLINs by NMCPs e so as to have at least one LLIN for every two household members, a ratio believed to be sufficient to achieve universal coverage in a population [58].

The increase in demand for LLIN has attracted interest from several pesticide companies into producing new LLIN products. Several companies have developed and manufactured LLIN products that have been prequalified by the WHO PQT.

LLINs are classified into two types depending on the technology involved in its production [59]. The first is called incorporation technology; this uses polyethylene or polypropylene as material where pyrethroid insecticide is incorporated into the polymer before fibre extrusion. With this

technology, insecticide will migrate to the surface of the fiber and will be regenerated from the reservoir after the surface insecticide is washed off or lost by friction or abrasion during use.

The second type is where a resin-based polymer coating bound to the surface of the multifilament polyester netting material is used as the insecticide reservoir for replacement of surface insecticide. This is called coating technology.

Good example of LLIN of incorporation type is the Olyset® Net (Sumitomo, Osaka, Japan) which is LLIN based on polyethylene incorporation technology with permethrin as an active ingredient. Olyset® net is treated at the factory with permethrin 2% incorporated into the yarns of the netting and the insecticide diffuses constantly over time to the surface of the yarn. The bioavailability of the insecticide is sufficient to kill mosquitoes. Efficacy trials of Olyset® Nets in Cambodia, Viet Nam, Tanzania, Solomon Islands, Malaysia, Senegal, Cote d'Ivoire and India reported LLINs to have long- lasting insecticidal efficacy[20, 51, 56, 60-68].

PermaNet® 2.0 is a good example of LLINs with deltamethrin insecticide coated within a polymer resin around the fibre at toxic concentrations for control of malaria vectors. PermaNet® 2.0 coated with deltamethrin at the factory have been evaluated for bio-efficacy in Pakistan, Tanzania, India, Uganda, and a several other countries, and reported to perform well after repeated washing [18, 42, 47, 49, 69]. Another insecticidal mosquito net of Tianjin Yorkool (China), as part of the requirements for extension of WHO specifications for deltamethrin long-lasting (coated) insecticidal mosquito net [52]. Interceptor™ long-lasting insecticidal nets (LN) is another example of LLIN of coating type that contain Fendozin textile auxiliary, a finishing product that binds the α-cypermethrin insecticide to the fibres of the net in a special coating were evaluated for bio-efficacy in Tanzania, India, Uganda, and Liberia and reported to perform well after repeated washing and after several years of field usage [70-73].

Despite manufacture claims, it is a pre-requisite for any new LLIN product to pass through a series of evaluation stages prior to interim or full recommendation by the WHOPQT (formerly known as the WHO Pesticide Evaluation Scheme) before use by the community. Interim recommendation is given to a LLIN product after it has been successfully passed phase I and II

WHOPQT evaluations while full recommendation is given to a LLIN product after it has successfully passed phase III evaluations. The WHOPQT evaluation phases (I-III) are described in WHO document [74].

There are several brands of manufactured LLIN which claimed to be wash-resistant and several of them are already in use in some countries. Thus far, WHO has granted a full recommendation to several products [75].

1.2.Long lasting treatments

Long lasting insecticide treatment of nets is relatively recent technology from agrochemical industry developed in response to the acknowledged problem of low re-treatment rates of the prototype Insecticide Treated Nets. Long-lasting insecticidal nets (LLINs) have become the most important tool for malaria vector control since their early development in the first decade of the millennium. However, since the number of LLINs delivered from manufacturers through NMCPs are still insufficient or intermittent [57], many households use locally sourced bed nets through the commercial retail sector which are neither ITNs nor LLINs, which require annual re-treatment with insecticide when insecticide becomes depleted after repeated washing. Retail surveys have shown that most of locally sourced bed nets available through the commercial retail sector are not LLINs and those which are in use from this source have either never been treated or were treated only on purchase [76-78]. This highlights a need for a long-lasting insecticide treatment kit that could convert untreated nets into ITNs that can withstand repeated washing without the need for re-treatment.

Such treatment of nets is meant to turn a conventional net into a long-lasting insecticide treated net (LLIN). While factory based long lasting treatment technology involves two technologies - the addition of a resin ‘coating’ to an insecticide formulation at the time of net treatment or the ‘incorporation’ of insecticide into the polymer during extrusion of the fiber during manufacture - only the first methodology can be done by communities or families. Here, the resin acts as a special binder that enhances holding of the insecticide on net, turning it into a long-lasting wash-resistant treatment. The technology offers the prospect of conventional nets being converted into LLIN through a dipping process that can be done post-manufacture under field conditions or

in the home [79]. A dip-it-yourself long-lasting treatment could solve the problem of having to regularly retreat conventional ITN through extending insecticidal life of the ITN. With incorporation technology, this can only be done under factory conditions during manufacture. Despite having the long-lasting technology already developed, with products such as Olyset™ and PermaNet® in place, the long-lasting treatment technology is very desirable in ensuring that those conventional untreated nets already in use are turned into LLINs over their remaining lifetime. Such an insecticide kit could also be sold together with untreated nets on purchase in local retail shops and enable local producers that lack LLIN manufacturing technology to produce an ITN which could contribute usefully to malaria control and address local LLIN shortages [57, 76].

Two brands of long-lasting treatment kit have so far been developed: KO-Tab 1-2-3 developed by Bayer Environmental Sciences [79] and ICON Maxx developed by Syngenta [80]. ICON Maxx is based on the slow-release capsule suspension (CS) formulation of lambda-cyhalothrin that has previously been evaluated under WHOPES and recommended for treatment of mosquito nets [81].

1.3.The insecticide resistance threat

Malaria vector control has been feasible using insecticides delivered by insecticide-treated nets (ITNs), indoor residual spraying (IRS) or larval source management (LSM) [82].

The success achieved by insecticidal control of malaria vectors (i.e., through IRS and ITN) relies on the continued susceptibility of *Anopheles* mosquitoes to a limited number of insecticides.

Fourteen insecticides from seven classes (organochlorines, organophosphates, carbamates, pyrrole, pyrethroids, neonicotionoids and meta-diamines) have been recommended and approved for IRS, until recently only pyrethroids have been approved for bed net use [75]. All these classes with the exception of the pyrrole chlorfenapyr are nerve poisons and they either target acetylcholinesterase in the synapses between neurons or the voltage-gated sodium channels on insect neurons and axons. Pyrethroids and DDT are neurotoxins that act on the voltage-gated sodium channels (VGSC) by modifying their gating kinetics which result in the

prolonged opening of individual channels leading to paralysis and death of the insect. Organophosphates and carbamates bind to acetylcholinesterase in synapses and block transmission of nerve impulses.

Since the mid-1950s, there have been many reports of reduced susceptibility of *Anopheles* mosquito populations to insecticides such as DDT, malathion, fenitrothion, propoxur and bendiocarb [83, 84]. Among these, pyrethroids was the sole insecticide class recommended for ITNs until the advent of next -generation nets (i.e. PBO and Dual AI ITNs) although pyrethroids remain a component of these nets as well. To date, resistance to four classes of insecticide has been reported in *Anopheles* species in different regions of Africa [85-92]. The spread of insecticide resistance among major malaria vectors threaten the sustainability of the current gains towards decreasing malaria in Africa.

Four types of resistance mechanisms against insecticides have been described. These include metabolic resistance, target site resistance, penetration resistance and behavioral resistance [93].

Among the four, target site resistance and metabolic resistance due to elevated levels of detoxifying enzymes are the main mechanisms by which insect pests develop resistance to insecticides [94]. Both mechanisms have been detected in various species of insects [93, 95, 96]. Alone or in combination these mechanisms cause resistance to all classes of insecticides.

One of the mechanisms by which mosquitoes becomes resistant is by altering the target site of the insecticide thereby preventing it from binding effectively, hence the insecticide has little or no effect on the insect. An alanine to serine substitution at position 302 or 296 of the γ-amino-butyric acid (GABA) receptor is widely found in the dieldrin-resistant (rdl) insect species including *An. gambiae* [78] and *An. funestus* [79]. Mutations in the gene coding for acetylcholinesterase (ace-1), the target site of OP and carbamate insecticides [80], reduces the inhibition effect of these insecticides on these synaptic receptors [81, 82]

An important target site mechanism for pyrethroid resistance is caused by mutation of the target-site that reduces the action of this class of insecticide [97, 98]. This target site resistance

mechanism is known as knock down resistance (*kdr)* and is due to mutations in the voltage gated sodium channel (VGSC), the target site for pyrethroids and DDT, so these insecticides can no longer bind to its target [131]. Target site resistance to pyrethroids and DDT in *An. gambiae* is due to a substitution at a single codon in the sodium channel gene. Two *kdr* alleles occur in *An. gambiae*, a leucine to phenylalanine (L1014F) substitution, known as West *kdr* [95] and a leucine to serine (L1014S) substitution known as East *kdr* [96]. The former mutation which leads to the substitution of a leucine codon (TTA) for phenylalanine (TTT) is widespread in West Africa where it is strongly associated with *An. gambiae* sensu lato [s.l.] [95, 99], whereas the latter (East Africa kdr) mutation leads to the substitution of a leucine (TTA) to a serine (TCA) are been described from East African populations *An. gambiae* sensu lato [s.l.]. Both types of *kdr* mutation are associated with DDT and pyrethroid resistance phenotypes in wild *An. gambiae s.l.* populations [95, 96, 99, 100]. N'Guessan et al. [101] were the first to report an association between pyrethroid resistance caused by site-insensitivity *kdr* and mixed function oxidases (MFOs); this led to reduced efficacy of LLINs in West Africa (Benin). Mutations in the gene coding for the neurotransmitter acetylcholinesterase (ace-1^R), the target site of organophosphates and carbamates is also a target site resistance that reduces toxicity of organophosphates and carbamates by reducing the inhibitory effect of the insecticides on this enzyme [132]. An alanine to serine substitution at position 302 or 296 of the γ-amino-butyric acid (GABA) receptor is a target site resistance that is found in the dieldrin-resistant (rdl) Anopheline vectors [133, 134].

Metabolic resistance on the other hand occurs when increased activity or overproduction of one or more enzymes results in adequate proportion of the insecticide being sequestered, metabolized or detoxified before it reaches the target site therefore impairing the toxicity of the insecticide [102, 103]. Three protein families are largely responsible for insecticide metabolism: the cytochrome P450-dependent monooxygenase (P450s), carboxylesterases (COEs), and glutathione transferases (GSTs).

The cytochrome P450-dependent monooxygenase (P450s) is the primary enzyme family responsible for pyrethroid metabolism in insects [97]. To date insect P450s have been assigned to six families: five are insect-specific and one, CYP4, has sequence homologies with families in

other organisms. Increased transcription of genes belonging to the CYP4, CYP6, and CYP9 families has been observed in insecticide-resistant strains in different insect species [104, 105]. In *An. gambiae* these 3 gene families have 111, 31 and 51 members respectively [106]. Although there are over hundred P450 enzymes in *An. gambiae* [106], as in other insects, only a minority of these enzymes are involved of detoxifying insecticides.

P450 enzymes have been reported to be responsible for pyrethroid resistance in *An. gambiae* and *An. funestus* in several places, these include pyrethroid resistant *An. funestus* [91] from Southern Mozambique in which studies have shown that P450 enzymes as being the chief candidate for conferring pyrethroid resistance in this species [107]. A recent study by Jones *et.al.* reported that a P450 candidate gene CYP4G16 was associated with resistant *An. arabiensis* from Pemba, Tanzania [108]. A study by Corbel *et. al*. using biochemical assays implicated the detoxification enzymes in conferring resistance to permethrin, DDT, OP and carbosulfan in *An. gambiae* and *Culex quinquefasciatus* from four localities in Benin including rural, agricultural and urban sites [109]. P450s have also been associated with an increase in permethrin tolerance in *An. gambiae* in Kenya [110]. It appears that P450 enzymes (also known as mixed function oxidases MFOs) may also act in consort with *kdr* to create a pyrethroid resistance that could cause control failure of *Anopheles gambiae* in parts of West Africa [101, 109, 111]. Likewise, MFOs enzymes were responsible for the pyrethroid resistance that evolved in *Anopheles funestus* in South Africa [91, 112]. However multi-country study coordinated by WHO to assess the importance of insecticide resistance on disease control showed continued efficacy of LLINs even in areas with resistance [139]. Recent studies have reported a reduced efficacy of the current malaria vector control tools (LLINs and IRS) in the areas with pyrethroid resistant mosquitoes. Studies have reported the loss of insecticidal efficacy [101] and protective efficacy of pyrethroid-treated nets [113] in Benin. A multi-centre study reported that deltamethrin coated LLIN did not kill as many mosquitoes in areas with metabolic resistance or target-site resistance as in susceptible areas [114]. The loss of insecticidal and protective efficacy of pyrethroid treated bed nets to resistant *An. gambiae s.s.* populations have also been reported in other places apart from southern Benin [115]. Pyrethroid resistance that evolved in *An. funestus* was the first that led to the failure of IRS campaigns in South Africa [91, 112].

The well documented spread of insecticide resistance among major malaria vectors not only threatens the sustainability of the current gains towards decreasing malaria morbidity in Africa but also stresses the pressing need for alternative strategies to fight against resistant mosquitoes [116]. Pyrethroid-DDT cross-resistance in particular, presents a major challenge for malaria vector control in Africa as pyrethroids represent the class of insecticide most used for treating bed nets, and including in the next generation nets (i.e. PBO and Dual AI ITNs) [117], thus calling for alternative insecticides and resistance management strategies.

1.4. Resistance management

The main aim of insecticide resistance management (IRM) strategies is to prevent or delay the evolution of resistance, or to assist to retrieve the susceptibility status in which insecticide resistance has already been well-established.

Resistance management has a theoretical foundation in population genetics that goes back four decades [118, 119]. More recently, the WHO provided a generic guidance for countries to manage insecticide resistance using the Global Plan for Insecticide Resistance Management (GPIRM) in malaria vectors produced in 2012 [135]. The GPIRM describe practices aimed at reducing the potential for mosquitoes to become resistant to insecticides and it urges all malaria endemic countries to develop strategies for monitoring and managing insecticide resistance to ensure that the limited numbers of insecticides available for vector control are protected and their effectiveness are maintained.

There are several different strategies that have been proposed to tackle the insecticide resistance crisis including the novel rotations of alternative insecticide with different mode of action in time or in space, and application of mixtures of unrelated insecticides.

1.4.1. Insecticide mixtures

Insecticide mixture refers to the resistance management technique whereby two or more unrelated compounds are mixed within the same product or formulation so that the mosquito is guaranteed to come into contact with both at the same time. Simulation modelling has shown that the most promising way to delay the selection of resistance is by means of application of

mixtures of unrelated insecticides [120]. The idea behind mixtures is that insects resistant to one insecticide should be killed by the second insecticide provided insects are not resistant to both. When resistance is present at low frequency (as when it first evolves) double resistance will be rare and selection of each type of resistance should be delayed or prevented. Recently, Indoor residual spraying (IRS) that contains different classes of insecticide formulation in Fludora® Fusion (Bayer) (the neonicotinoid insecticide clothianidin in mixture with deltamethrin) presents a promising example of insecticide mixture. Interceptor G2 LN (IG2) is a Dual-AI LLIN developed by the manufacturer BASF SE designed to provide protection against pyrethroid-resistant mosquitoes by means of a mixture of chlorfenapyr (pyrrole class) and alpha-cypermethrin (pyrethroid) in a long-lasting wash-resistant formulation.

1.4.2. Insecticide Combinations

The use of two insecticides applications within the same building, e.g., one insecticide on the walls and another on nets in the same household can achieve the same objective. Insecticide combinations differ from insecticide mixtures in that the same insect is likely, but not guaranteed, to come in contact with both insecticides. Combinations can be applied spatially as two insecticides in a 2-in-1 (mosaic) format. Insecticide mosaics refers to a technique whereby compound A is sprayed in one area and compound B in another area, so that the same mosquito populations are exposed sequentially to A and B in space.

With a net this will be a case in which the pyrethroid is restricted to the sides and the alternative insecticide to the top of the net [124]. For the 2-in-1 net to work as a resistance management tactic, mosquitoes should contact both the top and sides sequentially so that any pyrethroid resistant mosquito that survives contact with the pyrethroid stands a high chance of being killed by the alternative insecticide or vice-versa. Most mosquitoes do in fact contact the top first [125] possibly in response to odour plumes or concentration gradients, and this gives the 2-in-1 concept its validity.

1.4.3. Insecticide rotations

The other commonly proposed technique for managing insecticide resistance is the rotation of chemically different classes of insecticides in time rather than in space. IRS rotations can be

applied at different times thereby restricting use of a single product or class and hence preserving susceptibility [121].

1.4.4. Synergists

Another approach to combating resistance is to use a chemical synergist with the pyrethroid on part or all the bed net. A synergist is a substance which does not by itself have insecticidal properties, but when mixed or applied with insecticides of a particular class, considerably enhances its potency, for example by inhibiting an enzyme that normally has detoxifying activity against the insecticide.

Synergists have been in use for many years and have contributed to enhance the efficacy of insecticides [126, 127]. This is due to their enzyme-inhibiting action, restoring the susceptibility of insects to the chemical which would otherwise require higher levels of the toxicant for their control [128]. Synergists are also useful for laboratory investigation of resistance mechanisms through their ability to inhibit specific metabolic pathways [128].

One type of synergist capable of inhibiting MFOs is piperonyl butoxide (PBO). PBO is commonly used in commercial aerosols for potentiating pyrethroid activity against flying or domestic insect pests [129]. PBO is an inhibitor of mixed function oxidases with potential to reduce activity of enzymes associated with pyrethroid resistance [128]. Combination nets treated with both pyrethroid, and synergist may have application against resistant mosquitoes, particularly those whose resistance is based on oxidative metabolism. There may also be enhanced activity against susceptible mosquitoes since mixed function oxidases are involved in many metabolic activities including activation and as enhancing penetration of insecticide across the insect cuticle [130].

1.4.5. Alternative insecticides

Alternative insecticides to pyrethroids have been tested on nets for effect against wild, pyrethroid resistant mosquito populations and are now being used widely as IRS [100, 122]. Most alternatives lack the excito-repellency of pyrethroids, a characteristic important for reducing biting rates or for providing personal protection to occupants of insecticide treated nets. This limitation is another reason for combining the alternative insecticide with a pyrethroid that is still

capable of providing repellency. Older classes of insecticide, not previously adopted for use for public health, ITN in particular, like the organophosphates (OP) and carbamates that are still widely used in agriculture have proven to be also effective in public health as residual treatments [123]. Because the mode of action (acetylcholinesterase inhibition) differs from that of pyrethroids (Na^+ channel interference), they have the potential to overcome *kdr* and other forms of pyrethroid resistance when used on nets. Studies in experimental huts showed that OP (pirimiphos methyl PM) on nets perform better than pyrethroids in killing most or all *An. gambiae* with *kdr* but do not fully protect sleepers from being bitten if holes are present [29, 100]. This is because OP lack the irritancy of pyrethroids, which drives mosquitoes away after short contact. The carbamate carbosulfan produces high kill (92%) which is higher than any pyrethroid against even susceptible populations. It inhibited blood feeding by 70%, showed residual activity after several months of use, and did not select *kdr* genotypes (unlike the pyrethroids). The problem with carbosulfan, however, is its break-down product, carbofuran, which is highly toxic to mammals (LD_{50} 8mg/kg, WHO class I) and rules out its use on ITN at normal dosages [123]. Carbamates such as bendiocarb have the same problem of high human toxicity [123]. Certain OP insecticides, such as chlorpyrifos methyl (CM), malathion and pirimiphos methyl could potentially to replace the pyrethroids since their mammalian toxicity is lower than the two cyano-pyrethroids, deltamethrin and lambda-cyhalothrin, commonly used on nets. The problem is their unpleasant odour and solubility in water, a problem that can be solved by microencapsulation, as in Actellic CS.

Rationale

Phased evaluation of LLIN by WHOPQT/WHOPES

For insecticide treated nets (ITNs) to be eligible for the donor financed market they must demonstrate their effectiveness for malaria vector control after passing through a series of tests organized by WHO Prequalification Team (WHOPQT, formerly WHOPES who produced the guideline) to ensure their quality and performance through standard testing procedure for biological and chemical efficacy, and physical integrity (hole index and net durability), against mosquito vectors [74]. In undertaking the evaluation procedures WHOPQT have established

evaluation guidelines for all three phases of testing [74]. The WHOPQT LLIN evaluation guideline rely on the 3 minutes exposure cone bioassays or the tunnel test at phase I for passing or failing LLIN product in phase III trials [74]. The 3 minutes cone bioassay and tunnel tests does not account for friction and general wear and tear which can contribute to attrition of insecticide residues. Also, among the shortcomings of the cone bioassay is that of underestimating the efficacy of the LLIN with insecticides which have repelling effects like pyrethroid. The repellence phenomenon of insecticides tends to influence and underestimate the results of this method as the mosquitoes may be repelled and move to rest onto the plastic sides of the cone or cotton swab. This shortens the exposure time to less than three minutes. The overnight tunnel test allows the mosquito to realistically behave *ad libitum* which the Muheza lab made a mainstream bioassay test for WHO.

The most realistic way to study the efficacy of the net is controlled household studies such as the experimental hut trials which allow the mosquitoes to behave freely or naturally around the house structure [74].

Phase II LLIN evaluations in experimental huts are used to provide interim recommendation to a new LLIN product. It is important to calibrate the outcomes between phase I and phase II and phase III outcomes. For example, it is important to find the correlation between the arbitrary 20 washes that LLIN are purposefully subjected to in WHOPES Phase II to see if it is a good approximation of Phase III after 3 years under household use where nets would be subjected to more vigorous challenges than just washing, for example the removal of surface insecticide through friction and abrasion in everyday use, but over the 36 months. It important to fully verify or justify that this removal in field condition would add up, or be equivalent to, the artificial 20 washes of Phase II.

Resin coated LLIN and incorporation LLIN.

Long-lasting nets are factory-treated, and their insecticides are either incorporated into the yarns or coated to their surface [59]. In both cases, the amount of insecticide is divided into a smaller fraction on the fibre surface and into a larger fraction as a reservoir inside the fibre or in the coating [138]. Understanding of similarities and differences between the LLINs of different

technologies (polyester or polyethylene) in terms of killing efficacy and personal protection are important for LLIN procurer's decision-making on which polymer to buy. Although a number of studies have reviewed the similarities and differences of between the two types ('incorporation' and 'coated') of LLIN technologies, most of these studies involved comparison of bio-efficacy and material durability between Olyset® and PermaNet® [47]. However, the most common LLINs, Olyset net and PermaNet, contain different active ingredients (i.e., permethrin and deltamethrin respectively). Thus, the reported differences in bio-efficacy can be due attributed to differences in the performances of the active ingredients rather than differences in polymer or production technology. Comparison of the differences in efficacy between the two LLIN polymers are better explored in studies where the two technologies contain the same active ingredient. This is vital information to international donor and NIMCPs when it comes to effective procurement decision making.

Humanitarian emergencies and private sector nets

With epidemiological success of hand treated ITNs and the development of factory produced LLINs, LLINs are still facing several technical and logistical challenges that compromise their effectiveness in field. First, it is recommended that coverage should be at least 80% for optimal community protection (mass effect) that is an important attribute of LLINs. However, as LLIN supply is a donor-dependent and supplied at a particular interval (in most cases at 3-year intervals), studies have reported inadequate LLIN coverage between universal coverage campaigns; even the continuous distribution channels (i.e. ANC, School nets etc) still have not adequately cover the gap hence many communities in endemic countries tend to cover this gap in coverage through purchasing nets that are untreated from the local retail sector [136, 137]. The inapplicability of LLINs in humanitarian emergencies and complex political situations is the other challenge facing LLINs that is also compounded with insecticide resistance that presents a threat to continued efficacy of the LLINs. The availability of post-manufacture long-lasting treatment kits would raise the prospect of it becoming an all-purpose formulation for such purposes as military clothing, civilian bed covers and curtains, or for blankets, tarpaulins and tents distributed in epidemics, disasters or humanitarian emergencies, and may provide an answer to the problem of reduced LLIN coverage between distribution campaigns, by turning

commercial retail-sourced untreated nets into LLINs through simple home or community treatment.

The pyrethroid resistance in mosquito vectors that has been reported in many African countries and is spreading rapidly across Africa could reduce the impact of our two most successful malaria prevention interventions – indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs). The impact of pyrethroid resistance on malaria transmission and the efficacy of control tools is not consistent and may differ from one area to another [25]. Establishment of the impact of insecticide resistance on the efficacy of standard pyrethroid only long-lasting insecticide treated nets is of paramount importance for resistance management and for effective malaria control decision making by the Malaria Control Programs of all malaria endemic countries.

Dual-AI LLINs

It is envisaged that resistance will eventually erode the efficacy of pyrethroid-only LLINs. Thus, for malaria elimination to remain a realistic prospect, innovation in the LLIN market is essential so to maintain the efficacy of this preventative measure for as long as possible. Resistance management has its foundation in population genetics. There is established evidence that the most promising way to control resistant mosquitoes or delay the selection of resistance is to apply mixtures of unrelated insecticides. It is important to ascertain if improved vector control and insecticide resistance management could be achieved using novel LLIN products that contain mixtures and combination of pyrethroid and non-pyrethroid insecticides or synergists.

Aim and objectives of the thesis.

The general objective of this study is to evaluate in laboratory, experimental huts and field trials new standard pyrethroid LLIN products, long-lasting treatment kits, and mixture/combination LLIN for the purpose of validation and improvement of the evaluation guidelines, suggestions for new approaches for improved LLIN efficacy for vector control and for the purpose of studying the added efficacy with the use of combination and alternative insecticides for use on bed nets to overcome problems of insecticide resistance

Specific objective 1

To evaluate the efficacy and wash-fastness of new long-lasting pyrethroid nets

The aim of this is to evaluate new pyrethroid LLIN products for the purpose of:

a. WHO recommendation and for purpose of validation and possible input into the LLIN evaluation guidelines.
b. correlate efficacy between phase II and phase III outcomes.
c. investigate similarities and differences in efficacy between the LLINs made of different polymer technologies.

The evaluations were performed in the field in a small-scale in experimental hut studies under well-controlled conditions for some of the new products. The trials were performed on natural vector populations using formulated LLIN products. This phase may also involve small-scale studies of user acceptability and appropriate use of long-lasting nets. Other products under this objective undertook evaluation on a larger scale, for example in household randomised controlled trials.

Specific objective 2

To evaluate long lasting net treatment kits – insecticide impregnation processes that enable nets to remain effective for longer.

An objective of this work was to evaluate long-lasting treatment kit products for their wash fastness, efficacy and longevity on different fabrics used in malaria vector control. This was done to investigate whether improved vector control can be achieved by the use of long-lasting treatment kits to meet the challenges that may threaten effectiveness of nets by:

a. improving inadequate LLIN coverage between coverage campaign through converting retail untreated nets into long-lasting nets
b. catering for the inapplicability of LLINs in humanitarian emergencies and complex political situations through treating with long-lasting treatment kits the tarpaulins and tents distributed in epidemics, disasters or humanitarian emergencies.

c. providing for potential for insecticide resistance management plan through treating standard pyrethroid only LLINs post-manufacture alternative insecticides with no cross resistance

The evaluation of products under this objective was performed on laboratory reared mosquitoes using a variety of standardized bioassays and in the field on a small scale (e.g. experimental huts) under well controlled conditions and in field phase III community trials.

Specific objective 3

To assess the impact of insecticide resistance on the efficacy of pyrethroid long-lasting insecticidal nets (LLIN):

The aim of this was to evaluate standard pyrethroid-only LLINs with the purpose of finding the impact of insecticide resistance on the entomological efficacy of LLINs. The trials under this objective were performed in the field on a small scale (e.g. at a household level in experimental huts) whereby LLINs products were tested against wild free flying pyrethroid susceptible and resistant mosquitoes.

Specific objective 4

To identify and evaluate a) synergist-pyrethroid combinations, and b) alternative insecticides for use in combination with pyrethroids to imitigate pyrethroid resistance in local vectors.

The non-pyrethroid AI may be the synergist piperonyl butoxide PBO which neutralizes the enzymes responsible for resistance, or an insecticide unrelated to pyrethroid to which there is no resistance. The partner AI may be spatially separated on the net or mixed with the pyrethroid.

The aim was to evaluate combination LLINs with the purpose of finding their bility to mitigate and for providing evidence-based advice to National Control Programmes. Under this objective, products were evaluated in the laboratory using a variety of standardized resistance strains and bioassays, and in the field in experimental huts under controlled conditions.

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PART TWO

Research question: Evaluation of a pyrethroid standard long-lasting insecticide LLIN, Interceptor® LN: with recommended suggestions for WHO laboratory (Phase I), experimental huts (Phase II) and field (Phase III) LLIN evaluation guidelines improvements.

Prologue:

Bed nets treated by the user with pyrethroid insecticides (conventionally treated Insecticide-treated nets ITNs) proved to be an efficacious and cost-effective measure to reduce malaria morbidity and mortality in the 1990s.

However, as conventional ITNs need to be re-treated with insecticide at least once a year, or after two or three washes, their effective protection over the longer-term may become compromised.

Long-lasting insecticidal nets (LLINs) which can withstand repeated washing, overcame the retreatment constraint that compromised conventionally treated nets. As a result, insecticide treated nets still remain the most sustainable and effective malaria control tool in endemic countries (Kleinschmidt et al., 2018). Approximately 663 million cases of malaria, were prevented by LLINs since the year 2000, representing 69% of the total cases averted by all interventions used for malaria control (Bhatt, Weiss, Cameron, et al., 2015; Kleinschmidt et al., 2018).

Long-lasting nets are factory-treated, and their insecticides are either incorporated into the textile or coated on to their surface. In both cases, the amount of insecticide can be divided into a smaller fraction available at the yarn surface and a larger amount as a reservoir inside the yarn or coating. (Skovmand et al., 2021).

The high demand for LLINs has attracted interest from many pesticide manufacturers into producing new LLIN products. For a LLIN to be eligible for the donor financed market they must demonstrate their effectiveness for malaria vector control after passing through a series of tests organized by WHO (WHO Pesticide Evaluation Scheme or Pre-Qualification Team) to ensure their quality and performance through standard testing for chemical, physical and biological efficacy against mosquito vectors. Interim recommendation is given to a LLIN product after it has been successfully passed phase I and II WHOPES evaluations while the full recommendation is given to a LLIN product after it has successfully passed phase III evaluation. The WHOPES evaluation phases (I-III) are described in WHO Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets 2013 [1].

Statement of the problem

The WHOPES LLIN evaluation guideline mostly relies on the 3 minutes exposure cone bioassays for passing or failing LLIN product in phase III. Although the 3 minutes cone bioassay is a good test for evaluation of the biological activity and wash resistance of nets as it is capable of revealing small variations in residual efficacy between treatments, the standard cone test does not account for friction and general wear and tear which can also contribute to attrition of insecticide residues. Also, among the shortcomings of the cone bioassay is that of underestimating the efficacy of the LLIN with insecticides which have repelling effects like permethrin. The repellence phenomenon of insecticides tends to influence and underestimate the results of this method as the mosquitoes may easily move upward and rest on the plastic wall of the cone or cotton plug. This shortens the exposure time to less than three minutes.

The best way to study the real effect of washing and ageing of the personal protection of LLINs are the controlled field studies such as the experimental hut studies where all key aspects of mosquito behavior, insecticide toxicity and ageing of LLINs are assessed.

The aim of this part of the thesis is to assess the efficacy and to answer the question how does the protection given by an LLIN decline as it loses insecticide and gains holes, and what is the interaction between insecticide loss and hole acquisition for possible improvement of the current WHOPES guideline on testing and verification of new LLIN products.

Justification

Although WHOPES phase III community trials are best for the assessment of the survivorship and fabric integrity of LLINs in various environments and cultural settings, their sole reliance on the 3 minutes cone bioassays to conclude what will happen in real life is not adequate.

The best way to study the full effect of washing and ageing on the personal protection of LLINs are the controlled household studies such as the experimental hut studies. While improving laboratory procedures for testing nets is particularly important for estimating personal protection and malaria control, the hut trial can be included in the phase III trials for estimating realistic effect of washing and ageing of nets on the personal protection of LLINs.

Findings reported here might not be enough to drive their adoption into the WHO testing procedures, but the evidence will provide additional evidence-based scientific information to inform policy on the need to appraise inclusion of experimental hut trials on nets taken annually from phase III LLINs trials. Experimental Hut Trials (EHT) will complement laboratory based 3-minutes cone bioassays and tunnel tests on the same netting.

General objective

To undertake full (phase I-III) evaluation of Interceptor LLIN in accordance with the WHOPES guideline and to assess the rate at which the protection given by an LLIN declines as it loses insecticide and gains holes, and the interaction between insecticide loss and hole acquisition.

Before assessment for the interaction between insecticide loss and hole acquisition, the validation of the LLINs that are to be taken annually and tested in the huts is of importance to confirm that the products meet WHO LLINs standards for interim and full recommendations. It is a pre-requisite for any new LLIN product to pass through a series of evaluation stages prior to its interim or full approval by the WHOPES/WHOPQ to be used by the community. Hence specifically, this part of the thesis sought:

Specific objectives

I. To evaluate the wash fastness and long-lasting efficacy of the Interceptor LN in controlled WHO experimental hut trials (standard WHO Phase I and II trials).
II. To evaluate the long-lasting effectiveness of the Interceptor LN in laboratory conditions over three years using cone and tunnel test bioassays (standard WHO Phase III trials).
III. To evaluate the efficacy of the Interceptor LN after 1, 2 and 3 years of household usage under experimental hut trials EHT rather than laboratory bioassay (i.e., re-testing nets used in Phase III trials in controlled household conditions in experimental huts).
IV. To evaluate similarities and differences in efficacy between long lasting insecticidal nets treated with similar insecticides but different technologies (coating or impregnation) or polymers (polyethylene and polyester).

Studies to address objectives above are presented and discussed in three separate chapters and one appendix as follows.

Appendix 2: Evaluation of the long-lasting insecticidal net Interceptor® laboratory and experimental hut studies against anopheline and culicine mosquitoes in northeastern Tanzania.

This paper was led by a senior author (R Malima) in which the present author was the second author. Appendix 2 gives critical technical insight to understanding other Part 2 chapters led by Tungu (chapters 2, 3 and 4) and is recommended to be read before reading the rest of Part 2.

Chapter 2: Interceptor® long-lasting insecticidal net: phase III evaluation over three years of household use and calibration with Phase II experimental hut outcomes

Chapter 3: Comparative efficacy of long-lasting insecticidal nets of alpha-cypermethrin incorporation (polyethylene) and coated formulations (polyester) in experimental hut trials of DuraNet® LN and Interceptor® LN

Chapter 4: Assessment of the impact of holes and declining insecticide to the protective efficacy of a LLIN: experimental huts trial of Interceptor LLINs after 1, 2 and 3 years of field usage

Chapter 2: Interceptor® long-lasting insecticidal net: phase III evaluation over three years of household use and calibration with Phase II experimental hut outcomes

Prologue:

Before the granting of interim or full recommendation by the WHO, candidate LLIN must demonstrate their effectiveness for malaria vector control after passing through a series of tests organized by WHOPES (WHO Pesticide Evaluation Scheme) to ensure their quality and performance through standard testing procedure for chemical, physical and biological efficacy sufficiently against mosquito vectors.

Due to the length of this thesis, results of phase I and phase II experimental hut trials (EHT) and laboratory evaluation of Interceptor® LLIN are presented and discussed in Appendix 2. Thus, before reading this chapter (chapter 2) go to appendix 2 that describe and discuss results of the Interceptor phase I and phase II study results that study contributed to the evidence that led to granting of interim approval to Interceptor LN by WHO.

For a LLIN to be eligible for the donor international or country NMCPs market they must have received mainly full recommendation after demonstrating effectiveness and meeting WHOPQT LLIN efficacy criteria.

This chapter presented and discussed the results of Phase III evaluation of Interceptor® LN in terms of biological efficacy over 36 months in line with WHOPES guidelines and procedures to determine their efficacy, longevity, integrity, wash resistance and household acceptability under field conditions. This study was done in Muheza, northeastern Tanzania.

Chapter 2: Interceptor® long-lasting insecticidal net: phase III evaluation over three years of household use and calibration with Phase II experimental hut outcomes after 20 washes

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The material presented in this chapter has been published as:

Patrick Tungu, Matthew Kirby, Robert Malima, William Kisinza, Stephen Magesa, Caroline Maxwell, Benard Batengana, Olivier Pigeon and Mark Rowland: **Interceptor® long-lasting insecticidal net: phase III evaluation over three years of household use and calibration with Phase II experimental hut outcomes**. *Parasites & Vectors* (2016) 9:204

Cover sheet for each 'research paper' included in research thesis.

1. For a 'research paper' already published

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1.2. When was the work published? **2016**

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Abstract

Background

Long-lasting insecticidal nets (LN) are an effective tool for malaria prevention. The World Health Organization Pesticide Evaluation Scheme has established evaluation criteria to facilitate registration for public use. A household randomized trial was conducted in Tanzania according to WHOPES Phase III procedures to evaluate the alpha-cypermethrin coated Interceptor® LN (BASF) over three years' use. Outcomes were calibrated against results of Phase II experimental hut trials.

Methods

Interceptor LN (200 mg/m2 alpha-cypermethrin) and conventionally treated nets CTN (40 mg/m2 alpha-cypermethrin) were randomly distributed to 934 households. At 6-monthly intervals, household surveys recorded net use, durability, adverse effects, user acceptance and washing practices. Concurrently, 30 nets of each type were collected and tested for knock-down and kill of *Anopheles gambiae* mosquitoes in cone and tunnel bioassays. Alpha-cypermethrin content of nets was assessed annually.

Results

At 12 months 97 % of Interceptor LN met the efficacy criteria by cone or tunnel test; this pass rate declined to 90 % at 24 months and 87 % at 36 months. In contrast only 63 % of CTN met the efficacy criteria at 12 months, 14 % at 24 months and 0 % at 36 months. The alpha-cypermethrin content at 36 months on Interceptor LN was 20 % (42 ± 13 mg/m2) of the initial content but on CTNs only 4 % (1.3 ± 1.6 mg/m2) remained. Interceptor LN was reported to be used year-round

and washed 4.3 times/year. A few recalled facial tingling during the first days of use but this did not deter usage. The average number of holes at 36 months was 18, hole area per net was 229 cm2 and hole index was 332. Insecticide content and cone bio efficacy of LN and CTN after 36 months' use were similar to that of LN and CTN used in earlier Phase II hut trials, but while the 20 times washed LN tested in experimental huts gave adequate personal protection the 20 times washed CTN did not.

Conclusions

More than 80 % Interceptor LN fulfilled the WHOPES Phase III criteria at 36 months and thus the LLIN was granted full WHO recommendation. Phase III outcomes at 36 months were anticipated by Phase II outcomes after 20 standardized washes.

Keywords: Long-lasting insecticidal net, LLIN, Anopheles gambiae, Tanzania, Randomised controlled trial, Alpha-cypermethrin

Background

Long-lasting insecticidal nets (LLIN) that repel or kill mosquitoes that contact the netting are the primary method of preventing malaria in many countries of Africa south of the Sahara and Asia [1–3]. The retention of this biological activity, through 20 washes and 3 years of field use without need for re-treatment, is ultimately what defines and distinguishes a long-lasting insecticidal net (LLIN) from a conventionally treated net (CTN) [4, 5]. Preservation of bio-efficacy is achieved during the manufacturing through one of three treatment processes: a) the active ingredient is incorporated into the synthetic fibre materials before the yarn is extruded; b) the extruded yarn is coated with insecticide and polymer binding agent before the nets are sewn; c) pre-sewn nets are mechanically sprayed with the insecticide plus binder. Formal evaluations of LLIN started more than a decade ago [6, 7]. More recently the WHO Global Malaria Programme has urged national malaria control programmes to purchase, promote and scale-up the coverage of LLINs [4], effectively phasing out CTN that require multiple re-treatments during the lifetime of use. Today, the World Health Organization reports that almost half of the African population at risk from malaria has access to insecticide treated nets (mainly LLIN) in the home and an estimated

44 % were sleeping under treated nets compared to 2 % in 2004 [8]. Several brands of LLIN are recommended by WHOPES. One of these is Interceptor® LN (BASF Corporation, Germany) [9], which even after 20 standardized washes demonstrates high killing effect (> 75 %) and personal protection (> 75 %) against malaria vectors in Phase II experimental hut trials [9, 10]. However, less is known of the longevity, physical integrity, attrition rate, persistence of bio-efficacy and insecticide content of LLIN under household conditions. For donors and procurement organizations, such information is vital to the planning of LLIN distribution and replacement campaigns.

Interceptor LN nets contain a textile auxiliary Fendozin® (BASF) as a finishing product that binds the alpha-cypermethrin insecticide to the polyester fibres in a resin-based polymer coating [9, 10]. This coating can withstand multiple washes and yet allows the slow release of the alpha-cypermethrin to the net surface where it rapidly knocks down and kills mosquitoes as they contact the net.

Some field studies of Interceptor® LN have shown encouraging efficacy and acceptability outcomes over 1–3 years of use [11–14]. In Liberia, a prospective study showed a low rate of insecticide loss and high acceptability of Interceptor LN [11]; however, these outcomes were measured for only 1-year post-distribution. In north-eastern India, two groups of three [12] and six [14] villages received Interceptor LN in field trials, which resulted in large reductions in vector mosquito population densities. However, in these studies the intervention villages were compared with villages that received untreated nets or focal spraying of DDT instead of conventionally treated nets. As such, neither the study design nor the outcome measure (reduction of vector abundance) satisfies the requirements of a WHOPES Phase III trial or WHOPES criteria for full recommendation. A full WHOPES recommendation is only granted after demonstrating that the candidate LLIN still meets specific efficacy criteria after 3 years of regular household use in clearly defined phase III trials [5, 15].

The overall objective of this study was to carry out a Phase III evaluation of Interceptor LN in line with WHOPES guidelines and procedures to determine their efficacy, longevity, integrity, wash

resistance and household acceptability under field conditions. The specific objectives were a) to evaluate Interceptor LN in terms of biological efficacy at 7 time points over 36 months in comparison with conventional alpha-cypermethrin treated nets used under similar field conditions, b) to determine chemical content at annual intervals up to 3 years of use, c) to monitor net integrity, d) to assess household acceptance and use of Interceptor LN, and e) to calibrate the outcomes of Phase III household trials with outcomes of Phase II experimental hut trials.

Methods

Study areas

The study site was comprised of 3 villages containing 15 hamlets in Muheza district, Tanga region, northeast Tanzania (Fig. 1). The household demographic survey and baseline census were conducted in 2008. Magila village consists of 5 hamlets (Kibaoni, Kwedunda, Magazini, Potwe, Seluka), 391 houses and a population of 2,959. Ubembe village consists of 8 hamlets (Mianzini, Majengo, Ubembe, Misufini, Mbuyuni, Mgombezi A and B, Mkinga), 335 households and a population of 1,478. Mikwamba village consists of 2 hamlets (Mikwamba, Mangachini), 208 households and a population of 943.

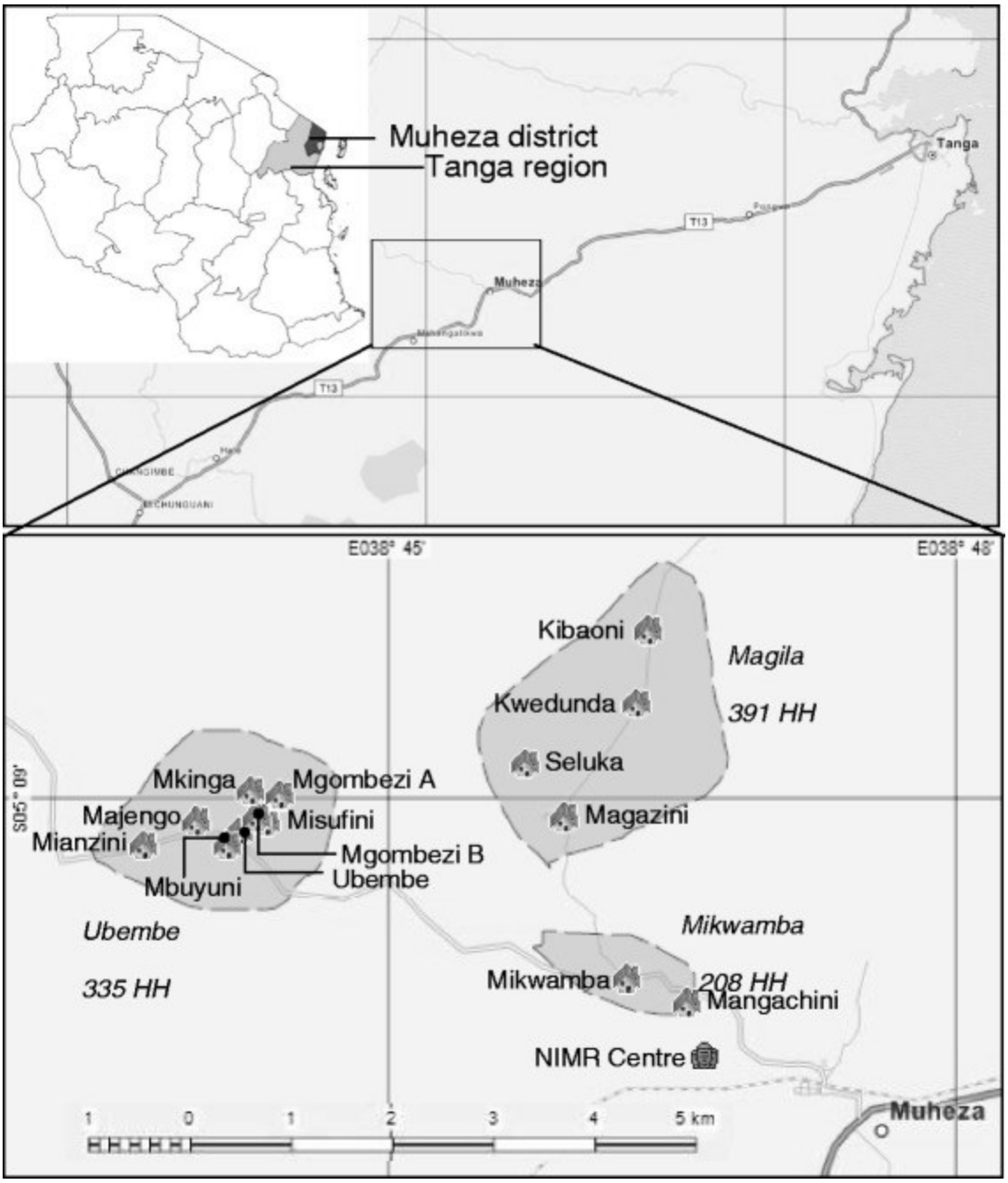

Fig. 1: Location of study villages and hamlets within Muheza district, Tanzania

The area experiences a long rainy season between April and August and a short rainy season between December and January. During the rainy seasons *Anopheles gambiae* sensu lato predominates. *An. funestus* becomes more common in the dry season. The area has been shown

in the past to have high entomological inoculation rates, estimated between 300 and 1,000 infective bites per person per year [16].

Study design

A 3-year community randomized trial was conducted with the household as the unit of randomization and with the mosquito nets as the unit of observation. The efficacy of Interceptor LN was monitored over 3 years of continuous use. Conventionally treated nets were used for comparative purposes. Initially it was proposed to replace the CTNs with LNs 1 year into the trial as recommended in the WHOPES 2005 guidelines for laboratory and field testing of LN [15]. Unfortunately, thieves raided the store in which the replacement nets were stockpiled, and all the nets were stolen. After reviewing the decision was made to continue monitoring the CTN until a randomly selected net failed to meet the cut-off bioassay criteria (see section Insecticide bioassay efficacy of nets), at which point all the nets from that household were replaced with LN. This study pre-dated the 2013 revised WHOPES guidelines for laboratory and field testing of LN, which recommended that a candidate LN is field evaluated against an existing WHO-recommended LN rather than a CTN [5].

Household randomisation

A pre-distribution baseline census collected details of residents including the number of sleeping places per household, sizes of beds, net ownership and net usage. A household was defined as a group of related or unrelated persons living together in the same dwelling, acknowledging one adult as the household head. Each household was given a unique identification number, and the house was physically labeled with this number to facilitate revisits. The household number was used to randomly allocate the Interceptor LNs or CTNs to selected households, stratified by hamlet so that both net types were well represented within each hamlet (Fig. 2). The allocation of nets to each household was dependent on the number of sleeping places. Every bed or sleeping place had to be covered by a net in all hamlets to ensure the community was adequately protected.

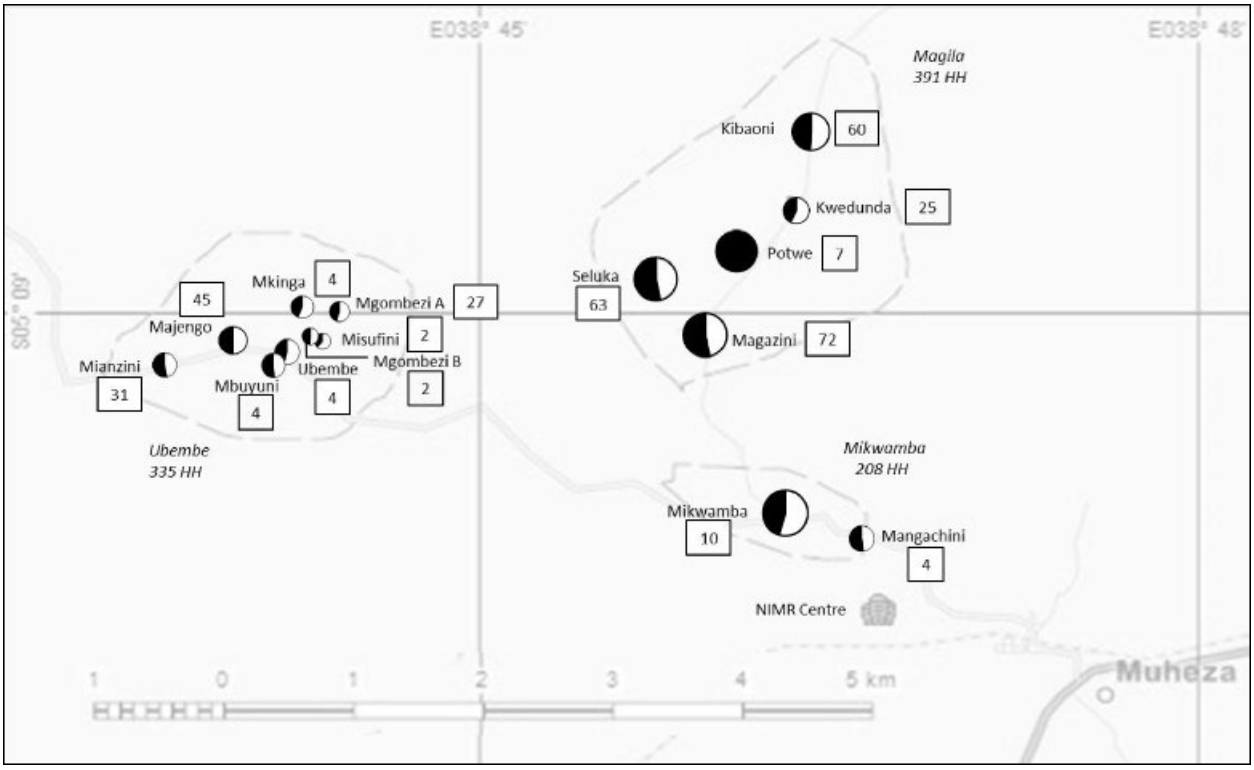

Fig. 2: Distribution of study nets within the 15 hamlets. Pie chart diameter is proportional to the total number of nets distributed in each hamlet (black = LN, white = CTN). Numbers in squares are the total nets (LN + CTN) destructively sampled from each hamlet.

Net treatment and distribution

Interceptor® LNs and untreated polyester nets of 75 denier were supplied by BASF Corporation (Ludwigshaven, Germany) in a range of sizes in width (4, 5 and 6 feet). Interceptor LN was treated with alpha-cypermethrin (coated onto filaments) at a target dose of 6.7 g AI/kg of netting material for 75-denier yarn, corresponding to 200 mg alpha-cypermethrin per m2 of polyester fabric (with a tolerance limit of ±25 %). Polyester nets of the same denier and supplier were treated individually at the NIMR Amani Medical Research Centre using an aqueous solution of alpha-cypermethrin (Fendona 10SC, BASF), with volume dependent on the size of the net using the formula of Pleass et al. [17] to achieve a target dosage of 40 mg/m2. Nets were laid flat over plastic sheeting to dry in shaded conditions and rolled over periodically until the insecticide solution had dried. A unique code number was stenciled onto each net using a permanent marker. Nets were also marked with a cross in water-soluble ink for the assessment of washing practices.

A total of 1,953 Interceptor LN and 1,593 CTN were distributed to the selected households in 2008. The household number was used as the unit to randomly allocate the Interceptor and CTNs. The distribution teams and the recipients were blinded to the identity of the nets received by each household. Individual households received either Interceptor LNs or CTNs and not a mix of types. Enough nets were distributed to cover all sleeping spaces in the house. Householders were informed about the need for reporting adverse effects during net use, as well as advised on appropriate use and maintenance of nets. Assistance in hanging up the nets over the sleeping area was given where needed.

Household surveys and net sampling

Thirty nets of each type were sampled at baseline (pre-distribution) and during cross sectional surveys of households carried out after 6, 12, 18, 24, 30 and 36 months of field use. The 60 households sampled per survey were selected randomly from the household ID master list. If the selected house could not be surveyed for various reason, next house from the randomized list was selected for the survey. The selected households received a replacement LN and were removed from the study. Figure 2 shows the number of nets sampled from each hamlet. At the time of collection, a questionnaire was applied to assess current net use, acceptability, washing practices and any adverse effects.

Net integrity and durability

Net integrity and durability surveys were carried out for Interceptor LN at baseline and 6-, 12-, 18-, 24-, 30- and 36-months post-distribution and for CTNs at baseline, 6- and 12-months post-distribution. The CTN arm was withdrawn after 12 months as they were found to have lost their efficacy at this time interval. At each survey point 30 nets per arm were sampled. The nets were hung over a wooden frame and scored for the size and distribution of holes, repairs (stitches, knots and patches) and open/failed seams. Cleanliness assessment was done simultaneously, and nets categorized according to grade of cleanliness/dirtiness.

Hole sizes were categorized as size 1 – smaller than a thumb, size 2 – larger than a thumb but smaller than a fist, size 3 – larger than a fist but smaller than a head, size 4 – larger than a head. Hole index, hole area and hole circumference were estimated using the following formulae:
Hole index = (no. of size 1 holes × 1) + (no. of size 2 holes × 23) + (no. of size 3 holes × 196) + (no. of size 4 holes × 578).

Hole area = (no. of size 1 holes × 0.25π) + (no. of size 2 holes × 9π) + (no. of size 3 holes × 25π).
Hole circumference = (no. of size 1 holes × 1π) + (no. of size 2 holes × 6π) + (no. of size 3 holes × 10π)

In the formula for hole index, the multipliers used assume that the hole size equates to the mid-point of the range for each hole size category using the method described by WHO [18]. The formulae for calculating the hole area and hole circumference were based on the area and circumference of a circle: size 1 holes were of 0–2 cm diameter (midpoint = 1 cm). Size 2 holes were 2–10 cm diameter (midpoint = 6 cm). Because no size 4 holes were observed and few holes categorized as size 3 were wider than 10 cm, the average diameter of size 3 holes was set at the lower limit of 10 cm diameter. Thus, the estimate of hole area gives a slightly more conservative value when compared to the hole index. Hole circumference was included as it might be the more biologically relevant indicator: mosquitoes walking or skipping across the surface of net must encounter the edge of a hole before penetrating the net.

Insecticide bioassay efficacy of nets

Thirty Interceptor LNs and 30 CTNs were sampled at baseline and at 6-, 12-, 18-, 24-, 30- and 36-months post-distribution. Five netting pieces (25 cm × 25 cm) were cut from the five panels of each net in accordance with WHOPES guidelines [15]. Cone bioassay protocol described is in detail in appendix 1, section 2.3.1.1. Only the net piece closest to average mortality of the net was used for the tunnel test. Any net meeting the cone criteria of ≥ 80 % mortality or ≥ 95 % knockdown or tunnel test criteria of ≥ 80 % mortality or ≥ 90 % blood-feeding inhibition was considered to have met the WHOPES criteria.

Chemical analysis

From each of the 30 LN and 30 CTN sampled at baseline and surveys at 12, 24 and 36 months, five additional pieces of netting (30 cm × 30 cm) were cut for chemical analysis. As before, the pieces were cut from the five panels of each net and the piece closest to the mattress line was excluded as per WHOPES guidelines. All Interceptor pieces and the baseline and 12-month CTN pieces were sent to the WHO-collaborating Centre Wallon de Recherches Agronomiques (CRA-W) in Belgium for chemical analysis. The net pieces from each individual net were pooled, cut into small pieces and homogenized, and alpha-cypermethrin was extracted from an aliquot by heating under reflux with tetrahydrofuran in accordance with the CIPAC method for alpha-cypermethrin in coated LNs. Dioctyl phthalate was added as an internal standard; alpha-cypermethrin content of each individual net was determined using gas chromatography with flame ionization detection (GC-FID). Pieces from CTN at 24 and 36 months were analyzed by high-performance liquid chromatography at The London School of Hygiene and Tropical Medicine (LSHTM) using the method described by Yates et al. [19].

Data analysis

Data were double entered into Microsoft Access 2007 and analysed in STATA version 10.1 Proportional data such as the 1 h knockdown and 24 h mortality was transformed using square root arc sign method before analysis. Categorical data was analysed using Chi-square, and assessment of net efficacy over successive surveys was analysed using Chi-square tests for trend. Continuous data was analysed using Wilcoxon rank sum test where the data was not normally distributed.

Ethical considerations and approval

Ethical clearance was received from the Medical Research Coordination Committee of the National Institute of Medical Research, which is the National Ethics Committee of the Ministry of Health in Tanzania. The project also obtained ethical clearance from the Research Ethics Committees of LSHTM and WHO.

Results

Household surveys

A total of 3,546 sleeping places were identified across all study hamlets in the baseline survey, and 77 % of these contained beds. Most beds were size 5′ × 6′ (2,066) or 6′ × 6′ (660). Nets of appropriate size were given to cover all sleeping places. During the net-sampling cross sectional surveys, households were asked about house characteristics, net use and net washing practices. Most houses had palm thatched roofs (range between surveys 50–64 %), though corrugated iron was also common. Most householders were farmers (range 43–97 %) and most (65–79 %) had received 7 or more years of primary school education but less than 10 % had received secondary or further education. Over a third of households lived on less than $1 per day; the highest salary recorded was only $3 per day and the mean income was $1.75 US per day.

Net use and washing

Reported use of both types of net was high throughout the study. At 12-, 24- and 36-month post-distribution, all respondents indicated using their nets year-round and every night. The placement of nets provided corroboration; 98 % (127/130) of Interceptor LN were found hung above beds and the remaining 3 LNs were observed suspended over floor mattresses. Similarly, in the CTN group 99 % (118/119) of nets were seen hung over a bed.

Interceptor LNs and CTNs were washed on average 4.3 times per year. Despite this it was observed that 70 % of Interceptor LN and 77 % of CTN had accumulated some dirt after 6 months, and this proportion increased after 12 months (Table 1). After 36 months only 10 % of the Interceptor LNs were scored as clean and 27 % were scored as very dirty. No differences were reported between the washing practices of families using Interceptor LN and families using CTN. All respondents reported washing their nets in cold water. Nets were soaked by 20–37 % of respondents; soaking times ranged from 10 min to 2 h. Nets were reported to be washed using commercial bar soap (53–62 %), commercial detergent powder (17–27 %) or both (8–30 %). Most nets (68–90 %) were rinsed after washing and most (75–95 %) were dried outside. No one reported rubbing nets against rocks or stones during washing.

Table 1: Washing frequency and net appearance.

	Interceptor LN						alpha-cypermethrin CTN					
			% General aspect of nets						% General aspect of nets			
Survey (month)	No. nets	Mean no. of washes[a]	Clean	Slightly dirty	Dirty	Very dirty	No. nets	Mean no. of washes[a]	Clean	Slightly dirty	Dirty	Very Dirty
0	30	0	100	0	0	0	30	0	100	0	0	0
6	30	3	30	40	30	0	30	4	23	47	27	3
12	30	3	13	30	47	10	30	2	20	27	40	13
18	30	2	3	0	97	0	-	-	-	-	-	-
24	30	1	23	0	73	4	-	-	-	-	-	-
30	30	2	10	37	43	10	-	-	-	-	-	-
36	30	2	10	20	43	27	-	-	-	-	-	-

[a]**Mean number of washes during the six-monthly periods**

Physical integrity

The baseline survey found no holes or open seams on any of the sampled Interceptor LNs or CTNs (Tables 2 and and3).3). After 6 months, 63 % of Interceptor LN and 83 % of CTN had at least one hole; these were mainly of size 1 and the mean number of holes was only 5 per net for Interceptor LN and 9 for CTN. By 24 months, 83 % of Interceptor LN had at least one hole and the mean number of holes per net was 22. After 36 months, the percentage of nets with at least one hole and the mean number of holes per net did not increase relative to the 24-month survey. From the 6th month survey onwards most holes were size 1, approximately a quarter were size 2 and a minority were size 3. The vast majority of holes were always to be found in the lowest section of the net, at body level, where the net is tucked under the mattress (if present). The number and the position of holes did not differ between net types (both types being of the same 75 denier material). The physical integrity of the Interceptor nets deteriorated between 12 and 24 months with respect to hole index (Wilcoxon rank sum test $Z = -2.797$, $P = 0.005$), hole area ($Z = -2.797$, $P = 0.005$) and hole circumference ($Z = -2.827$, $P = 0.005$) but between 24 and 36 months no further deterioration was evident (hole index $Z = -0.296$, $P = 0.77$; hole area $Z = -0.222$, $P = 0.82$; hole circumference $Z = -0.015$, $P = 0.99$) (Table 4).

Table 2 Physical condition of Interceptor LN and CTN by survey round – holes by size category.

	Interceptor LN						alpha-cypermethrin CTN					
Survey (month)	No. nets	Mean (SD) holes/net	% holes by size category				No. nets	Mean (SD) holes/net	% holes by size category			
			1	2	3	4			1	2	3	4
0	30	0 (0)	0	0	0	0	30	0 (0)	0	0	0	0
6	30	5 (9)	64	26	10	0	30	9 (20)	75	22	3	0
12	30	6 (9)	71	16	13	0	30	11 (18)	72	22	6	0
24	30	22 (23)	68	25	7	0	-	-	-	-	-	-
36	30	18 (20)	67	27	6	0	-	-	-	-	-	-

Table 3: Physical condition of Interceptor LN and CTN by survey round – holes by distribution

	Interceptor LN							alpha-cypermethrin CTN						
Survey (month)	No. nets	% Nets with ≥ 1 hole	% Holes by distribution[a]			Mean no. of open seams	% Nets with any repairs	No. nets	% Nets with ≥ 1 hole	% Holes by distribution[a]			Mean no. of open seams	% Nets with any repairs
			Lower	Upper	Roof					Lower	Upper	Roof		
0	30	0	0	0	0	0	0	30	0	0	0	0	0	0
6	30	63	73	18	9	0	0	30	83	93	4	2	0	0
12	30	60	75	11	14	0.1	3	30	67	79	16	5	0.2	7
24	30	83	84	10	6	2.2	3	-	-	-	-	-	-	-
36	30	83	70	17	13	1.4	20	-	-	-	-	-	-	-

[a]Location of holes: lower = lower half of side panels; upper = upper half of side panels; roof = top panel

Table 4: Physical integrity – comparison of estimates of the average hole index, hole area and hole circumference for a) Interceptor LN; b) alpha-cypermethrin CTN

a)	Interceptor LN

Survey (month)	Hole index			Hole area (cm^2)			Hole circumference (cm)		
	Mean (1SD)	Median (IQR)	Geometric mean	Mean (1SD)	Median (IQR)	Geometric mean	Mean (1SD)	Median (IQR)	Geometric mean
0	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
6	139 (351)	2 (0–83)	7	83 (193)	2 (0–69)	6	59 (113)	6 (0–48)	8
12	170 (630)	2 (0–68)	7	88 (263)	1 (0–75)	6	54 (118)	5 (0–65)	7
24	442 (696)	78 (3–533)	46	282 (393)	84 (3–404)	37	194 (240)	63 (11–326)	45
36	332 (442)	126 (30–549)	70	229 (283)	102 (33–346)	60	162 (187)	127 (36–212)	54
b)	alpha-cypermethrin CTN								
Survey (month)	Hole index			Hole area (cm^2)			Hole circumference (cm)		
	Mean (1SD)	Median (IQR)	Geometric mean	Mean (1SD)	Median (IQR)	Geometric mean	Mean (1SD)	Median (IQR)	Geometric mean
0	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
6	121 (324)	5 (1–51)	12	91 (257)	4 (1–60)	10	73 (186)	16 (4–53)	15
12	205 (458)	8 (0–173)	14	134 (253)	6 (0–87)	12	95 (161)	19 (0–76)	14
24	-	-	-	-	-	-	-	-	-
36	-	-	-	-	-	-	-	-	-

Analysis of chemical content

At baseline, the mean concentration of alpha-cypermethrin was 204 mg/m2 for Interceptor LN and 32 mg/m2 for CTN (Fig. 3). These values were within 25 % of the target dosages (200 and 40 mg/m2 respectively). The mean concentration of alpha-cypermethrin in the Interceptor LNs had decreased to 117 mg/m2 after 12 months, to 68 mg/m2 after 24 months and to 42 mg/m2 after 36 months (Fig. 3). The mean concentration of alpha-cypermethrin in the CTNs was 9.6 mg/m2 after 12 months' field use, 0.7 mg/m2 after 24 months and 1.3 mg/m2 after 36 months. At some time points a difference was apparent in insecticide concentration between nets which passed the bioassay criteria (≥80 % mortality) and those which failed it: among the CTN at 12 months the mean concentration was 15.8 mg/m2 for those which passed and just 7.6 mg/m2 for those which failed; among the LN at 24 months the mean concentration was 74.5 mg/m2 for those which passed but only 55.1 mg/m2 for those which failed. However, it is interesting to note that 40–50 % of mosquitoes were still being knocked down and killed by the CTN sampled after 36 months despite very low insecticide concentrations on the nets.

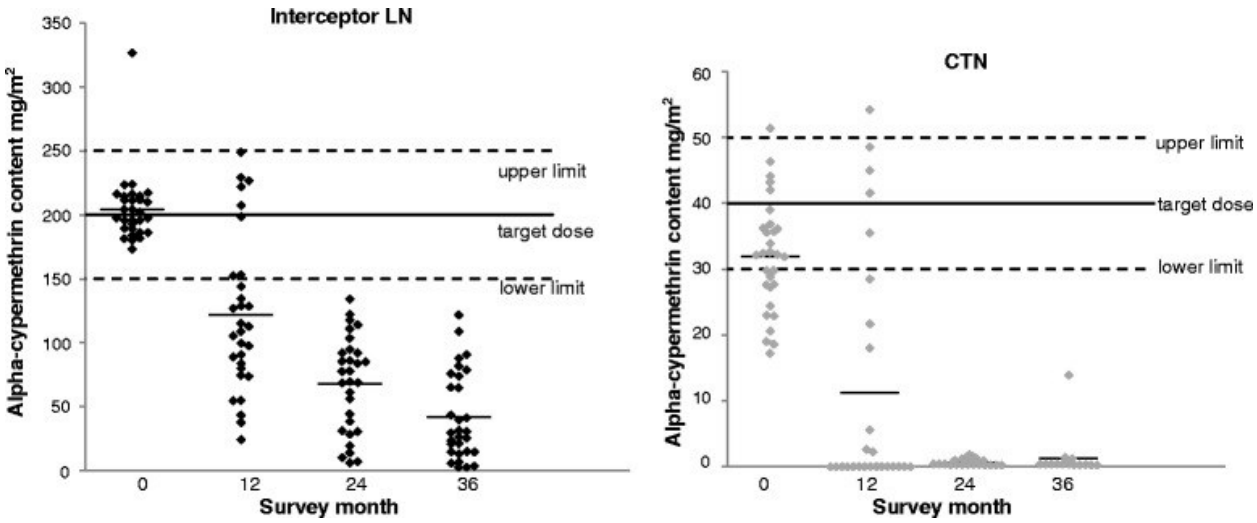

Fig. 3: Alpha-cypermethrin content (mg AI/m2) on individual Interceptor® LN and CTN samples at baseline and after 12-monthly intervals of field use. Mean concentrations for each time point are indicated by the thin horizontal lines. The target dose and upper and lower limits are for alpha-cypermethrin content at baseline indicated as solid and dashed lines.

Net efficacy through bioassay

A total of 210 Interceptor LNs and 210 CTNs were sampled for bioassays and chemical analysis at 6 monthly intervals during the 3 years. Cone bioassay tests on Interceptor LN and CTN at baseline

(before distribution) resulted in knock down of 100 % and mortality of > 99 % on all pieces tested (Figs. 4 and and5).5). After 6 months' use the mean percentage mortality (± C.I.) was 92 % (88–96) on the Interceptor LNs and 80 % (74–87) on the CTNs (t = 5.25, df = 223, P = 0.0001; t-test) (Fig. 5). Similarly, knockdown was 95 % (92–98) on the Interceptor LNs compared to 85 % (80–90) on the CTNs (t = 6.03, df = 223, P = 0.0001; t-test) (Fig. 4). Two of the Interceptor LNs and 10 of the CTNs failed to meet the WHOPES criteria for the cone test. When the tunnel test was applied, all Interceptor LNs (100 %) and all but two of the CTNs (93 %, 28/30) met the WHOPES criteria (Fig. 6).

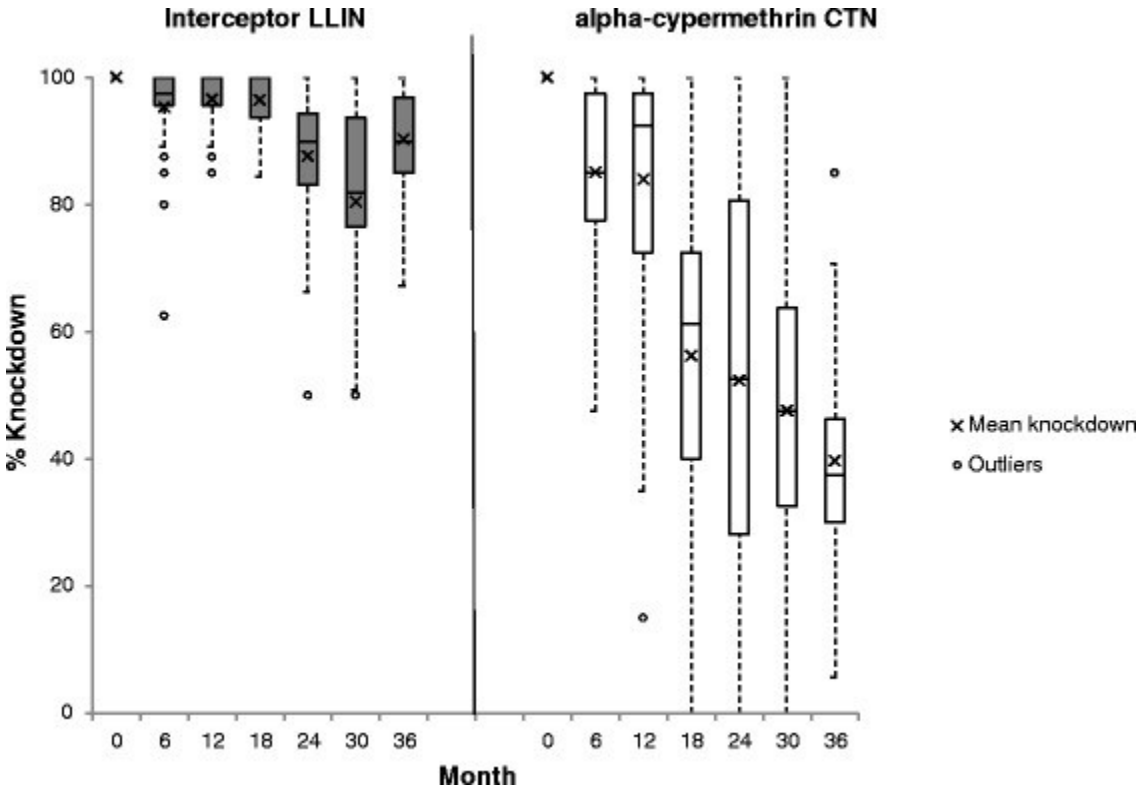

Fig. 4: Median (IQR) and mean percentage *An. gambiae* (s.s.) (Kisumu) knockdown 1 h post-exposure to Interceptor LN and CTN pieces in cone bioassays

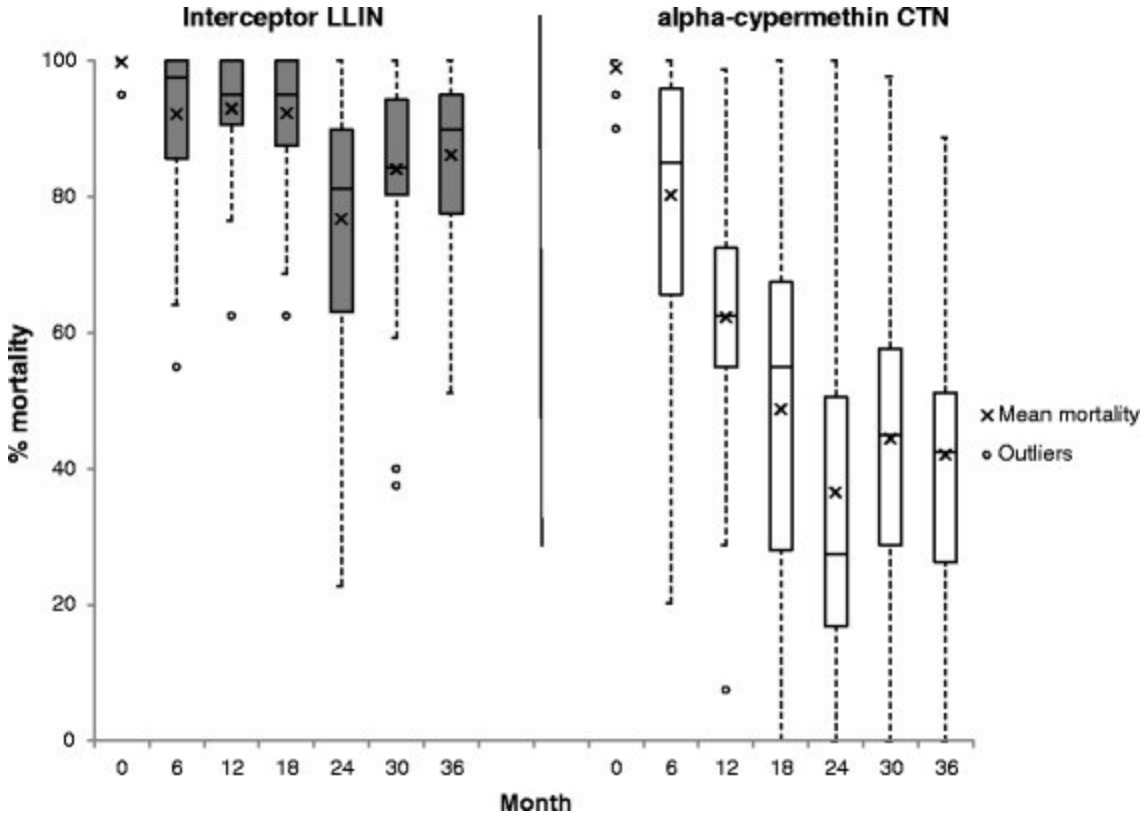

Fig. 5: Median (IQR) and mean percentage *An. gambiae (s.s.)* (Kisumu) mortality 24 h post-exposure to Interceptor LN and CTN pieces in cone bioassays

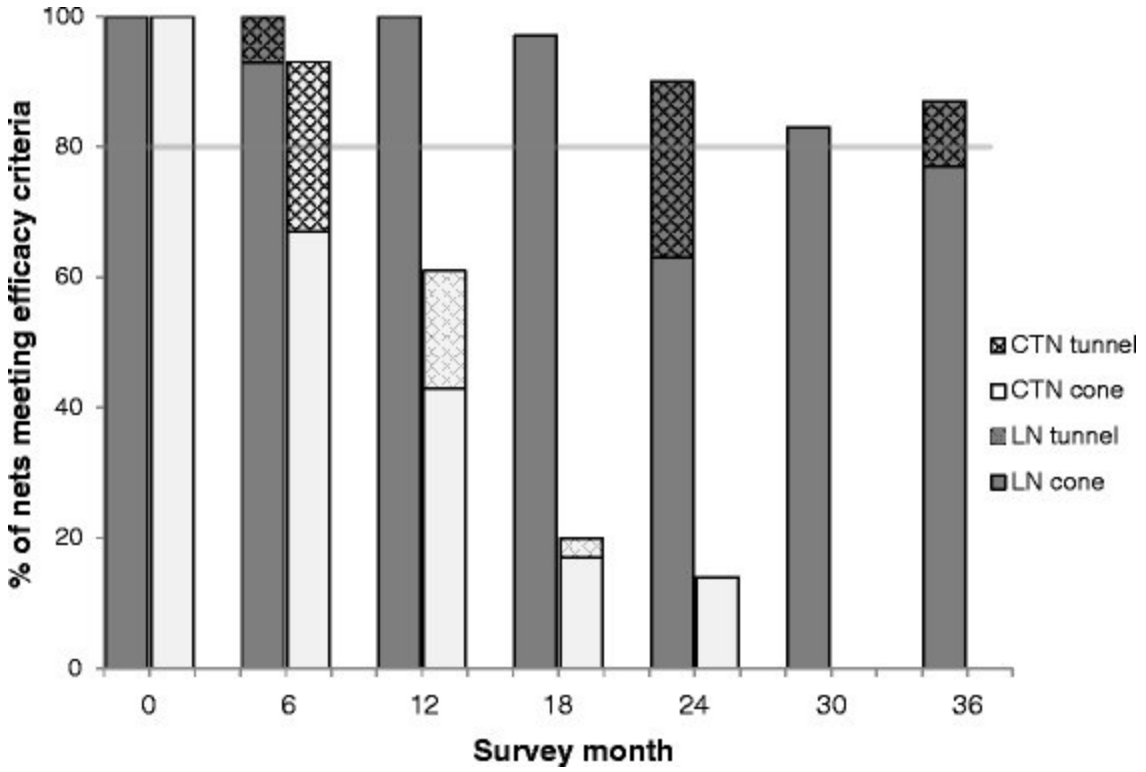

Fig. 6
Percentage Interceptor LN & alpha-cypermethrin CTN meeting WHO efficacy criteria (solid bar = cone test, hatched bar = tunnel test1) by survey round. The horizontal line represents the acceptability cut-off for WHOPES full approval of the LN. No CTN passed at 30 or 36 months. 1WHO criteria: cone test: ≥ 80 % mortality and/or ≥ 95 % knockdown; tunnel test: ≥ 80 % mortality and/or ≥ 90 % blood feeding inhibition where control tunnel test > 35 % penetration into host chamber. Tunnel tests were carried out on nets that did not satisfy the cone test criteria.

At 12 months, 97 % (29/30) of the Interceptor LNs but only 63 % (16/27) of the CTNs met the WHOPES criteria for cone and tunnel tests (Fisher's exact X2 = 12.0, df = 1, P = 0.001). Only 3 % of Interceptor LNs failed the cone test but 56 % of CTNs failed the cone test at 12 months (Fig. 6). This difference between Interceptor LN and CTNs is also reflected in the mean percentage mortality of 93 % (90–96) for Interceptor LNs and 62 % (56–69) for the CTNs (t = 12.93, df = 223, P = 0.0001; t-test) in the cone bioassay tests (Fig. 5), and similarly in the percentage knockdown of 97 % (95–98) for the Interceptor LNs and 84 % (77–91) for the CTNs (t = 5.51, df = 223, P = 0.0001; t-test) (Fig. 4).

At 18 months, 97 % (29/30) of Interceptor LNs met the WHOPES criteria by either the cone or the tunnel test (Fig. 6). This figure declined to 90 % (27/30) at 24 months; at this sampling point fewer

nets passed the cone bioassay criteria (63 %, 19/30) compared to before, but the majority (8/11) of nets that failed met subsequently the tunnel test criteria. At 30 and, crucially, 36 months Interceptor LN met the cone and tunnel test criteria with combined pass rates of 83 % (25/30) and 87 % (26/30) respectively; overall the incremental decrease in pass rate over the 36 months was small but significant (X2 for trend = 11, df = 1, P = 0.001). By contrast the efficacy of the CTN decreased after 12 months, with only 20 % (6/30) meeting the criteria at 18 months, 14 % (4/30) at 24 months, and none at 30 or 36 months (X2 for trend = 125, df = 1, P = 0.0001). The major differences in the pass rates of Interceptor LN and CTNs after 1 year is also reflected in their percentage mortality and knockdown scores in cone bioassay tests between 12 months and 36 months (Figs. 4 and and55).

Calibration of net efficacy and insecticide content in Phase III household trials and Phase II experimental huts trials

The Phase II experimental hut trial results of Interceptor LN and alpha-cypermethrin CTNs evaluated at the NIMR Amani Centre and presented in Table 5 were taken from Malima et al. [10]. The average alpha-cypermethrin content of Interceptor LNs and CTNs collected after 36 months in the present Phase III household trial was similar to the average alpha-cypermethrin contents of 20 times washed LNs and CTNs used in the Phase II experimental huts that led to the initial WHO interim recommendation for Interceptor LN (Table 5). Twenty times washed Interceptor LN (consistent with Interceptor LNs after 36 months field use) continued to demonstrate satisfactorily high levels of personal protection and mosquito mortality, both of which were significantly greater than the inadequate levels of protection and mortality recorded for 20 times washed CTNs. It follows that CTNs after 36 months field use would not provide adequate protection to users of such nets.

Table 5: Calibration of alpha-cypermethrin content and entomological outcomes in Phase II experimental hut trial (Malima et al. [10]) with alpha-cypermethrin content of nets in Phase III household randomised trial

	Interceptor LN	Interceptor LN	CTN
Number of washes in Phase II trial	0	20	20

	Interceptor LN	Interceptor LN	CTN
% Mortality corrected for control*	92[a]	76[b]	44[c]
% Personal Protection*	79[a]	76[a]	6.4[b]
Mean concentration of alpha-cypermethrin (mg/m^2) in Phase II trial	147	41	1.2
Mean concentration of alpha-cypermethrin (mg/m^2) in Phase III trial	204[1]	42[2]	1.3[2]

Percentages followed by the same letter superscript do not differ at 0.05 level.
1at baseline before distribution
2after 36 months

Adverse effects

Few adverse effects were reported by net users. At 12 months post-distribution, 8.4 % (21/249) of respondents recalled experiencing adverse effects during the first few days of use. The most common events were facial tingling (2 %), headache (1.6 %) and irritation (1.2 %). Adverse effects were slightly higher among users of the Interceptor LN compared to CTNs (11.5 % versus 5 %). Respondents reported that symptoms stopped once the net had been washed and nobody was deterred from using their nets. No adverse effects were reported in any of the subsequent surveys.

Discussion

This WHOPES sponsored Phase III trial evaluated the efficacy of Interceptor LN over 36 months of household use using the standard WHO cone bioassay criteria of knockdown and mortality and the tunnel test criteria of mortality and blood feeding inhibition [5, 15]. At the conclusion of the trial 87 % of LNs sampled at 36 months met one or more of these efficacy criteria, and thus the LN product exceeded the 80 % threshold required to attain WHO full recommendation [20]. Each criterion contributed to determining whether a sampled batch of nets achieved the WHOPES threshold or not. For example, at 36 months 30 % (9/30) of nets reached the threshold based on both cone mortality and knockdown criteria, a further 40 % (12/30) passed on mortality criteria only (having failed on knockdown criteria), and 2/30 (7 %) passed on knockdown criteria only (having failed on mortality criteria). Thus, cone mortality made by far the larger contribution to

the overall pass rate. At 24 months the contribution of cone mortality was greater still: 63 % passed based on cone mortality, 27 % passed on the basis of knockdown, but no nets passed on knockdown alone which means that knockdown rates made no contribution to the overall pass rate. A similar story emerged at 12 months with 93 % passing on the mortality criterion and only 3 % passing based on knockdown alone. This indicates the major contribution of mortality over knockdown to the evaluation of pyrethroid LN efficacy.

Tunnel tests also made an important contribution. At 36 months 77 % of samples passed based on cone criteria but critically an additional 10 % (3/30) passed on the basis of tunnel test criteria, lifting the overall pass rate to above the WHO threshold of 80 %. Tunnel tests can also be an important validation check on the veracity of the interpretation in the rare circumstances where anomalous results are recorded in the cone bioassays. For example, at 24 months an unexpectedly low 63 % of nets passed based on cone criteria, but a further 27 % subsequently passed based on tunnel test criteria.

At 36 months 26/30 (87 %) of LN had reached the required standard. It is sobering to reflect that had 3 LNs of this batch by chance did not meet the required standard Interceptor LN would have failed to reach the pass rate of 80 %. Just a few nets can exert great leverage around the 80 % threshold when only 30 nets form the basis of the decision. In response to this, WHOPES has decided to increase the sample size at the all-important 36-month time point from 30 to 50 nets to improve statistical power and precision [5].

The CTNs were monitored beyond the anticipated 12 months' end point because of theft of Interceptor LNs from the store. In-use CTN continued to be followed up for efficacy and chemical content. After 12 months' use the insecticide content of the CTNs had decreased by 66 % relative to baseline; however, most nets still met the efficacy criteria. From that point on the situation changed profoundly: after 24 months the insecticide content of the CTNs decreased by 94 % and few CTNs met the WHO efficacy criteria. Despite this, it is notable that while only a milligram per m2 alpha-cypermethrin residue remained on the average CTN, the nets still killed about 40 % of mosquitoes in cone bioassay. A similar observation was made during the Phase II experimental

hut trial conducted in the same locality 3 years earlier; while after 20 standardized washes only a milligram of alpha-cypermethrin per m2 remained on the CTNs, mortality of 68 % was being recorded in cone bioassays and 44 % of free flying An. gambiae were still being killed by these nets in experimental huts [10]. However, the level of personal protection from mosquito biting from these nets was, at 6 %, insignificant both statistically and in terms of protection [10] and this provides a strong argument for always deploying LLIN over CTN.

The rate of loss of insecticide over time was more gradual in the Interceptor LNs and was remarkably constant year by year. After 12 months of use the insecticide content of the LNs had decreased by 43 % of the initial content of 204 mg/m2, after 24 months it had decreased by a further 42 %, and after 36 months it had decreased by a further 38 %. At 36 months the average insecticide content was 42 mg alpha-cypermethrin per m2; this was remarkably similar to the 41 mg/m2 alpha-cypermethrin content observed in Interceptor LNs after 20 standardized washes in the Phase II experimental hut trials done in the same locality 3 years previously [10]. This similarity in chemical content between a Phase III household randomized trial and Phase II experimental hut trial indicates that the 20 standardized washes which LLINs undergo before testing in experimental huts is a fair approximation to the average loss of insecticide due to wear and tear, abrasion and washing that LLINs undergo during 3 years of household use at least in this area. The outcomes of Phase II experimental hut trials would appear to be a reasonable prediction of the outcome of Phase III trials conducted in the community. While this correlation is encouraging, more LN products need to be evaluated and compared in Phase II experimental hut and Phase III household trials before this conclusion can be fully verified or justified. It is nevertheless encouraging – even fortuitous - that the arbitrary 20 washes that LLIN are purposefully subjected to in WHOPES Phase II seem a good approximation to Phase III after 3 years. In practice the number of washes that a net is subjected to during 3 years of household use may fall short of the 20 washes of Phase II; in the present Interceptor LN trial the average net was estimated to be washed 4.3 times a year or only 13 times over the 36 months. Under household use the average net would be subjected to more vigorous challenges than washing – the removal of surface insecticide through friction and abrasion in everyday use, for example -

but over the 36 months this removal would seem to add up, or be equivalent to, the 20 washes of Phase II.

Taking the logic of the Phase II and Phase III calibration one step further, a typical Interceptor LN after 3 years of household use and alpha-cypermethrin content of 42 mg/m2 should, as predicted by experimental hut trials, continue to kill up to 78 % of hosting seeking *An. gambiae* that contact the net and would still provide 76 % protection to the occupants [10]. Given the major loss of efficacy and protection observed with the average CTN after 3 years, discussed above, this concludes the argument for always deploying LLIN over CTN as retreatment proved to be logistically very challenging.

At all-time points, trial participants reported a high frequency of net use all year round; this assertion was corroborated by the high proportion of nets observed hanging above the beds. After 36 months in the field most nets had incurred damage: few were without holes (only 17 %) and most were dirty or very dirty (70 %). The Tanzanian nets were in worse condition than the Interceptor LNs studied in Uganda where after 36 months, 27 % were without holes and 29 % were scored as dirty or very dirty [13]. The bio efficacies were similar between the Tanzanian and Ugandan studies, and therefore the accumulation of dirt or soot on the nets may not affect the toxicity of the pyrethroid, as noted by Kayedi et al. [21]. Nevertheless, the trials do highlight the issue of durability and the importance of high denier netting to achieve that durability (the Interceptor LN issued were only 75 denier). The majority of holes were size 1 and most were found on the lower half of the nets where abrasion caused by tucking under the mattress was more likely to occur. While the attrition of nets (loss of nets from households) was not monitored, the trend in hole index over time indicates that it stabilized after 24 months. Nets were probably being discarded once they had become highly holed, so the residual population of nets maintained a more regular pHI after 24 months. A ‘steady state’ hole index after 24 months’ use has been observed during sequential household surveys in the Phase III evaluations of PermaNet 2.0 and Olyset LN [22–24]. A threshold hole index or hole area which a given proportion of LN are expected to reach after 3 years are important criteria for WHOPES to establish and encourage manufacturers to improve the durability and longevity of their products.

Although some adverse effects were reported during the first weeks of net usage, these were rare and short lived and did not deter Interceptor LN use.

The Tanzanian trial was one of three trials commissioned by the WHO Pesticide Evaluation Scheme [20], while a fourth non-WHOPES trial was conducted independently in Uganda [13]. After 36 months, the percentage of nets that met the WHO efficacy criteria was 98 % at one site in India (Gujarat), 73 % at a second site in India (Chhattisgarh) and 83 % in Uganda. The data from Gujarat had to be discounted as the majority of Interceptor LN exceeded the tolerance limit of alpha-cypermethrin content at baseline and the study in Chhattisgarh failed after 36 months as only 73 % of the nets passed the threshold bioassay criteria [25]. In the two trial locations where the Interceptor LNs were within the acceptable range for alpha-cypermethrin content – Tanzania and Uganda – the LNs did meet the WHOPES efficacy criteria after 3 years of use [20].

Conclusion

In this WHOPES Phase III household randomized trial conducted in Tanzania, Interceptor LN succeeded in meeting the WHOPES efficacy criteria for long-lasting insecticidal nets after 36 months of use. Based on this trial and one other non WHOPES trial where the LNs were within the acceptable range of alpha-cypermethrin content at baseline, Interceptor LN obtained WHO full recommendation. The calibration of Interceptor LNs at 36 months and Interceptor LNs with similar levels of alpha-cypermethrin content tested in Phase II experimental hut studies predicts that such nets would continue to give high levels of personal protection and mosquito control after 3 years of household use provided net integrity is maintained.

Recommendation from the WHO Pesticide Evaluation Scheme (WHOPES) that a LLIN brand is suitable for malaria prevention has been the criteria used in the purchase of LLIN. These as discussed in this and next (Chapter 3), recommendations are based on the fulfilment of WHO efficacy criteria in laboratory and field trials, necessitate that LLINs retain effective insecticidal activity for at least 20 laboratory washes and after 3 years of useful life. WHOPES phase III community trials are proper for the assessment of the survivorship and fabric integrity of LLINs in various environments and cultural settings, it is not valid to rely on the bioassays to conclude

what will happen in the real life. While there is standard method of quantifying the number of holes in a bed net, lack of a standardized method to define a threshold of functional bed net in terms of physical integrity in the field based on the decay in LLIN`s personal protection is a significant limitation of present guidelines in studies evaluating the effectiveness of bed nets. Threshold net integrity criteria that a LN should reach in order to obtain recommendation should be established by WHOPES to improve LN durability The proper way to study the real effect of washing and aging on the personal protection of LLIN's are the controlled field studies such as the hut studies.The next chapter (Chapter 3) is about the study that evaluated the biological performance against wild free flying mosquitoes in experimental huts of the nets collected from field after 1,2 and 3 years of field household usage with the purpose ascertain and for recommendation for the adaptation of LLIN integrity criteria in the phase III LLIN evaluation guidelines.

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Chapter 3: **Comparative efficacy of alpha-cypermethrin long-lasting insecticidal nets made of pyrethroid-incorporated polyethylene or pyrethroid-coated polyester: experimental hut trials of DuraNet® LN and Interceptor® LN.**

Prologue:

Long-lasting nets (LLINs) are factory-treated, and their insecticides are either incorporated into the polymer or coated on to the polymer surface. In both cases, the amount of insecticide can be divided into a smaller fraction available on the yarn surface and a larger proportion as a reservoir inside the yarn or in the resin coating.

Although a number of studies have reviewed the similarities and differences between the two types ('incorporation' and 'coated') of polymer technology, most of this involved comparison of bio-efficacy and textile durability between Olyset® LN and PermaNet® 2.0 LN. However, Olyset net and PermaNet contain different active ingredients (i.e., permethrin and deltamethrin respectively). Thus, the reported differences in bio-efficacy can be attributed to differences in the performances of the active ingredient rather than differences in polymer or production technology. Comparison of the differences in efficacy between the two LLIN technologies are better explored in studies where the two technologies contain the same active ingredient, i.e., no confounding by intrinsic differences in AI. Findings from this comparison are expected to present important evidence to international donors and NIMCPs when it comes to effective procurement decision-making between polymers.

This chapter compares the efficacy of DuraNet® LN (alpha-cypermethrin incorporated) to the efficacy of Interceptor® LN (alpha-cypermethrin coated) reported in previous chapters in trials which were done in the same huts at similar time.

Chapter 3 Comparative efficacy of alpha-cypermethrin long-lasting insecticidal nets made of pyrethroid-incorporated polyethylene or pyrethroid-coated polyester: experimental hut trials of DuraNet® LN and Interceptor® LN.

Abstract

Background:

Long-lasting insecticidal nets (LLINs) are produced in two formats: with the pyrethroid 'incorporated' into fibers (polyethylene) during extrusion of the filaments in manufacture, and with the pyrethroid 'coated' in a polymer resin on the surface of the fibres (polyester) post-manufacture. LLIN are mainly produced from the two polymers, polyethylene and polyester. New types of LLIN need to meet quality and efficacy standards set by the World Health Organization. Both types of polymers are WHO-recommended but insecticide and textile manufacturers tend to specialize on making one or the other formats of netting. The present study comparatively assesses efficacy and wash-resistance of DuraNet® which is a LLIN with alpha-cypermethrin incorporated into the polyethylene fibers, and Interceptor® which is a LLIN with alpha-cypermethrin coated on to the surface of polyester fibers within a polymer resin.

Methods:

The bio-efficacy and retention of pyrethroid in DuraNet LN and Interceptor LN were evaluated using WHO approved methodology, namely experimental hut trials (EHT), against pyrethroid-susceptible *Anopheles gambiae* and *An. funestus* in consecutive years. In the two EHTs, mosquito mortality, blood feeding inhibition and personal protection was compared between zero washed (0W) and 20-times washed (20W) LLIN of both polymer types.

Results:

The biological and behavioral effects of the LLINs in EHT showed similar trends against *An. funestus* and *An. gambiae* across each of the treatment technologies. Percentage mortality of *An. gambiae* after zero and 20 washes were similar in incorporation (96% after 0W, 83% after

20W) and coated nets (92%, 76%) respectively. Personal protection after zero and 20 washes was similar in incorporation (71%, 79%) and coated nets (79%, 76%). Blood-feeding inhibition after zero and 20 washes was similar in incorporation (63%, 73%) and coated nets (74%, 66%). None of these differences were statistically different between LLIN types.

Conclusions:

These studies confirm that polyethylene (DuraNet LN) and polyester (Interceptor LN) polymers both meet the bio-efficacy criteria required by WHO. The similarities in outcomes observed between alpha-cypermethrin incorporation and coating LLIN indicate that the type of polymer used for netting is less pertinent to efficacy as compared to the type of pyrethroid and dosage applied, provided the manufacturing and testing quality meets high WHO standards.

Keywords: Long-lasting insecticidal net, DuraNet LN, Interceptor LN, alpha-cypermethrin, *Anopheles gambiae*, *Anopheles funestus,* experimental hut trial, Tanzania.

Background

Long-lasting insecticidal nets (LLINs) are an important tool for vector control against malaria and other mosquito-borne diseases (WHO, 2014b). There has been a growing demand for LLINs within the frame of national malaria control programmes with a target of at least one LLIN for every two household members, a ratio believed to be sufficient to achieve universal coverage in a population (WHO, 2014a). The demand for LLINs has attracted interest of several pesticide companies into producing new LLINs.

It is pre-requisite for any new LLIN to pass through a series of evaluation stages prior to gaining interim or full recommendation by the WHO to be used in the community. The interim recommendation is given to a LLIN product after it has successfully passed phase I and II WHO evaluations while the full recommendation is given to a LLIN product after it has passed phase III evaluation (WHO, 2013a).

LLINs are classified into two categories depending on the technology involved in their treatment and production (WHO, 2003). The first is called 'incorporation' technology, in which pyrethroid

insecticide is incorporated into the netting polymer during production and gradually migrates to the surface of the fiber. The second is based on a 'coating' of resin in which the pyrethroid is bound to the surface of the multifilament polyester netting. With this second technology, insecticide is regenerated from the resin matrix after the surface-bound insecticide is washed off. This is called 'coating' technology.

An example of incorporation type of LLIN widely used is Olyset Net® (Sumitomo Co, Osaka, Japan) which is based on polyethylene incorporation technology with permethrin as an active ingredient. While a review of Olyset nets conducted by WHOPES in 2009 on the physical status of Olyset nets in different countries reported it to be in good condition up for up to 5 years of field usage (WHO, 2009), other studies has reported it to be insecticidal and intact for up to seven years of fields usage (Malima et al., 2008; Tami et al., 2004). Based on the available evidence Olyset Net® was recommended as a long-lasting insecticidal net for malaria prevention by the WHO in 2001 (WHO, 2001).

PermaNet® 2.0 is a widely used LLIN with deltamethrin insecticide coated around the fibre within a resin formulation. Several studies showed high efficacy of PermaNet 2.0, based on laboratory wash resistance and experimental hut trials (Gimnig et al., 2005; Graham et al., 2005; Lindblade et al., 2005). PermaNet 2.0 received a preliminary WHOPES recommendation in 2004 (WHO, 2004). Five years later, based on results from several field trials (Graham et al., 2005; Kilian et al., 2011; Lindblade et al., 2005) and WHOPES review trials, full recommendation was granted to PermaNet 2.0® (WHO, 2008b).

Although a number of studies have reviewed the similarities and differences between the two types of treatment technologies ('incorporation' and 'coated'), most of these studies involved comparison of bio-efficacy and material durability between Olyset® and PermaNet®. While most consistently showed that Olyset® Net was more durable and less bio-effective compared to PermaNet® 2.0 (Kayedi et al., 2007; Mejia et al., 2013; Allan et al., 2012; Tsuzuki et al., 2011), the two LLIN contain different active ingredients (i.e., permethrin and deltamethrin respectively). Thus, the reported differences in bio-efficacy could be due to differences in the performances of the active ingredients, or to differences in production technology than to the polymer used to

make the net. Comparison of the differences in efficacy between the two LLIN technologies or the two treatment technologies are better explored in studies where the two contain the same active ingredient and concentrations.

DuraNet® long-lasting net, formerly manufactured by Clarke Mosquito Control (USA), as an alpha-cypermethrin, incorporated into polyethylene filaments LLIN (WHO, 2008a) has been evaluated in several countries (Atkinson et al., 2009; Denham et al.; Gunasekaran et al., 2014). Based on results from several laboratory and field evaluation trials, DuraNet LN received WHO full approval in 2013 (WHO, 2013b).

Interceptor® LN, on the other hand, is a long-lasting insecticidal mosquito net made with alpha-cypermethrin active ingredient coated in a resin matrix on to polyester fabric, manufactured by BASF (Germany). Laboratory and field evaluation trials have been carried out in several countries (Banek et al.; Bhatt et al.; Dev et al.; Kilian et al., 2011; Malima et al., 2013). Based on results from these and WHOPES supervised phase III trials, full approval was granted to Interceptor by WHOPES in 2012 (WHO, 2012).

The present paper presents the results of the experimental hut and laboratory evaluations of DuraNet® LN compared to Interceptor LN in Muheza, northeastern Tanzania. Both trials were undertaken as part of WHO-commissioned phase II evaluations in verandah trap experimental huts of the National Institute for Medical Research (NIMR) against wild free-flying *An. gambiae* and *An. funestus* mosquitoes. The studies compare the efficacy of DuraNet® LN (alpha-cypermethrin incorporated polyethylene) to the efficacy of Interceptor LN (alpha-cypermethrin coated polyester) in trials which were done in the same huts (Malima et al., 2013; WHO, 2007) at almost same time as the DuraNet® LN (WHO, 2007b). Both studies contributed to WHO interim recommendations for the bio-efficacy of these brands of LLIN. The outcomes of the two studies are relevant to all net studies that follow, as they compare efficacy of two brands of LLIN which are made of different polymers (polyethylene and polyester), different treatment technologies (incorporation and coating) while at the same time both contain the same insecticide alpha-cypermethrin at similar concentration, so the studies are not confounded by different active

ingredients or dosage. Polyethylene and polyester continue to be the predominant polymers in long lasting nets.

Methods

Study area and experimental huts

The experimental hut trials were conducted at the NIMR field station at Zeneti village “5° 13' S latitude, 38° 39' E longitude" and 193 m altitude; where *An. gambiae* s.s. and *An. funestus* are the major malaria vectors (Mboera and Magesa, 2001). Insecticide susceptibility tests carried out by NIMR in this area at the time of the two (DuraNet and Interceptor) hut trials, the vector populations were 95-100% susceptible to alpha-cyano pyrethroids (Kabula et al.).

The huts were constructed to a design described by WHO (WHO, 2005), based on the original verandah-hut design developed in Tanzania (Smith, 1965; Smith and Webley, 1968) with minor modification. Modification included a reduced eave gap of 2 cm, a wooden ceiling, a roof of corrugated iron, and a concrete floor surrounded by a water-filled moat. The huts had open eaves with verandah traps and window traps on each side. The working principle of these huts has been described previously (Malima et al., 2008).

Technical specifications of the nets, net preparation and washing

Technical specifications of the nets

DuraNet net is manufactured by Clarke Mosquito Control (USA) as an alpha-cypermethrin (incorporated into filaments) LN. Alpha-cypermethrin is incorporated into 150-denier, monofilament, high-density polyethylene fibers, with the target dose of 5.8 g/kg AI, corresponding to 261 mg of alpha-cypermethrin per LN m^2 (WHO, 2008a).

The Interceptor™ LN net is manufactured by BASF (Germany) as an alpha-cypermethrin long-lasting (coated) mosquito net (LN) with the target treatment dose of 200 mg per m2 of the polyester fibres (WHO, 2007). The specifications of Interceptor nets used in the trial were multifilament with 75 denier and mesh size of 24 holes/cm^2. The nets measured 180 cm in length, 160 cm width and 150 cm height.

Nets used for conventional treatment (CTN) were polyester multifilament, 75 denier, mesh 156 holes/inch^2 and dimensions 180cm long, 150cm high, 130cm wide, manufactured by SiamDutch Mosquito Netting Co., Bangkok, Thailand. Treatment with alpha-cypermethrin was done using aqueous solution of Fendona® 10% SC at a target dosage of 40mg ai/m^2. Impregnation of nets used the method described by Pleass (Pleass et al., 1993).

To simulate wear and tear six 4 cm x 4 cm holes were cut into each net (two holes on each side and one hole at each end). The long-lasting insecticidal nets and conventional alpha-cypermethrin treated nets (CTN) were washed according to WHO Phase II protocols. Washing of the nets followed the WHO standardized procedure described in appendix 1, section 2.4.

The CTN washed to the 'point of insecticide exhaustion' served as a positive control in the EHT. The determination of point of exhaustion is described in detail in appendix 1, Section 2.5.

The bioassay procedure was adopted for an Interceptor and Duranet LN to determine the number of washes to less than 80% mortality or 95% knock-down in WHO cone bioassays.

Exploratory bioassay tests on DuraNet LLIN and CTN washed up to 20 times.

WHO cone bioassays were also carried out using *Anopheles gambiae s.s* Kisumu on five positions on DuraNet LN after 0, 5, 10 ,15 and 20 washes.

Experimental hut study design

The DuraNet trial entailed four treatment arms: (i) Unwashed DuraNet LN (0 W), (ii) DuraNet LN net washed 20 times (20 W), (iii) Polyester net conventionally treated with alpha-cypermethrin at SC10% at 40 mg/m² and washed six times (CTN 6 W), (iv) Polyester net conventionally treated with alpha-cypermethrin at SC10% at 40 mg/m² and washed 20 times (CTN 20 W), (v) Untreated unwashed polyester net (0 W)**.** The Interceptor EHT trial followed a similar design.

For the purpose of comparison between LLINs of incorporation and coated types, results of four treatment arms from the Interceptor LN huts trial were included and compared to those of the DuraNet LN. The Interceptor arms included are: (i) Unwashed Interceptor LN (0 W), (ii) Interceptor LN net washed 20 times (20 W), (iii) Polyester net conventionally treated with alpha-

cypermethrin at SC10% at 40 mg/m² and washed 20 times (CTN 20 W), (iv) Untreated unwashed polyester net (0 W).

The primary outcomes were deterrence, treatment-induced exiting (exophily), mortality, overall killing effect, blood-feeding inhibition and % personal protection.

These outcomes are described in more detail in appendix 1, section 2.6.2.

DuraNet LN trial took place for 36 nights between May and July 2007 while Interceptor LN trial was undertaken for 66 days between May and August in 2006. Treatment arms were rotated twice through each hut according to a Latin Square design. A treatment was assigned at random to a particular hut for 6 nights' observation before being transferred to the next hut. Between 19:30 and 6:30 hours male volunteers slept on beds under the nets. The sleepers were rotated through the huts on consecutive nights. Six nets were available per treatment arm and each net was tested on consecutive nights during the six-night rotation. At the end of the weekly rotation the huts were cleaned and aired for one day before starting the next rotation. Each morning dead and live mosquitoes were collected from the verandahs, room and window traps. Live mosquitoes were provided with 10% sugar solution. Delayed mortality was recorded after 24h. Mosquitoes were identified to species and gonotrophic status as unfed, blood-fed, semi-gravid or gravid. Samples of *An. gambiae s.l.* were identified to species by PCR (Scott et al., 1993). Species identification recorded 100% *An. gambiae s.s.* from Zeneti, Muheza (N=60). Based on these results all specimens collected in the hut trials were recorded as *An. gambiae s.s.*

The criteria for new LLIN set by WHO is that the LLIN washed 20 times should perform equal to or better than the CTN washed until just before exhaustion. Twenty washes are set by WHO as an approximate number of washes a LLIN is likely to incur during its lifetime.

Assessment of toxicity of nets used in the experimental hut trial.

WHO cone bioassays were performed on a randomly selected net from each of the six treatment arms of the DuraNet trial using laboratory reared *Anopheles gambiae* Kisumu at three intervals: before any washing, after completion of the washing cycles, and after completion of the hut trial. Four pieces measuring 30 cm x 30 cm were cut along a diagonal transect on the 4 side panels and

a further piece was cut from the top panel. Two replicate bioassay tests were carried out on each side panels and on the top panel using 5 mosquitoes per replicate.

Chemical analysis

Netting samples were taken for insecticide residue analysis by HPLC on three occasions: before washing, after completion of the washes, and after conclusion of the trial. 75 samples 15 from the six treatment arms of DuraNet ^{LN} and Interceptor LN trials cut from 4 side panels and 1 top panel as described by WHO (WHO, 2005): 25 samples pre-washing, 25 samples post-washing, and 25 samples post hut trial. From each net sample (3 pieces measuring 10 cm x 5 cm), a piece measuring 5cm x 5cm was cut with scissors. HPLC analyses were carried out on each piece, the average amount of pyrethroid estimated and the dosage per m^2 calculated.

Ethics, consent and permission

Ethical clearance was obtained from the ethics committees of the NIMR Tanzania (Ref: NIMR/HQ/R.8a/Vol X/86) and London School of Hygiene and Tropical Medicine (LSHTM). Written informed consent was obtained from all volunteers participating in the study and each was provided with chemoprophylaxis (Malarone) and monitored daily for fever or possible adverse events due to insecticide exposure from the nets.

Statistical analysis

The principal aim was to compare the efficacy of DuraNet LN and Interceptor LN washed 0 and 20 times to a conventional treated net washed to just before cut-off point. The key outcomes were the overall proportions of mosquitoes' blood-feeding or dying relative to the untreated control. Logistic regression was used to estimate proportional outcomes of treatments (mortality, blood-feeding, exiting), and negative binomial regression was used to analysis counts of mosquitoes entering the huts and blood-feeding (personal protection), after adjusting for clustering by day and for variation between individual sleepers and hut position.

Results

Determination of regeneration time of the CTN:

In the Duranet trial, tests on CTN, mortality decreased below 80% after 7 washes. Cut-off point of the alpha-cypermethrin CTN was therefore set at 6 washes. In the Interceptor trial it occurred after 4 washes, meaning the alpha-cypermethrin CTN washed 20 times was set as cut-off point wash interval.

Supporting bioassay tests on DuraNet LLINs and CTNs used in the trial before and after washing.

A random net in each treatment arm was cone bio-assayed before and after completion of washes. Before washing knockdown and mortality was 100% on all treatments (Table 1). After washing 20 times knock-down fell to 5% and mortality to 6% in the CTN. Knock-down and mortality in the DuraNet LLIN washed 20 times was 86% and 90% respectively.

Table 1 Knockdown and mortality of *Anopheles gambiae* Kisumu exposed in three-min cone bioassays on hut nets before and after washing.

Treatment Arm	Number tested	Before washing		After washing	
		% Knockdown	% Mortality	% Knockdown	% Mortality
Untreated Net	50	0	0	4	2
Unwashed DuraNet® LN	50	100	100	100	100
DuraNet® LN washed 20X	50	100	100	86	90
CTN washed 6X	50	100	100	30	20
CTN washed 20X	50	100	100	5	6

Mosquito entry and exiting from experimental huts.

During the DuraNet trial *An. funestus* was 3 times more abundant than *An. gambiae* (Table 2). During the Interceptor trial *An. gambiae* was the only species available in numbers sufficient for statistical analysis. Therefore, only *An. gambiae* results from DuraNet (incorporated type) and Interceptor (coated type) were used in comparative analysis of efficacy of incorporated and coated LLIN types.

There was no evidence of significant deterrence of *An. gambiae* with DuraNet arms at 0 and 20 washes compared to the untreated control (Table 2). The deterrence recorded with the unwashed DuraNet was similar to that recorded by the unwashed Interceptor treatments (p=0.125). There was also similarity in deterrence between Interceptor and DuraNet arms with the LLIN washed 20-times treatment arm (p=0.498) relative to untreated control.

Table 2 Number of mosquitoes entering and deterrence of wild *An. gambiae* and *An. funestus* huts during DuraNet (Incorporated) and Interceptor (Coated) trials.

		Untreated net	LN	LN
Number of washes		0	0	20
Anopheles gambiae				
Incorporation LN	**Total females caught**	**143**	**112**	**112**
	Geometric mean per night	2.8	2	2
	Deterrence (%)	$0^{a,1}$	$21.7^{a,1}$	$21.7^{a,1}$
Coating LN	**Total females caught**	**171**	**134**	**122**
	Geometric mean per night	2.6	2	1.8
	Deterrence (%)	$0^{a,1}$	$20.4^{b,1}$	$21.1^{b,1}$
Anopheles funestus				
Incorporation LN	**Total females caught**	**529**	**587**	**490**
	Geometric mean per night	11.9	14.1	11.8
	Deterrence (%)	$0^{a,1}$	$0^{a,1}$	$7.4^{a,1}$

Note:

1. Percentage deterrence, exiting and 95% CIs are back transformed from values calculated by the blocked logistic regression model.
2. Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression (P<0.05).
3. Within each *An. gambiae* row, column not sharing a superscript number differ significantly by blocked logistic regression (P<0.05

Percentage exophily from each treatment arm in the East African huts were 80% or higher (Table 3). Natural exophily was very high. The exiting rates recorded by unwashed Interceptor and DuraNet LLINs were statistically not statistically different (p=0.068). There was no evidence for any increase in alpha-cypermethrin induced exiting at 0 washes or 20 washes for either LLIN product when mortality was so high. *An. funestus* results showed similar trends to that of *An. gambiae* with each treatment.

Table 3 Experimental huts results: Number exiting and % exiting of wild *An. gambiae* and *An. funestus* huts during DuraNet (Incorporated) and *An. funestus* huts during Interceptor (Coated) trials.

		Untreated net	LN	LN	CTN	CTN
Number of washes		0	Unwashed	20	6 (cut off)	20
Anopheles gambiae						
Incorporation	% Exiting	$88.1^{abc,1}$	$79.5^{b,1}$	$82.1^{bc,1}$	89.5^{ac}	$91.0^{a,1}$
Coating	% Exiting	$86^{a,1}$	$86.6^{a,1}$	$92.6^{a,2}$	-	$92.8^{a,1}$
Anopheles funestus						
Incorporation	% Exiting	$85.4^{a,1}$	$86.9^{a,1}$	$84.7^{a,1}$	94.9^{b}	$85.3^{a,1}$

Note:

1. Percentage deterrence, exiting and 95% CIs are backtransformed from values calculated by the blocked logistic regression model.
2. Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression ($P<0.05$).
3. Within each An. gambiae row, column not sharing a superscript number differ significantly by blocked logistic regression ($P<0.05$).

Mortality and overall killing effect.

The unwashed DuraNet LLIN and Interceptor induced 96.2% and 92.5% mortality of *An. gambiae* respectively that entered the hut. This fell to 83.1% and 77.9% mortality after 20 washes (Figure 1). Mortality induced by the 0 washed and 20 times washed Interceptor (polyester) against *An. gambiae* (91.9% and 76.2% when corrected for control, respectively) was similar to mortality induced by the 0 washed and 20 times washed DuraNet (polyethylene) (91.9% and 76.2% when corrected for control, respectively) (Table 4, Figure 1). The mortality induced by the 20 times washed CTN used in DuraNet trial was similar statistically ($p=0.636$) to 20 times washed CTN used

in the Interceptor (coated) trial (with corrected mortality of 42.7% and 42.1% respectively) (figure 1, Table 4. Overall killing effect of the unwashed DuraNet LLIN was similar statistically (p=0.098) to that of unwashed Interceptor LLIN (Table 4) while the killing effects recorded by the CTNs washed 20 times used in the DuraNet and Interceptor trials were also similar (p=0.603) statistically (Table 4).

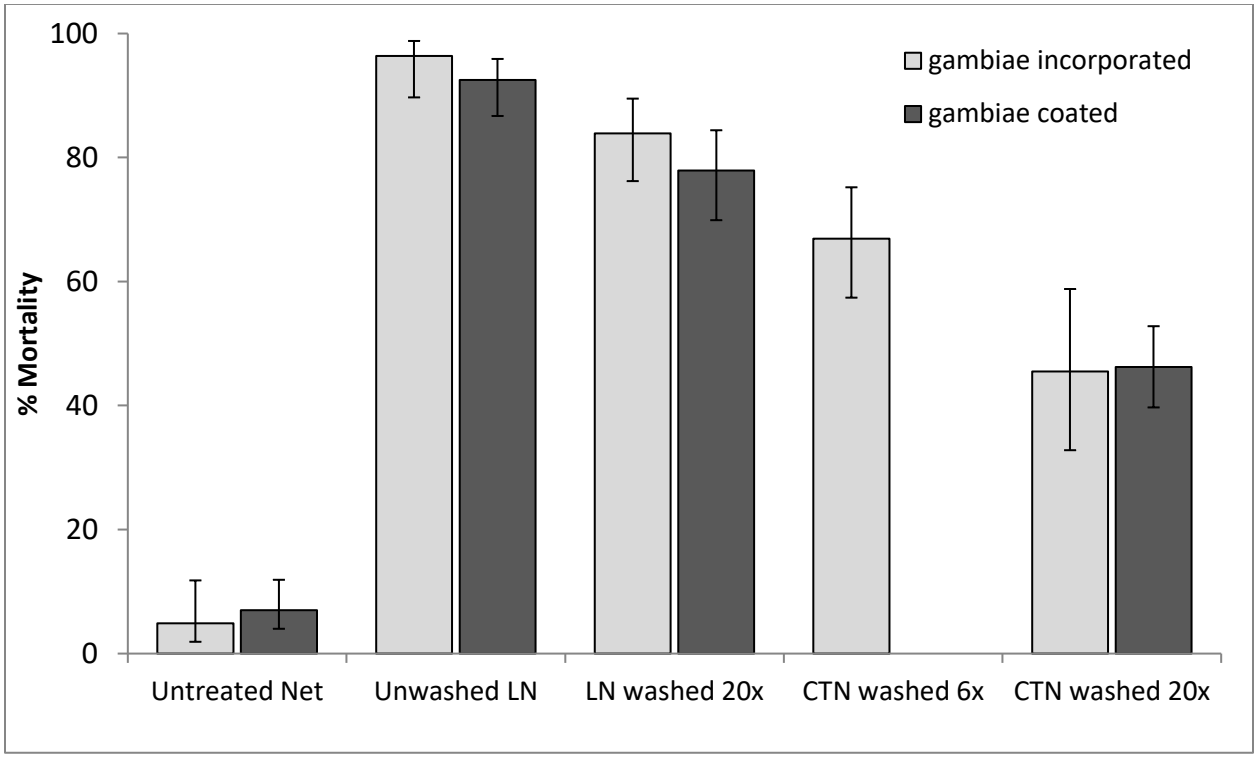

Figure 1. Mean mortality rates of *An. gambiae* in experimental huts with DuraNet (incorporated) trial and Interceptor (coated) trial at different wash intervals versus untreated control.

Table 4 Experimental huts results: %mortality corrected for control, killing effect of wild *An. gambiae* and *An. funestus* huts during DuraNet (Incorporated) and Interceptor (Coated) trials.

	Untreated net	**LN**	**LN**	**CTN**	**CTN**
Number of washes	0	0	20	6 (cut off)	20
Anopheles gambiae					

Incorporation	% Mortality corrected for control	0[a]	96.2[b,1]	83.1[c,1]	65.2[e]	42.7 [d,1]
Coating	% Mortality corrected for control	0[a]	91.9[b,1]	76.2[c,1]	-	42.1[d,1]
Incorporation	% Overall Killing Effect	0[a]	69.2[b,1]	60.8[b,1]	53.1[bc]	37.8[c,1]
Coating	% Overall Killing Effect	0[a]	70.4[b,1]	52.2[c,1]	-	57.2[c,1]
Anopheles funestus						
Incorporation	% Mortality corrected for control	0[a]	92.8[b,1]	81.3[c,1]	68.5[d]	39.7 [e,1]
	% Overall Killing Effect	0[a]	98.3[b,1]	71.1[c,1]	67.1[cd]	47.8[d,1]

Notes:

1. **Percentage deterrence, exiting and 95% CIs are backtransformed from values calculated by the blocked logistic regression model.**
2. **Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression (P<0.05).**
3. **Within each An. gambiae row, column not sharing a superscript number differ significantly by blocked logistic regression (P<0.05).**

Blood feeding inhibition (BFI) and personal protection.

The percentage reduction in blood feeding recorded with the 0 washed DuraNet and Interceptor (63.5% and 73.8% respectively) were similar statistically (p=0.318) (Figure 2 & Table 5). Compared to untreated nets the percentage change in feeding recorded with DuraNet and Interceptor after 20 washes were also similar (72.4%, 65.8%)

The personal protection recorded by Duranet and Interceptor before (71.4%, 79.5%) and after (78.6%, 75.6%) 20 washes were also similar statistically (p=0.494 and p=0.804 respectively) (Table 5). Similar levels of personal protection (p=0.342) were recorded with the CTN washed 20 times which were compared in the two (DuraNet and Interceptor) trials as positive controls to the two types of LLIN (Table 5).

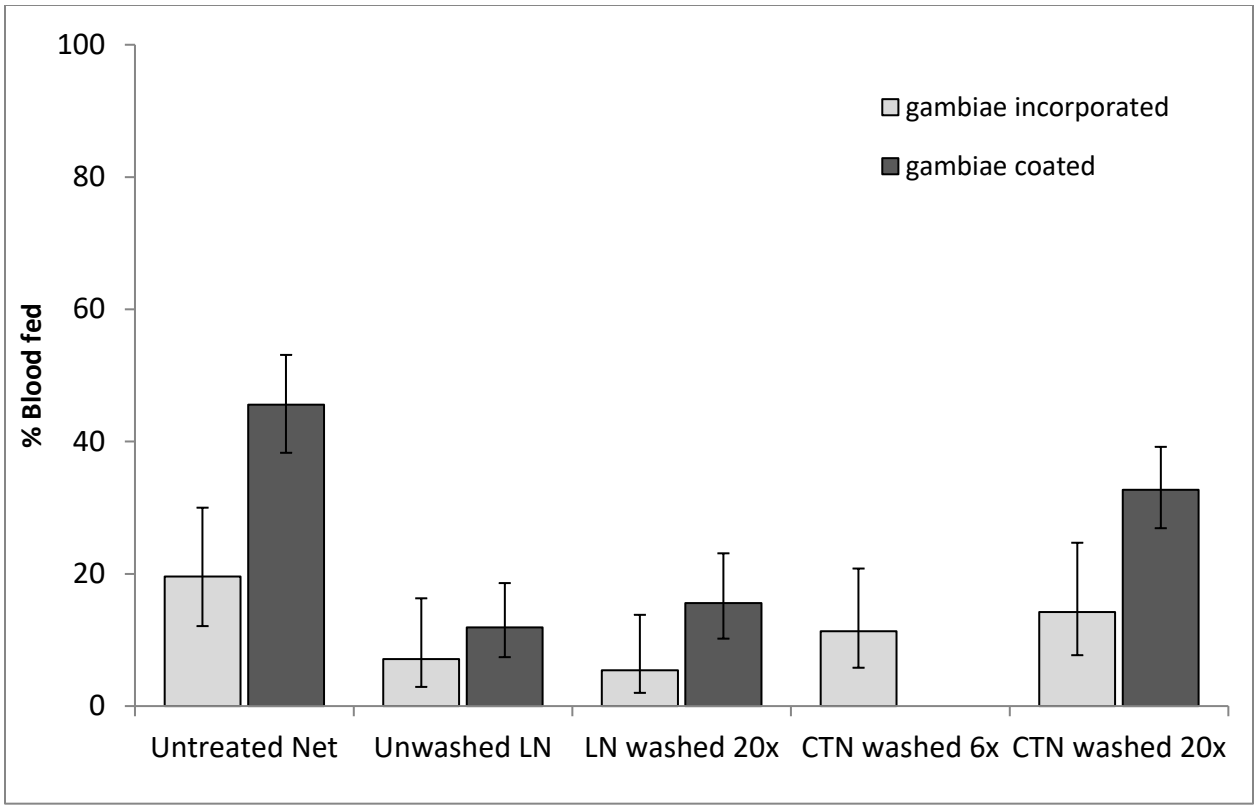

Figure 2. Percentage blood feeding success in *An. gambiae* in experimental huts with DuraNet (incorporated) and Interceptor (coated) arms at different wash intervals versus untreated control.

Table 5 Experimental huts results: % Blood feeding inhibition and personal protection of wild *An. gambiae* and *An. funestus* huts during DuraNet (Incorporated) and Interceptor (Coated) trials.

		Untreated net	LN	LN	CTN	CTN
Number of washes		0	0	20	6	20
Anopheles gambiae						
Incorporation	% Blood feeding inhibition	0^{a}	$63.5^{bc,1}$	$72.6^{c,1}$	42.3^{bc}	$27.5^{b,1}$
Coating	% Blood feeding inhibition	0^{a}	$73.8^{b,1}$	$65.8^{b,1}$	-	$28.3^{c,1}$
Incorporation	% Personal Protection	0^{a}	$71.4^{bc,1}$	$78.6^{c,1}$	50^{abc}	$32.1^{ab,1}$
Coating	% Personal Protection	0^{a}	$79.5^{b,1}$	$75.6^{b,1}$	-	$6.4^{a,1}$

Anopheles funestus						
Incorporation	% Blood feeding inhibition	0^{a}	$73.9^{b,1}$	$62.1^{b,1}$	52.3^{b}	$0^{a,1}$
	% Personal Protection	0^{a}	$71.1^{b,1}$	$64.9^{b,1}$	50.9^{b}	$0^{a,1}$

Notes:

1. **Percentage deterrence, exiting and 95% CIs are backtransformed from values calculated by the blocked logistic regression model.**
2. **Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression (P<0.05).**
3. **Within each *An. gambiae* row, column not sharing a superscript number differ significantly by blocked logistic regression (P<0.05).**

Chemical analysis.

Chemical residue analysis showed an average content of 227 mg/m^2 (5.04 g/kg) for the unwashed DuraNet LN, which complies with the declared content 261mg/m^2 (5.8 g/kg) +/- 20% (Table 6). After 20 washes, the content fell to 153mg/m^2 (3.4 g/kg), corresponding to an overall retention value of 67.5%. On the same net, variation between samples was low (0.9 to 5.9% relative standard deviation, RSD). The observed dose for conventionally treated nets (44.8 mg/m2) was close to the target dose (40mg/m2). After two washes of the conventionally treated nets, no insecticide could be detected.

The average content results of Interceptor nets used in Zeneti Tanzania was 132 mg/m^2 which fell to 41 mg/m^2 after 20 washes, an AI retention of only 31% (Malima et al., 2013). The observed dose for conventionally treated nets (144 mg/m^2). After twenty washes of the conventionally treated nets, this fell to 1.2 mg/m^2 a retention of 0.8%.

In summary the chemical analysis results shows that the retention rate with the DuraNet LN (incorporated) was twice as much (67.5%) of that of the Interceptor (coated) (31%).

Table 6. Chemical analysis of alpha-cypermethrin, mg/m² (± 95% CI) estimated on nets before and after 20 washes.

Treatment	Concentration of alphacypermethrin (mg/m^2)

	Before washing	After 20 washes (% retention)
Interceptor LN	132	41 (31%)
DuraNet® LN	302	177 (58%)
CTN washed 20 times (Interceptor)	144	1.2 (0.8%)
CTN washed 20 times (DuraNet)	49	0 (0%)

Discussion

The studies compared the efficacy of alpha-cypermethrin incorporated polyethylene net (DuraNet® LN) to the efficacy of alpha-cypermethrin coated polyester net (Interceptor LN) in trials which were done in the same huts within a year of each other.–Previous studies comparing efficacy of LLIN of incorporation and coated type involving Olyset® (incorporation) and Permanet® 2.0 (coated) nets were confounded by differences in active ingredient (Gunasekaran et al., 2014; Hawley et al., 2003; Mosha et al., 2008) and to differences in treatment technology. Moreover, use of PermaNet 3.0 was doubly confounded by being part polyethylene (on the roof panel) and part polyester (on the side panels). This study`s comparative advantage over previous studies were the different treatments (incorporated versus coating) and polymers (polyethylene versus polyester) were carried out at the same site, within the same experimental huts (East African design), using the same study design (approved by WHO), using the same active ingredient (alpha-cypermethrin) at similar concentration, within months of each other.

This study`s comparative analysis revealed similarities in personal protection and mortality between incorporation and coated LLINs when unwashed and after being washed twenty times.

The similarity in efficacy between DuraNet and Interceptor has been reported also in India with a very different climate to E Africa (Gunasekaran et al., 2014).

High alpha-cypermethrin content after washing was reported in the chemical analysis results of DuraNet LN. By contrast, the level of alpha-cypermethrin in conventionally treated nets declined considerably with 20 washes.

To obtain WHO full approval both products, DuraNet and Interceptor, undertook WHO phase III field trials which evaluates wash fastness, insecticidal efficacy and net fabric integrity over 30-36 months of household use (WHO, 2013a). The WHO phase III efficacy criteria for LLIN are that after 3 years of routine household use, at least 80% of nets tested meet the threshold criteria for either the WHO cone bioassay test or the tunnel test (WHO, 2013a). The percentage of DuraNet meeting WHOPES criteria for either the cone or tunnel test through 36 months of use in Ghana and India were 82% and 100% respectively (WHO, 2013b), while for Interceptor the percentage nets meeting these criteria in trials conducted in three countries (Tanzania, Uganda and India) ranged between 83-97% (WHO, 2012); all well above the WHO threshold. In the two trials over the 3 years of use the average loss of alpha-cypermethrin content in DuraNet ranged from 47% to 68% (WHO, 2013b), for the Interceptor the average loss of alpha-cypermethrin in the two trials in India and Tanzania was 79% and 82% respectively. These results from phase III show that the nets of incorporation type (DuraNet) retain more insecticide in the fibre compared to the coated type (Interceptor). However, surface bioavailability of the insecticide in both types was adequate since the bio-efficacy of both after 36 months of field usage was comparable, that is, both incorporation and coating types met the WHOPES efficacy criteria required after 3 years of usage.

In phase III trials the loss of physical integrity was measured by the percentage of nets with holes and the proportionate hole index (pHI). In WHOPES supervised trials, the percentage of DuraNet LNs with at least one hole after 36 months of use was 74.0% and 86.3% in India and Ghana respectively, and the mean pHI after 36 months of use was 87 and 93 respectively. This is comparable between the two nets. Among the four sites, the percentage of Interceptor LNs with at least one hole after 36 months of use ranged between 77.4% and 93.3% whereas the mean pHI after 36 months of use ranged between 116 and 377.

Generally, the multicentre phase III trials confirmed bio-efficacy and wash fastness over the three-year lifetime, based on WHO LN guidelines and efficacy criteria, noting that full recommendation was granted to both DuraNet (WHO, 2013b) and Interceptor (WHO, 2012).

The results of the present Phase II study on wash resistance of these nets in laboratory conditions and Phase II experimental hut trial with no significant loss of efficacy of DuraNet between zero and 20 washes, and the similarity in efficacy between the DuraNet and Interceptor, corresponded well with their retention of biological activity in the community when using LLINs under field conditions as both of this product passed WHOPES phase III efficacy criteria for LLIN. Thus, the current WHOPES phase II evaluation study successfully predicted the outcome of the three-year Phase III household trial.

After these trials (and partly based on the variable results with CTN washed to cut-off), WHO changed the guidelines to comparing the test LLIN against a currently approved LLIN after zero and 20 washes rather than against CTN. As a marker the determination of the CTN washed to just before <80% mortality was too variable between testing sites and centers to be a useful reference point. It was more relevant to compare new candidate LLIN versus an established approved LLIN: the latter was less subject to error than the CTN method and helped to raise standards.

In conclusion, the similarity in bio-efficacy recorded in this and other studies between a good incorporation LLIN (DuraNet) and a good coating LLIN (Interceptor) both treated with same insecticide (alpha-cypermethrin), against local free flying *Anopheles gambiae s.l.,* after comparative analysis of the results of the trials, signify that the type of net is less relevant to efficacy at 0 and 20 washes if the insecticide is the same and the net quality is high.

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Part two: summary of key findings:

In conclusion, the following are the Part two key findings and recommendations.

On the basis of results described in appendix 2, which formed part of an official WHOPES evaluation, Interceptor LN received interim recommendation as an approved LN. Earlier in 2013 the WHOPES guidelines for testing of LN were revised to include as a positive control a WHOPES-recommended LN with similar specifications to the candidate LN in terms of insecticide, treatment technique, netting material, and washing frequency (0 and 20 times) [6]. The revised guidelines were issued after the current trial and, indeed, Interceptor now constitutes a LN appropriate to use as a positive control against new candidate LN. The Phase I laboratory bio- and chemical assays confirmed that the Interceptor LN insecticide binding process imparts strong wash-retention characteristics.

In WHOPES Phase III household randomized trial conducted in Tanzania described in chapter 2, Interceptor LN succeeded in meeting the WHOPES efficacy criteria for long-lasting insecticidal nets after 36 months of use. Based on this trial and one other non WHOPES trial where the LNs were within the acceptable range of alpha-cypermethrin content at baseline, Interceptor LN obtained WHO full recommendation. Cone mortality criteria making by far the larger contribution to the overall pass rate. Tunnel tests also made an important contribution, and it was noted that tunnel tests can also be an important validation check on the veracity of the interpretation in the rare circumstances where anomalous results are recorded in the cone bioassays.

At 36 months the average insecticide content was 42 mg alpha-cypermethrin per m2; this was remarkably similar to the 41 mg/m2 alpha-cypermethrin content observed in Interceptor LNs after 20 standardized washes in the Phase II experimental hut trials done in the same locality 3 years previously [10]. This similarity in chemical content between a Phase III household randomized trial and Phase II experimental hut trial indicates that the 20 standardized washes which LLINs undergo before testing in experimental huts is a fair approximation to the average loss of insecticide due to wear and tear, abrasion and washing that LLINs undergo during 3 years

of household use. The outcomes of Phase II experimental hut trials would appear to be a reasonable prediction of the outcome of Phase III trials conducted in the community. While this correlation is encouraging, more LN products need to be evaluated and compared in Phase II experimental hut and Phase III household trials before this conclusion can be fully verified or justified. It is nevertheless encouraging – even fortuitous - that the arbitrary 20 washes that LLIN are purposefully subjected to in WHOPES Phase II seem a good approximation to Phase III after 3 years.

The similarities in outcome observed between incorporation LLIN (DuraNet) and coating LLIN (Interceptor) indicate that the type of net is not relevant if the type of pyrethroid used is the same and the manufacturing quality meets high WHO standards.

Lastly, the ability of alpha-cypermethrin-treated nets to kill pyrethroid-resistant *Anopheles gambiae* is limited, thus this raises a need for a new generation of nets treated with a pyrethroid plus a synergy or an insecticide to which *Anopheles gambiae* shows no resistance for the purpose of restoring it protective efficacy. Part four of this thesis describes in detailed the context of the impact of insecticide resistance on the entomological indices.

Due to its proved LLINs effectiveness as discussed in the chapters in this part of the thesis, the World Health Assembly (WHA) set a target of 85% of those at risk of malaria should benefit from preventive interventions. Despite this achievement, not all households have enough nets to meet family needs: an estimated 71% of households have insufficient ITNs to protect all household members and one-third of households do not own even a single ITN [7].

Thus, there is a need for a long-lasting insecticide treatment kit which could convert untreated nets into ITNs that can withstand repeated washing without the need for re-treatment. The coming part of the thesis contains chapters about treatment kits and their potential role not only in addressing the coverage challenge but also in meeting the need of malaria control in emergency situations like refugee camps where the use of LLINs is not practical.

PART THREE

Research question: Can wider coverage, improved vector control and insecticide resistance management be achieved through the additional use of long-lasting net treatment kits; insecticide impregnation processes that enable nets and other impregnated materials to remain effective for longer?

Prologue:

Conventional insecticide treated nets became important for malaria control in West and East Africa, and South and South-East Asia, after large scale vector control trials in the 1990s. The initial trials were conducted in the Gambia, Kenya and Tanzania in Africa, and in Pakistan, India and Thailand in Asia using treated-by-hand using formulations which lasted less than a year. Epidemiological success led to the problem of scale-up of coverage, and the need for research into longer-lasting formulations and treatments with longer lasting treatment kits. In truth, they were addressing a similar question, and research into what became long-lasting insecticidal nets (LLIN) was done mostly in parallel with research into long-lasting treatment kits.

Statement of the problem

With epidemiological success of hand treated ITNs and the development of factory produced LLINs, LLINs were still facing several technical and logistical challenges that seem to compromise their effectiveness in the field. Major challenges facing LLIN intervention include the following:

a. Inadequate coverage of LLINs.

   For LLINs to be able to provide community wide protective effect, adequate supply and coverage is the key factor. It is recommended that coverage should be at least 80% for optimal community protection (mass effect) that is an important attribute of LLINs. However, as LLIN supply is a donor dependent and supplied at a particular interval (in most cases a 3–4-year intervals), studies have reported inadequate LLIN coverage between universal coverage campaigns hence most communities in endemic countries tend to cover this gap in coverage through purchasing nets that are untreated from the local retail sector.

b. The inapplicability of LLINs in humanitarian emergencies and complex political situations.

   LLINs are already promoted widely as a means of preventing malaria transmission and can reduce malaria morbidity and mortality considerably. However, during acute emergencies or epidemics nets may be difficult to obtain, and they may also be unsuitable for refugees sleeping under plastic shelters or tents in refugee camps.

c. Insecticide resistance. Insecticide resistance is also a threat to continued efficacy of the LLINs.

Justification

Establishment of the long-lasting efficacy and wash fastness of the long-lasting treatment kits on nets and more so on other fabrics and materials used for making mosquito nets and other materials like blankets, tents and curtains retention is of importance for improving vector control.

General objective

Purpose of this part of the thesis is to ascertain if improved vector control and insecticide resistance management be achieved through the use of long-lasting net treatment kits.

Specifically, this part sought:

IV. To evaluate wash fastness and long-lasting efficacy of long-lasting treatment kits in experimental huts.

V. To evaluate the long-lasting effectiveness of the long-lasting treatment kits in real life conditions over three years.

VI. To evaluate the efficacy of treatment kits on various types of polymer fabrics used in netting or curtain materials that could be harnessed in malaria control.

Studies to address objectives above are presented and discussed in three separate chapters as follows.

Chapter 4: Evaluation of ICON Maxx, a long-lasting treatment kit for mosquito nets: experimental hut trials against anopheline mosquitoes in Tanzania

Chapter 5: Effectiveness of a long-lasting insecticide treatment kit (ICON® Maxx) for polyester nets over three years of household use: a WHO phase III trial in Tanzania.

Chapter 6: Bio-efficacy and wash-fastness of a lambda-cyhalothrin long-lasting insecticide treatment kit (ICON® Maxx) against mosquitoes on various polymer materials

Chapter 4: Evaluation of ICON Maxx, a long-lasting treatment kit for mosquito nets: experimental hut trials against anopheline mosquitoes in Tanzania

Prologue:

It has been explained in previous Parts and Chapters of this thesis that criteria for use of public funds on the purchase of LLIN as practiced by all major funders is the recommendation from the WHO PQT that a LLIN brand is suitable for malaria prevention after it passed WHOPQT evaluation phases. The evaluation of specific products by the then WHOPES comprises three phases of testing. Phase I consists of laboratory testing of wash resistance and insecticide regeneration on the surface of the net. This is followed by small-scale field trials usually using experimental huts to test wash-resistance and efficacy in phase II. Finally large-scale field trials under "real life conditions" are done in phase III testing of long-lasting efficacy, community acceptance and safety observations [32]. If a product has fulfilled the testing criteria of phase I and II of resisting at least 20 WHO standard washes it usually receives an interim recommendation while full recommendation is given after it has been shown to remain effective for at least three years of field use during phase III.

Purpose of this chapter (chapter 4) and the following chapter (chapter 5) was to assess the suitability of Icon Maxx long-lasting treatment for malaria prevention in accordance with the 2013 WHOPES/WHOPQT guidelines criteria.

ICON Maxx is not a long-lasting net but a formulation in kit form to treat or re-treat nets. WHOPES guidelines were followed in these evaluations.

This chapter describes a small-scale experimental huts trial (WHOPES phase II) to evaluate wash-resistance and efficacy of the ICON Maxx long-lasting treatment kit.

Chapter 4: Evaluation of ICON Maxx, a long-lasting treatment kit for mosquito nets: experimental hut trials against anopheline mosquitoes in Tanzania

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The material presented in this chapter has been published as:

Tungu PK, Malima R, Mosha FW, Lyimo I, Maxwell C, Kaur H, Kisinza WN, Magesa SM, Kirby MJ, Rowland M: **Evaluation of ICON Maxx, a long-lasting treatment kit for mosquito nets: experimental hut trials against anopheline mosquitoes in Tanzania**. *Malar J* **14**, 225 (2015). https://doi.org/10.1186/s12936-015-0742-z

Cover sheet for each 'research paper' included in research thesis.

1. For a 'research paper' already published

1.1. Where was the work published? **Malaria Journal**

1.2. When was the work published? **February 2015**

1.2.1. If the work was published prior to registration for your research.

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Abstract

Background

Insecticide-treated nets are the primary method of preventing malaria. To remain effective, the pyrethroid insecticide must withstand multiple washes over the lifetime of the net. ICON® Maxx is a 'dip-it-yourself' kit for long-lasting treatment of polyester nets. The twin-sachet kit contains a slow-release capsule suspension of lambda-cyhalothrin plus binding agent. To determine whether ICON Maxx meets the standards required by the World Health Organization Pesticide Evaluation Scheme (WHOPES), the efficacy and wash fastness of ICON Maxx was evaluated against wild, free-flying anopheline mosquitoes.

Methods

ICON Maxx was subjected to bioassay evaluation and experimental hut trial against pyrethroid-susceptible *Anopheles gambiae, Anopheles arabiensis* and *Anopheles funestus*. Mosquito mortality, blood feeding inhibition and personal protection were compared between untreated nets, conventional lambda-cyhalothrin treated nets (CTN) washed either four times (cut-off threshold) or 20 times, and ICON Maxx-treated nets either unwashed or washed 20 times.

Results

In bioassays, ICON Maxx demonstrated superior wash resistance to the CTN. In the experimental hut trial, ICON Maxx killed 75 % of *An. funestus*, 71 % of *An. gambiae* and 47 % of *An. arabiensis* when unwashed and 58, 66 and 42 %, respectively, when 20 times washed. The CTN killed 52 % of *An. funestus*, 33 % of *An. gambiae* and 30 % of *An. arabiensis* when washed to the cut-off threshold of four washes and 40, 40 and 36 %, respectively, when 20 times washed. Percentage mortality with ICON Maxx 20 times washed was similar (*An. funestus*) or significantly higher (*An. gambiae, An. arabiensis*) than with CTN washed to the WHOPES cut-off threshold. Blood-feeding

inhibition with ICON Maxx 20 times washed was similar to the CTN washed to cut-off for all three species. Personal protection was significantly higher with ICON Maxx 20 times washed (66-79 %) than with CTN washed to cut-off (48-60 %).

Conclusions

Nets treated with ICON Maxx and washed 20 times met the approval criteria set by WHOPES for Phase II trials in terms of mortality and blood-feeding inhibition. This finding raises the prospect of conventional polyester nets and other materials being made long-lastingly insecticidal through simple dipping in community or home, and thus represents a major advance over conventional pyrethroid treatments.

Background

Insecticide-treated nets (ITNs) are the most effective and feasible means of preventing malaria in Africa south of the Sahara [1]. Because conventional ITNs need to be re-treated with pyrethroid insecticide at least once per year to maintain their efficacy, several manufacturers of nets have developed long-lasting insecticidal nets (LLINs) in which wash-resistant formulation of insecticide is coated or incorporated into the netting fibres during production [2]. With good LLIN technology, insecticidal efficacy can be maintained against anopheline mosquitoes for at least three years without need for further re-treatment [2]. The advent of LLINs provided a technical solution to the problem of low annual re-treatment rates of conventional ITNs after initial distribution and washing [3] and henceforth LLINs would become the most important tool for malaria prevention in Africa and Asia.

In 2005 the World Health Assembly (WHA) set a target of 85 % of those at risk of malaria should benefit from preventive interventions by the end of 2015 [4]. This led to increased demand for LLINs by national malaria control programmes (NMCPs) to meet the target of at least 85 % protected by 2015 and led to international donors opting for LLINs as their preferred choice of net [5, 6]. The proportion of the population with access to ITNs has increased markedly in sub-Saharan Africa over the ten years since the WHA set the agenda. Based on data from household surveys and reports on ITNs delivered by manufacturers and distributed by NMCPs, an estimated

49 % of the population at risk had access to an ITN in their household in 2013, compared to 3 % in 2004 [7]. Despite this achievement, not all households have enough nets to meet family needs: an estimated 71 % of households have insufficient ITNs to protect all household members and one-third of households do not own even a single ITN [7]. More needs to be done to reach all families with ITNs and supply enough ITNs for all household members [7].

While the main emphasis has been to treat LLINs during manufacture, the majority of ITNs available through the commercial retail sector are not LLINs and those which are in use from this source have either never been treated or were treated only on purchase [2, 8, 9]. Many households still use locally sourced nets which are not LLINs, and which require regular re-treatment with insecticide when insecticide becomes depleted after repeated washing. Thus, there is a need for a long-lasting insecticide treatment kit which could convert untreated nets into ITNs that can withstand repeated washing without the need for re-treatment. Such an insecticide kit could also be bundled with untreated nets on purchase and enable local producers that lack LLIN manufacturing technology to produce an ITN which could contribute usefully to malaria control and address local LLIN shortages [2, 10].

Two brands of long-lasting treatment kit have so far been developed: KO-Tab 1-2-3 developed by Bayer Environmental Sciences [10] and ICON Maxx, developed by Syngenta [11]. ICON Maxx is based on the slow-release capsule suspension (CS) formulation of lambda-cyhalothrin that has previously been evaluated by WHOPES and recommended for treatment of mosquito nets [12]. ICON Maxx is presented as a twin-sachet pack, containing lambda-cyhalothrin 10CS and binding agent, sufficient for the treatment of an individual polyester mosquito net. The target dose depends on the net size and can range from 50 mg AI/m2 for a large family-size net to 83 mg AI/m2 for a single-size net. A safety assessment of ICON Maxx concluded that no unacceptable exposures were found in the preparation, maintenance, and use of the nets [12].

To determine whether ICON Maxx treated nets meet the standards required by WHOPES, the efficacy and wash fastness of ICON Maxx was evaluated in laboratory and field conditions against wild, free-flying anopheline mosquitoes. This paper reports upon the Phase II experimental hut

evaluations undertaken in Tanzania by the National Institute for Medical Research (NIMR) in Muheza against *Anopheles gambiae* and *Anopheles funestus* and by the Kilimanjaro Christian Medical College (KCMUCo) in Moshi against *Anopheles arabiensis*. Together, these trials contributed to the WHOPES recommendation for use of ICON Maxx as a long-lasting, wash-resistant treatment for polyester mosquito nets.

Methods

Study areas and experimental huts

The study made use of experimental hut sites in two districts of Tanzania: Muheza in Tanga region and Moshi in Kilimanjaro region. The Muheza trial was conducted at the NIMR field station at Zeneti village 5° 13' S latitude, 38° 39' E longitude and 193 m altitude, where *An. gambiae s.s.* and *An. funestus* are the major malaria vectors [13]. Insecticide susceptibility tests carried out by NIMR showed that the vector populations were 95-100 % susceptible to alphacyano pyrethroids [14]. The Moshi trial was conducted at the field site of KCMUCo in an area of rice irrigation 3° 23' S latitude, 37° 20' E longitude and 800 m altitude, where *An. arabiensis* is the vector species. Insecticide susceptibility tests indicated susceptibility to alphacyano pyrethroids [15].

The huts at both sites were constructed to a design described by World Health Organization (WHO) [16] based on the original verandah-hut design developed in Tanzania [17, 18]. Modifications included a reduced eave gap of 2 cm, a wooden ceiling, a roof of corrugated iron, and a concrete floor surrounded by a water-filled moat. The huts had open eaves with verandah traps and window traps on each side. The working principle of these huts has been described previously [19].

Net preparation and washing

ICON Maxx is a twin-sachet kit, with one containing 7.3 ml of lambda-cyhalothrin 10 % CS and the other containing 7.7 ml of binding agent. The target dose of lambda-cyhalothrin on a family size (130 × 180 × 150 cm) polyester mosquito net is 55 mg AI/m2 (corresponding to 1.55 g AI/kg for a 100-denier net). The ICON Maxx kits and a white colored 100-denier family-size nets used

in the study were supplied by Syngenta (Basel, Switzerland). During treatment, the contents of both sachets were mixed with 500 ml of water, sufficient to saturate an individual polyester family-size net.

Conventionally treated family-size nets were treated with lambda-cyhalothrin 2.5 % CS (Iconet®, Syngenta; Basel, Switzerland) to a target dose of 15 mg/m2 recommended by WHO [20]. To simulate wear and tear a total of six 4 cm × 4 cm holes were cut into each net (two holes on each side and one hole at each end). The long-lasting insecticidal nets (LN) and conventional lambda-cyhalothrin treated nets (CTN) were washed according to WHOPES Phase II washing protocols [16]. Each net was washed individually in 10 l of tap water containing 2 g/l of soap ('Savon de Marseille'), subjected to 20 rotations per min for 6 min during a 10 min immersion, then rinsed twice. The interval between washes was one day, which is the established regeneration time for ICON Maxx [12]. The washing schedule was stepped to ensure that the final wash of all treatment arms of the trial was completed on the same day.

The CTN washed to the 'point of insecticide exhaustion' served as a positive control against which to assess ICON Maxx performance. The determination of point of exhaustion is described in detail in appendix 1, Section 2.5.

Exposure was for 3 min, knock-down was scored after 60 min and mortality was scored 24 hr later. The same procedure was adopted for an ICON Maxx treated net to determine the number of washes which ICON Maxx treatment causes less than 80 % mortality and 95 % knock-down in WHO cone bioassays conducted after each wash.

Experimental hut study design

The following five treatment arms were tested in the huts: (i) unwashed ICON Maxx net, (ii) ICON Maxx net washed 20 times, (iii) polyester net conventionally treated with lambda-cyhalothrin at 15 mg/m2 and washed four times, (iv) polyester net conventionally treated with lambda-cyhalothrin at 15 mg/m2 and washed 20 times, (v) untreated unwashed polyester net.

The primary outcomes were Deterrence, Treatment-induced exiting (exophily), mortality, Overall killing effect, Blood-feeding inhibition and %Personal protection. These outcomes are described in more detail in appendix 1, Section 2.6.2.

Each morning dead and live mosquitoes were collected from the verandahs, rooms and window traps. Live mosquitoes were provided with 10 % sugar solution. Delayed mortality was recorded after 24 hours. Mosquitoes were identified to species and gonotrophic status was recorded as unfed, blood-fed, semi-gravid, or gravid.

Experimental hut trials were conducted in Muheza and Moshi to similar study design. Latin squares were adopted to adjust for any variation between hut position, volunteer sleeper attractiveness and individual nets. The treatment arms were rotated once through each of the huts: a treatment was assigned at random to a particular hut for six (Muheza) or four (Moshi) nights of observation before being transferred to the next hut. Between 19:30 and 06:30 hours adult volunteers slept on beds under the nets. The sleepers were rotated through the huts on consecutive nights. Two to three nets were available per treatment arm and each net was tested for two nights during the four- or six-night rotation. At the end of the rotation the huts were cleaned and aired for one day before starting the next rotation. Data were collected for 36 nights in the Muheza trial and for 24 nights in the Moshi trial.

Random samples of *An. gambiae* s.l. from the huts were identified to species by polymerase chain reaction (PCR) [21]. Species identification recorded 100 % An. gambiae s.s. from Zeneti, Muheza (N = 60) and 100 % *An. arabiensis* from Lower Moshi (N = 60). Based on these results all specimens collected in the hut trials were recorded as *An. arabiensis* in Moshi and as *An. gambiae* s.s. in Muheza.

The criterion for efficacy was that the ICON Maxx washed 20 times should perform equal to or approximate number of washes a LLIN is likely to incur during its lifetime.

Chemical analysis

The lambda-cyhalothrin content of ICON Maxx and CTN nets used in the hut trials was estimated from netting samples (four per net) cut before and after washing according to WHO guidelines [15]. Lambda-cyhalothrin was extracted using acetonitrile and injected onto high performance liquid chromatography (HPLC) (Dionex Summit, Surrey, United Kingdom), separated on a 120 Å column, eluted with a 10 % acetonitrile aqueous solution and passed through a PDA-100 detector at 275 nm. From the calibration curve, the lambda-cyhalothrin content and the dosage per m2 was calculated.

Supporting bioassay tests on ICON Maxx nets and CTNs used in the trials.

Cone bioassays

Efficacy of ICON Maxx and CTN was assessed using WHO cone bioassays after treatment, after completing the washing cycles and at the end of the hut trials. Bioassay tests were conducted using a total of 50 *An. gambiae* Kisumu (pyrethroid susceptible), two to five days of age, on five sections of the net as per WHO guidelines in conditions of 25 ± 2 °C and 75 ± 10 % humidity. Mortality was recorded 24 hours after exposure.

Tunnel tests

The tunnel tests were carried out on pieces of ICON Maxx and CTN netting taking from the hut trials nets after 0, 20 and 30 washes. The additional washing to 30 washes was to determine whether the long-lasting treatment could withstand more than the standard 20 washes. The tests were conducted at the KCMUCo Moshi site using laboratory-reared *An. arabiensis* Doldotha strain (pyrethroid susceptible).

The standard WHO tunnel test was conducted. The dimensions and procedure for tunnel test adhered in this study is well described above (appendix, section 2.3.1.2).

Ethics, consent and permission

Ethical clearance was obtained from the ethics committees of the NIMR Tanzania (Ref: NIMR/HQ/R.8a/Vol X/86) and London School of Hygiene and Tropical Medicine (LSHTM). Written informed consent was obtained from all volunteers participating in the study and each was

provided with chemoprophylaxis and monitored daily for fever or possible adverse events due to insecticide exposure from the nets.

Statistical analysis

The main outcomes were the comparisons of efficacy of the ICON Maxx unwashed and 20 times washed relative to the CTN washed to cut-off in terms of the proportions of mosquitoes' blood-feeding or killed by the treatments. Logistic regression analysis was used to estimate proportional outcomes (mortality, blood-feeding, exiting) and negative binomial regression was used to analyze counts of mosquitoes' blood feeding (personal protection) or dying (overall insecticidal effects) relative to the untreated control, after adjusting for variation between individual sleepers and hut position. Laboratory bioassay data was analysed using logistic regression.

Results

Determination of the cut-off number of washes for conventional treated net

The cut-off point, sometimes known as the 'point of insecticide exhaustion', is the number of washes at which cone bioassay mortality using *An. gambiae* Kisumu still causes ≥80 % mortality [16]. At four washes, mortality fell below the critical threshold with the CTN (Fig. 1) meaning that lambda-cyhalothrin CTN washed three times was the standard reference. With the ICON Maxx treated net the mortality did not fall below the critical thresholds until 26 washes.

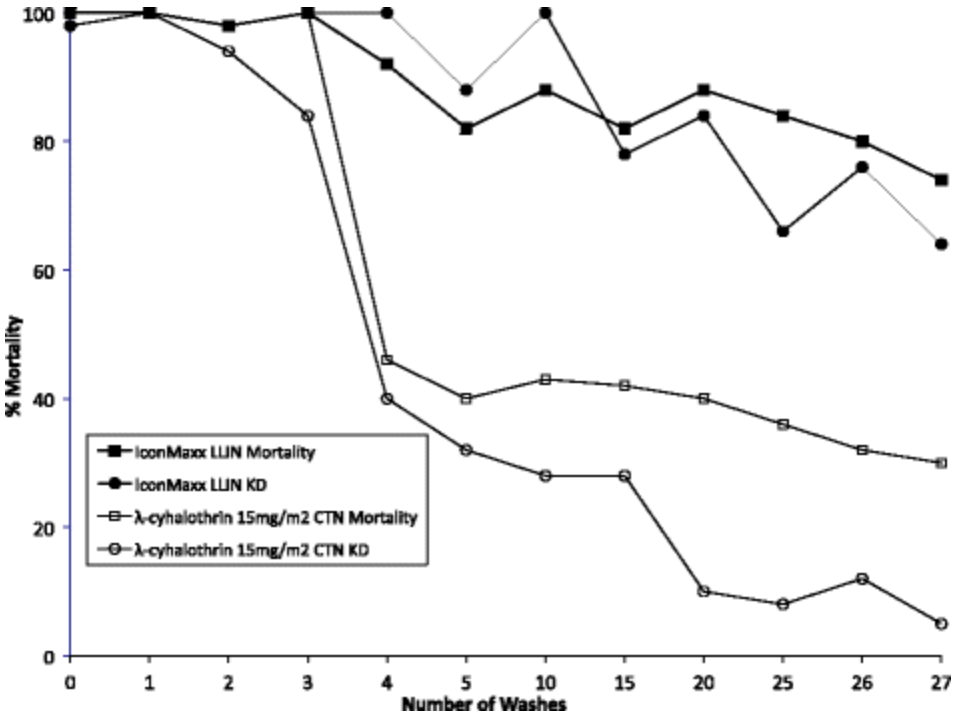

Fig. 1 Mortality of *Anopheles gambiae* Kisumu exposed in three-min cone bioassays to ICON Maxx LN and lamda-cyhalothrin CTN at 15 mg/m^2

Phase II - experimental hut trials

Mosquito entry and exiting from experimental huts.

The numbers and proportion entering and exiting the hut are shown in Table 1. During the trial in Muheza, 97 An. gambiae and 222 *An. funestus* were collected in the control huts. Percentage deterrence of An. gambiae was similar with treatments ICON Maxx unwashed or ICON Maxx 20 times washed (58 vs 61 %) and these were not significantly different to CTN washed to the cut-off point (41 %). Deterrence was lowest with the CTN washed 20 times (12 %). With *An. funestus*, the deterrence effect was significantly higher with ICON Maxx 20 times washed compared with the CTN washed to cut-off (66 and 25 %, respectively, P = 0.001). Deterrence was negligible with the CTN washed 20 times (1.8 %). During the trial in Moshi, 483 *An. arabiensis* were collected in the control huts. No significant deterrent effect was observed for any treatment arm.

Table 1 Anopheline mosquitoes collected and exiting into verandah and window traps in the ICON Maxx experimental hut trials in Muheza and Moshi, Tanzania in 2008

	Untreated net	ICON Maxx	ICON Maxx	CTN	CTN
Number of washes	0	0	20	Cut-off	20
Anopheles funestus					
Total females caught	222	122	76	167	218
Average catch per night	6.2[a]	3.4[b]	2.1[b]	4.6[a]	6.1[a]
% deterrence	-	45	65.8	24.8	1.8
Total females exiting	200	106	71	164	200
% exiting	90.1[a]	86.9[a]	93.4[a]	98.2[b]	91.7[a]
Anopheles gambiae					
Total females caught	97	41	38	57	85
Average catch per night	2.7[a]	1.1[b]	1.1[b]	1.6[bc]	2.4[ac]
% deterrence	-	57.7	60.8	41.2	12.4
Total females exiting	79	34	33	56	75
% exiting	81.4[a]	82.9[a]	86.8[a]	98.3[b]	88.2[a]
Anopheles arabiensis					
Total females caught	483	369	533	573	424
Average catch per night	20.1[a]	15.4[a]	22.2[a]	23.9[a]	17.7[a]
% deterrence	-	23.6	0	0	12.2
Total females exiting	392	319	469	450	352
% exiting	81.2[ac]	86.4[ab]	88.0[b]	78.5[c]	83.0[ac]

Numbers in the same row sharing a letter superscript do not differ significantly (P >0.05)

Exiting rates of *An. gambiae* and *An. funestus* from huts were high with untreated nets (81 and 90 %, respectively). A significant insecticide-induced exophily occurred for both species only with the CTN washed to the cut-off point (P = 0.02 for *An. gambiae* and P = 0.003 for *An. funestus*). The majority of *An. arabiensis* (81 %) exited the control huts during the night, and no insecticide-induced exophily was apparent.

Mortality and overall killing effect.

Percentage mortality by treatment is shown in Fig. 2 and mortality corrected for control and overall killing effect is shown in Table 2. With *An. gambiae*, mortality with ICON Maxx treated nets was not significantly less at 20 washes (66 %) than at zero washes (71 %) (P = 0.95) and was twice as high as the mortality observed with CTN washed to cut-off point (33 %) (P = 0.001). Unwashed ICON Maxx treated nets induced 75 % mortality of *An. funestus*. The mortality of *An. funestus* was not significantly higher with ICON Maxx washed 20 times compared with the conventionally treated nets washed to the cut-off point (58 and 52 %, respectively; P = 0.058). During the Moshi trial, the mortality of *An. arabiensis* with ICON Maxx treated nets washed zero times (47 %) and 20 times (42 %) were significantly higher than the mortality observed with conventionally treated nets washed four and 20 times (30 %, 36 %). No significant difference in mortality was observed between unwashed and washed nets of either treatment. With the CTN 20 times washed, considerable mortality was still observed across all three species, ranging between 36 and 40 %.

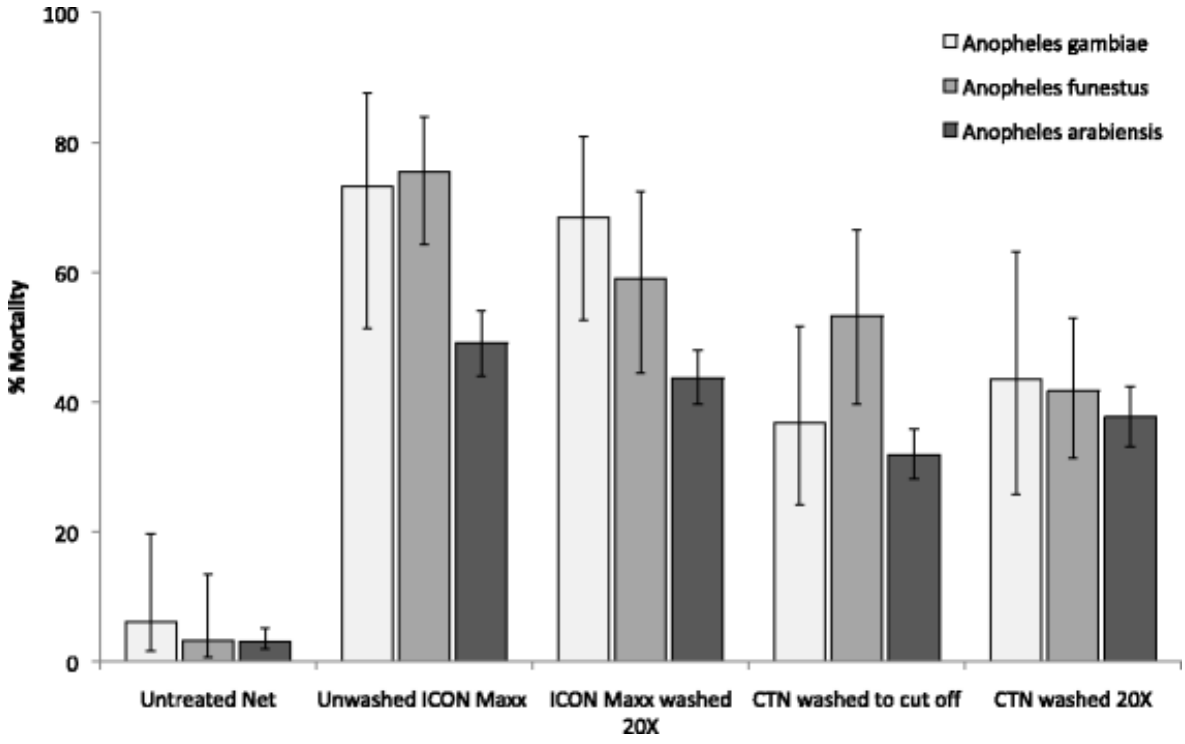

Fig. 2 Percentage mortality of *Anopheles gambiae*, *Anopheles funestus* and *Anopheles arabiensis* in experimental hut trials of ICON Maxx treated nets and lambda-cyhalothrin CTN.

Table 2 Mortality and blood-feeding outcomes of anopheline mosquitoes collected in the ICON Maxx experimental hut trials in Muheza and Moshi, Tanzania in 2008

	Untreated net	ICON Maxx	ICON Maxx	CTN	CTN
Number of washes	0	0	20	Cut off	20
Anopheles funestus					
Total dead	8	92	45	89	91
% mortality corrected for control	0^{a}	74.6^{b}	57.7^{bc}	51.6^{c}	39.6^{d}
% overall killing effect	0^{a}	37.8^{bc}	16.7^{b}	34.5^{c}	37.4^{bc}
Total blood-fed	81	23	20	41	88
% blood-feeding inhibition	0^{a}	48.3^{b}	27.9^{b}	32.7^{b}	0^{a}
% personal protection	0^{ab}	71.6^{c}	75.3^{c}	49.2^{ac}	0^{b}
Anopheles gambiae					
Total dead	6	30	26	21	37
% mortality corrected for control	0^{a}	71.4^{b}	66.3^{b}	32.7^{c}	39.8^{c}
% overall killing effect	0^{a}	30.4^{b}	25.3^{b}	19^{b}	39.2^{b}
Total blood fed	48	3	10	25	34
% blood-feeding inhibition	0^{a}	85.2^{b}	46.8^{a}	11.3^{a}	19.2^{a}
% personal protection	0^{a}	93.8^{b}	79.2^{bc}	47.9^{ac}	29.2^{a}
Anopheles arabiensis					
Total dead	15	181	233	183	160
% mortality corrected for control	0^{a}	47.4^{b}	41.9^{b}	29.8^{c}	35.7^{c}
% overall killing effect	0^{a}	34.4^{b}	45.1^{b}	34.8^{b}	30^{b}

	Untreated net	ICON Maxx	ICON Maxx	CTN	CTN
Total blood fed	131	40	44	52	54
% blood-feeding inhibition	0[a]	60[bc]	69.6[b]	66.5[bc]	53[c]
% personal protection	0[a]	69.5[b]	66.4[b]	60.3[b]	58.8[b]

Numbers in the same row sharing a letter superscript do not differ significantly (P >0.05)

As a significant deterrence effect was observed with most treatments against *An. gambiae* and *An. funestus*, the overall killing effect was usually less than the percentage mortality of mosquitoes collected from the huts except with the CTN washed 20 times, which showed no deterrence effect. The overall killing effect was similar across most treatments because there was a trade-off between high mortality and high deterrence with the ICON Maxx treatments and low mortality and low deterrence with the CTN treatments. As no significant deterrence effect was observed against *An. arabiensis*, the overall killing effect and percentage mortality were quite similar to each other. The majority of dead mosquitoes were collected from window and verandah traps rather than the room.

Blood feeding inhibition (BFI) and personal protection

Percentage blood feeding by treatment is shown in Fig. 3 and blood-feeding inhibition and personal protection is shown in Table 2. In the Muheza trial, significant blood-feeding inhibition was observed in both species with ICON Maxx treated nets unwashed or 20 times washed but BFI was generally less in *An. funestus* (48 and 28 %, respectively) than in *An. gambiae* (85 % and 47 % respectively). Blood-feeding inhibition of the ICON Maxx treated nets 20 times washed was not significantly different to that in the CTN washed to cut-off against either *An. funestus* (28 vs 33 %, P = 0.247) or *An. gambiae* (47 vs 11 %, P = 0.173). In the Moshi trial, all insecticide treatments provided significant blood-feeding inhibition (ranging from 53 to 70 %). Blood-feeding inhibition for ICON Maxx treated nets 20 times washed was similar to that of the conventionally treated nets washed to the cut-off point (70 and 67 %, respectively).

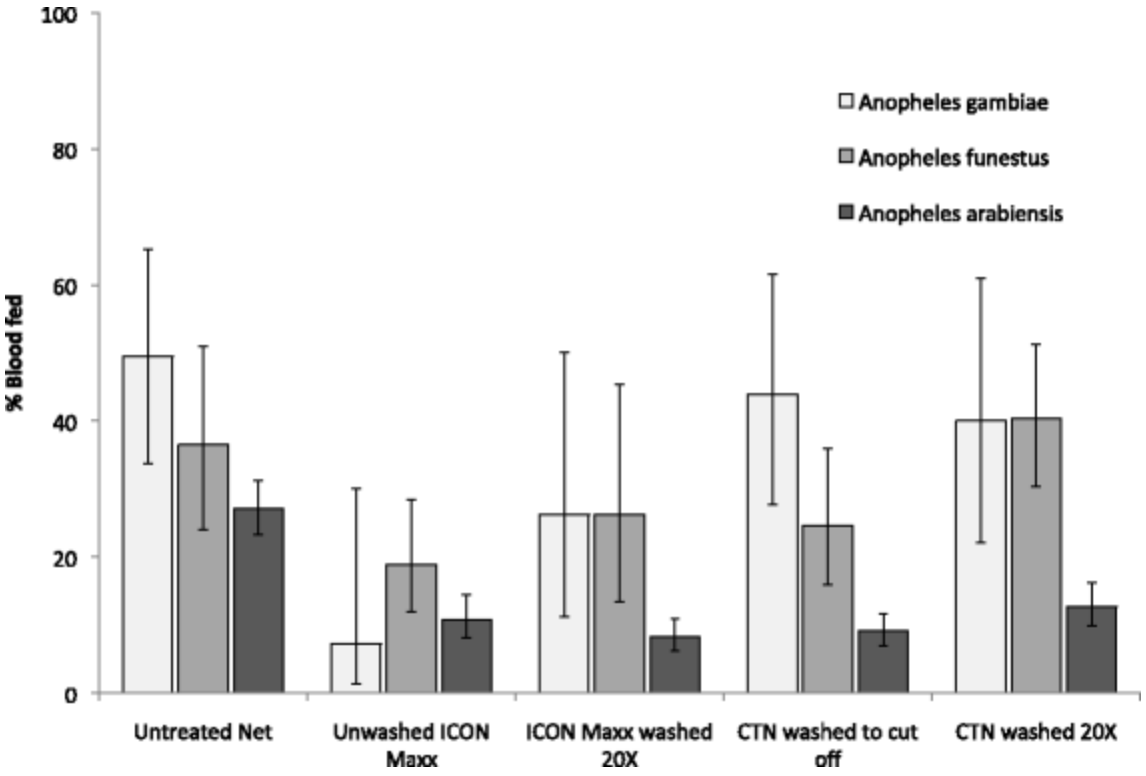

Fig. 3 Percentage blood feeding of *Anopheles gambiae*, *Anopheles funestus* and *Anopheles arabiensis* in experimental hut trials of ICON Maxx treated nets and lambda-cyhalothrin CTN.

The personal protective effect against the biting *An. gambiae* was 79 % with the ICON Maxx treated nets 20 times washed and 48 % with the CTN washed to cut-off (P = 0.059). Against *An. funestus*, these values were 75 and 49 %, respectively (P = 0.114), and against *An. arabiensis* they were 66 and 60 %, respectively (P = 0.395).

Chemical analysis

The chemical analysis (Table 3) showed that mean (±95 % CI) lambda-cyhalothrin content of ICON Maxx and CTN samples was 59.7 ± 29 mg/m2 and 13.2 ± 6.1 mg/m2, respectively. Both means were close to the target application rates of 55 mg/m2 and 15 mg/m2, respectively. Twenty washes removed 48.5 % of lambda-cyhalothrin from the ICON Maxx netting and 98.5 % from the CTN. The lambda-cyhalothrin remaining on the CTN washed to cut-off was 3.8 % (0.5 mg/m2) and on ICON Maxx washed to cut-off (27 washes) it was 10.5 % (6.3 mg/m2).

Table 3 Chemical analysis of lambda-cyhalothrin on the ICON Maxx and CTN in the experimental hut trial in Muheza, Tanzania in 2008

Number of washes	Concentration of alpha-cypermethrin (mg/m^2)	
	ICON Maxx	CTN
0	59.7 ± 29.1	13.2 ± 6.1
4[a]	-	0.5 ± 0.1
20	29.0 ± 18.3	0.2 ± 0.1
27[b]	6.3 ± 3.3	-

1. [a]cut-off wash number for CTN
2. [b]cut-off wash number for ICON Maxx

Supporting bioassay tests on ICON Maxx nets and CTNs used in the trials.

Cone bioassay tests

ICON Maxx and lambda-cyhalothrin CTN nets were tested by cone bioassay using *An. gambiae* Kisumu on five sections of the net (n = 50) before washing, after washing 20 times (before the trial) and after the hut trial. Before washing, mortality was 100 % for both treatments. After washing, the ICON Maxx and CTN induced 88 and 50 % mortality, respectively, and at the end of the trial they induced 92 and 12 %, respectively.

Tunnel tests

Tunnel tests using *An. arabiensis* Doldotha (pyrethroid susceptible) strain on ICON Maxx and CTN netting washed zero and 20 times are shown in Fig. 4. The proportion penetrating the unwashed ICON Maxx and CTN netting was less than 20 %, the proportion killed was 100 % and the proportion blood-fed was less than 2 %. With 20 washes, the proportions penetrating the ICON Maxx and CTN were 25 and 79 %, the proportions blood feeding were 10 and 78 %, and the proportions killed were 100 and 9 %, respectively. In all three criteria ICON Maxx was significantly superior to the CTN ($p < 0.01$).

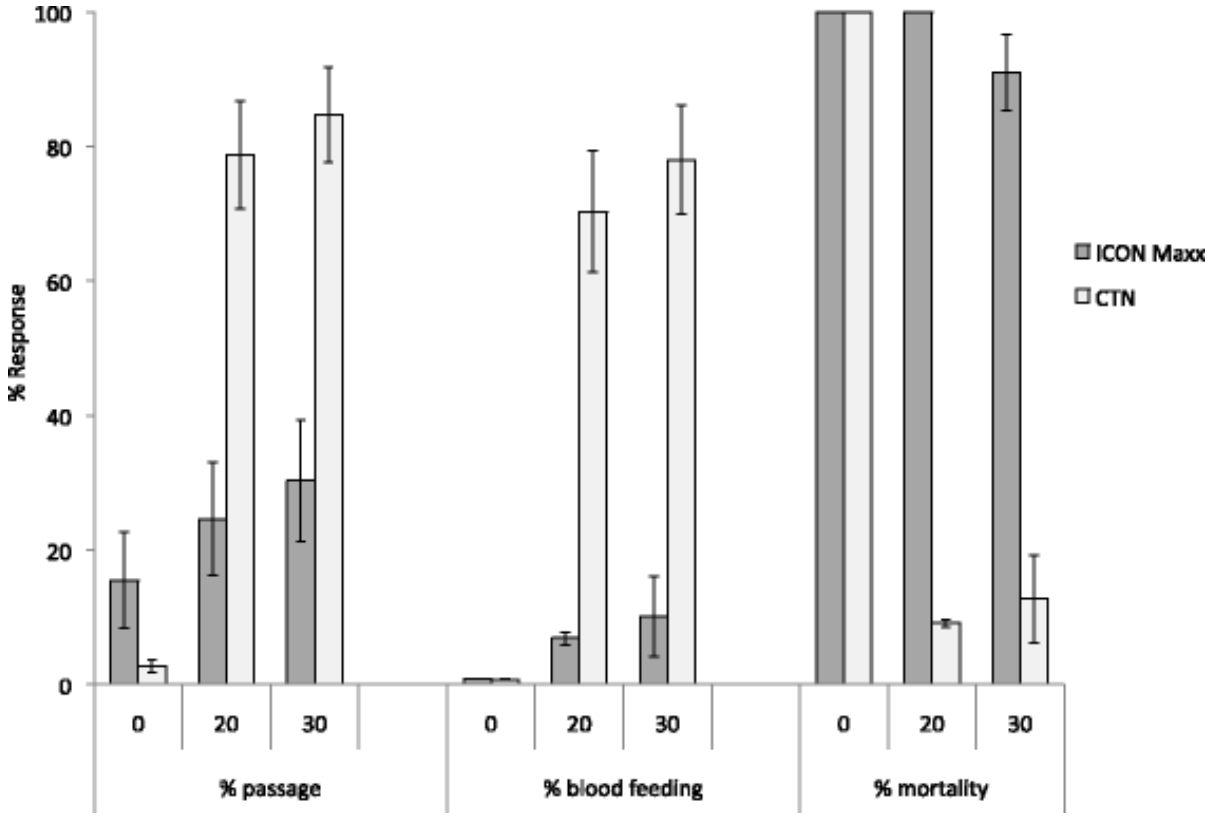

Fig. 4 Tunnel test results with ICON Maxx and lambda-cyhalothrin CTN before and after washing against *Anopheles arabiensis* Doldotha pyrethroid susceptible strain.

Discussion

The Phase II experimental hut trials performed in Tanzania on anopheline populations susceptible to lambda-cyhalothrin (*An. funestus*, *An. arabiensis* and *An. gambiae*) demonstrated that ICON Maxx induced significantly higher mortality and similar rates of blood-feeding inhibition compared to a conventional lambda-cyhalothrin treated net washed to cut-off, and therefore fulfilled the WHOPES criterion of a long-lasting insecticidal treatment. In a further WHOPES-supervised Phase II trial in Burkina Faso against a population of *An. gambiae* that was pyrethroid resistant the mortality induced by unwashed Icon Maxx against free-flying mosquitoes was less than 30 % compared to the 71 % mortality generated against the population in Tanzania, which were susceptible [12]. Despite the high-level of pyrethroid resistance (due to knock-down resistance frequency of 0.7-0.9 and probably metabolic mechanisms too) in Burkina Faso and the

low mortality recorded in the trial, Icon Maxx was shown to be superior to the CTN washed to cut-off in the huts [12]. Given the efficacy and resistance to washing of nets treated with ICON Maxx in both studies, WHOPES recommended that an interim recommendation be granted to ICON Maxx as a long-lasting treatment [12]. The one caveat was the nets sampled in Burkina Faso did show an unexpectedly high variation both between and within nets and therefore WHOPES concluded that given the heterogeneity in lambda-cyhalothrin concentration on the surfaces of the nets, ICON Maxx cannot be recognized as equivalent to a WHOPES-recommended, factory-produced LLIN where greater attention can be paid to quality assurance during production. Because only a limited number of nets could be analysed chemically in the Tanzania trial, it was not possible to assess variation in application rate to the same degree as in the Burkina Faso trial. Overall, the lambda-cyhalothrin retention index after 20 washes in the Tanzania and Burkina Faso trials was 51.5 and 28.2 %, respectively, and both were significantly superior to the CTN retention index. Crucially, biological performance against free-flying anophelines did not significantly deteriorate after 20 washes in either trial and therefore any heterogeneity in concentration across the surface of the net does not translate to a loss of biological efficacy if mosquitoes are sampling a range of insecticide concentrations across the surface as they attempt to gain access to the host. A third experimental hut trial was conducted with ICON Maxx in Côte d'Ivoire in which over 60 % of free-flying An. gambiae were killed but as the resistance status was undetermined this result is difficult to put into context [22].

The WHOPES guidelines for testing of LN were revised in 2013 to include as a positive control a WHOPES-recommended LN with similar specifications to the candidate LN in the type of insecticide, treatment technique, netting material and wash number (0 and 20 times) [23]. LN manufacturers are not necessarily keen to have their established LN product compared against another LN and, by necessity, the reference LN often needs to be obtained from the free market. Some recent WHOPES trials have re-instated the CTN washed to cut-off, in addition to the reference LN washed 20 times, as a second comparison arm to check that the equivalence/superiority of the reference LN is being maintained through quality assured production. The present trial was undertaken before the revised guidelines were introduced. In

view of the quality assurance issues, it is important to retain the CTN in WHOPES Phase II hut trials as one of the positive control arms.

The laboratory biological and chemical assays confirmed that the ICON Maxx insecticide binding process imparts strong wash-retention characteristics. The Phase II washing regime stripped 96 % of the lambda-cyhalothrin from the conventionally treated net within just a few washes as demonstrated by the surface content falling from 13.2 to 0.5 mg/m2 at cut-off and to 98 % reduction after 20 washes. And yet in hut trials the CTN was still killing up to 40 % of all three species of Anopheles after 20 washes. A similar finding was observed in Phase II experimental hut trials of Interceptor LN, with the alpha-cypermethrin CTN washed 20 times killing between 40 % and 50 % of anophelines in the hut trials [24]. The plausible explanation is that alphacyano-pyrethroids, such as lambda-cyhalothrin and alpha-cypermethrin, have strong binding affinity to polyester filaments so that even after multiple washes a thin layer of pyrethroid of less than 1 mg/m2, barely detectable by HPLC, must remain bound to the fibres and be sufficiently bio-available to induce mortality in free-flying mosquitoes in experimental huts. This explanation is supported by the cone test results on CTN which showed a 60 % decrease in mortality over the first four washes and then little or no further decrease in mortality in tests over the next 20 washes.

Lower rates of mortality were recorded in the huts with *An. arabiensis* than with *An. gambiae* and *An. funestus*. Differential mortality between these species has been observed before with other types of pyrethroid in other trials of ITNs [25]. *Anopheles arabiensis* is less anthropophilic than *An. gambiae* and *An. funestus* and the favoured hypothesis is that *An. arabiensis* is likely to be less persistent at the surface of the net and more likely to be repelled by the pyrethroid. There was no evidence that *An. arabiensis* is more resistant to lambda-cyhalothrin than An. gambiae or *An. funestus* or shows differential response to ITN bioassay, as all three species showed greater than 95 % mortality in 3-min cone tests [25]. The lower mortality of *An. arabiensis* has been proposed as a possible explanation for the species shift in favour of *An. arabiensis* over *An. gambiae,* which has coincided with the universal coverage campaigns of LLINs in Tanzania in recent years [26].

The demonstration of retention of efficacy and wash fastness with ICON Maxx raises the prospect of long-lasting pyrethroid treatment of textile materials other than mosquito nets, such as curtains, canvas tents or blankets either in or outside the factory. There is great diversity in the fabrics and materials used for making mosquito nets; insecticide-treated blankets, tents and curtains have also shown protection against malaria in trial settings [27–29]. The, yet unexplored question is whether this formulation makes other types of material long lasting. The efficacy and wash resistance of ICON Maxx therefore needs to be confirmed on materials made from other types of polymers such as cotton, nylon and polyethylene before it can have the widest possible application or impact against malaria.

In Phase III trials, recently completed, ICON Maxx demonstrated efficacy criteria expected of long-lasting net after 30–36 months of household use, whereas the CTN fell short of the efficacy criteria within just 12 months of use [30]. WHOPES distinguishes between long-lasting insecticide treatments that are carried out in the community and LLINs that are produced in the factory and expected to meet higher standards of quality control and homogeneity of application [12]. The Phase III trial of ICON Maxx, recently completed by NIMR/LSHTM in Muheza, Tanzania, was the first demonstration of a long-lasting treatment, as opposed to a long-lasting factory-treated net, providing efficacy and wash fastness over the three-year expected lifetime of the net [30]. The outcome of the present Phase II experimental hut trial, with no significant loss of efficacy of ICON Maxx between zero and 20 washes, successfully predicted the outcome of the three-year household trial.

Conclusion

Consequent to this Phase II experimental hut trial, ICON Maxx obtained interim approval from WHO and has since achieved full recommendation after Phase III household trials. It is the first long-lasting treatment kit to obtain full WHOPES recommendation.

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Chapter 5: Effectiveness of a long-lasting insecticide treatment kit (ICON® Maxx) for polyester nets over three years of household use: a WHO phase III trial in Tanzania.

Prologue:

Phase II Small-scale field experimental huts trial of ICON Maxx to test wash-resistance and efficacy described in the previous chapter (Chapter 5) showed that ICON Maxx successfully met the WHOPQT phase II efficacy criteria and merited WHO interim approval.

To attain full recommendation from WHO ICON Maxx had to remain effective for at least three years of field use in a large-scale field trials under “real life conditions“ in phase III, testing the long-lasting efficacy, community acceptance and safety observations [32].

Current chapter describe and discuss the results of the study whose main objective of the present study was to evaluate ICON Maxx treated nets in line with WHO guidelines for field testing of LLIN to determine insecticidal efficacy, wash fastness, acceptability, net integrity and net survivorship under East African household conditions over 3 years of use in comparison with a standard lambda-cyhalothrin 10% CS conventionally treated net without binder. As a result of this trial and others like it in other countries, Icon Maxx was granted full recommendation by WHO.

Chapter 6: Effectiveness of a long-lasting insecticide treatment kit (ICON® Maxx) for polyester nets over three years of household use: a WHO phase III trial in Tanzania.

The material presented in this chapter has been published as:

Patrick K. Tungu, Wema Sudi, William Kisinza and Mark Rowland: Effectiveness of a long-lasting insecticide treatment kit (ICON® Maxx) for polyester nets over three years of household use: a WHO phase III trial in Tanzania. *Malar J* 20, 345 (2021). https://doi.org/10.1186/s12936-021-03871-3

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Abstract

Background

ICON® Maxx (Syngenta) is an insecticide treatment kit of pyrethroid and binding agent for long-lasting treatment of mosquito nets. Interim recommendation for use on nets was granted by the World Health Organization (WHO) after successful evaluation in experimental huts following multiple washes. A full WHO recommendation is contingent upon demonstration of continued bio-efficacy after 3 years of use.

Methods

A household-randomized prospective study design was used to assess ICON Maxx-treated nets over 3 years in north-eastern Tanzania. Conventional treated nets (with lambda-cyhalothrin, but without binder) served as a positive control. At 6-monthly intervals, cross-sectional household surveys monitored net use and physical integrity, while cone and tunnel tests assessed insecticidal efficacy. Pyrethroid content was determined after 12 and 36 months. A parallel cohort of nets was monitored annually for evidence of net deterioration and attrition.

Results

After 12 months' use, 97% of ICON Maxx-treated nets but only 67% of CTN passed the WHO efficacy threshold for insecticidal durability (> 80% mortality in cone or tunnel or 90% feeding inhibition in tunnel). After 24- and 36-months use, 67% and 26% of ICON Maxx treated nets met the cone criteria, respectively, and over 90% met the combined cone and tunnel criteria. Lambda-cyhalothrin content after 36 months was 17% (15.8 ± 4.3 mg/m2) of initial content. ICON Maxx nets were used year-round and washed approximately 4 times per year. In cross-sectional survey after 36 months the average number of holes was 20 and hole index was 740 cm2 per net. Cohort

nets had fewer holes and smaller hole index than cross-sectional nets. However, only 15% (40/264) of cohort nets were not lost to follow-up or not worn out after 36 months.

Conclusions

Because more than 80% of nets met the WHO efficacy criteria after 36 months use, ICON Maxx was granted WHO full recommendation. Cross-sectional and cohort surveys were complementary and gave a fuller understanding of net durability. To improve net usage and retention, stronger incentives and health messaging should be introduced in WHO LLIN longitudinal trials. Untreated polyester nets may be made long-lasting insecticidal in Africa through simple household treatment using ICON Maxx pyrethroid-binder kits.

Background

Long-lasting insecticidal nets (LLINs) are an important tool for malaria vector control. With LLIN technology, insecticidal efficacy is expected to be sustained against Anopheline mosquitoes for at least 3 years without need for further retreatment [1]. The proportion of the population with access to insecticide-treated nets (ITNs) and LLINs has increased markedly in sub-Saharan Africa over the past two decades. Manufacturers' delivery data for 2004–2020 show that over 2.3 billion ITNs and LLINs were supplied globally in that period, of which 1.9 billion (86%) were supplied to sub-Saharan Africa [2]. By 2019, 68% of households in Africa had at least one ITN/LLIN, increasing from about 5% of households in 2000. The percentage of the population sleeping under ITNs or LLINs has increased from less than 2% in 2000 to 46% in 2019 [2]. Although highest numbers of LLINs are being delivered to sub-Saharan countries, 1 in 4 children live in households with no access to ITN or protection by indoor residual spraying [3]. To achieve one LLIN for every two household members, a ratio considered sufficient to achieve universal coverage [4], an estimated 200–300 million replacement nets are required each year to achieve and maintain universal access [3].

Owing to the insufficient number of LLINs being delivered through NMCPs and NGOs, many households use nets sourced locally through commercial and retail sectors. The majority of these are not LLINs and surveys show most have either never been treated or were treated only once

on purchase [5,6,7]. These nets may be made from a variety of synthetic polymers or natural fibres. This emphasizes the need for long-lasting insecticide treatment kits that can be used to convert untreated nets into products that withstand repeated washing without need for annual retreatment. Such insecticide kits could be bundled together with untreated nets, sold from shops, and enable local net producers that lack LLIN manufacturing technology to produce a long-lasting ITN, which could address local LLIN shortages and contribute usefully to malaria control [1, 3].

Two brands of long-lasting treatment kit have been developed: KO-Tab 123 by Bayer Environmental Sciences [8] and ICON Maxx by Syngenta [9]. KO-Tab 123 treated nets remained insecticidal for 15–20 washes in WHO Phase II evaluation trials [8]. ICON Maxx is a twin-sachet treatment kit for treatment of individual family-sized polyester nets based on a slow-release capsule suspension formulation of lambda-cyhalothrin 10% CS and binding agent. Following bio-efficacy and wash-fastness studies in Phase I laboratory and Phase II experimental hut studies in Tanzania and Burkina Faso, WHO interim recommendation was granted to ICON Maxx [9, 10]. Further hut trials were run in Côte d'Ivoire [11]. Full WHO recommendation is only granted after demonstrating the candidate LLIN or long-lasting treatment kit meets specific efficacy criteria after 3 years of regular household use in large scale Phase III longitudinal trials [12, 13].

The main objective of the present study was to evaluate ICON Maxx treated nets in line with WHO guidelines for field testing of LLIN to determine insecticidal efficacy, wash fastness, acceptability, net integrity, and net survivorship under East African household conditions over 3 years of use in comparison with a standard lambda-cyhalothrin 10% CS conventionally treated net without binder. Running in parallel with this Phase III longitudinal study presented here, a series of Phase I laboratory studies with ICON Maxx were run on a variety of polymer netting materials (polyethylene, polyester, nylon, cotton) to assess the treatment kit's versatility on other types of netting and household substrates.

Methods

Study areas

The trial was conducted in the two coastal villages, Tongoni and Mwarongo, in Muheza and Tanga districts (5°10′ 0S; 38°46′ 0E) (Fig. 1). In the household demographic census survey, Tongoni village comprised 5 hamlets and 484 houses, Mwarongo village comprised 2 hamlets and 335 houses. The residents subsisted on maize, cassava and rice with some working on sisal plantations and others on orange plantations and animal husbandry. Annual rainfall was bimodal: short rains from October to December, long rains from March to June, ranging from 800–1400 mm per annum. Malaria transmission occurred most of the year, and there were two seasonal mosquito peaks during and after the long and short rainy seasons [14, 15]. During the rains *Anopheles gambiae* sensu stricto (s.s.) predominated, and in the dry season *Anopheles funestus* was more common. Malaria transmission is classified as holoendemic although some areas of the districts have a long history of ITN use [14, 16] and LLIN universal coverage campaigns took place in 2011 [7].

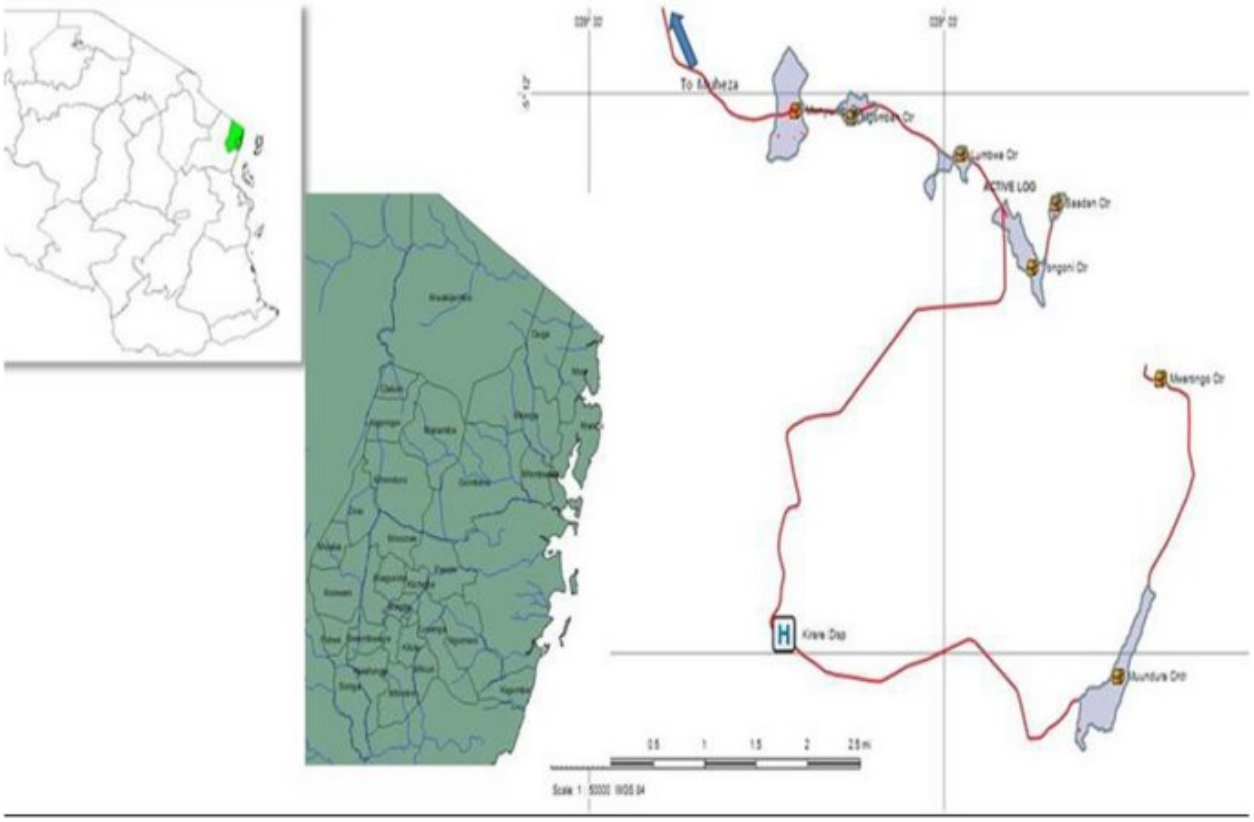

Fig. 1 The Map of Tanzania (top left), Muheza district (middle) and the GPS-generated map of study area showing hamlet boundaries.

Study design

The efficacy of ICON Maxx (Syngenta, Switzerland) treated polyester nets and nets conventionally treated with lambda-cyhalothrin CS (Iconet CS, Syngenta, Switzerland) at WHO recommended dosages were compared under field conditions in a two-arm household randomized trial with the household as the unit of randomization and mosquito nets as the unit of observation. The conventionally treated nets (CTN) were studied for one year after which all households with the Iconet-treated CTN were replaced with ICON Maxx treated nets [12]. The efficacy of ICON Maxx treated nets were monitored for up to 3 years of continuous use. It should be noted that the study coincided with the publishing of the 2013 revised WHO Guidelines for Laboratory and Field Testing of LLIN which recommended that a candidate LLIN or long-lasting treatment kit should be field evaluated with reference to an existing WHO-recommended LLIN rather than a CTN [13]. The WHO made an exception for Phase III trials already in progress.

Seven cross sectional surveys were undertaken, the first was carried out 1 month after net distribution, subsequent surveys took place every 6 months. A random sample of 30–50 households from each arm was selected every 6 months from the master list of participating households and subjected to physical integrity inspection, cone bioassay and tunnel tests. In year 1, both arms were surveyed, in years 2 and 3 only the ICON Maxx arm was surveyed.

Additional cohorts of 250 nets from a randomly selected 100 households from each arm were followed up annually for 3 years in the case of ICON Maxx and for 1 year in the case of Iconet CTN for assessment of survivorship and attrition in accordance with the revised WHO guideline [13].

Treatment of nets with ICON Maxx and Iconet

A total of 2500 polyester nets were treated individually with long-lasting ICON Maxx (Syngenta, Switzerland) from twin sachet packs, containing 7.3 ml of lambda-cyhalothrin 10% CS and 7.7 ml of binding agent. Nets were available in three widths (180, 150 and 120 cm) according to family needs and sleeping arrangements and were all 180 cm long and 150 cm high. ICON Maxx solution was applied by hand individually in basins according to the manufacturer’s instructions using the appropriate volume of solution for each size of net to give a target dose of 62 mg AI/m2. A safety

assessment concluded that no unacceptable exposures were found in the preparation, maintenance and use of the nets over the prescribed dose range of 50 (for family net) to 83 (for single net) mg AI/m2 [9].

A further 1250 conventionally treated polyester nets were treated with lambda-cyhalothrin 10% CS sachet (Iconet 10% CS, Syngenta Switzerland) without binder to a recommended target dose of 15 mg AI/m2 [8]. Treatment of nets was carried out by a trained team of field workers under supervision of the principal investigator. During treatment field workers wore personal protective clothing including gloves and masks. Treatment was done outdoors in the open air and nets were dried on plastic sheeting under shade. Nets were turned over periodically until dripping stopped and then hung-over washing lines to complete the drying.

Household randomization and net distribution

Houses were numbered during a village and household census. After the census, the ICON Maxx nets and CTNs were distributed to each household door-to-door in late June and early July 2011. A total of 1250 ICON Maxx nets and 1250 CTNs were distributed. A further 1250 ICON Maxx nets were held back to replace the 1250 CTNs at the end of the first year. The distribution was stratified by household so that each net type was present in each hamlet in a 1:1 ratio. Individual households received either ICON Maxx or Iconet CTNs rather than a mix of types. A unique code number was written on each net using a permanent marker. Sufficient nets were distributed to cover all sleeping spaces in each house. Householders were informed about the need for reporting adverse effects during net use and advised on proper use and maintenance. Assistance in hanging nets over the sleeping places was given where needed.

Household surveys and net integrity (cross-sectional surveys)

During cross-sectional household surveys nets were sampled from each treatment arm at six-monthly intervals. Both ICON Maxx and CTNs were randomly sampled at 6 and 12 months after distribution; thereafter, only ICON Maxx nets were sampled after 18, 24, 30 and 36 months of use. The Iconet arm was discontinued after 12 months as the net failed efficacy criterion.The household with Iconet were given IconMax nets but they were not surveyed henceforth. The 30 households sampled per survey (50 in the final 36-month survey) were selected at random using

the household ID master list, one net from each household was sampled, the selected household received a replacement ICON Maxx net and then removed from the study. At the time of each cross-sectional survey and net collection, a household questionnaire was applied to assess net use, acceptability, washing practices and any adverse effects.

Net integrity surveys were carried out every 6 months for 36 months in the randomly selected ICON Maxx houses and after 6 and 12 months in the randomly selected CTN houses. Each selected net was hung over a wooden frame and scored for size and distribution of holes, repairs (stitches, knots and patches) and open/failed seams. Assessment of cleanliness was done concurrently, and nets categorized according to their degree of dirtiness. Hole sizes were categorized as size 1—smaller than a thumb, size 2—larger than a thumb but smaller than a fist, size 3—larger than a fist but smaller than a head, size 4—larger than a head. Hole index was estimated using the method defined by the WHO, which assumes that the hole size equates to the mid-point of the range for each hole size category [13]. The estimate of hole area gives a slightly more conservative value when compared to the hole index [17].

Net attrition and functional survivorship (cohort surveys)

Two cohorts of 100 houses from ICON Maxx and Iconet CTN arm were selected, censused and nets checked at the end of each study year (after 12, 24 and 36 months) for condition, attrition, and functional survivorship. Study nets were recorded as present, discarded due to damage (wear and tear, rodent and burn holes) or lost to follow up. Households where the inhabitants were absent or where nets were recorded as given away, used elsewhere, stolen, or lost were not included in the estimates of functional survivorship. Functional net survivorship was based on damage only and did not include in numerator or denominator nets lost to follow up due to family movement, theft, gifted or sold as these nets might still be functional.

Chemical analysis

From each of the ICON Maxx and CTN sampled at baseline and surveyed at 12, 24, 30 and 36 months, five additional pieces of netting (30 cm × 30 cm) were cut for chemical analysis from each of the five panels of each net. The piece closest to the mattress line was excluded as per WHO guidelines [12]. All pieces were sent to the WHO-collaborating at the Centre Wallon de

Recherches Agronomiques (CRA-W) for chemical analysis. The net pieces from each net were pooled, cut into small pieces and homogenized, and lambda-cyhalothrin was extracted and quantified using gas chromatography CIPAC 463/LN/M/3 [18].

Bio-efficacy and residual activity of nets

From the 30 to 50 ICON Max nets and CTNs sampled every 6 months, 5 netting pieces measuring 25 cm × 25 cm were cut from the five panels of each net in accordance with WHO guidelines [12]. Cone bioassay tests were carried out on the netting pieces at the NIMR Amani Centre using 2–5-day old, unfed, female *An. gambiae* s.s. (Kisumu). Twenty mosquitoes were exposed in 4 replicates of 5 mosquitoes to pieces from positions 2–5 of each net (total of 80 mosquitoes per net) for 3 min in WHO plastic cones; the piece where abrasion was greatest (tucked under the mattress) was excluded as recommended by WHO [12]. After exposure the mosquitoes were held in paper cups at 26 °C and 80% relative humidity and given access to 10% glucose solution. Knockdown was recorded 1 h after exposure and mortality after 24 h. When knockdown was < 95% and mortality was < 80%, the net was subjected to tunnel testing [12]. The net piece closest to average mortality of the net was tested in the tunnel. Any net meeting the cone criteria of ≥ 80% mortality or ≥ 95% knockdown or tunnel test criteria of ≥ 80% mortality or ≥ 90% blood-feeding inhibition was considered to have met the required threshold.

Data analysis

Data were double entered into Microsoft Access and analysed in STATA version 10. Comparison of chemical content between net types over time was analysed using analysis of variance and t-tests. Wilcoxon rank sum test was used to analyse continuous data that was not normally distributed. Logistic regression was used to analyse the association between percentage knockdown and 24-h mortality with washing, net usage and insecticide content at baseline and at after 12, 24 and 36 months of field usage. Chi-squared test for trend was used to analyse net efficacy over successive surveys. Poisson regression was used to the test for association between hole index and time, number of washes and net usage.

Ethics, consent and permissions

Ethical clearance was obtained from the ethics committees of the NIMR Tanzania (Ref: NIMR/HQ/R.8a/Vol X/86) and London School of Hygiene and Tropical Medicine. Written informed consent was obtained from all household heads of participating families.

Results

Household surveys

A total of 705 households were identified in the baseline survey; 70% had mud walls and 75% palm thatched roofs. Other roofing materials included corrugated iron; some walls were made of brick. The mean ages of household heads in ICON Maxx and Iconet households were 43 and 52 years respectively. Most householders were farmers (33% ICON Maxx, 55% Iconet); the remainder were employed as fishermen, teachers, nurses, students or unemployed. The majority (65% ICON Maxx, 78% Iconet) had received 7 years of primary school education, 7% and 20% had received secondary education and others had not gone to school at all.

Both the ICON Maxx nets and Iconet CTNs were well-accepted by the communities. Reported net use was 100%. Respondents indicated using their nets year-round and every night. Almost 90% of surveyed nets were found hanging above beds and 10% were observed suspended over floor mattresses. 72% and 78% of sampled populations stated their reason for using nets was to protect themselves from mosquito biting while 13% and 28% stated for protection from malaria. The frequency of net washing was found not to differ between ICON Maxx net surveys. Estimated washing frequency was 4 times per year (Table 1).

Table 1 Washing frequency and net appearance

Survey (month)	IconMaxx						lambda-cyhalothrin CTN					
	No. nets	Mean (SD) no. of washes[a]	General aspect of nets %				No. nets	Mean (SD) no. of washes[a]	General aspect of nets %			
			Clean	Slightly dirty	Dirty	Very Dirty			Clean	Slightly dirty	Dirty	Very Dirty
Cross section survey												
0	30	0	30	0	0	0	30	0	30	0	0	0
6	30	3 (2.0)	7	21	69	3	30	2 (1.7)	18	26	52	4

Survey (month)	IconMaxx						lambda-cyhalothrin CTN					
	No. nets	Mean (SD) no. of washes[a]	General aspect of nets %				No. nets	Mean (SD) no. of washes[a]	General aspect of nets %			
			Clean	Slightly dirty	Dirty	Very Dirty			Clean	Slightly dirty	Dirty	Very Dirty
12	30	1 (0.3)	45	0	39	16	30	1 (0.4)	36	3	36	25
24	30	4 (1.5)	13	13	55	19	–	–	–	–	–	–
36	50	4 (2.6)	16	6	56	22	–	–	–	–	–	–
Cohort survey												
0	264	0	100	0	0	0	266	0	266	0	0	0
12	98	6 (2.0)	29	7	54	10	113	6 (2.0)	36	5	52	7
24	46	6 (3.2)	28	14	46	12	–	–	–	–	–	–
36	40	7 (3.5)	26.5	26.5	15	32	–	–	–	–	–	–

[a] **Mean number of washes per year**

All respondents in all surveys reported washing their nets in cold water. Nobody reported rubbing nets against rocks or on washing stones. Nets were pre-soaked by 18–40% of respondents; soaking times ranged from 10 min to 4 h. Nets were reported washed using commercial bar soap (30–85%), detergent powder (14–50%) or both (14–32%). Most nets (92–98%) were rinsed after washing and most (92–98%) were dried outdoors.

Despite householders reporting high frequency of ICON Maxx net washing, it was observed that only 45% and 16% were scored as clean at 12 months and 36 months respectively and 19% and 22% were scored as very dirty at 24 months and 36 months respectively. There was no association between the alpha-cypermethrin content remaining on the nets at 36 months and the reported number of washes ($F_{1,48} = 1.2$, $P = 0.30$). Nor was there any association between the reported number of washes over 36 months and the proportion of nets failing the cone bioassay criterion ($F_{1, 48} = 0.3$, $P = 0.85$).

Physical integrity of nets in cross-sectional surveys

The same brand of 100-denier nets was used in ICON Maxx and Iconet CTN arms. In the baseline survey there were no holes or open seams on any of the sampled nets in the ICON Maxx or CTN arms. After 6 months, approximately half of the nets (52% of ICON Maxx nets, 56% of Iconet CTNs) had at least one hole (these were mainly of the smallest size category) (Table 3); the mean number of holes per net was 4.3 for ICON Maxx nets and 5.5 for CTN (Table 2). The number of holes increased between 6 and 12 months to a mean of 9 for ICON Maxx arm and 7 for Iconet arm; most holes were always found in the lower part of the panels (Table 3). Comparison of physical integrity between ICON Maxx and CTN nets confirmed there was no difference between either arm at 12 months when the CTNs were disused (Tables 2, 3). After 24 months, while most ICON Maxx nets (84%) had a least one hole, these remained of the smallest hole category (Tables 2, 3); the mean number of holes per net had increased to 14.5 (Tables 2, 3), the mean proportionate hole index (HI) was 589, the median HI was 197 (IQR = 352) and the geometric mean number was 71 (Table 4). After 36 months, most hole indices had increased: 82% of nets were holed (Tables 2, 3), the majority were still size 1 (59%), the mean HI was 740, the median HI was 417 (IQR = 615) and the geometric mean number was 59 (Table 4).

Table 2 Physical condition of IconMaxx and CTN by survey round holes by size category

Survey (month)	ICON Maxx						lambda-cyhalothrin CTN					
	No. of nets	Mean no. of holes per net	%Percentage of holes per size				No. of nets	Mean no. of holes per net	%Percentage of holes per size			
			1	2	3	4			1	2	3	4
Cross section survey												
0	30	0	0	0	0	–	30	0	0	0	0	–
6	30	4	58	29	13	–	30	5	44	26	30	–
12	30	9	72	21	7	–	30	7	57	30	13	–
24	30	14	53	36	11	–	–	–	–	–	–	–
36	50	20	59	31	8	2	–	–	–	–	–	–
Cohort survey												
0	264	0	0	0	0	–	266	0	0	0	0	–

Survey (month)	ICON Maxx						lambda-cyhalothrin CTN					
	No. of nets	Mean no. of holes per net	%Percentage of holes per size				No. of nets	Mean no. of holes per net	%Percentage of holes per size			
			1	2	3	4			1	2	3	4
12	98	5	54	34	12	–	113	3	56	32	12	–
24	46	11	66	25	9	–	–	–	–	–	–	
36	40	15	41	50	7	2	–	–	–	–	–	–

Table 3 Physical condition of IconMaxx and CTN by survey round holes by distribution

Survey (month)	ICON® MAXX LLIN							lambda-cyhalothrin CTN						
	No. of nets	% nets w/at least 1 hole	% Holes by distribution			Mean no. of open seams	% nets with any repairs	No. of nets	% nets w/at least 1 hole	% holes by distribution[a]			Mean no. of open seams	% nets with any repairs
			Lower	Upper	Roof					Lower	Upper	Roof		
Cross section survey														
0	30	0	0	0	0	0	0	30	0	0	0	0	0	0
6	30	52	82	12	6	0.3	20	30	56	81	14	5	0.6	0
12	30	74	89	3	8	0.6	30	30	72	90	9	1	0.5	0
24	30	84	67	22	11	1.2	13	–	-	-	-	-	-	-
36	50	82	74	11	15	0.9	32	-	-	-	-	-	-	-
Cohort survey														
0	264	0	0	0	0	0	0	266	0	0	0	0	0	0
12	98	48	86	5	9	0.3	6.7	113	42	84	11	5	0.3	2.6
24	46	67	77	12	1	0.6	5	-	-	-	-	-	-	-
36	40	85	67	21	12	1.4	20	-	-	-	-	-	-	-

[a]Location of holes: lower, lower half of side panels; upper, upper half of side panels; roof, top panel

Table 4 Physical integrity–comparison of estimates of the average hole index, hole area between cross-sectional and cohort surveys for ICON Maxx and Lambda-cyhalothrin CTN

Survey type	Survey (month)	ICON Maxx LLIN							lambda-cyhalothrin CTN						
		No. of nets	Hole index			Hole area cm^2			No. of nets	Hole index			Hole area cm^2		
			Mean (SD)	Median (IQR)	GM[a]	Mean (SD)	Median (IQR)	GM[a]		Mean (SD)	Median (IQR)	GM[a]	Mean (SD)	Median (IQR)	GM[a]
Cross section	0	30	0	0	0	0	0	–	30	0	0	0	0	0	0
Cohort		264	0	0	0	0	0	–	266	0	0	0	0	0	0
Cross section	12	30	513 (2048)	13 (162)	17	244 (865)	15 (162)	13	30	214 (425)	24 (265)	19	126 (206)	29 (163)	16
Cohort		98	166 (326)	0 (196)	8	101 (183)	0 (138)	7	113	114 (261)	0 (57)	6	67 (139)	0 (65)	5
Cross section	24	30	589 (1017)	197 (352)	71	373 (767)	86 (352)	49	–	–	–	–	–	–	–
Cohort		46	271 (435)	27 (415)	23	164 (247)	31 (204)	19	–	–	–	–	–	–	–
Cross section	36	30	740 (986)	417 (615)	59	483 (632)	226 (615)	101	–	–	–	–	–	–	–
Cohort		40	535 (614)	300 (876)	133	382 (393)	271 (738)	80	–	–	–	–	–	–	–

The age of nets (number of months of use) was positively associated with net HI ($F_{1,345} = 9.31$, $P = 0.002$). There was no association between the reported number of washes per net and net HI ($R2 = 0.015$, $P < 0.4027$), suggesting that frequency of reported washing was not associated with net durability. Nor did any differences in type of washing agent used have any association with net durability ($F = 0.03$, $P < 0.969$).

While the mean number of holes per net, the hole index and hole area showed an increasing trend between 0 and 36 months, no more than 13% of holes were ever greater than size 2 (Tables 2, 3, 4).

Net efficacy through bioassay

Baseline cone bioassay tests on ICON Maxx and CTN (Iconet) after treatment but before distribution resulted in 100% knockdown and 100% 24-h mortality on all pieces tested (Figs. 2 & 3). After six months of use the mean percentage mortality (± 95% CI) was significantly greater on ICON Maxx than on CTN (87.2%, CI 82–92 vs 63.9%, CI 56–87, $p < 0.0001$); similarly, mean percentage knockdown on ICON Maxx was significantly greater than that on the CTN (97.7%, CI 96–99 vs 86%, CI 78–94, $p < 0.004$) (Figs. 2 & 3). The survey after 12 months of use continued to show differences between ICON Maxx and CTN treatments in mean percentage mortality (93%, CI 86–96, vs 72%, CI 62–81, $p < 0.0004$) and mean percentage knockdown (97%, CI 94–99, vs 85%, CI 78–91, $p < 0.0005$) (Figs. 2 & 3). With respect to pass rate, 1 ICON Maxx and 8 CTN failed the cone after 6 months of use (Fig. 4). Some nets that failed the cone test subsequently passed the tunnel test criteria (1 ICON Maxx and 2/8 CTN), producing combined test pass rates of 100% for ICON Maxx and 80% (24/30) for CTN at 6 months (Fig. 4, Table 5). After 12 months of use, whilst the combined test pass rates remained high for ICON Maxx nets at 96.7% (29/30), it was much lower for the CTN at 66.7% (20/30) (Fig. 4, Table 5). Bioassays on the CTN were discontinued forthwith. Subsequent surveys focused on the ICON Maxx nets. After 18 months fewer ICON Maxx nets passed the cone test (70%, 21/30) but combined cone and tunnel testing produced an overall pass rate of 96.7% (29/30) similar to the pass rate at 12 months (Fig. 4, Table 5). After 24 months the combined pass rate remained high at 90% (27/30). After 30 months, although fewer nets passed the cone test criteria (33%, 10/30), most nets that failed the cone tests achieved the

tunnel test criterion (80%, 16/20) producing an overall pass rate of 86.7% (26/30). At 36 months, although only 26% (13/50) of ICON Maxx nets passed the cone criterion, 86.5% (32/37) of nets that failed the cone achieved the tunnel test criterion producing an overall pass rate of 90% (45/50) (Fig. 4). The incremental decrease in pass rate over the full 36 months was small but significant (χ2trend = 11, P = 0.001). The incremental decrease in cone test pass rate was highly significant over the full 36 months (χ2trend = 34, P = 0.001).

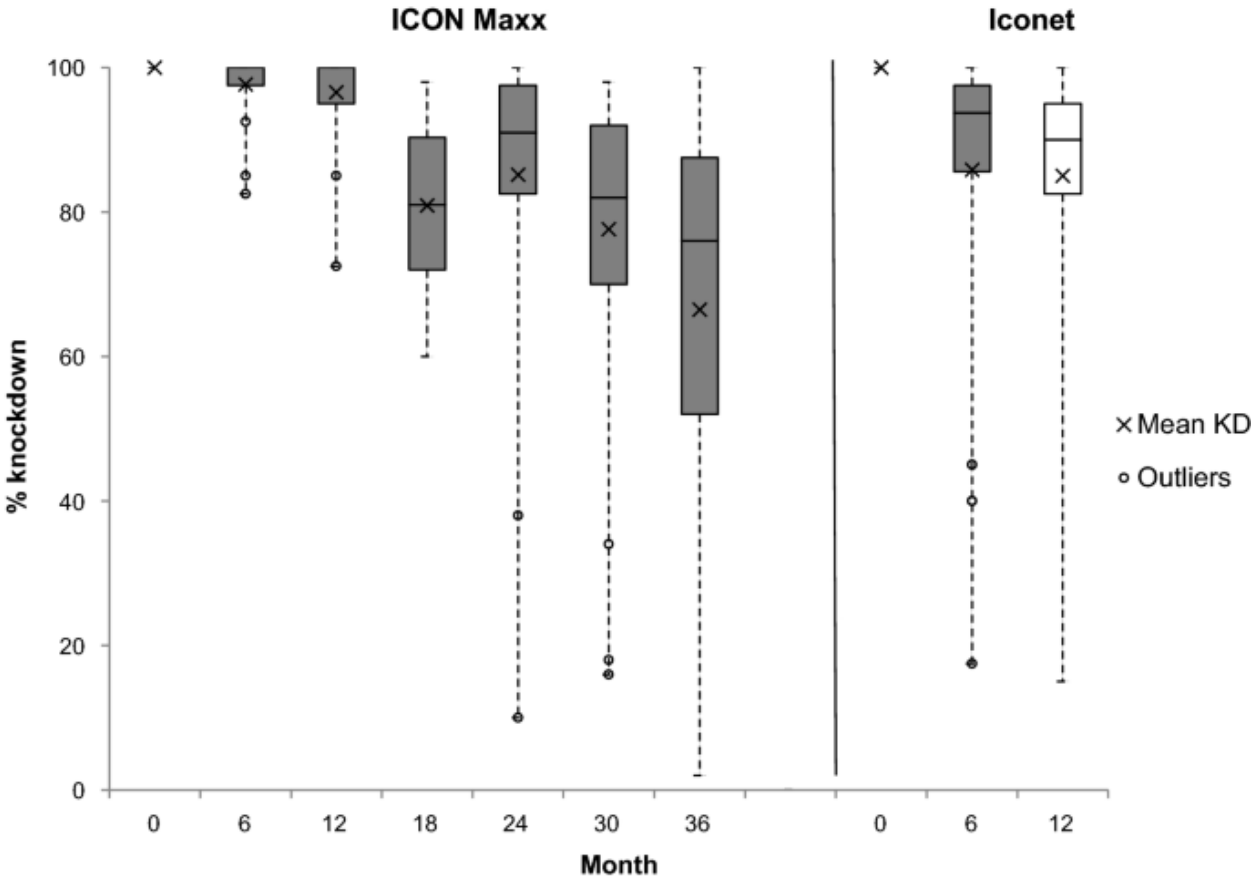

Fig. 2 Median (IQR) and mean percentage An. gambiae s.s. (Kisumu) knockdown 1 h post-exposure to ICON Maxx and CTN pieces in cone bioassays

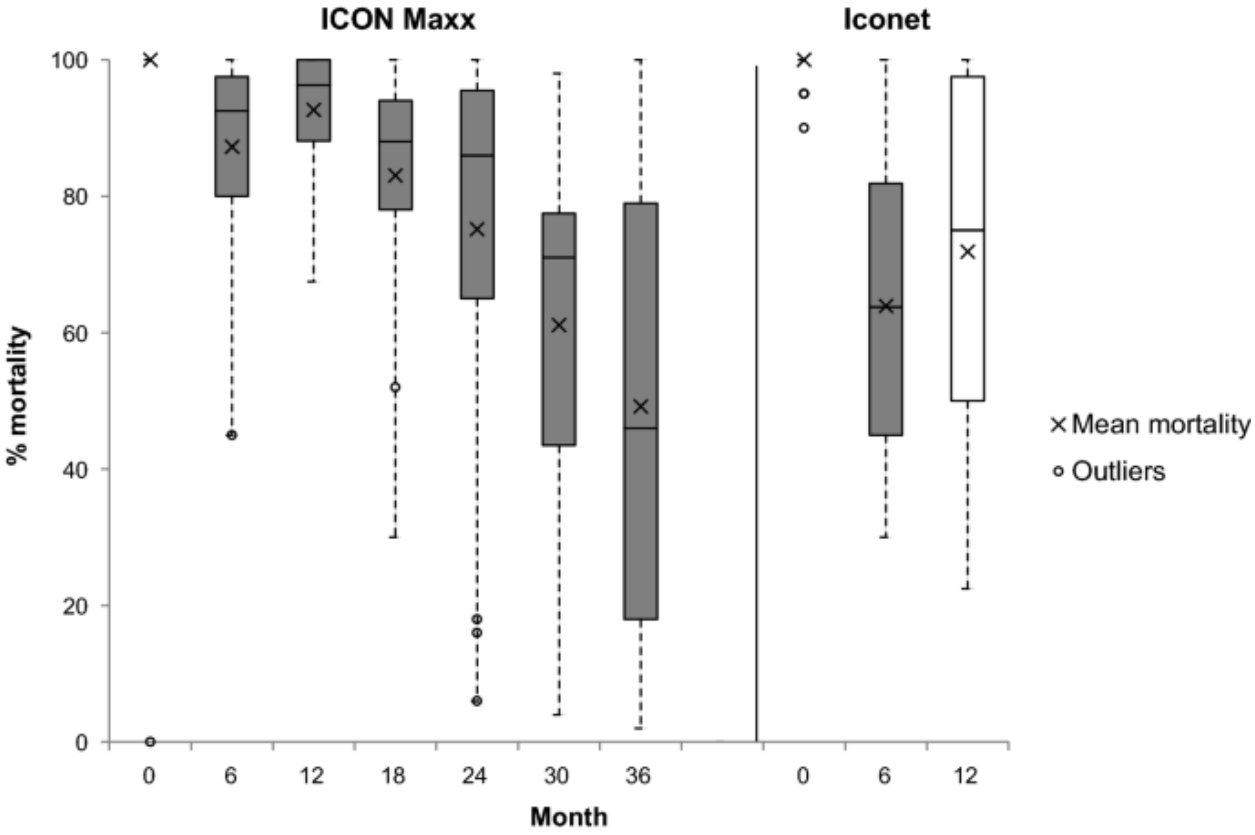

Fig. 3 Median (IQR) and mean percentage *An. gambiae s.s.* (Kisumu) mortality 24 h post-exposure to IconMaxx and CTN pieces in cone bioassays

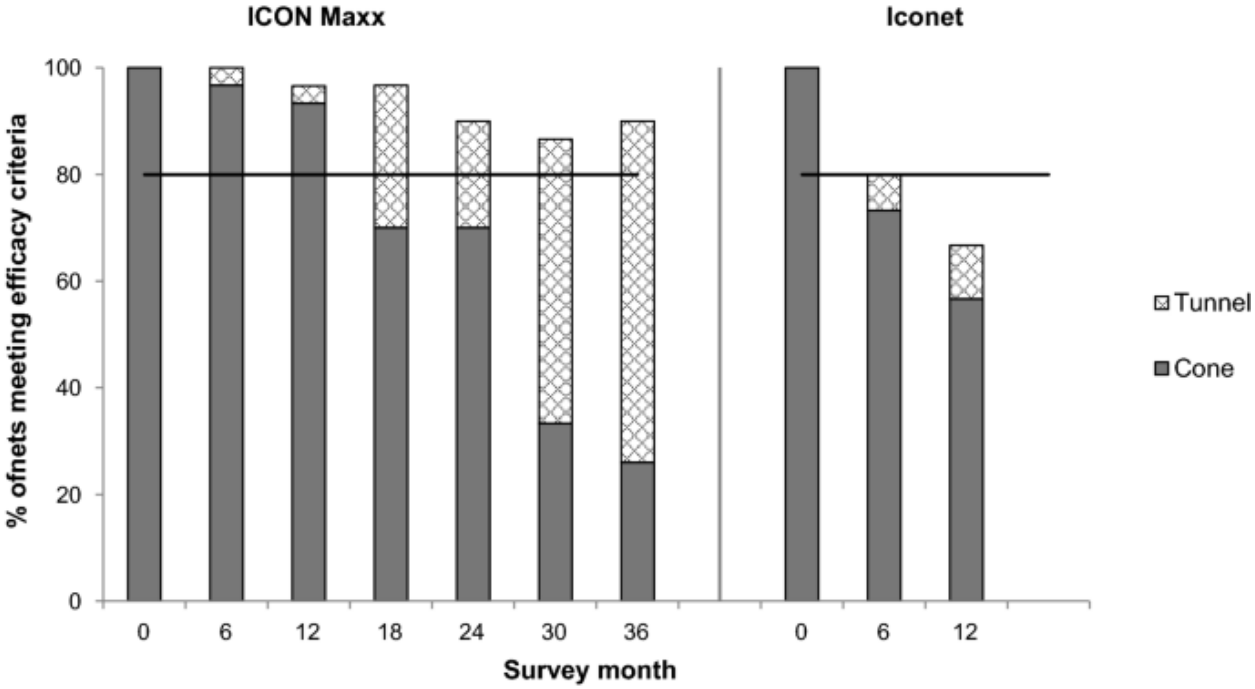

Fig. 4 Percentage ICON Maxx & CTN meeting WHO efficacy criteria (solid bar = cone test, hatched bar = tunnel test) by survey round. The horizontal line represents the acceptability cut-off for WHOPES full approval of the LN.

Table 5 Percentage of ICON Maxx and Iconet CTN meeting WHO efficacy criteria by survey round

Survey (month)	ICON Maxx			lambda-cyhalothrin CTN		
	Cone bioassays	Tunnel tests	Cone and tunnel tests combined	Cone bioassays	Tunnel tests	Cone and tunnel tests combined
0	100 (30/30)	–	100 (30/30)	100 (30/30)	–	100 (30/30)
6	96.7 (29/30)	100 (1/1)	100 (30/30)	73.3 (22/30)	25 (2/8)	80 (24/30)
12	93.3 (28/30)	50.0 (1/2)	96.7 (29/30)	56.7 (17/30)	23 (3/13)	66.7 (20/30)
18	70.0 (21/30)	88.9 (8/9)	96.7 (29/30)	–	–	–
24	70.0 (21/30)	66.7 (6/9)	90.0 (27/30)	–	–	–
30	33.3 (10/30)	80.0 (16/20)	86.7 (26/30)	–	–	–
36	26.0 (13/50)	37.0 (32/37)	90.0 (45/50)	–	–	–

Analysis of chemical content and insecticide retention (Fig. 5 and Table 6)

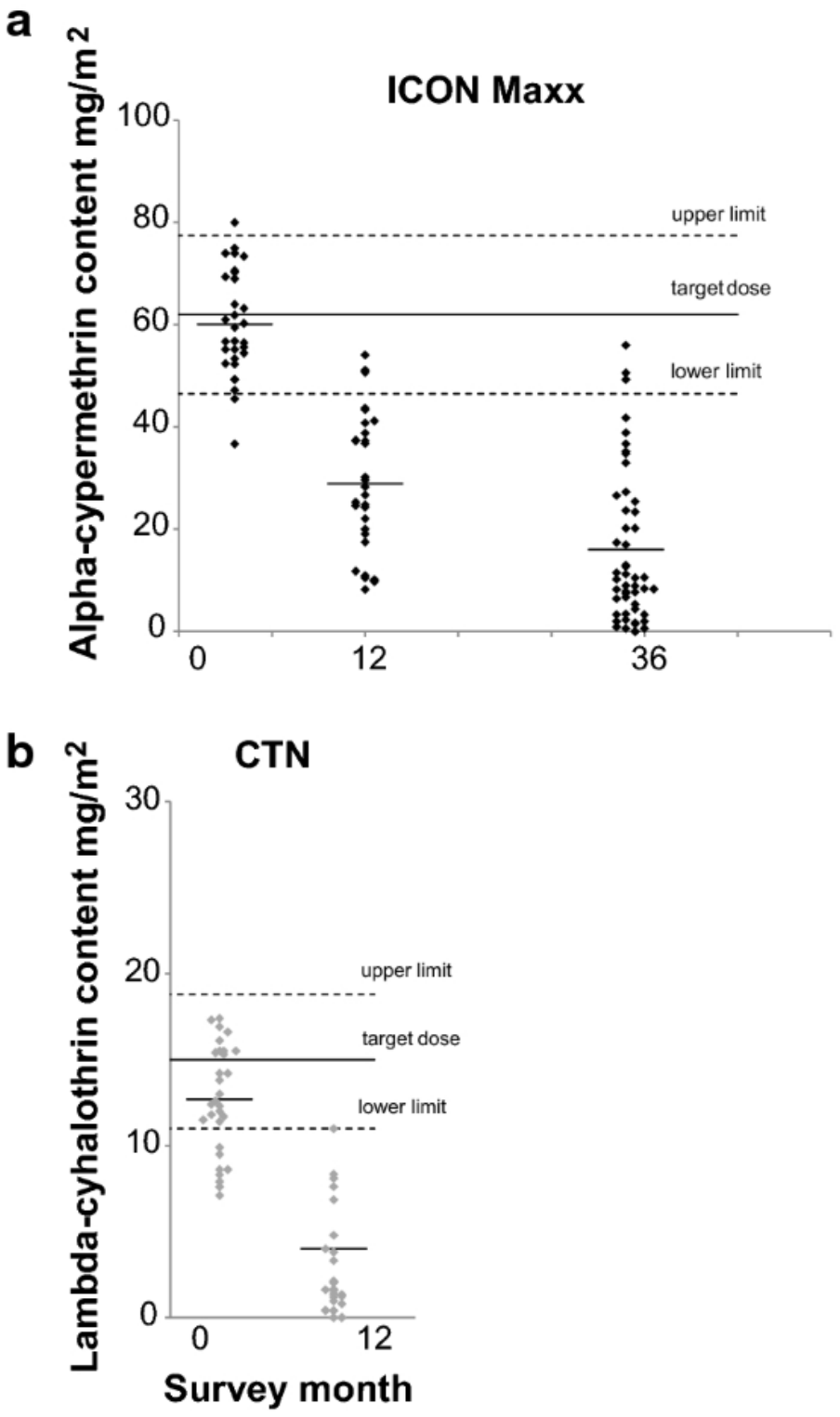

Fig. 5: a Lambda-cyhalothrin content (mg AI/m2) on individual IconMaxx nets samples at baseline after 12 and after 36 months of field usage for IconMaxx nets. Mean concentrations for each time point are indicated by the thin horizontal lines. The target dose and upper and lower limits are for lambda-cyhalothrin content at baseline indicated as solid and dashed lines. b Lambda-cyhalothrin content (mg AI/m2) on individual CTN samples at baseline after 12-monthly intervals of field use for Iconet. Mean concentrations for each time point are indicated by the thin horizontal lines. The target dose and upper and lower limits are for lambda-cyhalothrin content at baseline indicated as solid and dashed lines.

Table 6 Lambda-cyhalothrin content (mg AI/m^2) on ICON Maxx nets and CTN at baseline and after field use

Survey (month)	ICON Maxx-treated nets			Iconet CTNs		
	N	Mean mg AI/m^2	%AI loss	N	Mean mg AI/m^2	%AI loss
0	30	60.1 (56.3–63.9)	–	30	12.7 (11.5–13.9)	–
12	30	28.9 (23.9–33.9)	52%	30	4 (0.9–7.1)	68%
36	50	15.8 (11.5–20.1)	74%			

At baseline, the mean lambda-cyhalothrin content of ICON Maxx treated nets was 60.1 mg AI/m2 (Fig. 5a); this was close to the target of 62 mg AI/m2. At baseline, the mean lambda-cyhalothrin content among CTN was 12.7 mg AI/m2, well within the acceptable limits of the target dose of 15 mg AI/m2 (Fig. 5b). After 12 months of household use the lambda-cyhalothrin content of ICON Maxx had decreased to 28.9 mg AI/m2 corresponding to 52% loss of the baseline AI content (Fig. 5a); the content of CTN decreased to 4 mg AI/m2 after 12 months corresponding to 68% of baseline AI content (Fig. 5b). After 36 months the mean lambda-cyhalothrin content on the surveyed ICON Maxx nets was 15.8 mg AI/m2 (n = 50, RSD = 93.4%) corresponding to 73.7% loss of the original content (Table 6).

The mean lambda-cyhalothrin concentration on ICON Maxx treated nets that passed the cone bioassay criteria at 36 months was 30.4 (21.4–39.4) mg AI/m2, while content on nets that failed

the cone criteria was 10.7 (7.5–13.9) mg AI/m2; the difference in AI content between failing nets and those passing cone test criteria was significant ($F_{1,48} = 26.1$, $P = 0.0001$).

The mean concentration on nets that failed the tunnel test criteria was 5.7 mg/m2 and on those that passed was 16.9 mg/m2; the difference in AI content between pass and fail was significant ($t = 2.5$, $P = 0.009$).

Adverse effects among staff treating nets and families using nets.

Three attendants were responsible for treating nets at the start of the project at a rate of 60 nets per person per day for 2 weeks. All attendants who treated the nets reported sneezing and facial itching, and one reported fever. The adverse effects were more common after treating with Iconet (CTN) than after treating with ICON Maxx, even though the treatment dose was higher for ICON Maxx. One of the attendants regularly reported irritation to facial skin (paraesthesia). The effect took about 3 h to subside on each occasion. The discomfort was not so severe that the individual took time off work. All proper precautions were taken while treating the nets including wearing of masks and gloves.

Of the 60 households included in the first week and first month post-treatment surveys, only a small proportion reported experiencing any adverse effects and only during the first few days of net use. Similar proportions of Iconet CTN users (10%) and ICON Maxx net users (6%) reported these effects which included bad odour, sneezing, skin itching, nasal discharge and facial itching. The effects were transient and did not deter users from continuing to use the nets. No adverse effects were reported after one month of use. During the 6 months survey the interviewees reported that all symptoms stopped after the net had been washed once. At no stage did any of the adverse events require medical attention.

Net attrition and survivorship rate

Net survivorship due to loss of integrity (accumulated holes) caused by physical deterioration or damage fell from 100 to 78% after 12 months, to 70% after 24 months and to 68% after 36 months (Fig. 6 & Table 2b). A remarkably high proportion of nets distributed (72%, 189/264) were lost not due to loss of integrity but to more mundane reasons such as moving house to outside

the study area, hut collapse, or nets being given away during the 36 months. For most of the nets that were lost to follow up this occurred between 0 and 24 months.

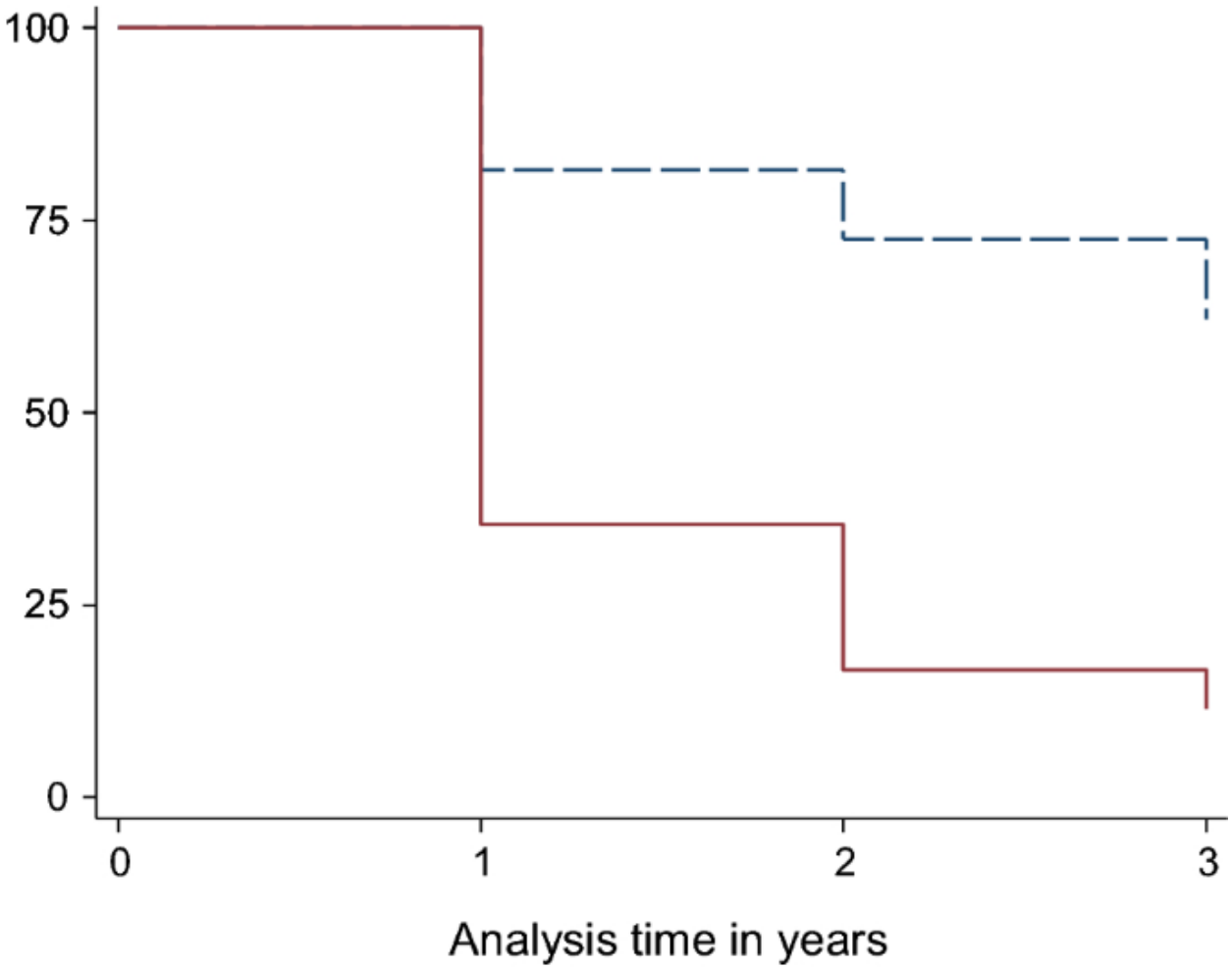

Fig. 6: Kaplan–Meier Nets Survival Curve; Hatched line = Net discarded due to damage, Solid line = Net discarded due to damage or lost to follow-up)

In the comparison of physical integrity of nets between the cohort-longitudinal surveys and cross-sectional surveys, the majority of holes in the cohort surveys were found in the lower part of the panels (Table 3 & Fig. 7). In all three cohort surveys the hole indices were significantly lesser ($Z = 2.46$; $P = 0.014$ for 12th month, $Z = 2.6$; $P = 0.009$ for 24th month and $Z = 6.14$; $P = 0.001$ for 36th month surveys) than in the cross-sectional surveys after the corresponding periods of use (Table 3 & Fig. 7). This may reflect cohort members' self-awareness that they were being monitored more closely than other recipients of ICON Maxx treated nets.

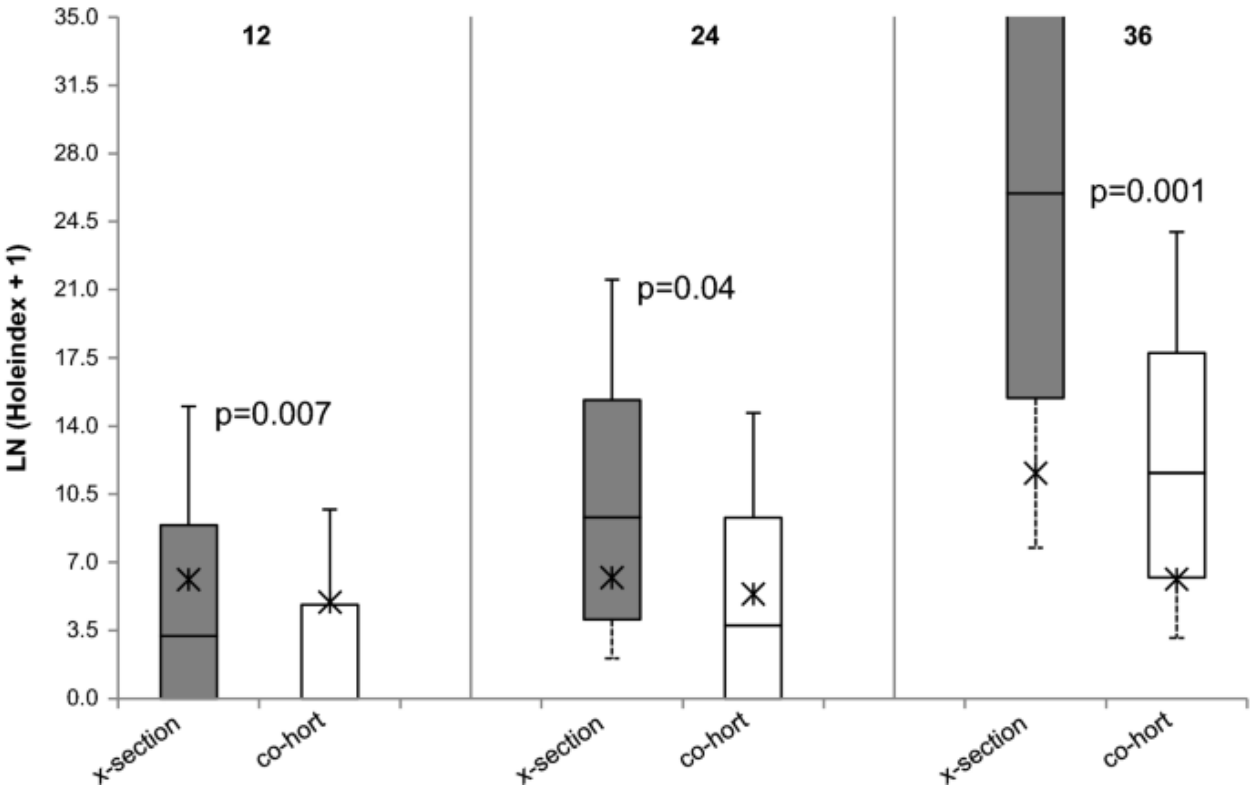

Fig. 7: Comparison of median (IQR) and mean ln (hole index + 1) of the ICON Maxx nets between cohort and cross section surveys at 12-, 24- and 36-month's survey points.

Attrition and physical integrity of the ICON Maxx-treated nets were monitored as recommended in the WHO LLIN testing guidelines. As a home-treatment kit, it should be noted that net integrity and hole index were never part of the product claim of ICON Maxx. Nevertheless, it was important to compare net integrity and bio-efficacy as part of the evaluation of ICON Maxx and to correlate the formulation performance with polyester net condition, as well as consider other types of material that ICON Maxx might be called upon to treat.

Discussion

Testing, bio efficacy and recommendation

The present study evaluated the efficacy of ICON Maxx treated nets for up to 36 months of household use using the standard WHO cone bioassay criteria of knockdown and mortality and the tunnel test criteria of mortality and blood feeding inhibition. According to WHO testing guidelines [13] a candidate LLIN or long-lasting treatment kit is deemed to meet the requisite

threshold for WHO recommendation if, at the end of 3 years use, at least 80% of the sampled nets retain bio-efficacy using any of the WHO bioassay criteria [13].

Applying the WHO criteria, ICON Maxx failed to achieve the 80% threshold at the 36-month sampling point based on cone bioassay alone. On inclusion of the tunnel test data, more than 90% of ICON Maxx treated nets met the efficacy criteria for the combined cone test and tunnel test. While the bio-efficacy of ICON Maxx treated nets remained high throughout the 36-month study period, the comparator arm of Iconet CTN was dropped from the trial after 12 months because at that sampling point it fell short of the required 80% pass rate and was considered unethical to continue. At that point, the CTN households were provided with ICON Maxx sachets and instruction leaflets and verbal guidance given.

Examining the bioassay methods individually, the ICON Maxx nets met the WHO cone efficacy criteria for the first 12 months. In the 2nd year fewer than 80% of nets met the WHO cone criteria, and the tunnel test played an increasingly important role. By the 36-month survey point, 74% of sampled ICON Maxx nets were failing the cone test and the tunnel testing was required to achieve the 80% pass rate. Contrast the long-lasting treatment kit results with other net products tested in Phase III trials at NIMR Muheza, such as Interceptor LN [17], a factory produced long lasting insecticidal net. After 36 months, a much higher proportion of Interceptor LN, 77% (23/30), met the cone criteria and only a few cone-failures needed to go forward to tunnel testing criteria to take Interceptor LN over the line. By contrast, with the ICON Maxx sampled at 36 months only 26% (13/50) met the cone criteria and a further 86% (32/37) had to go forward to tunnel testing to take ICON Maxx over the line. Why the difference? Was it differences in binder constituents between ICON Maxx and Interceptor LN or was it differences in binding process: factory versus community? The two-Phase III studies were comparable: both were done at the NIMR Amani Centre by the same scientific group in consecutive years. But because the two alphacyano-pyrethroids (alpha-cypermethrin in Interceptor LN and lamba-cyhalothrin in ICON Maxx) and the loading dosages (200 mg/m2 in Interceptor LN and 62 mg/m2 in ICON Maxx) were not the same in the two products, the differences in efficacy cannot be attributed with any certainty to differences in binder technology (factory machine versus field hand treatment) or binder

composition. However, the proportions of loading concentration lost over 12 to 36 months in the field were in fact remarkably similar: 49% in Interceptor and 48% in ICON Maxx after 12 months, and 82% in Interceptor and 74% in ICON Maxx after 36 months [19, 20]. It may not be a question of quality of binding agent or within-net heterogeneity between factory treatment versus community treatment but simply a question of different pyrethroid and the higher concentration of AI applied under factory conditions. Notwithstanding the true reason, the Tanzanian ICON Maxx samples did meet the bio-efficacy criteria, the overall study was reviewed by the WHO and ICON Maxx was given full recommendation as a long-lasting net treatment for up to 3 years of use.

Besides Tanzania, the only other significant Phase III trial of ICON Maxx sponsored by WHO was conducted in India in Odisha state [19, 21]. Relatively poorer performance of ICON Maxx nets was reported, with only 59% of the ICON Maxx nets meeting efficacy criteria with the combined cone test or tunnel test after 36 months. While 80% passed the combined cone-tunnel after 30 months, a much smaller proportion of the tested *Anopheles stephensi* responded adequately (41% mortality in the tunnel after 36 months and only 17% of nets passing the tunnel criteria) as compared to the 81% mortality in the tunnel with An. gambiae after 36 months in Tanzania and 86% of nets passing the tunnel criteria. Another difference notably at odds with the Tanzanian trial was high AI retention of lambda-cyhalothrin in ICON Maxx nets after 36 months (34 mg/m2 or 55% of loading content), potentially due to the shorter season of net use in India each year, as compared to 16 mg/m2 or 26% of loading content retained after 36 months and the much longer period of use in Tanzania. While the tunnel test is a highly realistic bioassay and simulator of experimental hut trials, there are clearly outstanding questions about vector responsiveness to bait in the tunnel and comparative performance of the different mosquito species that need to be resolved.

Beyond ICON Maxx, several brands of standard LLIN have achieved full WHO recommendation. Among the polyester LLIN they include PermaNet 2.0 LN and Interceptor LN [9, 22]. Among the polyethylene LLIN they include Olyset LN and Duranet LN [20, 23]. A further four brands have obtained WHO full recommendation on the basis of equivalence to the aforementioned brands,

and a further nine have obtained WHO interim recommendation after demonstrating bio-efficacy in Phase II experimental hut trials [24]. The main purpose of ICON Maxx, in having achieved full recommendation, is not to rival these brands of LLIN (which it could do) but to facilitate the treating of untreated nets, acquired commercially, in community or home, or for re-treating currently used nets between universal coverage campaigns if the gap is proving too long.

As mentioned, net integrity and hole index is not part of the product claim. Nevertheless, as part of the evaluation it was important to compare net integrity and bio-efficacy with what owners' report. This is particularly important when, as a cultural norm, the net recipients may not be allowed free access to the home to inspect the nets in situ. For example, nets became quite dirty within a year, and stayed dirty despite owners claiming to wash them every few months. There was no association between the reported number of washes at 36 months and the proportion of nets passing the cone bioassay criteria or with how much alpha-cypermethrin remained on the net. There was no association between the reported number of washes per net and net integrity (hole index). The only correlation observed was between the reported frequency of net use and net integrity or hole index. Clearly the hole index is a key indicator to retain with longitudinal studies of this kind. The positive association between bioassay outcome and AI content was reassuring to observe, as was the association between hole index and loss of bioassay efficacy or loss of AI content over time. Net cleanliness and reported number of washes were not reliable. The respondents may for personal reasons feel bound to give responses which they think will encourage the interviewer even when this is not the aim; for example, they may wish to report relatively higher number of washes with the purpose of showing that the net is being well cared for. The study did also highlight that washing was not the sole cause of insecticide removal; physical abrasion and friction during daily use were also contributing factors.

Net integrity and durability

While many LLIN brands may achieve the requisite WHO bio-efficacy, few LLINs can withstand the abrasion and wear and tear of 3 years of field use. Survival will depend on the local environment and conditions of use. After 36 months, most treated nets were lost or damaged: 18% were without holes and most were dirty. The condition of the polyester nets was consistent

with that of the factory treated polyester LLIN previously evaluated in the same district where only 17% were without holes and 70% were dirty [17]. Both cross-sectional and cohort longitudinal surveys were used in monitoring of net integrity and the accumulation of holes. Nets sampled during cross-sectional surveys were always in worse condition as compared to cohort nets surveyed at the same time point. One possible explanation is that cohort households were better informed from the outset of their involvement in the longitudinal study than were cross sectional households. With the insight that their nets would be periodically inspected, this might have influenced cohort households to look after their nets better than the cross-sectional households did. Another possible reason was cultural. According to the norms of the society living in the study area, most householders were not willing to allow access to field workers to their home to randomly select the net for cross sectional survey. In most cases it was the householders who sampled the net to give to the field workers. Their awareness that the sampled net would be replaced by a new one could influence them to select the net of worse condition as a way of discarding a net nearer its end of life in most households that wouldn't allow for random sampling of a net. This might have led to net sampling bias during cross sectional surveys leading to samples of worse condition compared to cohort nets.

As shown by the present trial, nets are lost to follow-up for a variety of reasons apart from deterioration or attrition. This might include migration of trial families and giving away or misuse of nets. Attrition due to reasons other than loss of integrity is a drain on the trial in terms of time and resources and creates the risk of leaving the trial underpowered for measuring true attrition due to loss of integrity. In some cases, losses to follow-up may make up 70% of the nets distributed at the start of a trial. Most of these were given away to relatives or moved with the family members who relocated outside the study area. Consequently, it is desirable to devise new procedures to limit such losses to follow-up.

How can net retention be improved? Any form of coercion would be unethical and impossible to enforce in practice. In the current WHO LLIN trial procedures, participating families are under no obligation to use or retain their nets. However, it might be possible to specify terms in the participant consent form that would help improve net retention while not affecting participants'

right to withdraw at any stage of the trial. In response to this issue, which arose from consideration of the present trial, the WHO proposed modifications to the consent forms used in Phase III trials and suggested the following new procedures:

a) Cohort surveys

Study participants/families enrolled into the cohort component of the trial would be requested to consent to the following:

- Participants would not give away or sell the study nets.

- Participants would retain the freedom to stop using the nets at any time but should let investigators know the reasons when asked during the follow-up survey.

- Investigators would inform participants that the nets will be replaced after 3 years (at the end of the trial period and not before) regardless of net condition but only on production of the trial net which may be stored in the meantime for inspection.

- If participants stop using the trial net for any reason, including accumulation of holes, they must store the net for replacement after 3 years, or give it to the investigators who will replace it after the 3-year trial period has elapsed.

Such consent by participants would fulfil the needs of the trial and may reduce non-attritional losses but would not affect participants' right to stop using their nets at any time for whatever reason.

b) Cross-sectional surveys

Other families who are eligible to be selected for cross-sectional surveys would have their nets replaced at the time of destructive sampling and would not be eligible for a second substitution at the end of the trial. Their consent form would be amended differently. They would be informed:

- That they should not give away or sell the study nets; and

- That they retain the freedom to stop using the nets at any time but are required to let investigators know the reasons when asked during the follow-up survey.

These were adopted by the WHO [19] and shall be included in the next edition of the WHO LLIN guidelines.

Treatment of other polymer nets and materials

While this Phase III field trial evaluated the bio-efficacy and wash-fastness of ICON Maxx on polyester nets, a parallel Phase I study assessed ICON Maxx treatments on netting made of cotton, polyethylene, nylon, white and dyed polyester. The aim was to widen the range of household materials that vector mosquitoes may encounter in broader range of settings, and which could be rendered insecticidal without having to specify target product profiles or create bespoke products which may not justify the cost of investment as specific products or interventions.

Evaluation compared WHO cone, cylinder and tunnel tests using An. gambiae. ICON Maxx treated polyester and polyethylene netting met the WHO cone and tunnel test bio-efficacy criteria for LLIN after 20 standardized washes, and nylon and cotton netting passed the WHO tunnel test criterion of 80% mortality after 20 washes. The correlation of these findings with the current Phase III data on polyester raises the prospect of using ICON Maxx as an effective approach for converting untreated nets, curtains, military clothing, blankets, top-sheets and tents and tarpaulins, as used in disasters and humanitarian emergencies, into effective long-lasting insecticidal products for vector control of malaria [25,26,27], leishmaniasis [28] and dengue [29]. It may also provide a solution to the problem of reduced LLIN coverage between universal coverage campaigns by enabling conversion of commercially sourced untreated polyester and polyethylene nets into LLINs via community treatment. It may also rise open a new door to binding of non-pyrethroid insecticides to nets and textile materials for control of pyrethroid resistant vectors.

Conclusion

This WHO Phase III household randomized trial of ICON Maxx treated polyester nets conducted in Tanzania achieved the combined cone and tunnel test efficacy criteria after 36 months of use. Based on this and other trials and noting the overall bio-efficacy of the ICON Maxx long-lasting treatment for polyester nets, full recommendation was granted with an estimated duration of insecticidal efficacy of 36 months depending on the local setting. To guarantee efficacy, ensure proper net treatment and to minimize losses to reasons other than physical integrity, health education leaflets and packages should be provided concurrently with ICON Maxx sachet distribution.

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Chapter 6: Bio-efficacy and wash-fastness of a lambda-cyhalothrin long-lasting insecticide treatment kit (ICON® Maxx) against mosquitoes on various polymer materials

Prologue:

Preceding chapter described a Phase III field trial that evaluated the bio-efficacy and wash-fastness of ICON Maxx on polyester nets. On the basis of this and other studies like this carried out in parallel in other countries full approval was granted to ICON Maxx by WHO pesticide evaluation scheme [33]. ICON Maxx offers the prospect of converting conventional polyester nets into LLIN through a dipping process that can be done post-manufacture under field conditions or in the home.

Knitted polyester is presently the more widely used material (WHO, 2003), but it is not the only material used in nets. Mosquito nets made from cotton are still widely used in West Africa, Iran and Pakistan. The global market for cotton nets in local retail sector remains high. Nylon nets are also used in India and Africa. The use of polyethylene nets is increasing and nets with finer weave are now available. Also, there is great diversity in the fabrics and materials used for making mosquito nets, blankets, tents and curtains. It is therefore important to demonstrate that ICON Maxx can fulfil the criteria of treatment on a range of netting materials other than polyester.

The still unexplored question still is, can this formulation make other types of material long-lasting? High efficacy and wash fastness reported, ICON Maxx might therefore also be potential in treating non-net substrates such as curtains, canvas tents or blankets for malaria control. Therefore, efficacy and wash resistance of ICON Maxx needs to be confirmed on nets made of cotton, nylon, polyethylene and other synthetic materials before this product can have the widest possible application or impact against malaria.

The present chapter report the findings of laboratory evaluation for the efficacy and wash-fastness of ICON Maxx on nets made of cotton, polyethylene, and nylon. The study also did evaluation of ICON Maxx on white and dyed polyester nets. This was done in comparison to the same netting

material but conventionally treated with lambda-cyhalothrin 2.5% CS (ICONET), a commercial microencapsulated formulation ('Icon', Syngenta UK) only without a binder.

Chapter 6: Efficacy of ICON® Maxx treatment kit on different polymer netting materials and the assessment of comparative performance of bioassay techniques

The material presented in this chapter has been published as:

Patrick K. Tungu, Wema S. Sudi, Harparkash Kaur, Stephen M. Magesa and Mark Rowland: Bio-efficacy and wash-fastness of a lambda-cyhalothrin long-lasting insecticide treatment kit (ICON® Maxx) against mosquitoes on various polymer materials: *Malar J* **20**, 387 (2021). https://doi.org/10.1186/s12936-021-03909-6

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2.3. Stage of publication – Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I supervised/performed the study, analysed the data, co-interpreted the findings and drafted the manuscript.

[redacted]

Candidate's signature_____

Supervisor or senior author's signature to confirm role as stated in (3) [redacted]

Abstract

Background

Long-lasting efficacy of insecticide-treated nets is a balance between adhesion, retention and migration of insecticide to the surface of netting fibres. ICON® Maxx is a twin-sachet 'home-treatment kit' of pyrethroid plus binding agent, recommended by the World Health Organization (WHO) for long-lasting, wash-fast treatment of polyester nets. While knitted polyester netting is widely used, fine woven polyethylene netting is increasingly available, and nets made of cotton and nylon are common in Africa and Asia. It is important to investigate whether ICON Maxx can fulfill the WHO criteria of long-lasting treatment on a range of domestic fabrics to widen the scope for malaria protection.

Methods

This study was a controlled comparison of the bio-efficacy and wash-fastness of lambda-cyhalothrin CS, with or without binder, on nets made of cotton, polyethylene, nylon, dyed and undyed polyester. Evaluation compared an array of bioassays: WHO cone and cylinder, median time to knockdown and WHO tunnel tests using Anopheles mosquitoes. Chemical assay revealed further insight.

Results

ICON Maxx treated polyethylene and polyester netting met the WHO cone and tunnel test bio-efficacy criteria for LLIN after 20 standardized washes. Although nylon and cotton netting failed to meet the WHO cone and cylinder criteria, both materials passed the WHO tunnel test criterion of 80% mortality after 20 washes. All materials treated with standard lambda-cyhalothrin CS without binder failed to meet any of the WHO bio-efficacy criteria within 5 washes.

Conclusion

The bio-efficacy of ICON Maxx against mosquitoes on netting washed up to 20 times demonstrated wash durability on a range of synthetic polymer and natural fibres: polyester, polyethylene, nylon and cotton. This raises the prospect of making insecticide-binder kits into an effective approach for turning untreated nets, curtains, military clothing, blankets—and tents and tarpaulins as used in disasters and humanitarian emergencies—into effective malaria prevention products. It may provide a solution to the problem of reduced LLIN coverage between campaigns by converting commercially sourced untreated nets into LLINs through community or home treatment. It may also open the door to binding of non-pyrethroid insecticides to nets and textiles for control of pyrethroid resistant vectors.

Background

Insecticide-treated mosquito nets (ITNs), developed during the 1980s proved highly effective in reducing malaria-related morbidity and mortality [1]. Operationally, however, ITNs suffered several challenges in the field; these included the logistical problem of having to retreat nets every 12 months, the recurrent cost of annual retreatment and the unavailability of insecticides in remote places [2].

The advent of long-lasting insecticidal nets (LLIN) that do not require insecticide retreatment over a 3-years' lifespan provided a technical solution to the logistical challenge of low retreatment coverage [3,4,5,6]. LLIN have since become the essential tool for vector control and malaria prevention in sub-Saharan Africa. The World Health Organization (WHO) recommends and promotes universal coverage of 1.0 LLIN for every 1.8 persons in populations at risk in malaria endemic countries [7]. The push towards this target has led to increased demand for LLIN by

national malaria control programmes, international malaria control agencies and institutional buyers who have increasingly opted for LLIN as their preferred choice of malaria prevention [2, 8].

Thus far, international malaria control agencies have spent over two billion dollars on the provision of LLINs, leading to scale-up of access, which currently exceeds 50% of the population of sub-Saharan Africa [7]. The target of universal coverage is critical to success and while 50% is an impressive achievement, malaria elimination remains a distant prospect, and millions of African households remain unprotected particularly in the later stages, between universal coverage campaigns [9].

LLINs are treated with insecticide during net manufacture. However, the majority of ITN that are available through the commercial retail sector are not LLIN [9] and those nets in use, sourced from retail outlets, have either never been treated or were treated only once at the time of purchase [9, 10]. Locally sourced nets, which are not LLIN, may lose efficacy prematurely, long before the nets physically perish from wear and tear [9, 10]. This raises a need for treatment kits that can convert these nets post-manufacture into long-lasting insecticidal nets through simple household or community dipping.

Progress has been made with long-lasting treatment kits that can transform untreated nets into long-lasting treated nets by combining a conventional insecticide with a binding agent and the simple act of immersion into aqueous solution of the mixture. With this technology the untreated or conventionally treated nets already in use may be transformed into LLINs by the community post-manufacture under field conditions.

ICON Maxx is a long-lasting insecticide formulation developed by Syngenta in kit form [11]. Thus far, ICON Maxx is the only long-lasting insecticide treatment kit that has full recommendation of the World Health Organization for use on polyester nets [12, 13]. The kit is based on a slow-release capsule suspension (CS) formulation of lambda-cyhalothrin previously evaluated by the WHO and recommended for treatment of mosquito nets. ICON Maxx is presented as a twin sachet pack, containing lambda-cyhalothrin 10CS and binding agent, sufficient for the treatment

of an individual mosquito net. The target dose of ICON Maxx on a family-size polyester mosquito net is 62 mg AI/m2. The actual dose received depends on the net size and can range from 50 mg AI/m2 (for a large family-size net) to 83 mg AI/m2 (for a single-size net). Efficacy and wash fastness of ICON Maxx has been demonstrated in several laboratory, experimental hut and field trials [14,15,16]. In all these studies the demonstration was mad0e on nets made of polyester netting [14,15,16,17]. Although polyester is currently more widely used [15] it is not the only polymer used. Use of polyethylene nets is increasing, and nets of fine polyethylene weave are now available. Mosquito nets made traditionally from cotton are also common in countries of West Africa and South Asia. The global local retail market for cotton nets remains high. It is estimated that over 50% of nets sold in Iran and Pakistan are made of cotton. Nylon nets are used in India and Africa. There is also great diversity in the fabrics, and synthetic polymers used in curtains, blankets and other barriers to mosquitoes that are potentially treatable in the home.

The question is whether binder formulations can make these other types of polymers, aside from polyester, long-lasting. The efficacy and wash resistance of ICON Maxx needs to be confirmed on nets made of cotton, nylon, polyethylene and other synthetic materials before this product can have the widest possible application or impact on malaria.

Polyester and other netting materials come in a range of colours. There is some evidence that dye may affect the uptake and retention of conventional insecticide formulations during immersion [15]. It is important to confirm that uptake and retention of insecticide-plus-binder is not adversely affected by textile finishing.

The present study reports on the laboratory evaluation of bio-efficacy and wash-fastness of ICON Maxx on netting made of cotton, polyethylene, nylon, white and dyed polyester nets. This was done in controlled comparison with the same netting materials conventionally treated with lambda-cyhalothrin CS, a microencapsulated formulation (‘Iconet’, Syngenta UK) that does not include the long-lasting binder component.

Methods

Netting and treatment

Polyester white, polyester blue, polyethylene, cotton and nylon netting materials were used as substrates. Cotton nets were sourced from a manufacturer in Pakistan that supplies the national army, the polyethylene and nylon nets were sourced from manufacturers in India and the polyester nets were supplied by Vestergaard Frandsen. The absorbency of each material was determined using a test solution of ICON Maxx in de-ionized water. Solutions of ICON Maxx were specially prepared to match each material's absorbency to achieve a similar target loading dose per unit surface area of 62 mg/m2 for ICON Maxx and 15 mg/m2 for Iconet. The nets were considered treated when all solution had been absorbed and all areas of the net were visibly wet without any dripping. The nets were dried horizontally in a darkened room at 30 °C on polythene sheeting and turned over every 10 min until completely dry. Each material was then cut into five 60 cm × 40 cm samples. Various positive and negative controls were introduced. Untreated samples of each material were retained as negative controls. Netting of each material treated with lambda-cyhalothrin 2.5% CS (Iconet), without binder formulation, served as positive controls for the ICON Maxx treated materials. ICON Maxx treated polyester white was used as the reference arm as it had already received recommendation by the WHO [12, 13].

Washing procedure

Samples of each material were washed 0, 5, 10, 15 or 20 times. All samples were washed as 60 cm × 40 cm pieces except the polyethylene which was stiffer and harder to immerse and had to be cut into two to ensure thorough washing. The standard WHO Phase 1 laboratory washing procedure was adopted [14]. A soap solution of 2 g/l was produced using the soap Savon de Marseille and de-ionized water. Each net was placed in a 1 l bottle and immersed in 500 ml of soap solution before placement in a water bath. All samples were shaken at a rate of 155 movements per minute and remained immersed at 30 °C for 10 min. Each swatch was rinsed twice in de-ionized water under the same water bath conditions. A piece of each treated material was kept unwashed to serve as the zero-washed sample. Washing started on 20-wash pieces 20 working days (4 working weeks) before testing was due to start on 15 wash pieces 5 days later, down to 5-wash pieces 5 days before pieces were due to be tested in rotation.

Mosquitoes

All mosquitoes used were insectary reared non-blood fed female pyrethroid susceptible Anopheles gambiae sensu stricto (s.s.) (Diptera: Culicidae) mosquitoes (Kisumu strain), susceptible to all pyrethroids, reared in the National Institute for Medical Research, Ubware Centre. Pyrethroid susceptible mosquitoes were used as these were most sensitive for showing changes in binding affinity of the formulations on polymers over multiple washes. Pyrethroid resistant mosquitoes are less sensitive/suitable for demonstrating pyrethroid-binder retention/loss when using mortality/or knockdown is the outcome measures.

Cone bioassays

To evaluate the efficacy of ICON Maxx and Iconet treated netting materials, standard WHO cone bioassays were performed, based on the WHO Phase I protocol [14] against insectary-reared pyrethroid-susceptible An. gambiae Kisumu strain. Four WHO cones were fixed to each netting sample and 5 mosquitoes aged 2–5 days old were introduced into each cone. After 3 min exposure the mosquitoes were transferred to holding cups. Control mosquitoes were exposed to untreated netting. The 20 mosquitoes tested per replicate were provided with a pad of glucose solution for nourishment. Tests were done at 25 °C and 70% RH. Knock-down (KD) was recorded 1-h post-exposure and mortality 24 h later. Five replicates were carried out per sample, 100 mosquitoes per treatment. If control mortality exceeded 10% on any day the results were discounted and the test repeated; this procedure was followed in all bioassay tests described. All replicates of the various textile-wash treatments were carried out in strict rotation using Latin squares to adjust for any variation in insect batch or test conditions.

Cylinder bioassays

In preparation for this assay, treated and washed samples of each material were cut and stapled to pieces of plain paper measuring 12 cm × 15 cm before insertion inside WHO susceptibility test cylinders and securing with metal rings. Ten 2–3-days old female mosquitoes were introduced to each holding chamber and transferred into the test chamber where they were exposed for 3 min. After exposure, the mosquitoes were returned to the holding chambers and given access to sugar solution. The number knocked down was recorded after 60 min and the number dead was

recorded 24 h later. A negative control of mosquitoes exposed to untreated netting material was conducted in parallel each test.

Median time to knock down (MTKD)

In the MTKD bioassay eleven mosquitoes 2–3 days old were introduced into a WHO wire ball frame covered with the treated material [14]. Knock down was defined as a mosquito lying either on its back, side or no longer able to support itself. The time taken for each mosquito to knockdown was recorded up to the median (6th) mosquito. Nine replicates were conducted for each treatment, material and wash number, using Latin squares. Untreated net of each material was used as a negative control.

Tunnel tests

Tunnel tests were used to assess unwashed treated netting and netting washed 20 times as a proxy for 3 years of field use, as per WHO guidelines [14]. Design, dimensions and procedure of experimental huts is described in detail in appendix 1, section 2.3.1.2.

Chemical analysis

High pressure liquid chromatography HPLC was used to determine the concentration of insecticide on each treated piece of net after washing the requisite number of times. Four pieces measuring 5 × 5 cm of each net treatment were cut and placed in a borosilicate glass vial with 1 ml of acetronitrile. The vials were sonicated for 10 min; the solution was removed and placed in HPLC vials. The HPLC analysis was conducted at the London School of Hygiene and Tropical Medicine using a Dionex Summit range of equipment and software (Camberley, Surrey, UK). The samples were separated using an AcclaimR C18 120 (250 × 4.6 mm, Dionex, UK) column eluted with water/acetonitrile (90:10%; v/v) at a flow rate of 2 ml/min and passed through the photodiode array detector (PDA-100, Dionex) set at 27 nm. The authenticity of the detected peaks was determined by comparison of retention time, spectral extraction at 275 nm and spiking the sample with commercially available standards.

Statistical analysis

Mixed effects generalized linear models using STATA® 15 (Stata Corporation, Collage Station, TX, USA 2005) were used for analysis. The independent variables included treatment (ICON Maxx, Iconet), net material (the 5 types of polymers), number of washes (0, 5, 10, 15, 20 for Icon Maxx or 0, 5, 10 for Iconet), number of replicates adjusting for group size, and the interactions between net type and number of washes. Mixed effects linear regression was used to analyse the content of lambda-cyhalothrin in netting samples (AI retention index) extracted by HPLC, adjusting between netting materials, treatments and number of washes. Mixed effects logistic regression models were used to analyse the change in biological responses (proportions killed, knocked down) after washing of materials, and testing with cone, cylinder or tunnel tests.

Ethical clearance

Approval was obtained from the ethics committees of the London School of Hygiene and Tropical Medicine and the Tanzanian National Institute of Medical Research (Ref: NIMR/HQ/R.8a/Vol. X/86). The procedure for use of guinea pigs in tunnel tests conformed to criteria established in EC Directive 86/609/ECC regarding protection of animals used for experimental purposes.

Results

Chemical analysis

Despite applying the target dose of 15 mg/m2 lambda-cyhalothrin, chemical analysis by HPLC of the Iconet CS treated materials (without binder) showed that cotton and undyed polyester (white) had higher affinity for lambda-cyhalothrin CS as compared to dyed polyester (blue), nylon and polyethylene (mixed effects linear regression $F(4, 15) = 26.5$, $p = 0.005$). Within 5 washes almost all detectable lambda-cyhalothrin was removed from the two polyester nettings and less than 1 mg/m2 was detectable on polyethylene and nylon. More insecticide (4.8 mg/m2) was retained in cotton fibres than in other nettings at 5 washings (Mixed effects linear regression $F(4, 15) = 207.9$, $p = 0.0001$) (Fig. 1a).

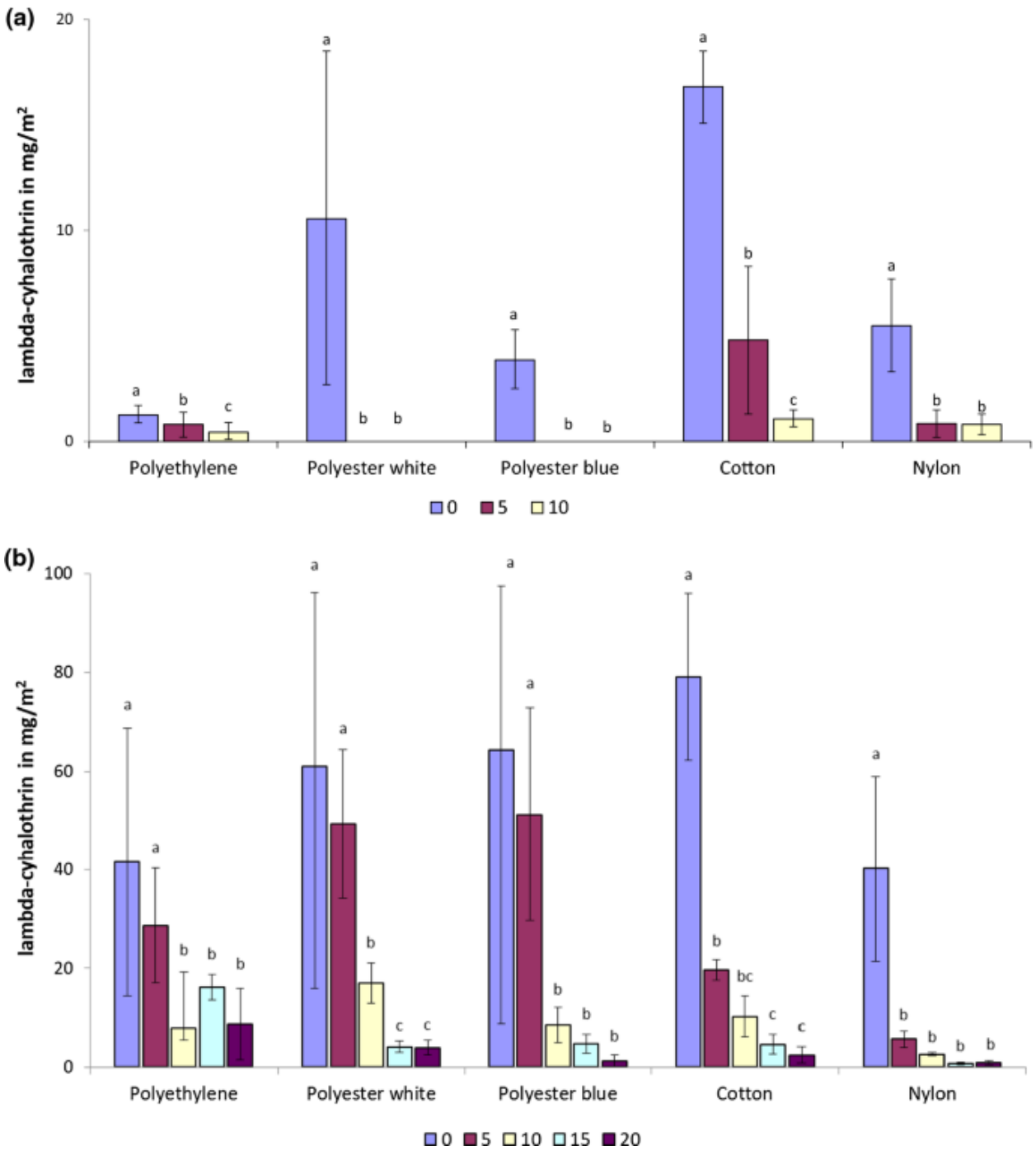


Fig. 1 a Mean lambda-cyhalothrin content (± 95% CI) for netting materials treated with Iconet and washed up to 10 times. b Mean lambda-cyhalothrin content (± 95% CI) for netting materials treated with ICON Maxx and washed up to 20 times

Chemical analysis of the ICON Maxx treated materials (with binder) showed that all materials had higher affinity for the pyrethroid with binder than for pyrethroid without binder as compared with the Iconet formulation (Mixed effects linear regression, $F(1, 38) = 60{,}8$, $p = 0.0001$). Within

the ICON Maxx treatments, cotton and polyester undyed and dyed (white and blue) showed higher affinity or absorption (79, 61, 64 mg/m2, respectively), with loading dosages of lambda-cyhalothrin similar to the target dose of 62 mg/m2, whilst polyethylene and nylon showed lower loading dosages of only 42 and 40 mg/m2, well below the intended target (Fig. 1b). After washing 0–5 times, the two polyesters and the polyethylene showed particularly high retention of insecticide, with over 70% of the initial lambda-cyhalothrin content remaining (Fig. 2): none of these 3 materials showing a significant decline in content after 5 washes (Fig. 1b). The mixed effects linear regression showed that the loss of insecticide was significantly greater in cotton ($t = -17.0$, $p = 0.001$) and nylon ($t = -9.2$, $p = 0.001$) at 5 washes (Fig. 1b) with neither material retaining more than 25% of loading dose (Fig. 2). Comparing polyester blue and polyester white over all 0–20 washes there was no evidence that polyester blue showed less affinity for ICON Maxx on impregnation or retained less AI than polyester white over 0–20 washes ($t = 0.20$, $p = 0.845$) (Figs. 1b, 2) in the mixed effects linear regression. The only material of the 5 assessed in which binding and retention of AI over 20 washes was significantly less than the other materials in the analysis was nylon ($t = -3.69$, $p = 0.001$).

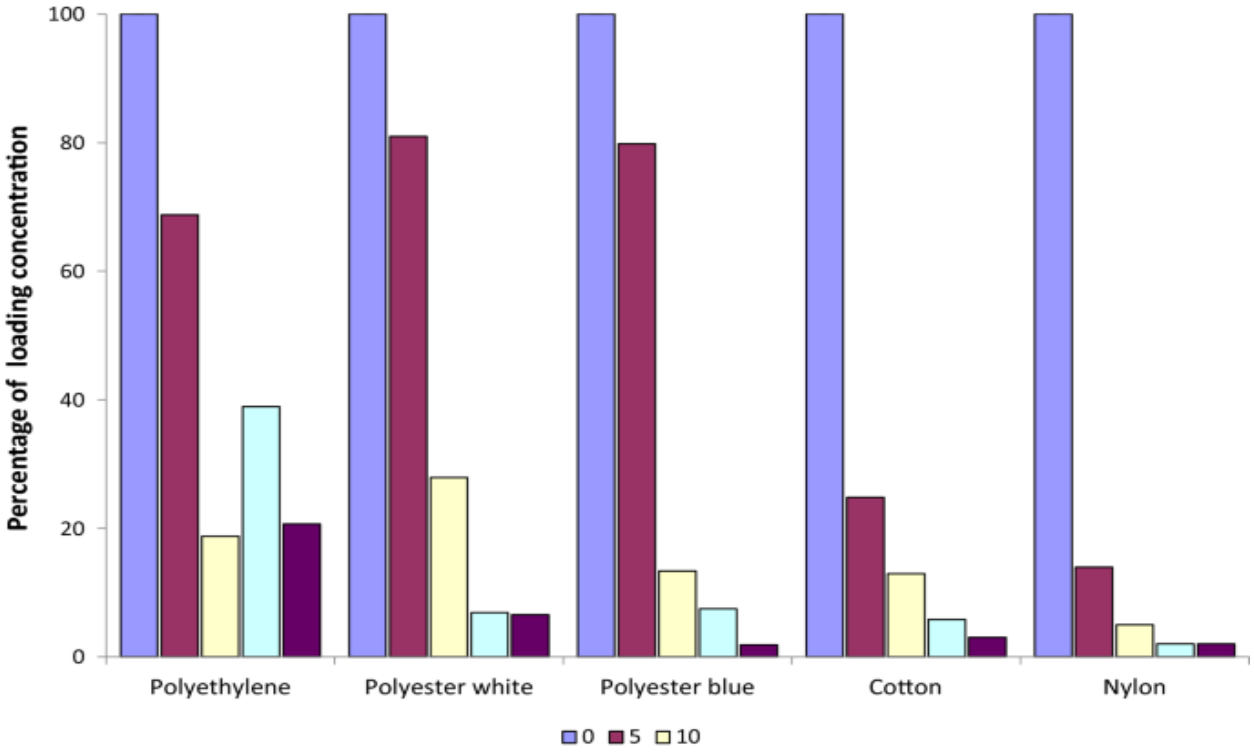
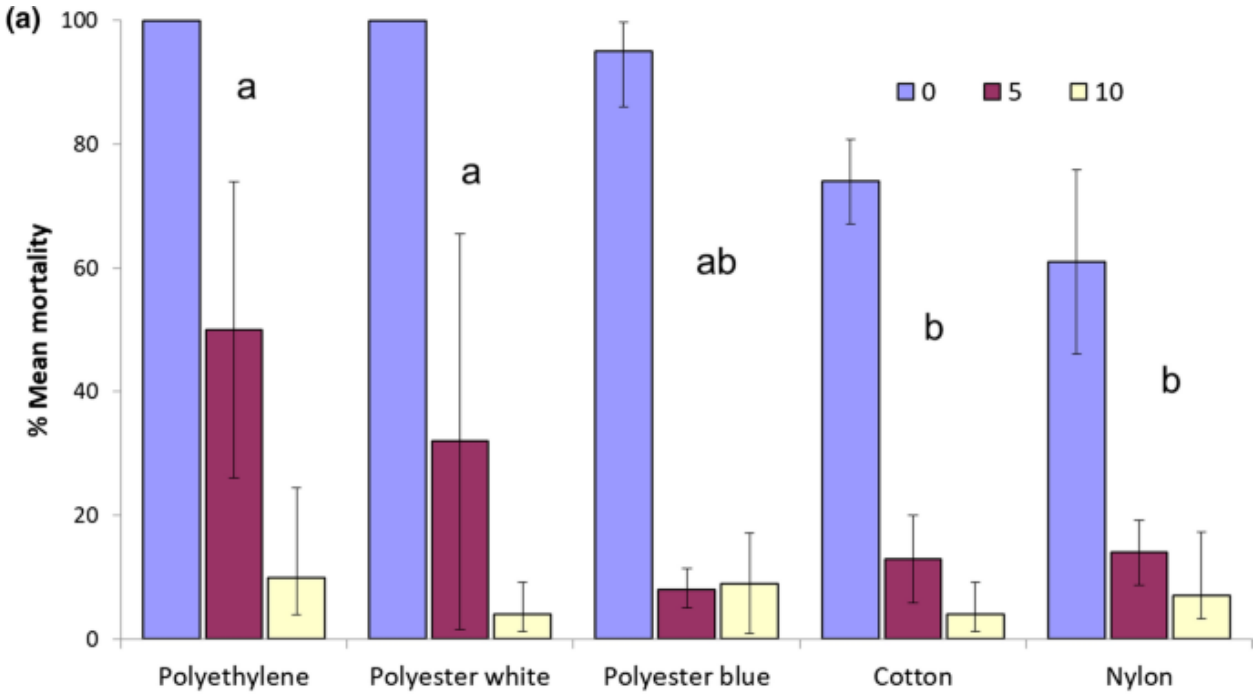


Fig. 2 **Content of lambda-cyhalothrin as a percentage of the ICON Maxx loading dose over 20 washes.**

Cone bioassays

Iconet treated materials.

The mosquito mortalities induced by Iconet treated polyester white, polyester blue and polyethylene in cone tests all exceeded 90% after loading, whilst cotton and nylon only induced between 60 and 80% mortality after treatment (Fig. 3a). Comparing all materials (using mixed effects logistic regression, adjusting for sample size in replicate tests), polyethylene and polyester white recorded higher mortality than other materials across the first 5 washes ($z = -1.75$, $p = 0.001$). Mortality decreased to less than 10% after 10 washes across all materials. The knockdown trend was consistent with the mortality trend (Table 1).



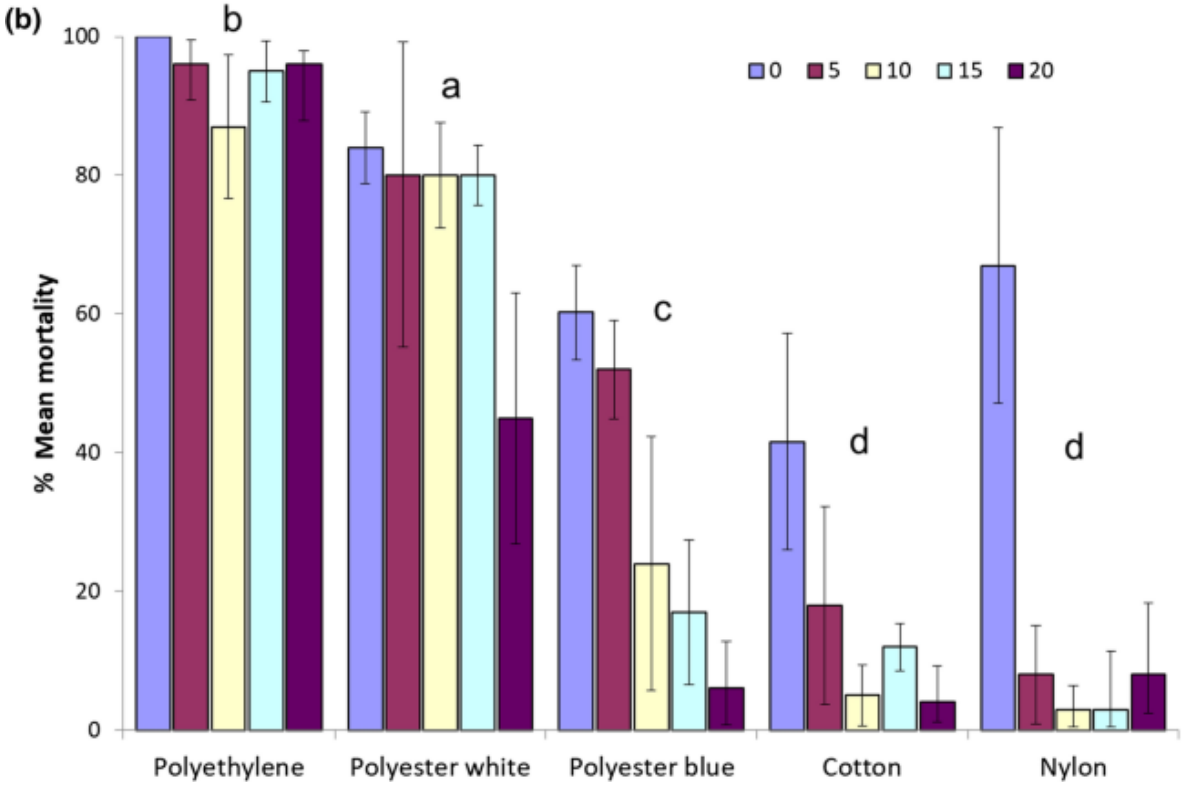


Fig. 3 a Cone bioassay: Percentage mortality (± 95% CI) at 24 h after exposure for netting materials treated with Iconet and washed up to 10 times. b Cone bioassay: Percentage mortality (± 95% CI) at 24 h after exposure for netting materials treated with ICON Maxx and washed up to 20 times

Table 1 Cone bioassays: percentage knockdown at 60 min after exposure to netting materials treated with Iconet or ICON Maxx and washed up to 10 times or 20 times, respectively.

Washes	Polyethylene	Polyester white	Polyester blue	Cotton	Nylon
Iconet					
0	100	100	90	100	96
5	70	56	14	13	9
10	40	15	5	4	0
IconMaxx					
0	98	72	90	65	100
5	99	70	81	39	76
10	64	72	36	6	22
15	99	66	24	15	0
20	99	39	1	2	3

ICON Maxx treated materials.

The mortality induced by ICON Maxx treated polyethylene netting in cone bioassays was 100% after loading and exceeded 90% mortality after 20 washes. Mortality on polyethylene was significantly higher than on all other materials across each wash point including polyester white ($z = 5.8$, $p = 0.001$). Mortality induced by polyester white exceeded 80% over 0–15 washes decreasing to 45% only after 20 washes. Mortality induced by polyester blue was only 60% at loading decreasing to 24% at 10 and 6% at 20 washes, thus confirming the poorer adhesion and retention of the binder formulation on dyed compared to undyed polyester ($z = -10.6$, $p = 0.001$). Mortality of ICON Maxx on cotton and nylon while initially efficacious was not sustained after 5 washes, and retention on these materials was lowest of all ($z = -14.3$, $p = 0.0001$).

The knockdown trend was consistent with mortality; polyethylene recorded higher knockdown than any other material, followed by polyester white and then polyester blue. Percentage knockdown on treated cotton and nylon decreased after 10–15 washes (Fig. 3b; Table 1).

Cylinder bioassays

Iconet treated materials.

As in cone tests, the highest mortality recorded in cylinder tests with Iconet treated netting was with polyethylene, polyester white, polyester blue and cotton, followed by nylon (71%). Within 5 washes efficacy was inadequate.

With respect to knockdown, all unwashed materials recorded 100% knockdown at 60 min except nylon at 85%. However, at 5 washes, percentage knockdown on all materials except polyethylene had decreased to 50% or less (Fig. 4a; Table 2).

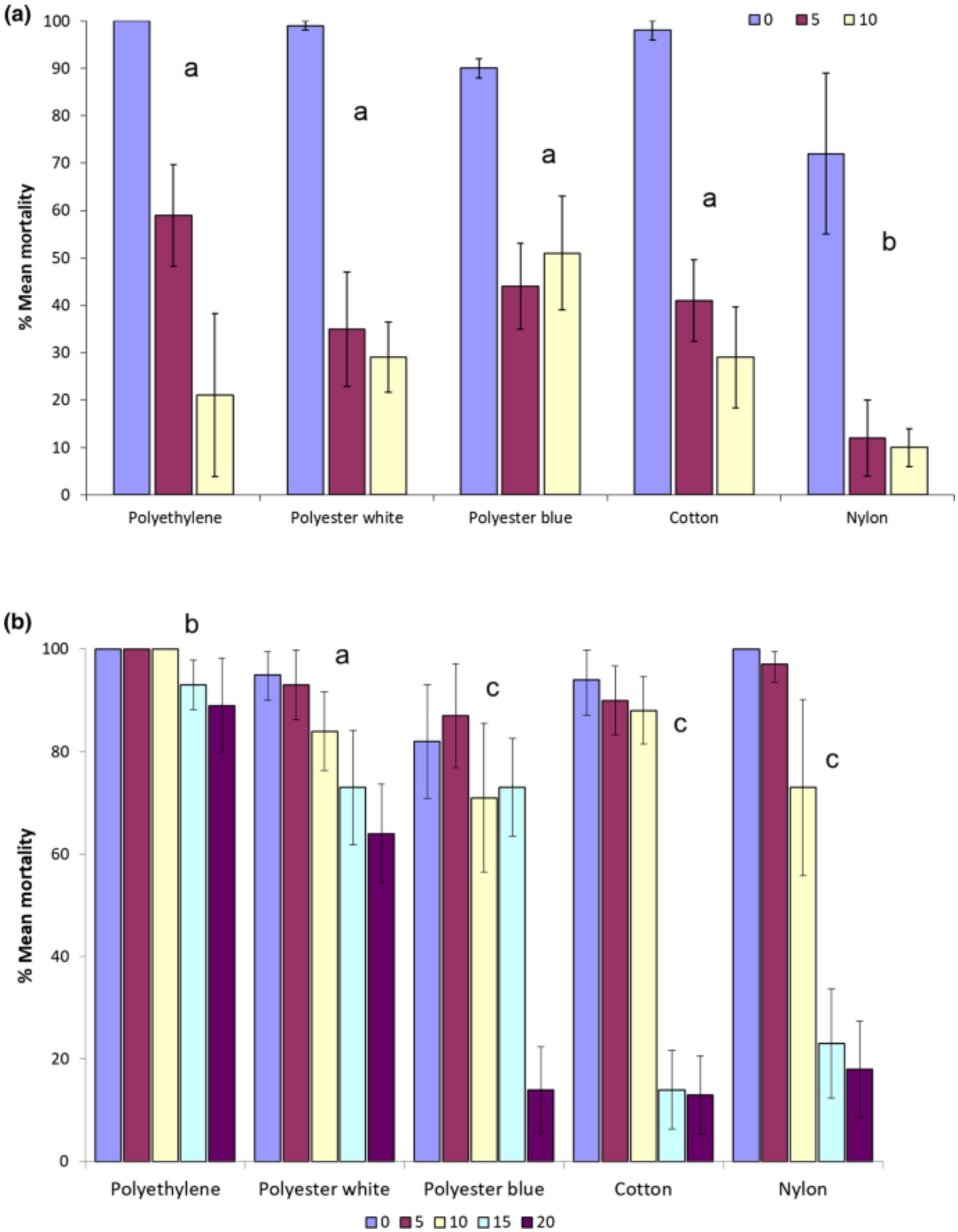


Fig. 4 a Cylinder bioassay: Percentage mortality (± 95% CI) at 24 h after exposure for netting materials treated with Iconet and washed up to 10 times. b Cylinder bioassay: Percentage mortality (± 95% CI) at 24 h after exposure for netting materials treated with ICON Maxx and washed up to 20 times

Table 2 Cylinder bioassays: percentage knockdown 60 min after exposure to netting materials treated with Iconet or ICON Maxx and washed up to times or 20 times respectively.

Washes	Polyethylene	Polyester white	Polyester blue	Cotton	Nylon
Iconet					
0	100	100	100	100	85
5	70	15	29	50	6
10	38	1	25	35	1
IconMaxx					
0	100	100	97	99	100
5	100	100	97	99	100
10	100	93	94	97	75
15	100	94	85	49	20
20	100	88	10	35	21

ICON Maxx treated materials.

With all netting materials, cylinder mortality was exceptionally high (> 95%) after treatment (0 washes) and at 5 washes. Polyethylene recorded significantly higher performance than all other materials with 100% mortality at 0, 5 and 10 washes and was the only material to exceed 80% mortality at 20 washes ($z = 8.18$, $p = 0.0001$). Polyester white and cotton exceeded 80% mortality at 10 washes. Polyester blue exceeded 70% at 15 washes. Polyester white recorded significantly higher mortality than polyester blue from 0 to 20 washes ($z = 4.34$, $p = 0.001$). Polyester white decreased below 80% after 15 washes and blue after 10 washes. Mortalities recorded with nylon and cotton decreased below 20% at 15 washes (Fig. 4b). The only materials that incurred loss of activity at 15 washes were cotton and nylon.

With respect to 60-min knockdown, every material recorded between 100 and 97% knockdown after 0 and 5 washes. After 20 washes, only polyethylene showed 100% knockdown and only polyester white recorded 80% knockdown. Knockdown on cotton and nylon showed decrease between 10 and 15 washes (Table 2).

ICON Maxx comparative efficacy in cone and cylinder bioassays

Comparing all treated materials, higher mortality was recorded in the cylinder bioassay as compared to the cone bioassay at each wash point (mixed effects logistic regression, $z = 10.6$, $p = 0.001$) (Figs. 5a, b). As exposure time was the same in cone and cylinder, this higher mortality was probably due to a higher ratio of netting covered surface to uncovered plastic surface in the cylinder as compared to the cone. Mortality was consistently high in the cylinder at 0 washes (> 80% mortality) for each material tested compared to the cone (Fig. 5a). At 20 washes the difference in mortality between cylinder and cone was smaller and yet consistent for each material. The difference in mortality between cone and cylinder was due to variation in AI retention between the 5 materials ($z = 4.8$ to 9.0, $p = 0.001$) in addition to differences in efficacy between cylinder and cone ($z = 10.6$, $p < 0.001$) (Fig. 5b).

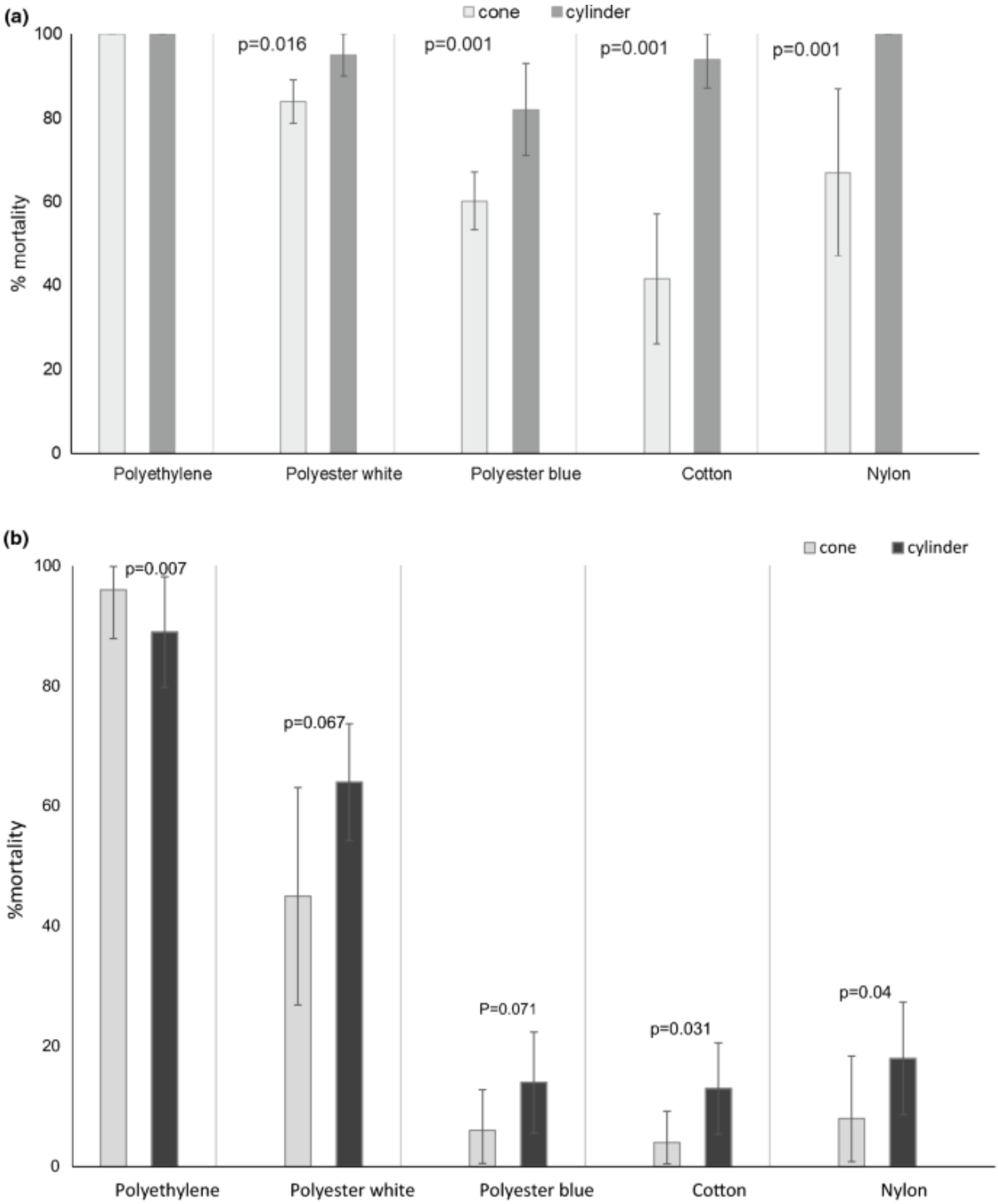


Fig. 5 a Cone and cylinder bioassay comparison: percentage mortality (± 95% CI) at 24 h after exposure for polyester white nets treated with ICON Maxx at 0 wash point. b Cone and cylinder bioassays comparison: % mean mortality at 24 h post exposure (± 95% confidence intervals) for polyester white nets treated with ICON Maxx at 20 wash point

Tunnel tests

With the unwashed ICON Maxx treated materials, blood-feeding inhibition was higher than the WHO 90% threshold with polyethylene, polyester white and polyester blue. Percentage mortality with all 5 materials ranged from 91 to 96%, well above the 80% threshold. After 20 washes, polyethylene, nylon and polyester white all passed the WHO criterion of 90% blood-feeding inhibition (Table 3). Percentage mortality with polyethylene, cotton and nylon ranged from 83 to 88%; hence all these polymers passed the WHO mortality criterion. Mortality was less than the 80% threshold with only polyester white (the reference net) and polyester blue.

Table 3 Tunnel tests: percentage passage, blood-feeding inhibition and mortality after exposure to ICON Maxx treated netting at 0 and 20 washes.

Number of washes	Material	Passage inhibition %	Blood-feeding inhibition %	Mortality %
0	Untreated net	56	–	0
	Polyethylene	57	90	95
	Polyester white	82	100	96
	Polyester blue	64	93	91
	Cotton	37	82	94
	Nylon	16	100	94
20	Polyethylene	29	90	86
	Polyester white	22	100	54
	Polyester blue	25	86	65
	Cotton	24	75	83
	Nylon	14	100	88

Comparing cylinder and tunnel at 0 washes, all materials recorded well over 90% mortality in both assays. At 20 washes (the critical threshold) all materials (except polyethylene) recorded below 20% mortality in cone and cylinder and yet all materials passed the tunnel test except for polyester white and blue. Not surprisingly perhaps, there was no association between the results

of tunnel tests and the results of cone and cylinder tests; the only exception was polyethylene which passed the thresholds of all the bioassays.

Median time to knock down bioassays.

Median time to knock down (MTKD) is considered a good indicator of surface AI. By noting the median responder in an MTKD assay and running several replications, it is possible to generate the confidence interval around the mean of the medians. MTKD with all unwashed Iconet treated materials were statistically similar except for nylon, which took 1.5–2 times longer to reach than other materials. After 5 washes and 30 min testing, no Iconet treated materials reach median knockdown.

With all ICON Maxx treated materials, the difference in MTKD between 0- and 5-times washed materials did not differ significantly; thus, the binder was retaining the lambda-cyhalothrin on the netting surfaces (Table 4). With polyethylene white and polyester blue, MTKD was not showing significant differences between 0 and 10 washes, although there was some indication of MTKD taking longer to reach 10 washes. With cotton and nylon, MTKD was not reached within 30 min exposure on 10-times washed netting indicating loss of bioavailability of surface AI on these samples (Table 4).

Table 4 Median time to knock down in minutes (95% CI) on ICON Maxx treated materials after 0, 5 and 10 washes.

Material	0 washes	5 washes	10 washes
Polyethylene	$10^{a,1}$ (0.9)	$11.8^{a,1}$ (0.9)	$14.2^{a,2}$ (0.6)
Polyester white	$14.6^{b,1}$ (0.6)	$13.7^{b,1}$ (0.9)	$14.7^{a,1}$ (0.6)
Polyester blue	$9^{a,1}$ (1.3)	$10.3^{a,1}$ (1.6)	$14.1^{a,2}$ (0.8)
Cotton	$14.5^{b,1}$ (0.8)	$14.1^{b,1}$ (0.8)	$> 30^{c,2}$ (0)
Nylon	$10.3^{a,1}$ (1.1)	$14.2^{b,2}$ (0.7)	$> 30^{c,3}$ (0)

Within each wash-point column, materials sharing same letter superscripts do not differ statistically ($p \geq 0.05$). Within each wash-point row, materials sharing same numeric superscripts do not differ statistically ($p \geq 0.05$)

Regression analysis showed surface content of insecticide had significant effect on MTKD; for every 1 mg decrease in insecticide content there was 3 s increase in MTKD ($F_{1,50} = 6.27$, $p = 0.0156$).

Discussion

There were four objectives. The primary objective was to determine whether the pyrethroid lambda-cyhalothrin bound within a polymer resin could improve the wash fastness of the insecticide on nets made from a range of synthetic polymers, natural fibres and dye finishes. To complement the study, controlled comparison was made with a standard pyrethroid CS treatment which lacked the binder. The second objective was to take the treatments through the WHO LLIN evaluation process to determine which insecticide-treated substrates would withstand 15–20 washes and potentially achieve WHO recommendation. The third objective was to introduce new types of bioassays and compare against the cone test to test their potential utility. The fourth, but not least, was to consider some currently neglected humanitarian contexts badly in need of vector borne disease control and consider whether insecticide-binder treated substrates could provide a solution.

According to WHO, LLIN evaluation guides, polyethylene, cotton and nylon treated with ICON Maxx met the tunnel test criteria of > 80% mortality after 20 washes in Phase I, which is a recognized surrogate for 3 years of pyrethroid durability on household LLIN [18]. Polyester white (undyed), the positive control, and polyester blue fell short of the WHO tunnel criteria in Phase I tests but elsewhere in other studies they did achieve the WHO threshold [16, 17, 19], emphasizing the importance of multiple trials before coming to a consensus conclusion. Polyethylene also met the required criterion of > 80% mortality in cone tests and was the best performing polymer of the four tested. In all bioassays except the tunnel test, cotton and nylon netting did not reach the WHO threshold. Most of the textiles performed well in one or more types of bioassays and none can be ruled out as a suitable substrate for vector control treatment. Even polyester white, the positive control, which failed to meet the required threshold in the Phase I, fared well in Phase II (experimental hut) trials and Phase III (field trials of insecticide

durability) in the same locality [12, 13, 16]. Therefore, the Phase I tests reported here are best viewed comparatively, one textile versus another, rather than as pass or fail.

By contrast, none of the nettings—polymer or natural fibre—withstood more than a few washes, when treated with a standard lambda-cyhalothrin CS formulation. There is no question of the superiority of the binder formulation, which is a genuine technical advance for a variety of potential malaria control substrates or contexts [12].

Nylon

Nylon showed poorer adhesion of ICON Maxx on loading and poorest wash retention of all materials, losing 84% of insecticide content within 5 washes and 98% within 20 washes. In cone bioassay, mortality decreased by 92% within 5 washes and yet in cylinder bioassay where most of the interior was netting-covered, mortality stood at 97% after 5 washes and only decreased to low level after 10–15 washes. In tunnel test, ICON Maxx treated nylon passed the WHO criterion of > 80% mortality at 20 washes. Of all the materials tested, nylon was the most unpredictable. While its efficacy in tunnel was encouraging, nylon failed as a substrate of preferred choice for ICON Maxx treatment due to the poorer absorption, adhesion and wash-resilience. If there is a choice of material, the better option would be substitution of nylon with a better adhering or wash-tolerant polymer. On ICON Maxx treated nylon net curtains, as a barrier against Aedes and for prevention of Aedes borne arboviruses, it may have potential, warranting further studies in household conditions.

Cotton

Owing to the high absorptive property of cotton, the cotton samples contained the highest loading dose of ICON Maxx initially. However, as on nylon, adhesion and retention of the insecticide was poor, content decreasing by 75% after 5 washes and by 96% after 15 washes. Cone bioassay recorded only 40% mortality at 0 washes and 4% at 20 washes but, as was the case with nylon, mortality in cylinders was high between zero and 10 washes and only decreased sharply at 15 washes. As with nylon, cotton passed the tunnel test criterion for LLIN at 20 washes.

The presence of a high dose of lambda-cyhalothrin together with a low insecticidal activity suggests that bioavailability on the surface of cotton netting fibres is low, that is, most of the insecticide remained locked within the cotton fibres and failed to contact mosquito tarsi. This was not the case with synthetic fabrics such as polyester and polyethylene on which the insecticide is readily bio-available on the surface of fibres. Other studies have also reported the low insecticidal property of pyrethroids on cotton as compared to other fabrics [20, 21]. However, with the tunnel test, results with cotton netting exceeded 80% mortality after twenty washes, so bringing cotton into line with WHO criteria for recommendation [14].

Polyethylene

As with nylon, polyethylene treatment demonstrated a relatively low loading dosage (40 mg/m2) but in contrast to nylon and cotton, polyethylene showed better retention at 5 washes and a more regular loss rate over the course of 0–20 washes. Mortality in cone and cylinder bioassay was consistently high (~ 95%) over the course of 20 washes, and thus a completely different trajectory compared with nylon and cotton. ICON Maxx seemed to stay bound to the polyethylene, which remained fully toxic whereas the binder seemed lost from nylon and cotton during washing. As with nylon and cotton, polyethylene exceeded the tunnel test criteria at 20 washes.

Polyethylene seems an ideal substrate for ICON Maxx. In some studies, polyethylene netting materials were shown to be strong and able to tolerate five years of field use [3, 22]. More recently in larger scale surveys polyethylene has shown poor durability [23], somewhat improved by changing the knitting weave [23].

Polyester

The superiority of ICON Maxx on polyethylene compared to undyed polyester white was a surprise since the latter was the positive control and the polymer netting that ICON Maxx was designed for originally. While both polyester white and blue fell consistently short of polyethylene in an array of bioassay tests, ICON Maxx did attain WHO recommendation for use

on polyester over 15–20 washes which is a significant increase in wash-tolerance compared to the standard CS formulation tested in this paper. After Phase II (experimental hut) trials and Phase III (three-year field trials of insecticide durability) ICON Maxx did attain WHO recommendation as a polyester long-lasting treatment [13]. Comparing undyed and dyed polyester netting, the chemical analysis indicated similar loading dosages, implying that the binder in ICON Maxx had largely overcome the poor adherence induced by the dye of earlier formulations on polyester [18]. At most wash points the rate of loss of insecticide was similar between polyester blue and polyester white treated with ICON Maxx. While polyester white tended to record greater mortality than polyester blue in some bioassays, the differences were marginal and not consistent between all types of bioassays.

Comparison of ICON Maxx with KO-Tab 123

ICON Maxx is not the first wash-resilient formulation to be developed [12]. KO-Tab 123 was a wash-resilient formulation of deltamethrin (25 mg/m2) and binder rather than lamba-cyhalothrin (55 mg/m2) and binder in ICON Maxx [24]. Its development coincided with the development of factory produced LLIN and it was not taken forward to Phase III field trials. Had it done so, it might have proven as effective as ICON Maxx, which did go on to Phase III evaluation and obtained WHO full recommendation for 2.5–3 years of effective field use [12]. When compared with ICON Maxx on the same materials as tested in the present paper, it showed similarity in characteristics over 20 washes: high insecticide retention and bio-efficacy on undyed polyester and polyethylene and poorer retention and bio-efficacy on cotton and nylon [25].

Choice of testing methodology: cone, cylinder or tunnel

Despite having the same 3-min exposure, the mortality/knockdown responses differed considerably between cone and cylinder tests. The purpose of the comparison was to identify whether the cylinder should supplant the cone as the primary WHO insecticide bioassay. Both are WHO bioassays. The cone bioassay was initially designed for assessment of IRS bio-efficacy and residual activity on hard flat wall and ceiling surfaces of sprayed houses. Only later was it re-purposed for use as an ITN/LLIN bioassay. The IRS bioassay exposes mosquitoes for 30 min; this

gives a mortality similar to that of free-flying mosquitoes entering and exiting IRS sprayed experimental huts [26] and is, therefore, appropriate as an exposure time. For ITN testing the cone has limitations: contact time is shorter, and it is difficult to 'settle' the mosquitoes on cone netting for the prescribed 3 min. If the purpose of a residual bioassay is to manage undesirable variables, then control of exposure time is essential in a short exposure assay. In this respect, the cylinder is an improvement over the cone; when cylinder and cone mortality are compared, mortality is higher in the cylinder than in the cone due to higher ratio of netting to plastic. This was particularly evident at zero washes. But is the cylinder any less variable than the cone? The mortality at 20 washes for the different polymers tested would suggest not. Mortality rose and fell between the cylinder and cone in synchrony depending to the attributes of the netting surface and AI concentration retention rather than with other attributes of the test method. If exposures longer than 3 min are required, for example when testing resistant strains, the cylinder would be the better, more precise tool to use than the cone as they guarantee maximum contact with the treated surface of the tested mosquitoes.

On the other hand, if the aim is to simulate natural host-seeking behaviour on and around the net then the overnight tunnel test is the more realistic bioassay than either the cone or cylinder. In the 3 min cone or cylinder bioassay the mosquito is standing on the netting or flitting around it, whereas in the night-time tunnel test the host-seeking mosquito is trying the penetrate through the net. Different anatomical parts may be in contract with the net and for different lengths of time in the tunnel. The correlations between cone and tunnel and between cylinder and tunnel remain weak.

In these tests, susceptible mosquito strain was deliberately used as they are the most sensitive tool to investigate the properties of surface binder and pyrethroid over changing concentration before and after washing. In the wild of course, many mosquito populations will also contain insecticide resistant mosquitoes, and these may, or may not, be killed by the surface pyrethroid. If not killed, they may still be repelled or inhibited from blood-feeding. Eighty per cent of LLIN in use for malaria control are still standard pyrethroid-only LLIN and only a fraction will contain a second active ingredient. One of our aims was not just to facilitate treatment of untreated nets

with a wash-tolerant pyrethroid but to encourage manufacturers to produce sachets of alternative active ingredients with which to make standard pyrethroid LLINs to become more effective 'mixture nets'.

Future uses

The obvious use for ICON Maxx and other treat-it-yourself long lasting pyrethroid kits is to bundle the sachets with the hundreds of thousands of untreated nets that continue to be sold in retail markets, rural and urban. Conical nets, at the 'luxury' end of the market are rarely bundled with kits, and the wholesalers and retailers of untreated conical and rectangular nets will need regular supplies of kits.

This is a timely reminder for beneficiaries of free distributions of LLIN, who may know little or nothing about LLIN production, that LLIN are special because of the insecticide they contain, and the nets need to be used with care and respect. Older nets can be made more protective with a top-up of insecticide, especially if the next universal coverage campaign is delayed, giving older nets a further 2–3 years of protective use. Universal campaigns are often supplemented with top-ups of new LLINs in the interval between campaigns, and if LLIN numbers are in short supply, untreated nets and older LLINs that are still serviceable would continue to provide benefit if re-treated.

Mosquito nets are not the only household product which might obtain benefit from long-lasting insecticide treatment. Curtains made of nylon, polyethylene or cotton could provide family protection from Anopheles vectors of malaria and Aedes vectors of dengue, chikungunya and yellow fever. These could be immersed in ICON Maxx solution like the netting described in this article or sprayed with deltamethrin 62 SC-PE (polymer-enhanced suspension concentrate formulation), a product specially derived from KO-Tab-123 technology as an aqueous spray formulation (K-Othrine Polyzone, Bayer Crop Sciences, Germany) [28].

Armed services have favoured the use of permethrin on combat clothing for personal protection because of its high repellence [29, 30]. Alphacyano-pyrethroids such as ICON Maxx might be

preferred in certain locations due to its higher toxicity compared to permethrin. To prevent skin irritation the treated material might be separated from skin contact by a non-treated inner layer of material [31].

Disasters and humanitarian emergencies

The same arguments apply to civilian bedding and to top-sheets and blankets treated and distributed in epidemics, disasters or emergencies [32, 33]. Standard issue in humanitarian emergencies are blankets, tents and polyethylene tarpaulins [31, 33, 34] particularly for refugee populations on the move, i.e., situations where nets are dysfunctional or where sprayable housing is absent. Acute phase emergencies are a niche, which has proven difficult to supply with adequate vector control protection. The problem is compounded by the sectorial nature of international aid. Blankets and tents in emergencies are administered by the shelter sector, vector control is administered by the health sector. Blankets, sheets and shelters are also location-specific, and utility and material will depend on climate and ambient temperature. The solution might be to coordinate the shelter and public health sectors to treat whatever shelter or material is provided on-site with a long-lasting insecticide or repellent formulation mixed with binder formulation and UV protectant, applied by immersion, spray pump, or treated at source during manufacture. Bespoke factory manufactured products may not justify the investment in stockpiling, bespoke long-lasting formulations that can used to treat a variety of products could, on the other hand, justify the investment and be shifted fast to where it is needed.

The treatment of polyethylene tarpaulins or shade cloth with pyrethroid plus binder as used in emergency shelter has formed the basis of the insecticide treated wall liner concept of protection in the home [35].

Dual-AI LLIN and non-pyrethroid long-lasting treatment kits

The first Dual Active Ingredient LLINs were the PBO-synergist nets PermaNet 3.0 [36] and Olyset Plus [37]. Whilst the pyrethroid in all WHO recommended LLINs should remain effective for 3 years, WHO is now referring to Dual-AI nets as ITNs because it is not clear whether the PBO

component will last a full 3 years of field use [38]. Whilst Olyset Plus, the first in class pyrethroid–PBO net, has demonstrated effectiveness for two years, it is not yet clear in the ongoing cluster randomized trial whether the PBO will remain effective for the full 3 years. If it falls short of 3 years, there is an opportunity here to apply PBO via a PBO-binder long-lasting kit after 2 years to take it through the third year. Similarly, there is an opportunity for a PBO-binder long-lasting kit to be applied to any pyrethroid LLIN to convert those to pyrethroid-PBO LLIN. This could apply equally to other partner AI, such as pyriproxyfen or chlorfenapyr which are being used with pyrethroid in other types of Dual-AI LLIN should these fall short of 3 years' effectiveness [39]. In environments with high pyrethroid resistance, it would be a mistake to allow Dual AI nets to revert to a pyrethroid-only LLIN in their third year as users would only be part-protected.

Conclusion

In all tests performed, ICON Maxx treated polyethylene recorded greater performance than the positive control (polyester white) and other netting materials tested. Although the efficacy of ICON Maxx on cotton and nylon netting were low compared to other materials, they still met WHO criteria for LLIN. All ICON Maxx treated materials demonstrated insecticidal efficacy after twenty washes and met WHO criteria for long-lasting insecticidal treatment in one or more bioassays described here. Chemical analysis confirmed that lambda-cyhalothrin was more strongly retained in the ICON Maxx-treated than in Iconet treated materials. The high efficacy, wash-fastness and versatility of ICON Maxx raises the prospect of it becoming an all-purpose formulation for such purposes as military clothing, civilian bed covers and curtains, or for blankets, tarpaulins and tents distributed in epidemics, disasters or humanitarian emergencies, rather than dream of bespoke long-lasting insecticidal products for niche markets that may not be viable investment for manufacturers. ICON Maxx or treatment kits like ICON Maxx may provide an answer to the problem of reduced LLIN coverage between distribution campaigns, by turning commercial retail-sourced untreated nets into LLINs through simple home or community treatment.

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Part three: summary of key findings and new findings since publication

In concluding part three, here are the key findings and recommendations:

Nets treated with ICON Maxx and washed 20 times met the approval criteria set by WHOPES for Phase II entomological trials in terms of mortality and blood-feeding inhibition. This finding raises the prospect of conventional polyester nets and other materials being made long-lastingly insecticidal through simple dipping in community or home, and thus represents a major advance over conventional pyrethroid treatments.

WHOPES concluded that given the heterogeneity in lambda-cyhalothrin concentration on the surfaces of the nets, ICON Maxx cannot be recognized as equivalent to a WHOPES-recommended, factory-produced LLIN where greater attention can be paid to quality assurance during production. For example, ICON Maxx sampled in Burkina Faso showed an overly high variation between and within nets.

Crucially, biological performance against free-flying anophelines did not significantly deteriorate after 20 washes in either trial and therefore any heterogeneity in concentration across the surface of the net does not translate to a loss of biological efficacy if mosquitoes are sampling a range of insecticide concentrations across the surface as they attempt to gain access to the host.

In Phase III trials, ICON Maxx demonstrated efficacy criteria expected of long-lasting net after 30-36 months of household use, whereas the CTN fell short of the efficacy criteria within just 12 months of use. WHOPES distinguishes between long-lasting insecticide treatments that are conducted in the community and LLINs that are produced in the factory and expected to meet higher standards of quality control and homogeneity of application. The Phase III trial of ICON Maxx demonstrated a long-lasting treatment, as opposed to a long-lasting factory-treated net, providing efficacy and wash fastness over the three-year expected lifetime of the net.

While factory made Interceptor nets achieved the 36-month efficacy threshold using 77% cones and 10% tunnels, hand dipped ICON Maxx sampled in the same district of Tanzania only achieved it by 26% cones and 64% tunnels. This suggests that ICON Maxx would have not gone any further than 36 months, whereas the factory-treated Interceptor had plenty of tunnel tests to spare after 36 months.

There is great diversity in the fabrics and materials used for making mosquito nets; insecticide-treated blankets, tents and curtains have also shown protection against malaria in trial settings. The high efficacy, wash-fastness and versatility of ICON Maxx raises the prospect of it becoming an all-purpose formulation for such purposes as military clothing, civilian bed covers and curtains, or for blankets, tarpaulins and tents distributed in epidemics, disasters or humanitarian emergencies, rather than dream of bespoke long-lasting insecticidal products for niche markets that may not be viable investment for manufacturers.

ICON Maxx or treatment kits like ICON Maxx may provide an answer to the problem of reduced LLIN coverage between distribution campaigns, by turning commercial retail-sourced untreated nets into LLINs through simple home or community treatment.

Similarly, there is an opportunity for a PBO-binder long-lasting kit to be applied to any pyrethroid LLIN to convert those to pyrethroid-PBO LLIN. This could apply equally to other partner AI, such as pyriproxyfen or chlorfenapyr which are being used with pyrethroid in other types of Dual-AI LLIN should these fall short of 3 years' effectiveness. In environments with high pyrethroid resistance, it would be a mistake to allow Dual AI nets to revert to a pyrethroid-only LLIN in their third year as users would only be part-protected. There is no such thing as a non-pyrethroid AI treatment kit. There ought to be. Such a kit would have wide utility. It could be used for control of pyrethroid resistant mosquitoes. Also, as a means of converting pyrethroid-only nets into long lasting mixture nets, a challenge that will be discussed in detailed in the chapter that follows in Part 5

that will describe a study to investigate the impact of insecticide resistance on the efficacy of LLINs.

PART FOUR

Research question: Does insecticide resistance undermine entomological efficacy of the Standard Pyrethroid-only Long-lasting insecticide treated bed nets in Tanzania?

Prologue:

Long-lasting net (LLINs) have become an important tool for vector control against malaria and other mosquito-borne diseases.

There is evidence that the use of LLINs on a large scale in areas with insecticide-susceptible mosquito populations, decreases malaria related morbidity and mortality even among non-users of nets [34, 35]; In short, the nets have a community effect by reducing the longevity of malaria vector mosquitoes [36]. For this reason, the use of ITNs has been considered an important tool in the Roll Back Malaria (RBM) strategy.

One of the challenges that is facing LLINs today is the evolution of pyrethroid resistance in *Anopheles* mosquitoes. The pyrethroid resistance in mosquito vectors has been reported in many African countries [37-41] and is spreading rapidly across Africa. The signs are that resistance could reduce the impact of our two most successful malaria prevention interventions – indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) [37, 41, 42].

Statement of the problem

The impact of pyrethroid resistance on malaria transmission and the efficacy of control tools is not consistent and may differ from one area to another [25]. In some areas insecticide resistance has been reported to reduce the impact LLINs and IRS, as shown by findings of decreased efficacy of LLINs in parts of West Africa, East Africa, South Africa [17, 26-30]. Elsewhere, pyrethroid resistance has been reported to have little or no effect on the efficacy of pyrethroid-LLIN [25, 31, 32]. Mathematical models predict increased malaria incidence but real-world evidence for this is lacking [62].

This evidence shows that while WHO resistance assays might be useful in place of resistance surveillance tools, they offer petite understanding into the functional implication of this resistance. Thus, merely characterizing a population as resistant

according to the WHO test criteria does not mean there are automatically any consequences for the effectiveness of ITNs.

In Tanzania, national surveillance of resistance has been undertaken annually by NIMR at sentinel sites around the country to monitor for any changes in the frequency of resistance in response to universal LLIN coverage campaigns thus in Tanzania, progress has been made on understanding the presence, distribution and causes of pyrethroid resistance via annual national sentinel site surveys of insecticide susceptibility [18-20]. However, a high frequency of resistance as indicated by WHO susceptibility test results does not necessarily translate into vector control failure, nor may it necessarily extrapolate to negative operational impact on vector control by LLINs [21].

Rationale and justification

Insecticide resistance is well established in all malaria endemic countries including Tanzania. Although substantial progress has been made on understanding the causes of pyrethroid resistance, remarkably few studies have focused on either entomological or epidemiological impact of resistance on current malaria control activities. As we move into the next malaria eradication era, it is vital that the implications of insecticide resistance are understood and strategies to mitigate these effects are implemented. Thus, establishment of the impact of insecticide resistance on the efficacy of standard pyrethroid-only long-lasting insecticide treated nets is of paramount importance for resistance management and for effective malaria control decision making by the Malaria Control Programs of all malaria endemic countries including Tanzania.

General objective

Purpose of this part of thesis is to ascertain the impact of insecticide resistance on entomological efficacy of standard pyrethroid Longa-lasting insecticidal treated nets.

Study to address objective above are presented and discussed in chapter 8 of this thesis as follows;

Chapter 7: Reduced efficacy of LLINs after selection of pyrethroid-resistant *Anopheles gambiae s.l.* and *funestus s.l.* in NE Tanzania: a longitudinal meta-analysis of experimental hut studies

Abstract

Background:

The extent to which insecticide resistance is affecting malaria vector control in community and home is not fully understood. This study assessed the implications of insecticide resistance for entomological efficacy of LLINs against wild free-flying field *Anopheles gambiae* s.l. and *Anopheles funestus* s.l. in experimental hut trials (EHT) in NE Tanzania before and after the evolution of pyrethroid-resistance.

Methods:

Evaluations of LLIN efficacy were conducted following World Health Organization (WHO) guidelines in a series of ten trials commissioned by WHO between 2006 and 2017, before and after resistance development. The evaluation criteria were based on mortality, blood feeding inhibition, induced exophily, personal protection, mass killing effect and deterrence. WHO bioassay methods were used to detect resistance while PCR molecular diagnostics detected resistance alleles and identified mosquitoes to species.

Results:

An. gambiae s.l. and *An. funestus s.l.* mosquitoes were fully susceptible to pyrethroids until 2010-2013 when they showed resistance. The VGSC L1014S point mutation *kdr* was detected in *An. gambiae s.s.* at the allelic frequency of 47%. Synergist tests with PBO restored efficacy, indicating added involvement of metabolic mechanisms. Meta-analysis of the 10 trials showed that mortality of susceptible *An. gambiae s.l.* was 6.7 and 5.2-fold greater on zero-times ($z = 6.6$, $p = 0.001$) and 20-times washed LLINs ($z = 2.3$, $p = 0.023$) than on resistant *An. gambiae s.l.* The mortality of unwashed and washed LLINs against

susceptible *An. funestus s.l.* was 3.3 (z = 2.8, p = 0.004) and 2.6 (z = 2.9, p = 0.004) fold greater than on resistant *An. funestus s.l*. Resistant *An. gambiae s.l.* were more likely to exit the huts as compared to susceptible *An. gambiae s.l*. (z = 2.79, p = 0.005). The transition from susceptibility to resistance on changes to blood-feeding rates was non-significant for either species.

Conclusions:

Reduced mortality induced by LLINs after selection of pyrethroid resistance indicates that resistance may undermine household and community control of vector populations. Personal protection parameters seemed less affected by the transition to resistance. Meta-analysis, comparing the same net brands before and after selection of resistance, reveal which control parameters seem most affected by resistance.

Keywords: Long-lasting insecticidal net, resistance, *Anopheles gambiae*, *Anopheles funestus,* experimental hut, Tanzania.

Background

Long-lasting insecticidal nets (LLINs) are the primary tool for malaria vector control in equatorial and sub-Saharan Africa [1, 2]. The use of LLINs on a large scale not only decreases malaria morbidity and mortality [3] but also provides community protection by reducing the longevity of malaria vector mosquito populations [4].

Over the last decade LLINs have been massively scaled-up in sub-Saharan Africa [5] initially by targeting pregnant women and children under five years of age as the individuals most vulnerable to malaria [6], and more recently by aiming for universal coverage of communities [7]. Several net delivery systems have been tested in endemic African countries, which have proven effective in scaling up coverage [8].

Ever since the World Health Organization (WHO) approved the use of LLINs as a primary strategy for malaria control [9], long-lasting formulations of pyrethroid, owing to wash-durability, residual activity and cost effectiveness have been the primary class of insecticide used on nets [10]. Resistance to pyrethroid insecticide in Anopheline mosquitoes is caused by a variety of mechanisms, most notably the target site insensitivity mechanisms *kdr* [11, 12] and metabolic mechanisms caused by mixed function oxidases (MFOs) [13-15]. Both types of mechanism are now firmly established in many African countries as resistance continues to spread [16, 17].

In Tanzania, progress has been made on understanding the presence, distribution and causes of pyrethroid resistance via annual national sentinel site surveys of insecticide susceptibility [18-20]. However, a high frequency of resistance as indicated by WHO susceptibility test results does not necessarily translate into vector control failure, nor may it necessarily extrapolate to negative operational impact on vector control by LLINs [21]. The substantiation of resistance, as demonstrated by WHO susceptibility tests requires corroboration on the impact of control tools in household use as measured using other standard WHO approaches, such as Phase II experimental hut trials (EHT) against wild host-seeking resistant mosquitoes [22], and longitudinal multi-site village trials through which surveillance for increasing malaria transmission can be monitored [23, 24].

The impact of pyrethroid resistance on malaria transmission and the efficacy of control tools is not consistent and may differ from one area to another [25]. In some areas insecticide resistance has been reported to reduce the impact LLINs and IRS, as shown by findings of decreased efficacy of LLINs in parts of West Africa, East Africa, South Africa [17, 26-30]. Elsewhere, pyrethroid resistance has been reported to have little or no effect on the efficacy of pyrethroid-LLIN [25, 31, 32].

In Tanzania, national surveillance of resistance has been undertaken annually by NIMR at sentinel sites around the country to monitor for any changes in the frequency of resistance in response to universal LLIN coverage campaigns. Most intensive use of

pyrethroid IRS and LLIN for malaria control has been concentrated around Lake Victoria in Kagera and Misungwi led by the National MCP supported by PMI (President's Malaria Initiative), with field research support from NIMR and LSHTM [24]. Concurrently, over the period 2006 to 2017, NIMR and LSHTM have been commissioned by WHO under the auspices of Pesticide Evaluation Scheme (WHOPES) and the Prequalification Team for Vector Control (WHO/PQT-VC) to undertake a series of LLIN product evaluations according to standardized methodology in suites of experimental huts of 'the East African design' in NE Tanzania (WHO 2013). The main objective of the WHO hut trials was to assess product efficacy and wash-fastness before and after standardized washing to determine whether the candidate LLIN met the efficacy thresholds established by WHO against wild, free-flying mosquitoes under standardized household conditions. This involves recording of mosquito entry and exiting into window and verandah traps and recording of host feeding and mosquito mortality. Whilst the EHT site location, Zeneti (Muheza), has not changed over this period, the composition of the mosquito populations may have altered under selection pressure from the intermittent campaigns of universal net coverage led by the National MCP. Thus, the LSHTM/NIMR project on LLIN product development and evaluation for WHO provides a unique opportunity to study the evolution of insecticide resistance over the decade under natural household conditions, and its effect on behavior and survival of *An. gambiae s.l.* and *An. funestus s.l*.

In short, the fixed hut location and changing resistance status of the vectors interact to provide a unique opportunity to study the changing effectiveness of standard pyrethroid nets, and their capacity to control mosquitoes that have become increasingly resistant over the years.

Methods

Study area and experimental huts

The experimental hut trials were conducted at the NIMR field station at Zeneti village “5° 13' S latitude, 38° 39' E longitude" and 193 m altitude; where *An. gambiae* s.s. and *An. funestus* are the major malaria vectors [33], with entomological inoculation rates (EIRs) of 34–405 infective bites per person per year [34]. During the trials between 2006 and 2010 the local population of *Anopheles gambiae s.l.* were 100% susceptible to pyrethroids [35, 36]. However, during the hut trials conducted around 2011 and 2013 the vector species in this study area has become resistant to pyrethroid [19].

The huts were constructed to a design described by WHO [37], based on the original verandah-hut design developed in Tanzania [38, 39] with some significant modifications. These included reduction of the eave gaps to 2 cm, and introduction of wooden, hessian-lined ceiling, roofs of corrugated iron, concrete floors surrounded by water-filled moats and unidirectional baffles over eave gaps [40]. The huts had open eaves with verandah traps and window traps on each side. The working principle of these huts has been described by WHO [36]. Baffles were introduced in 2014. On each day of the trial, two opposite sides of the huts have window traps and closed verandahs that are screened to capture mosquitoes leaving via the eaves. The other two verandahs are unscreened so that mosquitoes can enter through eaves, but are restricted from exiting by eave baffles (Oxborough et al. 2015a).

While most experimental hut trials were premeditated and commissioned by WHO, the meta-analysis was opportunistic and took advantage of the evolution of resistance to open a window on what resistance may mean for vector control. The brands of LLIN assessed over the 10 years are listed in Table 1. All were approved by WHO. Some LLIN are made of polyethylene, others of polyester. Some are coated with the AI formulation; others have the formulation incorporated in the fibre during manufacture. All contain pyrethroid as the active ingredient at different concentrations, some with deltamethrin, others with permethrin, alpha-cypermethrin or lambda-cyhalothrin. The original aim was to test LLIN capacity to meet the WHO criteria after 20 washes; and all those cited did.

Table 1: Description of trials and treatment arms

Sno.	Year of the trial	LLIN product tested	Wash status arms included	Status of local *An. gambiae S.L.*
1	2006	Interceptor ™	Unwashed & 20 times washed	Susceptible
2	2008	DuraNet	Unwashed & 20 times washed	Susceptible
3	2008	PermaNet 2.0	Unwashed & 20 times washed	Susceptible
4	2010	Olyset Net	Unwashed & 20 times washed	Susceptible
5	2013	Olyset Net	Unwashed only	Resistant
6	2013	Interceptor ™	Unwashed only	Resistant
7	2013	PermaNet 2.0	Unwashed & 20 times washed	Resistant
8	2014	PermaNet 2.0	Unwashed & 20 times washed	Resistant
9	2015	Olyset Net	Unwashed only	Resistant
10	2017	Interceptor	Unwashed & 20 times washed	Resistant

Net preparation and washing

The long-lasting insecticidal nets were washed according to WHOPES Phase II washing protocols [37]. Each net was washed individually in 10 litres of tap water containing 2 g/l of soap ('Savon de Marseille'), subjected to 20 rotations per minute for 6 min during a 10-min immersion, and then rinsed twice. The interval between washes was in accord with the regeneration time of the candidate net, as determined by WHO. The washing schedule was stepped to ensure that the final wash of all treatment arms was completed on the same day. To simulate wear and tear a total of six 4 cm x 4 cm holes were made in each net (two holes cut on each side and one hole at each end).

Experimental hut study design

The 10 experimental huts trials included in the study were conducted between 2006 and 2017 and employed standard LLIN treatment arms and the same two suites of huts. Each trial employed the same basic study design in which Latin square rotation adjusted for any variation between hut position, individual volunteer sleeper attractiveness and individual net. All ten hut trials had at least the following three arms that were analyzed comparatively in the study: (i) unwashed LLIN (0W), (ii) LLIN washed 20 times (20W), (iii)

untreated unwashed polyester net (0W)**.** The details of the trials and treatment arms are listed in Table 1**.**

The primary outcomes compared between trials were: (i) deterrence - the reduction in entry into treatment hut relative to the control huts (i.e. those containing untreated nets); (ii) treatment induced exiting - the proportion of mosquitoes found in exit traps of treatment huts relative to the same proportion in control huts; (iii) mortality - the proportion of mosquitoes killed relative to the total catch size; (iv) overall killing effect - the numbers killed by a treatment relative to the untreated control, as derived from the formula: *killing effect (%) = 100(Kt-Ku)/Tu* where Kt is the number killed in the huts with treated nets, Ku is the number dead in the huts with untreated nets, and Tu is the total entering the huts with untreated nets; (v) blood-feeding inhibition - the proportional reduction in blood feeding in huts with treated nets relative to controls with untreated nets; (vi) personal protection - the reduction in mosquito biting by treated nets relative to untreated nets, as derived from the formula: *% Personal protection=100(Bu-Bt)/Bu* where Bu is the total number blood-fed mosquitoes in the huts with untreated nets, and Bt is the total number blood-fed in the huts with treated nets.

Treatment arms were rotated once or twice through each hut according to a Latin Square design. A treatment was assigned at random to a particular hut for 6 nights' observation before being transferred to the next hut. Male volunteers slept on beds under the nets between the hours of 19:30 and 6:30. The sleepers were rotated through the huts on consecutive nights. Six nets were available per treatment arm and each net was tested on consecutive nights during the six-night rotation. At the end of the weekly rotation the huts were cleaned and aired for one day before starting the next rotation. Each morning dead and live mosquitoes were collected from the verandahs, room and window traps. Live mosquitoes were provided with 10% sugar solution. Delayed mortality was recorded after 24h. Mosquitoes were identified to species and gonotrophic status as unfed, blood-

fed, semi-gravid or gravid. Samples of *An. gambiae s.l.* were identified to species by PCR [41].

WHO insecticide susceptibility tests

During each of the trials, susceptibility tests were carried out using WHO test kits for adult mosquitoes [42-44] lined with test papers impregnated with 0.75% permethrin, 0.05% deltamethrin or 0.05% alphacypermethrin. The quality of the test paper was checked against the laboratory susceptible *An. gambiae s.s.* Kisumu strain. Mosquitoes used in these tests were the 2-5 days old female F1 progeny of the mosquitoes which were collected from Zeneti during each of these trials. The testing procedure was done according to WHO protocols [42-44].

The resistance or susceptibility status was evaluated based on the WHO criteria, i.e., 98-100% mortality indicates susceptibility; 90-97% mortality required confirmation and less than 90% mortality indicates possible resistance. When the control mortality was recorded between 5% and 20%, the mean observed mortality was corrected using Abbott's formula.

Piperonyl butoxide (PBO) synergist tests were conducted on mosquitoes that were found to be resistant to permethrin and/or deltamethrin according to the WHO protocol [44]. The aim of this test was to ascertain the involvement of mixed function oxidases in the observed phenotypic resistance. In this test, 2–5 days old F1 adult mosquitoes were pre-exposed to 4% piperonyl butoxide (PBO) paper for 1 h then exposed to 0.75% permethrin or 0.05% deltamethrin for 1 h.

Molecular species identification of the *Anopheles gambiae* complex

Anopheles gambiae sibling species identification was carried out according to the standard polymerase chain reaction (PCR) method [41].

Detection of knock down resistance (*kdr*) alleles in *Anopheles gambiae* complex.

The Taqman assay technique of Bass [45] was used for the detection of the VGSC L1014S or L1014F *kdr* alleles. In some samples, detection of *kdr* alleles were done using conventional PCR method. All *An. gambiae s.l.* mosquitoes collected from huts were analyzed for L1014S or L1014F *kdr* alleles.

Ethics, consent and permission

Ethical clearance was obtained from the ethics committees of the NIMR Tanzania (Ref: NIMR/HQ/R.8a/Vol X/86), London School of Hygiene and Tropical Medicine (LSHTM) and WHO. Written informed consent was obtained from all volunteers participating in the study, and each was provided with chemoprophylaxis and monitored daily for fever or possible adverse events due to insecticide exposure from the nets.

Statistical analysis

The initial objective, and first analysis, was to compare the efficacy of each LLIN product when zero washed and 20 times washed (trials done between 2006-2017) as per standard WHO Phase II criteria for LLIN analysis (WHO 2005, 2013). The key outcomes were the overall proportions of mosquitoes' blood-feeding or killed and the proportions entering or exiting relative to the untreated control treatment. Mixed effects logistic regression (with hut, sleeper, treatment being fixed effects and time of trial being a random effect) was used to estimate proportional outcomes of treatments (proportions killed [mortality], blood-feeding [inhibition], exiting [exophily]), and negative binomial regression was used to analyse counts of mosquitoes entering the huts (deterrence), blood-feeding (personal protection) or dying (mass killing effect), after adjusting for clustering by day and for variation between individual sleepers and hut position.

The second objective was to compare bio-efficacy and behaviour before and after development of pyrethroid resistance using meta-analysis methods. In the meta-analyses, risk ratios of the proportions dying (mortality), blood-feeding and exiting were pooled using a random-effects meta-analysis model STATA® statistical software version

16 (Stata corporation, College Station, Texas 77845 USA, 2019) before and after the development of resistance. Overall heterogeneity across trials was calculated using Cochrane's Q test with a P value < 0.05 to indicate statistical heterogeneity and quantified heterogeneity using the I^2 statistic.

Results

An. gambiae s.l. and *An. funestus s.l.* susceptibility tests

From 2006 to 2010 WHO resistance tests using 0.05% deltamethrin, 0.75% permethrin and 0.05% alphacypermethrin test papers were conducted directly on adults collected from huts. These tests showed 100% mortality indicating that *An. gambiae s.l.* was susceptible. Thereafter, in 2013, 2014 and 2015, observing WHO guidance, susceptibility tests using 0.05% deltamethrin were conducted on F1 progeny of *An. gambiae s.l.,* collected during the trials from huts with treated and untreated nets; these tests produced reduced mortality rates of 73%, 71.1% and 61.3% on test papers (figure 1). Susceptibility tests with 0.05% deltamethrin, 0.75% permethrin and 0.05% alphacypermethrin test papers were conducted on F1 of *An. gambiae s.l.* collected from the trial in 2015 from both treated and untreated huts; these tests recorded mortality of 61%, 45% and 50% respectively. Similar trends in resistance were observed with *An. funestus s.l.* (figure 1).

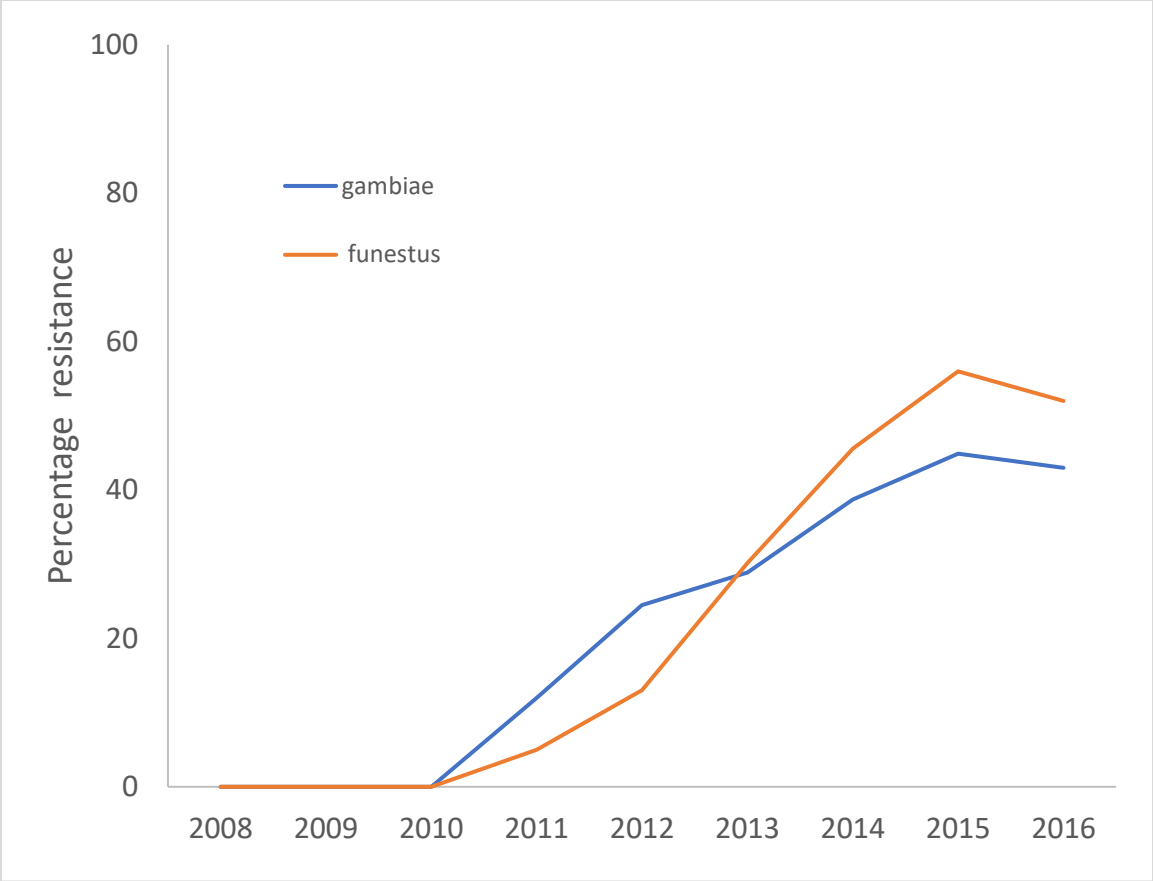

Figure 1: Zenet field *An. gambiae s.l* and *An. funestus s.l* population 2008-2017 permethrin resistance trend.

With both permethrin and deltamethrin, PBO restored efficacy (>97% mortality) in all *An. gambiae s.l.* and *An. funestus s.l.* mosquitoes tested. This observation suggests that partially in the case of *An. gambiae* or fully in the case of *An. funestus*, metabolic resistance might have contributed to the observed phenotypic resistance to pyrethroids.

Molecular characterization of mosquitoes.

A total of 1122 mosquitoes collected from the huts during the trials were identified using PCR. 94% of the analyzed *Anopheles gambiae s.l.* mosquitoes were *An. gambiae s.s.* and 6% were *An. arabiensis.* All analyzed *Anopheles funestus s.l*. were identified as *An. funestus* s.s.

A total of 833 *An. gambiae s.s.* and 40 *An. arabiensis* were analysed for VGSC L1014S and L1014F kdr allele. All 40 *An. arabiensis* were homozygous susceptible of 833 *An. gambiae s.s.* 252 (30%) were homozygous susceptible, 387 (47%) were heterozygous resistant with VGSC L1014S and 194 (23%) were homozygous resistant for VGSC L1014S. From these *An. gambiae,* 24 randomly selected mosquitoes were analyzed for the molecular form: all were of "S" form.

Phase II – experimental hut trials

Mortality and overall killing effect.

The proportions killed with the LLIN treatments and dying in the untreated control are shown in Tables 1a-c. For the deltamethrin LLIN, PermaNet 2.0, experimental hut trials were conducted in 2008, 2013 and 2014. In these trials, all insecticide treated nets recorded significantly greater mortality against *An. gambiae s.s.* than were recorded with untreated control nets (Table 2a). In the 2008 trial, when *An. gambiae s.s.* was fully susceptible, the unwashed PermaNet 2.0 recorded significantly greater mortality than was recorded with PermaNet 2.0 washed 20 times and CTN washed 3 times (97.7%, 84.7% and 68.7% control-corrected mortality (ccm), respectively). During the 2013 trial when *An. gambiae s.s.* had become resistant, the unwashed PermaNet 2.0, PermaNet 2.0 washed 20 times and CTN washed 3 times recorded reduced mortality against *An. gambiae s.s.* (23.9%, 21.6% and 18.3% ccm) compared to 2008. A year later, in the 2014 trial, mortality rates against *An. gambiae s.s.* across all 3 treatments never reached above 20% (Table 2a).

The overall killing effect against *An. gambiae s.s.* (the numbers of mosquitos killed by each treatment) recorded during 2008, 2013 and 2014 trials mirrored the percentage mortality trends (Table 2a).

Table 2a Experimental huts results: %mortality corrected for control, killing effect, Blood feeding, blood feeding inhibition and personal protection of wild *An. gambiae* *s.l.* huts during PermaNet 2.0 hut trials conducted at Zeneti in 2008, 2013 and 2014.

		Untreated net	PermaNet® 2.0	PermaNet® 2.0	CTN
Initial dose of Deltamethrin (mg/m^2)		0			25
Number of washes		0	Unwashed	20	3
Anopheles gambiae					
% Mortality corrected for control	**2008**	- ^{a1}	94.7^{b1}	84.7^{c1}	68.7^{d1}
	2013	- ^{a1}	23.9^{b2}	21.6^{b2}	18.3^{b2}
	2014	- ^{a1}	6.8^{b3}	0^{a3}	0^{a3}
% Overall Killing Effect	**2008**	-^{a1}	60.9^{b1}	55.6^{b1}	41.9^{b1}
	2013	-^{a1}	12.4^{a2}	26.3^{b2}	16.8^{b2}
	2014	-^{a1}	24^{a2}	0^{a2}	3.4^{a3}
% Blood fed (95% C.I.)	**2008**	27.9^{a1}	10.3^{b1}	9.2^{b1}	10.5^{b1}
	2013	25.8^{a2}	5.9^{b2}	12.4^{c2}	12.8^{c2}
	2014	34.5^{a2}	14.8^{b3}	30.8^{c3}	27.4^{c3}
% Blood feeding inhibition	**2008**	-^{a1}	63.21^{b1}	67.02^{b1}	62.29^{b1}
	2013	-^{a2}	77.2^{b2}	51.9^{c2}	50.4^{c2}
	2014	-^{a2}	57.1^{b3}	10.7^{ac3}	20.6^{c3}
% Personal Protection	**2008**	-^{a1}	70.8^{b1}	73.3^{b1}	70.8^{b1}
	2013	-^{a1}	85.2^{b1}	40.9^{c2}	49.6^{c2}
	2014	-^{a1}	10^{a1}	0^{a3}	0^{a3}

Note:

1. **Percentage deterrence, exiting and 95% CIs are backtransformed from values calculated by the blocked logistic regression model.**
2. **Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression (P<0.05).**
3. **Within each *An. gambiae* row, column not sharing a superscript number differ significantly by blocked logistic regression (P<0.05).**

For the permethrin LLIN, Olyset Net, experimental hut trials were conducted in 2010, 2013 and 2015. All insecticide treatments recorded significantly greater mortality against *An. gambiae s.s.* than in the untreated control (Table 2b). With the permethrin LLIN, the

mortality recorded against the unwashed and 20-times washed Olyset Nets (100% and 71.9% ccm) was significantly greater in the 2010 trial, when *gambiae* s.s. was susceptible, than was recorded with Olyset Net during 2013 and 2015 trials when the vector was resistant (Table 2b). However, the overall killing effect of the unwashed Olyset Net was not significantly greater during the 2010 trial than the killing effects recorded during trials in 2013 and 2015, possibly due to repellency of permethrin (Table 2b).

Table 2b Experimental huts results: % mortality corrected for control, killing effect, Blood feeding, blood feeding inhibition and personal protection of wild *An. gambiae s.l.* huts during Olyset huts trials conducted at Zenet in 2010, 2013 and 2015.

		Untreated net	OlysetNet	OlysetNet	CTN
Initial dose of alphacypermethrin (mg/m^2)		0			40
Number of washes		0	Unwashed	20	3
Anopheles gambiae					
% Mortality corrected for control	**2010**	0^{a1}	$100^{b,1}$	$71.9^{c,1}$	$76.4^{c,1}$
	2013	0^{a1}	$5.1^{b,2}$	-	$3^{ab,2}$
	2015	$0^{a,1}$	$12.1^{a,2}$	-	-
% Overall Killing Effect	**2010**	0^{a1}	$10.3^{b,1}$	$38.2^{bc,1}$	$69.1^{c,1}$
	2013	0^{a1}	$5.9^{b,1}$	-	$5^{b,2}$
	2015	0^{a1}	$5.2^{b,1}$	-	-
% Blood fed (95% C.I.)	**2010**	$72^{a,1}$	$0^{b,1}$	9^{c}	$12^{d,1}$
	2013	$25.8^{a,2}$	$5.3^{b,2}$	-	$12.4^{c,1}$
	2015	$24.1^{a,2}$	$11.1^{b,2}$	-	-
% Blood feeding inhibition	**2010**	0^{a}	$100^{b,1}$	87.5^{c}	$83.3^{d,1}$
	2013	0^{a}	$79.4^{b,2}$	-	$51.9^{b,2}$
	2015	0^{a}	$54.3^{b,2}$	-	-
% Personal Protection	**2010**	0^{a}	$100^{b,1}$	92^{c}	$73.5^{d,1}$
	2013	0^{a}	$77.2^{b,2}$	-	$38.6^{c,2}$

	2015	0^{a}	$71.4^{b,2}$	-	-

Note:

1. **Percentage deterrence, exiting and 95% CIs are backtransformed from values calculated by the blocked logistic regression model.**
2. **Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression (P<0.05).**
3. **Within each *An. gambiae* row, column not sharing a superscript number differ significantly by blocked logistic regression (P<0.05).**

For the alpha-cypermethrin LLIN (Table 2c), Interceptor LN, in the 2006 and 2008 trials when *An. gambiae* was susceptible, the unwashed Interceptor LN arms recorded significantly greater mortality (91.9% and 96.2% respectively) than Interceptor washed 20 times (76.2 and 83.1 respectively), indicating some loss of A.I. to washing. Mortality recorded with the unwashed and 20-times washed Interceptor LN against *An. gambiae* s.s. in 2006 (91.9% and 76.2% ccm) and in 2008 (96.2% and 83.1% ccm) when *An. gambiae* was susceptible were significantly greater than mortality recorded with the unwashed Interceptor LN during the 2013 trial (5.1%) when the vector was resistant (Table 2c). Results of the killing effect of the unwashed Interceptor net mirrored that of the percentage mortality both before and after selection of pyrethroid resistance (Table 2c).

Table 2c Experimental huts results: % mortality corrected for control, killing effect, Blood feeding, blood feeding inhibition and personal protection of wild *An. gambiae* *s.l.* huts during Interceptor and DuraNet hut trials conducted at Zenet in 2010, 2013 and 2015.

		Untreated net	**Interceptor**	**Interceptor**	**CTN**
Initial dose of alphacypermethrin (mg/m^2)		0			40
Number of washes		0	Unwashed	20	3
Anopheles gambiae					
% Mortality corrected for control	**2006**	0^{a1}	$91.9^{b,1}$	$76.2^{c,1}$	-
	2008	0^{a1}	$96.2^{b,1}$	$83.1^{c,1}$	65.2^{d}
	2013	$0^{a,1}$	$5.1^{b,2}$	-	$3^{b,2}$
% Overall Killing Effect	**2006**	0^{a1}	$70.4^{b,1}$	$52.2^{c,1}$	-
	2008	0^{a1}	$69.2^{b,1}$	$60.8^{b,1}$	53.1^{bc}

	2013	0^{a1}	$6.3^{b,2}$	-	$5^{b,2}$
% Blood fed (95% C.I.)	**2006**	32.1^{a1}	16.2^{b1}	16.1^{b1}	-
	2008	$19.6^{a,2}$	$7.1^{b,2}$	$5.4^{b,2}$	$11.3^{b,2}$
	2013	$25.8^{a,2}$	$7.8^{b,2}$	-	$12.4^{c,2}$
% Blood feeding inhibition	**2006**	0^{a}	$73.8^{b,1}$	$65.8^{b,1}$	-
	2008	0^{a}	$63.5^{b,1}$	$72.6^{b,1}$	$42.3^{b,1}$
	2013	0	$69.9^{b,1}$	-	$51.9^{b,1}$
% Personal Protection	**2006**	0^{a}	$79.5^{b,1}$	$75.6^{b,1}$	-
	2008	0^{a}	$71.4^{b,1}$	$78.6^{b,1}$	$50^{b,1}$
	2013	0^{a}	$64.9^{b,1}$	-	$38.6^{b,1}$

1. **Percentage deterrence, exiting and 95% CIs are backtransformed from values calculated by the blocked logistic regression model.**
2. **Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression (P<0.05).**
3. **Within each *An. gambiae* row, column not sharing a superscript number differ significantly by blocked logistic regression (P<0.05).**

Meta-analysis pooled estimate of mortality risk

Meta-analysis across the 10 EHT trials showed that with unwashed LLIN the pooled relative risk for mortality of susceptible versus resistant *An. gambiae s.s.* was 6.68 (3.81 – 11.70) fold greater (z = 6.64, p = 0.001) (Fig 2a). With the washed LLIN the pooled mortality relative risk rate between susceptible and resistant *An. gambiae s.l.* was 5.23 (1.26 – 21.76) indicating mortality ratio induced in the washed LLINs was lower with resistant compared to susceptible *An. gambiae s.s.* (z = 2.26, p = 0.023) (Fig 2a). Generally resistant *An. gambiae s.l.* mosquitoes were significantly less likely to be killed by both unwashed (z = 6.64, p = 0.001) and 20 times washed (z = 2.28, p = 0.023) LLINs compared to susceptible *An. gambiae s.l*. mosquitoes (Fig 2a)

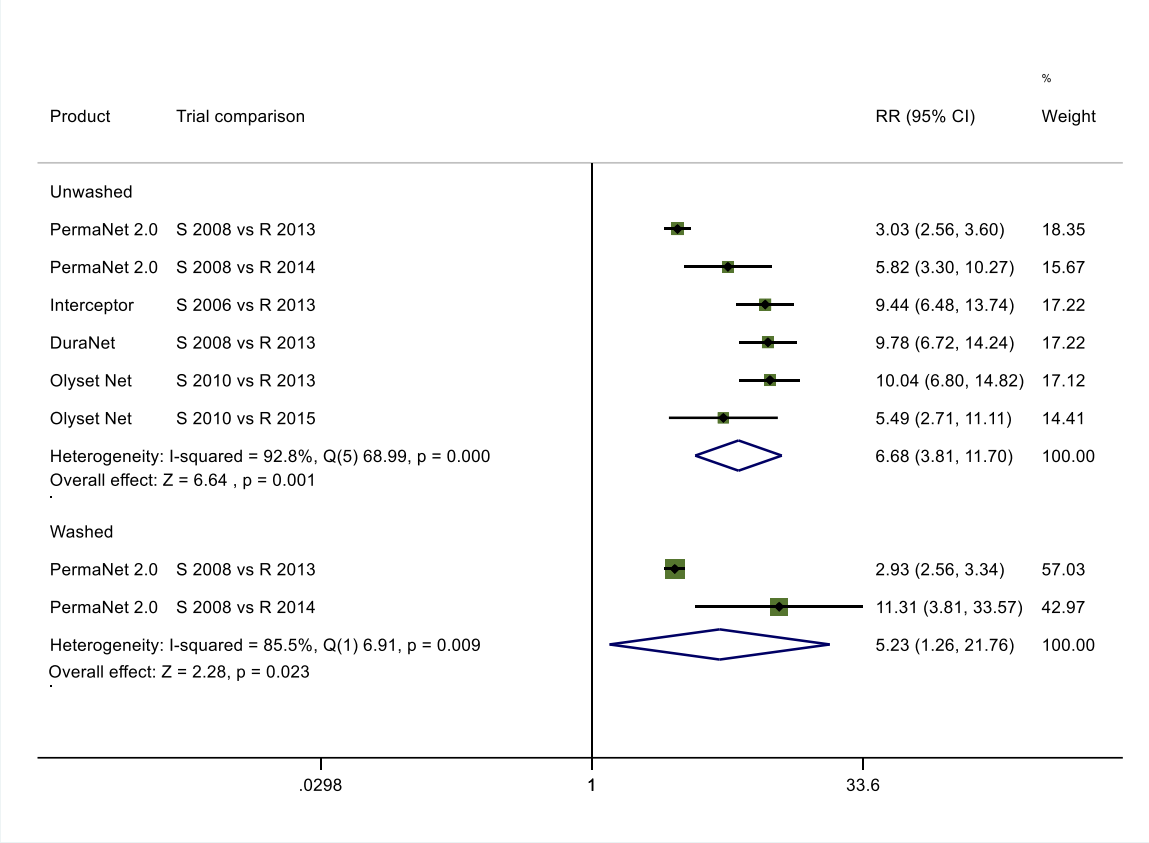

Figure 2a. Metanalysis of the efficacy results of washed and unwashed LN efficacy in trials done when vectors were susceptible and those done when vectors where resistant: *An. gambiae s.l.*: mortality.

The pooled estimate of relative risk for mortality of wild free-flying susceptible versus resistant *An. funestus s.l.* was 3.31 (1.45 – 7.54) fold with unwashed LLIN and 2.58-fold (1.36 – 4.90) with washed LLIN. Therefore, pooled mortality risk of susceptible vs resistant *An. funestus s.l.* was also lessened with washing of the LLIN. The differences in mortality risk between susceptible to resistant *An. funestus s.l.* was significant in both unwashed (z = 2.8, p = 0.004) and washed LLINs (z = 2.89, p = 0.004) (Fig 2b). Resistant *An. funestus s.l.* were significantly less likely to be killed by both unwashed (z = 2.85, p = 0.04 and 20 times washed (z = 2.89, p = 0.089) LLINs compared to susceptible *An. funestus s.l*. (Fig 2b).

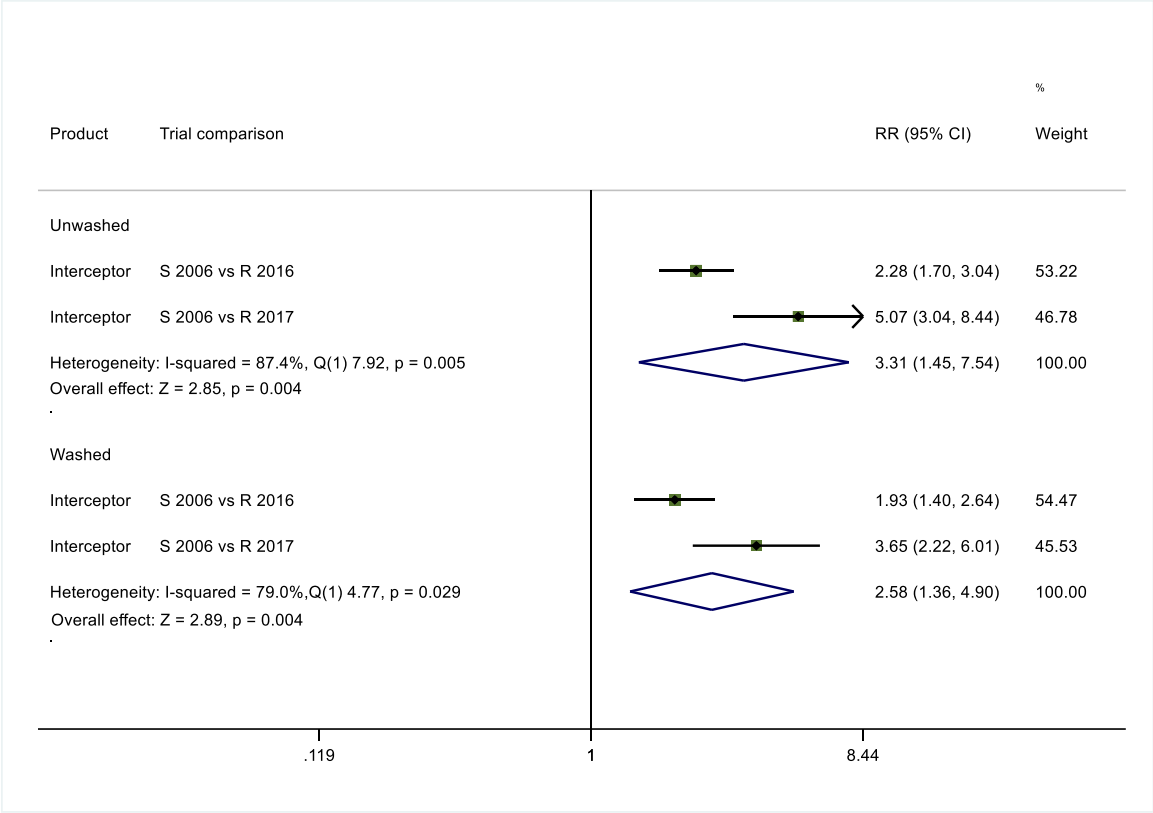

Figure 2b. Metanalysis of the efficacy results of washed and unwashed LN efficacy in trials done when vectors where susceptible and those done when vectors where resistant: *An. funestus s.l.:* mortality.

Logistic regression analysis

While the meta-analysis compares mortality risk to mosquitoes between specific LLIN products in relation to the transition from susceptibility to resistance, the logistic regression analysis pools all product data and shows broad temporal trends over the 10 years spanning the transition from susceptibility to resistance. Results shows that there was significant association between insecticide resistance in *An. gambiae s.l.* and the decline in the killing efficacy of LLIN (t = -9.5, p = 0.001) (Fig 2). With *An. funestus s.l.* also significant association was recorded between resistance in *An. funestus s.l.* with the decline in the killing efficacy of LLINs (t = -6.65, p = 0.001) (Fig. 2).

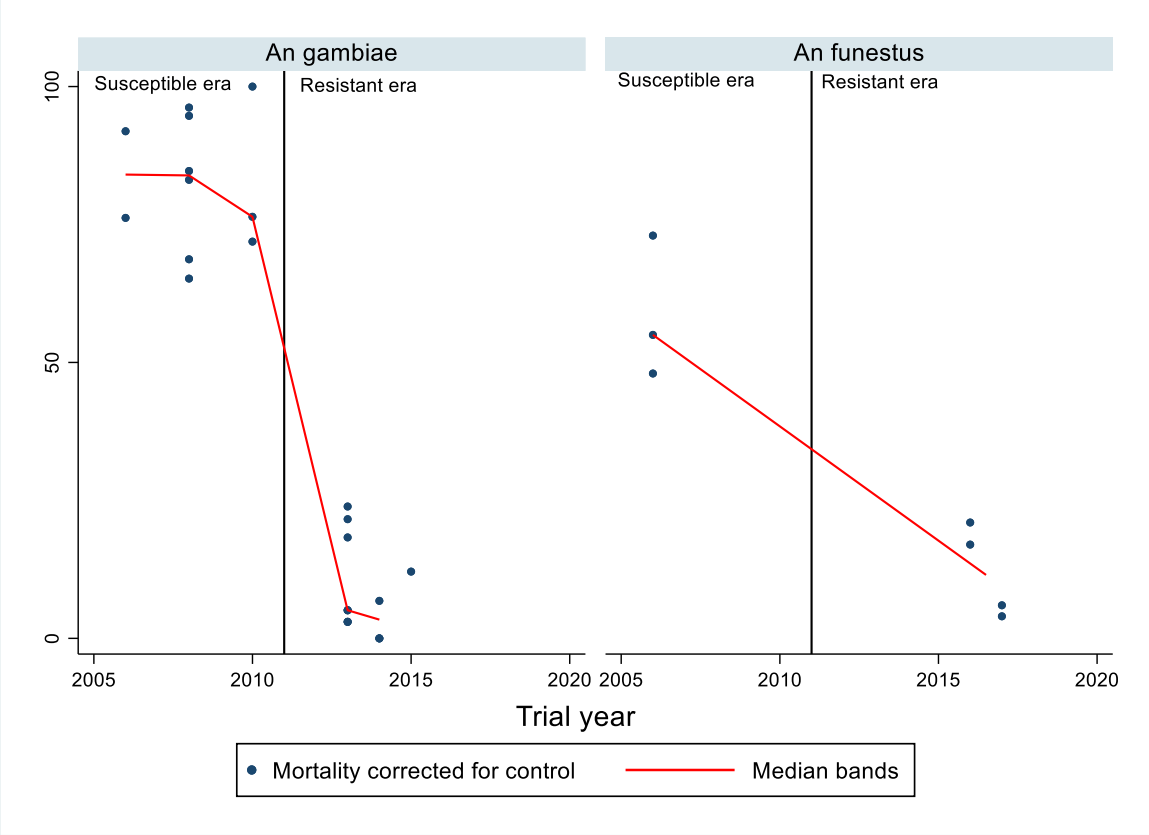

Figure 3: Mortality corrected for control of wild free flying *An. gambiae s.l.* and *An. funestus s.l.* entered into experimental during trials. Each point denotes the average mortality for each trial at 95% confidence interval. The line graph shows trend of median values for the control corrected mortalities in various trials.
NOTE: Vertical blue lines demarcate period of transition from susceptibility to resistance

Percentage blood feeding, blood feeding inhibition (BFI) and personal protection

With PermaNet 2.0 in all trials (2008, 2013 and 2014), all 3 insecticide treatments (unwashed PermaNet 2.0, PermaNet 2.0 washed 20 times, and deltamethrin CTN), recorded significant lower blood-feeding rates as compared to the untreated control (Table 2a).

In 2008 when vectors were susceptible, the unwashed, 20 times washed and the CTN washed 3 times recorded similar percentage blood feeding of *An. gambiae* (10.3%, 9.2% and 10.5% respectively). During 2013 trial when vectors were resistant, unwashed PermaNet 2.0 also recorded significantly lower percentage blood feeding (5.9%) compared to PermaNet washed 20 times and CTN washed 3 times (12.4% and 12.8%

respectively). With 2014 trial, when *An. gambiae* was highly resistant, the unwashed PermaNet 2.0 also recorded significantly lower blood feeding rate of (14.8%) compared to PermaNet 2.0 washed 20 times and CTN washed 3 times (30.8% and 27.4% respectively). The last two were similar statistically (Table 2a).

Comparing the 20 times washed PermaNet 2.0 in 2008, 2013 and 2014, the BFI was 67% when susceptible, 52% when moderately resistant but only 11% when highly resistant in 2014. The BFI in the 3-times washed CTN also fell during this period (62% in 2008, 50% in 2013, and 21% in 2014) (Table 2a).

Consistent with the BFI results, there was decrease in personal protection recorded across the three trials over this period (2008, 2013 and 2014) (Table 2a). Personal protection was 70.8% and above when the *An. gambiae* population was susceptible whether PermaNet 2.0 was unwashed, or 20 times washed. But when the *An. gambiae* became resistant in 2013 personal protection fell to 40.9% when 20 times washed and to 0% in 2014 when the population was more resistant (Table 2a).

With Olyset Net trials (2010, 2013 and 2015), all 3 insecticide treatments (unwashed, washed 20 times, CTN 3 times washed) recorded significantly lower percentage feeding rates as compared to the untreated control (Table 2b). Percentage blood feeding rate recorded during the 2010 trial (0%), when vectors were susceptible was significantly lower than the feeding rates recorded in 2013 and 2015 when resistant (5.3% and 11.1% respectively). The feeding rate between trials in 2013 and 2015 was statistically similar. Similar trend of significant increase in feeding rates between trials were also recorded with CTN washed 3 times. In Olyset trials, trends of blood-feeding inhibition recorded mirrored that of personal protection (Table 2b).

With the Interceptor trials (2006, 2008 and 2013), all 3 treatments also recorded significantly lower percentage Anopheles *gambiae* feeding rates compared to the untreated control (Table 2c). In the comparison between trials, percentage blood feeding

rate recorded between trials (2006, 2008 and 2013 trials) were also statistically similar. The trend in blood feeding inhibition mirrored that of personal protection (Table 2c).

Meta-analysis pooled estimate

The meta- analysis`s pooled estimates of relative risks between the percentage feeding of wild free flying susceptible and resistant *An. gambiae s.l.* were not significantly different for either unwashed (RR = 1.01, 0.95 – 1.08) (z = 0.42, p = 0.677) or washed LLINs (RR = 0.97, 0.90 – 1.05) (z = 0.79, p = 0.429) (Fig. 4a).

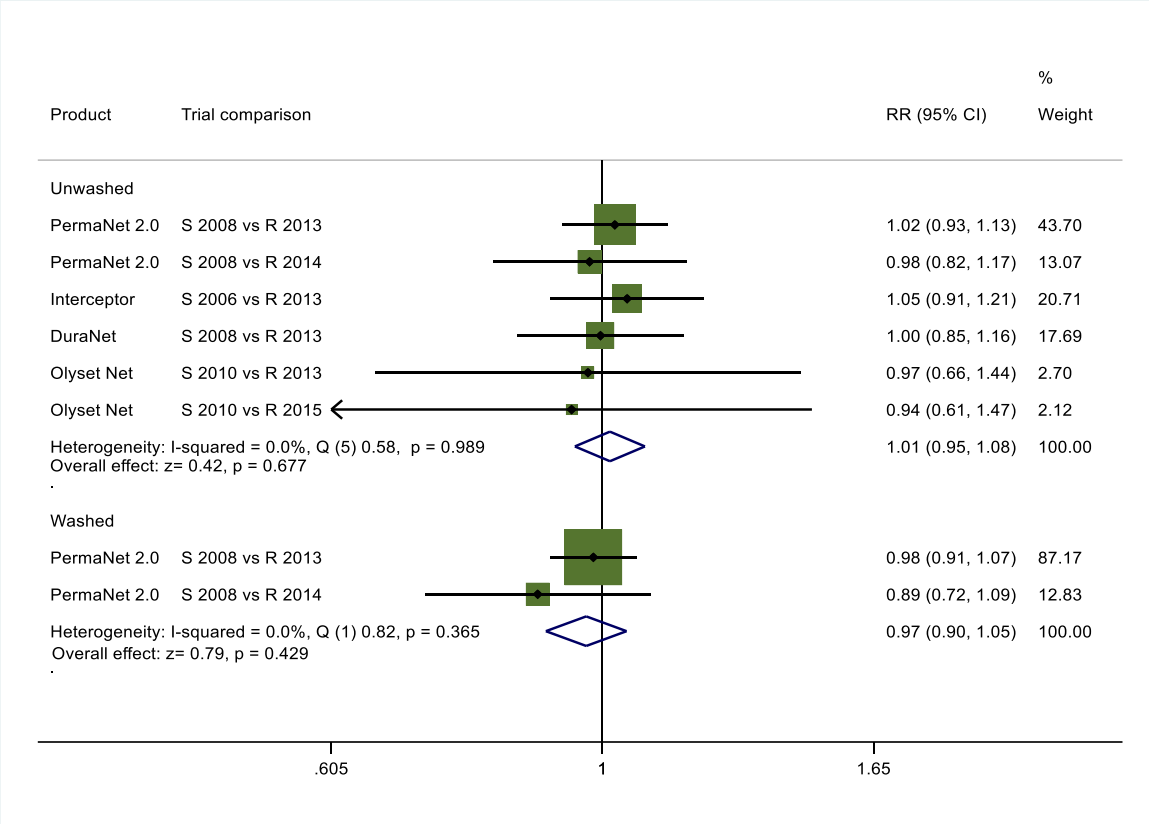

Figure 4a. Metanalysis of the efficacy results of washed and unwashed LN efficacy in trials done when vectors where susceptible and those done when vectors where resistant: *An. gambiae s.l.:* feeding.

The differences between pooled estimates of relative risks between the percentage feeding of wild free-flying susceptible and resistant *An. funestus s.l.* were also not significant for either unwashed (RR = 1.00, 0.86 – 1.17) (z = 0.05, p = 0.983) or washed (RR = 0.99, 0.86, - 1.15) (z = 0.08, p = 0.939) LLINs (Fig 4b).

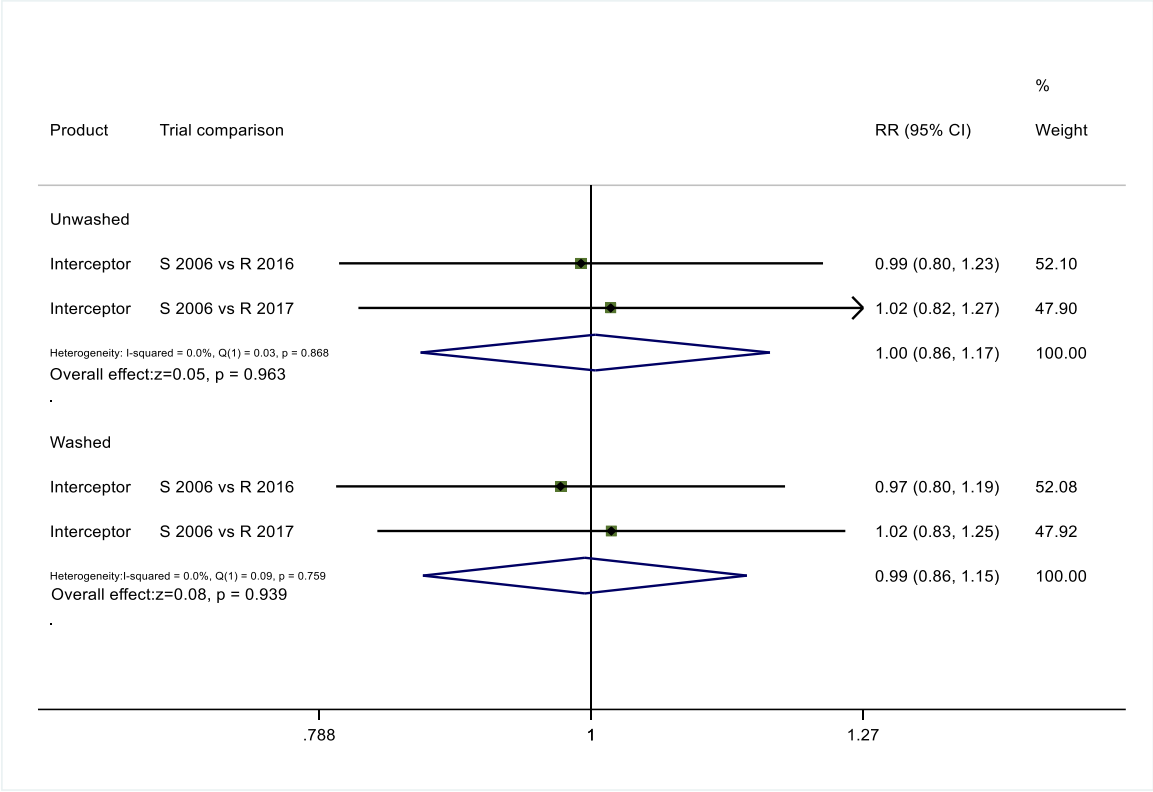

Figure 4b. Metanalysis of the efficacy results of washed and unwashed LN efficacy in trials done when vectors where susceptible and those done when vectors where resistant: *An. funestus s.l.* feeding

Logistic regression analysis

The blood-feeding logistic regression analysis indicated that resistance was not associated with a significance change in *An. gambiae s.l.* feeding rate (t = 0.41, p = 0.686). With *An. funestus s.l.* the resistant mosquitoes seemed less likely to feed as compared to susceptible *An. funestus s.l.* but this decrease was also not significant (z = -1.48, p = 0.178) (Fig. 5).

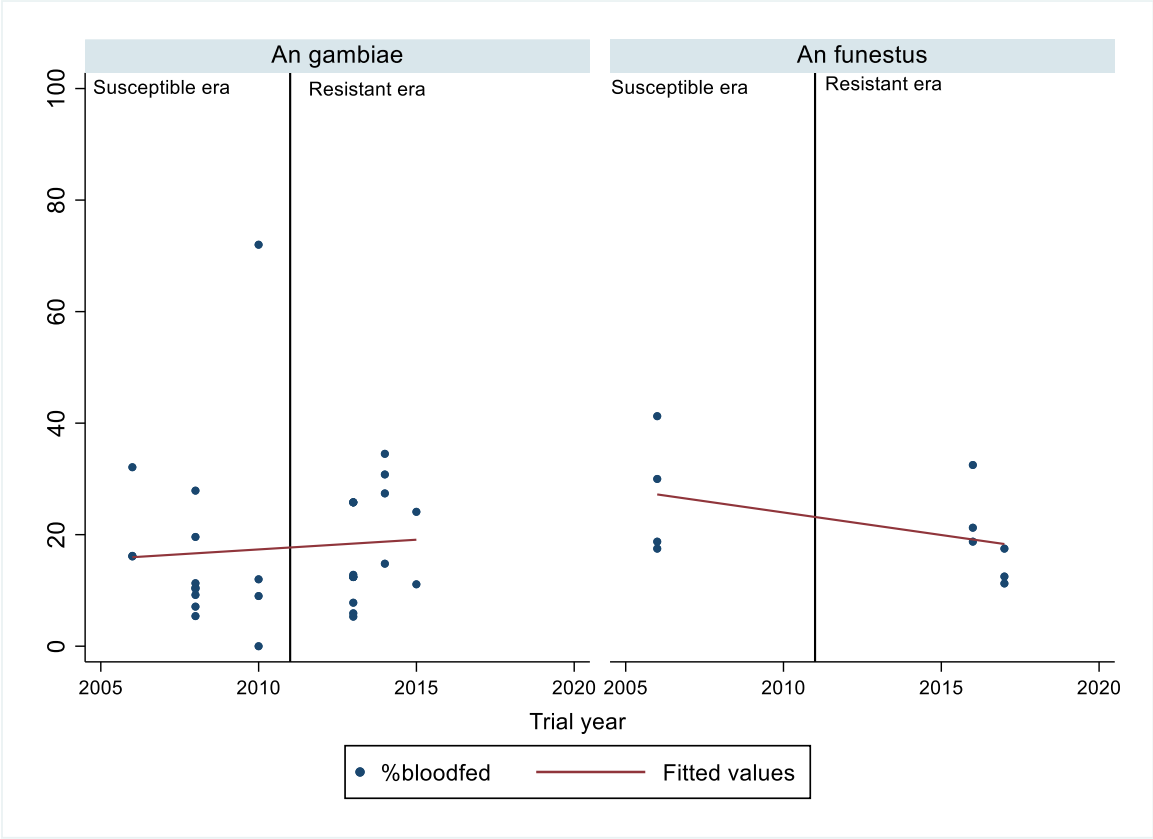

Figure 5: Percentage blood feeding of wild free flying *An. gambiae s.l.* and *An. funestus s.l.* during EHT. Each point denotes the percentage blood feeding for each trial. The line graph shows best fit regression line trend o for the percentage blood feeding in various trials.
NOTE: Solid line demarcates trials done when vectors where susceptible to those done when vectors were resistant

Mosquito exiting from experimental huts.

In the 2008 PermaNet 2.0 trial, when the vectors were susceptible, a naturally high proportion of *An. gambiae* were collected each morning from the verandah and window traps of huts with untreated nets. Insecticide induced exophily of susceptible *An. gambiae* from huts with treated nets compared to huts with untreated nets was not significant because most of the latter exited naturally each night from the huts (Tables 2a). However, in the 2013 trial when vectors were resistant, both the unwashed and washed PermaNet 2.0 LLINs recorded significantly more survival/exiting compared to when mosquitoes were susceptible (Table 3a).

Table 3a Experimental huts results: Number entering, %deterrence, exiting and % exiting of wild *An. gambiae s.l.* huts during PermaNet 2.0 huts trails conducted at Zeneti in 2008, 2013 and 2014.

		Untreated net	PermaNet 2.0	PermaNet 2.0	CTN
Initial dose of Deltamethrin (mg/m^2)		0			25
Number of washes		0	Unwashed	20	3
Trial year					
2008	**Total females entering**	**723**	**574**	**586**	**560**
	Geometric Mean females caught/night (95% C.I.)	13.4	10.6	10.9	10.4
	%Deterrence	0^{a1}	20.6^{a1}	18.9^{a1}	22.5^{a1}
2013	**Total females entering**	**445**	**289**	**548**	**452**
	Mean females caught/night (95% C.I.)	20.4	10.8	27.3	16.9
	%Deterrence	0^{a1}	35.1^{b1}	0^{a1}	0^{a1}
2014*	**Total females entering**	**29**	**61**	**39**	**73**
	Geometric Mean females caught/night (95% C.I.)	1.7	1.7	1.5	1.9
	%Deterrence	0^{a1}	0^{a1}	0^{a1}	0^{a1}
2008	% Exiting	85.5^{a1}	85.5^{a1}	88.4^{a1}	92.0^{b1}
2013	% Exiting	73.9^{a1}	94.1^{b1}	90.9^{b1}	89.2^{b1}
2014	% Exiting	69^{a1}	88.5^{b1}	92.3^{b1}	65.8^{c1}

Notes:

1. **Percentage deterrence, exiting and 95% CIs are backtransformed from values calculated by the blocked logistic regression model.**
2. **Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression (P<0.05).**
3. **Within each *An. gambiae* row, column not sharing a superscript number differ significantly by blocked logistic regression (P<0.05).**

In 2008 when vectors were susceptible, unwashed Olyset net recorded induced deterrence. In 2013 and 2015, in trials with resistant vectors, unwashed Olyset net

recorded significantly higher exiting rates (96% and 100% respectively) relative to untreated control arms (68.8% and 81% respectively) (Table 3b).

Table 3b Experimental huts results: Number entering, %deterrence, exiting and % exiting of wild *An. gambiae s.l.* huts during Olyset net huts trials conducted at Zeneti in 2010, 2013 and 2015.

		Untreated net	OlysetNet	OlysetNet	CTN
Initial dose of permethrin (mg/m^2)		0			500
Number of washes		0	Unwashed	20	3
Trial year					
2010	**Total females entering**	**68**	**13**	**43**	**107**
	Geometric Mean females caught/night	0.6	0.1	0.5	0.8
	% Deterrence	$0^{a,1}$	$80.9^{b,1}$	$36.8^{c,1}$	$0^{a,1}$
2013	**Total females entering**	**221**	**245**		**282**
	Geometric Mean females caught/night	3.4	4		4.7
	% Deterrence	$0^{a,2}$	$0^{a,2}$	-	$0^{a,2}$
2015	**Total females entering**	**58**	**36**		
	Geometric Mean females caught/night	0.5	0.3		
	% Deterrence	$0^{a,3}$	$37.9^{b,1}$	-	-
Trial year					
2010	% Exiting	$47.1^{a,1}$	$30.8^{b,1}$	$81.4^{c,2}$	$88.8^{c,1}$
2013	% Exiting	$68.8^{b,2}$	$96.3^{b,2}$	-	$87.6^{c,1}$
2015	% Exiting	$81^{c,3}$	$100^{a,3}$	-	-

Notes:

1. **Percentage deterrence, exiting and 95% CIs are backtransformed from values calculated by the blocked logistic regression model.**
2. **Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression (P<0.05).**

3. **Within each *An. gambiae* row, column not sharing a superscript number differ significantly by blocked logistic regression (P<0.05).**

Induced exophily recorded by unwashed and washed Interceptor LN in 2006 and 2008 trials when vectors were susceptible ranged between 79-93 but was not significantly higher to the rates recorded by untreated control. In 2013 when vectors were resistant, unwashed Interceptor recorded higher exophily (94.5%) than that recorded by the untreated control arm (68.8%) (Table 3c).

Table 3c Experimental huts results: Number entering, % deterrence, exiting and % exiting of wild *An. gambiae s.l.* huts during Interceptor LN huts trails conducted at Zeneti in 2006, 2008, 2013 and 2017.

		Untreated net	Interceptor	Interceptor	CTN
Initial dose of alphacypermethrin (mg/m^2)		0			40
Number of washes		0	Unwashed	20	3
Trial year					
2006	**Total females entering**	**171**	**134**	**122**	**224**
	Geometric Mean females caught/night (95% C.I.)	2.8	1.9	2.1	2.5
	% Deterrence	0^{a,1}	21.7^{a,1}	21.7^{a,1}	13.3^{a}
2008	**Total females entering**	**143**	**112**	**112**	**124**
	Geometric Mean females caught/night (95% C.I.)	2.6	2	1.8	3.4
	% Deterrence	0^{a,1,2}	20.4^{b,1,2}	21.1^{b,1}	0^{a,1}
2013	**Total females entering**	**221**	**255**		**282**
	Geometric Mean females caught/night (95% C.I.)	3.4	3.9		4.7
	% Deterrence	0^{a2}	0^{a2}	-	0^{a2}
2017	**Total females entering**	**221**	**255**		**282**
	Geometric Mean females caught/night (95% C.I.)	3.4	3.9		4.7
	% Deterrence	0^{a2}	0^{a2}	-	0^{a2}
2006	% Exiting	86^{a,1}	86.6^{a,1,2}	92.6^{a,2}	92.8^{a,1}

2008	% Exiting	$88.1^{abc,1}$	$79.5^{b,1}$	$82.1^{bc,1}$	$89.5^{ac,1,2}$
2013	% Exiting	$68.8^{a,2}$	$94.5^{b,2}$	-	$87.6^{c,2}$
2017	% Exiting	$68.8^{a,2}$	$94.5^{b,2}$	-	$87.6^{c,2}$

Notes:

1. **Percentage deterrence, exiting and 95% CIs are backtransformed from values calculated by the blocked logistic regression model.**
2. **Within each column, rows not sharing a superscript letter differ significantly by blocked logistic regression (P<0.05).**
3. **Within each *An. gambiae* row, column not sharing a superscript number differ significantly by blocked logistic regression (P<0.05).**

There were no significant differences in exiting rate of *An. funestus* from the huts with untreated nets as compared to that recorded from the huts with treated nets (unwashed or 20 times washed Olyset Net), thus no insecticide induced exiting rate of *An. funestus* was observed in this hut trial.

Meta-analysis pooled estimate

Results from meta-analysis also showed that with unwashed LLINs different levels of deterrence were recorded between susceptible and resistant *An. gambiae s.l.* ($z = 2.78$, $p = 0.005$) meaning resistant *An. gambiae s.l.* were more likely to exit the huts with the unwashed LLINs as compared to susceptible *An. gambiae s.l.*; the increase in exophily of the resistant mosquitoes was significant with the unwashed LLINs ($z = 2.78$, $p = 0.005$) (Fig. 6a). However, with washed LLINs same levels of deterrence were recorded ($z = 1.45$, $p = 0.148$) (Fig. 6a).

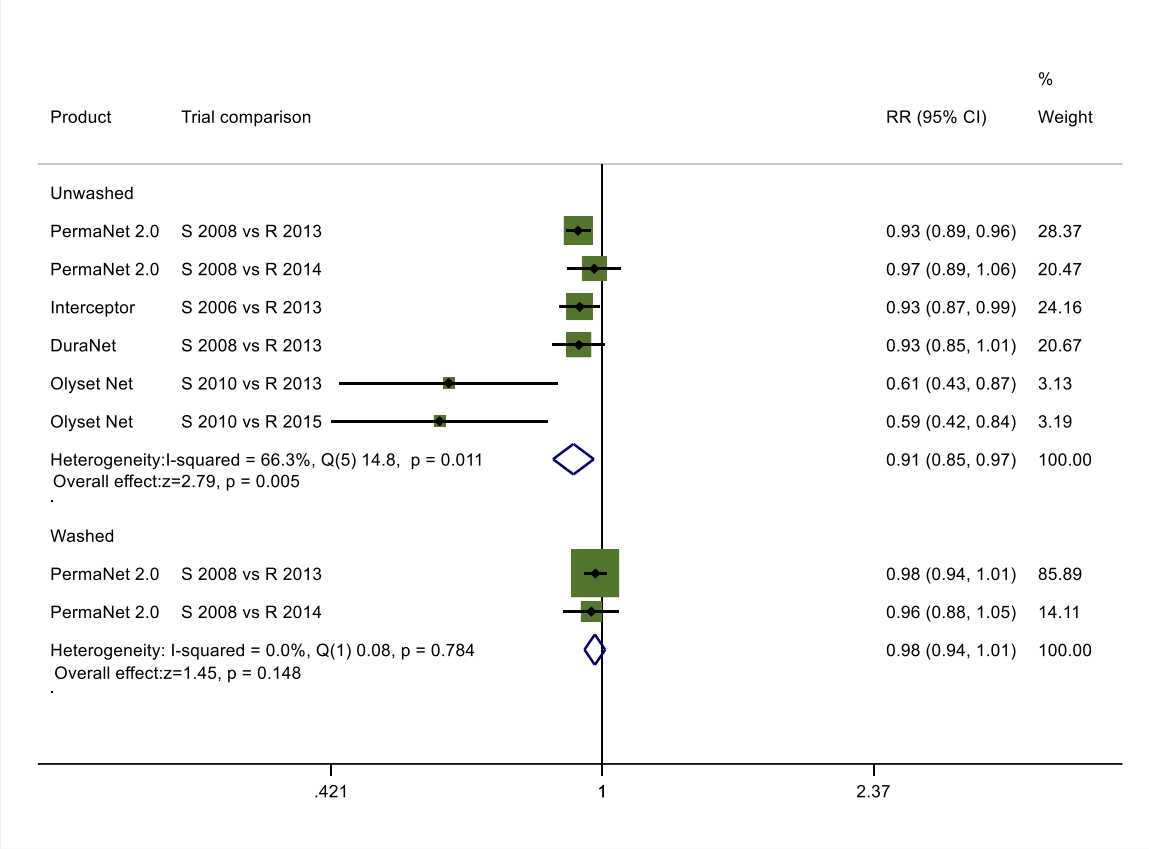

Figure 6a. Metanalysis of the efficacy results of washed and unwashed LN efficacy in trials done when vectors where susceptible and those done when vectors where resistant: *An. gambiae s.l.:* exophily.

Meta-analysis pooled estimate

Resistant *An. funestus s.l.* with unwashed and 20 times washed LLLIN were 1.21-fold and 1.25-fold respectively less likely to exit as compared to susceptible *An. funestus s.l.* (z = 3.9, p = 0.001) and (z = 1.98, p = 0.047) with unwashed and washed LLINs respectively (Fig. 6b).

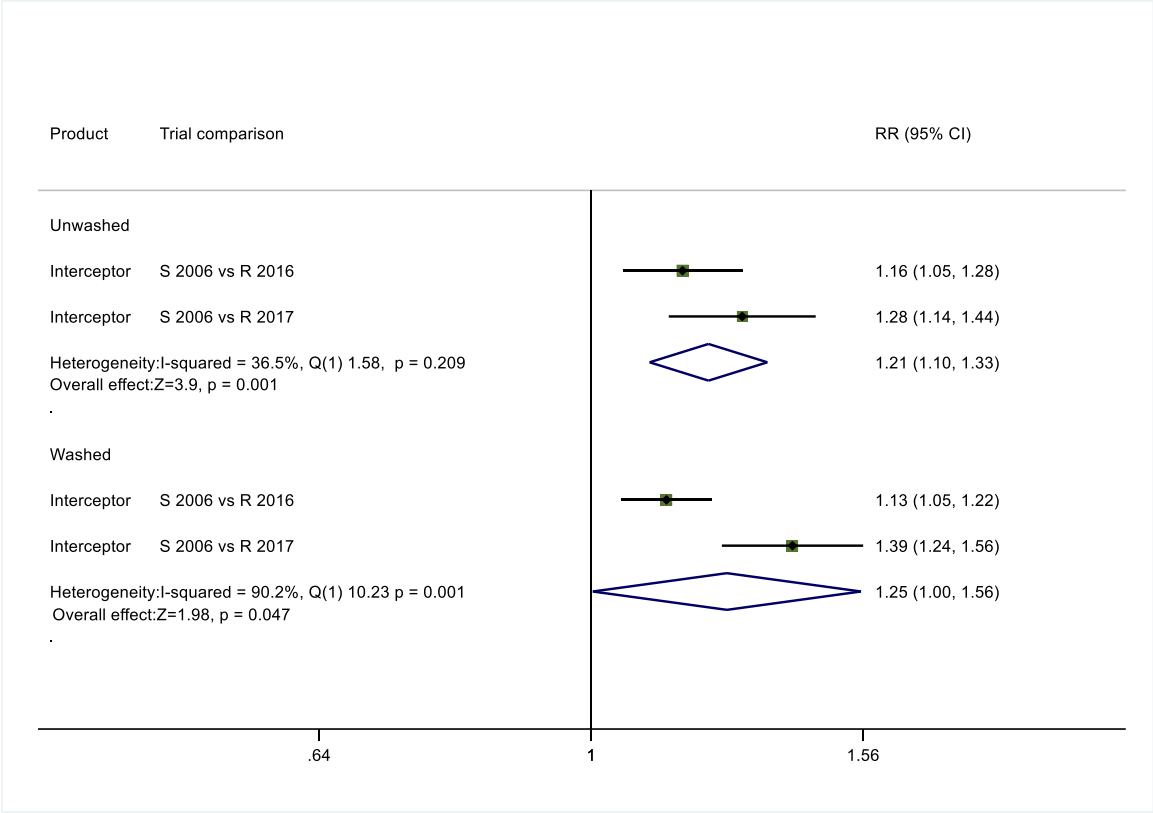

Figure 6b. Metanalysis of the efficacy results of washed and unwashed LN efficacy in trials done when vectors where susceptible and those done when vectors where resistant: *An. funestus s.l.:* exophily.

Logistic regression analysis

Regression analysis of the trials showed that though there was decrease in exophily with resistant mosquitoes, the decrease was not significant, hence resistance was not associated with any change in LLIN-induced exophily of wild free flying *An. gambiae s.l.* (t = -0.30, p=0.764). With *An. funestus s.l.* the decline in exophily with resistant mosquitoes as compared to susceptible mosquitoes was significant, hence resistance was significantly associated with any change in LLIN-induced exophily of wild free flying *An. funestus s.l.* (t = 3.19, p=0.013) (Fig 4).

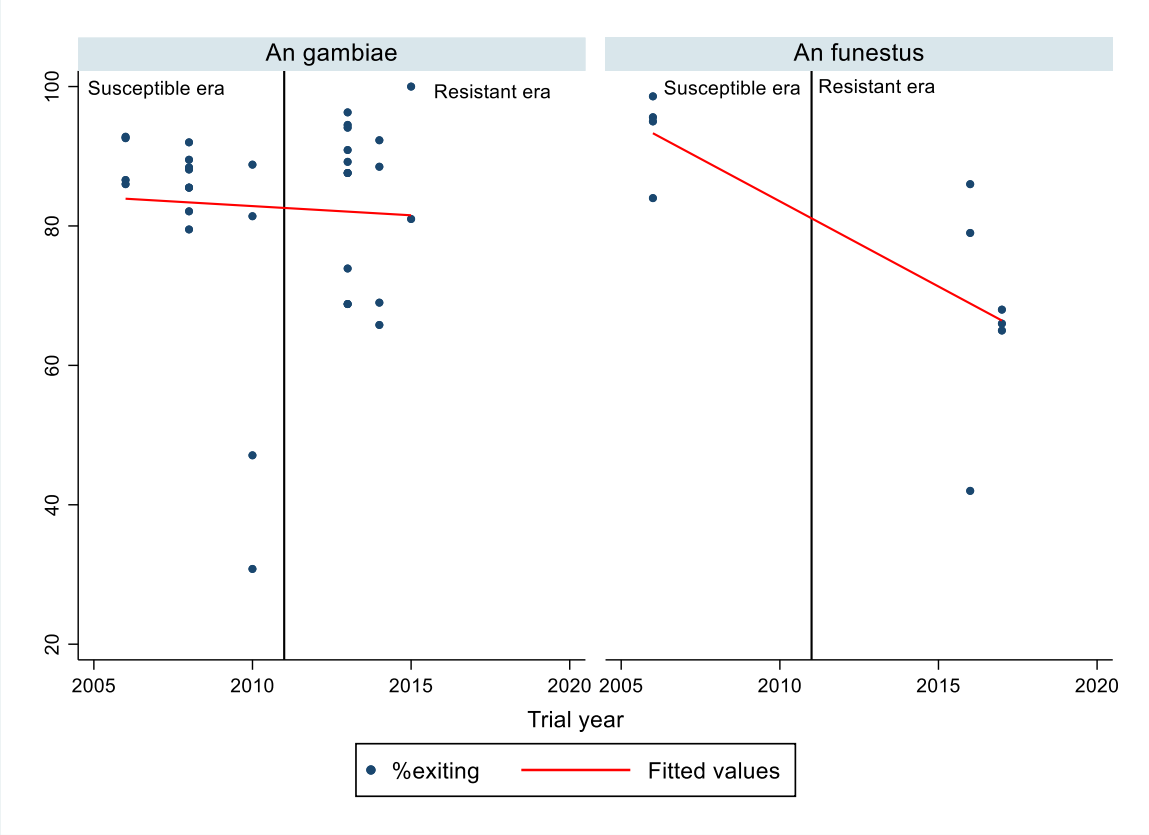

Figure 7: Percentage exiting of wild free flying *An. gambiae s.l.* and *An. funestus s.l.* entered into experimental during trials. Each point denotes the percentage exiting for each trial. The line graph shows best fit regression trend line for the percentage exiting in various trials.
NOTE: Solid line demarcates trials done when vectors where susceptible to those done when vectors were resistant

Mosquito entry into experimental huts

The numbers and proportions caught entering the hut are shown in Table 3a-c. The geometric mean number (GMN) of *An. gambiae* collected per hut per night during Permanent 2.0 trials varied between 10-13, 11-27 and 1.5-1.9 during 2008, 2013 and 2014 trials, respectively (Table 3a). The GMN of *An. gambiae s.l.* collected during the Olyset Net trials ranged between 0.6-0.8, 3-5 and 0.3-0.5 during trials in 2010, 2013 and 2015 (Table 3b). GMN collected during Interceptor LN trials ranged between 2-2.8, 2-3.4 and 3.4-4.7 per hut per night for trials in 2006, 2008 and 2013 (Table 3c). The PermaNet 2.0 and Olyset Net trials in 2014 and 2015 were conducted during short rainy season, therefore relatively

fewer numbers of *An. gambiae* were collected, the GMN ranging between 1.5-1.7 and 0.3-0.5 respectively).

Compared to the totals collected in the huts with untreated control net, there were no major differences in the number of mosquitoes collected from the huts with treated nets (unwashed or 20 times washed) in 2006 and 2013 Interceptor LN trials, 2008 and 2014 PermaNet 2.0 LN trials, and in 2013 Olyset Net LN trial; hence little evidence of deterrence between treatments within each of the trials. The differences in the number of mosquitoes collected in each treatment arm between trials was also not significant (Table 3a-c). There were significant differences ($P<0.05$) in the number of mosquitoes collected from the huts with treated nets in 2008 Interceptor LN trial, 2013 PermaNet 2.0 LN trial and 2010, 2015 Olyset Net LN trials compared to numbers collected in huts with untreated control net.

Meta-analysis pooled estimate

Results from meta-analysis also showed that similar levels of deterrence were recorded between susceptible and resistant *An. gambiae s.l.* against both unwashed ($z = 1.47$, $p = 0.141$) and washed LLINs ($z = 0.09$, $p = 0.951$) (Fig 8a). Conversely there was significant reduction in deterrence with resistant *An. funestus s.l.* compared to susceptible *An. funestus s.l.* against both unwashed ($z = 2.72$, $p = 0.006$) and washed LLINs ($z = 7.66$, $p = 0.001$) (Fig 8b).

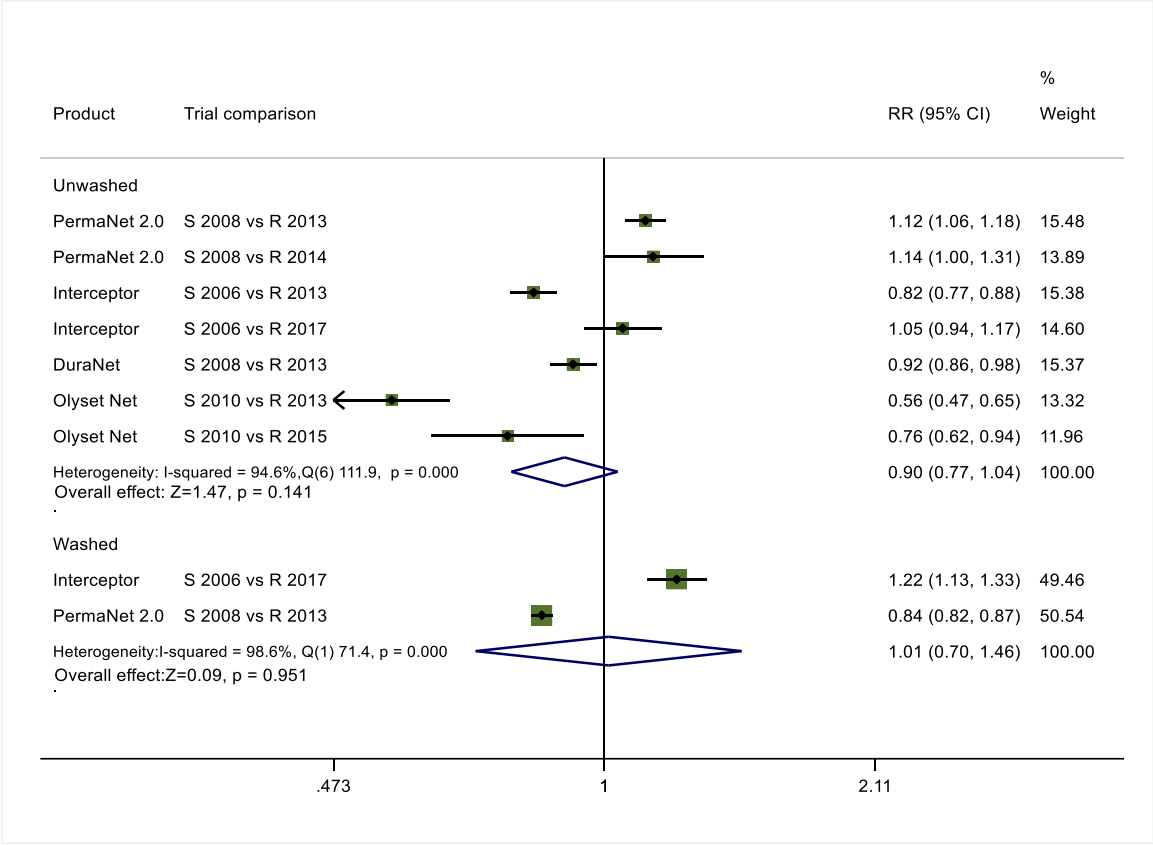

Figure 8a. Metanalysis of the efficacy results of washed and unwashed LN efficacy in trials done when vectors where susceptible and those done when vectors where resistant: *An. gambiae s.l.*: deterrency.

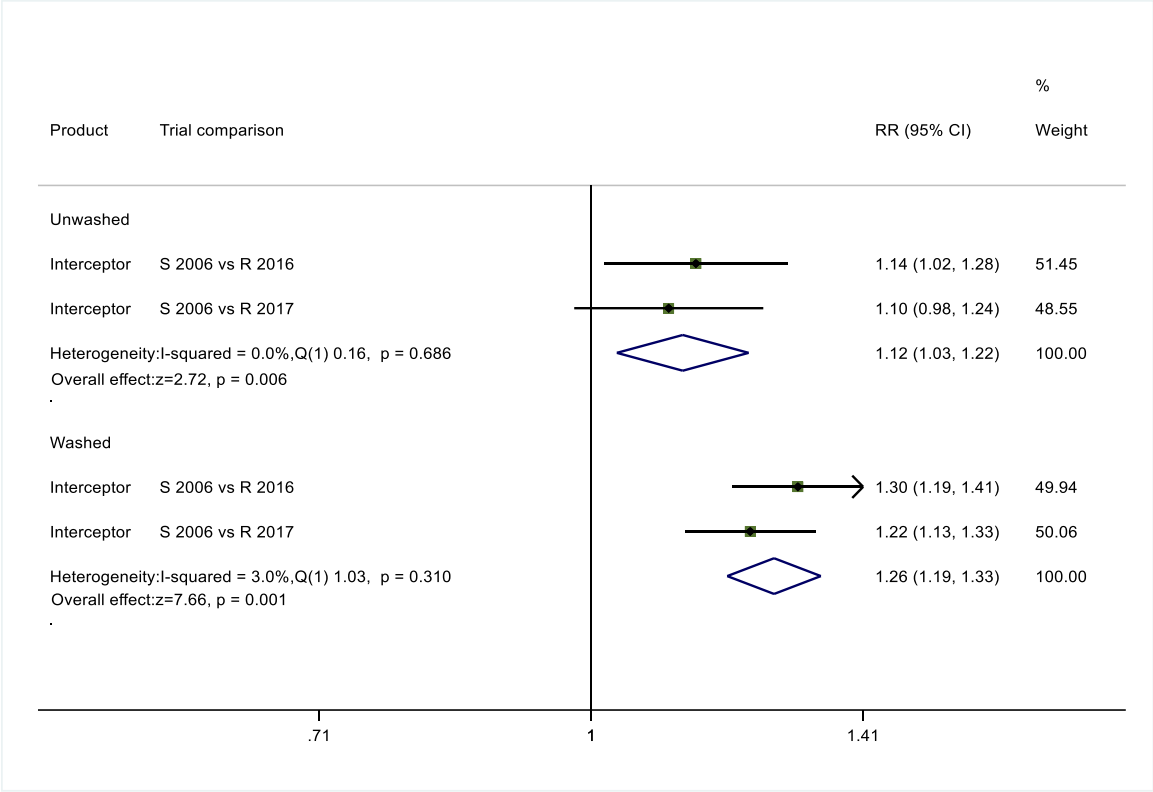

Figure 8b. Metanalysis of the efficacy results of washed and unwashed LN efficacy in trials done when vectors where susceptible and those done when vectors where resistant: *An. funestus s.l.*: deterrency.

Logistic regression analysis

The regression analysis showed that the transition from susceptibility to resistance was not associated with any change in LLIN-induced deterrence of wild free flying *An. gambiae s.l.* (t = -1.35, p=0.186) or *An. funestus s.l.* (t = 1.85, p=0.101) (Fig 9).

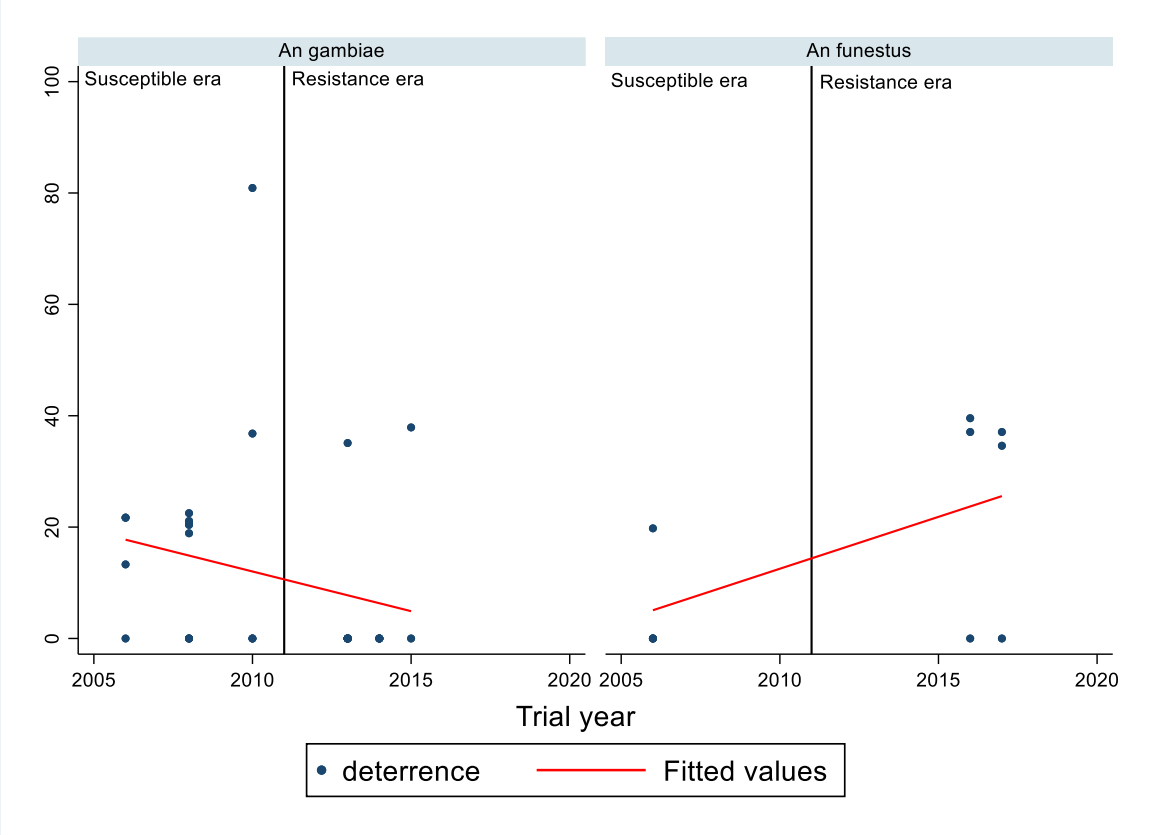

Figure 9: Percentage deterrence of wild free flying *An. gambiae s.l.* and *An. funestus s.l.* Each point denotes number entering relative to control for each trial. The line graph shows best fit regression trend line for the percentage exiting in various trials
NOTE: Solid line demarcate trials done when vectors where susceptible to those done when vectors were resistant.

Discussion

The study assessed the impact of insecticide resistance on the efficacy of pyrethroid LLINs in a series of ten LLIN evaluations in experimental huts trials in north-eastern Tanzania between 2006 and 2017. The early trials were conducted when *An. gambiae s.l.* and *An. funestus s.l.* were susceptible to pyrethroids, later trials were conducted after the development of pyrethroid resistance in the two vectors. The fixed hut location and changing resistance status of the vectors provided a unique opportunity to study the changing effectiveness of standard LLIN and their capacity to control mosquitoes that had become increasingly resistant during the recent decade.

Regression analysis showed that resistant *An. gambiae s.l* and *An. funestus s.l* mosquitoes were less likely to be killed by LLIN as compared to free-flying susceptible mosquitoes. The meta-analysis of trials, before and after resistance, on the same brands of net was a novel approach showing that mortality risk ratio of susceptible versus resistant free-flying *An. gambiae s.l.* was 6.9 times higher with unwashed LLIN and 5.2 times higher with the 20 times-washed LLIN tested against a variety of pyrethroids, net brands and textile materials. The meta-analysis on free flying *An. funestus s.l.* showed mortality risk ratios were 3.3 and 2.6 times higher on the same nets for susceptible compared to resistant mosquitoes. These findings depict the pyrethroid LLINs' declining efficacy after prolonged use (Standardized washing is a crude proxy for an ageing net) to control pyrethroid resistant *An. gambiae s.l.* and *An. funestus s.l.* that contain *kdr and/*or metabolic resistance mechanisms in NE Tanzania. These findings may imply a loss of mass population effect on mortality after the evolution of high-level resistance.

Reductions in efficacy of insecticidal interventions, due to resistance, have been reported in several countries in Africa, including Benin and Burkina faso in West Africa [25] and Ethiopia [46] and Uganda [47] in East Africa, to name but a few locations. Contrasting findings from other studies that show continued efficacy of LLIN against *An. gambiae* [48], probably mark the transition over the years between selection of mono-factorial resistance due to one mechanism (e.g., *kdr*) to selection of multiple mechanisms such as kdr *and* MFOs as has occurred in Benin [29, 49]. Other reasons may be due to differences in the biology or species present at the location where the trials were conducted; for example, in Kenya and Tanzania *An. gambiae s.s.* predominates and in Ivory Coast and Benin *An. coluzzii* forms the majority. Differences in resistance intensity and *kdr* frequencies between these sites might also explain the contrasting findings [50].

The study also provides evidence of association between pyrethroid resistance and LLIN-induced exophily, whereby resistant *An. gambiae s.l.* were more likely to survive and exit the huts. The meta-analysis comparing the induced exophily of resistant versus

susceptible *An. gambiae s.l.* showed exophily rate. was 9% and 3% greater than the rate recorded on the unwashed and 20 times washed LLINs. By contrast, the meta-analysis found no evidence for an association between resistance in *An. funestus s.l.* and LLIN-induced exophily; quite the reverse, *An. funestus* appeared to become *more* endophilic once resistant.

The trial meta-analyses found little evidence for major differences in blood-feeding rates of resistant compared to susceptible *An. gambiae s.l.* or in blood-feeding rates of resistant and susceptible *An. funestus s.l*. The meta-analyses confirmed that any difference was non-significant for both species, whether with unwashed or 20-times washed LLIN. Personal protection due to pyrethroid irritability or net barrier effect would be expected to continue after resistance has evolved in the population, indicating some continued personal protection until individual nets become highly holed.

The study outcomes present further evidence that resistance in Tanzania may undermine the efficacy of LLINs. Although the study presents evidence of insecticide resistance having a negative entomological impact on LLIN efficacy, it did not include an analysis on malaria epidemiology and therefore should not be interpreted as LLIN failure in controlling malaria disease burden. The interaction between resistance and changes to mosquito physiology and behavior is complex and may or may not lead to LLIN failure [51-54]. In this study, for example, resistant *An. gambiae s.l.* were significantly more likely to exit the huts as compared to susceptible *An. gambiae s.l.*, and the meta-analysis showed that with unwashed LLINs exophily rate of resistant *An. gambiae s.l.* was significantly higher by 9% than the exophily rate recorded by free flying susceptible *An. gambiae s.l.* The increase in exiting behaviour in resistant *An. gambiae s.l.* combined with stasis in feeding behavior led to resistance induced LLINs avoidance behavior which was also reported by others [55]. Thus, although mosquitoes might not be killed by the standard LLIN due to resistance, the change in behaviour may lessen the capacity for mosquitoes to continue probing around the net and possibly feed on a human that might occupy a

slightly torn net. This, together with other physiological factors, such as the reduction in the rate of malaria oocyst development in pyrethroid-exposed resistant infected mosquitoes [56], the possibility of delayed mortality beyond the 24-h standard holding interval when pyrethroid-resistant malaria vectors are exposed to pyrethroids [57], and resistant mosquitoes being less fit compared to susceptible ones [57, 58], may combine to lead to reduced lifespan and fewer older infective mosquitoes. These resistance-induced behavioral/physiological factors may operate additively to reduce the negative epidemiological impact that resistance may otherwise have, and lead to continued efficacy of LLIN in controlling malaria mosquitoes in areas with resistant mosquitoes, as reported in epidemiological studies that show LLIN-users protected against malaria compared with non LLIN-users [21, 51, 52, 54, 59, 60].

On the other hand, the recent 4-arm randomized controlled trials that has evaluated new-generation PBO-pyrethroid or Dual-AI LLINs in locations of high resistance have noted that not only the new generation LLIN shows improved entomological and epidemiological effect compared to the standard pyrethroid-only LLIN reference arms, the pyrethroid-only LLINs are failing sooner than before, just as they acquire holes and the net barrier is breached [23]. The latter is evidence that resistance not only undermines entomological efficacy, morbut e importantly, it may undermine the epidemiological argument for issuing standard LLINs. While the latter is the single, most powerful argument against distributing pyrethroid-only LLIN in areas of high resistance, a few individuals still argue that some PBO LLIN show higher pyrethroid content or show increased diffusion of pyrethroid to explain PBO LLIN superiority [61]. While this line of argument may be true to some extent, higher pyrethroid concentration does not necessarily lead to higher effect; a saturation point on mortality would soon be reached. The more plausible explanation for improved effect is the synergistic action of PBO or absence of resistance to the partner insecticide. This highlights the need for more new insecticide classes with novel mode of action, new control tools that can better control resistant mosquitoes, and new strategies for effective, sustainable resistance management.

Conclusion

The reduced efficacy of pyrethroid-treated nets (LLINs) on entomological indices in an area with pyrethroid resistant *An*. *gambiae s.l. and An. funestus s.l.* populations confirm that, in Tanzania, resistance may limit and undermine the effectiveness of malaria vector control interventions especially pyrethroid LLINs. To safeguard insecticide-based vector control, new insecticide classes with novel mode of action need to be scaled-up to achieve the targets of the WHO Global Technical Strategy for Malaria 2016-2030.

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Part four summary of key findings:

In concluding part four here are the key findings and recommendations:

Regression analysis showed that resistant *An. gambiae s.l.* and *An. funestus s.l.* mosquitoes were less likely to be killed by LLIN as compared to free-flying susceptible mosquitoes. The meta-analysis of trials, before and after resistance, on the same brands of net was a novel approach showing that mortality risk ratio of susceptible versus resistant free-flying *An. gambiae s.l.* was 6.9 times higher with unwashed LLIN and 5.2 times higher with the 20 times-washed LLIN tested against a variety of pyrethroids, net brands and textile materials. The meta-analysis on susceptible versus resistant free flying *An. funestus s.l.* showed mortality risk ratios were 3.3 and 2.6 times higher using the same nets.

Reduced mortality induced by LLINs after selection of pyrethroid resistance indicates that resistance may undermine household and community control of vector populations. Personal protection parameters seemed less affected by the transition to resistance. Meta-analysis, comparing the same net brands before and after selection of resistance, reveal which control parameters seem most affected by resistance.

Personal protection due to pyrethroid irritability or net barrier effect would be expected to continue after resistance has evolved in the population, indicating some continued personal protection until individual nets become highly holed.

Although the study presents evidence of insecticide resistance having a negative entomological impact on LLIN efficacy, it did not include an analysis on malaria epidemiology and therefore should not be interpreted as LLIN failure in controlling malaria disease burden.

To safeguard insecticide-based vector control, new insecticide classes with novel mode of action need to be scaled-up to achieve the targets of the WHO Global Technical Strategy

for Malaria 2016-2030. The issue of insecticide resistance management and control of insecticide resistant vectors will be discussed in the next chapter in Part 5 studies to investigate the role of synergists and novel insecticides on nets.

PART FIVE

Research question: Can improved vector control and insecticide resistance management be achieved when synergist-pyrethroid combination LLINs or LLINs with dual insecticides are used against pyrethroid resistant malaria vector populations?

Prologue:

The chemical agents that make malaria vector control feasible are the pyrethroids. The best tools for delivering pyrethroids are long-lasting insecticidal nets (LLIN) and indoor residual spraying (IRS). But coinciding with the increased coverage of LLIN and IRS is the development and spread of resistant mosquitoes that may undermine the effectiveness of the two tools.

Statement of the problem

Long-lasting net (LLINs) have become a crucial tool for vector control against malaria and other mosquito-borne diseases. Unfortunately, resistance to pyrethroids, the class of insecticide that is widely used in almost all of the currently prequalified LLINs, is now widespread in African malaria vectors [1]. At the time of this first study with PBO LLIN in Tanzania, the impact of pyrethroid resistance in Tanzania was limited or unquantified. More resistance was recorded in West Africa where it occurred at quite high levels. Resistance impact in Tanzania was largely unquantified at the time and varied depending on the level of resistance and location, malaria endemicity, and proportion of the human population using LLINs [2]. A multi-Centre trial found no strong evidence that pyrethroid resistance reduced the personal protection provided by use of LLINs [3]. This subject remained controversial until comparatively recently when it was demonstrated convincingly in a cluster RCT that PBO LLIN improved malaria control relative to standard LLIN [4]. Now it is generally accepted the development of insecticide resistance is probably the biggest threat to capacity to control malaria vectors or sustain any drive towards malaria elimination.

Justification

It is generally envisaged that resistance will eventually erode the efficacy of pyrethroid-only LLINs. Thus, for malaria elimination to remain a realistic prospect, innovation in the LLIN market is essential so to maintain the efficacy of this preventative measure for as long as possible.

Resistance management is founded on population genetics. There is evidence that the most promising way to delay the selection of resistance is to apply mixtures of unrelated insecticides.

Most alternative insecticides to pyrethroids alternatives lack the excito-repellency of pyrethroids, a characteristic important for reducing biting rates or providing personal protection to users of insecticide-treated nets (ITNs). This curb is the main reason for combining the alternative insecticide with a pyrethroid that can add repellency to the product. Rather than use a non-pyrethroid insecticide to overcome resistance, a valid approach is to deploy a chemical synergist on the fibres. Synergists overcome resistance by inhibiting the enzymes responsible for certain types of resistance.

General objective

Purpose of this part of thesis is to ascertain if improved vector control and insecticide resistance Management be achieved using novel LLIN products that contain a combination of pyrethroid and non-pyrethroid insecticides or synergists.

Specifically, this part sought:

I. To evaluate wash fastness and long-lasting efficacy of new dual-active LLINs treated with a combination of pyrethroid and piperonyl-butoxide (PBO) (treated only on the roof panel of the net) against pyrethroid-resistant malaria vectors in experimental hut trials.

II. To evaluate wash fastness and long-lasting efficacy of new dual-active LLINs treated with a combination of pyrethroid and piperonyl-butoxide (PBO) (treated only on the whole net) against pyrethroid-resistant malaria vectors in experimental hut trial.

III. To undertake a comparative meta-analysis of the efficacy of the LLINs treated with a combination of pyrethroid and piperonyl-butoxide (PBO) between those with PBO restricted on the roof panel only and those with PBO treated on whole net.

IV. To evaluate wash fastness and long-lasting efficacy of new dual-active LLINs treated with a combination of pyrethroid and chlorfenapyr (CFP) against pyrethroid-resistant malaria vectors in experimental hut trials.

Studies to address the objectives above are presented and discussed in four separate chapters as follows:

Chapter 8: Evaluation of PermaNet 3.0 a deltamethrin-PBO combination net against *Anopheles gambiae* and pyrethroid resistant *Culex quinquefasciatus* mosquitoes: an experimental hut trial in Tanzania

Chapter 9: Field evaluation of Veeralin, an alpha-cypermethrin + PBO long-lasting insecticidal net, against natural populations of *Anopheles funestus* in experimental huts in Muheza, Tanzania

Chapter 10: Comparative and meta-analysis of the efficacy of standard and PBO synergist long-lasting nets before and after development of insecticide resistance against *Anopheles gambiae* and *Anopheles funestus* mosquitoes: experimental hut trials in Tanzania

Chapter 12: Efficacy of interceptor® G2, a long-lasting insecticide mixture net treated with chlorfenapyr and alpha-cypermethrin against *Anopheles funestus*: experimental hut trials in north-eastern Tanzania.

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Chapter 8: Evaluation of PermaNet 3.0 a deltamethrin-PBO combination net against *Anopheles gambiae* and pyrethroid resistant *Culex quinquefasciatus* mosquitoes: an experimental hut trial in Tanzania

Prologue:

One of the most promising ways to manage insecticide resistance is to apply combination /mixtures of unrelated insecticides or a mixture of insecticide and a chemical synergist.

With Long-lasting insecticidal treated nets, combinations can be applied either as a mixture of the two insecticides or as a two-in-one (mosaic) format, in which the pyrethroid is restricted to the sides and the alternative insecticide to the top of the net [29, 31]. For the two-in-one net to work as a resistance management tactic mosquitoes should contact both the top and sides so that any pyrethroid resistant mosquito that survives contact with the pyrethroid stands a high chance of being killed by the alternative insecticide. There is evidence that host-seeking mosquitoes of the *An. gambiae* complex do in fact contact the top before the sides [32] possibly in response to human odour plumes or concentration gradients, and this gives the two-in-one concept a degree of credibility.

This chapter describes and discusses the results of the small-scale field experimental huts trial to evaluate wash-resistance and efficacy of the PermaNet 3.0 a deltamethrin-PBO combination net (that has PBO treated on the roof panel only) against *Anopheles gambiae* and pyrethroid resistant *Culex quinquefasciatus* mosquitoes. This trial was done during a period when mosquitoes were still susceptible to pyrethroids, and use of PBO was in its infancy on LLINs. In this circumstance, there was less benefit be gained from any use of PBO.

Chapter 8: PermaNet 3.0 a deltamethrin-PBO combination net against *Anopheles gambiae* and pyrethroid resistant *Culex quinquefasciatus* mosquitoes: an experimental hut trial in Tanzania

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The material presented in this chapter has been published as:

Tungu P, Magesa S, Maxwell C, Malima R, Masue D, Sudi W, Myamba J, Pigeon O, Rowland M: **Evaluation of PermaNet 3.0 a deltamethrin-PBO combination net against Anopheles gambiae and pyrethroid resistant Culex quinquefasciatus mosquitoes: an experimental hut trial in Tanzania.** *Malar J* 2010, **9:**21. http://doi:10.1186/1475-2875-9-21

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Abstract

Background:

Combination mosquito nets incorporating two unrelated insecticides or insecticides plus synergist are designed to control insecticide resistant mosquitoes. PermaNet 3.0 is a long-lasting combination net incorporating deltamethrin on the side panels and a mixture of deltamethrin and synergist piperonyl butoxide (PBO) on the top panel. PBO is an inhibitor of mixed function oxidases implicated in pyrethroid resistance.

Method:

An experimental hut trial comparing PermaNet 3.0, PermaNet 2.0 and a conventional deltamethrin-treated net was conducted in NE Tanzania using standard WHOPES procedures. The PermaNet arms included unwashed nets and nets washed 20 times. PermaNet 2.0 is a long-lasting insecticidal net incorporating deltamethrin as a single active.

Results:

Against pyrethroid susceptible *Anopheles gambiae* the unwashed PermaNet 3.0 showed no difference to unwashed PermaNet 2.0 in terms of mortality (95% killed) but showed differences in blood-feeding rate (3% blood-fed with PermaNet 3.0 versus 10% with PermaNet 2.0). After 20 washes the two products showed no difference in feeding rate

(10% with 3.0 and 9% with 2.0) but showed small differences in mortality (95% with 3.0 and 87% with 2.0). Against pyrethroid resistant *Culex quinquefasciatus*, mediated by elevated oxidase and kdr mechanisms, the unwashed PermaNet 3.0 killed 48% and PermaNet 2.0 killed 32% but after 20 washes there was no significant difference in mortality between the two products (32% killed by 3.0 and 30% by 2.0). For protecting against Culex PermaNet 3.0 showed no difference to PermaNet 2.0 when either unwashed or after 20 washes; both products were highly protective against biting. Laboratory tunnel bioassays confirmed the loss of biological activity of the PBO/deltamethrin-treated panel after washing.

Conclusion:

PermaNet products were highly effective against susceptible *Anopheles gambiae*. As a long-lasting net to control or protect against pyrethroid resistant mosquitoes PermaNet 3.0 showed marginal improvement over PermaNet 2.0 against *Culex quinquefasciatus*.

Background

The development of insecticide resistance is probably the biggest threat to capacity to control malaria vectors or sustain any drive towards malaria elimination. The chemical agents that make malaria vector control feasible are the pyrethroids. The best tools for delivering pyrethroids are long-lasting insecticidal nets (LLIN) and indoor residual spraying (IRS) [1]. Latest trends confirm that the scale up of these two tools is making inroads into the malaria burden in many African countries [2–6]. This has stimulated new discussion about malaria elimination which a few years ago seemed inconceivable [7–11]. But coinciding with the increased coverage of LLIN and IRS is the development and spread of resistant mosquitoes that may undermine the effectiveness of the two tools [12–16]. For elimination to remain a realistic prospect, it is essential to preserve the pyrethroids for as long as possible because no other insecticide class can match the pyrethroids for effectiveness, safety, cost per unit dose, acceptability or suitability for LLIN and IRS [17, 18].

Resistance management has a theoretical foundation in population genetics that goes back three decades [19–21]. Simulation modeling has shown that the most promising way to delay the selection of resistance is to apply mixtures of unrelated insecticides [22–25]. The idea behind mixtures is that insects which develop resistance to one insecticide should be killed by the second insecticide provided they are not resistant to both and a proportion of each generation escapes exposure altogether. When resistance is present at low frequency – such as when it first evolves – double resistance will be rare and selection of each type of resistance should be delayed or prevented.

The same principle has been adopted in the strategy to preserve anti-malarial drug efficacy known as combination therapy [1, 26]. Adoption of combination therapy on the Thai-Burmese border has prevented or delayed the selection of drug resistance when in the preceding decade chloroquine, SP and mefloquine monotherapy were each rendered ineffective by sequential evolution of resistance [27]. It is time a similar strategy was adopted for preserving insecticides for malaria vector control.

Alternative insecticides to pyrethroids have been tested on nets for effect against wild, pyrethroid resistant mosquito populations but only under limited experimental conditions in the field [28–31]. Most alternatives lack the excito-repellency of pyrethroids, a characteristic important for reducing biting rates or providing personal protection to users of insecticide-treated nets (ITNs). This limitation is the main reason for combining the alternative insecticide with a pyrethroid that can add repellency to the product. Combinations can be applied either as a mixture of the two insecticides or as a two-in-one (mosaic) format, in which the pyrethroid is restricted to the sides and the alternative insecticide to the top of the net [29, 31]. For the two-in-one net to work as a resistance management tactic mosquitoes should contact both the top and sides so that any pyrethroid resistant mosquito that survives contact with the pyrethroid stands a high chance of being killed by the alternative insecticide. There is indirect evidence that host-seeking mosquitoes of the *An. gambiae* complex do in fact contact the top [32] in

response to odor plumes or concentration gradients, and this gives the two-in-one concept a degree of credibility.

Rather than use a non-pyrethroid insecticide to overcome resistance, another approach is to deploy a chemical synergist on the fibres. Synergists overcome resistance by inhibiting the enzymes responsible for certain types of resistance. Resistance to pyrethroids in Anopheline mosquitoes appears to be caused by two primary mechanisms: a target site insensitivity mechanism known as kdr, and a metabolic mechanism caused by mixed function oxidases (MFOs). MFOs are responsible for the pyrethroid resistance that evolved in *Anopheles funestus* and which led to the failure of IRS campaigns in South Africa [12, 33]. It appears that MFOs may also act in consort with kdr to create a pyrethroid resistance, which is causing control failure of *Anopheles gambiae* M form in parts of West Africa [13, 14, 34]. Both MFOs and kdr together are responsible for pyrethroid resistance in *Culex quinquefasciatus* [35, 36]. One type of synergist capable of inhibiting MFOs is piperonyl butoxide (PBO). PBO is commonly used in commercial aerosols for potentiating pyrethroid activity against flying or domestic insect pests [18].

PBO has potential to combat the growing problem of pyrethroid resistance in An. gambiae and other vector species. PermaNet 3.0 is a long-lasting insecticidal net developed by Vestergaard Frandsen in which the PBO together with the pyrethroid deltamethrin are incorporated into the polyethylene fibres on the roof panel of the net. The sides of PermaNet 3.0 are made of polyester and coated with a long-lasting formulation of deltamethrin like the pyrethroid-based LLIN, PermaNet 2.0 but with a strengthened lower part. By restricting PBO to the roof of the net the concept of PermaNet 3.0 is to have the insect contact the synergist on the roof, mediated by the odour plume, before making further contact with pyrethroid on the sides during exploration.

PermaNet 3.0 was submitted by Vestergaard Frandsen to the WHO Pesticide Evaluation Scheme (WHOPES) for formal evaluation. The current paper reports upon a WHOPES-sponsored experimental hut trial conducted against wild, free flying An. gambiae and *Cx*

quinquefasciatus in Muheza, Tanzania, together with supporting laboratory data and chemical analysis.

Methods

Long-lasting insecticidal nets

PermaNet 3.0 LN (Vestergaard Frandsen SA, Denmark) is a LLIN consisting of a top panel made of monofilament polyethylene (100 denier) fabric incorporating deltamethrin at 4 g/kg (approx. 180 mg/m^2) and piperonyl butoxide at 25 g/kg (approx. 1.1 g/m^2), plus side panels made of multifilament polyester (75 denier) fabric with a strengthened border treated with deltamethrin at 2.8 g/kg (approx. 118 mg/m2).

PermaNet 2.0 LN (Vestergaard Frandsen SA, Denmark) is a LLIN made of multifilament polyester (75-100 denier) fabric, factory treated with a wash-resistant formulation of deltamethrin at 1.8 g/kg (for 75 denier) (approx. 62 mg/m2).

The conventionally treated net (CTN) is a multifilament polyester (100 denier) fabric treated with deltamethrin (K-Othrine SC, Bayer) at 25 mg/m2; the net was treated by hand, on site, in an aqueous solution of the formulation.

The size of the PermaNet 2.0 and standard nets was 130 cm wide, 190 cm long, 150 cm high. PermaNet 3.0 nets measured 120 cm wide, 190 cm long, 150 cm high. Hence the top panel of PermaNet 3.0 containing the PBO plus deltamethrin constituted 19.7% of the overall surface area whereas the remaining 80.3% on the sides contained only deltamethrin as an active ingredient.

Mosquito strains

Anopheles gambiae sensu stricto (s.s.) Kisumu, a laboratory insecticide susceptible strain, originally from Kenya.

Culex quinquefasciatus TPRI, a laboratory insecticide susceptible strain, maintained by the Tropical Pesticide Research Institute, Tanzania.

Culex quinquefasciatus Masimbani, a multiple resistant strain from northeast Tanzania, containing elevated oxidase and kdr pyrethroid resistance mechanisms. In WHO resistance tests the strain showed survival after one hour exposure to test papers of permethrin (47% survival) deltamethrin (48%), DDT (58%), malathion (27%) and propoxur (46%).

Exploratory bioassay tests on PermaNet 3.0, PermaNet 2.0 and CTN washed up to 20 times.

Cone bioassays

Three min exposure bioassay tests were conducted using *An. gambiae* Kisumu (pyrethroid susceptible) on PermaNet 3.0, PermaNet 2.0 and the CTN after 0, 10 and 20 washes. Similarly, *Cx. Quinquefasciatus* Masimbani (pyrethroid resistant) was exposed in 3 min bioassay tests to netting taken from the roof and sides of the PermaNet 3.0 after 0, 10 and 20 wash intervals. Washing was conducted on entire nets using the WHO Phase II washing protocol [37]. Bioassays were conducted with WHO cones on netting samples, using five 3–5-day old mosquitoes per replicate and 10 replicate tests per treatment [37, 38]. After the 3 min exposures mosquitoes were aspirated from the cones and held in paper cups and provided with 10% glucose solution. Mortality was recorded after 24 h.

Tunnel tests

The tunnel tests were carried out on samples of PermaNet 3.0 netting cut from the roof and lower sides of the net after 0, 10 and 20 washes using the Phase II washing procedure [37]. The tests were conducted using laboratory-reared *An. gambiae* Kisumu (insecticide susceptible), *Cx. quinquefasciatus* TPRI (insecticide susceptible) and *Cx. quinquefasciatus* Masimbani (pyrethroid resistant). Tunnel tests were replicated three times.

The dimensions and procedures for tunnel tests are described in detail in appendix 1, section 2.3.1.2.

Experimental hut trial

Determination of the point of 'insecticide exhaustion'

A polyester net conventionally treated with deltamethrin at dosage 25 mg/m2 was washed until just before 'insecticide exhaustion' as defined by WHO [37]. The conventionally treated net (CTN) treatment serves as a positive control to judge PermaNet 3.0 performance against. The point of exhaustion is the point at which the CTN showed less than 80% mortality or 95% knock down in WHO cone bioassays conducted after each wash. The standardized WHO washing protocol requires the net to be stirred in 10 litres of soap solution (2 g/litre of 'Savon de Marseille') for 6 min, during a 10 min washing cycle at ambient temperature. Nets were rinsed and dried and left for one day between washes. Determination of the 'point of exhaustion' was carried out by exposing unfed An. gambiae Kisumu in 10 replicates of 5 mosquitoes per replicate at each wash interval on the five panels of each net. Exposure was for 3 min and mortality was scored 24 h later.

Study area and hut design

The six veranda trap huts were situated at Zeneti village, Muheza district, NE Tanzania (5°13'S and 38°39'E). Detailed descriptions of the study area and experimental huts are provided in appendix 1, section 2.6.1

Anopheles gambiae s.s., *An. funestus* and *Cx.* Quinquefasciatus are the predominant mosquito species in the area. The *An. gambiae* and *An. funestus* are susceptible to pyrethroids; *Cx.* Quinquefasciatus is resistant to pyrethroids, mediated by enhanced oxidase and site insensitivity mechanisms [[36], Malima & Rowland, unpublished data]. The timing of the trial was set during a period when both *An. gambiae* and *Cx.*

Quinquefasciatus were abundant. The wild adult mosquitoes were characterized for resistance by testing with deltamethrin 0.05% papers in WHO test kits.

Study design

The following six treatment arms were compared:

1. Unwashed PermaNet 3.0
2. PermaNet 3.0 washed 20 times
3. Unwashed PermaNet 2.0
4. PermaNet 2.0 washed 20 times
5. Polyester net, conventionally treated with deltamethrin at 25 mg/m2, washed until just before exhaustion
6. Untreated polyester net

Each net was deliberately holed with six 4 cm × 4 cm holes to simulate a worn net. The trial took place between 7 July and 4 October 2008. The treatment arms were rotated 3 times through the huts according to a Latin Square design. A treatment was assigned at random to a particular hut for 3 nights' observation before being rotated to the next hut. Male volunteers slept on beds under the net which were tucked under the mattress. The six sleepers were rotated through the six huts on consecutive nights. Data were collected for 54 nights. Three nets were available per treatment arm and each net was tested on consecutive nights during the three-night rotation. At the end of each rotation the huts were cleaned and aired for one day and the treatments moved to the next hut.

White sheets were laid over the veranda and room floors to ease the collection of knocked-down mosquitoes. Each morning after dawn, mosquitoes were collected using aspirators from the floor, walls, exit traps and inside the nets, scored as dead or alive and as fed or unfed and identified to species using a binocular microscope. Live mosquitoes were held for 24 h with sugar solution in paper cups to determine delayed mortality.

The primary outcomes were Deterrence, Treatment-induced exiting (exophily), mortality, Overall killing effect, Blood-feeding inhibition and %Personal protection. These outcomes are described in more detail in appendix 1, Section 2.6.2.

The criteria for approval were that the PermaNet 3.0 LN washed 20 times or more should perform according to these outcomes equal to or better than a conventionally treated net washed till just before exhaustion. Twenty washes are set by WHO as the average number of washes a LLIN is likely to incur during its life, assuming nets are washed 4 times a year and last up to 3 years.

Assessment of toxicity of nets used in the experimental hut trial.

WHO cone bioassays were performed on a randomly selected net from each of the six treatment arms using laboratory reared An. gambiae Kisumu at three intervals: before any washing, after completion of the washing cycles, and after completion of the hut trial. Four pieces of netting measuring 30 cm × 30 cm were cut along a diagonal transect on the four side panels and a further piece was cut from the top panel. Three replicate bioassay tests were carried out on each side panel and 10 replicate tests on the top panel using five mosquitoes per replicate.

Chemical analysis of nets used in the experimental hut trial.

Chemical analysis was conducted on PermaNet 2.0, PermaNet 3.0 and CTN from the 5 treatment arms before washing, after washing and after the hut trial. Taking one net per treatment arm, five 30 cm × 30 cm samples were cut from the four side panels and the one top panel of each net before and after washing and post hut trial. From each sample pieces were also taken for determination of density or homogenized, and an analytical portion of 300 mg taken for determination of deltamethrin, deltamethrin R-isomer and/or PBO.

Deltamethrin, deltamethrin R-isomer and piperonyl butoxide were extracted by heating under reflux for 60 minutes with xylene and determined by gas chromatography with flame isonisation detection (GC-FID) using the internal standard calibration.

Analysis

The analysis of experimental hut data was carried out using logistic regression for proportional data (proportions blood-feeding, dying and exiting each night) and negative binomial regression for numeric data (numbers collected, dying and feeding each night) after adjusting for the effects of individual huts and sleepers. Data was analysed using Stata 9 software (Stata Co., College Station, TX, USA).

Proportional data from laboratory bioassay tests (cone tests and tunnel tests) were normalized using arcsine square root transformation and the replicate test data analysed using analysis of variance [40].

Ethical clearance

Approval was obtained from the ethics review committees of the London School of Hygiene and Tropical Medicine, the Tanzanian National Institute of Medical Research (Ref: NIMR/HQ/R.8a/Vol. X/86) and the World Health Organization. Each trial participant gave written informed and was offered chemoprophylaxis during and for one month after the experimental hut trial.

The procedure for use of guinea pigs in tunnel tests conformed with criteria established in EC Directive 86/609/ECC regarding protection of animals used for experimental purposes. The procedure accorded with published guidelines of the World Health Organization and was approved by the Tanzanian National Institute of Medical Research Project Review Committee.

Results

Exploratory bioassay tests on PermaNet 3.0, PermaNet 2.0 and CTN washed up to 20 times.

Cone bioassays

After 20 washes PermaNet 3.0 induced 100% mortality, PermaNet 2.0 induced 98% mortality and the deltamethrin-treated CTN induced 8% mortality in *An. gambiae* Kisumu (Figure 1).

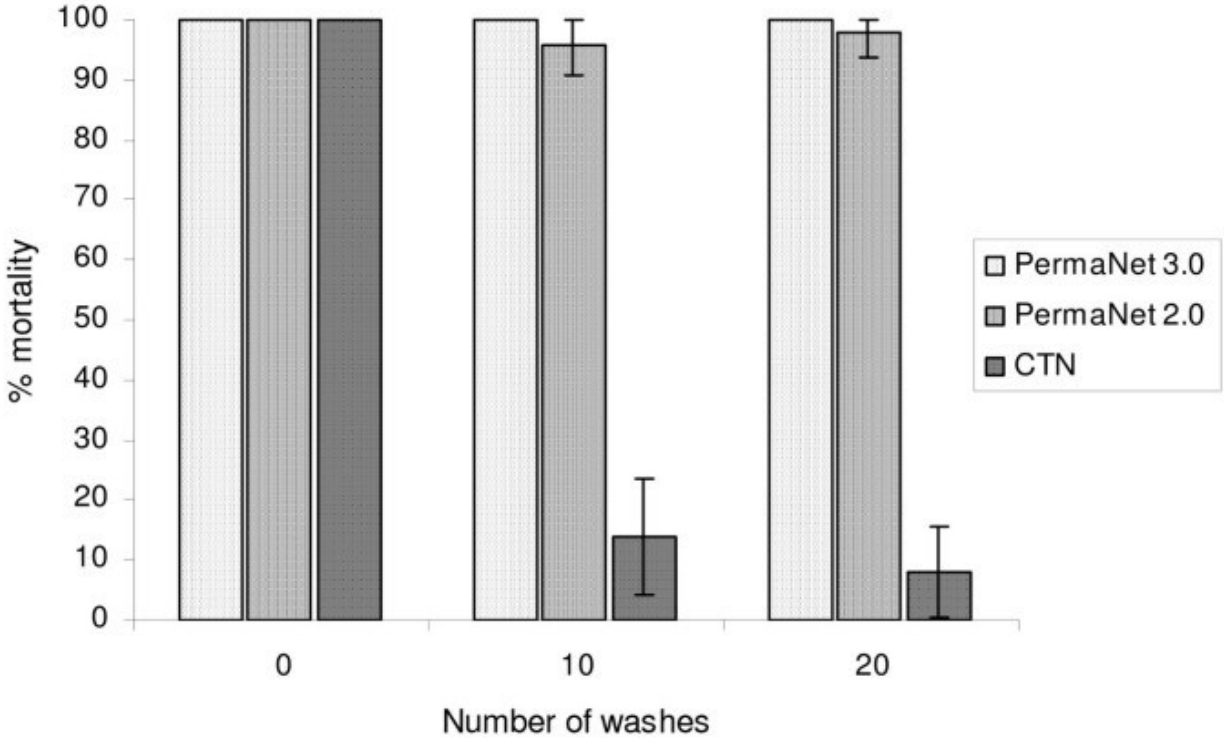

Figure 1: Efficacy of treated netting after washing as determined by WHO cone bioassay tests with *Anopheles gambiae* Kisumu.

The percentage mortality induced by PermaNet 3.0 against the *Cx. Quinquefasciatus* Masimbani pyrethroid resistant strain differed between roof and sides (Figure 2). The side netting induced only 33% mortality before washing, decreasing to 4% mortality after 20 washes. The roof netting induced higher rates of mortality than the sides: 86% at 0 washes, decreasing to 15% after 20 washes (P = 0.01). Thus, the PBO was synergistic before washing but the effect was mostly lost between 10 and 20 washes (Figure 2).

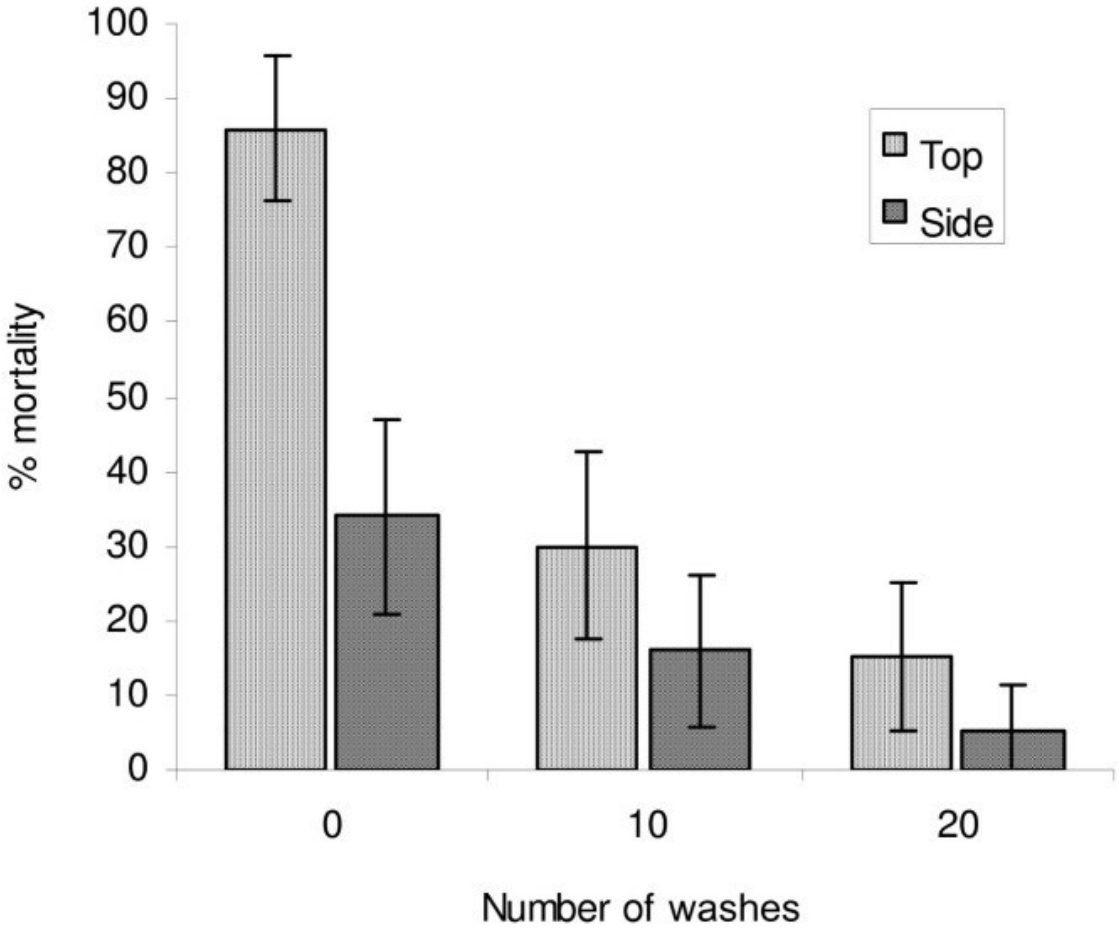

Figure 2: Efficacy of PermaNet 3.0 top and side panels against pyrethroid resistant *Culex quinquefasciatus* Masimbani as determined by WHO cone bioassay tests.

Tunnel tests

Passage

Each mosquito strain (*An. gambiae* Kisumu, *Cx. Quinquefasciatus* TPRI and *Cx. Quinquefasciatus* Masimbani) showed over 90% penetration through the untreated netting. All insecticide treatments inhibited passage of mosquitoes through the netting but less so for the pyrethroid resistant *Cx. Quinquefasciatus* Masimbani (P = 0.014) (Figure 3). After 20 washes, passage of each strain was more inhibited through the PBO/deltamethrin-treated top netting than through the deltamethrin-treated side

netting (P = 0.04); passage through the PBO treated top netting was just as inhibited after 20 washes as at 0 washes (P = 0.45).

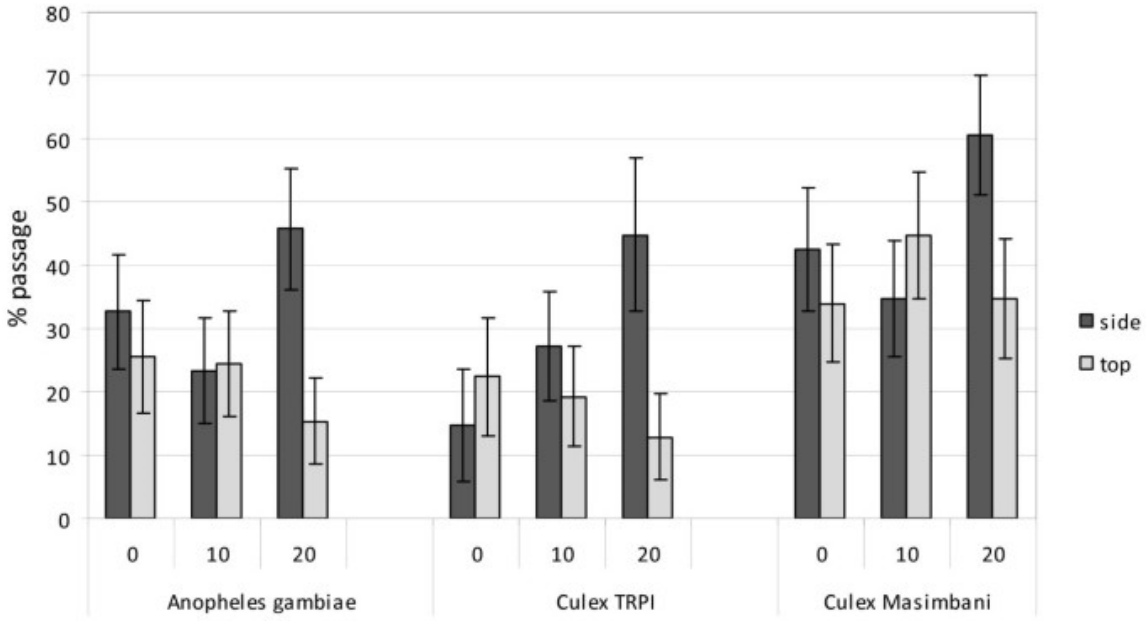

Figure 3: Tunnel tests using PermaNet 3.0 top and side netting before and after washing (10 or 20 times) against pyrethroid susceptible (*Anopheles gambiae* and *Culex* TPRI) and resistant (*Culex* Masimbani) mosquitoes – Percentage passage through the holed netting.

Blood feeding

All three strains showed a high rate of feeding (78% or more) through the untreated netting. The blood-feeding trends for each of the treatments (Figure 4) mirrored that for passage trends (Figure 1a).

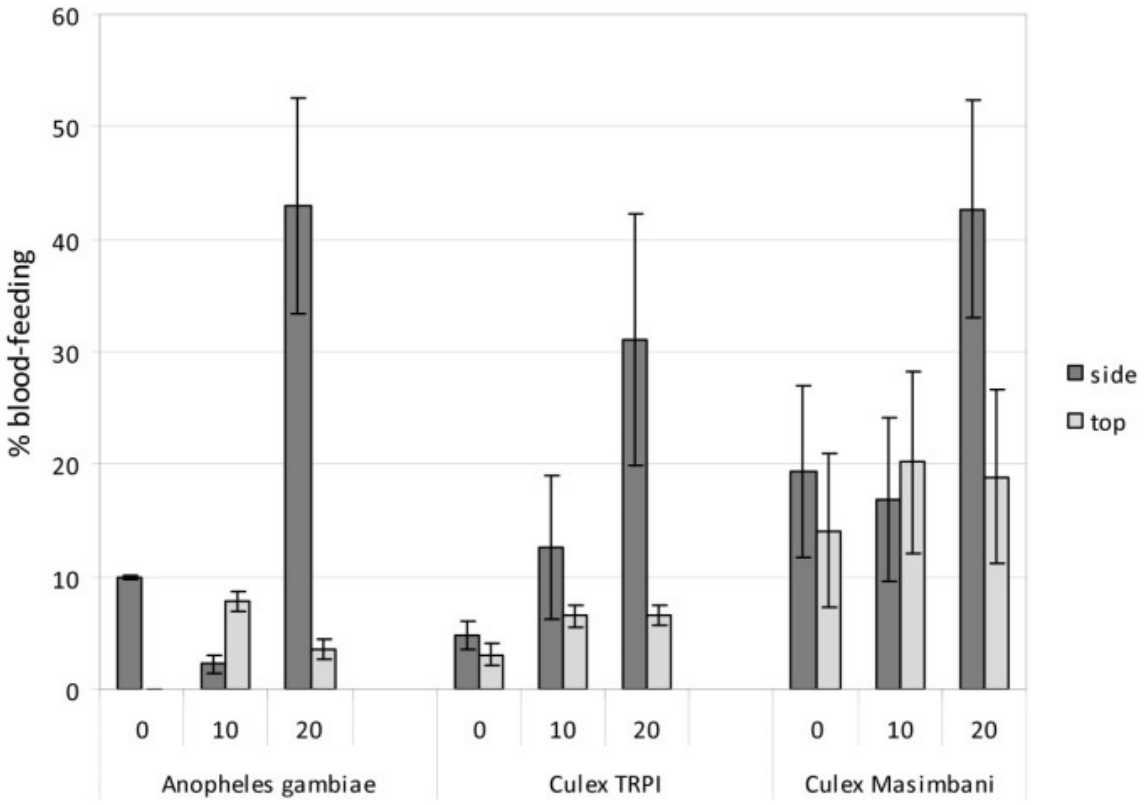

Figure 4: Tunnel tests using PermaNet 3.0 top and side netting before and after washing (10 or 20 times) against pyrethroid susceptible (*Anopheles gambiae* and *Culex* TPRI) and resistant (Culex **Masimbani) mosquitoes – Percentage blood-feeding**.

The *An. gambiae* Kisumu and *Cx. Quinquefasciatus* TPRI susceptible strains showed low rates of feeding through the unwashed deltamethrin-treated side netting but higher rates of feeding through side netting washed 20 times (P = 0.07). The feeding rate associated with the PBO-deltamethrin impregnated netting was highly inhibited both in unwashed and in 20 washed samples.

The resistant *Cx. Quinquefasciatus* Masimbani strain showed higher feeding rates than the susceptible *Cx. Quinquefasciatus* TPRI strain (or *An. gambiae*) through treated netting at each wash interval (P = 0.01). The feeding rate of the resistant *Cx. Quinquefasciatus* Masimbani was notably higher through the deltamethrin-treated side netting after 20 washes. The feeding rate observed with PBO/deltamethrin-impregnated top netting did

not change significantly with washing (P = 0.34) and after 20 washes was only half that observed with the deltamethrin side netting.

The trend in feeding rate among mosquitoes penetrating the holed netting showed a gradual increase over the course of 20 washes regardless of whether the netting was treated with deltamethrin or PBO-deltamethrin (Figure 5). For the two susceptible strains tested, the proportion feeding was always less with the PBO-deltamethrin netting than with the deltamethrin netting. The pyrethroid resistant *Cx. Quinquefasciatus* Masimbani strain showed little or no difference in the proportion feeding between the deltamethrin-treated and PBO/deltamethrin-treated netting.

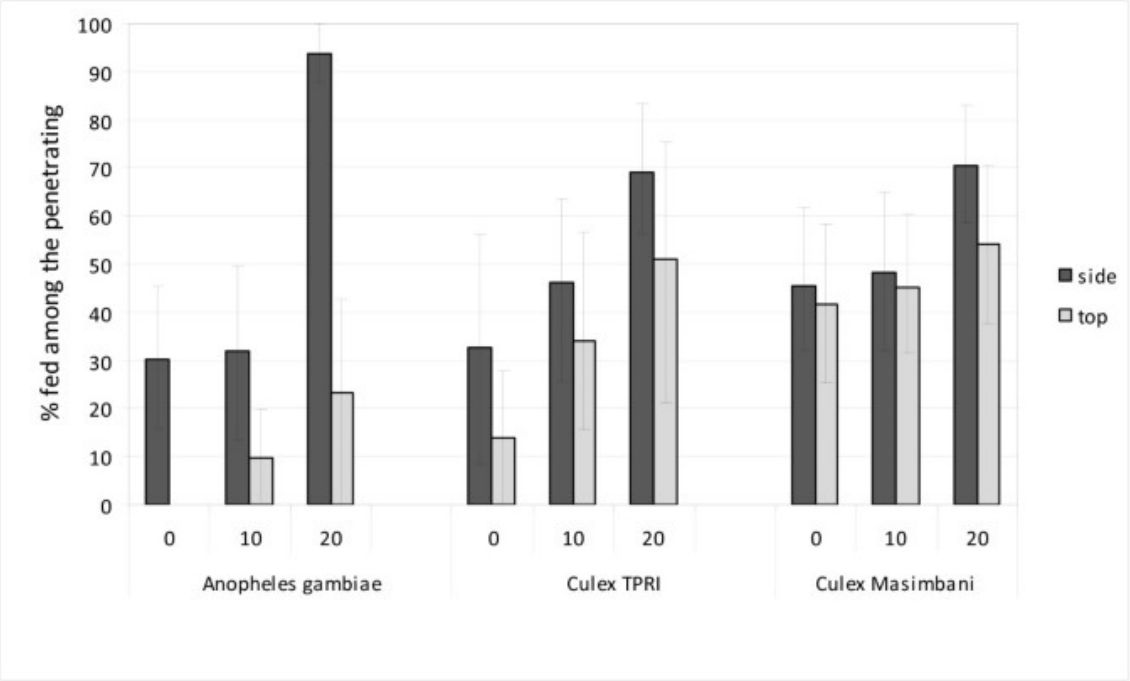

Figure 5: Tunnel tests using PermaNet 3.0 top and side netting before and after washing (10 or 20 times) against pyrethroid susceptible (*Anopheles gambiae* and *Culex* TPRI) and resistant (*Culex* Masimbani) mosquitoes – Percentage blood-feeding among those mosquitoes that penetrated the netting.

Mortality

The untreated nets recorded zero mortality against all three strains. Both types of treated netting induced greater mortality against *An. gambiae* Kisumu than against *Cx. Quinquefasciatus* susceptible and resistant strains (P = 0.001) (Figure 6). The mortality

rate against *An. gambiae* Kisumu was greater with PBO-deltamethrin netting than with deltamethrin netting (P = 0.04). Washing the two types of netting up to 20 times did not have a significant effect on mortality of the highly susceptible *An. gambiae* Kisumu (P = 0.21).

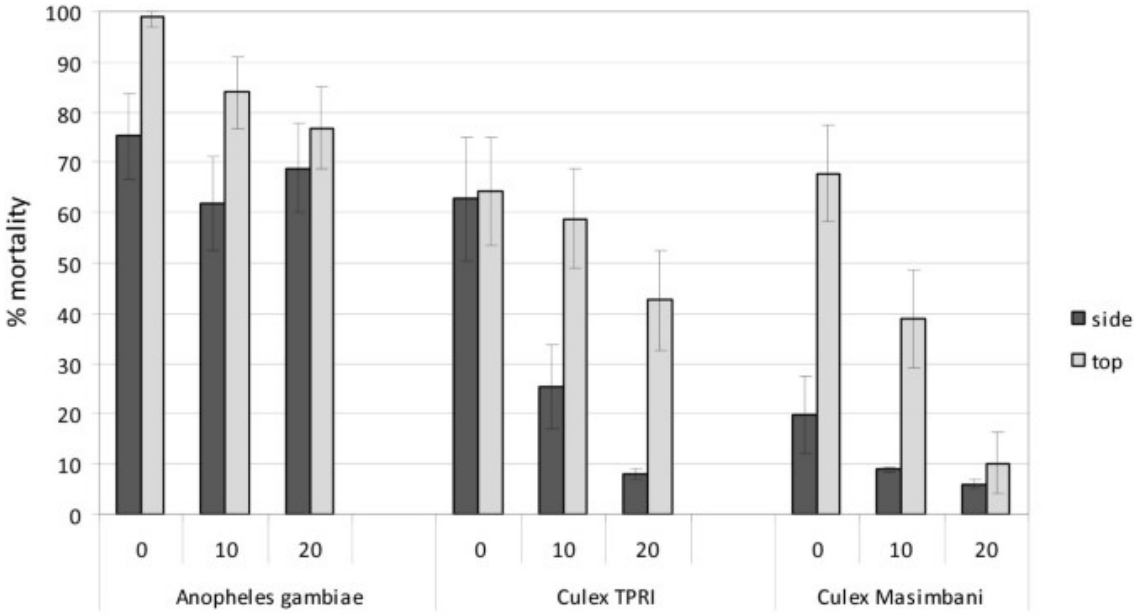

Figure 6: Tunnel tests using PermaNet 3.0 top and side netting before and after washing (10 or 20 times) against pyrethroid susceptible (*Anopheles gambiae* and *Culex* TPRI) and resistant (*Culex* Masimbani) mosquitoes – Percentage mortality.

The pyrethroid susceptible *An. gambiae* Kisumu strain recorded greater mortality than susceptible *Cx. quinquefasciatus* TPRI strain on each type of netting at the corresponding wash interval (P = 0.01). The pyrethroid resistant *Cx. quinquefasciatus* Masimbani strain recorded lower mortality (20%) than the susceptible TPRI strain (62%) on unwashed deltamethrin-treated side netting (P = 0.047). Mortality rates in both strains decreased after washing, indicating removal of deltamethrin by the washing process. With the side nettings, the difference in mortality between resistant and susceptible Culex strains became progressively smaller with washing (17% difference at 10 washes) and was barely evident at 20 washes (2% difference). Hence the contact time of *Cx quinquefasciatus* with

the deltamethrin-treated net at 20 washes was insufficient to induce mortality in either strain.

The unwashed PBO-deltamethrin top netting induced an almost identical level of mortality in susceptible (64.2%) and resistant (67.7%) *Cx quinquefasciatus*, indicating that PBO was synergizing the pyrethroid resistance in *Cx quinquefasciatus* Masimbani. Over the course of 20 washes of the PBO-deltamethrin netting there was a partial loss of activity against the susceptible strain and a near complete loss of activity against the resistant strain. After 20 washes there was no significant difference in mortality induced against *Cx quinquefasciatus* Masimbani by the top or side nettings (P = 0.68). This indicates that the surface concentration of PBO was largely removed by washing so no further synergy was evident against the resistant Masimbani strain and any PBO replenishment from the core of the fibres after washing was insufficient to regain toxic activity.

Experimental hut trial

Determination of point of 'insecticide exhaustion'

The point of exhaustion is the number of washes at which cone bioassay mortality of *An. gambiae* Kisumu decreases to less than 80%. Mortality decreased below 80% after four washes, and hence CTNs washed three times were used as reference nets in the hut trials.

Resistance status

WHO resistance tests with deltamethrin 0.05% test papers on adults collected from huts at the start of the trial indicated that An. gambiae was susceptible (100% mortality) and *Cx. Quinquefasciatus* was resistant (52% mortality).

Assessment of toxicity of nets used in the experimental hut trial.

Before washing of the trial nets, the percentage knockdown and mortality of *An. gambiae* Kisumu were recorded as 100% for each treatment arm. After 20 washes, PermaNet 3.0 still scored 100% mortality whereas PermaNet 2.0 scored 96% mortality. At the end of the

36-day trial, mortality in PermaNet 3.0 and 2.0 arms stood at 96% or more and in the CTN washed to just before exhaustion mortality stood at 90%.

Number of mosquitoes collected in the experimental huts.

Anopheles gambiae sensu strictu was the only member of the *An. gambiae* complex present at Zeneti village. *An. gambiae* were more abundant than *Cx. Quinquefasciatus* during the trial (Tables 1 and 2). The average number of An. gambiae per treatment ranged from 8 to 14 females per night. *Culex quinquefasciatus* ranged from 1 to 1.8 per night. Fewer *An. gambiae* females were collected from the huts with treated nets, but the difference compared to the untreated control was not significant except for the unwashed PermaNet 3.0 which showed 41% deterrence (P = 0.03).

Table 1 Experimental hut summary for *Anopheles gambiae*.

	Untreated net	PermaNet 3.0 Unwashed	PermaNet 2.0 Unwashed	PermaNet 3.0 washed 20 times	PermaNet 2.0 washed 20 times	CTN washed 3 times
Total females caught	**723**	**425**	**574**	**558**	**586**	**560**
Females caught per night	13[a]	8[b]	11[ab]	10[ab]	11[ab]	10[ab]
% Deterrence	-	41	21	23	19	22
Total females in veranda and exit traps	**618**	**335**	**491**	**474**	**518**	**515**
% Exiting (95% C.I.)	86[a] (83-88)	79[b] (75-82)	86[a] (82-88)	85[a] (82-88)	88[a] (86-91)	92[c] (89-943.9)
Total females blood fed	**202**	**11**	**59**	**58**	**54**	**59**
% Blood fed (95% C.I.)	28[a] (25-31)	3[b] (1-5)	10[c] (8-13)	10[c] (8-13)	9[c] (7-12)	11[c] (8-13)

	Untreated net	PermaNet 3.0 Unwashed	PermaNet 2.0 Unwashed	PermaNet 3.0 washed 20 times	PermaNet 2.0 washed 20 times	CTN washed 3 times
% Blood feeding inhibition	-	91	63	63	67	63
% Personal protection	0[a]	95[b]	71[c]	71[c]	73[c]	71[c]
Total females dead	**108**	**407**	**548**	**531**	**510**	**411**
% Mortality (95% C.I.)	15[a] (13-18)	96[b] (93-97)	96[b] (93-97)	95[b] (93-97)	87[c] (84-90)	73[d] (70-77)
% Mortality corrected for control	-	95	95	94	85	69
% Overall killing effect	0[a]	41[b]	62[b]	59[b]	56[b]	42[b]

Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).

Table 2 Experimental hut summary for Culex quinquefasciatus.

	Untreated net	PermaNet 3.0 Unwashed	PermaNet 2.0 Unwashed	PermaNet 3.0 washed 20 times	PermaNet 2.0 washed 20 times	CTN washed 3 times
Total females caught	**81**	**70**	**52**	**96**	**87**	**68**
Females caught per night	1.5[a]	1.3[a]	0.9[a]	1.8[a]	1.6[a]	1.3[a]
% Deterrence		14	36	0	0	16
Total females in veranda and exit traps	**46**	**65**	**51**	**95**	**84**	**61**
% Exiting (95% C.I.)	57[a] (46-67)	93[bc] (84-97)	98[bc] (88-100)	99.0[c] (93-100)	97[bc] (90-99)	90[b] (80-95)
Total females blood fed	**41**	**4**	**3**	**0**	**0**	**10**

	Untreated net	PermaNet 3.0 Unwashed	PermaNet 2.0 Unwashed	PermaNet 3.0 washed 20 times	PermaNet 2.0 washed 20 times	CTN washed 3 times
Total females caught	**81**	**70**	**52**	**96**	**87**	**68**
% Blood fed (95% C.I.)	51[a] (40-61)	6[bc] (2-14)	6[bc] (2-16)	0[c]	0[c]	15[b] (8-25)
% Blood feeding inhibition	-	89	89	100	100	71
% Personal protection	0[a]	90[b]	93[b]	100[b]	100[b]	76[b]
Total females dead	**5**	**36**	**19**	**35**	**30**	**26**
% mortality (95% C.I.)	6[a] (3-14)	51[b] (40-63)	37[c] (25-50)	37[c] (28-46)	34[c] (25-45)	38[c] (28-50)
% Mortality corrected for control	-	48	32	32	30	34
% Overall killing effect	0[a]	38[b]	17[b]	37[b]	30[b]	26[b]

Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).

Exiting rates

The proportion collected each morning from the veranda and window traps of huts with untreated nets was higher for *An. gambiae* (85.5%) than for *Cx. Quinquefasciatus* (56.8%). Insecticide induced exiting from huts with treated nets relative to the huts with untreated nets was evident for *Cx. Quinquefasciatus* but not for *An. gambiae*, because most of the latter exited naturally each night from the huts with untreated nets (Tables 1 and 2).

Blood-feeding

In huts with untreated nets, there was a higher rate of blood-feeding among *Cx. Quinquefasciatus* than among *An. gambiae* (Tables 1 and 2). There was a significantly

lower rate of blood-feeding among *Cx. Quinquefasciatus* and *An. gambiae* in huts with treated nets (P = 0.0001).

Anopheles gambiae showed the lowest blood-feeding rate in huts with the unwashed PermaNet 3.0. However, there was no significant difference in the *An. gambiae* feeding rates between PermaNet 3.0 washed 20 times, PermaNet 2.0 washed 20 times, PermaNet 2.0 unwashed or CTN washed to just before exhaustion.

The feeding rates of *Cx. Quinquefasciatus* did not differ in huts with unwashed PermaNet 3.0 or unwashed PermaNet 2.0 (P = 0.73). Feeding rates after 20 washes were 0% for both PermaNet 3.0 and PermaNet 2.0. Huts with CTN washed three times recorded a feeding rate of 15%, a significantly higher rate than that recorded for PermaNet 3.0 washed 20 times or PermaNet 2.0 washed 20 times.

The PermaNet 3.0 and PermaNet 2.0 washed 20 times, and the CTN washed three times, scored similar levels of personal protection against *An. gambiae* (71%, 73% and 71% respectively). The personal protection recorded against *Cx. Quinquefasciatus* with the PermaNet 2.0, PermaNet 3.0 and CTN were not significantly different from one another.

Mortality

The huts with LLINs and CTNs recorded much greater levels of mortality of *An. gambiae* and *Cx. Quinquefasciatus* than huts with untreated nets.

Against *An. gambiae* there was no difference between the proportions killed by the unwashed PermaNet 3.0 or unwashed PermaNet 2.0 (P = 0.79); both induced greater than 95% mortality. Against *Cx. Quinquefasciatus*, the proportion killed by the unwashed PermaNet 3.0 (51%) was greater than the proportion killed by the unwashed PermaNet 2.0 (37%) (P = 0.05). The mortality associated with PermaNet 3.0 fell to 37% after 20 washes. The mortality associated with PermaNet 2.0 remained a consistent 36% and 35% at 0 and 20 washes respectively; both these values were virtually identical to the mortality induced by PermaNet 3.0 after 20 washes (37%) (P = 0.77). This means that any synergized

toxicity by PBO in PermaNet 3.0 against *Cx. Quinquefasciatus* was no longer evident after 20 washes. The mortality associated with PermaNet 3.0 and PermaNet 2.0 after 20 washes against *Cx. Quinquefasciatus* was similar to that of the positive control (CTN washed three times) (P = 0.85) (Table 2).

Against *An. gambiae* the PermaNet 3.0 recorded higher mortality than PermaNet 2.0 after 20 washes (P = 0.001). The difference was small (8%) but may indicate that the PBO was not completely depleted but could still exert a limited effect against highly susceptible mosquitoes or may be these were killed by the remaining deltamethrin. Both PermaNet 3.0 and PermaNet 2.0 washed 20 times induced significantly greater mortality against *An. gambiae* than the conventionally treated net washed three times (P = 0.001).

The overall killing effect against *An. Gambiae* (the proportion of mosquitoes killed by the treated nets relative to the number entering untreated huts) was similar in PermaNet 3.0 (59%) and PermaNet 2.0 (56%) when washed 20 times (P = 0.87). These overall killing effects were not significantly greater than that for the CTN washed three times (42%) (P = 0.89).

Chemical analysis

The chemical analysis is presented in Figure 7. The loading dosage of deltamethrin on the polyester side panels of PermaNet 3.0 was recorded as 103 mg/m2 in one sample and 119 mg/m2 in a second sample, within the acceptable range. After 20 washes the quantity on the sides had decreased to 53 mg/m2. After the trial was completed, the quantity had decreased still further to 28 mg/m2. The loading dosage of deltamethrin on the polyethylene top panel of PermaNet 3.0 was 136 mg/m2. After 20 washes it was recorded as 132 mg/m2 and after the trial was completed it was recorded as 135 mg/m2. The lack of any evident decrease in content was presumably due to the deltamethrin on the top panel being incorporated or locked into the polyethylene fibre as opposed to being coated on the surface of the fibre on the polyester sides during manufacture.

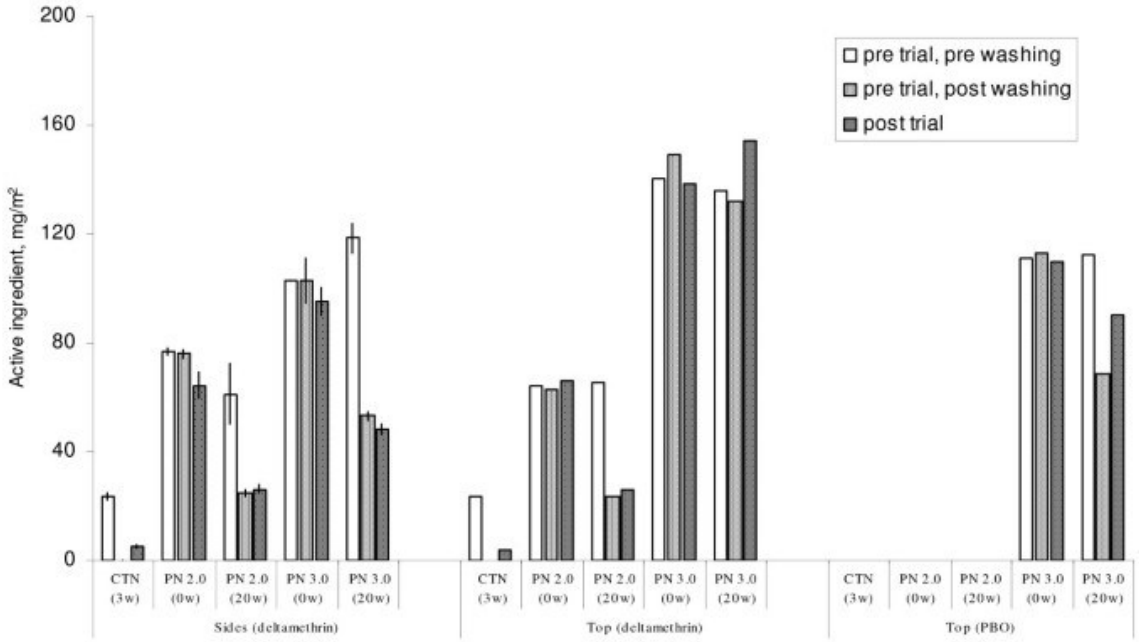

Figure 7: Chemical analysis of deltamethrin and PBO content of PermaNets used in the hut trial. Content of samples were analyzed pre and post washing and post-trial. Number of washes are recorded in parenthesis on X axis. PBO content was 10 times the values recorded on the Y axis. Confidence intervals reported for deltamethrin on side netting.

The loading dose of deltamethrin in PermaNet 2.0 was 61 mg/m2 in one sample and 77 mg/m2 in another. After 20 washes the dose had decreased to 25 mg/m2; no further loss was recorded at the completion of the trial.

The PBO on the top panel of PermaNet 3.0 was 1142 mg/m2 before washing, 684 mg/m2 after 20 washes, and 1013 mg/m2 at the end of the trial. The variation partly reflects the limited number of samples taken for analysis (only one sample taken post-washing and one post-trial). There was no evidence of any real change in PBO content due to washing.

Discussion

Laboratory tunnel tests with PermaNet 3.0 produced results consistent with experimental hut trials and help to explain the trends in blood feeding and mortality associated with PermaNet 3.0 and PermaNet 2.0 in the field. The tunnel tests demonstrated a synergistic interaction of PBO and deltamethrin on roof netting against susceptible *An. gambiae* and both susceptible and resistant *Cx. Quinquefasciatus* relative to netting from side panels

treated with deltamethrin alone. This synergy was manifested in higher mortality, reduced passage through the holes and reduced feeding rates with netting treated with PBO-deltamethrin. The synergy in tunnels against pyrethroid resistant *Cx. Quinquefasciatus* was progressively lost over 10 washes and fully lost after 20 washes. Cone bioassays on resistant *Cx. Quinquefasciatus* confirmed the loss of synergy over 20 washes.

In the experimental hut trial both PermaNet 2.0 and PermaNet 3.0 induced high rates of mortality against pyrethroid susceptible *An. gambiae* at 0 and 20 washes and both rates exceeded that of the CTN washed to just before cut off point. Based on this result PermaNet 3.0, like PermaNet 2.0 before it, warrants interim approval by WHO as a LLIN [41]. It was encouraging that the *An. gambiae* blood feeding rate associated with the zero washed PermaNet 3.0 was lower than with the zero washed PermaNet 2.0. After 20 washes, however, the feeding rates between PermaNet 3.0 and PermaNet 2.0 no longer differed, indicating a loss of activity under field conditions.

It was initially encouraging that the mortality rate of pyrethroid resistant *Cx. quinquefasciatus* in huts with zero washed PermaNet 3.0 was higher than that with zero washed PermaNet 2.0. This indicated the PBO in PermaNet 3.0 was exerting a partial synergism against *Cx. Quinquefasciatus*. As per tunnel test results the synergism in the huts was fully lost after 20 washes. Blood feeding rates in *Cx. Quinquefasciatus* in the huts did not differ between PermaNet 2.0 and 3.0 either in unwashed or 20 washed nets. According to Khayrandish & Wood [35], WHO used synergists and nerve recordings to explore the resistance in *Cx. Quinquefasciatus* from this region, enhanced oxidases and a nerve insensitivity mechanism, probably kdr, are responsible for pyrethroid resistance.
The roof netting showed minor change in chemical content (either in deltamethrin or in PBO) after twenty washes. Certainly, any slight change observed was not sufficient to explain the significant difference in efficacy (mortality) between unwashed and 20 times washed PermaNet 3.0 in cones, tunnel tests or experimental huts. This indicates that

deltamethrin, the PBO or both compounds are depleted from the surface of the fibre after 20 washes and fail to migrate sufficiently from the core to the surface to allow full regeneration. It would seem that PBO rather than deltamethrin is the compound that remains locked in the fibre. The evidence for this stems from the lack of difference in mortality in the tunnel tests between the 20 times washed PermaNet 3.0 and the 20 times washed PermaNet 2.0 against resistant *Cx. Quinquefasciatus* taken together with the higher mortality with 20 times washed PermaNet 3.0 against susceptible *Cx. Quinquefasciatus,* deltamethrin must still be present on the surface of both PermaNet 2.0 and 3.0 and causing some mortality of susceptible *Cx. Quinquefasciatus* but there seems little or no PBO left on the surface of PermaNet 3.0 netting to allow synergy in resistant *Cx. Quinquefasciatus*.

It is possible insufficient time was given between washing and tunnel testing for regeneration of PBO to occur. However, the evidence from the hut trial suggests this is not the reason since over the six weeks in the huts the PermaNet 3.0 washed 20 times showed no difference in performance to the PermaNet 2.0 washed 20 times, but during this six-week interval there was plenty of time for the PBO to migrate to the surface of fibres. The lack of difference in *Cx. Quinquefasciatus* mortality between PermaNet 3.0 washed 20 times and PermaNet 2.0 washed 20 times suggests a failure to regenerate.
It is possible that the higher mortality initially seen with PermaNet 3.0 relative to PermaNet 2.0 is due more to the higher loading dose of deltamethrin than to any contribution of PBO. An appropriate control to test this hypothesis – a 'PermaNet 3.0' loaded with the same dosage of deltamethrin but containing no PBO – was not available for testing. Such a control should always be considered in future testing of combination nets.

At present there is limited evidence that mosquitoes contact the roof of the net while seeking access to the host. This may not hold for all species, and more corroborative observations are required. Unless the majority of mosquitoes respond to host odour or

convection plumes in this way, the 2-in-1 concept as a tactic for managing resistance management tactic is flawed. The higher mortality of Culex in huts with unwashed PermaNet 3.0 versus unwashed PermaNet 2.0 does, however, provide some support to the concept.

It is important to note that the laboratory tests and phase II trials reported here refer to efficacy before and after standardized washing rather than to performance under long term household use. There is limited temporal dimension to this work because the interval between the start of washing and the completion of the trials was only three months. Because pyrethroids used on nets have low vapour pressure a pyrethroid LLIN that showed high efficacy after 20 Phase II washes might, quite reasonably, be expected to remain efficacious for at least three years of household use, as reported recently for PermaNet 2.0 and Olyset LLINs [41, 42]. We have no information on how long the PBO component of PermaNet 3.0 would remain effective in the field as the synergist incorporated into netting may have different physical characteristics to pyrethroids. By contrast there is reasonable expectation based on current knowledge that the pyrethroid in conventional LLIN would last for three years or more [37].

The marginal difference in mortality between PermaNet 3.0 and 2.0 against *An. gambiae* in huts either before or after washing would seem unlikely to provide additional control of *An. gambiae* populations; besides, mortality with PermaNet 2.0 was already high. With pyrethroid resistant *Cx. Quinquefasciatus* almost half survived exposure to PermaNet 3.0 in the huts and this proportion increased to 64% after washing. As a combination net designed to control pyrethroid resistance mediated by mixed function oxidase mechanisms the capacity of PermaNet 3.0 to control pyrethroid multiple resistant mosquitoes or prevent selection of resistance appears limited.

Conclusion:

PermaNet products were highly effective against susceptible *Anopheles gambiae*. As a long-lasting net to control or protect against pyrethroid resistant mosquitoes PermaNet 3.0 showed marginal improvement over PermaNet 2.0 against *Culex quinquefasciatus*.

At present there is limited evidence that mosquitoes contact the roof of the net while seeking access to the host. This may not hold for all species, and more corroborative observations are required. Unless the majority of mosquitoes respond to host odour or convection plumes in this way, the 2-in-1 concept as a tactic for managing resistance management tactic is flawed. The higher mortality of Culex in huts with unwashed PermaNet 3.0 versus unwashed PermaNet 2.0 does, however, provide some support to the concept.

Contrary to this chapter that discussed the results of the PermaNet 3.0 (A combination LLIN with PBO treated on roof panel only), the next chapter (Chapter 12) focusses on describing the results of the superiority trial that comparatively evaluated the efficacy of the PBO LLIN that had PBO treated on whole net relative to PermaNet 3.0 (A combination LLIN with PBO treated on roof panel only).

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Chapter 9: Field evaluation of Veeralin, an alpha-cypermethrin + PBO long-lasting insecticidal net, against natural populations of *Anopheles funestus* in experimental huts in Muheza, Tanzania

Prologue:

Previous chapter discussed the efficacy of the combination PBO LLIN (PermaNet 3.0) with pyrethroid (deltamethrin) restricted to the sides and the synergist (PBO) to the top of the net. For this treatment paten of two-in-one net to work as a resistance management tactic mosquitoes should contact both the top and sides so that pyrethroid could be potentiated by the synergy (PBO) hence restoration of its efficacy against resistant mosquito.

There is some evidence that mosquitoes contact the roof of the net while seeking access to the host. This may not hold for all species, and more corroborative observations are required. Unless the majority of mosquitoes respond to host odour or convection plumes in this way, the 2-in-1 concept as a tactic for managing resistance management tactic is flawed. The higher mortality of Culex in huts with unwashed PermaNet 3.0 versus unwashed PermaNet 2.0 does, however, provide some support to the concept.

To provide more evidence, this chapter describes and discusses results of the small-scale field experimental huts to evaluate wash-resistance and efficacy of the pyrethroid -PBO combination that has PBO on whole net in comparison to PermaNet 3.0 discussed in previous chapter that has PBO treated on the roof panel only against resistant *Anopheles funestus* mosquitoes.

Chapter 9: Field evaluation of Veeralin, an alpha-cypermethrin + PBO long-lasting insecticidal net, against natural populations of *Anopheles funestus* in experimental huts in Muheza, Tanzania

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The material presented in this chapter has been published as:

Tungu PK, Waweru J, Karthi S, Wangai J, Kweka EJ: **Field evaluation of Veeralin, an alpha-cypermethrin + PBO long-lasting insecticidal net, against natural populations of Anopheles funestus in experimental huts in Muheza, Tanzania.** *Curr Res Parasitol Vector Borne Dis* 2021, **1:**100030. https://doi.org/10.1016/j.crpvbd.2021.100030

Cover sheet for each 'research paper' included in research thesis.

1. For a 'research paper' already published

1.1. Where was the work published? **Current Research in Parasitology & Vector-Borne Diseases**

1.2. When was the work published? **May 2021**

1.2.1. If the work was published prior to registration for your research.

degree, give a brief rationale for its inclusion **N/A**

1.3. Was the work subject to academic peer review? **YES**

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Abstract

Background

The success of long-lasting insecticidal nets (LLIN) as the primary method for preventing malaria is threatened by pyrethroid resistance in Anopheles vectors. New generation long-lasting nets incorporating PBO synergist (piperonyl butoxide) with pyrethroid are designed to control insecticide-resistant mosquitoes.

Methodology

The efficacy of Veeralin® PBO LLINs was evaluated in experimental huts against wild free-flying pyrethroid-resistant *Anopheles funestus (s.l.)*. Mosquito mortality, blood-feeding inhibition and personal protection were compared between untreated nets, standard LLINs and PBO/pyrethroid combination nets.

Results

Blood-feeding rates recorded with 20-times washed Veeralin were not significantly different from those with 20-times washed PermaNet 3.0 LLIN, a WHO Pre-Qualification Team (PQT) approved PBO/pyrethroid LLIN. This provides evidence that Veeralin LLIN provides similar blood-feeding inhibition to the standard approved LLIN and thus meets WHO PQT criteria for blood-feeding. Results show significantly higher mortality for Veeralin PBO LLINs against pyrethroid-resistant *Anopheles funestus* (s.l.) compared to

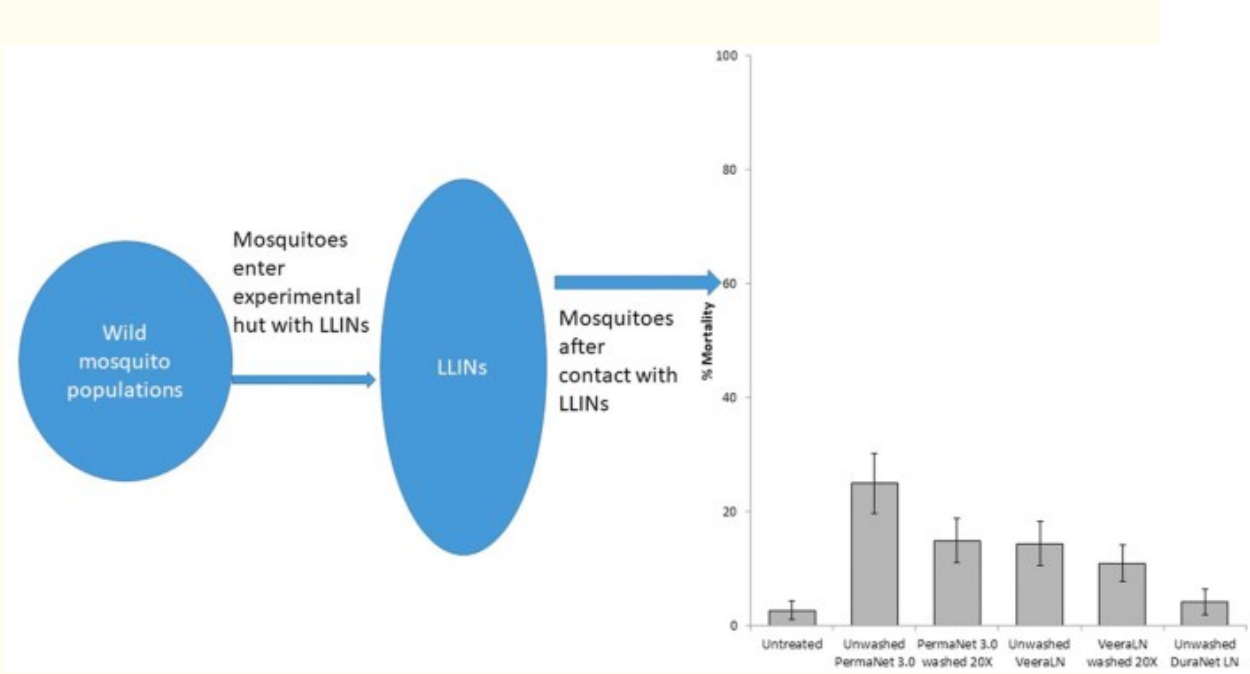
DuraNet, a WHO PQT approved standard pyrethroid-only LLIN, both when unwashed and washed 20 times.

Conclusion

The improved efficacy over a standard pyrethroid-only LLIN can be attributed to the effect of PBO in the Veeralin LLIN, hence meeting the Vector Control Advisory Group (VCAG) criteria for a resistance breaking LLIN. The non-inferiority of Veeralin LLIN to PermaNet 3.0 shows that PBO LLN will have same efficacy regardless of treatment patterns.

Keywords: Long-lasting insecticidal nets, *Anopheles gambiae* (s.l.), *Anopheles funestus*, Mortality, Knockdown, Exophily, Blood-feeding

Graphical abstract



Introduction

Long-lasting insecticide-treated nets (LLINs) have played a vital role in the decline of malaria incidences and vector populations across malaria endemic countries (WHO, 2019, 2020). In Africa, there has been an increased percentage of the population sleeping under an ITN between 2000 and 2020, for the whole population (2–46%), for pregnant women (3–52%) and for children aged under 5 years (3–52%) (Bhatt et al., 2011, 2015; WHO, 2019, 2020).

The World Malaria Report in 2020 showed the stagnation in control occurred between 2015 and 2019 with some countries reporting increased malaria cases (WHO, 2020). The rise in malaria vector species resistance to multiple insecticides is a current major concern (WHO, 2020). There is experimental evidence linking vector resistance to factors suggesting reduced efficacy of nets against resistant mosquitoes in Benin and Muheza, Tanzania (N'Guessan et al., 2007; Asidi et al., 2012; Kweka et al., 2019). Furthermore, sustained malaria transmission has been recorded in areas with high resistance to multiple insecticides (Surendran et al., 2020; Bartilol et al., 2021; Soma et al., 2021). The frequency of pyrethroid resistance and underlying mechanisms may predict decline in the protection of LLINs against resistant populations of malaria vectors (Asidi et al., 2012; Yewhalaw & Kweka, 2016).

Combination LLINs with pyrethroid and synergist compound piperonyl butoxide (PBO) have been developed and deployed as an alternative tool against pyrethroid-resistant mosquitoes (Kweka et al., 2017a, b). PBO is a synergist compound that inhibits cytochrome P450 metabolic enzymes which often play a key role in the detoxification of insecticides (Bingham et al., 2011; Stevenson et al., 2011; Yewhalaw & Kweka 2016; Kweka et al., 2017a, b). Pyrethroid-PBO nets have been shown to have improved efficiency to reduce malaria cases in areas with resistant populations of malaria vectors in Muleba, Tanzania, and across Uganda (Protopopoff et al., 2018; Staedke et al., 2020).

The use of pyrethroid-PBO nets was advocated as a new step with an interim recommendation by the World Health Organization (WHO, 2019, 2020) for the efficient control of pyrethroid-resistant mosquito vectors. There are several pyrethroid-PBO net products in the market, some of which have PBO applied on the top of nets or on the whole net. Veeralin® LLINs produced by VKA Polymer Ltd, Tamil Nadu, India, is a new brand of LLINs alpha-cypermethrin-PBO, with PBO-treated on the whole net.

This study reports the phase II experimental hut trial of Veeralin LLINs in Muheza, Tanzania, an area with pyrethroid-resistant populations of malaria vectors *Anopheles gambiae* (s.l.) and *An. funestus*.

Materials and methods

Description of the test product

Veeralin LLIN is an alpha-cypermethrin pyrethroid + PBO (incorporated into polyethylene) LLIN made of monofilament yarn (150 denier) containing 6.0 ± 25% g/kg alpha-cypermethrin and 2.2 ± 25% g/kg PBO. Veeralin LLIN is manufactured by Vector Control Innovations Pvt Ltd., India, and has recently passed WHOPES Phase I efficacy criteria with 20 standard washes. Comparison of Veeralin LLIN was done against PermaNet 3.0 LLIN, which is a WHO PQT recommended pyrethroid + PBO LLIN with a top panel made of monofilament polyethylene (100 denier) fabric incorporating deltamethrin at 4 g/kg (c.180 mg/m^2) and piperonyl butoxide at 25 g/kg (c.1.1 g/m^2). The side panels have 85 mg/m2 in the netting and 115 mg/m2 in a 70 cm border (i.e., 2.8 g/kg deltamethrin). DuraNet® is a WHO PQT approved pyrethroid-only LLIN manufactured by Clarke Mosquito Control (USA). Alpha-cypermethrin is incorporated into 150-denier, monofilament, high-density polyethylene fibres, with a target dose of 5.8 ± 25% g/kg AI, corresponding to 261 mg of alpha-cypermethrin per m^2.

Description of the trial site and hut design

The experimental huts are located at a field site in Zeneti village, 30 km from Muheza District town, in Tanga region, northeastern Tanzania (5°13'24"S, 38°39'96"E), at an altitude of 192.9 m above sea level (Kweka et al., 2019a). The area around Muheza is characterized by high malaria prevalence caused by *Plasmodium falciparum* which is transmitted by *Anopheles gambiae* (s.s.) during the rainy season, and by *An. funestus* (s.s.) during the dry season (Mboera & Magesa, 2001). The huts are made to a standard traditional East African veranda trap-hut design, with brick walls plastered with mud on the inside, a wooden ceiling lined with hessian sackcloth, corrugated iron roof, open eaves, with window traps and veranda traps on each side. The huts are built on concrete plinths and surrounded by a water-filled moat to deter entry of scavenging ants. There are two screened veranda traps on both sides of the huts to capture any mosquitoes that exit via the unbaffled open eaves (baffles are funnel-shaped wooded structures that allow mosquito entry but prevent their exit). The eaves of the two open verandas are baffled inwardly to funnel host-seeking mosquitoes into the hut and to deter exiting through these openings. With this modified hut design there is no need to make any correction for escaping mosquitoes (Kweka et al., 2017b; Mahande et al., 2018).

Bioassays on Veeralin LLIN preparation and washing procedure.

The nets were washed according to a protocol adapted from the standard WHO washing procedure used in Phase I. The nets were washed in individual aluminum bowls, one bowl for each net type containing 10 litres of filtered water from the local water provider with a pH of 6.0 and containing 2 g/l of Jamaa palm oil soap, which was grated to create flakes and then dissolved completely in warm water before addition to the basin using manual agitation. Each net was agitated for 3 min, left to soak for 4 min and re-agitated for 3 min. Agitation was done by stirring the net and gently submerging it by hand (wearing heavy duty rubber gloves) with 20 rotations/submersions per minute. Rinsing was performed twice using clean water (10 l per rinsing, i.e., 20 l per net). Nets were dried vertically in the shade then packed in polythene bags, placed inside of metal boxes and stored at 27 ± 2 °C between washes. The interval of time between washes (i.e., regeneration time: the

time required to restore the biological efficacy of a net when the surface insecticide has been depleted by washing) was 5 days for Veeralin LLIN and 1 day for PermaNet 3.0 LLIN (WHOPES, 2011).

Cone bioassays

Bioassays were performed according to standard WHO procedures (WHOPES, 2013). From each treatment arm, one net was sampled. From each of the sampled nets in the baseline and washing evaluation, four pieces of 25 × 25 cm were cut from net sides as per the WHO LLIN guidelines, with the same procedure repeated after the trial. On each netting sample, a standard WHO cone was held in place using masking tape. An untreated control with 5 cones was run for each net. Five laboratory-bred fully pyrethroid susceptible *An. gambiae* (Kisumu strain) female mosquitoes (sugar-fed, 3-day-old) were introduced into each cone and exposed for 3 min. Thereafter they were removed from the cone using a manual aspirator and placed into paper cups supplied with 10% sugar solution provided on cotton wool. Therefore, 25 mosquitoes were exposed to each netting sample. All cone bioassays and subsequent holding periods were conducted at 27 ± 2 °C and 80 ± 10% relative humidity. Outcomes measured were knockdown after 60 min and mortality after 24 h.

WHO insecticide susceptibility tests

The susceptibility tests were carried out using the WHO test kits for adult mosquitoes (WHO, 1998, 2016). Test papers impregnated with the WHO-recommended discriminating dosage of 0.75% permethrin were used because alphacypermethrin and deltamethrin test papers (which would match the net insecticides) were not available. The quality of the test papers was checked against a laboratory susceptible *An. gambiae* Kisumu strain. Wild mosquitoes used in the tests were F1 of adults *An. funestus* and *An. gambiae* (s.l.) collected from the untreated experimental huts during and just after this trial. For each test, batches of 15–20 adult females were aspirated from paper cups and transferred into the holding tubes where they were held for 1 h before testing in exposure

tubes lined with the test papers. Mosquitoes were exposed for 1 h and the number of mosquitoes knocked down was recorded after 60 min. At the end of exposure period mosquitoes were transferred into holding tubes (lined with untreated papers) and provided with cotton pad soaked in 10% sugar placed on top of the holding tube. Mortality was scored 24 h post-exposure and each test was replicated depending on the number of mosquitoes collected. WHO permethrin and deltamethrin-treated papers at 5× and 10× of the discriminating concentrations were also tested to assess higher intensity of resistance for *An. gambiae* (s.l.) and *An. funestus*.

Experimental huts field study

Washed and unwashed candidate LLINs were evaluated using 6 East African experimental huts for their effects on wild *An. funestus* (s.s.) mosquitoes and for their ability to deter entry, drive mosquitoes out of houses, induce mortality and inhibit blood-feeding (WHO, 1998, 2013, 2016). Other mosquito species were also collected but numbers were too few for statistical analysis. Before testing in the experimental huts, preliminary catches (without any treatment) were performed for 4 nights to ensure the field team was fully versed in collection procedures.

The following 6 treatment arms were compared: (i) unwashed Veeralin; (ii) unwashed PermaNet 3.0; (iii) unwashed DuraNet LLIN; (iv) Veeralin washed 20 times; (v) PermaNet 3.0 washed 20 times; and (vi) untreated polyester net.

After every 6 nights, the treatment arms were rotated among the huts according to a Latin square scheme. Six nets were used per treatment arm and each of the 6 nets was tested for one night during the 6 consecutive nights. At the end of the 6 nights, the huts were carefully cleaned and aired to remove potential contamination. Sheets and pillows were also washed to prevent potential contamination. The treatment arm was then rotated to a different hut. The study was performed for 6 rounds over 6 weeks to ensure complete rotation through the huts. The number of mosquitoes that were collected per night was assessed for sufficiency as calculated by power analysis (Johnson et al., 2014)

assuming a 70% reduction in feeding inhibition. Nets were stored in labelled polythene bags in the shade at an average temperature of 28 °C between testings.

Six (4 × 4 cm) holes were made in each net, 2 holes in each of the long side panels, and one hole at each end (head- and foot-side panels). Each net was individually coded and labelled with a long-lasting label attached to the one of the hanging loops and bagged in polythene bags labelled with the same code. Adult male volunteers (> 18-years-old) in good health, who are experienced in these kinds of studies, slept under the nets. Male participants were recruited among the inhabitants of the village close to the site of the experimental huts. Participants were informed on the objectives of this study and recruited upon written informed consent in national language (Kiswahili). Sleepers rotated randomly among huts each night of the study. They entered the hut at 18:00 h and remained inside until 06:00 h. Each morning of the study at 06:00 h, mosquitoes were collected from inside the nets, the floor and walls of the hut as well as from the exit traps (window and eave traps) following the WHO standard operating procedure (WHO, 1975). Mosquitoes were collected from within nets and exit traps using mouth aspirators and from inside the huts (walls, ceilings and floors) using manual aspirators (WHO, 1975).

Data analysis

The primary outcomes were Deterrence, Treatment-induced exiting (exophily), mortality, Overall killing effect, Blood-feeding inhibition and %Personal protection. These outcomes are described in more detail in appendix 1, Section 2.6.2.

The main analyses were performed using IBM SPSS version 26 (IBM Corp., Armonk, NY, USA) using blocked logistic regression (logit estimation for grouped data) for proportional data and Poisson regression (Negative binomial) for numerical data. Variance estimates were adjusted for clustering by each hut night of collection. The primary criteria in the evaluation were blood-feeding inhibition and mortality.

Results

Cone bioassay tests

Cone bioassays were conducted using the Kisumu susceptible mosquitoes for all experimental arms: before washing; after washing but before hut trials; and after the hut trials. Before washings, both 1-h knockdown and 24-h mortality for all treated nets was 100%. After washing, but before the hut trial, the 1-h knockdown was 96% and 88% for 20 times washed Veeralin and PermaNet 3.0, respectively (Table 1), and after hut trials the mortality was 100% for all treatments except for the untreated control arm which recorded 0% (Table 1).

Table 1: Cone bioassays of six arms of nets before washing, after washing, before hut trial and after experimental hut trials

Treatment	Before washing.			After washing, before hut trial			After hut trial		
	No. of mosquitoes tested	% Knockdown (60 min)	% Mortality (24 h)	No. of mosquitoes tested	% Knockdown (95% CI)	% Mortality (24 h)	No. of mosquitoes tested	% Knockdown (95% CI)	% Mortality (24 h)
UTN	125	0	0	125	0	0	125	0	0
Veeralin, UN	125	100	100				125	100	100
Veeralin, WA	125	100	100	125	96.0 (93.2–99.1)	100	125	96.0 (90.3–97.0)	100
PermaNet 3.0, UN	125	100	100				125	100	100
PermaNet 3.0, WA	125	100	100	125	88.0 (83.2–93.1)	100	125	76.0 (71.0–82.2)	100
DuraNet, UN	125	100	100				125	98.0 (96.1–100)	100

Abbreviations: UN, unwashed net; WA, 20 times washed net; UTN, untreated polystyrene net (a negative control); CI, confidence interval.

Susceptibility test of *An. funestus* (s.l.) and *An. gambiae* (s.l.) from untreated huts

WHO susceptibility tests on F1 of the adult *An. funestus* collected from the experimental huts with untreated nets and tested with permethrin papers recorded a mortality rate of 44% (31.8–56.2%), indicating that *An. funestus* (s.l.) was resistant to pyrethroids. Susceptibility tests on F1 *An. gambiae* collected from untreated huts recorded

percentage mortality of 27% (16.4–37.5%) to permethrin. Insecticide resistance intensity testing showed Zeneti village wild *An. gambiae* displayed over 10-fold resistance to permethrin and deltamethrin (Table 2).

Table 2: Permethrin and deltamethrin resistance intensity results

Treatment	N	Mortality (%)	SE
Permethrin 5×	80	83.75	2.4
Permethrin 10×	80	78.75	3.1
Deltamethrin 5×	80	80.75	3.1
Deltamethrin 10×	80	88.50	1.4

Abbreviations: N, number of mosquitoes used; SE, standard error.

Number of mosquitoes collected from huts.

Anopheles funestus (s.l.) were more abundant than *Anopheles gambiae* during the trial. The average (geometric mean) number of *An. funestus* per hut per night varied between 6 and 8 (Table 3). The number of *An. gambiae* species collected were too few (range: 0.1–0.3 mosquitoes per hut per night) for any meaningful conclusions, and hence have not been included in this paper. All treated arms induced significant deterrence in reference to untreated control arm (Negative binomial, range for Z: 2.7–9.1, df = 11, all $P < 0.05$) except Veeralin LLIN washed 20× (Negative binomial, $Z = 0.294$, df = 11, $P = 0.769$).

Table 3: Results for experimental huts against Zeneti wild free-flying *Anopheles funestus* (number entering, proportions deterred, exiting, blood-feeding, BFI and personal protection)

Treatment	Untreated net	PermaNet 3.0	PermaNet 3.0	Veeralin	Veeralin	DuraNet
No. of washes	**0**	**Unwashed**	**20**	**Unwashed**	**20**	**Unwashed**
Total no. of females caught	374	256	328	319	364	288
Geometric mean females caught/night (95% CI)	8 (5.2–10.8)	5.9 (3.7–7.1)	7.7 (5.7–9.7)	7.4 (5.1–9.7)	7.8 (5.1–10.5)	6.3 (3.8–9.8)
% Deterrence	–[a]	31.5[b]	12.3[b]	14.7[b]	2.7[a]	0[a]
Total no. of females in verandah and exit traps	134	179	211	223	275	220
% Exophily (95% CI)	35.8[a] (31.0–41.0)	70[bc] (64.3–75.5)	64.3[c] (59.2–69.5)	69.9[cd] (64.9–74.9)	75.5[bd] (71.1–80.0)	76.4[bd] (71.5–81.3)
Total no. of blood-fed females	111	52	43	49	41	34
% Blood-fed (95% CI)	29.7[a] (25.1–34.3)	20.3[b] (15.4–25.2)	13.1[bc] (9.5–16.8)	15.4[bc] (11.4–19.3)	11.3[c] (8.0–14.5)	11.8[c] (8.1–15.5)
% Blood-feeding inhibition	–	31.6	55.9	48.1	62.6	40.3
% Personal protection	–[a]	53.2[a]	61.3[a]	55.6[a]	63.1[a]	70.3[a]

Note: Within a row, treatments not sharing a superscript letter differ significantly by blocked logistic regression (P < 0.05).
Abbreviation: CI, confidence interval.

Exiting rates

All treated arms recorded significantly higher *An. funestus* (s.l.) exiting rates as compared to that recorded by the untreated control arm (Logistic regression, range for Z: 7.0–10.9, df = 15, all $P < 0.05$). Exiting rates of Veeralin washed 20 times were significantly higher than that of PermaNet 3.0 washed twenty times (Logistic regression, $Z = 3.9$, df = 15, $P \leq 0.001$) (Table 3).

Blood-feeding

Blood-feeding rates recorded in all treatment arms were significantly lower than the untreated control arm, thus providing evidence that all the pyrethroid nets provided protection against mosquito bites in this pyrethroid-resistant population. Moreover, the blood-feeding rate recorded by Veeralin nets after being washed 20× (11.3%) was similar statistically (Logistic regression, $Z = -1.27$, $df = 11$, $P = 0.205$) to that recorded by 20× washed PermaNet 3.0 (13.1%), suggesting that Veeralin LLIN washed 20× is as protective as the 20× washed PermaNet 3.0 net which is the WHO PQT approved PBO/pyrethroid bi-treated LLIN (Table 3). Furthermore, blood-feeding rate recorded for Veeralin LLIN after being washed 20× (11.3%) was statistically similar to unwashed DuraNet LLIN (11.8%) (Logistic regression, $Z = 0.60$, $df = 11$, $P = 0.549$) meaning also that Veeralin LLIN washed 20× provides similar feeding inhibition as the WHO PQT approved standard pyrethroid-only LLIN. Although there were differences in personal protection recorded between treated nets, these differences were not significant (Negative binomial, range for Z: -0.2–0.4, $df = 11$, $P > 0.05$).

Mortality

Mortality of *An. funestus* (s.l.) recorded in all treated arms was significantly higher (Logistic regression, range for Z: 4–7, $df = 15$, $P < 0.05$) as compared to the untreated control arm (Table 4). Mortality recorded for unwashed PermaNet 3.0 LLIN (22.9%) was significantly higher than other treatments; however, mortality recorded for PermaNet 3.0 and Veeralin after being washed 20 times (12.6% and 8.5%, respectively), were not significantly different. Moreover, mortality rates induced by the unwashed Veeralin (12.1%) and 20× washed Veeralin (8.5%) were significantly higher (Logistic regression, $Z = 3.6$ and 5.0, respectively, $df = 15$, $P < 0.05$) than the rates recorded by the WHO approved pyrethroid-only standard unwashed DuraNet LLIN (1.5%). Other net treatments had comparable results except for the DuraNet which recorded significantly lower killing effect (Table 4).

Table 4: Experimental huts results: percentage mortality and killing effect of *Anopheles funestus*

Number of washes	Untreated net	PermaNet 3.0	PermaNet 3.0	Veeralin	Veeralin	DuraNet
	0	Unwashed	20	Unwashed	20	Unwashed
Total no. of females caught	374	256	328	319	364	288
Total no. of females dead	10	64	49	46	40	12
% Mortality corrected for control (95% CI)	–[a]	22.9[b] (17.8–28.1)	12.6[c] (9.0–16.2)	12.1[c] (8.5–15.6)	8.5[c] (5.7–11.4)	1.5 (−0.8–3.9)
% Overall killing effect	–[a]	14.4[bc]	10.4[bc]	9.6[bc]	8[c]	0.5[e]

Notes: Percentage mortality and 95% Cis are backtransformed from values calculated by the blocked logistic regression model. Within a row, treatments not sharing a superscript letter differ significantly by blocked logistic regression (P < 0.05).

4. Discussion

This study was designed to investigate the efficacy of the Veeralin LLIN after being exposed to 20 washings according to the WHO standardized washing procedure (WHO, 1998). Contrary to most studies of PBO nets that have been evaluated in areas dominated by *An. gambiae* (s.s.) or *An. arabiensis* (N'Guessan et al., 2010; Koudou et al., 2011; Pennetier et al., 2013), this evaluation trial was conducted in an area predominated by *An. funestus* (s.s.) malaria vectors (Derua et al., 2015; WHO, 2017; Kweka et al., 2018, 2020). The assessment of the laboratory-based knockdown effect (using susceptible *An. gambiae* (s.s.)) before washing, after 20 washes and after hut trials have shown the Veeralin PBO LLINs elicit high knockdown and mortality. Results presented here of bioassays done after 20 washes following the WHO standard washing protocol (WHO) revealed efficacy of Veeralin LLIN nets in producing > 80% mortality in all test mosquitoes up to 20 washes, thus meeting WHO wash-fastness criteria for a LLIN.

The present study shows that the predominant population of malaria vector mosquitoes during this trial was *An. funestus* (95.01%) with *An. gambiae* (s.l.) population (0.52%) and *Culex quinquefasciatus* (4.47%) being too low for a formal statistical analysis. This study revealed similar findings to previous studies conducted in the same area, where *An. funestus* (s.l.) dominated in the experimental huts (Tungu et al., 2015; Kweka et al., 2019, 2020). There are studies from different ecological areas of Tanzania indicating that recently *An. funestus* (s.s.) has emerged as the dominant malaria vector, replacing *An. arabiensis* and *An. gambiae* (s.s.), perhaps related to differential effects of intervention tools among species (Lwetoijera et al., 2014; Kweka et al., 2020).

The deterrence effect observed for the Veeralin unwashed (14.7%) and Veeralin washed 20 times (2.7%) was found to be low, as in previous studies where PermaNet 3.0 and DuraNet LLINs were evaluated in experimental huts against wild populations in Magugu, Moshi and Muheza (Tungu et al., 2010; Mahande et al., 2018; Kweka et al., 2019). The findings of the present study contrast with the findings in M'bé, Côte d'Ivoire, West Africa, where unwashed Veeralin, and Veeralin washed 20 times showed deterrence rates of 65.3% and 64.2%, respectively (Oumbouke et al., 2019b). The difference in the experimental hut outcomes from Côte d'Ivoire and Tanzania might be attributed to differences in the degree of pyrethroid resistance and vector species composition, whereby typically East African mosquitoes are less resistant to pyrethroids compared to those in West Africa and are thus more likely to be killed than deterred (Oumbouke et al., 2017, 2019a, b), or to the differences in hut designs.

The Veeralin washed 20 times had a higher exophily rate (75.5%) in the experimental hut compared with the PermaNet 3.0 washed 20 times (64.3%). Unwashed Veeralin and unwashed Permanent 3.0 had similar exophily rates of 70%. The highest (but similar to Veeralin washed 20 times, see Table 3) exophily value was recorded by a pyrethroid-only net (DuraNet). The pyrethroid nets with PBO (PermaNet 3.0, Veeralin) and pyrethroid-only LLIN (DuraNet) induced higher exophily in this study than in a similar study conducted

in West Africa where the Veeralin washed 20 times had an exophily effect of 64.7% and unwashed had 55.5% effect (Kweka et al., 2019). The exophily assessed for DuraNet in the study by Kweka et al. (2019) is similar (78.4% vs 76.4%) in the present study.

The highest blood-feeding inhibition was found for Veeralin (62.6%) followed by 55.9% for PermaNet 3.0, both washed 20 times. In addition, another study conducted with Magnet LLIN (alphacypermethrin without PBO) in the same area as the present study recorded a similar blood-feeding inhibition of 58.1% against the *An. funestus* (s.l.) population (Kweka et al., 2019).

The percentage mortality corrected for control was found to be lowest in pyrethroid-only treated net (DuraNet) unwashed (1.5%). In 2007 when the alphacypermethrin-only DuraNet LLIN was evaluated in the Muheza huts for WHOPES and *An. funestus* (s.l.) and *An. gambiae* were still pyrethroid-susceptible, the percentage mortality corrected for control of *An. funestus* and *An. gambiae* were respectively 93% and 96% with the unwashed DuraNet and 83% and 81% with the 20 times washed DuraNet (Kweka et al., 2019). This major difference in mortality with the alphacypermethrin-only LLIN can be attributed to elevation of pyrethroid resistance in Muheza in both *An. funestus* (s.l.) and *An. gambiae* over the interim period (Kweka et al., 2019). Mortality induced by 20 times washed Veeralin was significantly higher than that for the unwashed DuraNet LLIN, a WHO PQT approved standard pyrethroid-only LLIN. Mortality induced by 20 times washed Veeralin LLIN was similar to that recorded for the unwashed and 20 times washed PermaNet 3.0, a PBO LLIN and a positive control in this trial. Taken together, these results provide evidence that Veeralin LLINs have met PQT mortality criteria for a LLIN.

A candidate LLIN meets the WHO PQT phase II efficacy criteria if it performs as well as or better than the reference LLIN when washed 20 times in terms of blood-feeding inhibition and mortality. The WHO Vector Control Advisory Group (VCAG) advises the WHO on new vector control tools and stipulates that for use against pyrethroid-resistant vector populations, the mixture LLIN should demonstrate efficacy (mosquito mortality or

prevention of blood-feeding) significantly greater than standard pyrethroid-only LLIN. Significantly higher mortalities of pyrethroid-resistant *An. funestus* (s.l.) were recorded by the unwashed and 20 times washed Veeralin compared to DuraNet LLIN in experimental hut; the incorporation of PBO in Veeralin has shown increased mortality impact than in non-PBO DuraNet LLIN. High killing effect similar to WHO-approved standard PBO LLINs against pyrethroid-resistant mosquitoes have been recorded by Veeralin in a previous study (Oumbouke et al., 2019b). The significantly higher mortalities over pyrethroid-resistant *An. funestus* (s.l.) recorded by both unwashed and 20 times washed Veeralin compared to the unwashed ordinary DuraNet LLIN can be attributed to the effect of Piperonyl Butoxide (PBO), a chemical synergy contained in the whole Veeralin net thus meeting the VCAG criteria for a resistance-breaking LLIN.

Although this study reports superior efficacy of the Veeralin LLIN than a pyrethroid-only treated net over pyrethroid-resistant mosquitoes, the improvement, though significant, was 12–22% (corrected) mortality compared to 1.5% (for the non-PBO net) which may not be meaningful for public health impact. This apparently marginal improvement of PBO nets in hut trials was also observed last year in a non-WHOPES trial of Olyset Plus against Olyset in Muheza (Dr Patrick K. Tungu, personal communication). In Bagamoyo, Tanzania, another experimental hut study detected a similar result with Veeralin nets (WHO, 2016). However, the results from a cluster randomized trial have shown that PBO-LLINs provide better control of malaria than standard nets in Tanzania, whereby Olyset plus (PBO) nets provided a 33% malaria case reduction compared to Olyset nets at 21 months (Protopopoff et al., 2018). This indicates that the marginal improvement documented in the hut trials may indeed translate into a major improved in malaria control. Indeed, the study conducted in Muleba, Tanzania, provided sufficiently compelling evidence for efficacy of PBO-LLINs in reducing malaria cases for the WHO to give an evidence-based interim decision to approve PBO-LLIN to be used for malaria control (WHO, 2017).

5. Conclusion

The findings of this study have shown that blood-feeding rates recorded for 20 times washed Veeralin LLIN were statistically similar to those of the 20 times washed PQT-approved PBO/pyrethroid and a positive control in this trial. Also, the mortality induced by 20 times washed Veeralin LLIN was statistically similar to that recorded for the 20 times washed PermaNet 3.0 LLIN. This provides evidence that efficacy of VeeraLIN LLIN is statistically similar to PermaNet 3.0, the WHOPQT listed PBO LLIN and thus meeting WHOPQT mortality criteria for LLIN.

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Epilogue

The non-inferiority of Veeralin LN (5 panel treated) to PermaNet 3.0 (upper panel treated) provides further evidence that most mosquitoes respond to host-odour convection plumes, thus giving more support to the 2-in-1 concept as a tactic for managing resistance.

The present chapter (chapter 9) shows the similarity in efficacy between a PBO LN with PBO restricted on the roof panel compared to PBO LN with PBO on all 5 panels. However, it is important to quantify the actual effect size gained by deploying PBO LN as opposed to standard pyrethroid only LN in an area with resistant mosquitoes. With the purpose of quantifying the effect size gained by the use of PBO, the following chapter (Chapter 10) compares the meta-analysis of standard LLIN versus PBO synergist LLIN before and after development of insecticide resistance with *Anopheles gambiae* and *Anopheles funestus* over a decade of experimental hut trials.

Chapter 10: Effectiveness of PBO synergist long-lasting nets over a decade, before and after development of insecticide resistance, against *Anopheles gambiae* and *Anopheles funestus* in north-eastern Tanzania: experimental hut trials at a single location.

Abstract

Background

The future of pyrethroid long-lasting insecticidal nets (LLIN) for preventing malaria is threatened by the selection of pyrethroid resistance. Long-lasting nets incorporating the synergist piperonyl butoxide (PBO) are designed to prevent malaria transmitted by resistant mosquitoes, mediated by cytochrome P450 mono-oxygenase mechanisms. The efficacy of PBO-pyrethroid LLIN was evaluated in a series of experimental hut trials (EHT) before and after development of pyrethroid resistance, between 2005 and 2017, in northeast Tanzania during a period of increasing LLIN usage and insecticide resistance.

Methods

PBO-pyrethroid LLIN and standard pyrethroid LLIN, treated with permethrin or deltamethrin were evaluated in a series of EHT trials in Muheza, Tanzania against wild *Anopheles gambiae* and *Anopheles funestus*. Mosquito mortality, blood-feeding and exophily were compared between untreated nets, standard pyrethroid LLINs and PBO-pyrethroid LLINs. The outcomes were analysed by logistic regression after adjusting for covariates. Yearly trends between EHT were analysed by meta-analysis. Supplementary bioassay tests on LLIN assessed natural contact-time mortality response, contact irritability and repellence.

Results

Before 2010, *An. gambiae* and *An. funestus* were susceptible to pyrethroids. Between 2010 and 2013 resistance frequency increased from 0% to 57.3% in *An. gambiae* and from 0% to 47.4% in *An. funestus*. Resistance intensity reached 28-fold in *funestus*. In EHT, before resistance, wild free flying *An. gambiae* and *funestus* killed by standard and PBO LLIN treatments ranged up to 100% mortality. After selection of resistance, there was major

reduction in numbers killed: standard LLIN killed only 8-18% and PBO LLIN killed only 20-26% in EHT. In the meta-analysis of paired standard and PBO LLINs, there was mortality relative risk of 1.68 (1.08, 2.62) with *gambiae* and 1.83 (1.05, 3.17) with *funestus*. This low-level synergy cannot explain the major effect of PBO LLIN on malaria prevalence in some cluster randomised controlled trials (cRCT). In supplementary bioassays using wild-caught *gambiae*, 3-minute exposure to standard LLIN killed fewer than 12%, whereas 3 min exposure to PBO LLIN killed 97%. WHO irritability tests indicated the mean (median) contact time before taking first flight was shortest for PBO LLIN, 22 (15) seconds, intermediate for pyrethroid nets, 64 (27) seconds, and longest for untreated nets 110 (50) seconds. This shows repellence recorded was 3 times quicker ($Z = -3.437$, $p = 0.001$) with PBO-permethrin LLIN compared to standard permethrin LNs at the mean point and 2 times quicker at the median point. Contact irritability and repellence may also explain the reduced blood feeding and enhanced personal protection observed with holed PBO LLIN compared to holed standard LLIN.

Conclusions:

There was only partial restoration of efficacy with PBO LLIN against pyrethroid resistant *An. gambiae* and *funestus* compared to standard pyrethroid only LLIN. Contact irritancy and repellence may have a larger role in PBO-pyrethroid mediated efficacy than appreciated hitherto. Other factors may contribute to PBO efficacy not currently detected in EHT.

Keywords Long-lasting insecticidal nets, piperonyl butoxide, PBO, Resistance, *Anopheles gambiae*, *Anopheles funestus,* experimental huts, Tanzania

Background

Long-lasting insecticidal nets (LLINs) are an essential tool for malaria vector control in most endemic countries [1]. The universal distribution of LLINs free of charge across all age groups has led to a marked increase in the population sleeping under LLINs in sub-Saharan Africa, from less than 2% in 2000 to an estimated 55% in 2015, and to the proportion of children aged under five sleeping under nets to an estimated 68% [1].

Consequently, by 2014 pyrethroid resistance had been detected in the primary malaria vectors in most African countries [1]. The evolution and spread of resistance in the major vectors were affecting the efficacy of LLINs and undermining malaria control across the continent [2-5]. In Tanzania, the main focus of this paper, insecticide resistance in malaria vectors was first detected in 2006 in Kilimanjaro in the north of the country where *Anopheles arabiensis* first showed reduced susceptibility to permethrin [6]. Spreading across Tanzania, resistance was reported in 10 regions in 3 species: *Anopheles gambiae s.s*., *arabiensis* and *funestus* [7-9]. In *Anopheles gambiae s.s*. two types of resistance mechanisms were recorded: voltage gated sodium channel VGSC L1014S target site insensitivity and metabolic mechanisms due to enhanced P450 oxidases [8, 10-12]. Although other underlying factors had been identified, such as insecticide runoff from crops and rice into irrigation systems [13], the massive increase in insecticide-based malaria vector control over the last 15 years has major contribution in insecticide selection pressure on Anopheline populations across the country (Moshi, Jackie 2018, WHO 2015).

Among the approaches to combat pyrethroid resistance was the switch from standard LLIN to LLIN that incorporates the synergist piperonyl butoxide (PBO) and pyrethroid in the fibres of mosquito nets during manufacture. Synergists target resistance by inhibiting the enzymes responsible for metabolising insecticides. Cytochrome P450 mono-oxygenases are the main enzymes responsible for pyrethroid resistance in mosquitoes (WHO, 2006). PBO has long been used in commercial aerosols for potentiating pyrethroid activity against flying or domestic insect pests [14]. Pyrethroid-PBO combination LLINs have application against resistant mosquitoes whose resistance is based on oxidative metabolism of insecticides [15-17]. PBO is less effective against VGSC L1014S target site insensitivity mechanisms.

Several pyrethroid-PBO LLINs, developed to provide additional efficacy against pyrethroid-resistant mosquitoes, have been granted WHO recommendation. Amongst these, PermaNet® 3.0 and DawaPlus® 3.0 [17-22] are mosaic nets, with PBO and pyrethroid deltamethrin incorporated into the polyethylene fibres on the upper panel and with side panels made of polyester and coated with long-lasting deltamethrin. By restricting PBO to the roof of the net, the aim is to maximize insect contact with the synergist on the roof panel [23]. On other PBO net brands: Olyset® Plus, Veeralin®, DawaPlus 4.0 (TsaraBoost®) and Yorkool G3, the synergist and pyrethroids are incorporated in all five polyethylene panels [15, 16, 24, 25].

In several EHT trials, in various countries, PBO LNs have shown increased entomological impact against resistant mosquitoes compared to standard pyrethroid-only LLINs [16-18, 20-22, 26, 27]. Initially, the only evidence for epidemiological impact of the PBO LNs was derived from simulation models based on experimental hut data [19, 28]. Such evidence did not justify a complete switch from pyrethroid-only LLINs to PBO LNs, and consequently PBO nets were not deployed as standard control tool in areas against resistant mosquitoes [29, 30]. According to WHO, correlation between bioassay and experimental hut using mathematical models is not sufficient to justify epidemiological predictions [29]. To be categorized as a new paradigm (a class of LLIN whose public health value is yet unproven [30]), PBO LLIN, like all novel vector control products, are required to demonstrate greater impact on the epidemiology of malaria than exists at the time. A cluster randomised trial underway in Muleba, in northwest Tanzania by LSHTM and PAMVERC partners enabled the required epidemiological evidence to be produced, and WHO to grant an exception convergent with policy (WHO 2018).

While WHO has now granted recommendation to PBO LLIN, there is still much to understand about this class of net. The purpose of this paper is to present a chronological series of experimental hut trials of PBO synergist LLIN carried out between 2005-2017 in Muheza in northern Tanzania before and after development of pyrethroid-resistance in local Anopheline mosquitoes, analysed by meta-analysis, and backed up by supplementary bioassay tests to explore the activity of PBO-pyrethroid on mosquitoes in greater depth.

Methods

Study area and experimental huts

The studies made use of entomological testing facility at NIMRI, Ubwari and the suites of experimental huts situated in Muheza district, Tanga region. The EHT trial was conducted at the NIMR field station at Zenet village 5° 13' S latitude, 38° 39' E longitude, 193 m altitude; where *An. gambiae* s.s. and *An. funestus* s.s. and *An. arabiensis* are the major malaria vectors [31, 32].

Insecticides and mosquito nets

Olyset® Net LN specifications with respect to permethrin contains 2% (w/w) as the sole AI, corresponding to 20 g AI /kg. Net size was similar to Olyset Plus.

Olyset® Plus LN is a LLIN manufactured by Sumitomo Chemical Japan, made of 150-denier mono-filament polyethylene containing technical permethrin 2% (w/w) as active ingredient (AI), corresponding to 20 g AI /kg, and piperonyl butoxide (PBO) 1% (w/w), as synergist corresponding to 10 g/kg (400 mg of AI/m^2). Net size 180 cm long, 160 cm wide and 210 cm high.

PermaNet 2.0 LN (Vestergaard Frandsen SA, Denmark) is made of multifilament polyester (100 denier) fabric, factory treated with a wash-resistant formulation of deltamethrin at 55mg/m^2 and 2.4g/kg. Net size 130 cm wide, 190 cm long, 150 cm high.

PermaNet 3.0 LN (Vestergaard Frandsen SA, Denmark) has a top panel made of monofilament polyethylene (100 denier) incorporating PBO 4 g/kg (180 mg/m^2), and side panels made of polyester similar to PermaNet 2.0 with strengthened lower panel.

DawaPlus 3.0 LN from Tana Netting, comprised of side and end panels made of knitted poly-filament polyester fibres coated with 3 g ai/kg deltamethrin (120 mg/m^2), and a roof panel made of polyethylene (40 g/m^2) incorporating 3 g ai/kg deltamethrin (120 mg ai/m^2) and 11 g/kg PBO (440 mg/m^2).

DawaPlus 4.0 LN from Tana Netting, polyethylene (40 g/m^2) incorporating 3 g ai/kg deltamethrin (120 mg ai/m^2) and 11 g/kg PBO (480 mg/m^2) on all panels.

PandaNet LN is a long-lasting insecticidal net containing 1.8 g/kg of deltamethrin incorporated in monofilament (110 denier) polyethylene fibre.

Veeralin PBO LLIN (VKA India). Alpha cypermethrin incorporated into 130 denier monofilament polyethylene fibres with the target dose of 6.0g AI /kg corresponding to 216mg AI/m2 of alphacypermethrin and 2.2 g/Kg corresponding to 79mg/m2 of PBO.

Mosquito strains

Anopheles gambiae s. s. Kisumu, a laboratory pyrethroid insecticide susceptible strain, originally from Kenya.

Anopheles gambiae s.s. RSP, a laboratory pyrethroid insecticide resistant strain, homozygous for VGSC L1014S (*kdr* east).

Anopheles arabiensis a laboratory pyrethroid insecticide resistant strain from Moshi, Kilimanjaro region, Tanzania, with resistance to permethrin due to increased oxidase and esterase activity [11, 12].

Anopheles gambiae s.s. Muleba, a pyrethroid resistant strain from Muleba, Kagera region, resistant to lambda-cyhalothrin, deltamethrin and permethrin. It contains the L1014S resistance gene at a high allelic frequency (0.98) [8, 9].

Anopheles gambiae s. l Zeneti, a pyrethroid resistant strain from Zeneti village near Muheza, Tanzania, containing the L1014S knockdown resistance gene (*kdr* east*)* pyrethroid resistance mechanism, [31] and 30-fold resistance at LD50 relative to permethrin compared to susceptible *An. gambiae* Kisumu.

Culex quinquefasciatus Masimbani, a pyrethroid resistant strain from Muheza, Tanzania, containing elevated oxidase and *kdr* pyrethroid resistance mechanisms, showing 23% mortality in WHO tests using permethrin 0.75% test papers (Malima & Matowo unpublished). Earlier tests using synergists PBO and DEF implicated *kdr* and mixed function oxygenase mechanisms [33].

Experimental huts

Study area and experimental huts are described in more detail in appendix 1, Section 2.6.1.

Experimental hut study design

Experimental huts trials were undertaken in 2005, 2009, 2010, 2013, 2015, 2016 and 2017 (Table 1). These recorded up to six of the following treatment arms: (i) Unwashed Olyset Net, (ii) Unwashed OlysetPlus Net, (iii) Unwashed PermaNet 2.0, (iv) Unwashed PermaNet 3.0, (v) Unwashed PandaNet LN, (vi) Unwashed DawaPlus 3.0 LN, (vii) Unwashed DawaPlus 4.0 LN, (viii) Veeralin, (ix) Untreated unwashed polyester. Each of these trials had six treatment arms. In some trials the nets were washed 20 times. Rotation using Latin squares was used to adjust for any variation between hut position, sleeper attractiveness and individual nets. Mosquito collection, scoring and recording procedures during these trials have been described previously [39] or described in WHOPES LLIN guidelines (2013). Depending on mosquito density recorded at the time of the trial, a decision was taken to collect data for either 36 or 72 nights.

The primary outcomes were: (i) deterrence – the reduction in entry into treatment hut relative to the control huts (i.e. those containing untreated nets); (ii) treatment induced exiting – the proportion of mosquitoes found in exit traps of treatment huts relative to the proportion in control hut exit traps; (iii) mortality – the proportion of mosquitoes killed relative to the total catch.

Table1. Experimental huts trials; Year and treatment arms

Trial name	Trial year	Control	Standard LN 1	Standard LN 2	PBO LN1	PBO LN 2	PBO LN 3	PBO LN 4
Olyset 7 years	2005	Untreated net	New Olyset net	7 years Olyset net				
PermaNet 3.0	2009	Untreated net	Unwashed PermaNet 2.0	PermaNet 2.0 washed 20X	Unwashed PermaNet 3.0	PermaNet 3.0 washed 20X		
Olyset Plus	2010	Untreated net	Unwashed OlysetNet	OlysetNet washed 20X	Unwashed Olyset Plus	Olyset Plus washed 20X		
Olyset Plus	2013	Untreated net	Unwashed OlysetNet		Unwashed Olyset Plus	Olyset Plus washed 20X		
OlysetPlus/Actellic CS	2015	Untreated net	Olyset Net		Unwashed Olyset Plus			
DawaPlus 3&4	2016	Untreated net	Unwashed PandaNet LN	PandaNet LN washed 20 X	Unwashed DawaPlus 3.0	Unwashed DawaPlus 4.0	DawaPlus 3.0 washed 20X	DawaPlus 4.0 washed 20X
DawaPlus 3&4	2017	Untreated net	Unwashed PandaNet LN	PandaNet LN washed 20 X	Unwashed DawaPlus 3.0	Unwashed DawaPlus 4.0	DawaPlus 3.0 washed 20X	DawaPlus 4.0 washed 20X
VeeraLIN	2018	Untreated net	Unwashed DuraNet LN		Unwashed PermaNet 3.0	PermaNet 3.0 Washed 20X	Unwashed Veeralin LN	Veeralin LN washed 20X

Synergist-insecticide test

To determine whether the mechanism of resistance was due to elevated mixed function oxidase enzymes, *Anopheles gambiae* and *Anopheles funestus* mosquitoes, collected from Zeneti village in 2016, were pre-exposed to the synergist, piperonyl butoxide (PBO), in 250 ml Wheaton bottles. The bottles were coated by adding 1 ml of a stock solution of 400 mg/L of PBO (corresponding to a dose of 400 ml/bottle) in acetone, rotating the bottles to ensure all surfaces were covered and then allowing the acetone to evaporate. Two to five days old female mosquitoes were added to the bottle and exposed for one hour in accordance with WHO protocol [40]. The mosquitoes were then removed and exposed to 0.05% deltamethrin or 0.75% permethrin in WHO tube assays for 1 hour as described above [40].

Molecular assays

For molecular characterization of Zeneti field *Anopheles gambiae* s.l, sibling species identification was carried out according to the standard polymerase chain reaction (PCR) method [41] in 2011 and 2013 and 2016. While *Anopheles funestus* species identification was carried out using conventional PCR assay, *Anopheles gambiae s.l* mosquitoes collected from huts were analysed for VGSC *kdr* using the Taqman assay technique of Bass [42] to detect L1014S or L1014F alleles.

Supplementary bioassay tests on Olyset and Olyset Plus used in the hut trials to characterize the behavior of the mosquitoes on the netting.

WHO Cone

To evaluate the efficacy of standard pyrethroid LLINs and Pyrethroid PBO-synergist combination LLINs against susceptible and resistant mosquitoes in the laboratory, standard WHO cone bioassays were performed, based on the WHO protocol [34], using

insectary-reared pyrethroid-susceptible *An. gambiae* Kisumu and pyrethroid-resistant *Anopheles gambiae s.s.* RSP, *Anopheles arabiensis,* and F1 generation of wild *An. gambiae s.l.* collected from Zeneti village.

Tunnel tests

Tunnel tests were carried out on net pieces cut from the top and side panels of the Olyset Plus, Olyset net, PermaNet 2.0 and PermaNet 3.0. The tests were conducted using laboratory-reared *Anopheles gambiae s.s.* Kisumu (pyrethroid susceptible), *Anopheles gambiae s.s.* Zeneti (pyrethroid resistant) and *Culex quinquefasciatus* Masimbani (pyrethroid resistant). The tunnel test was standard, comprised of a glass cylinder, 25 cm high, 21 cm wide, 60 cm long, divided into two by a transverse insert made of test netting. Nine 1-cm diameter holes were cut into the netting to allow passage of mosquitoes into the bait chamber, where a guinea pig was housed anaesthetized unconstrained in a cage and in the release chamber 50 unfed female mosquitoes aged five to eight days were released at dusk and left overnight in conditions of 25 ± 2ºC and 80 ± 10% humidity. The following morning the numbers of mosquitoes found alive or dead, blood fed or unfed in each compartment were recorded and delayed mortality recorded after a further 24 hours [34].

Effective exposure time

To determine the exposure time required to kill local resistant mosquitoes using standard and PBO LNs, WHO cone bioassay tests were conducted using laboratory reared susceptible *An. gambiae* Kisumu and F1 progeny of wild *An. gambiae s.l.* collected from Zeneti village exposed to Olyset and Olyset Plus for 0.5, 1, 2 and 3 min and mortality recorded after 24h. Ten replicates of five mosquitoes were tested for each exposure time.

Irritability Test

To characterize the excito-repellent properties of standard pyrethroid LLIN and PBO-Pyrethroid LLIN and to help explain the EHT results, fifty non-blood-fed, 2-5 day old

insectary-reared F1 generation of wild *An. funestus s.l.* collected as larvae from Zeneti village were individually introduced into plastic cones, containing either a piece of untreated netting, new Olyset LN or Olyset Plus LN, allowed to settle for 60 seconds, and time elapsing between the first landing and the next take-off of the mosquito was recorded, up to 360 seconds later. The relationship between net and percentage taking flight was analysed (FT_{50} and FT_{95}) using log dose probit regression software (Polo Plus, LeOra).

Chemical analysis

Chemical analyses were performed on Olyset and Olyset Plus netting sampled from the nets used in the trials. For each net, five pieces (25 cm x 25 cm) were cut from each net according to the WHO sampling method for LLINs and pooled for chemical analysis [34]. The average permethrin and PBO contents were determined using the CIPAC method 331/LN/M/3 (www.cipac.org). This method involved extraction of permethrin and PBO from the net samples in a water bath (85–90^{o}C) for 45 minutes with heptane in the presence of triphenyl phosphate as internal standard and determination by gas chromatography with flame ionization detection.

Statistical analysis

Data were entered into an Excel database and transferred to Stata 11 (Stata Corp LP, College Station, TX, USA) for processing and analysis. Data from cone bioassays were compared between each net using a Chi square test. Significance between treatments was set at 5% level. For the experimental hut trial, the principal aim was to compare the efficacy of the different treatments. The main outcomes were the comparisons of efficacy between treatments in terms of the proportions of mosquitoes' blood-feeding or killed by the treatments. Logistic regression analysis was used to estimate proportional outcomes (mortality, blood-feeding, exiting) and negative binomial regression was used to analyse counts of mosquitoes entering, blood feeding (personal protection) or dying

(overall insecticidal effect) relative to the untreated control, after adjusting for variation between individual sleepers and hut position.

Mortality results of trials were pooled using meta-analysis on odds of mortality using the fixed effects Mantel-Haenszel method. Overall heterogeneity across trials was calculated using Cochran Chi square tests and extended I^2 statistics which describe the variation in the study [43].

Ethics, consent and permission

Ethical clearance was obtained from the ethics committees of the NIMR Tanzania (Ref: NIMR/HQ/R.8a/Vol X/86) and London School of Hygiene and Tropical Medicine (LSHTM) for each of the hut trials. Written informed consent was obtained from all volunteers participating in the studies; each was provided with chemoprophylaxis and monitored daily for fever or possible adverse events due to insecticide exposure from the nets.

Results

Efficacy of Olyset Plus and Olyset LNs in bioassay against resistant strains of *Anopheles gambiae* and *Culex quinquefaciatus* mosquitoes.

The Cone test with 3 min exposure was used to compare efficacy of standard pyrethroid and PBO-pyrethroid LNs against susceptible and resistant mosquitoes in the laboratory. Both Olyset Plus and Olyset nets induced 100% mortality in tests against pyrethroid susceptible *Anopheles gambiae* Kisumu (Figure 1). Mortality of standard nets was broadly in line with the intensity of resistance in each of these strains. With the 4 pyrethroid resistant strains (An. *gambiae s.l.* RSP, *An. arabiensis* and *An. gambiae* Zeneti, *Culex quinquefasciatus* Masimbani), percentage survival was significantly higher with PBO-permethrin netting (Olyset Plus) than with permethrin-only treated netting (Olyset Net) (Fig 1).

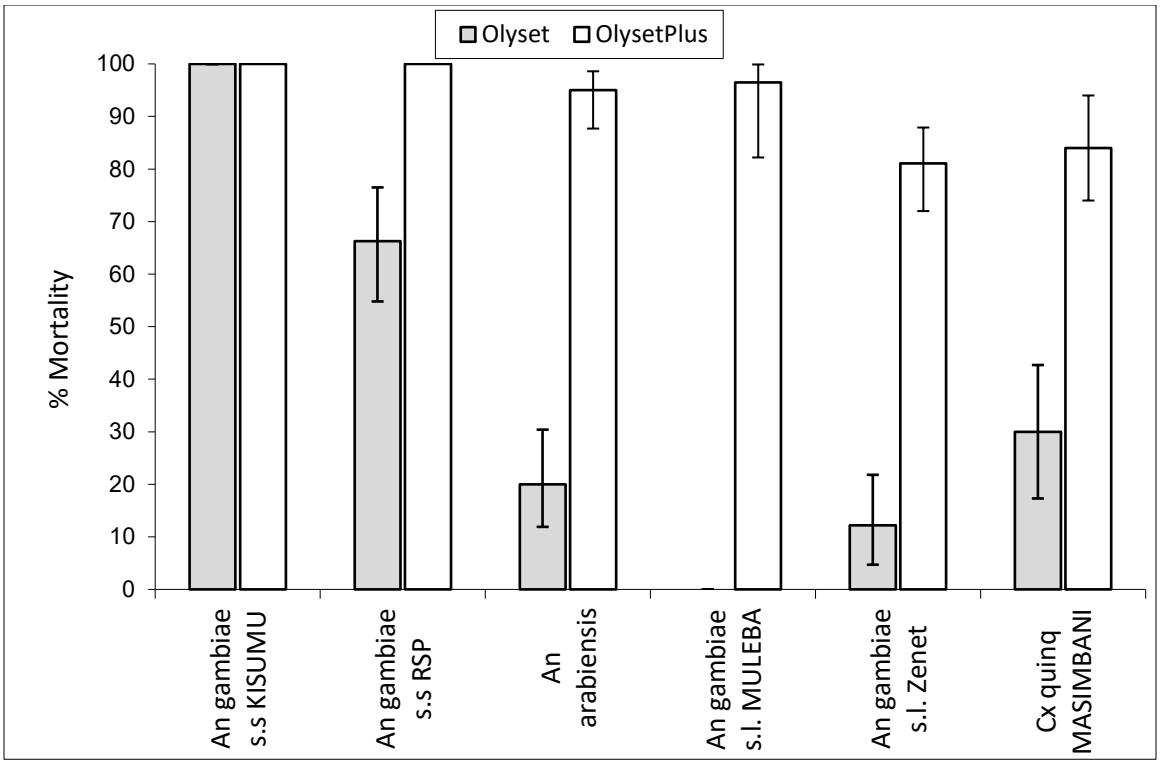

Figure 1. Cone bioassay test mortality after 3 min exposure and 24h holding to Olyset Plus (PBO-permethrin) and Olyset Net (permethrin) against pyrethroid susceptible (*Anopheles gambiae* KISUMU) and resistant (*An. gambiae* RSP*, An. gambiae* MULEBA, *An. gambiae* ZENETI, *An arabiensis* (MOSHI) and *Culex quinquefaciatus* (MASIMBANI) mosquitoes.

In Tunnel Tests, both standard and PBO LLIN inhibited penetration of the susceptible *An. gambiae*. The resistant *Culex* readily penetrated the standard LLIN (68%) but showed limited passage through the PBO LLIN (32%) (Fig 2). *An. gambiae* showed high blood-feeding with untreated netting (92%) but did not feed at all when exposed to standard or PBO netting. Resistant Culex recorded 62% feeding with untreated, 37% with standard but 0% with PBO LLIN netting (Fig 2). The susceptible *An. gambiae* recorded high mortality with both type of LLIN (Figure 2). *Culex* recorded 0% mortality with standard Olyset but high mortality (74.6%) with the PBO LLIN (Fig 2).

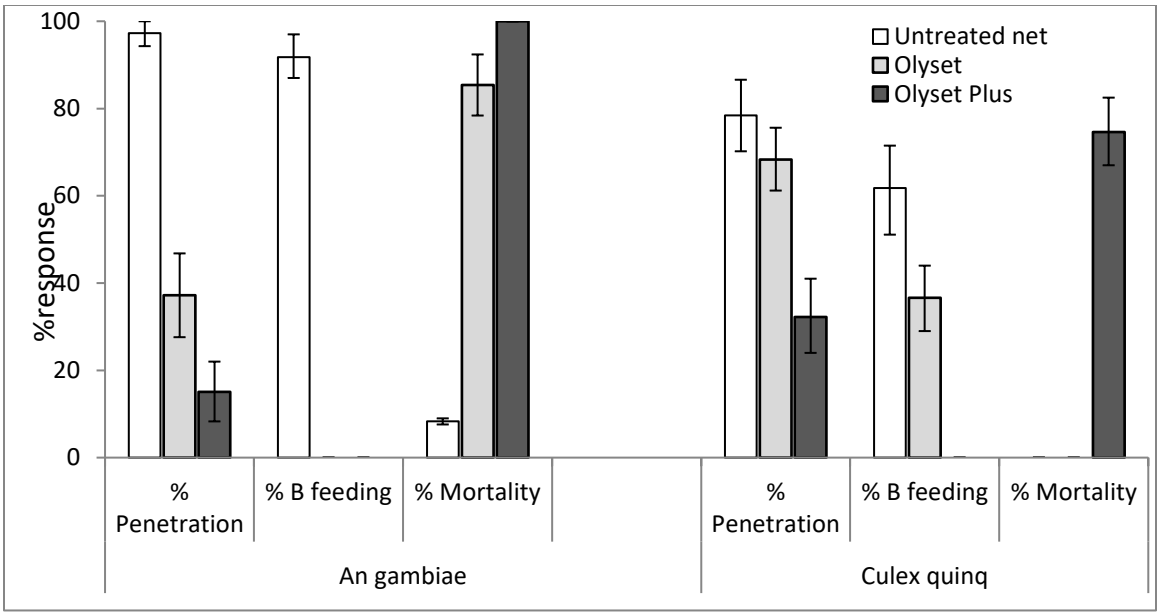

Figure 2: Tunnel tests using Olyset Plus and Olyset netting against pyrethroid susceptible (*Anopheles gambiae*) and resistant (*Culex* Masimbani) mosquitoes.

Phase II - experimental hut trials results

Species characterisation and insecticide resistance status

WHO susceptibility tests using WHO diagnostic dosages, conducted at the EHT site in Zeneti, Muheza showed that before 2010, *An. gambiae* was fully susceptible to pyrethroids (permethrin, deltamethrin). The test in 2010 showed signs of resistance to permethrin which gradually increased over the years to 43.6% resistance in 2016 (Figure 3). In 2014 *An. funestus s.s.* showed resistance to deltamethrin with 25% survival. In intensity bottle bioassays *An. gambiae* showed 30-fold resistance to permethrin.

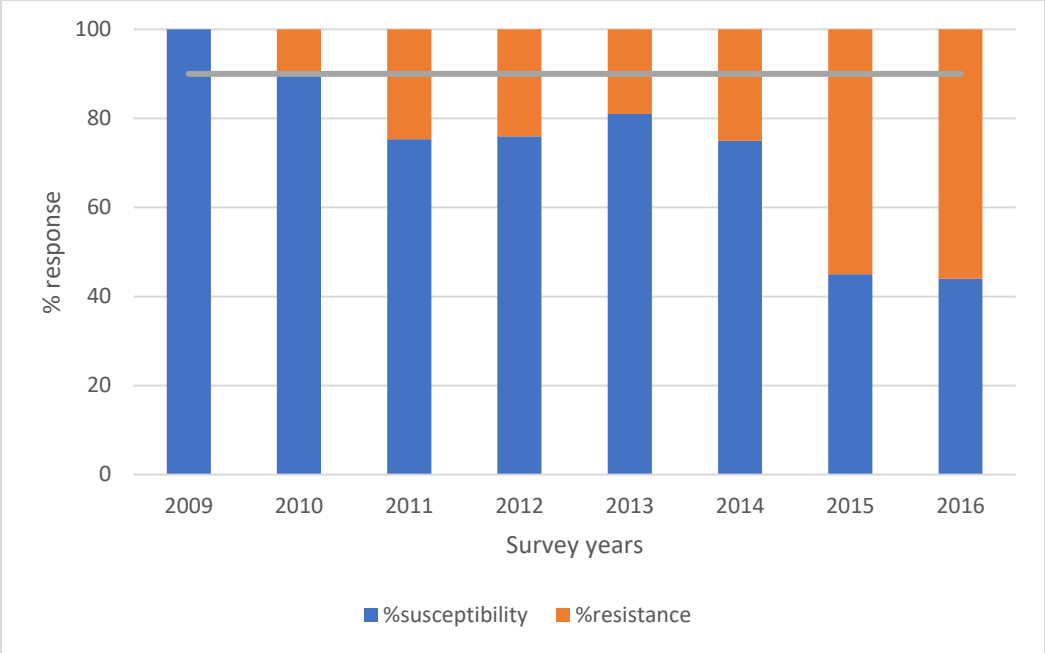

Figure 3. Zeneti wild mosquitoes' trend in resistance and susceptibility to permethrin over the years.

After exposure to the synergist PBO, percentage mortality of *An. gambiae* s.l. and *An. funestus* to 0.75% permethrin increased to 100%. The increase in pyrethroid susceptibility after exposure to PBO indicates elevated oxidase enzymes as the primary resistance mechanism. In 2011 all 80 sampled mosquitoes were *An. gambiae s.s.* with VGSC L1014S (*kdr east*) at allelic frequency of 20%.

In 2013 and 2016, of 1122 and 108 mosquitoes identified using qPCR, 94% and 96% were found to be *Anopheles gambiae s.s.* and 6% and 4% were *Anopheles arabiensis.* Of 833 and 101 *Anopheles gambiae s.s.* that analysed for *kdr-east,* 35% and 24% were homozygous susceptible, 24% and 57% were heterozygous resistant and 41% and 13% were homozygous resistant. The L1014S *kdr-east* allelic frequency in *Anopheles gambiae s.s.* were 0.46 and 0.44.

No *kdr* gene was detected in *Anopheles arabiensis.* All 40 *arabiensis* that analysed for *kdr east* were homozygous susceptible.

A sample randomly selected *An. gambiae s.s.* mosquitoes were analysed for the molecular form, and all were of "S" form.

Mosquito entry into experimental huts

Among the trials compared, the highest *Anopheles gambiae s.l.* collection was in the 2013 trial, where the average number of per hut per night ranged between 3-4 mosquitoes, and the lowest was recorded in the 2015 trial, where it ranged between 0.2-0.4 per night. Highest number of *Anopheles funestus* was recorded in the 2005 trial where the average collected per hut per night ranged between 4 and 5 mosquitoes. There were no *Anopheles funestus* collected during the trials in 2010 and 2013.

The outcome of the negative binomial regression analysis on the numbers caught per night revealed that in all trials there was no significant reduction in numbers entering the huts with standard LLINs compared to the huts with untreated control nets (Table 2). Significant ($p<0.05$) reduction in entry of *An. gambiae* and *An. funestus* in the huts with PBO LLIN relative to untreated control was observed in all trials except for the one in 2013 (Table 2). In both species there was no association between mosquito abundance and the transition from susceptibility to resistance or increasing resistance over time (Table 2).

Among the trials compared, the highest *Anopheles gambiae s.l.* collection was in 2013 trial, where the average number of *Anopheles gambiae* per hut per night ranged between 3-4 mosquitoes. The lowest collection of *Anopheles gambiae* was recorded in the 2015 trial where the average number of *Anopheles gambiae* per hut per night ranged between 0.2-0.4 only. The numbers of *Anopheles gambiae* collected during the trial in 2016 were too low for any meaningful statistical analysis, hence only *Anopheles funestus* were included. Highest number of *Anopheles funestus* was recorded in the trial in 2005 with average number of *Anopheles funestus* collected per hut per night ranging between 4 to 5 mosquitoes. There were no *Anopheles funestus* during the trials in 2010 and 2013. The outcome of the negative binomial regression analysis (performed with the number caught as dependent variable and the six experiment arms as independent variable) revealed

that in all trials no significant reduction in number of mosquitoes entering the huts with standard LNs relative to the huts with control untreated net (Table 5). Significant ($p<0.05$) reduction in entry of both species (*An. gambiae and An. funestus*) in the huts with PBO LLIN relative to untreated control was observed in all trials except the trial in 2013 (Table 5).

Table 2: Total number caught during the trial, average number per night (in parenthesis) and % deterrence (in square brackets) of susceptible and resistant *Anopheles gambiae* and *Anopheles funestus* in hut trials of Permethrin treated standard and PBO LNs.

	Year of the trial	Untreated net	Standard LN	PBO LN
Anopheles gambiae				
Before resistance				
	2009	723^{a} (13)	574^{ab} (11) [21]	425^{b} (8) [41]
	2010	68^{a} (1.9)	13^{a} (0.4) [80.9]	44^{bc} (1.2) [35.5]
After resistance				
	2013	221^{a} (3.4)	245^{a} (3.8) [0]	221^{a} (3.4) [0]
	2015	58^{a} (0.5)	36^{ab} (0.3) [37.9]	25^{b} (0.2) [56.9]
Anopheles funestus				
Before resistance	2005	315 (5.2)	222 (3.7) [29.5]	-
After resistance	2015	92^{a} (1.7)	64^{ab} (1.1) [30.4]	55^{b} (1) [39.1]
	2017	87^{a} (1)	116^{a} (1.2) [0]	110^{a} (1.2) [0]

***Treatment not tested**

Mortality

Percentage mortality by treatment arm is shown in Figure 4a and Table 3. During the period of susceptibility (2005-2010), high mortality of both Anopheles (*gambiae* and

funestus) were recorded with standard LLINs (ranging between 63-100%) and PBO LNs (98-100%) (Fig. 4a, Table 3).

After development of resistance there was a significant decrease in mortality induced by standard LLIN (ranging between 10-17%) and PBO LLIN (20%) against resistant *An. gambiae* and *An. funestus* using permethrin LLINs. In of the four trials conducted during the resistance period (2013-2018), there was a small but significant increase in mortality of *An. gambiae* and *An. funestus* induced by PBO LLIN as compared to standard LLIN (Figure 4a, Table 3). While the difference was significant, the increase in mortality (average 10.6%) recorded with PBO LLIN did not return mortality to the high levels seen against susceptible populations. Hence synergy by PBO only partially restored the situation to the one pre-resistance. Changing insecticide from permethrin and PBO (Olyset Plus) to deltamethrin and PBO (DawaPlus 4) did not change the pattern (Table 3).

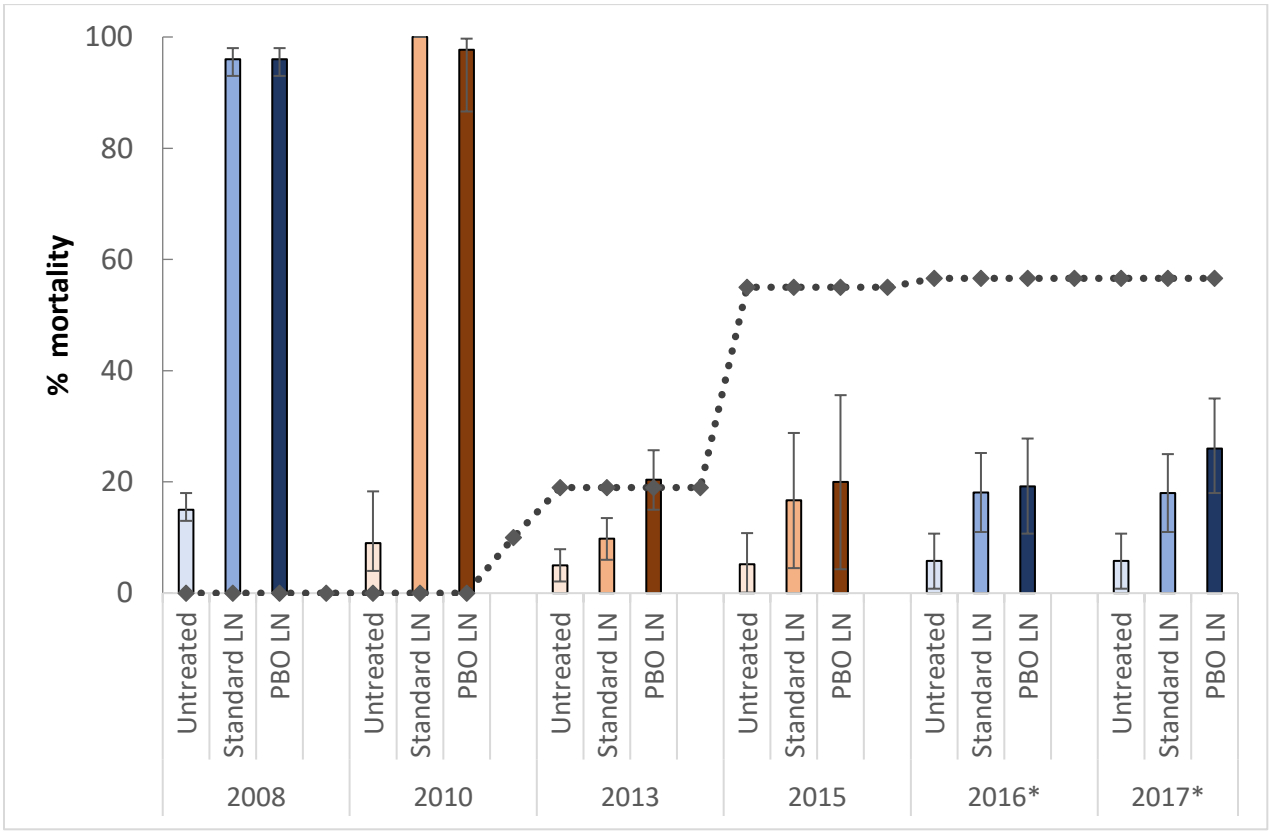

Figure 4a: Bar chart showing experimental huts % mortality with Untreated net, Standard and PBO LLINs with the trials before and after development of pyrethroid resistance by the local mosquitoes. Note: (i) Line graph showing trend of insecticide resistance over the years. (ii) Bars in blue coloration are trials with deltamethrin Standard LLINs and PBO LLINs while bars with brown coloration are the trials with permethrin Standard LLINs and PBO LLINs (iii) In X axis year with star (*) means the trial is with *An. funestus* s.l. (remainder are *An. gambiae*)

Table 3. Percentage mortality (95% CI) and total mosquito caught (in [ ]) in experimental huts trials of standard LN (Olyset) and permethrin PBO LN (Olyset Plus) before and after development of pyrethroid resistance in local mosquitoes.

	Untreated net	Standard LN	PBO LN
Anopheles gambiae			
Before resistance			
2009	15[a] (13-18) [723]	96[b] (93-97) [574]	96[b] (93-97) [425]
2010	9[a] (4-18.3) [68]	100[b] (100-100) [13]	97.7[c] (86-99.7) [44]
After resistance			
2013	5[a] (2.1-7.9) [221]	9.8[b] (6-13.5) [245]	20.4[c] (15-25.7) [221]
2015	5.2[a] (0.1-10.8) [58]	16.7[b] (4.5-28.8) [36]	20[b] (4.3-35.6) [25]
Anopheles funestus			
Before resistance			
2005	7.9[a] (4.9-10.9) [315]	73.9[b] (68.1-79.7) [222]	*
After resistance			
2015	3.2[a] (0.4-7) [92]	7.8[a] (1.1-14.6) [64]	19.6[b] (8.9-30.4) [56]
2017	5[a] (0.8-10.7) [87]	18[b] (11-25) [116]	26[c] (18-35) [110]

Meta-analysis of mortality

With susceptible *An. gambiae* mosquitoes, the relative risk of mortality between PBO LLINs versus standard pyrethroid LLINs was 1.00 (CI 0.98, 1.03) (Fig. 4b), indicating that the two treatments have a similar mortality effect size against susceptible *An. gambiae* mosquitoes (z = 0.23, P = 0.821).

With resistant *An. gambiae* mosquitoes, the relative risk of mortality between PBO LLINs versus standard pyrethroid-only LLINs was 1.68 (CI 1.08, 2.62) (z = 2.28, P = 0.023) (Fig. 7a or 4b), indicating that PBO LLINs showed a 1.68-fold significant increase in mortality of resistant mosquitoes compared to standard pyrethroid LLINs.

While this difference between PBO and standard LLINs was significant, the actual difference in mortality between the two types of net was quite minor compared to the major difference in mortality before and after selection of resistance.

Comparable results were shown by *An. funestus* mosquitoes from the same huts pre and post development of resistance (Figs. 4c).

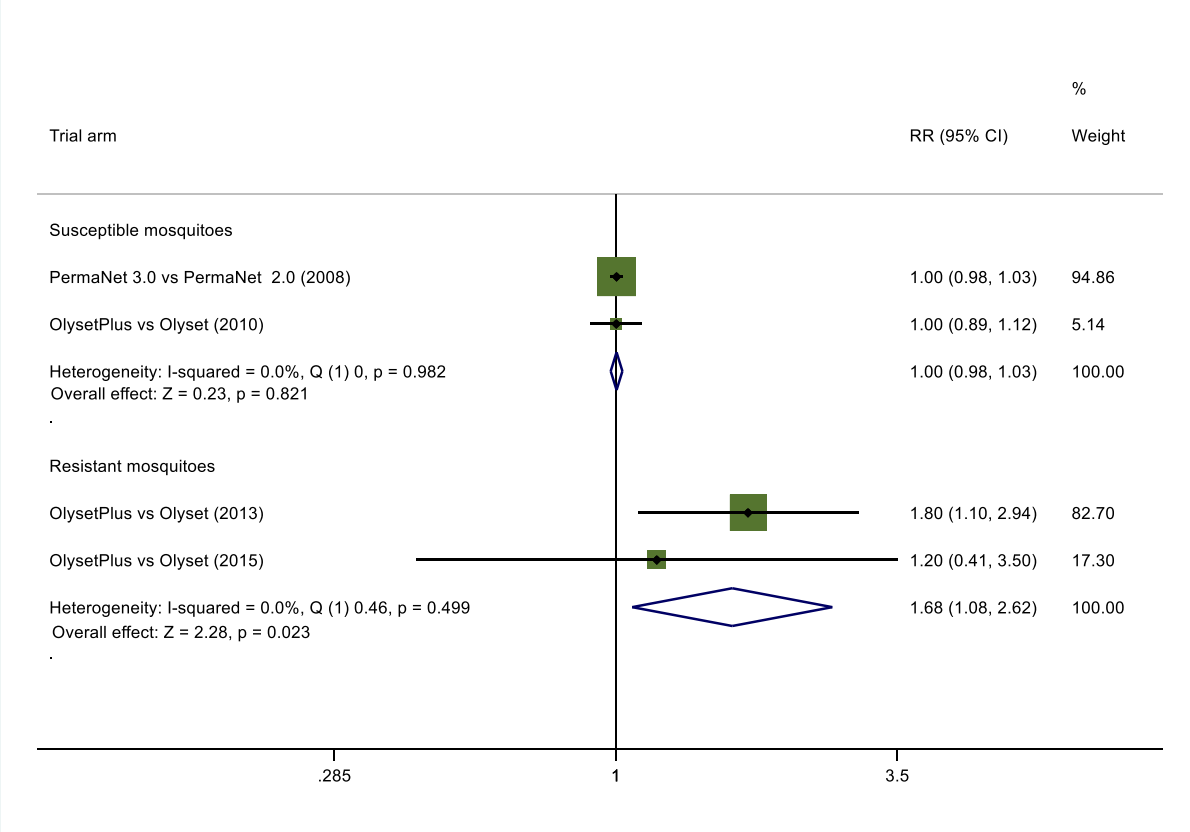

Figure 4b. Mortality meta-analysis forest plot: *An. gambiae s.l.*

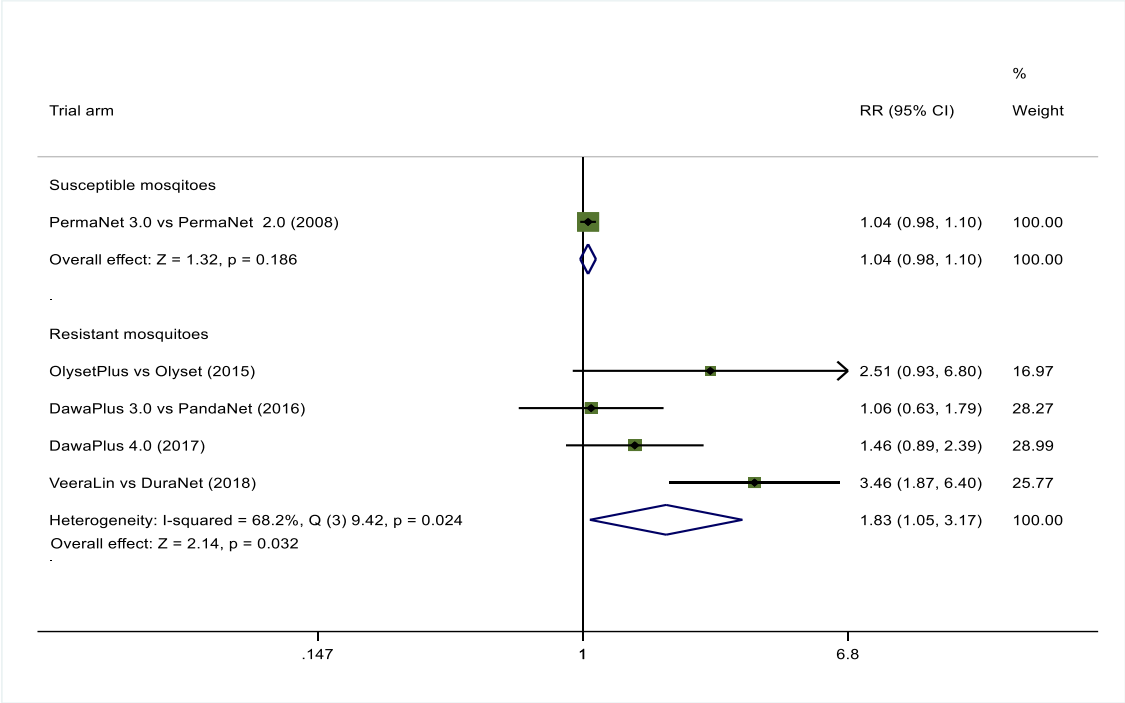

Figure 4c. Mortality meta-analysis forest plot: *An. funestus* s.l.

Blood feeding and blood feeding inhibition (BFI)

Percentage blood feeding and feeding inhibition results by treatment for trials are shown in Table 4. In all trials there was significantly higher percentage blood-feeding of *Anopheles gambiae* and *Anopheles funestus* in huts with the untreated nets as compared to the huts with the standard and PBO LLINs (Table 4). In all trials and with both species (*Anopheles gambiae* and *Anopheles funestus*), blood-feeding recorded with standard and PBO LLINs were statistically similar to standard LLINs ($p<0.05$) with exception of the trial in 2010 where standard LLINs recorded significantly lower *Anopheles gambiae* feeding rate (Table 4).

Table 4. Percentage Blood-feeding (95% CI), total number fed (shown in [ ]) and blood-feeding inhibition (% shown in bold type) in *Anopheles gambiae* and *Anopheles funestus* collected in experimental hut trials with permethrin treated standard LN (Olyset) and permethrin PBO LN (Olyset Plus)

		Untreated net	Standard LN	PBO LN
Anopheles gambiae				
Before resistance				
2005	% Blood feed (95%CI) [Total female caught]	27[a] (19-35) [30]	16[b] (10-21) [28]	*
	% Blood feeding inhibition	-	41	*
2010	% Blood feed (95%CI) [Total female caught]	72[a] (60-81) [49]	0[b] (0-0) [0]	9[c] (4-22) [4]
	% Blood feeding inhibition	-	100	88
After resistance				
2013	% Blood feed (95%CI) [Total female caught]	26[a] (20-32) [57]	5[b] (3-8) [13]	3[b] (0.6-4.5) [6]
	% Blood feeding inhibition	-	80	90
2015	% Blood feed (95%CI) [Total female caught]	24[a] (13-35) [14]	11[ab] (0.8-21) [4]	4[b] (0.01-12) [1]
	% Blood feeding inhibition	-	54	83
Anopheles funestus				
Before resistance				
2005	% Blood feed (95%CI) [Total female caught]	32[a] (27-38) [102]	16[b] (11-21) [36]	*
	% Blood feeding inhibition	-	50	*
After resistance				
2015	% Blood feed (95%CI) [Total female caught]	20[a] (11-28) [18]	3[b] (0.1-8) [2]	4[b] (0.1-8) [2]
	% Blood feeding inhibition	-	84	82

***Treatment not tested**

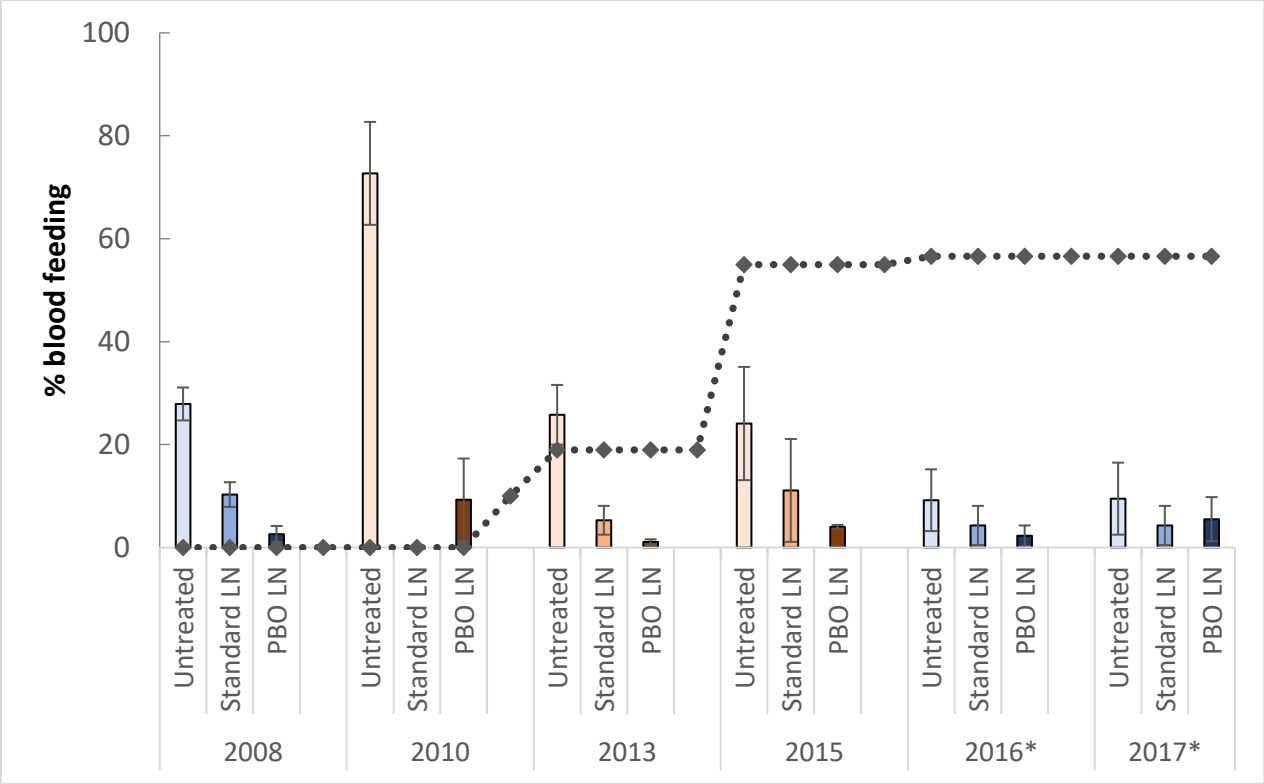

Figure 5a: Experimental hut trial trends: blood feeding with untreated, standard pyrethroid and PBO- pyrethroid LLINs before and after development of pyrethroid resistance.

Note: (i) Line graph showing trend in insecticide resistance over time.

(ii) results in green are EHT with deltamethrin nets while bars in purple are with permethrin nets Standard LLINs and PBO LLNs

(iii) In X axis, year with star (*) means the trial is with *An. funestus* s.l. (remainder are *An. gambiae*)

Meta-analysis of percentage blood feeding

In the meta-analyses of blood-feeding between the PBO LLINs and standard pyrethroid LLINs, with susceptible *Anopheles gambiae* mosquitoes, the comparison of relative risk between PBO LLINs versus the standard pyrethroid-only LLINs was 0.48 (CI 0.11, 2.09). There was high heterogeneity (I^2 89%), thus although this indicates over 50% decline in feeding with PBO LLIN as compared to standard LLINs, the decline was not significant.

With resistant *An. gambiae,* the comparison of relative risk between the PBO LLINs versus the standard pyrethroid-only LLINs showed that the feeding rate with PBO LLINs was reduced by 61% of that of standard pyrethroid only LLINs (0.39, CI 0.18, 0.82), and as the

95% confidence interval does not cross the line of no effect, this decrease was significant (Fig. 5b). *An. gambiae*

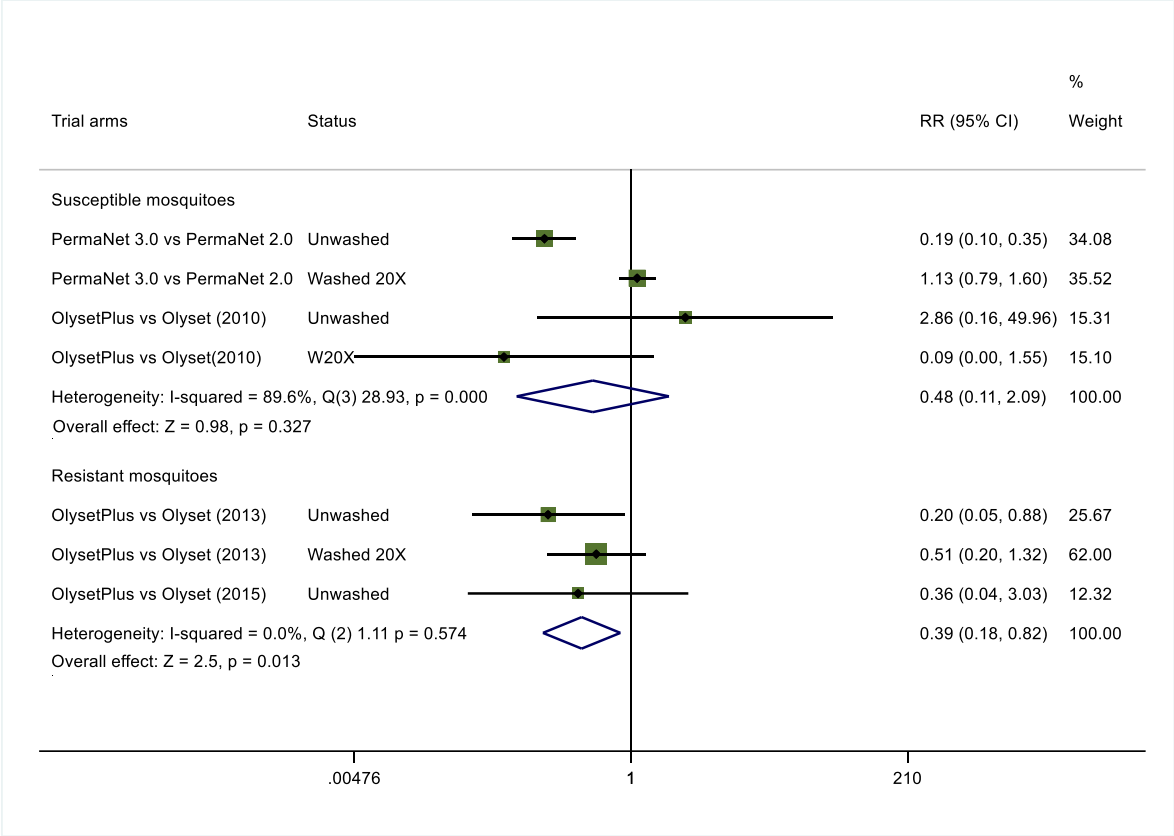

Figure 5b. Blood feeding meta-analysis forest plot: *An. gambiae* s.l.

Mosquito entry and exiting from experimental huts.

Among the trials compared, the highest *Anopheles gambiae s.l.* collection was in 2013 trial; in this trial the average number of *Anopheles gambiae* per hut per night ranged between 3-4 mosquitoes. The lowest collection of *Anopheles gambiae* was recorded in 2015 trial with the average number of *Anopheles gambiae* per hut per night ranged between 0.2-0.4 only. The numbers of *Anopheles gambiae* that was collected during the trial in 2016 were too low for any meaningful statistical analysis, hence only *Anopheles funestus* were included. Highest number of *Anopheles funestus* was recorded in the trial in 2005 with average number of *Anopheles funestus* collected per hut per night ranging

between 4 and 5 mosquitoes. There were no *Anopheles funestus* during the trials in 2010 and 2013. The outcome of the negative binomial regression analysis (performed with the number caught as dependent variable and the six experiment arms as independent variable) revealed that in all trials no significant reduction in number of mosquitoes entering the huts with standard LNs relative to the huts with control untreated net (Table 5). Significant ($p<0.05$) reduction in entry of both species (*An. gambiae and An. funestus*) in the huts with PBO LN relative to untreated control was observed in all trials except with the trial in 2013 (Table 5).

Table 5: Total number caught during the trial, average number per night (in parenthesis) and % deterrence (in square brackets) of susceptible and resistant *Anopheles gambiae* and *Anopheles funestus* in huts trials of Permethrin treated standard and PBO LNs.

	Year of the trial	Untreated net	Standard LN	PBO LN
Anopheles gambiae				
Before resistance				
	2009	723^{a} (13)	574^{ab} (11) [21]	425^{b} (8) [41]
	2010	68^{a} (1.9)	13^{b} (0.4) [35.3]	44^{c} (1.2) [80.9]
After resistance				
	2013	221^{a} (3.4)	245^{a} (3.8) [0]	221^{a} (3.4) [0]
	2015	58^{a} (*)	36^{ab} (0.3) [37.9]	25^{b} (0.2) [56.9]
Anopheles funestus				
Before resistance	2005	315 (2.9)	222 (2.1) [29.5]	*
After resistance	2015	79^{a} (1.4)	64^{ab} (1.1) [30.4]	55^{b} (1) [39.1]
	2017	87^{a} (1)	116^{a} (1.2) [0]	110^{a} (1.2) [0]
	2017	87^{a} (1)	116^{a} (1.2) [0]	101^{a} (1.1) [0]

***Treatment not tested**

Exophily

In all trials (before and after resistance), treatments (Standard and PBO LNs) induced significantly higher exophily ($p<0.05$) than the untreated control net (Table 6). Because

exophily was already high in untreated net arms (mostly greater than 70% mosquitoes exiting), insecticide induced exophily was not so evident before or after selection of resistance (table 6, Figure 6a).

Table 6. Percentage exophily (95% CI) of susceptible and resistant *Anopheles gambiae* and *Anopheles funestus* in hut trials of Permethrin treated standard and PBO LNs

	Year of the trial	Untreated net	Standard LN	PBO LN
Anopheles gambiae				
Before resistance				
	2009	86[a] (83-88)	86[a] (82-88)	79[b] (75-82)
	2010	47[a] (36-59)	31[b] (12-59)	86[c] (73-94)
After resistance				
	2013	69[a] (63-75)	96[b] (94-99)	95[b] (92-98)
	2015	81[a] (71-91)	100[b] (100-100)	96[c] (88-100)
Anopheles funestus				
Before resistance	2005	91[a] (88-95)	93[b] (89-96)	*
After resistance	2015	86[a] (79-93)	100[b] (100-100)	98.2[b] (95-100)
	2017	72[a] (63-82)	91[b] (86-97)	94[b] (89-98)
	2017	72[a] (63-82)	91[b] (86-97)	85[ab] (78-92)

***Treatment not tested**

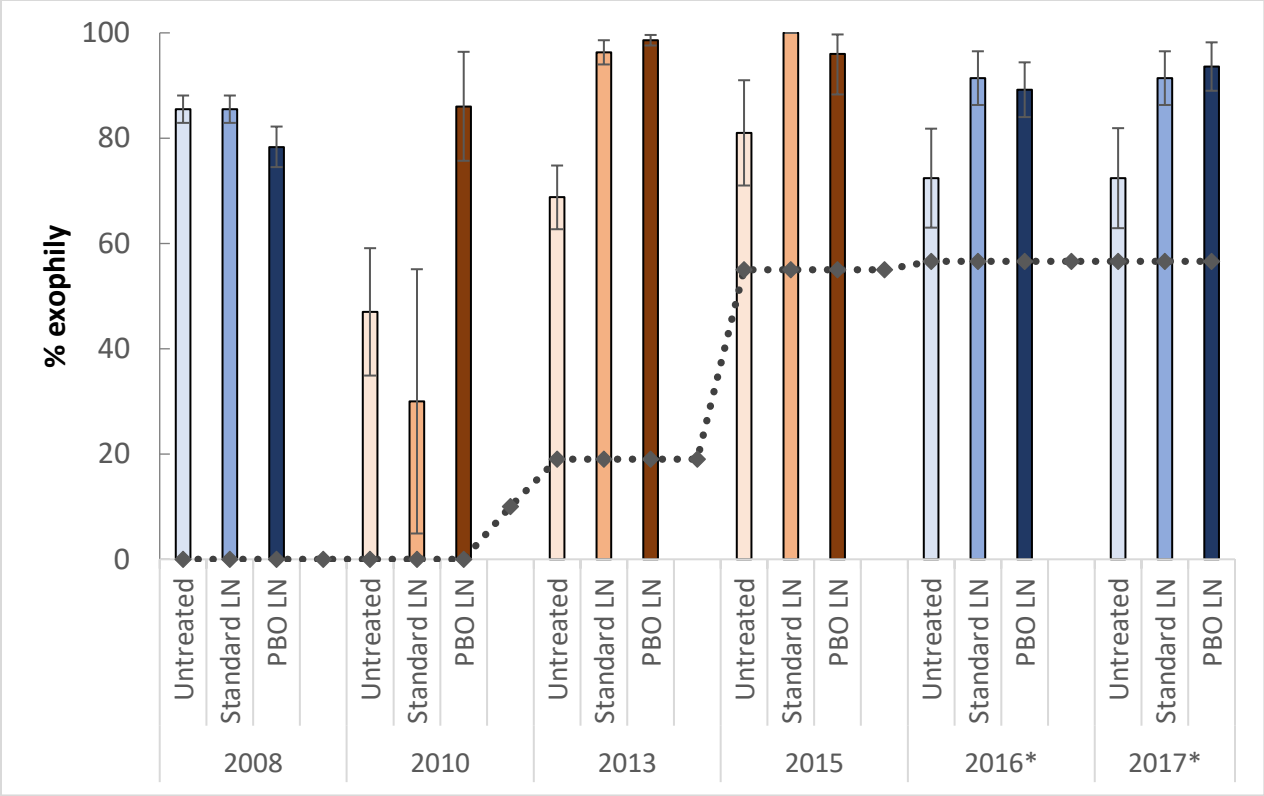

Figure 6a: Bar chart showing experimental huts <u>% exophily</u> with Untreated net, Standard and PBO LNs with the trials before and after development of pyrethroid resistance. Note: (i) Line graph showing trend of insecticide resistance over the years. (ii) Bars with blue coloration are the trials with deltamethrin Standard LLINs and PBO LLINs while bars with red coloration are the trials with permethrin Standard LLINs and PBO LLINs (iii) In X axis year with star (*) means the trial was with *An. funestus* s.l. (remainder are *An. gambiae*)

Meta-analysis of exophily

In the meta-analysis the pooled relative risk for exiting rates of susceptible *An. gambiae* mosquitoes showed a decline of 5% when using PBO LLINs compared to the that recorded by standard pyrethroid LLIN. However, the difference was not significant (z = 1.26, p = 0.208) (Fig. 6b). No difference was recorded in relative risk for exiting rates between PBO LLINs and standard pyrethroid only LLINs against resistant *Anopheles gambiae* mosquitoes (z = 0.25, p = 0.779) (Fig. 6b).

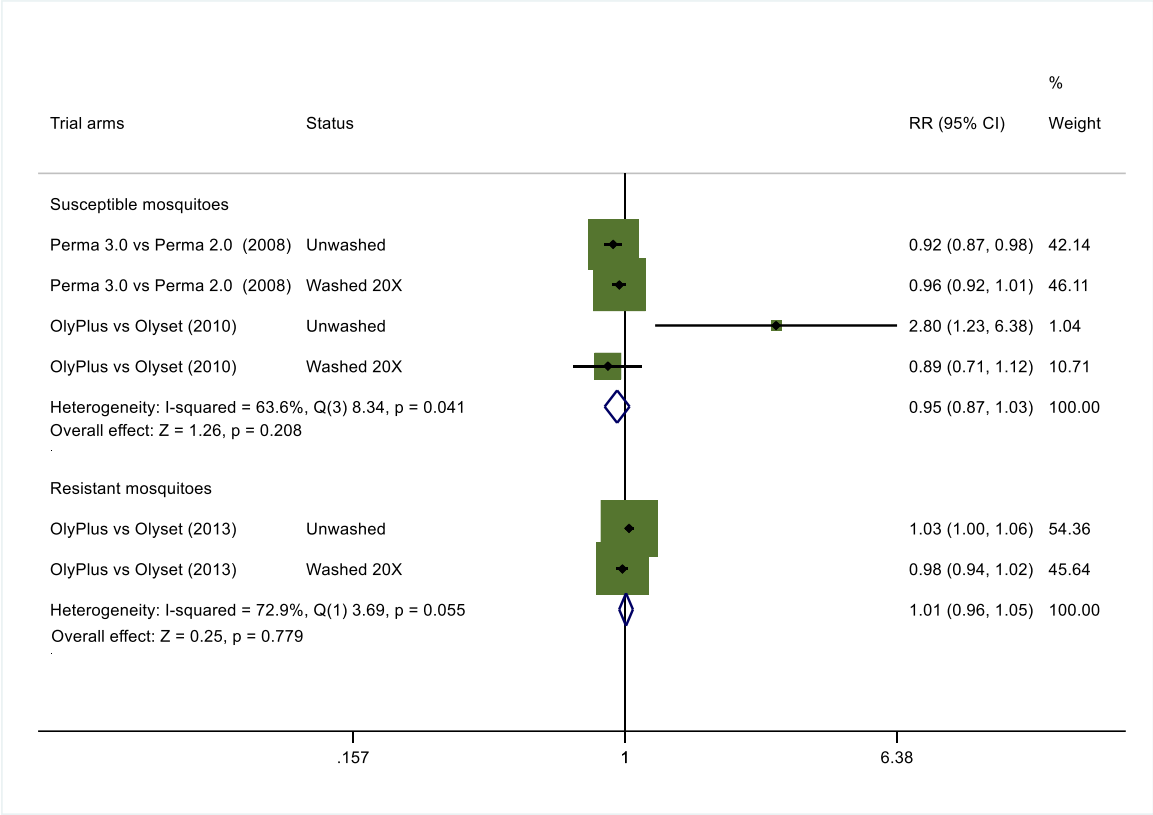

Figure 6b. Exophily meta-analysis forest plot: *An. gambiae* s.l.

Against susceptible *An. funestus* mosquitoes, the pooled relative risk of exiting showed a decrease of 11% when using PBO LLINs compared to standard pyrethroid LLIN. However, the difference was not significant (z = 1.33, p = 0.184) (Fig. 6c). The relative risk of exiting of resistant *Anopheles funestus* mosquitoes when using PBO LLINs compared to that recorded with standard pyrethroid LLIN was not significant (z = 0.26, p = 0.794) (Fig. 6c).

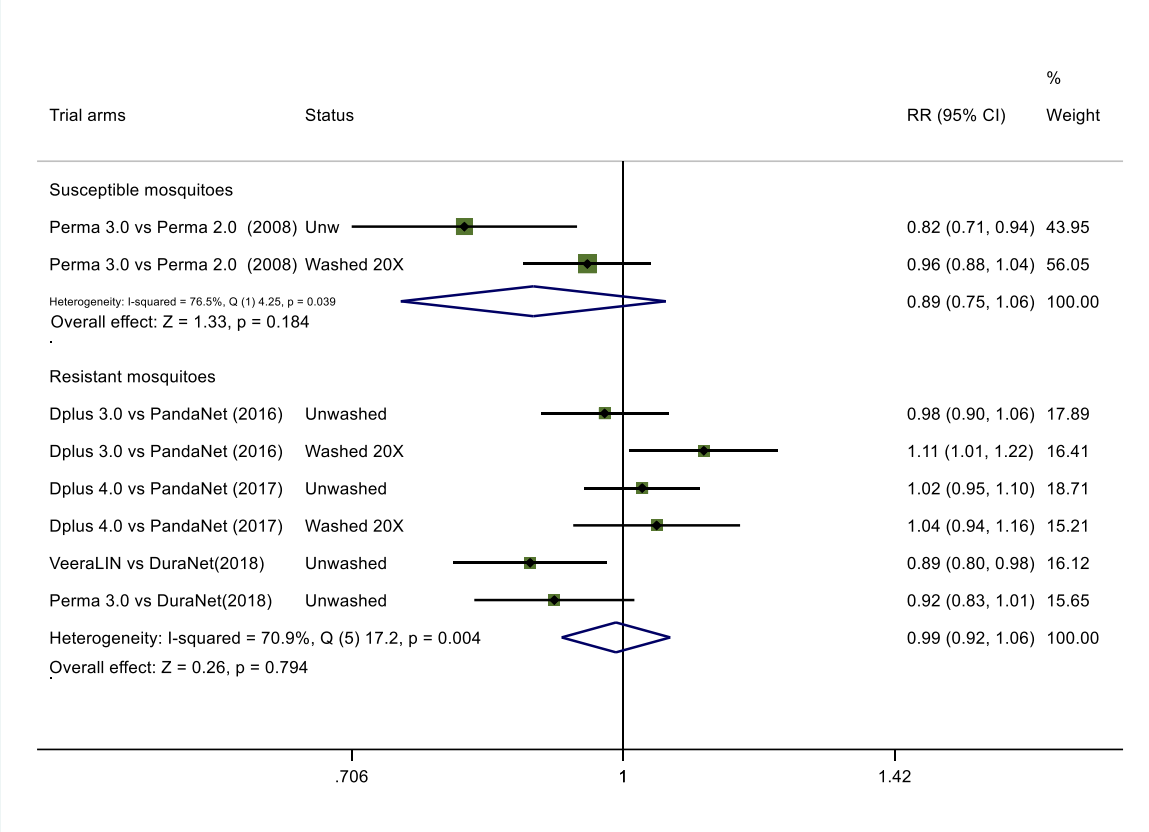

Figure 6c. Exophily meta-analysis forest plot: *An. funestus* s.l.

Chemical analysis of Olyset and OlysetPlus LNs used in 2010 trial.

Permethrin content in samples from 3 unwashed Olyset complies with the target dose of 20g/kg (± 3 g/kg). The permethrin content was 16.53 g/kg after 20 washes, corresponding to permethrin retention of 83%.

The permethrin content in 3 samples of unwashed Olyset Plus complied with the target dose of 20g/kg (± 25% g/kg). The permethrin content was 13.94 g/kg after 20 washes, corresponding to overall permethrin retention of 76% which was only slightly lower than that of Olyset net. The PBO content Olyset Plus complied with the target dose of 10g/kg (± 25% g/kg). The piperonyl butoxide content was 3.17 g/kg after 20 washes, corresponding to overall PBO retention of 38%.

Between washing and post EHT, the permethrin and PBO content in the tested Olyset and Olyset Plus did not decrease significantly.

Supporting bioassay tests on OlysetPlus LN, Olyset Net LN and CTNs used in the trials.

A series of contact bioassays was undertaken to help support or explain the results of the experimental hut trials.

Mortality-inducing exposure time in bioassay

Both Olyset net and Olyset Plus killed 100% of pyrethroid susceptible *Anopheles gambiae* Kisumu strain at all time points including the shortest exposure time (30 sec) (Figure 7). At the longest exposure time of 3 minutes on Olyset Plus, this was sufficient to kill nearly all pyrethroid resistant field *Anopheles gambiae* s.l. (97%). Only 40% of the F1 wild *Anopheles gambiae* s.l. resistant strain were killed by Olyset Plus at 30 second exposure time (Figure 7). The mortality of pyrethroid resistant F1 wild *Anopheles gambiae* s.l. strain induced by Olyset net never exceeded 12% even with the longest exposure time (3 minutes).

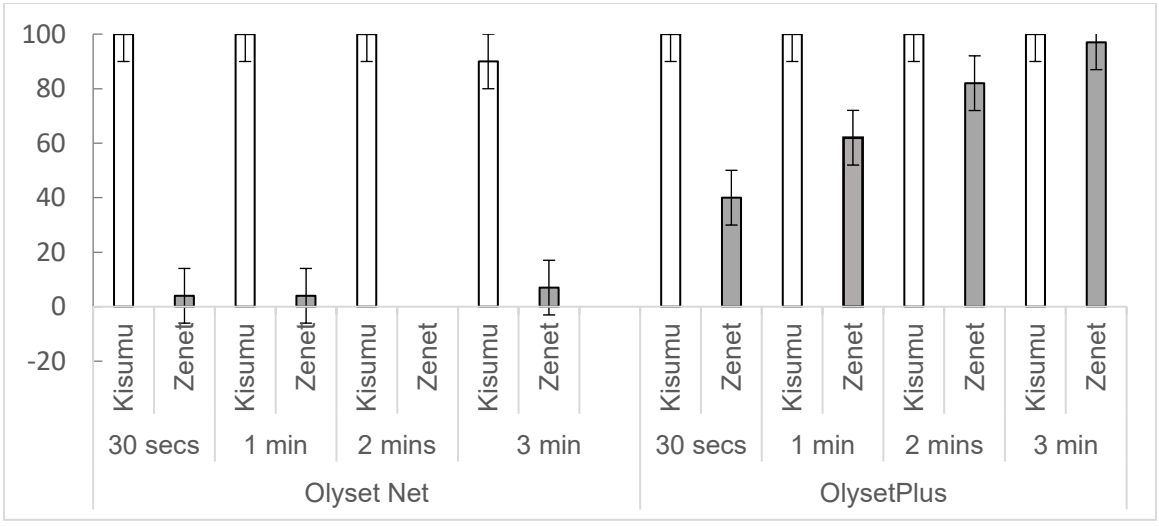

Figure 7. Cone bioassay mortality results of Unwashed Olyset Plus and Olyset against pyrethroid susceptible (*Anopheles gambiae* KISUMU) and resistant (*An. gambiae* Zeneti) mosquitoes at different (30 sec, 1min, 2mins and 3mins) exposure times

Irritability Tests

If it takes only 3 min exposure to kill 100% of field mosquitoes in cone bioassay as suggested in Fig 7, we hypothesized that to explain the hut trial results - where a much a much smaller proportion was killed by PBO-permethrin - contact time would be much shorter. A series of WHO irritability tests were therefore conducted on samples the Zeneti strain of *An. gambiae* on PBO-permethrin Olyset Plus LLIN and permethrin-only Olyset LLIN in which the time to first take-off was recorded. Mosquito irritability or excito-repellency was detected in response to Olyset net and OlysetPlus, as evidenced by significant difference in numbers of mosquitoes taking flight, over the six minutes, in response to the Olyset and OlysetPlus compared to the untreated control (P=0.002 and P=0.001 respectively). The mean (median) time to mosquito batch taking their first flight was untreated net 110 (50) seconds, Olyset Net 64 (27) seconds and Olyset Plus 22 (15) seconds. This shows repellence recorded with PBO-permethrin LLIN was 3 times faster as compared to standard permethrin LNs at the mean, and 2 times faster at the median. Between the untreated net LLIN compared to the PBO LN the difference was 7.8-fold faster at the median and 5.0-fold faster at the mean.

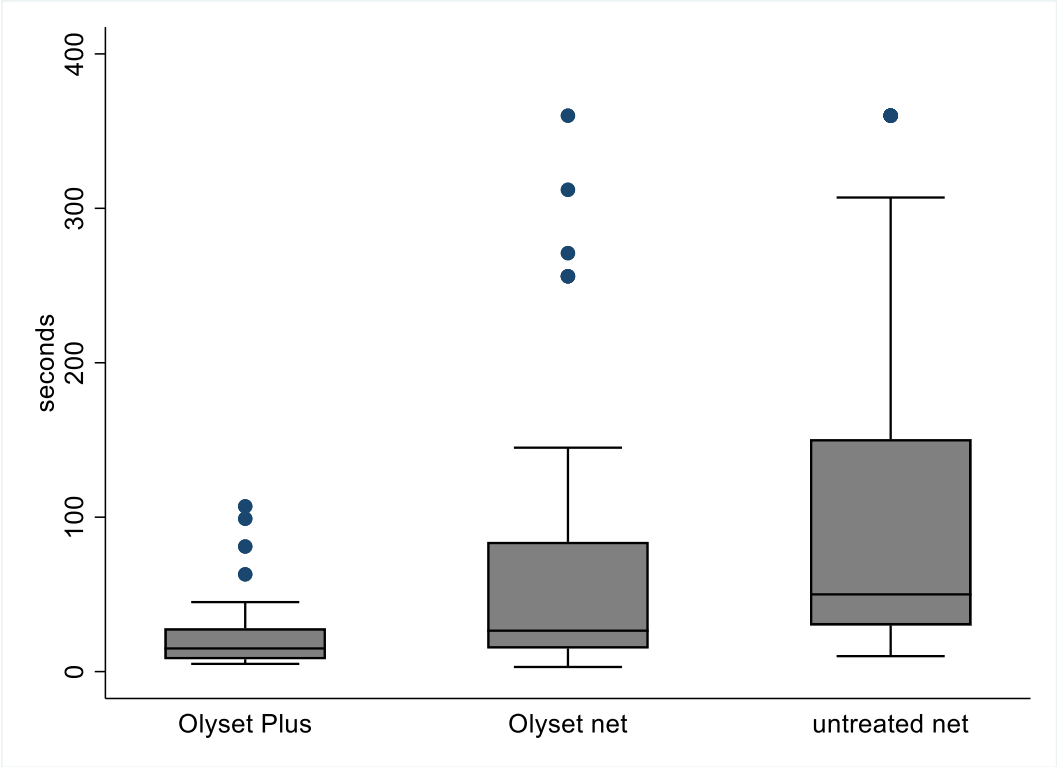

Figure 8. Time to first take-off of the F1 wild Zeneti *Anopheles gambiae s.l.* mosquitoes in response to Olyset and Olyset Plus nettings exposure in comparison to untreated netting

Discussion

This paper presents a longitudinal study, conducted over a decade, which sought to address how the evolution of insecticide resistance in *Anopheles funestus* and *An. gambiae* changes the efficacy of standard pyrethroid mosquito nets in experimental hut trials and how nets containing the synergist PBO might restore efficacy against resistant vectors.

Pre-resistance, high mortality of free-flying field susceptible mosquitoes was induced by the standard LLINs, ranging up to 100% mortality in EHT. Post-resistance, percentage mortality induced by standard LLINs against free-flying resistant *funestus* and *gambiae* was notably lower, with only 10-20% being killed. This decrease in mortality provided further evidence that pyrethroid resistance can seriously reduce the efficacy of pyrethroid-treated nets against malaria vectors. Apart from Tanzania, reduced levels of

vector mortality in countries with pyrethroid resistant vectors which use deltamethrin and permethrin LLINs as a public health intervention, have been recorded in Cote d'Ivoire, Southern Benin and Burkina Faso in West Africa, and Kenya, Malawi, and Uganda in E Africa [4, 44].

With PBO nets, when mosquito populations were still susceptible in Tanzania, mortality was unchanged compared to standard nets (RR = 1.00 for *An. gambiae* and 1.04 for A. *funestus*). But against resistant populations the meta-analysis showed a 1.68-fold and 1.83-fold significant increase in mortality risk compared to standard nets against *gambiae* and *funestus*. Compared to the major effect resistance had on vector survival in resistant populations, this extra effect of PBO LLIN on mortality seems limited: there was only a minor increase in mortality with PBO nets compared to the levels of mortality observed pre-resistance. Hence, synergy only partially restored the control pertaining before the evolution of resistance.

Other effects of PBO nets on free-flying resistant vectors were observed in the meta-analyses. Blood-feeding was reduced by 61% compared to standard LLIN (Relative risk 0.39, 95%CI 0.18, 0.82). This personal protection was especially evident with permethrin-PBO LLIN compared to standard permethrin LLIN. Reduced blood feeding was also observed with deltamethrin-PBO LLIN compared to deltamethrin LLIN and with alpha-cypermethrin-PBO LLIN compared to alpha-cypermethrin LLIN. Pre resistance, the effect of PBO compared to standard LLIN on blood feeding was not significant.

The effect of PBO LLIN on exophily was not evident either before or after development of resistance. This was probably because *An. gambiae* and *An. funestus* were both naturally exophilic and pyrethroid and PBO-pyrethroid both induced exophily. There was a trend of reduced entry with pyrethroid and PBO-pyrethroid LLINs but the difference was not significant.

In laboratory bioassays, where mosquitoes were less able to avoid contact with pyrethroid or PBO in WHO cone and tunnel tests compared to experimental huts, PBO-pyrethroid netting recorded higher mortality against resistant *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes compared to standard nets which had limited effect. In WHO cone bioassays, for example, where Olyset (standard pyrethroid net) and Olyset Plus (PBO LLIN) were exposed against various resistant strains of *An. gambiae s.s, An, arabiensis, An. gambiae s.l.* and *Culex quinquefaciatus* all of which expressed different levels of resistance (from low to high) and had different mechanisms of resistance (from metabolic to site-insensitivity or both), each strain responded as anticipated with mortality ranging between 0% to 65% with Olyset and 81% to 100% with Olyset Plus; this was in accordance to resistance expectation and synergistic interaction with PBO. It was less so in experimental huts.

The failure of the PBO nets to restore high efficacy and full synergy in experimental huts, in comparison in cone and tunnel tests might be due to higher irritability in resistant mosquitoes resulting in shorter contact time as compared to standard pyrethroid LLINs giving freedom of mosquitoes to fly freely out of huts. Laboratory irritability testing confirmed the exito-repellency of the two types of pyrethroid LLIN; time to first take-off was 5 times shorter (median) with the PBO LLIN (12.5 seconds) over the standard pyrethroid LLIN (63 seconds). In the context of holed-nets, this high level of excito-repellency on the surface of pyrethroid-PBO nets may prove a greater obstacle to penetration and blood-feeding and may represent a more repellent barrier to the host-seeking resistant mosquito than a standard pyrethroid LLIN does.

It is not clear whether the effects and outcomes observed in the present series of entomological studies would constitute greater epidemiological protection to a human population living in a malaria endemic area. In the later years of the longitudinal study, a factorial design trial was conducted by another LSHTM group in which 48 clusters were randomised to PBO-pyrethroid LLIN (Olyset Plus) and standard pyrethroid LLIN (Olyset

net) with or without IRS [46]. Cross sectional surveys were conducted each year to measure malaria infection prevalence in children aged under 14 years. At the end of the first year, PBO pyrethroid LLIN showed a 44% protective efficacy compared to standard LLIN, and at the end of the second year PBO pyrethroid LLIN showed a 33% protective efficacy compared to standard LLIN. This cRCT trial led to PBO LLIN taking half the global health market for LLIN [45]. The trials success led to world-wide change in the LLIN market towards PBO LLIN after many years of languishing on the side-lines for want of clear evidence of improved efficacy. This complex series of links takes us from conventional metabolic synergy to enhanced irritability and contact repellency, to malaria prevention and public health success. Whether or not the factors identified in our entomological study of PBO-permethrin versus permethrin LLIN are the sole contributors to the public health impact of PBO-pyrethroid LLIN may seem difficult to accept at first. But no other explanation seems likely. The evidence on global malaria burden is still being gathered as PBO LLINs are scaled up worldwide.

Conclusion

The higher mortality of pyrethroid resistant *An. gambiae* and *An. funestus* recorded by permethrin and deltamethrin PBO LLINs compared to standard permethrin and deltamethrin LLINs can be attributed to: 1. The effect of piperonyl butoxide on synergising of mixed function oxidase enzymes associated with resistance, and 2. The high irritability of PBO-pyrethroid which appears to increase contact-repellence and reduce host-seeking and blood feeding success compared to standard pyrethroid LLIN. Conventional notions of PBO synergy on disruption of metabolism seem unable to explain PBO contribution of the behavioral factors to entomological efficacy. Other unknown factors may be contributing to the greater effectiveness of PBO-pyrethroid LLIN on epidemiological outcomes.

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Chapter 11: Efficacy of Interceptor® G2, a long-lasting insecticide mixture net treated with chlorfenapyr and alpha-cypermethrin against *Anopheles funestus*: experimental hut trials in north-eastern Tanzania.

Prologue:

Insecticide resistance in malaria vectors is well established in almost all malaria endemic countries including Tanzania.

Chapters 9 and 10 show and discuss the advantage of using LLIN that contains a mixture of pyrethroid insecticides and piperonyl-butoxide synergist against resistant mosquitoes over standard pyrethroid only LLINs. Although piperonyl butoxide can restore susceptibility in pyrethroid-resistant mosquitoes, wherein the primary resistance mechanism is metabolic detoxification by oxidase enzymes, it has lesser impact on other mechanisms of pyrethroid resistance and thus its potential may be limited. The more promising way to manage insecticide resistance is to apply insecticide mixtures of pyrethroid plus insecticide with an alternative mode of action.

The best alternative strategy is to present two unrelated insecticides simultaneously. The WHO Global Plan for Insecticide Resistance Management recommends the use of two or more compounds of different insecticide classes to make a single product or formulation as an approach to combating resistance. The challenge is developing products that are not only effective against pyrethroid-resistant mosquitoes but are also safe to humans and are amenable to incorporation into the net fibres.

Interceptor G2 is a combination net that contains a mixture of pyrethroid insecticide (alpha-cypermethrin) together with pyrrole class of insecticide (chlorfenapyr).

This chapter discusses the results of two experimental huts trials of Interceptor G2 conducted in Northeastern Tanzania. With some novelty, they are presented as a meta-analysis of two EHT trials with the purpose of determining overall gain in entomological efficacy against insecticide resistant malaria vectors associated with the use of Interceptor G2 compared to standard pyrethroid only LLINs. Meta-analysis as demonstrated here is novel to WHOPQ and could be extended to other trials with IG2 done elsewhere in other countries. It could also be applied to any series of WHOPQ trials where merging of trials or meta-analysis of trials is required.

Chapter 11: Efficacy of Interceptor® G2, a long-lasting insecticide mixture net treated with chlorfenapyr and alpha-cypermethrin against *Anopheles funestus*: experimental hut trials in north-eastern Tanzania.

The material presented in this chapter has been published as:

Patrick K. Tungu, Elisante Michael, Wema Sudi, William W. Kisinza and Mark Rowland: **Efficacy of Interceptor® G2, a long-lasting insecticide mixture net treated with chlorfenapyr and alpha-cypermethrin against *Anopheles funestus*: experimental hut trials in north-eastern Tanzania.** *Malar J (2021) 20:180.* https://doi.org/10.1186/s12936-021-03716-z

Cover sheet for each 'research paper' included in research thesis.

1. For a 'research paper' already published

1.1. Where was the work published? **Malaria Journal**

1.2. When was the work published? **April 2021**

1.2.1. If the work was published prior to registration for your research.

degree, give a brief rationale for its inclusion **N/A**

1.3. Was the work subject to academic peer review? **YES**

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Abstract

Background

The effectiveness of long-lasting insecticidal nets (LLIN), the primary method for preventing malaria in Africa, is compromised by evolution and spread of pyrethroid resistance. Further gains require new insecticides with novel modes of action. Chlorfenapyr is a pyrrole insecticide that disrupts mitochondrial function and confers no cross-resistance to neurotoxic insecticides. Interceptor® G2 LN (IG2) is an insecticide-mixture LLIN, which combines wash-resistant formulations of chlorfenapyr and the pyrethroid alpha-cypermethrin. The objective was to determine IG2 efficacy under controlled household-like conditions for personal protection and control of wild, pyrethroid-resistant *Anopheles funestus* mosquitoes.

Methods:

Experimental hut trials tested IG2 efficacy against two positive controls—a chlorfenapyr-treated net and a standard alpha-cypermethrin LLIN, Interceptor LN (IG1)—consistent with World Health Organization (WHO) evaluation guidelines. Mosquito mortality, blood-feeding inhibition, personal protection, repellency and insecticide-induced exiting were recorded after zero and 20 washing cycles. The trial was repeated and analysed using multivariate and meta-analysis.

Results

In the two trials held in NE Tanzania, *An. funestus* mortality was 2.27 (risk ratio 95% CI 1.13–4.56) times greater with unwashed Interceptor G2 than with unwashed Interceptor LN ($p = 0.012$). There was no significant loss in mortality with IG2 between 0 and 20 washes (1.04, 95% CI 0.83–1.30, $p = 0.73$). Comparison with chlorfenapyr treated net indicated that most mortality was induced by the chlorfenapyr component of IG2 (0.96, CI 0.74–1.23), while comparison with Interceptor LN indicated blood-feeding was inhibited by the pyrethroid component of IG2 (IG2: 0.70, CI 0.44–1.11 vs IG1: 0.61, CI 0.39–0.97). Both insecticide components contributed to exiting from the huts, but the contributions were heterogeneous between trials (heterogeneity $Q = 36$, $P = 0.02$). WHO susceptibility tests with pyrethroid papers recorded 44% survival in *An. funestus*.

Conclusions:

The high mortality recorded by IG2 against pyrethroid-resistant *An. funestus* provides first field evidence of high efficacy against this primary, anthropophilic, malaria vector.

Background

Long-lasting insecticidal nets (LLINs) are essential for malaria transmission control in sub-Saharan Africa [1]. The halving of the malaria burden over the last 15 years is largely attributed to increasing coverage of pyrethroid LLIN, which culminated in universal free distribution across all age groups in Africa [2]. Concurrent with this public health achievement and cultural shift in sleeping behaviour has been the evolution and spread of pyrethroid resistance across Africa in the two primary vector mosquito species complexes.

Pyrethroids, owing to their efficacy, safety and low-cost were once the only insecticides approved for use on LLINs [3]. Since 2015, further reduction in the annual malaria burden has stalled, and pyrethroids are no longer deemed sufficient on their own [1]. The evolution of severe resistance was anticipated, and when the first signs of field failure

were reported in 2007 [4], steps had already been taken to identify alternatives [5, 6]. The first active ingredient (AI) to be developed by pesticide industry to enhance pyrethroid efficacy on nets was the synergist PBO [7, 8]. This supplemental compound, long used in domestic fly sprays to enhance pyrethroid toxicity, can neutralize metabolic mechanisms responsible for resistance to pyrethroids. Several brands of pyrethroid-PBO LLIN are currently being scaled up in countries where monooxygenase resistance mechanisms are contributing to impairment or loss of malaria control [9,10,11]. Pyrethroid-PBO LLIN is no panacea; it cannot neutralize all pyrethroid resistance mechanisms that have evolved, and this is no time for complacency. What is needed is an array of alternative insecticides that can complement the pyrethroids on Dual-AI LLIN. This is no trivial task as alternative insecticides for nets need to be safe to humans, toxic to mosquitoes, wash-tolerant on nets and exhibit no cross resistance to pyrethroids. One such insecticide, which is showing promise, is the pyrrole chlorfenapyr [12]. After 15 years of development and evaluation in laboratory and small-scale experimental hut trials against anopheline mosquitoes [13,14,15,16,17,18], the first cluster randomized trials (CRT) of a LLIN that combines chlorfenapyr with pyrethroid in a wash-tolerant formulation are currently underway and are due to report in 2021 in Tanzania, East Africa and in 2022 in Benin, West Africa. Epidemiological evidence of effectiveness against malaria in CRT is a prerequisite before the World Health Organization (WHO) will grant recommendation of any new class of LLIN for malaria control. Both CRTs are targeting the Anopheles gambiae complex: *An. gambiae* sensu stricto (s.s.) in NW Tanzania and *Anopheles coluzzii* in Benin. However, a third primary vector has re-emerged, *Anopheles funestus* [19], and this species is becoming the predominant vector along the eastern seaboard of Tanzania after a hiatus of several years when LLIN were first taken to scale in mass distribution campaigns and control of the then pyrethroid-susceptible *An. funestus* and An. gambiae was achieved [20, 21]. The return of both *An. funestus* and *An. gambiae s.s.* is in pyrethroid-resistant form.

The sibling species, vector competence and insecticide resistance status of the *An. funestus* complex has only recently been characterized in Tanzania [22]. Molecular identification of collections from 2005–2014 in NE Tanzania revealed *An. funestus s.s.* (97%) as the predominant species and *Anopheles rivulorum* (2%) and *Anopheles leesoni* (1%) as minor sibling species. *Plasmodium falciparum* CSP positivity was 8.3% for recently collected *An. funestus s.s.* [22]. *Wucheria bancrofti* infection rates decreased from 14.8% in the 2005–2007 archived specimens to only 0.5% in newly collected specimens, with 93% of filarial infections confined to *An. funestus s.s.* The high P. falciparum and decreasing W. bancrofti infections in *An. funestus s.s.* reflects infection levels of these parasites in the human population and confirms its vectorial importance [22]. Vector surveys further south in coastal Bagamoyo and Kilombero valley produced similar trends and species ratios as NE Tanzania. In Bagamoyo, there was 84% *An. funestus s.s.*, 13.6% *An. leesoni*, 1.5% *An. rivulorum*, and 0.6% *Anopheles parensis* [23]. In Kilombero valley, 97% were *An. funestus s.s.*, 2% *An. rivorulum* and 1% *An. leesoni* [24].

Anopheles funestus s.s. and *An. gambiae* s.s. in addition to being pyrethroid resistant are naturally highly anthropophagic and endophilic. These are the primary vector species to target with the new generation insecticides like chlorfenapyr. Unlike pyrethroids and other conventional public health insecticides which are neurotoxic, chlorfenapyr disrupts the oxidative pathways that enable proton transfer, conversion of ADP to ATP and cellular respiration in mitochondria [15, 25]. With its non-neurological mode of action, chlorfenapyr shows no cross resistance to insecticide classes normally used for vector control and hence is a leading candidate for targeting vector species resistant to standard neurotoxic insecticides [13, 17]. When evaluated on hand-treated mosquito nets against wild mosquitoes in experimental huts, chlorfenapyr showed improved control of mosquitoes resistant to WHO-approved insecticides [14, 26].

Interceptor G2 LN (IG2) is a Dual-AI LLIN developed by the manufacturer BASF SE which is designed to provide protection against pyrethroid-resistant mosquitoes by means of a

mixture of chlorfenapyr and alpha-cypermethrin in a long-lasting wash-resistant formulation. The first experimental hut trials of IG2, undertaken in Benin, Burkina Faso and Cote d'Ivoire in West Africa, targeted members of the *An. gambiae* complex: *An coluzzii*, *An. gambiae* s.s. and *Anopheles arabiensis* [15, 27, 28]. The present paper reports on sequential hut trials in NE Tanzania on the East African seaboard designed to assess the efficacy of Interceptor G2 LN against the primary East African vectors *An. funestus* s.s. and *Anopheles gambiae* s.s. IG2 was tested unwashed and after 20 standardized washes as proxy for an ageing net consistent with WHO guidelines for evaluating LLIN. Two other net types served as positive controls: the pyrethroid-only Interceptor LN (IG1) and a net hand-treated with chlorfenapyr SC formulation. While it was anticipated that pyrethroid resistant *An. funestus* s.s. and *An. gambiae* s.s. would both be present, on these two occasions only *An. funestus* s.s. was present in significant densities.

Methods

Study site and experimental huts

Two experimental hut studies were conducted in Muheza district, Tanga region, at the field station in Zeneti (5°13' S, 38°39' E, 193 m altitude), where *An. gambiae* s.s. and *An. funestus* s.s. are the major malaria vectors [20, 22]. Polymerase chain reaction sibling species analysis of 500 *An. funestus* collected from Zeneti between 2016–2017 results showed all were *An. funestus* s.s. In World Health Organization insecticide susceptibility tests using permethrin papers conducted on F1 adult mosquitoes from Zeneti in the year before the hut trials, mortality was 56% among *An. gambiae* s.s. and 62% among *An. funestus*. In intensity bottle bioassay *An. gambiae* s.s. showed 30-fold resistance to permethrin relative to susceptible Kisumu strain [30]. There was no resistance to carbamates or organophosphates.

The WHO Phase II evaluation of Interceptor G2 was conducted in 6 experimental huts of the East African design [31]. The operating principle of the huts is described in WHO LLIN evaluation guidelines [32]. The hut design allows host-seeking mosquitoes unfettered

access though two open eave gaps, 5 cm deep and 3 m wide, between wall and roof on two sides of the hut, attracted by the human host sleeping inside, and captures surviving mosquitoes exiting into window traps fitted on two of the walls or into verandah traps accessed through eave gaps above the walls. Other features include a ceiling lined with hessian sackcloth similar to thatch, a corrugated iron roof, a concrete plinth and water-filled moat to deny entry to scavenging ants. The eaves of the two unscreened verandahs were baffled inwardly to funnel host-seeking mosquitoes into the hut and to deter exiting through the same eave gaps. Two screened and closed veranda traps located on the other two sides of the hut, and two baffled window traps, capture any mosquito that exit the rooms via the two open eaves or windows. With this modification to the traditional verandah hut design there was no need to make any correction for escaping mosquitoes because all escapees are recorded [31].

Experimental hut trial design

Two experimental huts trials were undertaken. The first trial was conducted over 54 collection nights between November and December 2015, the second trial was conducted for 36 nights between May and July 2016. The following six treatment arms were included:

(i) Untreated polyester net,
(ii) Interceptor LN, unwashed
(iii) Interceptor LN, washed 20 times
(iv) Interceptor G2 LN, unwashed
(v) Interceptor G2 LN, washed 20 times
(vi) Polyester net, conventionally treated with chlorfenapyr SC formulation (Phantom 21% SC, BASF) at 200 mg/m2.

Washing of LLINs was done according to WHO Phase II protocols [32]. The interval between washes was 1 day which is the established regeneration time for Interceptor G2 and Interceptor LN [8]. Each net was cut with six holes of 4 cm diameter to simulate wear

and tear. For the washed nets, washing was done in 10 L of soap solution (2 g/l of Savon de Marseille). Nets were agitated for 3 min by stirring with a pole, then allowed to soak for four minutes, and then stirred again for 3 min. The nets were rinsed twice using the same procedure with clean tap water. All nets were 100-denier. Three nets were used per treatment arm.

Treatments were rotated between huts each week (3 nets tested 3 times over 9 days or 2 times over a 6-days) with sleepers rotated between huts and treatments each night using a randomized latin square design to adjust for variation in personal attractiveness to mosquitoes or hut positional effect. Each morning mosquitoes were collected and held for 72 h in cups with sugar solution to record any delayed mortality. All dead and surviving mosquitoes were retained on silica gel for molecular identification [33] and for genotyping of L1014S or L1014F kdr alleles using Taqman PCR [34].

The primary outcomes were Deterrence, Treatment-induced exiting (exophily), mortality, Overall killing effect, Blood-feeding inhibition and %Personal protection. These outcomes are described in more detail in appendix 1, Section 2.6.2.

Chemical analysis

Netting samples were cut from each net before and after washing and after completion of the trial for determination of insecticide content. Determination of alpha-cypermethrin and chlorfenapyr content was performed at BASF (1st trial) and Walloon Agricultural Research Centre (CRA-W) (2nd trial) using a draft CIPAC method jointly developed by CRA-W and BASF based on CIPAC 454/LN/M/3.1. The method involves extraction of alpha-cypermethrin and chlorfenapyr by ultrasonication at ambient temperature for 30 min in heptane in the presence of Di cyclohexyl phthalate as internal standard, by adding citric acid, and determination by gas chromatography with flame ionization detection (GCFID). The insecticide concentration of each sample (g/kg) was converted to mg/m2 before presentation.

Supplementary bioassay tests on nets used in the trials.

Mosquito strains

Anopheles gambiae s.s. Kisumu, a laboratory insecticide susceptible strain, originally from Kenya.

Anopheles gambiae s.s. Zeneti, a pyrethroid resistant strain of *An. gambiae* s.s. from Zeneti village containing the L1014S pyrethroid resistance knockdown allele (kdr east) [29] and showing 30-fold resistance to permethrin relative to susceptible *An. gambiae* Kisumu.

WHO cone bioassays.

These were conducted on standardized washed and unwashed nets to estimate the wash fastness of each net formulation. Five pieces were cut from each net and two replicates of five susceptible or resistant An. gambiae mosquitoes were exposed for 3 min. Mortality was scored at 24 h, 48 h and 72 h post-exposure.

Tunnel tests

These were conducted on standardised washed and unwashed pieces of Interceptor G2 LN netting after 0 and 20 washes. A total of 100 susceptible and resistant mosquitoes were tested in tunnel tests in replicates of 50 mosquitoes per test in accordance with WHO guidelines [32]. Dimensions and procedures for tunnel test are described in appendix 1, section 2.3.1.2

Statistical analysis

Data were entered into an Excel database and transferred to Stata 11 (Stata Corp LP, College Station, TX, USA) for processing and analysis. Cone bioassays and tunnel test data were analysed using logistic regression for grouped data adjusting for clustering within replicate tests.

Proportional outcomes in the experimental hut trial (mortality, blood-feeding, exiting) related to each treatment were assessed using logistic regression for grouped data adjusting for daily collected mosquitoes. In addition to the fixed effect of each treatment, each model included random effects to account for variation between the hut position and sleeper attractiveness. Comparison between numeric outcomes of treatments (personal protection, killing effect, deterrence) was analysed using negative binomial regression with adjustment for variation in the same covariates described above.

Risk ratios of mortality, blood-feeding and exiting rates the two trials were pooled using meta-analysis using a random-effects model STATA® statistical analysis software package version 16 (Stata corporation, Collage Station, Texas 77,845 USA, 2019). Overall heterogeneity across trials was calculated using Cochrane's Q test with a P value of less than 0.05 to indicate statistical heterogeneity and quantified heterogeneity using the I2 statistic [35, 36].

Results

Resistance status

WHO susceptibility tests using permethrin and alpha-cypermethrin treated papers were conducted against F1 progeny of mosquitoes collected from huts containing untreated nets before and during the trial. Mortality recorded using 0.75% permethrin papers was 46.7% for *An. gambiae* and 56.7% for *An. funestus* in the first trial (2015) and 43% and 52.6%, respectively in the second (2016), indicating resistance to pyrethroids in both species. Mortality using 0.05% alpha-cypermethrin papers was 52.7% for An. gambiae during the first trial. Alpha-cypermethrin papers were not available during the 2nd trial, but other alpha-cyano pyrethroids such as 0.05% deltamethrin and 0.05% lambda - cyhalothrin gave a similar 73.8% and 50.6% mortality respectively. Concurrent mortality using the same insecticide test papers against susceptible An. gambiae Kisumu was 100% in each case. Insecticide resistance intensity testing showed Zeneti field *An. gambiae* to

have over 30-fold resistance to pyrethroid (permethrin) compared to susceptible *An. gambiae* Kisumu.

Phase II—experimental hut trials

Mosquito entry into experimental huts

The average number of mosquitoes entering and exiting the hut are shown in Table 1. The geometric mean number of *An. funestus* collected during the first trial ranged from 0.6 to 1.5 per hut per night. During the second trial the geometric mean number of *An. funestus* ranged from 1.3 to 1.8 per hut per night. In both trials significantly fewer *An. funestus* were collected from the huts with chlorfenapyr CTN compared to the huts with the untreated nets. No consistent deterrent effect was observed with IG1 (alpha-cypermethrin alone) or IG2 compared to untreated nets.

Table 1 *Anopheles funestus* mosquitoes collected and exiting into verandah and window traps during the two experimental huts trials.

Trial	Effect	Untreated net	Interceptor LN	Interceptor LN	Interceptor G2	Interceptor G2	Chlorfenapyr CTN
		0 washes	0 washes	20 washes	0 washes	20 washes	0 washes
1	Total number caught (mean/night)	139 (2.6)	94 (1.7)	97 (1.8)	63 (1.2)	133 (2.5)	54 (1.0)
	Geometric mean per night (CI)	1.4[a] (0.9–1.9)	1.1[ab] (0.7–1.5)	1.3[a] (0.9–1.7)	0.9[ab] (0.6–1.2)	1.5[a] (1.0–2.1)	0.6[b] (0.4–0.9)
	% Deterrence	0	32.4	30.2	54.7	4.3	61.2
2	Total number caught (mean/night)	111 (3.1)	80 (2.2)	78 (2.2)	103 (2.9)	86 (2.4)	74 (2.1)
	Geometric mean per night (CI)	1.8[a] (1.1–2.8)	1.5[b] (1.0–2.2)	1.8[ab] (1.2–2.4)	1.6[ab] (0.9–2.4)	1.3[b] (0.8–2.0)	1.3[b] (0.8–2.0)
	% Deterrence	0	27.9	29.7	7.2	22.5	33.3

Trial	Effect	Untreated net	Interceptor LN	Interceptor LN	Interceptor G2	Interceptor G2	Chlorfenapyr CTN
		0 washes	0 washes	20 washes	0 washes	20 washes	0 washes
1	Total in verandah & window traps	59	74	83	51	107	32
	% Exiting (95% C.I.)	42[a] (27–59)	79[c] (67–87)	86[c] (73–93)	81[c] (68–90)	80[c] (69–89)	59[b] (39–77)
2	Total in verandah & window traps	75	53	51	48	46	58
	% Exiting (95% C.I.)	68[ac] (52–80)	66[ac] (51–79)	65[c] (54–75)	47[b] (31–63)	54[bc] (36–70)	78[a] (67–87)

The numbers in the same row sharing the same letter superscript do not differ significantly (p > 0.05)

Mortality and overall killing effect.

The overall percentage mortality by treatment arm is shown in Fig. 1. Because chlorfenapyr shows the property of delayed mortality, which reaches a maximum 72 h after mosquitoes enter the huts with chlorfenapyr treated nets, both 24 h and 72 h mortality are presented in Table 2. Percentage mortality corrected for untreated net control is also shown.

Trial	Effect	Holding period	Untreated net	Interceptor LN	Interceptor LN	Interceptor G2	Interceptor G2	Chlorfenapyr CTN
		Hours	0 washes	0 washes	20 washes	0 washes	20 washes	0 washes
1	Total number dead	24, 72	13, 29	14, 34	14, 33	30, 38	66, 92	23, 35
	% Mortality, overall	24	9^{a1} (4–19)	15^{a1} (8–27)	14^{a1} (8–24)	48^{b1} (36–59)	50^{b1} (41–58)	43^{b1} (30–56)
	% Mortality, control corrected	24	-	6^{a1} (0–19)	6^{a1} (0–17)	42^{b1} (30–54)	44^{b1} (35–54)	37^{b1} (30–52)
	% Mortality overall	72	21^{a2} (11–36)	37^{c2} (25–51)	34^{c2} (22–48)	60^{b1} (48–71)	70^{d2} (61–78)	65^{bd2} (48–78)
	% Mortality, control corrected	72	-	21^{a2} (5–33)	17^{a2} (2–34)	50^{b1} (34–62)	62^{c2} (51–72)	55^{bc2} (35–73)
2	Total number dead	24, 72	12, 14	10, 13	13, 14	31, 55	14, 42	20, 33
	% Mortality, overall	24	11^{a1} (5–17)	13^{a1} (5–20)	17^{ac1} (8–25)	30^{b1} (21–39)	16^{ac1} (9–24)	27^{bc1} (17–37)
	% Mortality, control corrected	24	-	2^{a1} (0–10)	7^{a1} (0–16)	22^{b1} (12–32)	6^{a1} (1–15)	18^{b1} (7–30)
	% Mortality overall	72	13^{a1} (7–19)	16^{a1} (8–24)	18^{a1} (9–27)	53^{b2} (44–63)	49^{b2} (38–59)	45^{b2} (33–55)
	% Mortality, control corrected	72	-	4^{a1} (0–13)	6^{a1} (0–16)	46^{b2} (35–58)	41^{b2} (29–53)	36^{b2} (23–48)
1	% Overall Killing Effect	24	-	5^{a}	5^{ab}	38^{b}	41^{c}	33^{ab}
		72	-	17^{b}	13^{b}	30^{a}	49^{a}	44^{a}
2	% Overall Killing Effect	24	-	0^{a}	1^{a}	17^{a}	2^{a}	7^{a}
		72	-	0^{a}	0^{a}	38^{b}	26^{b}	18^{b}

Table 2 Percentage mortality of *Anopheles funestus* corrected for control mortality 24 h and 72 h after exposure during the two experimental huts trials.

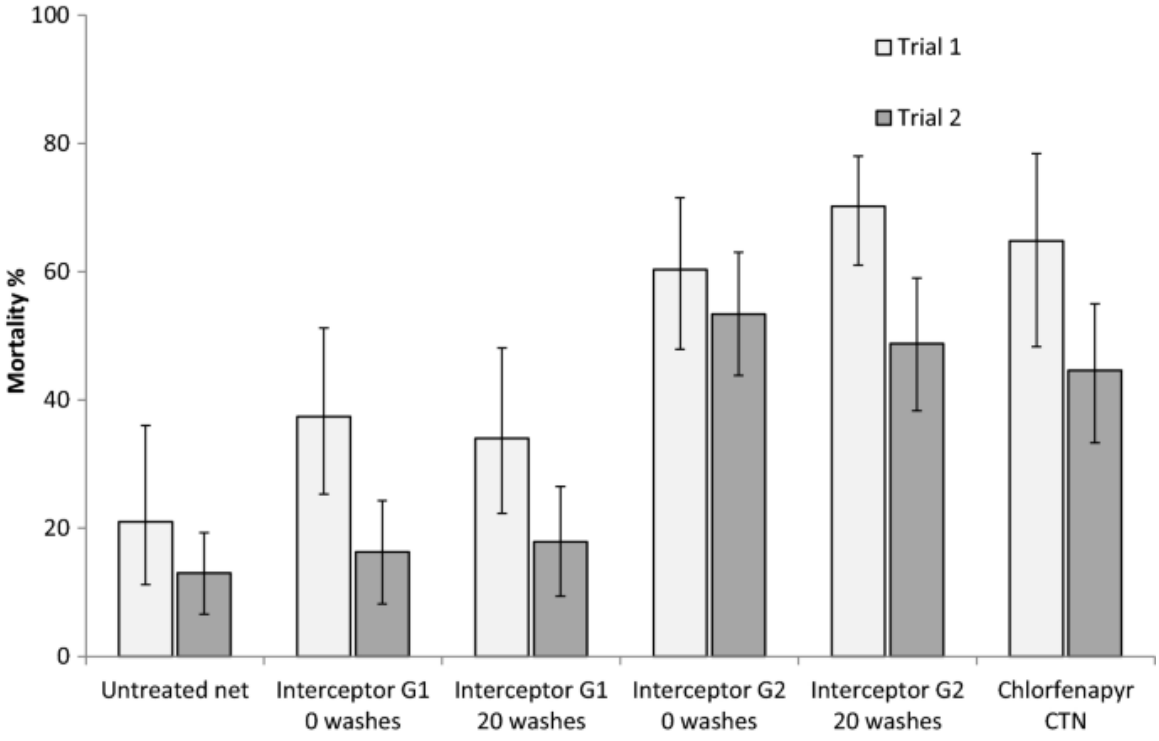


Fig. 1: Experimental hut trials of Interceptor G2 and Interceptor LN in NE Tanzania: mortality of free-flying *Anopheles funestus* after 72 h holding period.

In the first trial, control-corrected mortality of *An. funestus* after 24 h was 5–6% in the huts with the unwashed IG1 and in the huts with the IG1 washed 20 times (Table 2). Mortality in these treatment arms was significantly different from the mortality in the huts with the unwashed IG2 (42%), the IG2 washed 20 times (44%) and the chlorfenapyr CTN (37%). After 72 h, control corrected mortality was significantly higher than after 24 h across most of these treatments (Table 2). Mortality was significantly higher in the huts with the IG2 unwashed and washed 20 times compared with the IG1 unwashed and washed 20 times treatments. In the second trial, the trend was slightly different. Control corrected mortality significantly increased once again between 24 and 72 h with the unwashed IG2 (from 22 to 46%), the IG2 washed 20 times (from 6 to 41%) and the chlorfenapyr CTN (from 18 to 36%) (Table 2). But unlike the first trial, control-corrected mortality showed no significant change between 24 and 72 h with the unwashed IG1 (1.9% to 3.8%) and with the IG1 washed 20 times (6.6% to 5.6%). Therefore, delayed

mortality of An. funestus after 72 h was significantly pronounced only in the huts with the chlorfenapyr CTN, the unwashed Interceptor G2 and the Interceptor G2 washed 20 times.

Natural mortality of An. funestus after 72 h in the huts with the untreated nets in the first trial was significantly lower (21%) than the overall mortality in huts with the IG1 unwashed (37%) or IG1 washed 20 times (34%). In the second trial, natural mortality after 72 h in huts with the untreated nets was lower (13%) than in the first trial (21%), but on this occasion the untreated nets showed no difference in mortality compared to IG1 unwashed (16%) or IG1 washed 20 times (18%) which also stayed low. A further difference between the two trials: in the first, both IG2 and IG1 showed significantly delayed mortality between 24 and 72 h, in the second trial only IG2 showed significantly delayed mortality between 24 and 72 h and not IG1.

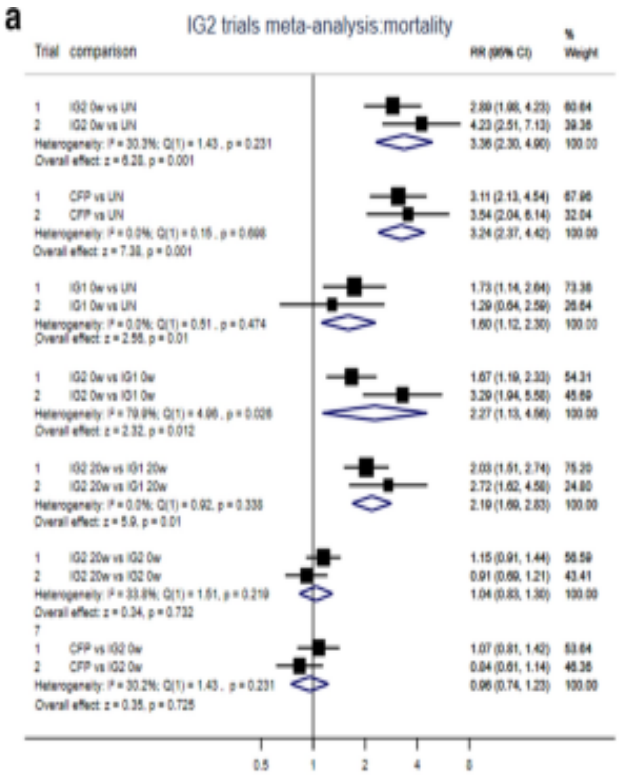
Because the untreated net control showed 21% (trial 1) and 13% (trial 2) mortality after the 72 h holding period, the observed mortality of IG2 presented in Fig. 1 after 72 h observation was considerably higher (range: 49–70%) than the control-corrected mortality (range: 41–62%) presented in Table 2.

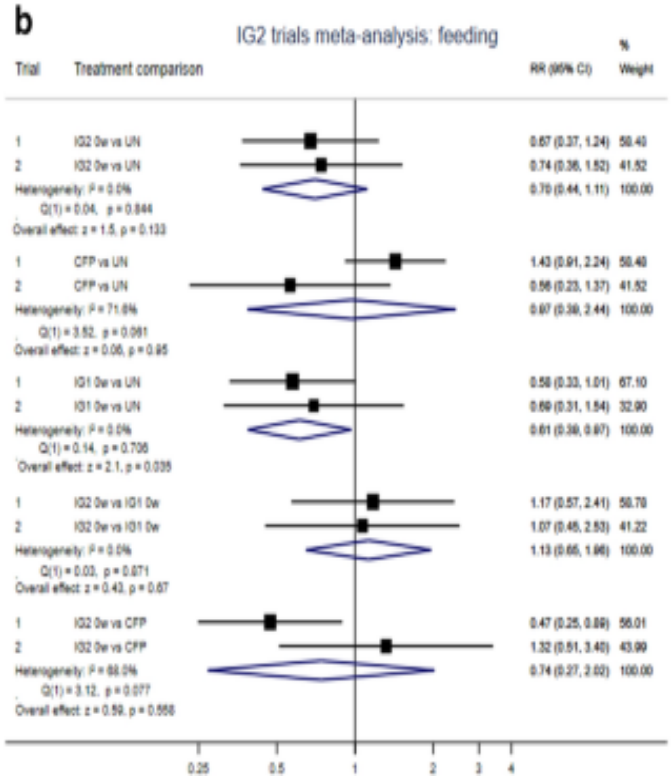
The ‘overall killing effect’ by the IG1 and IG2 interventions were consistent with percentage mortality of the IG1 and IG2 treatments observed in the huts. In the first and second trials, IG1 killed up to 16% and 0% of *An. funestus*, respectively, and IG2 killed up to 49% and 38%, respectively.

Meta-analysis of mortality

In the meta-analyses of mortality between the two trials, the comparison of relative risk between the unwashed IG2 and the untreated net was 3.36 (CI 2.3, 4.9) (P = 0.001). The comparison of mortality relative risk between the chlorfenapyr CTN and untreated net, 3.24 (CI 2.4, 4.2) (P = 0.001) was, therefore, quite similar to that of the unwashed IG2 and untreated net. The comparison of relative risk between the unwashed IG1 and the untreated net was less (1.60, CI 1.1–2.3) (P = 0.01), indicating a smaller effect size of

alpha-cypermethrin on mortality. The effect of the comparison between IG2 and IG1 was 2.27 (1.1, 4.6) (P = 0.012), confirming the greater contribution of chlorfenapyr than of alphacypermethrin to IG2 mortality. This was further confirmed by the comparison of chlorfenapyr CTN to IG2: the risk ratio was a not significant 0.96 (0.7, 1.23) (P = 0.231) implying that chlorfenapyr was making most of the contribution to mortality in IG2 and not alpha-cypermethrin. The similarity of relative risk between unwashed IG2 and IG2 after 20 washes (1.04, CI 0.8–1.3) (P = 0.73) indicated no loss of mortality effect in IG2 between 0 and 20 washes (Fig. 2a).


a
IG2 trials meta-analysis:mortality
Trial comparison
RR (95% CI)
% Weight
IG2 0w vs UN
IG2 0w vs UN
Heterogeneity: I² = 30.3%; Q(1) = 1.43 , p = 0.231
Overall effect: z = 6.28, p = 0.001
CFP vs UN
CFP vs UN
Heterogeneity: I² = 0.0%; Q(1) = 0.15 , p = 0.698
Overall effect: z = 7.38, p = 0.001
IG1 0w vs UN
IG1 0w vs UN
Heterogeneity: I² = 0.0%; Q(1) = 0.51 , p = 0.474
Overall effect: z = 2.58, p = 0.01
IG2 0w vs IG1 0w
IG2 0w vs IG1 0w
Heterogeneity: I² = 70.9%; Q(1) = 4.66 , p = 0.026
Overall effect: z = 2.32, p = 0.012
IG2 20w vs IG1 20w
IG2 20w vs IG1 20w
Heterogeneity: I² = 0.0%; Q(1) = 0.92, p = 0.338
Overall effect: z = 5.9, p = 0.01
IG2 20w vs IG2 0w
IG2 20w vs IG2 0w
Heterogeneity: I² = 33.8%; Q(1) = 1.51, p = 0.219
Overall effect: z = 0.34, p = 0.732
7
CFP vs IG2 0w
CFP vs IG2 0w
Heterogeneity: I² = 30.2%; Q(1) = 1.43 , p = 0.231
Overall effect: z = 0.35, p = 0.725
0.5
1
2
4
8


b
IG2 trials meta-analysis: feeding
Trial
Treatment comparison
RR (95% CI)
% Weight
IG2 0w vs UN
IG2 0w vs UN
Heterogeneity: I² = 0.0%
Q(1) = 0.04, p = 0.844
Overall effect: z = 1.5, p = 0.133
CFP vs UN
CFP vs UN
Heterogeneity: I² = 71.6%
Q(1) = 3.52, p = 0.061
Overall effect: z = 0.06, p = 0.95
IG1 0w vs UN
IG1 0w vs UN
Heterogeneity: I² = 0.0%
Q(1) = 0.14, p = 0.706
Overall effect: z = 2.1, p = 0.035
IG2 0w vs IG1 0w
IG2 0w vs IG1 0w
Heterogeneity: I² = 0.0%
Q(1) = 0.03, p = 0.871
Overall effect: z = 0.43, p = 0.67
IG2 0w vs CFP
IG2 0w vs CFP
Heterogeneity: I² = 68.0%
Q(1) = 3.12, p = 0.077
Overall effect: z = 0.59, p = 0.555
0.25
0.5
1
2
3
4

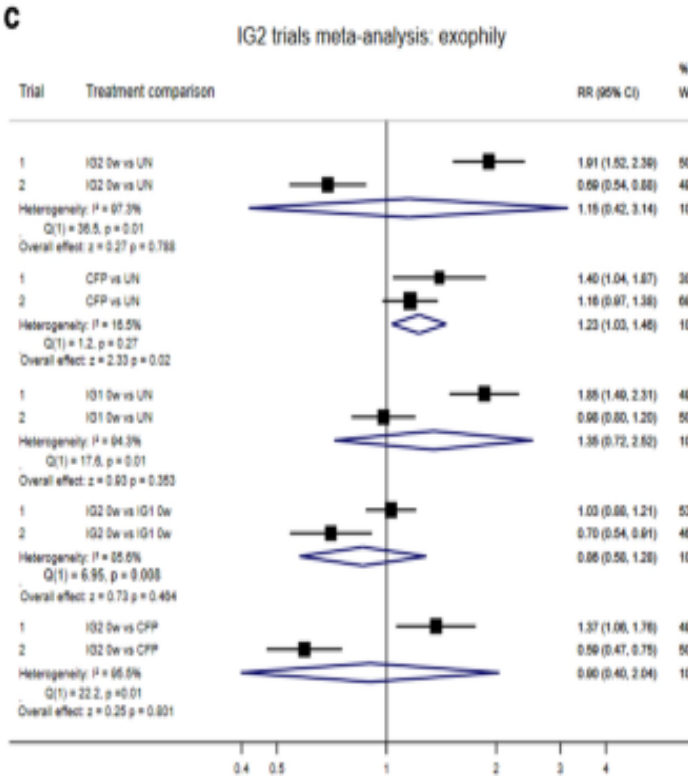

c
IG2 trials meta-analysis: exophily
Trial
Treatment comparison
RR (95% CI)
IG2 0w vs UN
IG2 0w vs UN
Heterogeneity: I² = 97.3%
Q(1) = 36.5, p = 0.01
Overall effect: z = 0.27 p = 0.788
CFP vs UN
CFP vs UN
Heterogeneity: I² = 16.5%
Q(1) = 1.2, p = 0.27
Overall effect: z = 2.33 p = 0.02
IG1 0w vs UN
IG1 0w vs UN
Heterogeneity: I² = 94.3%
Q(1) = 17.6, p = 0.01
Overall effect: z = 0.93 p = 0.353
IG2 0w vs IG1 0w
IG2 0w vs IG1 0w
Heterogeneity: I² = 85.6%
Q(1) = 6.95, p = 0.008
Overall effect: z = 0.73 p = 0.464
IG2 0w vs CFP
IG2 0w vs CFP
Heterogeneity: I² = 66.5%
Q(1) = 22.2, p =0.01
Overall effect: z = 0.25 p = 0.801
0.4
0.5
1
2
3
4

Fig. 2 a Interceptor G2 versus Interceptor LN: meta-analysis of the two hut trials: mortality. b Interceptor G2 vs Interceptor LN Metanalysis of the two trials: feeding. c Interceptor G2 vs Interceptor LN Metanalysis of the two trials: exophily

Blood feeding rates and personal protection

In the first trial, the percentage blood-feeding of *An. funestus* was significantly greater in the huts with the untreated net than in the huts with IG1 and IG2. There were no significant differences in blood-feeding rates between the huts with the IG1 or the IG2, with or without washing (Table 3). Neither was there significant difference in percentage blood-feeding between untreated net and chlorfenapyr CTN nor evidence of blood-feeding inhibition due to chlorfenapyr presence (percentage blood-feeding was greater in the huts with the chlorfenapyr CTN).

Table 3 Blood-feeding and blood-feeding inhibition of Anopheles funestus collected in the two experimental huts trials.

Trial	Effect	Untreated net	Interceptor LN	Interceptor LN	Interceptor G2	Interceptor G2	Chlorfenapyr CTN
		0 washes	0 washes	20 washes	0 washes	20 washes	0 washes
1	Total number blood fed	36	14	16	11	23	20
	% Blood fed (95% C.I.)	26^{ac} (20–33)	15^{b} (7–28)	17^{b} (8–30)	18^{bc} (10–29)	17^{b} (10–29)	37^{a} (22–55)
	% Blood feeding inhibition	0	42	36	32	33	0
	% Personal protection	0^{a}	61^{bc}	56^{bc}	69^{b}	36^{c}	44^{bc}
2	Total number blood fed	16	8	7	11	13	6
	% Blood fed (95% C.I.)	14^{a} (8–24)	10^{a} (5–20)	9^{a} (4–21)	11^{a} (4–25)	15^{a} (7–30)	8^{a} (3–20)
	% Blood feeding inhibition	0	31	38	26	0	44

Trial	Effect	Untreated net	Interceptor LN	Interceptor LN	Interceptor G2	Interceptor G2	Chlorfenapyr CTN
		0 washes	0 washes	20 washes	0 washes	20 washes	0 washes
	% Personal protection	0[a]	50[a]	56[a]	31[a]	19[a]	63[a]

The numbers in the same row sharing the same letter superscript do not differ significantly (p > 0.05)

In the second trial, while the percentage blood-feeding was greater in the huts with the untreated net than in the huts with the unwashed IG1 or IG1 washed 20 times the differences were not significant. Once again, no significant differences were evident between any of the IG1 and IG2 treatments. In the second trial, the difference between the untreated net and the chlorfenapyr CTN was also non-significant. Seven of the eight treatments that did show some degree of blood-feeding inhibition contained an alpha-cypermethrin component whether in IG1 or when twinned with chlorfenapyr in IG2.

In the first trial, personal protection in huts with IG1 and IG2 was significantly greater than in huts with the untreated nets. The chlorfenapyr net also showed significantly greater personal protection compared to untreated nets. In the second trial, while the numbers of *An. funestus* that were blood fed were also less in huts with the insecticide treated nets, neither the IG1, IG2 nor the chlorfenapyr treatments showed significant reduction in number blood-fed compared numbers blood-fed in huts with the untreated net. From these results it is not possible to conclude definitively that chlorfenapyr has no role in personal protection in huts with the chlorfenapyr treated net, but as regards personal protection in IG2, that the alphacypermethrin component has the major role mediated through reduced blood-feeding just as in IG1.

Meta-analysis of percentage blood feeding

In the meta-analyses of blood-feeding between the two trials, the comparison of relative risk between the unwashed IG2 versus the untreated net was 0.70 (CI 0.44, 1.11) (P = 0.133). The comparison of relative risk between the unwashed IG1 versus the untreated net was also quite similar (0.61, CI 0.39, 0.97) (P = 0.035) to that of IG2 above

(Fig. 2b). The comparison of relative risk between the chlorfenapyr CTN versus the untreated net was 0.97 (CI 0.39–2.44) (P = 0.95). Considering these results in reverse order: chlorfenapyr treatment seems to have no effect on blood-feeding compared to no treatment. Alpha-cypermethrin was the sole AI contributing to reduced blood-feeding in the comparison of IG1 to untreated net. The inference is the contributing active ingredient to reduced blood-feeding in IG2 versus untreated net is the alpha-cypermethrin rather than the chlorfenapyr. Further, the meta-analysis of relative risk of the comparison of IG2 versus chlorfenapyr CTN was 0.74 (0.3–2.0) (P = 0.67). This relative risk, being in the same direction as the relative risk between IG1 versus untreated net (0.61, 0.39–0.97) may support the interpretation that the chlorfenapyr has little or no role in blood-feeding in IG2 nor does it antagonize the positive effect alpha-cypermethrin has on reducing blood feeding in IG2 (Fig. 2b).

Exiting rates

In the first trial, mosquito exiting rates were significantly higher in the huts with IG1, IG2 and chlorfenapyr CTN treatments compared to the huts with untreated nets (Table 1). In the second trial the exiting rates from huts with IG1, 1G2 and chlorfenapyr CTN were not significantly different from exiting rates from huts with the untreated net nor from one another (Table 1).

Meta-analysis of enhanced exiting

In the meta-analysis these differences between the first and second trials led to heterogeneity in several of the comparisons of relative risk for exiting rates between treatments. No comparison between IG2 and any other treatment (untreated net, alpha-cypermethrin net, chlorfenapyr net) was significantly different from unity (Fig. 2c).

***Anopheles gambiae* sensu lato**

Abundance of *An. gambiae* was very low in trial 1 with only 42 mosquitoes collected from the six treatments over 54 nights. However, differences in mortality were observed at 72 h with significantly higher mortality observed in huts with unwashed IG2 and IG2 washed

20 times (14/16) compared to IG1 (4/11) or untreated nets (1/10) (Supplementary file), which is consistent with the *An. funestus* dataset trends. Insufficient *An. gambiae* were collected during trial 2 for formal analysis.

Chemical analysis

The mean alpha-cypermethrin content in unwashed IG2 for trial 2 (the WHO trial) was 2.81 g/kg (Table 4). The nets complied with the target dose of 2.4 g/kg ± 25% for 100 denier yarn. The mean chlorfenapyr content in unwashed IG2 for trial 2 was 5.22 g/kg. The nets complied with the target dose of 4.8 g/kg ± 25%. The within-net variation showed an acceptable homogeneity of active ingredient within the nets. After 20 washes the IG2 alpha-cypermethrin content for trial 2 was 1.65 g/kg, corresponding to an overall alpha-cypermethrin retention of 59%. The chlorfenapyr content was 1.66 g/kg after 20 washes, corresponding to an overall chlorfenapyr retention of 32% for trial 2. Netting samples were not kept back pre-washing in trial 1 for chemical analysis and therefore retention of chlorfenapyr and alpha-cypermethrin in IG2 after washing could not be accurately estimated. However, chemical analyses were conducted after the nets had been washed and tested in the huts and data was consistent with trial 2 post-trial retention estimates (see Table 4). The mean alpha-cypermethrin content in unwashed IG1from trial 2 was 5.55 g/kg. The alpha-cypermethrin content after twenty washes was 1.59 g/kg, corresponding to alpha-cypermethrin retention of 30% in IG1.

Table 4 Chemical analysis of insecticide on nets

Trial	Type of LLIN and wash treatment		Alpha-cypermethrin content (g/kg)				Chlorfenapyr content (g/kg)			
			Before washing*	After washing	Retention (%)	After trial	Before washing*	After washing	Retention (%)	After trial
1	Interceptor G1	0 wash	-	-	-	3.63	-	-	-	-
	Interceptor G1	20 washes	-	1.86	-	2.80	-	-	-	-

Trial	Type of LLIN and wash treatment		Alpha-cypermethrin content (g/kg)				Chlorfenapyr content (g/kg)			
			Before washing*	After washing	Retention (%)	After trial	Before washing*	After washing	Retention (%)	After trial
	Interceptor G2	0 wash	-	-	-	2.12	-	-	-	3.93
	Interceptor G2	20 washes	-	2.24	-	1.86	-	3.93	-	2.46
	Chlorfenapyr CTN	0 wash	-	-	-	-	-	-	-	6.29
2	Interceptor G1	0 wash	5.55	5.37	-	5.18	-	-	-	-
	Interceptor G1	20 washes	5.38	1.59	30%	1.52	-	-	-	-
	Interceptor G2	0 wash	2.81	2.77	-	2.85	5.22	5.12	-	5.08
	Interceptor G2	20 washes	2.79	1.65	59%	1.75	5.18	1.66	32%	1.79
	Chlorfenapyr CTN	0 wash	-	-	-	-	4.68	3.18	-	3.80

*** No individual samples were cut from nets pre-washing in the first trial**

Supporting bioassay tests on Interceptor and Interceptor G2 nets used in the hut trials.

The purpose of the supplementary bioassays was to sample netting from the IG2 and IG1 used in the experimental hut trials to 1) test bio-efficacy against pyrethroid resistant (Zeneti) and susceptible (Kisumu) strains in mosquito bioassay, 2) confirm the bio - efficacy of alpha-cypermethrin and chlorfenapyr components after multiple washing, 3) examine the capacity of tunnel tests to predict the performance IG2 netting under simulated hut conditions to control *An. gambiae* s.s.

Standard WHO Cone bioassay tests on nets with 3 min exposure of the susceptible strain and a 72-h holding period, induced mortality of 96% and 100% on the unwashed IG1 and the IG1 washed 20 times. With the chlorfenapyr CTN, mortality was 90%, 95% and 100%

after 24 h, 48 h and 72 h. For the unwashed IG2, mortality was 100% after 24 h exposure. For IG2 washed 20 times mortality was 62%, 72% and 86% after 24 h, 48 h and 72 h intervals (Fig. 3a).

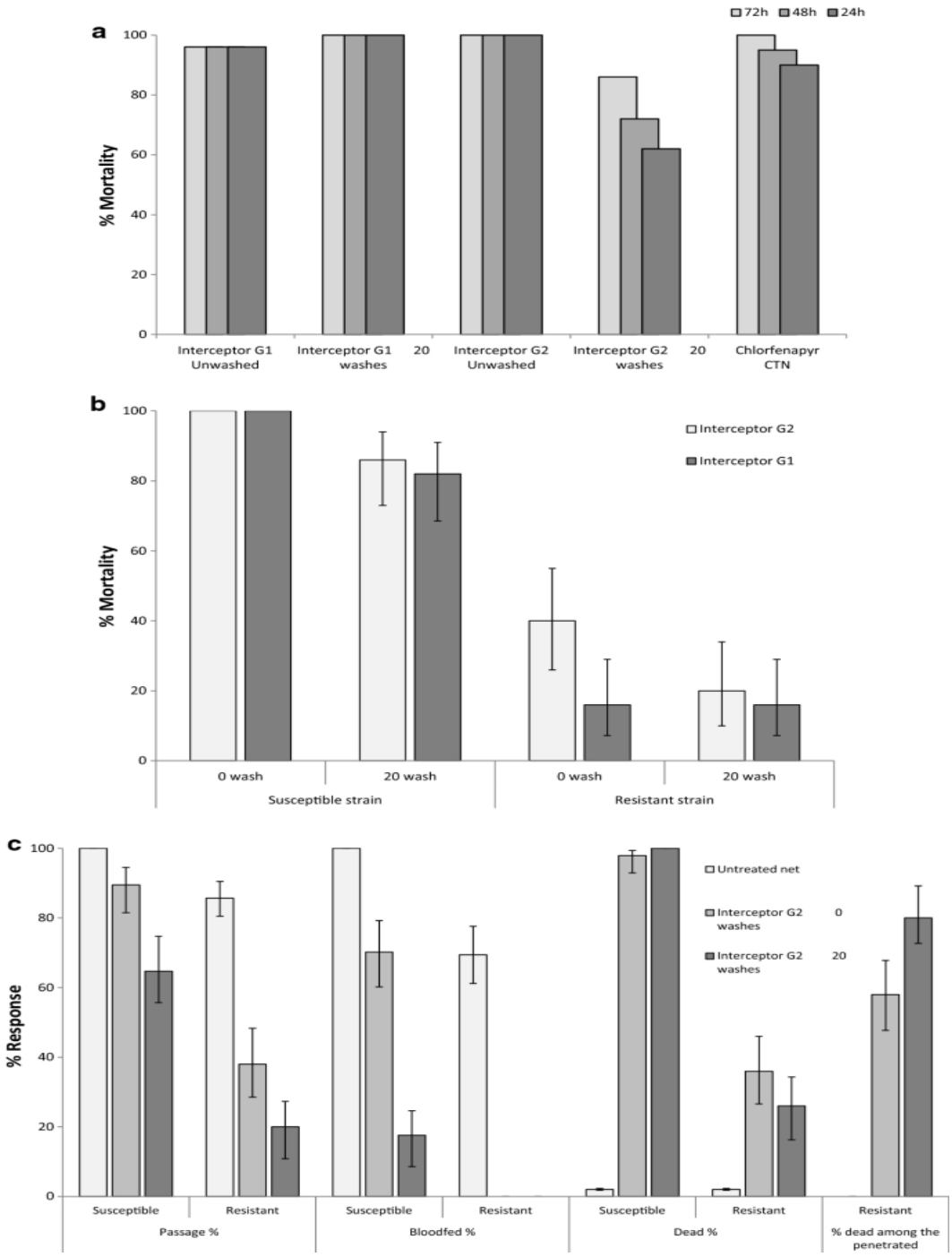


Fig. 3: a Cone bioassay test on unwashed and 20 times washed Interceptor G2 and Interceptor LN nets using pyrethroid susceptible *Anopheles gambiae* Kisumu. **b** Cone bioassay test mortality on unwashed and 20 times washed Interceptor LN and Interceptor G2 nets using *Anopheles gambiae* pyrethroid susceptible Kisumu and pyrethroid resistant Zeneti strain. Three-minute exposure and 72 h holding. **c** Tunnel tests with unwashed and washed Interceptor G2 netting against *Anopheles gambiae* pyrethroid susceptible (Kisumu) and pyrethroid resistant (Zeneti) mosquitoes: % passage, % feeding, % mortality

In further supplementary 3 min cone tests using the susceptible strain, mortality was 100% on unwashed IG1 and IG2, and on the IG1 and IG2 washed 20 times mortality was reduced to 82% and 86%, respectively. With the Zeneti pyrethroid resistant strain, cone mortality was reduced to 16% and 40% with unwashed IG1 and IG2, respectively, and 16% and 20% respectively after 20 washes (Fig. 3b).

Supplementary tunnel tests were conducted using susceptible and resistant strains tested on unwashed IG2 and IG2 washed 20 times (Fig. 3c). With untreated netting, 100% of the susceptible and 86% of the resistant mosquitoes penetrated the holes into the baited chamber, 100% of the susceptible and 69% of the resistant mosquitoes' blood-fed, and 2% of the susceptible and 2% of the resistant mosquitoes died. With unwashed IG2 netting fewer of the susceptible (89%) and resistant (38%) mosquitoes penetrated the holes, and even fewer susceptible (70%) and resistant (0%) mosquitoes' blood-fed. However, 98% of the susceptible and 36% of the resistant mosquitoes were killed by the unwashed IG2 their attempts to feed. With the IG2 washed 20 times, a smaller percentage of the susceptible (65%) and resistant (20%) mosquitoes penetrated the holes (surprisingly), fewer susceptible (18%) and resistant (0%) blood-fed, and yet 100% of susceptible and 26% of resistant mosquitoes were killed. The Zeneti strain was less adapted to the tunnel test, penetrating holed netting and responding/feeding on guinea pigs less well than did the long-established Kisumu.

Comparison of supplementary bioassay tests with hut trial results

Comparing the laboratory cone and tunnel bioassay results against the pyrethroid resistant *An. gambiae* s.s. strain and the experimental hut results against the wild pyrethroid resistant *An. funestus* population, both types of bioassay predicted the

response in the hut to the pyrethroid-only IG1: mortality was 16% in the cone and 13% in the hut against the unwashed IG1, and 16% in the cone and 11% in the hut against the IG1 20 times washed (averaged control-corrected mortality). When tested against the unwashed IG2, mortality was 40% in the cone, 36% in the tunnel and 51% in the hut; when tested with the 20 times washed IG2 mortality was 20% in the cone, 26% in the tunnel and 46% in the hut.

With the unwashed and washed IG2, percentage passage and percentage blood-feeding in the tunnel test were significantly lower with the newly colonized resistant Zeneti strain as compared to the long-established susceptible Kisumu strain. While up to 70% of Kisumu blood-fed after penetrating the IG2 netting, none (0%) of the Zeneti strain blood-fed through IG2. And while high mortality of Kisumu (up to 70%) was recorded with IG2, low mortality was recorded against unwashed and washed IG2 (36% and 26%, respectively). This very much reflected the new adaptation of the Zeneti strain to the tunnel test, possibly an avoidance or irritation of the treated net, or 'reluctance' to feed on guinea pigs. However, for those Zeneti strain mosquitoes that did penetrate the netting, mortality inflicted by unwashed and 20 times washed IG2 was high, 58% and 80% respectively, and more closely resembled mortality in experimental huts.

This series of bioassay tests demonstrates that the chlorfenapyr component of IG2 LN makes the major contribution to controlling pyrethroid resistant *An. gambiae* and *An. funestus*. The tunnel tests were more predictive of efficacy in experimental huts whilst cone bioassays were less predictive.

Discussion

Novel alternative insecticides which can complement the pyrethroids on LLIN and improve the control of pyrethroid resistant vectors are urgently needed to sustain progress against malaria. The objective of the present study was to determine the efficacy and wash-fastness of the chlorfenapyr-alphacypermethrin mixture net, Interceptor G2 LN, unwashed and after 20 washes, against the primary pyrethroid-resistant vectors *An.*

funestus and *An. gambiae* s.s. under household-like conditions compared to the standard pyrethroid-only net Interceptor LN (IG1). Previously this very team had participated in the development and evaluation of IG1 against *An. funestus* and *An. gambiae* s.s. 10–14 years ago when these species were pyrethroid susceptible in NE Tanzania [8, 20]. Latterly this team`s participation was extended to development and evaluation of the new generation long-lasting net IG2 against the *An. gambiae* sibling species *An. coluzzii* in Benin, W Africa, and *An. arabiensis* in Kilimananjaro, Tanzania, where the species had become pyrethroid resistant [13,14,15,16,17]. Two trials were more recently extended to Muheza, NE Tanzania, aimed at evaluating IG2 against pyrethroid resistant *An. gambiae* s.s. and *An. funestus*. Only *An. funestus* was caught in significant numbers. In the meta-analysis of the two trials, the mortality induced by IG2 against *An. funestus* was 3.4 times higher than with untreated nets and 2.3 times higher than with IG1. The comparison of mosquito mortality between the unwashed IG2 and IG2 washed 20 times produced a relative risk of 1.04 (CI 0.83–1.30) indicating no loss of efficacy of IG2 over 20 washes. This means IG2 exceeds by a factor of 2.3 the mortality criterion required by WHO PQT to grant the product LLIN status [32]. The comparison of chlorfenapyr CTN with IG2 confirmed that the chlorfenapyr component of IG2 was the main contributor to mosquito mortality and net efficacy. However, it was also confirmed that the pyrethroid continues to have a valuable role with respect to blood-feeding inhibition, repellency and personal protection. The pyrethroid contributed 39% protection against blood-feeding of pyrethroid-resistant *An. funestus* in IG1 and 30% protection in IG2 compared to untreated nets. This was not far short of the 32% blood-feeding inhibition shown by IG1 against pyrethroid-susceptible *An. funestus* in Zeneti hut trials over 10 years ago [20].

More important than the demonstration of equivalence of blood-feeding inhibition in resistant *An. funestus* was the clear demonstration of superior mortality of IG2 against resistant *An. funestus* that approached the mortality that IG1 once showed against An. funestus and *An. gambiae s.s.* in NE Tanzania before resistance evolved. In 2010, IG1 induced a control-corrected mortality of 80% among susceptible *An. funestus* when

unwashed and 60% after 20 washes. In 2015–2016, IG1 only induced a control-corrected mortality of 12% among pyrethroid resistant *An. funestus* unwashed and of 11% after 20 washes. In contrast, IG2 induced a control-corrected mean mortality of 49% among pyrethroid resistant *An. funestus* when unwashed and 52% after 20 washes. While the performance of unwashed IG2 did fall a little short of the mortality induced by unwashed IG1 in 2006 against susceptible *An. funestus*, the mortality that the 20-times washed IG2 demonstrated against resistant *An. funestus* in 2016 was comparable to the efficacy 20-times washed IG1 demonstrated against susceptible *An. funestus* in 2006. Other recent experimental hut trials in West Africa in which IG2 has generated high mortality include *An. coluzzii* in Benin (71%, 65%), in Burkina Faso (76%, 75%) and Côte d'Ivoire (90%, 82%) when unwashed and washed 20 times, respectively. This is comparable mortality to that achieved with IG1 and other pyrethroid-only nets in the 1990s and new millennium when standard ITN and LLIN were first demonstrating malaria control and personal protection [37]. Considering the impact of ITN and LLIN then, it is reasonable to anticipate that IG2 and other Dual-AI will achieve comparative control of pyrethroid-resistant mosquitoes as standard LLIN once did against susceptible mosquitoes.

It is certainly the case that high intensity resistance means that standard LLIN are no longer preventing malaria as they once did. In countries and regions bordering Lake Victoria, for example, standard LLIN no longer appear to be reducing malaria despite maintenance of high coverage [9, 38, 39]. A cluster randomised trial of standard pyrethroid LLIN conducted in the region of high resistance, Kagera, on the western shore of Lake Victoria, Tanzania, could only demonstrate stasis in 2018 after introduction of new pyrethroid-only LLIN [9] but in adjacent clusters which were randomised to receive pyrethroid-PBO synergist LLIN there was a significant reduction in entomological inoculation rate and malaria prevalence [10].

The only putative insecticide mixture LLIN on the horizon, apart from the pyrethroid-chlorfenapyr net IG2, is a net treated with pyrethroid and pyriproxyfen which is a

mosquito sterilant and insect growth regulator. In a stepped wedge cluster randomised trial conducted in Burkina Faso a reduced malaria incidence rate of 12% was observed in the intervention arm compared to the control, a standard pyrethroid-only LLIN [40, 41]. As a mixture of two adulticides, IG2 would appear to hold more promise. Owing to the diversity of novel AI and modes of action being tested on LLINs, the WHO is no longer willing to accept entomological evidence as generated in experimental hut trials as adequate evidence for recommendation of a novel LLIN class. Since 2017, the WHO has required all new classes of LLIN to be subject to cluster randomized trials (CRT) with malaria control outcomes before they can gain approval or recommendation for wide scale use as new methods of malaria control [42]. Chlorfenapyr is currently the only novel adulticide being evaluated on LLIN in a CRT. Such a trial takes at least 2 years to complete. This means that chlorfenapyr is a very precious AI, squandered at our peril. If chlorfenapyr fails due to evolution of resistance, there will be only PBO and pyriproxyfen left in the armoury for use on nets. Fortunately, chlorfenapyr is novel chemistry and there is no sign of resistance so far, but resistance will evolve just as it always does. What must be done now is to identify ways preserve this AI as much as it is used to good effect. There is temptation to use it as an IRS insecticide too. In hut trials, it appears less effective applied as an IRS adulticide and the WHO proposes cluster randomized trial evidence of malaria effect [43, 44]. Blanket IRS coverage may accelerate resistance selection, as was demonstrated after 7 years of pyrethroid IRS in Kagera region that led to premature loss of pyrethroid effectiveness in LLIN just as LLIN were being scaled up [9, 10]. What is needed is a far-sighted resistance management strategy which prioritizes PBO, chlorfenapyr, and the few AI that can be used safely on nets and reduces their use in other applications, like IRS, if there are good alternatives that can be used or rotated to reduce selection pressure on chlorfenapyr in IG2.

Conclusion

Novel alternative insecticides suitable that can complement the pyrethroids and improve the control of pyrethroid resistant malaria vectors are urgently required for sustaining LLIN as a means of malaria control. The mortality of pyrethroid resistant *An. funestus* induced by unwashed and 20 times washed Interceptor G2 appears to meet the entomological requirements set by the WHO for efficacy and wash-resistance. Thus far there is no epidemiological evidence to back up the entomological evidence nor any knowledge of how long Interceptor G2 LN or its chlorfenapyr component will remain effective under field conditions. Therefore, large-scale cluster randomized trials of Interceptor G2 with epidemiological endpoints are an essential next step. A CRT in NW Tanzania against *An. funestus* and *An. gambiae* is due to report in mid-2021 for recommendation as a new class of LLIN product to WHO.

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Part five summary of key findings and new findings since publication

In concluding part five here are the key findings and recommendations

Results in chapter 8 showed that as a long-lasting net to control or protect against pyrethroid resistant mosquitoes PermaNet 3.0 showed marginal but significant improvement over PermaNet 2.0 against pyrethroid resistant Culex mosquitoes. This indicated the PBO in PermaNet 3.0 was exerting a partial synergism against resistant mosquitoes. As per tunnel test results the synergism in the huts was fully lost after 20 washes. It would seem with PermaNet 3.0 that PBO rather than deltamethrin is the compound that remains locked in the fibre. That deltamethrin must still be present on the surface of both PermaNet 2.0 and 3.0 and causing some mortality of susceptible *Cx. quinquefasciatus* but there seems little or no PBO left on the surface of PermaNet 3.0 netting to allow synergy in resistant *Cx. quinquefasciatus*.

The analysis discussed in chapter 9, comparing PBO nets with two different treatments - that is PermaNet 3.0 with PBO treated only on the top panel of the net and Veeralin that has PBO treated in whole net – was within the margin of 'no significant difference'. Results also showed that after washing mortality induced by 20-times washed Veeralin LLIN was similar to that recorded for 20 times washed PermaNet 3.0, i.e., the two products, by that criteria were wash-fast.

The WHO Vector Control Advisory Group (VCAG) advises the WHO on new vector control tools and stipulates that for use against pyrethroid-resistant vector populations, the mixture LLIN should demonstrate efficacy (mosquito mortality or prevention of blood-feeding) significantly greater than standard pyrethroid-only LLIN. Significantly higher mortalities of pyrethroid-resistant *An. funestus* s.l. were recorded by the unwashed and 20-times washed Veeralin compared to positive control DuraNet LLIN in experimental hut; the incorporation of PBO in Veeralin has shown increased mortality impact than in non-PBO DuraNet LLIN. The significantly higher mortalities over pyrethroid-resistant *An.*

funestus s.l. recorded by both unwashed and 20 times washed Veeralin compared to the unwashed standard pyrethroid only DuraNet LLIN can be attributed to the effect of Piperonyl Butoxide (PBO), a chemical synergy contained in the whole Veeralin net thus meeting the VCAG criteria for a resistance-breaking LLIN. This further demonstrate the advantage of using PBO LN over standard pyrethroid LNs against resistant mosquitoes.

The similarity of Veeralin LN which is the PBO LN with PBO treated on whole net to PermaNet 3.0 not only shows the similarity in efficacy between a PBO LN with PBO restricted on the roof panel only to PBO LN with PBO treated on whole net but also provides yet another evidence that majority of mosquitoes respond to host odour or convection plumes thus mosquitoes contact the roof of the net while seeking access to the host, thus more support to the 2-in-1 concept as a tactic for managing resistance management.

Regarding actual efficacy effect size gained by deploying PBO LN as opposed to standard pyrethroid only LN in an area with resistant mosquitoes a comparative and meta-analysis of several experimental huts' efficacy trials of standard and PBO synergist long-lasting nets before and after development of insecticide resistance against *Anopheles gambiae* and *Anopheles funestus*. As reported in chapter 10 it was evident from meta-analysis results that in overall PBO LNs were associated with 69% and 83% significant increase effect size in terms of odds of mortality of resistant *An. gambiae* and *An. funestus* mosquitoes respectively compared to mortality recorded by standard pyrethroid LLINs, hence improved efficacy with PBO LLINs against resistant mosquitoes as compared to standard pyrethroid only LLIN.

Although this study reports superior efficacy of the PBO LNs over pyrethroid-only treated net against pyrethroid-resistant mosquitoes, the improvement, though significant, is is marginal which may not be meaningful for public health impact. However, the results from a cluster randomized trial have shown that PBO-LLINs provide better control of malaria than standard nets in Tanzania, whereby Olyset Plus (PBO) nets provided a 33%

malaria case reduction compared to Olyset nets at 21 months (Protopopoff et al., 2018). This indicates that the marginal improvement documented in the hut trials may indeed translate into a major improved in malaria control. Indeed, the study conducted in Muleba, Tanzania, provided sufficiently compelling evidence for efficacy of PBO-LLINs in reducing malaria cases for the WHO to give an evidence-based interim decision to approve PBO-LLIN to be used for malaria control (WHO, 2017).

An alternative way to safeguard insecticide-based vector control, new insecticide classes with novel mode of action need to be developed and scaled-up to achieve the targets of the WHO Global Technical Strategy for Malaria 2016-2030. Interceptor G2 LN (IG2) is a Dual-AI LLIN developed by the manufacturer BASF SE which is designed to provide protection against pyrethroid-resistant mosquitoes by means of a mixture of chlorfenapyr and alpha-cypermethrin in a long-lasting wash-resistant formulation. In the meta-analysis of the two trials, described in chapter 12, the mortality induced by IG2 against *An. funestus* was 3.4 times higher than with untreated nets and 2.3 times higher than with IG1. The comparison of mosquito mortality between the unwashed IG2 and IG2 washed 20 times produced a relative risk of 1.04 (CI 0.83–1.30) indicating no loss of efficacy of IG2 over 20 washes. This means IG2 exceeds by a factor of 2.3 the mortality of standard LLIN against resistant mosquitoes. Considering the impact of ITN and LLIN then, it is reasonable to anticipate that IG2 and other Dual-AI will achieve comparative control of pyrethroid-resistant mosquitoes as standard LLIN once did against susceptible mosquitoes.

Cluster randomized trials (CRT) of a LLIN that combines chlorfenapyr with pyrethroid in a wash-tolerant formulation in Tanzania, East Africa and in 2022 in Benin, West Africa have just been published. Both CRTs are targeted resistant *Anopheles gambiae* complex: *An. gambiae* sensu stricto (s.s.) in NW Tanzania and *Anopheles coluzzii* in Benin. Results from Tanzania study shows that a cohort of children aged 6 months to 10 years showed that the incidence of clinical malaria in areas using the Interceptor G2 was reduced significantly by an average of 44% over the 2 years of the trial compared with incidence

among those using pyrethroid-only LLINs. In addition, 2 years after LLIN distribution, malaria infection prevalence among children aged 6 months to 14 years was 25.6% in the group using the chlorfenapyr-containing LLINs compared with 45·8% in the group using pyrethroid-only LLINs (p=0·0001). These epidemiological evidence of effectiveness against malaria in CRT is a contributed to the evidence that recently (March 2023) convinced World Health Organization (WHO) to grant recommendation to Interceptor G2 as a new class of LLIN for malaria control in an area with insecticide resistant mosquitoes. These results also evidenced Interceptor G2 as more cost effective than standard pyrethroid only LLIN. All these provide crucial information for malaria control programmes in endemic areas and for donors who have been hesitant to purchase next-generation LLINs because of their relatively higher cost.

PART SIX

This chapter summarizes the outcomes and conclusions of the previous chapters, discusses their significance in relation to malaria vector control and insecticide resistance management, and discusses possible ways forward.

Chapter 12: General discussion and conclusions

Recap of main objectives:

1. To evaluate the efficacy and wash-fastness of new long lasting pyrethroid nets

2. To evaluate long lasting net treatment kits – insecticide impregnation processes that enable nets to remain effective for longer.

3. To assess the impact of insecticide resistance on the efficacy of pyrethroid long-lasting insecticidal nets (LLIN):

4. To identify and evaluate a) synergist-pyrethroid combinations, and b) alternative insecticides for use in combination with pyrethroids to overcome transmission problems due to pyrethroid resistance in local vectors.

Conclusions

Long-lasting insecticidal nets (LLINs) in which wash-resistant formulations of insecticide are either coated or incorporated into the netting fibres during production [2] is the most important tool for preventing malaria in Africa and Asia [12].

For a new LLIN product to be eligible for a WHO recommendation, manufacturers must demonstrate their effectiveness for malaria vector control by passing through a series of tests organized by WHOPQT (WHO Pre-Qualification Team) to ensure their quality and performance following standard testing procedures for chemical, physical and biological efficacy against mosquito vectors.

Despite their success in controlling malaria, LLINs still face technical and logistical challenges that may compromise effectiveness in field. Major challenges facing LLIN intervention include inadequate coverage, inappropriateness in humanitarian emergencies, and the ever-increasing growth in insecticide resistance.

The work described in this thesis was designed to evaluate in laboratory, experimental huts and field trials new standard pyrethroid LLIN products, long-lasting treatment kits, and mixture/combination LLIN for specific WHO approval and for purpose of validation and for suggestion for improvement of the WHO evaluation guidelines. Secondly, to make suggestions for new approaches for LLIN testing to improve vector control. Thirdly to investigate the impact of pyrethroid resistance on the efficacy of pyrethroid LLINs, and fourthly to ascertain if the efficacy LLINs can be maintained or improved by adopting synergists and alternative insecticides to overcome problems of insecticide resistance.

The results of the various studies performed have been discussed separately in the previous chapters. Hence the current chapter focuses on the key findings and conclusions of each objective proposed in the introduction and discussing their significance in relation to malaria vector control and insecticide resistance management in the present day:

1. Evaluation of a pyrethroid standard long-lasting insecticide LLIN, Interceptor LN.

One of the aims of this thesis was to evaluate new pyrethroid LLIN products for the following specific purposes, a) LLIN product quality control, for validation and possible input into the LLIN evaluation guidelines, b) correlate efficacy between phase II and phase III outcomes and c) investigate similarities and differences in efficacy between the LLINs made of different polymer technologies.

a) Evaluation of a pyrethroid standard long-lasting insecticide LLIN, Interceptor LN for: LLIN product quality control, for validation and possible input into the LLIN evaluation guidelines

This specific objective aimed at evaluation of the new LLINs products for quality control and for WHO recommendation was addressed by the work of this thesis as follows; Firstly results from appendix 1 informed the WHO evaluation in which Interceptor LN received interim recommendation. Chapter two of this thesis describes the first WHO Phase III household randomized trial of the Interceptor LN whereby results showed that Interceptor LN met the WHO efficacy criteria for long-lasting insecticidal nets after 36 months of use. On the basis of this trial and one other in India, Interceptor LN obtained

WHO full recommendation. This was the first time phase 2 and phase 3 trials had been conducted in this way; they had considerable impact on the content and methodology of 2013 WHOPES 'Guidelines for laboratory and field testing of LLINs.'

Secondly, the findings of these chapters showed the weakness of relying on a conventionally treated net that was washed just before cut of point (CTN) to achieve consistency between WHO sites in terms of the cut-off number of washes which varied greatly between sites. This might have been due to differences in vector susceptibility between species used at different sites, site-specific differences in the quality of the water, difference in the interpretation of the washing instructions between sites. A further discovery was that pyrethroids inherently adhered to polyester fibres with conventional formulations. Hence, there was wide variation in the number of washes for the CTNs between sites in Phase 2 trials. In 2013, the WHOPES Guidelines for testing of LLIN were revised to include, as a positive control, a WHO-recommended LN with similar specifications to the candidate LN in terms of insecticide, treatment technique, netting material, and washing frequency (0 and 20 times) [1]. The revised guidelines were published after the Interceptor trial, and have not been further revised by WHOPQ, except to recommend the use of a prequalified LN as a positive control against new candidate LN. Thus, results from the work of this thesis (Interceptor in Appendix 1 and Icon Maxx chapters 4-6) contributed evidence that led to the revised guidelines for testing of LN that included a LN as a positive control as opposed to CTN.

Thirdly, current WHOPQT guidelines for physical durability and attrition [2] provides only a standard approach of quantifying the number of holes on a net. Its limitation is it does not categorize measurement of holes as to whether the net is "effective" (still protective against mosquito bite as the new viable LLIN) or 'ineffective' (offering diminished or significantly lower protection from mosquito bites as compared to new viable LLIN) on the basis of LLIN personal protection. The current categorization of physical net integrity (good condition pHI <= 64, serviceable condition pHI = 65 – 642 and poor condition pHI >

642 specifically the upper limit is not based on strong evidence of insecticide durability as per the present Phase 3 community trial evidence. In the Interceptor Phase 3 there was only the CTN control up to 36 months and none of these passed beyond 12 month insecticidal efficacy. Physical durability was not measured in the Interceptor Phase 3 trial but it was in the IconMaxx trial for the first time.

b) Evaluation of a pyrethroid standard long-lasting insecticide LLIN, Interceptor LN for: correlate efficacy between phase II and phase III outcomes.

The second objective sought to correlate efficacy between phase II and phase III outcomes. Results from this study (chapter 2), shows that, after 36 months of field usage, the average insecticide content in Interceptor LNs sampled from phase III trial was 42 mg alpha-cypermethrin per m^2. This was remarkably similar to the 41 mg/m^2 alpha-cypermethrin content observed in Interceptor LNs after 20 standardized washes in the Phase II hut trials done in the same locality. The similarity in chemical content between the Phase III household-randomized and Phase II experimental hut trial indicates that 20 standardized washes was a fair approximation of the loss of insecticide to wear and tear, abrasion and washing LLINs during 3 years of household use. While the correlation as encouraging, more LN products need to be compared in Phase II hut and Phase III household trials before this conclusion is fully verified or justified.

c) Evaluation of a pyrethroid standard long-lasting insecticide LLINs: investigate similarities and differences in efficacy between the LLINs made of different polymer technologies.

LLINs are classified into two categories depending on the technology involved in their treatment and production (WHO, 2003): 'incorporation' technology, and 'coating' of resin in which the pyrethroid is bound to the surface of the multifilament polyester netting. The third objective of this thesis aimed at determining the similarities and differences in efficacy between LLINs with same AI but different impregnation technology. Results of this work (Chapter 3) shows that mosquito mortality after zero and 20 washes was similar in incorporation and coated nets as was was personal protection. The similarities in outcomes observed between incorporation (DuraNet) and

coating (Interceptor) LLIN indicates that the type of material (polymer) used to make the net is less important than the type of pyrethroid used. If the pyrethroid is the same and the concentration and diffusion properties are similar, and the manufacturing quality meets high WHO standards, then the type of net (polyester, polyethylene) seems to be less relevant to efficacy than AI and concentration. This is vital information to LLIN procurement, NMCP`s and international donors when it comes to effective decisions as far as LLIN procurement is concerned. However, this was a comparison of only two brands of alpha-cypermethrin net not a meta-analysis of several brands of net of each polymer, so it would be premature to make broad conclusions across all active ingredients and/or polymers.

2. To evaluate long lasting net treatment kits – insecticide impregnation processes that enable nets to remain effective for longer.

The objective of this work was to evaluate long-lasting treatment kit products for their wash fastness, efficacy and longevity on different fabrics used in malaria vector control. This was done to investigate whether improved vector control can be achieved by the use of long-lasting treatment kits to meet the challenges that may threaten effectiveness of nets by: a) improving inadequate LLIN coverage between coverage campaigns through converting retail untreated nets into long-lasting nets, b) catering for the inapplicability of LLINs in humanitarian emergencies and complex political situations and c) providing for potential for insecticide resistance management plan.

a) ***Evaluation of long-lasting net treatment kits for****: improving inadequate LLIN coverage between coverage campaigns through converting retail untreated nets into long-lasting nets*

Despite proven LLIN effectiveness, not all households have enough nets to meet family needs [3]. Results from this study showed that nets treated with ICON Maxx washed 20 times met the criteria set by WHOPQT for Phase II entomological trials in terms of mortality and blood-feeding inhibition. In Phase III trials, ICON Maxx demonstrated insecticidal efficacy up to 30-36 months of household use, whereas the CTN fell short of

efficacy criteria within just 12 months of use. With these results, ICON Maxx provided a potential solution to the problem of reduced LLIN coverage between distribution campaigns, by turning commercial retail-sourced untreated nets into LLINs through simple home or community treatment if promoted appropriately by NGOs and NMCPs. However, what arose instead was the evolution of pyrethroid resistance which NIMR (myself) has demonstrated to be widespread throughout Tanzania. This rendered the pyrethroid-only kits largely obsolete. What is needed now is a partner AI kit to convert pyrethroid LLIN to Dual AI ITN especially if the partner AI does not last for a full 3 years.

b) ***Evaluation of long lasting net treatment kits*** *:* as potential option catering for the inapplicability of LLINs in humanitarian emergencies and complex political situations

With its success in malaria control, LLINs are still facing several technical and logistical challenges that compromise their effectiveness in field. The inapplicability of LLINs in humanitarian emergencies and complex political situations is one challenge facing LLINs. There is great diversity in the fabrics and materials used for making mosquito nets, insecticide-treated blankets, tents and curtains have shown protection against malaria in trial settings. Results from this work (Chapters 4-6) have demonstrated not only high and long lasting efficacy of ICON Maxx on polyester netting materials but as well in variety of fabrics used as shelters in displaced populations in humanitarian emergencies such (i.e. nylon, polyethylene, cotton and polyester are all used). The new UNHCR tents are constructed from a mixture of high- and low-density polyethylene to provide strength while allowing pyrethroid to migrate to the outer surface. Interior tents are made from polyester. But this will have to be redeveloped for areas that have highly pyrethroid resistant vector populations. WHO in the latest 'WHO Handbook for Malaria Control in Humanitarian Emergencies' are promoting Organophosphate IRS spraying inside tents.

The high efficacy, wash-fastness and versatility of ICON Maxx raises the possibility of the formulation becoming an all-purpose formulation for such purposes as military clothing, civilian bed covers and curtains, or for blankets, tarpaulins and tents distributed in

epidemics even if the active ingredient has to be changed because of resistance. This opens up the possibility treatment kits formulation could also meet the needs of malaria control in emergency situations like refugee camps when the use of LLINs is not practical for IDPs supplied with tents. This can be done through treating with long-lasting treatment kits the tarpaulins and tents distributed in epidemics, disasters or humanitarian emergencies. This will require new formulation research with pesticide manufacturers or the IVCC.

c) ***Evaluation of long-lasting net treatment kits:*** As potential option for insecticide resistance management

One of the most promising ways to manage insecticide resistance is to apply combination /mixtures of unrelated insecticides or a mixture of an insecticide and a chemical synergist. The high and long-lasting insecticidal efficacy of ICON Maxx described in this thesis (Chapters 4-6) suggest that the Idea of log lasting treat yourself kits can be potential options for insecticide resistance management plan through treating standard pyrethroid only LLINs post-manufacture with alternative insecticides with no cross resistance. This presents an opportunity for pesticide companies to produce binders like PBO-binder long-lasting kit to be applied to any pyrethroid LLIN to convert those to pyrethroid-PBO LLIN. This could apply equally to other partner AIs, such as pyriproxyfen or chlorfenapyr which are being used with pyrethroid in other types of Dual-AI LLIN should these fall short of 3 years' effectiveness. In environments with high pyrethroid resistance, it would be a mistake to allow Dual AI nets to revert to a pyrethroid-only LLIN in their third year as users would only be part-protected. At the present time, there is no such thing as a non-pyrethroid AI treatment kit. Such a kit would have wide utility for control of pyrethroid resistant mosquitoes. An opportunity is being missed. It is not the role of manufacturers to lead public health initiatives. Instead, manufacturers will respond to public health initiatives and their preference is for one-off tenders. It is up to WHO, NGOs the Global Fund and other international agencies like PMI to drive the process and create markets

for such kits. Manufacturers will follow their lead if they create a market for kits or open tenders for such kits.

3. To assess the impact of insecticide resistance on the efficacy of pyrethroid long-lasting insecticidal nets (LLIN):

Insecticide resistance is well established in many malaria endemic countries including Tanzania. Although substantial progress has been made on understanding the causes of pyrethroid resistance, remarkably few studies have focused on either entomological or epidemiological impact of resistance on current malaria control activities. As we move into the next malaria eradication era, it is vital that the implications of insecticide resistance are understood and strategies to mitigate these effects are implemented. The third aim of this thesis evaluated standard pyrethroid-only LLINs with the purpose of finding the impact of insecticide resistance on the entomological efficacy of LLINs. Results described in chapter 7 of this thesis shows reduced efficacy of pyrethroid-treated nets (LLINs) in terms of entomological indices in an area with pyrethroid resistant *An. gambiae s.l. and An. funestus s.l.* populations. This confirms that, in Tanzania, insecticide resistance can limit and undermine the effectiveness of malaria vector control interventions especially pyrethroid LLINs. To safeguard insecticide-based vector control, new insecticide classes with novel modes of action need to be scaled-up to achieve the targets of the WHO Global Technical Strategy for Malaria 2016-2030.

4. To identify and evaluate a) synergist-pyrethroid combinations, and b) alternative insecticides for use in combination with pyrethroids to overcome transmission problems due to pyrethroid resistance in local vectors.

The concept of combining insecticides with unrelated modes of action can be used to enhance the control of pyrethroid resistant malaria vectors with LLINs and to manage insecticide resistance. Two major ways of achieving this are studied in this thesis: 1. combining pyrethroid LLINs with insecticide synergist and 2. mixing pyrethroids with non-pyrethroid compounds on bed nets. The studies in this thesis (chapter 7) were performed

in sites with resistant vectors to investigate how combinations could improve entomological efficacy of LLINs against insecticide resistant vector population.

a) **To identify and evaluate synergist-pyrethroid combinations for use on nets in combination with pyrethroids to overcome problems of insecticide resistance**.

Chapters 8 and 9 presents and discusses results of PBO nets (chapter 8 LLIN with PBO on roof panel only and Chapter 9, LLN with PBO on whole net). In both trials PBO LLINs showed marginal but significant improvement over standard pyrethroid only LLIN against pyrethroid resistant malaia vectors meeting the VCAG criteria for a resistance breaking LLIN. As reported in chapter 10 meta-analysis results showed that PBO LNs were associated with 69% and 83% significant increase in effect size in terms of odds of mortality of resistant *An. gambiae* and *An. funestus* mosquitoes respectively compared to mortality recorded by standard pyrethroid LLINs, hence improved efficacy with PBO LLINs against resistant mosquitoes compared to standard pyrethroid only LLINs. These plus the results from a cluster randomized trial have shown that PBO-LLINs provide better control of malaria than standard nets in Tanzania, whereby Olyset Plus (PBO) nets provided a 33% malaria case reduction compared to Olyset nets at 21 months (Protopopoff et al., 2018) post-distributiojn. This further demonstrates the advantage of using PBO LNs over standard pyrethroid LNs against resistant mosquitoes and demonstrate that the marginal improvement documented in the hut trials documented in this thesis may indeed translate into a major improved in malaria control.

The similarity of Veeralin LN which is the PBO LN with PBO treated on whole net to PermaNet 3.0 not only shows the similarity in efficacy between a PBO LN with PBO restricted on the roof panel only to PBO LN with PBO treated on whole net provides evidence that the 2-in-1 concept as a tactic for managing resistance management and for donors and NMCPs effective procurement decision making.

b) **To identify and evaluate dual pyrethroid with non-pyrethroid insecticide (chlorfenapyr) combination LLINs for improved control of pyrethroid resistant mosquitoes.**

In chapter 12 of this thesis, studies were performed to evaluate the efficacy of mosquito nets treated with a mixture of a pyrethroid and a non-pyrethroid for improved vector control with LLINs. The rationale for mixing pyrethroids with a non-pyrethroid insecticide on bed nets was to enhance toxicity to pyrethroid resistant mosquitoes through the non-pyrethroid compound while maintaining the protective excito-repellent property of the bed net through the pyrethroid.

In chapter 11 of this thesis which described a meta-analysis of two experimental hut trials, mortality induced by IG2 against *An. funestus* was 3.4 times higher than with untreated nets and 2.3 times higher than with IG1. The comparison of mosquito mortality between the IG2 unwashed and IG2 washed 20 times produced a relative risk of 1.04 (CI 0.83–1.30) indicating no loss of efficacy of IG2 over 20 washes. This means IG2 exceeds by a factor of 2.3x the mortality of standard LLIN against resistant mosquitoes. Considering the impact of ITNs and LLINs on malaria burden between 2000 and 2015, it is reasonable to anticipate that IG2 and perhaps other Dual-AI will achieve comparative control of pyrethroid-resistant mosquitoes as standard LLIN once did against susceptible mosquitoes. This depends on how universal the margin is shown by the chlorfenapyr component compared to other AI's. Recent evidence suggests that chlorfenapyr may act on the mitochondrial function of *Plasmodium* in the midgut as well as in mosquito flight muscle (N'Guessan et al, 2013). This shows something long suspected:- that chlorfenapyr is multifunctional in mode of action (killing parasites and insects by their capacity to disrupt mitochondrial function) and that experimental huts do not necessarily replicate all modes of action shown in the field.

Hence, cluster randomized trials (CRT) of IG2 [13-15], PBO LN and Pyriproxyfen LN [16] published in 2022 and last year , targeting resistant *Anopheles gambiae*, sensu stricto (s.s.) in Tanzania and *Anopheles coluzzii* in Benin, have both shown that among children aged under 10 years a 45% and 56% reduction in incidence of malaria under Interceptor G2 over the 2 years of the trial compared to pyrethroid-only LLINs correrate very much

to my EHT predictions that showed increase from 30% to 64% mortality for Interceptor to Interceptor G2 LLIN respectively. Interceptor G2 was considerably more effective that PBO-pyrethroid LLIN and PPF LN in these CRT trials.

This epidemiological evidence of effectiveness convinced World Health Organization (WHO) to recommend the Interceptor G2 as a new class of LLIN for malaria control in areas with insecticide resistant mosquitoes. Interceptor G2 was more cost effective than standard pyrethroid only LLIN. This provides crucial information for malaria control programmes in endemic areas and for donors who have been hesitant to purchase next-generation LLINs because of their relatively higher cost. The pyriproxifen-pyrethroid ITNs were also given a conditional recommendation by WHO on the basis of these trials.

Concluding points

The project was designed to evaluate in laboratory, experimental huts and field trials, new standard pyrethroid LLIN products, long-lasting treatment kits, and mixture/combination LLIN. This provided evidence for new WHO LLIN evaluation guidelines, suggestions for improved vector control, and for studying the added efficacy of combination and alternative insecticides for use on nets to overcome problems of insecticide resistance. Some useful conclusions can be drawn from the thesis:

a) The similarity in insecticide content between Phase III household randomized trial after 3 years and Phase II experimental hut trial indicates that the 20 standardized washes which LLINs undergo before testing in experimental huts is a fair approximation to the average loss of insecticide due to wear and tear, abrasion and washing that LLINs undergo during 3 years of household use. The 20 standardised washes that LLIN are subjected to in WHO Phase II are a good approximation to Phase III after 3 years and hence validate interim recommendation given to product after passing phase II evaluations. The similarities in outcome observed between incorporation LLIN (DuraNet) and

coating LLIN (Interceptor) indicate that the type of polymer and weave is less pertinent than the type of pyrethroid used, the manufacturing quality meets high WHO standards.

b) The efficacy and wash fastness of ICON maxx raises the prospect of conventional polyester nets and other materials being made long-lastingly insecticidal through simple dipping in community or home and thus represents a major advance over conventional pyrethroid treatment kits and may provide an answer to the problem of reduced LLIN coverage between distribution campaigns.

   The high efficacy, wash-fastness and versatility of ICON Maxx raises the prospect of it becoming an all-purpose formulation for such purposes as military clothing, civilian bed covers and curtains, or for blankets, tarpaulins and tents distributed in epidemics, disasters or humanitarian emergencies.

   As a means of converting pyrethroid-only nets into long lasting mixture nets, there is opportunity for a PBO-binder long-lasting kit to be applied to any pyrethroid LLIN to convert these to pyrethroid-PBO LLIN. This could apply equally to other partner AI, such as pyriproxyfen or chlorfenapyr which are being used with pyrethroid in other types of Dual-AI LLIN.

c) The longitudinal resistance study outcomes present evidence that resistance in Tanzania is undermining the efficacy of LLINs. Although the study presents evidence of insecticide resistance having a negative entomological impact on LLIN efficacy, use of standard LLIN was still better and preferable to untreated nets. Use should be restricted to areas of low resistance of which there are few. The entire country needs to switch to PBO and chlorenapyr nets. Comparison of PBO nets with two different treatment, that is PermaNet 3.0 with PBO treated only on the top panel of the net and VeeraLIN that has PBO treated in whole net showed no significant difference in efficacy between PBO LN that is partially treated (PBO only on the roof pane) compared to PBO LN that has PBO treated on whole net. This is important information for effective procuring decisions to NMCPs and all net producers.

d) PBO LLIN function by a combination of classical synergy (metabolism) and by irritability and contact repellency, which enhances mortality, reduces penetration of holes and reduces blood-feeding.IG2 exceeds by a factor of 2.3 the mortality of standard LLIN against resistant mosquitoes. it is reasonable to anticipate that IG2 and other Dual-AI will achieve comparative control of pyrethroid-resistant mosquitoes as standard LLIN once did against susceptible mosquitoes. If resistance to chlorfenapyr arises, as coverage is scaled up, there seem few other products on the horizon. Good PBO LLIN should be used first before IG2. IG2 is precious as it is the only insecticide alternative to pyrethroids on a net so needs to be preserved against selection for resistance.

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APPENDICES

Appendix 1: General Methodology

2.1. Field Site

All field studies took place in and around NIMR field station in Muheza Tanzania. The district topography provides a unique setting by having a wide variation of transmission patterns over an altitude gradient that rises from sea level to over 1500m above sea level over 70 km.

The field studies involve the use of experimental huts to evaluate product performance under natural conditions. The site has a strong history of such studies: Amani Medical Research Centre in Muheza is the hub of collaborations with the National Institute for Medical Research (NIMR). Muheza is an appropriate test site because of high intensity transmission and almost year-round *An. gambiae s.s.* productivity. *An. funestus*, another primary vector is present for several months a year, and work on these two species can be done concurrently. *Anopheles arabiensis* is also present in this area at a lesser extent.

While the primary vector in Africa is *An. gambiae*, in many areas *An. arabiensis* predominates or co-exists with *An. gambiae*. *An. arabiensis* is a less efficient vector than *gambiae* and tends to occur in less humid areas or during drier seasons of the year, as it possesses physiological adaptations that enable survival in more arid conditions [1, 2]. *An. arabiensis* is also associated with irrigated rice systems [3] and urban environments [4].

Therefore, by working in these project sites it is possible to target the main vector species and strains of *An. gambiae* insecticide.

The site employs entomologists and technicians working for Amani Medical Research Centre [22] who are experienced in bioassay techniques, mosquito identification and overnight evaluations at the field site.

NIMR Amani Centre has carried out research on malaria in Muheza district for over the last sixty years [23]. Several entomological studies have provided substantial data on vectors and transmission levels.

2.2.Mosquito rearing

Mosquitoes for bioassays performed in the laboratory were reared in the insectary at Ubwari field station which is under NIMR-Amani center. The author is also the manager of the insectary and supervises the mosquito rearing at this site. The mosquito species reared in this insectary include *Anopheles gambiae s.s.* (Kisumu), this is a pyrethroid susceptible strain originally from Kisumu Kenya.

Anopheles gambiae s.s. (Zeneti). This is a pyrethroid resistant strain originally sampled from Zeneti village in Muheza, the location of the experimental huts. This colony was colonized in 2016. The colony shows 40% resistance to pyrethroids with over 30-fold intensity of resistance. The colony has *kdr* gene at 44% allelic frequency and elevated P450 enzymes.

Culex quinquefasciatus (TPRI), insecticide susceptible strain maintained by the Tropical Pesticide Research Institute, Tanzania and *Culex quinquefasciatus* (Masimbani), a multiple resistant strain from NE Tanzania, expressing elevated oxidase and *kdr* pyrethroid resistance mechanisms. In WHO tests it shows 53% mortality on exposure to permethrin 0.75% test papers, 52% mortality on deltamethrin 0.05%, 42% mortality on DDT 4%, 73% mortality on malathion 5% and 54% mortality on propoxur 0.1% test papers. Insectary conditions are maintained at 27± 2°C and 80 ± 10% relative humidity for the adult holding room and experimental rooms.

Larvae are fed on a mixture of fish food (Tetramin®) for *Anopheles* larvae and cat food for *Culex* spp larvae. The larvae are reared from the first instar into batches of approximately 200 per bowl. Once a bowl contains pupae, they are collected into small containers and placed inside an adult

cage for emergence. For rearing adults to be used for experiments, the bowl was removed on the second day to ensure all adults in that cage were of a similar age. Other pupae were placed in stock cages for emergence. Adults were housed in 30cm^3 populations cages covered with mosquito netting. 10% glucose solution is provided in feeders and replaced once a week. Blood-meals normally after every three days, using anaesthetized rabbits after confining the animal and introducing them into the mosquito cages. A Petri dish lined with filter paper and with a small amount of water was provided for egg laying. Illumination was on a daily light/dark cycle of 12:12 hours. In the larval rearing room, the temperature is kept at 30 ± 2 °C and the humidity at 60 ± 5%.

2.3.Tests using adult mosquitoes.

WHO recommendations for the testing of bio-efficacy and residual activity of insecticides on treated surfaces are followed [5].

Insecticide-treated materials tested were evaluated under WHOPES phase I, phase II and phase III criteria [5]. Under the phase I evaluation criteria, bioassays were carried out with insectary reared mosquitoes, under the phase II evaluation criteria, products are tested in experimental hut trials which take place overnight with wild vectors.

This phase comprises bioassay tests, susceptibility tests and tunnel tests which will be performed on new and alternative potentially useful insecticide products and formulations using laboratory reared mosquitoes.

2.3.1.Bioassays

2.3.1.1.Three-minute WHO Cone Bioassays

Four WHO cones were affixed to each sample of netting cut from position 2 to 5 (Figure 2) and 5–10 mosquitoes were introduced into each cone. Mosquitoes used were 2-5-day-old female Anopheles gambiae (Kisumu) that were known to be susceptible to pyrethroid insecticides. Following three minutes exposure they were then transferred to plastic cups. Controls were exposed to untreated netting. Ten mosquitoes tested with the same net piece were held in a

netting-covered paper cup and provided with a pad of glucose solution for nourishment. One-hour post-exposure knocked down (KD) was recorded and the mortality-rate after 24 hours holding in the insectary adult room was also scored. Twenty replicates were carried out per sample giving 100 exposed mosquitoes per treatment. An untreated piece of each material was used as a negative control. If control mortality exceeded 20% on any day the results were disregarded and the test repeated, this applies to all tests described in this study. All bioassays were carried out on a rotation basis to avoid bias for any one treatment type due to variation in insect batch and local conditions.

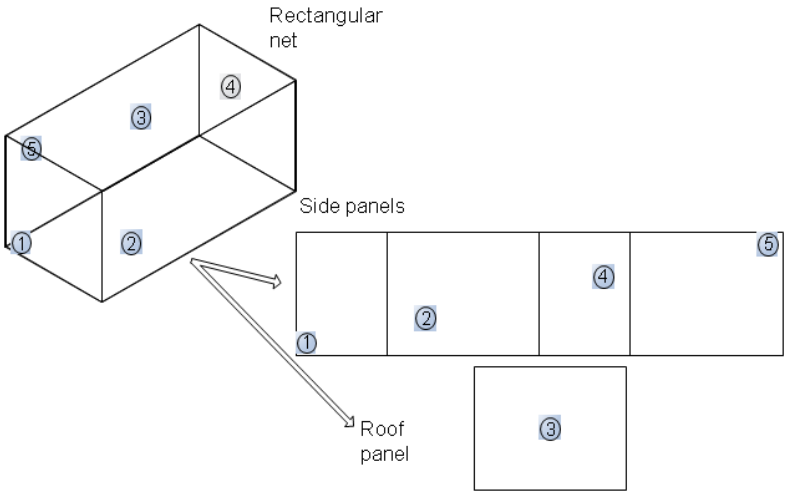

Figure 2: Numbering of net panels

2.3.1.2.Tunnel tests

These are carried out in apparatus described by WHO [5] with minor modifications. We used double barriers to prevent mosquitoes from contacting the net except after undertaking host orientation flights. The first barrier was a paper screen with a 1cm diameter hole separating the release chamber from the middle chamber. The second barrier was the test netting, with a total of nine 1cm diameter holes [6]. At each end of the tunnel, a 30-cm square cage is fitted and covered with polyester netting. In the first chamber at the other end of the tunnel, 100 non-blood-fed laboratory reared female mosquitoes, aged 5–8 days, are introduced at 18:00 hours. Females are free to fly in the tunnel but had to contact the treated piece of netting and locate

the holes in it before passing through to reach the bait. After a blood meal, they fly to the cage at the end of the short section of the tunnel. The following morning, at 08:00 hours, the mosquitoes are removed and counted separately from each section of the tunnel and the immediate mortality are recorded. Live females are placed in plastic cups with honey solution provided; delayed mortality is recorded after 24 hours. During tests, cages are maintained in a climatic chamber at 27±2ºC and 80±10% relative humidity under subdued light. Overall mortality is measured by pooling the immediate and delayed (24-hour) mortalities of mosquitoes from the two sections of the tunnel. Mortality rates in treated conditions are corrected using the Abbott formula and binomial exact 95% CIs for calculated for the corrected values.

Mosquito netting

Details of the netting materials used are given in the appropriate chapters.

Insecticides

Permethrin, deltamethrin, and alphacypermethrin were used for treatment of nets. All The following formulations of insecticide used are given in the appropriate chapters:

2.4.Phase II net washing procedures

Each net for experimental hut trials was washed individually in 10 liters of tap water containing 2 g/liters of soap ('Savon de Marseille'), subjected to 20 rotations per minute for 6min during a 10min immersion, then rinsed twice.

2.5.Determination of point of insecticide exhaustion or cut-off point

The point of insecticide exhaustion or cut-off point, as defined by WHOPES, is the number of washes at which the net causes less than 80% mortality and 95% knock-down in WHO cone bioassays conducted after each wash [16]. Determination of the point of exhaustion was carried out by exposing unfed *An. gambiae* s.s. Kisumu in ten replicates of five mosquitoes after each wash interval on the five panels of the CTN. Exposure was for 3 min, knock-down was scored after 60 min and mortality was scored 24 hr later.

2.6.Experimental hut trials on natural vector populations

This comprise of evaluation in experimental huts under controlled conditions. This was performed on insecticide products and formulations using wild free flying mosquitoes.

2.6.1.Study areas and experimental huts

The huts trials for this thesis used experimental huts Muheza and Moshi sites. The Muheza huts are located at a field site of the Amani Medical Research Centre in Zeneti village. The village is located 30 km from Muheza district northern eastern Tanzania between 5^{o}13’24” S and 38^{o}39’96” at an altitude of 192.9m above sea level. The area around Muheza is characterized with high malaria prevalence caused mainly by *Plasmodium falciparum* that is transmitted by *Anopheles gambiae* s.s. mosquitoes during the rain season and *Anopheles funestus* mosquitoes during the dry season [10]. The area has a typical entomological inoculation rate (EIRs) of 34–405 infective bites per person per year [11]. *Anopheles gambiae* s. s. is the predominant vector in the wet season while *Anopheles funestus* is predominant in the dry season [10]. Susceptibility studies carried out by the national insecticide resistance monitoring committee showed that up to 2009 the vector population was susceptible to pyrethroids [12] and it was after 2010 that resistance started to be recorded in the local vectors.

The experimental huts are identical in design at both sites. They are slightly modified from that described by Smith [13] constructed to a design described by the World Health Organization [7] and others [14-16]. They are made of fired mud brick walls plastered with mud on the inside, a wooden ceiling lined with hessian sackcloth. The roof is made of corrugated iron; there is an eave below the roof measuring approximately 4 cm around the hut. There is a window trap on each wall and a screened veranda on each side. The huts were built on concrete plinths and surrounded by a water-filled moat to deter entry of scavenging ants. The working principle of these huts has been described by Smith and Webley [17]. Each day of the trial, two opposite sides of the huts had closed verandas, screened to capture mosquitoes that left via the eaves. The other two verandas were left open such that mosquitoes could enter through the eaves. Each night’s collection inside the two screened veranda traps was multiplied by two and added to the room and window trap collections. In 2014 modification was done to the eave gape so that in

every day of the trial the two verandahs that are unscreened so that mosquitoes can enter through eaves but are restricted from exiting by eave baffles [24]. The multiplication was made to adjust for the unrecorded escapes through the two verandas which were left unscreened to allow routes for entry of wild mosquitoes via the 4cm gaps under the eaves. White cloth sheets are laid over the floor each night to ease the collecting of knocked down mosquitoes in the morning. Sugar solution is provided at night in verandahs and exit traps to reduce any control mortality.

At the end of each rotation, the north and south verandas were closed, and east and west sides opened, or vice versa, to compensate for possible selective exiting selectively in one compass direction.

2.6.2.Experimental Hut Evaluations

Adult men served as volunteer sleepers. Sleepers give informed consent prior to the trial. They sleep in the huts from 19:30 to 06:30 each night. All treatments and sleepers are rotated using a Latin square design according to a pre-arranged schedule to control for any variation due to differences in sleepers' individual attractiveness to mosquitoes. As described in specific chapters, normally six treatment arms and target application rates are evaluated.

Each net is deliberately holed with six holes (4 cm × 4 cm) to simulate a torn net. White sheets are laid over the verandah & room floors to ease the collection of knocked down mosquitoes. Each morning mosquitoes are collected from the floor, walls, exit traps and inside the nets, and scored as dead or alive and as fed or unfed. Live mosquitoes are held in paper cups supplied with cotton swab socked in glucose solution for 24 hours to assess delayed mortality. The huts are washed before each treatment rotation and windows and verandah's left open to reduce any risk of insecticide carry over.

2.6.3. Experimental huts trials entomological outcomes

The impact of each treatment was expressed in terms of the following entomological outcomes.

1. Deterrence – the reduction in entry into treatment hut relative to the control huts (i.e., those containing untreated nets).

2. Treatment-induced exiting - the proportion of mosquitoes found in exit traps of treatment huts relative to the same proportion in control huts.

3. Mortality - the proportion of mosquitoes killed relative to the total catch size.

3. Overall killing effect - the numbers killed by a treatment relative to the untreated control, as derived from the formula

- killing effect (%) =100(Kt−Ku) Tu
- where Kt is the number killed in the huts with treated nets, Ku is the number dead in the huts with untreated nets, and Tu is the total entering the huts with untreated nets.

4. Blood-feeding inhibition - the proportional reduction in blood feeding in huts with treated nets relative to controls with untreated nets

5. Personal protection - the reduction in mosquito biting by treated nets relative to untreated nets, as derived from the formula

- %Personal protection=100(Bu−Bt) Bu
- where Bu is the total number blood-fed mosquitoes in the huts with untreated nets, and Bt is the total number blood-fed in the huts with treated nets.

2.7. WHO insecticide susceptibility tests

The susceptibility tests were carried out using WHO test kits for adult mosquitoes [8] lined with using WHO test papers impregnated with discriminating dosages of 0.75% permethrin, 0.05% deltamethrin and 0.05% lambda-cyhalothrin (obtained from Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia). The quality of the test paper was checked against the laboratory susceptible *An. gambiae s.s.* Kisumu strain. Mosquitoes used in this test were the 2-5 days old female FI progeny of the mosquitoes. The rearing and testing were done in such a way that mosquitoes collected in the untreated control huts were tested separately to the mosquitoes collected from the huts with the treated nets. For each test, batches of 15- 25 adult 2-5 days old female F1 mosquitoes reared from field-collected larvae were aspirated from paper cups and transferred into the holding tubes where they were held for 1 hour before

transfer to exposure tubes lined with test papers and exposed for 1 hour. After exposure, the number of mosquitoes knocked down was recorded and the mosquitoes were then transferred into holding tubes. A cotton pad soaked in 10% sugar was placed on top of the holding tube.

The mortality was scored 24 hours post-exposure, and each test was replicated at least four times. The resistance or susceptibility status was evaluated based on the WHO criteria [8]. The resistance or susceptibility status were evaluated based on the WHO criteria i.e., 98-100% mortality indicate susceptibility; 90-97% mortality required confirmation and less than 90% mortality indicate possible resistance [8]. When the control mortality was scored between 5% and 20%, the mean observed mortality was corrected using Abbott's formula [9].

2.8.Molecular analysis

Molecular identification of members of the Anopheles gambiae species complex

Details of the molecular identification of members of the Anopheles gambiae species complex are given in the appropriate chapters.

Detection of knock down resistance (kdr) alleles in Anopheles gambiae complex.

Details of detection of knock down resistance (kdr) alleles in Anopheles gambiae complex.
are given in the appropriate chapters.

2.9.Data analysis

The effect of each treatment was assessed relative to the control in terms of deterrence (the number of mosquitoes caught in each hut), excito-repellency (the proportion of mosquitoes in the verandah traps), blood feeding inhibition and mortality rates. Proportional data were analyzed using logistic regression to estimate the outcomes within each trial, comparing results for treated and untreated nets clustering by day and adjusting for variation between individual sleepers and huts using STATA® statistical analysis software package version 13 (Stata Corporation, Collage Station TX, USA). Regarding non-normality of the data the numbers of blood-fed and dead mosquitoes and overall totals collected from each hut were compared using negative binomial regression.

An estimate for percentage personal protection of each treatment was derived from the proportions of mosquitoes deterred from hut entry and inhibited from blood-feeding relative to the control following formula.

Personal protection (%) = $100(B_u - B_t)/B_u$

Where, B_u = total number blood-fed in the huts with untreated nets.

B_t = total number blood-fed in the huts with treated nets.

An estimate for percentage the overall killing effect of the treatment was derived from number dead in treated nets and number dead in untreated hut out of total collection using the following calculation:

Insecticidal effect (%) = $100(K_t - K_u)/T_u$

Where K_t = number killed in the huts with treated nets,

K_u= number dead in the huts with untreated nets, and

T_u= total collected from the huts with untreated nets.

Abbott's formula was used to correct the observed mortality in adult susceptibility tests [9] when the mortality in control was between 5% and 20% [8]. Chi-square test was used to test for genotype-dependent mosquito survival in the susceptibility tests, genotype-dependent mosquito survival in huts and genotype-dependent mosquito blood feeding in the huts.

2.10.Ethical Clearance

Approval was obtained from the ethics review committees of the London School of Hygiene and Tropical Medicine, the Tanzanian National Institute of Medical Research (Ref: NIMR/HQ/R.8a/Vol. X/86) and the World Health Organization. Oral and written informed consent was obtained from all the volunteers recruited in the study as sleepers in the huts prior to the start of the experiment and were offered chemoprophylaxis during and for one month after the

experimental hut trial. During the trial all volunteers were monitored each day for signs of fever or possible side-effects of the ITNs/LLINs. The procedure accorded with published guidelines of the World Health Organization and was approved by the Tanzanian National Institute of Medical Research Ethical Review Committee.

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Appendix 2: Evaluation of the long-lasting insecticidal net Interceptor LN: laboratory and experimental hut studies against anopheline and culicine mosquitoes in northeastern Tanzania

Prologue:

Candidate LLIN must demonstrate their effectiveness for malaria vector control after passing through a series of tests organized by WHOPES (WHO Pesticide Evaluation Scheme) to ensure their quality and performance through standard testing procedure for chemical, physical and biological efficacy sufficiently against mosquito vector prior the gratification of interim or full approval by the WHO.

The interim approval is given to a LLIN product after it has been successfully passed phase I and II WHOPES evaluations.

This chapter present and discuss the results of phase I and phase II semi-field (experimental huts) and laboratory evaluation of Interceptor™ LLIN. This net is manufactured by BASF with 200 mg/m2 alpha-cypermethrin coated on polyester fibres [2] which was done as part of their WHOPES phase II evaluations in verandah trap experimental huts of the National Institute for Medical Research (NIMR) in Muheza, northeastern Tanzania, against wild free-flying *An. gambiae* and *An. funestus* mosquitoes.

This study was led by Dr R Malima as first author. P Tungu co-supervised the study, led the analysis and co-drafted the manuscript.

Evaluation of the long-lasting insecticidal net Interceptor® LN: laboratory and experimental hut studies against anopheline and culicine mosquitoes in north-eastern Tanzania

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The material presented in this appendix has been published by Robert Malima as:

Robert Malima, **Patrick K Tungu**, Victor Mwingira1, Caroline Maxwell, Stephen M Magesa, Harparkash Kaur, Matthew J Kirby and Mark Rowland: **Evaluation of the long-lasting insecticidal net Interceptor LN: laboratory and experimental hut studies against anopheline and culicine mosquitoes in north-eastern Tanzania.** *Parasites & Vectors* 2013, 6:296. Doi:10.1186/1756-3305-6-296

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Abstract

Background

Long lasting insecticidal nets (LN) are a primary method of malaria prevention. Before new types of LN are approved, they need to meet quality and efficacy standards set by the WHO Pesticide Evaluation Scheme. The process of evaluation has three phases. In Phase I the candidate LN must meet threshold bioassay criteria after 20 standardized washes. In Phase II washed and unwashed LNs are evaluated in experimental huts against wild, free flying anopheline mosquitoes. In Phase III the LN are distributed to households in malaria endemic areas, sampled over three years of use and tested for continuing insecticidal efficacy. Interceptor® LN (BASF Corporation, Germany) is made of polyester netting coated with a wash resistant formulation of alpha-cypermethrin.

Methods

Interceptor LN was subjected to bioassay evaluation and then to experimental hut trial against pyrethroid-susceptible *Anopheles gambiae sensu lato* (*s.l.*) populations and *Anopheles. funestus sensu lato* (*s.l.*) and resistant *Culex quinquefasciatus.* Mosquito mortality, blood feeding inhibition and personal protection were compared between untreated nets, conventional alpha-cypermethrin treated nets (CTN) washed 20 times and LNs washed 0, 20 and 30 times.

Results

In Phase I Interceptor LN demonstrated superior wash resistance and efficacy to the CTN. In the Phase II hut trial, the LN killed 92% of female *An. gambiae* when unwashed and 76% when washed 20 times; the CTN washed 20 times killed 44%. The LN out-performed the CTN in personal protection and blood-feeding inhibition. The trend for *An. funestus* was similar to *An. gambiae* for all outcomes. Few pyrethroid-resistant *Cx. quinquefasciatus* were killed and yet the level of personal protection (75-90%) against *Culex* was similar to that of susceptible *An. gambiae* (76-80%) even after 20 washes. This protection is relevant because *Cx. quinquefasciatus* is a vector of lymphatic filariasis in East Africa. After 20 washes and 60 nights' use the LN retained 27% of its initial insecticide dose.

Conclusions

Interceptor LN meets the approval criteria set by WHO and is recommended for use in disease control against East African vectors of malaria and filariasis. Some constraints associated with the phase II evaluation criteria, particularly the washing procedure, are critically reviewed.

Keywords: Long-lasting insecticidal net, LN, Interceptor LN, *Anopheles gambiae*, *Anopheles funestus*, *Culex quinquefasciatus*, Experimental hut

Background

Long lasting insecticidal nets (LN) are an ideal method of preventing malaria in Africa south of the Sahara and many Asian countries [1,2]. They provide good protection against mosquito bites when used regularly [3], they are relatively cheap compared to other methods of personal protection [3], they are simple to distribute to communities [4], and they are popular [2]. The market for LN has burgeoned in recent years. To ensure that LN competing for the market are fit for purpose the World Health Organization, through its Pesticide Evaluation Scheme (WHOPES), has set standards of quality and efficacy that require threshold criteria to be met or surpassed, regardless of the type of netting material, mode of manufacture, pyrethroid compound and long - lasting formulation. For any new LN to enter the market or be considered for tender by the main institutional buyers, it must attain WHOPES recommendation, which means in practice passing through a process of efficacy evaluation that has three phases [5,6]. During the Phase I evaluation

the candidate LN must, after determination of regeneration time, show efficacy in mosquito bioassay over 20 standardized washes. During Phase II, standardized washed and unwashed LN are evaluated against host seeking, free flying mosquitoes in experimental huts. At this juncture the LN may attain interim recommendation from WHOPES [5]. Phase III evaluation requires the LN to meet performance criteria after 3 years of use with families living in malaria endemic areas [6].

Interceptor is a LN developed and produced by BASF Corporation. The netting is a polyester fiber coated with a proprietary polymer containing the insecticide alpha-cypermethrin at 200 mg/m2. The polymer binds to the fiber and can withstand multiple washings, the active ingredient diffusing in a controlled manner to the surface of the polymer coat to maintain insecticidal efficacy [7-9].

This paper reports upon Phase I laboratory and Phase II experimental hut evaluations of Interceptor LN. The laboratory bioassays were conducted in the U.K. using laboratory-reared pyrethroid-susceptible *Anopheles stephensi* mosquitoes. The hut trials were undertaken at the National Institute for Medical Research (NIMR) in Muheza, northeastern Tanzania, against wild free-flying *An. gambiae* s.l, *An. funestus* s.l and *Culex quinquefasciatus* mosquitoes.

Methods

Phase I

Nets and washing process.

Interceptor LN nets were supplied by BASF Corporation (Ludwigshaven, Germany). The polyester net was treated with alphacypermethrin (coated onto filaments) at a target dose of 6.7 g AI/kg of netting material for 75-denier yarn, corresponding to 200 mg of alpha-cypermethrin per square metre of the polyester fabric (with a tolerance limit of ± 25%). Polyester nets of the same denier and source were treated by hand with an aqueous solution of alpha-cypermethrin (Fendona 10SC, BASF) at target dosages of 25 mg/m2 (hereafter CTN25) and 200 mg/m2 (CTN200) for use as positive controls. The washing procedure for Phase I testing of LNs followed the WHOPES guidelines [5,6]. Netting pieces (25 cm × 25 cm) were subjected to standardized

washing for intervals of 5, 10, 15 and 20 times, with a one-day interval between washes, using WHO-approved soap solution (Savon de Marseille) at 2g/L in deionized water at 30°C for 10 minutes in a shaker water bath set at 155 movements per minute. Pieces were then rinsed twice for 10 minutes in clean water.

Three-minute exposure (ball) bioassays

Netting pieces were fixed to a metal frame consisting of two interlocking rings of 11.5 cm diameter [5,10]. Ten 2–5-day old unfed female *Anopheles stephensi* (Beech: pyrethroid-susceptible strain) mosquitoes were exposed for 3 minutes then transferred to holding cups and supplied with a 10% glucose solution. The number knocked down was recorded after 60 minutes and the number dead after 24 hours. Test conditions were 25 ± 2°C and 80 ± 5% RH throughout.

Median knockdown time bioassay

In a separate series of assays, eleven 2–5-day old unfed females were introduced into the netted frame, and as each mosquito was knocked down it was removed using an aspirator. Knockdown was defined as either collapsed against the netting or fallen to the base, and not moving. The time for the median mosquito (6th) to be knocked down was the end point of the test [5,10].

Chemical analysis

Alpha-cypermethrin content of unwashed and washed nets was determined from net samples measuring 5 cm × 5 cm using the method described by Yates et al. [11]. Alpha-cypermethrin was extracted using acetonitrile and injected onto HPLC (Dionex Summit, Camberly, Surrey, UK), separated on a 120Å column, eluted with a 9:1 solution of water: acetonitrile and passed through a PDA-100 detector at 275 nm. From the calibration curve the amount of alpha-cypermethrin on the netting pieces was estimated and the dosage per m^2 calculated.

Phase II

Study area and experimental huts

This study used experimental huts in Muheza Tanzania. Study area and experimental huts are described in more detail in Appendix 1, Section 2.6.1.

Net preparation

The Interceptor LN, untreated nets and alpha-cypermethrin insecticide (Fendona 10SC) were supplied by BASF. All nets were 75 denier polyester and measured 2 m (L)×1.2 m (W) × 1.5 m (H). To simulate wear and tear a total of six 4 cm × 4 cm holes were cut into each net (two holes on each side and one hole at each end). The target concentration of alphacypermethin on the LNs and CTNs was 200 mg/m2. The LNs and CTNs were washed according to WHO Phase II washing protocols [5]. The washing protocol described in appendix 1, Section 2.4 was adhered to. The interval between washes was 1 day which is the established regeneration time for Interceptor LN [9]. The washing schedule was stepped to ensure that the final wash of all treatment arms was completed on the same day.

The CTN washed to the 'point of insecticide exhaustion' served as a positive control against which to assess Interceptor LN performance. The determination of point of exhaustion is described in detail in appendix 1, Section 2.5.

Experimental hut study design

The following five treatment arms were tested in the huts:

1. Unwashed Interceptor LN
2. Interceptor LN washed 20 times
3. Interceptor LN washed 30 times, in accordance with the manufacturer's claim for wash fastness
4. Polyester net conventionally treated with alpha-cypermethrin at 200 mg/m2 and washed 20 times
5. Untreated unwashed polyester net

The primary outcomes were Deterrence, Treatment-induced exiting (exophily), mortality, Overall killing effect, Blood-feeding inhibition and %Personal protection. These outcomes are described in more detail in appendix 1, Section 2.6.2.

Each morning dead and live mosquitoes were collected from the verandahs, room and window traps. Live mosquitoes were provided with 10% sugar solution. Delayed mortality was recorded

after 24h. Mosquitoes were identified to species and gonotrophic status was recorded as unfed, blood-fed, semi-gravid or gravid. Random samples of *An. gambiae s.l.* (n = 60) were identified to species by PCR [17].

The criteria for efficacy were that the Interceptor LN washed 20 times should perform equal to or better than the CTN washed until just before exhaustion. Twenty washes are set by WHO as an approximate number of washes a LN is likely to incur during its lifetime.

The trial took place between May and August 2006. The treatment arms were rotated twice through each hut according to a Latin Square design. A treatment was assigned at random to a particular hut for 6 nights' observation before being transferred to the next hut. Between 19:30 and 6:30 hours adult male volunteers slept on beds under the nets. The same five sleepers were rotated through the huts on consecutive nights. Six nets were available per treatment arm and each net was tested on consecutive nights during the six-night rotation. At the end of the weekly rotation the huts were cleaned and aired for one day before starting the next rotation. Data were collected for 60 nights.

Chemical analysis

Netting samples were taken for determination of alpha-cypermethrin content by HPLC on three occasions: before washing, after completion of the washes, and after conclusion of the trial as described by WHO [5]. Four netting pieces, each measuring 10 cm × 10 cm, were cut from the sides, end and top before and after washing from a 7th net taken from each study arm (these nets were not used in the hut trial), while a net taken from the huts from each study arm was sampled at the end of the trial. HPLC analyses were carried out on each piece as described for Phase I assays, the average amount of alpha-cypermethrin estimated and the dosage per m2 calculated.

Ethical clearance

Ethical clearance was obtained from the Ethics Committees of the National Institute for Medical Research Tanzania (Ref: NIMR/HQ/R.8a/Vol X/86), and the London School of Hygiene and Tropical Medicine (Ref: 8589). Written informed consent was obtained from all volunteers

participating in the study. The risks of malaria were explained, and all volunteers were provided with chemoprophylaxis. During the trial, each volunteer was monitored daily for fever or possible adverse effects due to the LNs or CTNs.

Analysis

The principal aim was to compare the efficacy of Interceptor LN washed 0 and 20 times to a CTN washed until 'exhaustion'. The key outcomes were the overall proportions of mosquitoes' blood-feeding or dying relative to the untreated control. Logistic regression was used to estimate proportional outcomes of treatments (mortality, blood-feeding, exiting), and negative binomial regression was used to analyze counts of mosquitoes entering the huts (personal protection, overall insecticidal effects), after adjusting for clustering by day and for variation between individual sleepers and hut position. Laboratory bioassay data was analysed using logistic regression. Median knock down tests and chemical analysis was analysed using analysis of variance. Further details are described in appendix 1, section 2.8.

Results

Phase I - laboratory tests

Ball bioassay tests: Bioassay tests were done on Interceptor LN and CTN25 (25 mg/m2). The percentage knockdown of *An. stephensi* decreased from 100% to 70% on exposure to the CTN25 washed 0 and 20 times ($p = 0.01$) and from 100% to 96% on exposure to the LN washed 0 and 20 times ($p = 0.03$) (Figure 1a). Percentage mortality was 55% on the CTN25 and 99% on the LN after 5 washes and decreased to 14% and 29% respectively after 20 washes ($p = 0.01$) (Figure 1b).

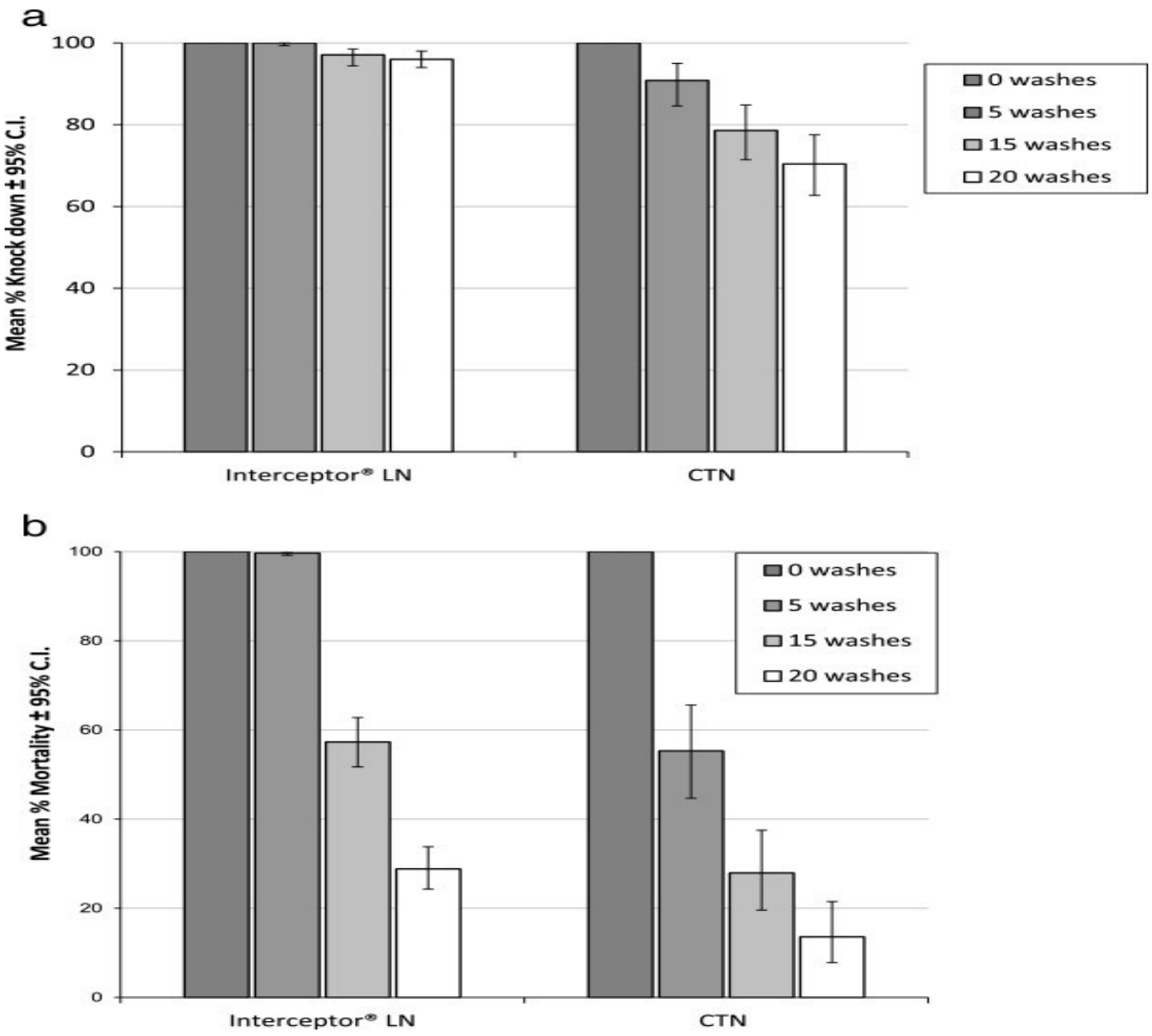

Figure 1
Response of *Anopheles stephensi* exposed in 3-minute ball bioassays to Interceptor LN and alpha-cypermethrin CTN25: a. Knockdown. b. Mortality.

Median knock down tests (MKDT): After each sequence of washes median knockdown time was extended, indicating the removal of surface insecticide. At each wash point MKDT took longer on the CTN25 than on the Interceptor LN indicating a lower surface concentration of insecticide on the CTN25 ($p = 0.01$) (Figure 2). There was correlation between percentage knockdown and the dosage of insecticide remaining on the netting after washing ($r2= 0.46$, $p < 0.001$) and between percentage mortality and dosage remaining ($r2= 0.61$, $p = 0.001$).

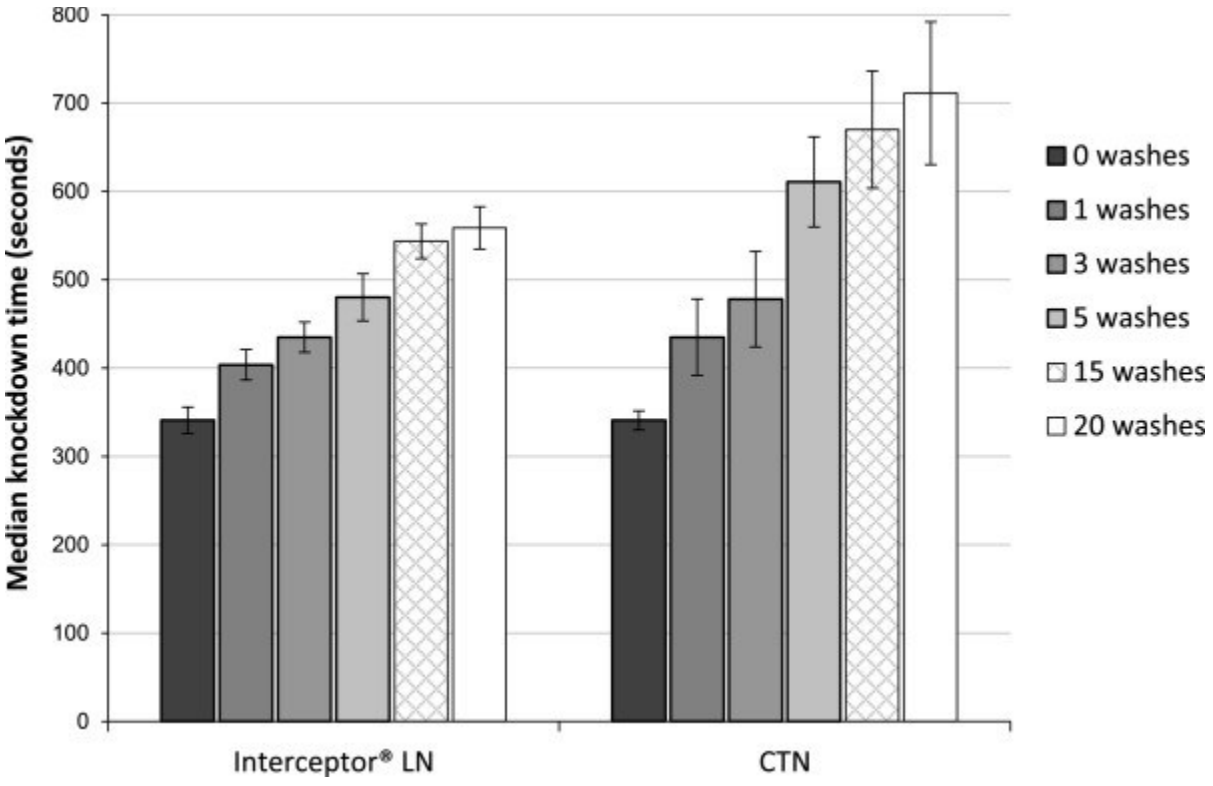

Figure 2
Median time to knockdown of *Anopheles stephensi* exposed in 3-minute ball bioassays to Interceptor LN and alpha-cypermethrin CTN25.

Chemical analysis: The alpha-cypermethrin content of the Interceptor LN decreased by 21% after 5 washes, from 209 mg/m2 to 166 mg/m2, and by 74% after 20 washes to 55 mg/m2 (Figure 3). The content of the CTN25 and CTN200, initially treated with 25 mg/m2 and 200 mg/m2, fell by 93% and 96% respectively after just 5 washes and by the 15th wash no alpha-cypermethrin was detectable by HPLC on either net.

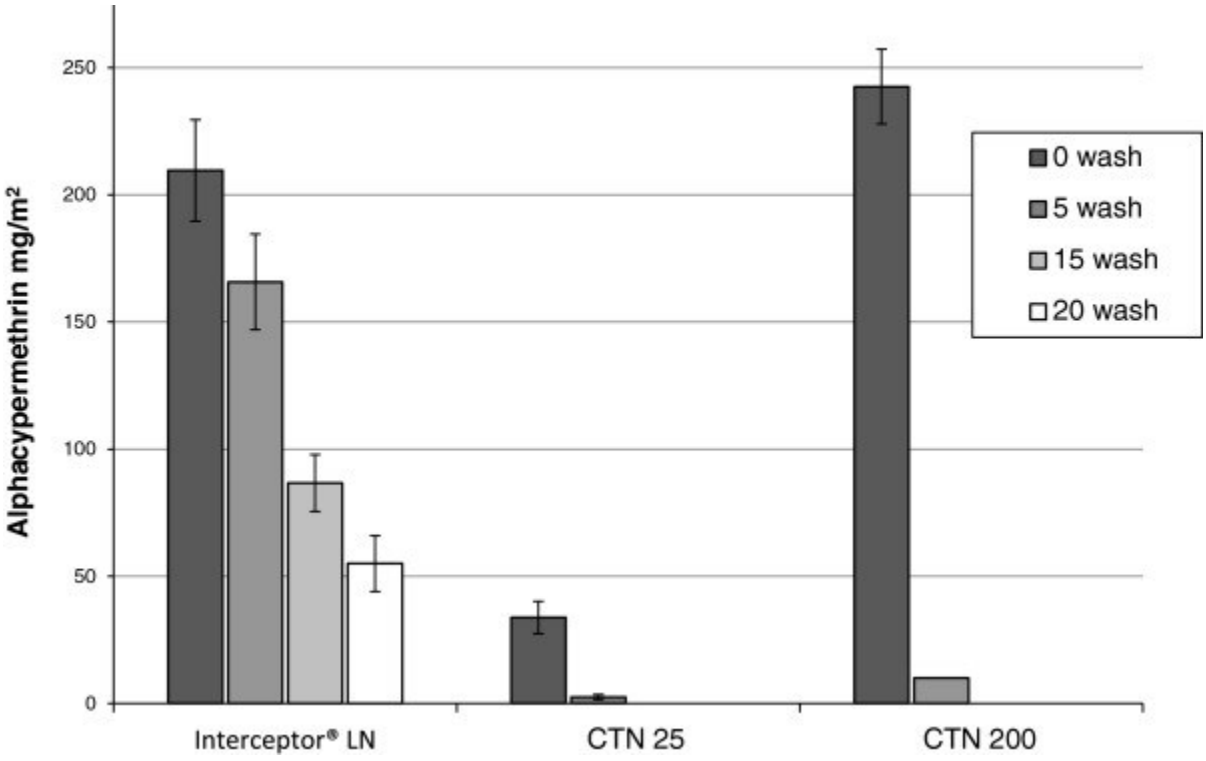

Figure 3
Chemical analysis of Phase I nets.

Phase II - experimental hut trial

Insecticide susceptibility

Susceptibility tests using WHO kits confirmed full susceptibility to alphacypermethrin in *An. gambiae* (100% mortality, N=351) and *An. funestus* (100% mortality, N=401). *Culex quinquefasciatus* were resistant to pyrethroid (52% mortality, N=234) and the ratio of time to 50% knockdown compared to a laboratory susceptible strain (TPRI) was 5.1 (95% confidence interval: 4.2 - 6.0).

Mosquito entry into the huts

A total of 1,836 female mosquitoes were collected over the 66 nights of the trial (Table 1). These consisted of 834 (45.4%) *Anopheles gambiae*, 440 (24.0%) *An. funestus* and 562 (30.6%) *Culex quinquefasciatus*. The mean number caught per night was 12.6 An. gambiae, 6.7 *An. funestus* and 8.5 *Cx. quinquefasciatus*. There was no straightforward evidence of deterred entry associated

with any of the treatments (Table 1). Relative to the untreated net there were fewer *An. gambiae* in huts with the unwashed LN and LN washed 20 times, but this trend was not apparent for the other species or for the CTN treatment.

Table 1
Number of wild mosquitoes entering the experimental huts during the trial of Interceptor LN

	Untreated net 0 W	Interceptor 0 W	Interceptor 20 W	Interceptor 30 W	CTN 20 W
Anopheles gambiae					
Total females caught.	171	134	122	183	224
Average catch per night	2.8[a]	2.2[b]	2.0[b]	3.0[a]	3.7[c]
Anopheles funestus					
Total females caught.	81	68	87	79	125
Average catch per night	1.3[a]	1.1[a]	1.4[a]	1.3[a]	2.1[b]
Culex quinquefasciatus					
Total females caught.	95	95	106	106	160
Average catch per night	1.6[a]	1.6[a]	1.8[a]	1.8[a]	2.7[b]

Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).

Blood feeding inhibition and personal protection

Blood feeding rates in huts with the holed untreated nets ranged from 32.1% for *An. funestus*, 45.6% for An. gambiae and 52.1% for *Cx. quinquefasciatus* (Figure 4). Relative to the untreated nets the blood feeding rates through the unwashed LN was 2.0 (32.1%/16.2%) times less for *An.*

funestus, 2.9 (45.6%/11.9%) times less for *An. gambiae* and 9.8 (52.1%/5.3%) times less for *Cx. quinquefasciatus*. The percentage blood feeding inhibition associated with unwashed LN was 49.6%, 73.8% and 89.8% respectively against these species (Table 2). The blood feeding rates with the LN washed 20 times did not differ significantly from the unwashed LN, and the levels of blood feeding inhibition also hardly changed. After 30 washes there were significant increases in percentage blood feeding relative to the unwashed LN; however, even after 30 washes the blood-feeding rates were still 50% less for An. gambiae, 64% less for *Cx. quinquefasciatus* and 25% less for *An. funestus* for the LN relative to the untreated net (Table 2). The percentage blood feeding inhibition associated with the CTN washed 20 times was significantly less than for the LN washed 20 times with respect to *An. gambiae* (p=0.01) and *Cx. quinquefasciatus* (p=0.01) but not to *An. funestus*, owing to the lower abundance.

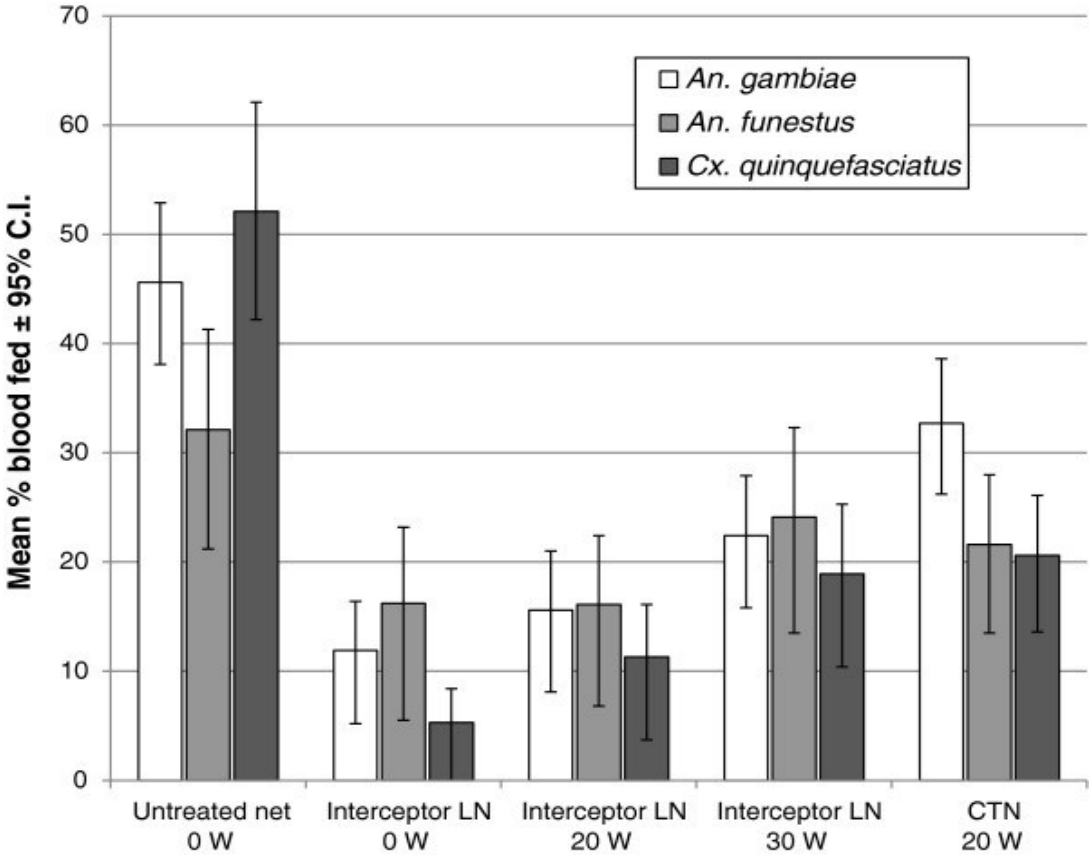

Figure 4

Blood feeding success in experimental huts with Interceptor LN and alpha-cypermethrin CTN versus untreated control.

Table 2
Blood-feeding inhibition and personal protection rates due to Interceptor LN and CTN in the experimental hut trial

	Untreated net 0 W	Interceptor 0 W	Interceptor 20 W	Interceptor 30 W	CTN 20 W
Anopheles gambiae					
Total blood fed	78	16	19	41	73
% Blood feeding inhibition	0^{a}	73.8^{b}	65.8^{bc}	50.9^{c}	28.3^{d}
% Personal Protection	0^{a}	79.5^{b}	75.6^{b}	47.4^{c}	6.4^{a}
Anopheles funestus					
Total blood fed	26	11	14	19	27
% Blood feeding inhibition	0^{a}	49.6^{b}	49.8^{b}	24.9^{ab}	32.7^{ab}
% Personal Protection	0^{a}	57.7^{b}	46.2^{b}	26.9^{b}	0^{a}
Culex quinquefasciatus					
Total blood fed	49	5	12	20	33
% Blood feeding inhibition	0^{a}	89.8^{b}	78.3^{bc}	63.7^{cd}	60.4^{d}
%Personal protection	0^{a}	89.8^{b}	75.5^{bc}	59.2^{c}	32.7^{d}

Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).

Personal protection examines the relative number of mosquitoes that blood feed in the presence of treated nets compared to untreated nets. Because there was little, or no deterrence associated with the alpha-cypermethrin treated nets the levels of percentage personal protection were

quite similar to percentage blood feeding inhibition of each treatment. Personal protection with the unwashed LN and the LN washed 20 times was over 75% against *An. gambiae* and *Cx. quinquefasciatus*, and over 45% against *An. funestus*. There was no evidence of personal protection from the CTN washed 20 times against the anophelines.

Mortality and overall killing effect.

The unwashed Interceptor LN killed 91.9% of *An. gambiae* that entered the hut (Figure 5, Table 3). This fell to 76.2% mortality after 20 washes and to 60.0% after 30 washes. This observed decline in efficacy was statistically significant (p=0.001). The mortality induced by Interceptor LN washed 20 times was significantly higher than the mortality induced by the alpha-cypermethrin CTN washed 20 times (p=0.01). *An. funestus* showed similar trends but with lower rates of mortality compared to *An. gambiae* for the Interceptor treatments (Figure 5, Table 3). The difference in mortality between the Interceptor LN washed 20 times and the CTN washed 20 times was not significant. The LNs and ITNs killed only 8-20% of *Cx. quinquefasciatus*, this species being resistant to pyrethroids.

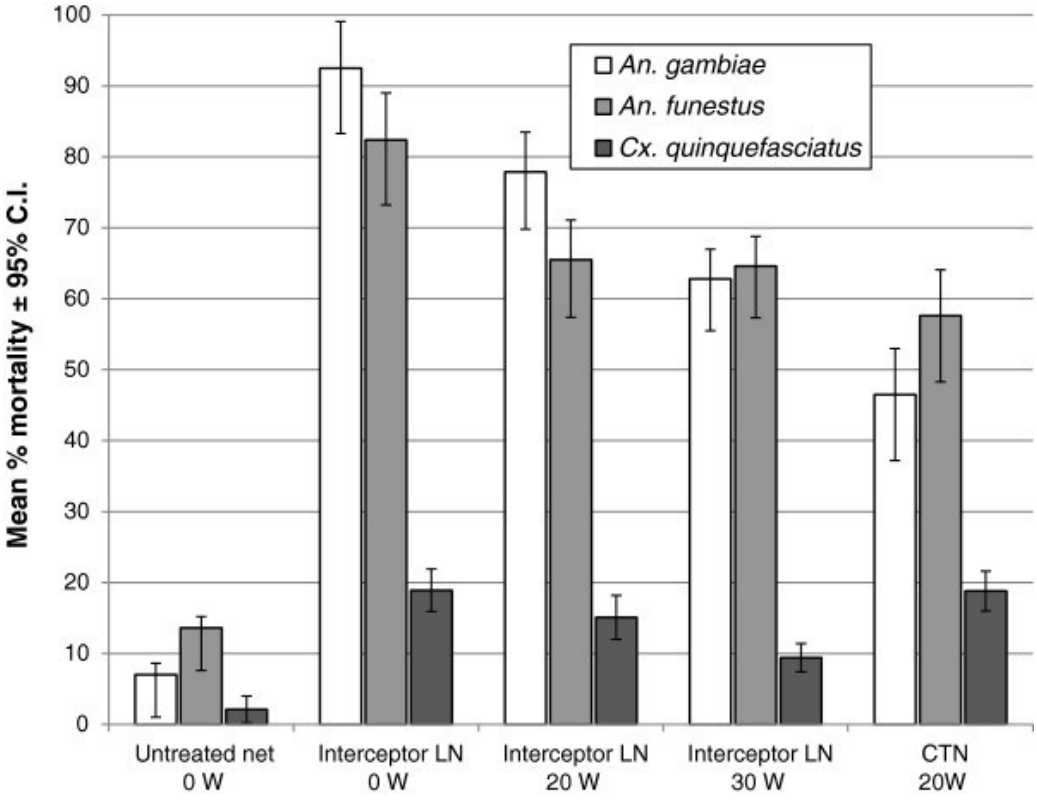

Figure 5 Mortality rates in experimental huts with Interceptor LN and CTN versus untreated control.

Table 3: Mortality and overall killing effect of Interceptor LN in the experimental hut trial.

	Untreated net 0 W	Interceptor 0 W	Interceptor 20 W	Interceptor 30 W	CTN 20 W
Anopheles gambiae					
Total females dead	12	124	95	115	103
% Corrected mortality	0[a]	91.9[b]	76.2[c]	60.0[d]	44.2[e]
% Overall killing effect	0[a]	70.4[b]	52.2[d]	64.8[bc]	57.2[cd]
Anopheles funestus					
Total females dead	11	56	57	51	72
% Corrected mortality	0[a]	79.6[b]	60.1[c]	59.0[c]	50.9[c]
% Overall killing effect	0[a]	64.3[b]	65.7[b]	57.1[b]	87.1[c]
Culex quinquefasciatus					
Total females dead	2	18	16	10	30
% Corrected mortality	0[a]	17.2[b]	13.3[b]	7.5[c]	17.0[b]
% Overall killing effect	0[a]	17.2[bc]	15.1[b]	8.6[b]	30.1[c]

Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).

The overall killing effect of the Interceptor LN against the two Anopheline species ranged between 70-50% over 0 and 30 washes but killed less than 20% of *Cx. quinquefasciatus* even when unwashed.

Exiting rates

The majority of *An. gambiae* and *An. funestus* (>86%) naturally exited the huts before dawn and were collected from the verandah and window traps (Table 4). The proportions exiting huts with LN were rather higher than from huts with untreated nets. In contrast to the Anophelines, around 50% of *Cx. quinquefasciatus* remained in the hut each morning (Table 4). However, in huts with pyrethroid nets, between 81% and 96% of Culex were induced to exit into the traps before dawn.

Table 4: Percentage of mosquitoes exiting huts into verandah and window traps in the Interceptor LN experimental hut trial

	Untreated net 0 W	Interceptor 0 W	Interceptor 20 W	Interceptor 30 W	CTN 20 W
Anopheles gambiae					
Total females exiting	147	116	113	174	207
% Exiting	86.0[a]	86.6[a]	92.6[ab]	95.6[b]	92.8[ab]
Anopheles funestus					
Total females exiting	70	65	86	76	120
% Exiting	86.4[a]	95.6[ab]	98.9[ab]	96.2[ab]	96.0[ab]
Culex quinquefasciatus					
Total females exiting	51	91	94	94	130

	Untreated net 0 W	Interceptor 0 W	Interceptor 20 W	Interceptor 30 W	CTN 20 W
% Exiting	54.0[a]	95.8[b]	88.7[bc]	88.7[bc]	81.2[cd]

Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).

Chemical analysis and bioassays on nets

The alpha-cypermethrin content on the nets before and after washing is summarized in Table 5. Chemical analysis showed that the initial dosages of the active ingredients were below the expected dosages, both for CTN (147 mg/m2 instead of 200 mg/m2) and for LN (132-142 mg/m2 instead of 200 mg/m2). The tolerance limit of alpha-cypermethrin on the LN is 200 mg AI per m2 ± 25% [6] and, as such, the alpha-cypermethrin content in unwashed Interceptor was found to be close to the lower tolerance limit. After 20 and 30 washes the concentrations on the LN had decreased to 41 mg/m2 and 21 mg/m2 respectively. In contrast, the concentration in alpha-cypermethrin CTN was only 1-3 mg/m2 after 20 washes. After 60 days of use in experimental huts, further decrease of alpha-cypermethrin was not evident.

Table 5: Chemical analysis of alpha-cypermethrin on the Interceptor LN and CTN before and after washing and at the end of the experimental hut trial

Treatment	Concentration of alpha-cypermethrin (mg/m^2 ± std dev)		
	Before washing	After washing	After hut trial
Untreated net	-	-	0
Interceptor LN unwashed	147 ± 2	-	140 ± 5
Interceptor washed 20 times.	132 ± 4	41 ± 5	36 ± 3
Interceptor washed 30 times.	-	21 ± 1	21 ± 1
CTN washed 20 times	144 ± 17	1.2 ± 0.4	1.0 ± 0.6

Cone bioassay tests were carried out on these same net samples before and after washing. Before washing the LN and CTN treatments both recorded 100% mortality and 100% knockdown in cone

tests. After 20 washes the LN recorded 100% mortality and 100% knockdown and the CTN recorded 68% mortality and 33% knockdown.

Discussion

The WHO Phase II experimental hut trial demonstrated that the blood-feeding inhibition (the proportional reduction in biting / blood feeding) and percentage mosquito mortality induced by Interceptor LN washed 20 times was superior to that of the CTN washed to exhaustion, and therefore the LN fulfilled the WHOPES criterion of a long-lasting insecticidal net [5]. Based on these results, which formed part of an official WHOPES evaluation, Interceptor LN received interim recommendation as an approved LN [9]. Earlier in 2013 the WHOPES guidelines for testing of LN were revised to include as a positive control a WHOPES-recommended LN with similar specifications to the candidate LN in terms of insecticide, treatment technique, netting material, and washing frequency (0 and 20 times) [6]. The revised guidelines were issued after the current trial and, indeed, Interceptor now constitutes a LN appropriate to use as a positive control against new candidate LN. Because more brands of LN are being submitted to WHO for recommendation, one of the purposes of the revision is to demonstrate that new candidate LN match or exceed the standards set by previously approved LN such as Interceptor. Recent WHOPES trials have included both a reference LN washed 20 times and a CTN washed to exhaustion as comparison arms to ensure that equivalence or superiority of the reference LN to the CTN is being maintained.

The Phase I laboratory bio- and chemical assays confirmed that the Interceptor LN insecticide binding process imparts strong wash-retention characteristics. The laboratory washing regime stripped the alpha-cypermethrin from the conventionally treated net within a few washes (surface content falling from 200 to 10 mg/m2) and to levels undetectable by HPLC within 15 washes. However, in bioassays on the same CTN (washed 15 times) the median mosquito was knocked down after just 11 minutes exposure and mortality reached 28% in the 3-minute bioassays. A similar finding was observed in the Phase II experimental hut trials: the CTN washed 20 times had a surface concentration of only 1.2 mg/m2 and yet this net was still able to kill 44% of An. gambiae and 51% of *An. funestus* that entered and came into contact with it. The only

explanation is that pyrethroids such as alpha-cypermethrin must have a strong affinity to the polyester netting fibres so that even after vigorous washing a thin layer of pyrethroid, virtually undetectable by HPLC yet sufficiently bioactive to induce knockdown and mortality, must remain bound to the fibres.

The performance of the CTN and the level of mortality and knockdown it induced after washing, while being surprising, were still not comparable to those of the LN. Interceptor LN retained a surface concentration of over 40 mg/m2 after 20 washes (30 times greater than the CTN's) and induced significantly higher levels of mortality than the CTN washed the same number of times. The comparison does, however, raise some issues and limitations concerning the WHOPES Phase II process. The Phase II preparation is designed to mimic the washing practices of net owning families; it cannot mimic the myriad ways in which insecticide is removed from the nets during a lifetime of use. An important source of removal during Phase III must be the abrasion a net is subjected to daily during 3 years of household use. In contrast, the only abrasion a net is subjected to during Phase II is the stirring and mashing during the 20 preparatory washes, and the 30–40 days of use during the hut trial. For this reason, a WHOPES Phase II cannot anticipate or predict the outcome of evaluation after 3 years of household use. Only a WHOPES Phase III - in which nets are distributed to householders and re-gathered for testing after 3 years - can show whether a LN really does justify its WHO recommendation. For this reason, the Phase II trial should only lead to an interim WHO recommendation. The WHO process is best seen as a series of gates with one phase setting a standard and leading to the next phase, rather than being predictive of the outcome of the next phase. The reality of this is demonstrated in the WHO report of the Phase III evaluation of Interceptor: it achieved the efficacy criteria of a true LN after 3 years of household use, whereas the CTN fell short of the efficacy criteria within just 1 year of use [9]. The failure of the CTN within a year of Phase III would not be predicted by the relative mortality shown by the CTN washed to exhaustion and Interceptor LN during Phase II. This raises the question of whether the Phase II preparatory procedure should include an accelerated abrasion process between washes that better mimics the wear and tear that a net is subjected to during a lifetime of use.

Culex quinquefasciastus and Anopheles gambiae were fully capable of feeding through holed untreated nets but when the nets were treated with pyrethroid the proportion that fed was reduced substantially, from 50% to 10-20%. The level of personal protection from the LN was 75-80% for An. gambiae and 75-90% for *Cx. quinquefasciatus*. The results for *An. gambiae* were expected, the results for *Cx. quinquefasciatus* results were not. This is because Tanzanian *Cx. quinquefasciatus* are highly resistant to pyrethroids due to site insensitivity and oxidase mechanisms [12], less than 20% are killed by the LN or CTN, and yet very few succeeded in blood feeding. In West Africa too, pyrethroid resistant *Cx quinquefasciatus* struggle to feed through holed LNs or ITNs [18,19]. In contrast, where An. gambiae has developed high level resistance due to a combination of kdr plus cytochrome P450 mechanisms [20] the proportion that manage to blood feed through holed LNs may increase to 60% or more [21,22]. LN lose their capacity to protect when anophelines become highly resistant yet seem to retain capacity to protect when *Cx quinquefasciatus* becomes resistant. The reason for the difference between genera is not clear but may be due to behavioral differences around the net. This is particularly relevant to East Africa because *Cx. quinquefasciatus* is an important vector of lymphatic filariasis there [23]. The evidence from the present trial is that an LN will provide protection against *Cx. quinquefasciatus*-borne filariasis despite the species being resistant to pyrethroids.

Conclusion

Consequent to this Phase II experimental hut trial Interceptor LN obtained interim approval from WHO being the first LN to contain the pyrethroid alpha-cypermethrin.

However, for the full approval a LLIN product must successfully passed phase III evaluations. The next chapter (Chapter 4) is about Interceptor WHOPES phase III evaluation trial; thus, the next chapter will present and discuss results of the Interceptor phase III trial.

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