A “lethal house lure” approach for the control of insecticide resistant African malaria vectors

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Thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy

University of London

October 2020

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Funded by the Bill & Melinda Gates Foundation

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Declaration

I, Welbeck Achille Oumbouke, 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.

Signed: **Date: 22 October 2020**

Abstract

It is now widely accepted that even with universal access and greater use of existing core malaria control measures, elimination of the disease will prove unattainable, especially in areas holoendemic for malaria. There is therefore a major public health imperative to identify new effective interventions to consolidate the major but fragile gains achieved over recent years. House improvement contributed to malaria elimination in developed countries but its potential as a vector control method remains generally underexploited in Africa. In2Care's electrostatic charged netting is a core component of the novel In2Care® EaveTubes vector control method, which is an example prototype belonging to a new house-based intervention class defined by the Vector Control Advisory Group (VCAG) as "lethal house lure". It is described as "Modifications made to a house to decrease exposure of inhabitants to vector". Although a range of laboratory and semi field studies provided some evidence on its potential to reduce malaria transmission, very little is known about its mode of functioning ("modus operandi") if deployed in mass at village level for vector control.

To fill this gap in knowledge, the present PhD project was designed as part of a cluster randomized controlled trial (CRT) evaluating the epidemiological impact of this new intervention in areas highly affected by pyrethroid resistance in central Côte d'Ivoire. A series of resistance genotyping, laboratory and experimental hut studies were performed: (i) to investigate initially the dynamics of insecticide resistance and associated genetic mechanisms in *Anopheles* mosquitoes from the trial area and explore its entomological impact on pyrethroid-only LLINs, (ii) to select and evaluate a long-lasting insecticide for use in In2Care® EaveTubes and investigate whether the community-wide deployment of EaveTubes treated with the chosen insecticide would exert any selection pressure on mosquitoes and (iii) explore whether existing vector control technologies including new generation LLIN or IRS insecticide formulations could be adapted to serve as alternative options for delivering insecticides in EaveTubes.

Resistance to insecticides from major classes was prevalent prior to the trial and was mediated primarily by target-site mutations and detoxification enzymes including cytochrome P450s and carboxylesterase. Pyrethroid resistance levels were extremely high and was shown to compromise the performance of pyrethroid-only LLIN in experimental huts. Although a wide range of insecticide classes could be deployed in EaveTubes for effective control of pyrethroid resistant mosquitoes, only the pyrethroid beta-cyfluthrin was durable and was associated with

~50% reduction in overnight mosquito survival in hut studies. However, the community-level use of beta-cyfluthrin treated EaveTubes resulted in a significant increase in the intensity of pyrethroid resistance over two years and, this was underpinned by a temporal increase in expression of metabolic genes coupled with the rise of cuticular genes over the course of the CRT. Alternative ways of delivering insecticides in EaveTubes by using netting from new generation LLIN or dipping the tube in insecticide solutions was shown to provide similar levels of control as with electrostatic netting despite low persistence.

These studies demonstrate the significant malaria control potential of EaveTubes in areas with extremely high pyrethroid resistance and stress the need for further optimization of the intervention.

Acknowledgements

First, I would like to extend my gratitude to my supervisors Raphael N'Guessan and Mark Rowland whose support was a milestone in the completion of this thesis. A special thanks to Raphael, my primary supervisor, who have ignited in me the passion for scientific research and for making this adventure enjoyable. I would not have completed this PhD without your unwavering commitment and dedication throughout. Mark, thank you for your contribution towards the review of manuscripts and the thesis.

I am most grateful to the Bill and Melinda Gates Foundation who funded the largest part of the research making up this thesis. I am also thankful to the former WHO Pesticide Evaluation Scheme (WHOPES) and V.K.A Polymers Ltd, India for providing additional support to the trials conducted in experimental huts.

I wish to thank my PhD advisors, Matthews Thomas, David Weetman and Jackie Cook for their invaluable guidance during my study.

Thank you to the Chair of the viva, Chrissy Roberts and my PhD examiners, Thomas Churcher and Seth Irish for agreeing to assess the thesis.

I am thankful to Dave Weetman's group at LSTM, especially Patricia Pignatelli who kindly trained me on state-of-the-art molecular techniques for insecticide resistance characterisation.

To Eleanore Sternberg for her advice on statistical analysis and for reviewing manuscripts. You have been an anonymous PhD advisor all these years but I would like to acknowledge here your contribution towards this work.

The work described in this thesis was performed at IPR in Côte d'Ivoire and I would like to thank the Director Kaba Dramane for his support. My gratitude also goes to Alphonsine Koffi and Ludovic P. Ahoua Alou for the collaboration and the technical team without whom none of this work would have been possible.

To God Almighty for giving me the opportunity to undertake this research study and to complete it satisfactorily.

To my friends: Barnabas Zogo, John Essandoh and Roseric Azondekon for their dedicated support.

My parents, Blaise Oumbouke and Solange Lokonon who have been a great source of support.

And finally to my wife, Aisse Métogbé and children, Errol, Anaelle and Oriel who have put up with my stress during these three years of study.

My role and contribution of co-authors in the thesis

Chapter 1 and 8: My contribution to these chapters is 100%.

Chapters 2, and 5. These studies were co-designed by me, my primary supervisor and other co-authors. I performed the phenotypic resistance testing and the molecular work at LSTM under Dave Weetman's guidance. I did the data analysis, again with support from Dave Weetman and wrote the draft manuscripts which were revised by co-authors.

Chapter 4: I contributed to the study design and participated in the data collection. I did the data analysis with guidance from Eleanore Sternberg. I wrote the manuscript which was reviewed by my supervisor Raphael N'Guessan, Mathews Thomas and Eleanore Sternberg before submission for publication.

Chapter 3 and 6: These studies were designed by Raphael N'Guessan (primary supervisor). I supervised the studies, analysed the data and interpreted the findings. I wrote the papers which were reviewed by my supervisors prior to submission for publication.

Chapter 7: I designed the study with contribution from study co-authors. I collected the data and did the statistical analysis. I wrote the paper with input from my supervisors and other co-authors.

Contents

List of abbreviations

ACE-1^R: Insensitive Acetyl cholinesterase gene

ACT: Artemisinin-based Combination Treatment

CDC: Centres for Disease Control

cDNA: Complementary DNA

CIPAC: Collaborative International Pesticides Analytical Council

COEs: Carboxylesterases

CPR: Cytochrome P450 Reductase

CPs: Chemosensory Proteins

CRT: Cluster Randomized Controlled Trial

CYP: Cytochrome P450

DDT: Dicloro-diphenyl-tricloroethane

DNA: De-oxyribonucleic acid

GC-FID: Chromatography with Flame Ionization Detection

GLM: Generalised Linear Model

GLMM: Generalised Linear Mixed Model

GPIRM: Global Programme for Insecticide Resistance Management

GSTs: Glutathione S-transferases

HDPE: High-Density Polyethylene

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

LSM: Larval Source Management

NMCP: National Malaria Control Programme

OCP: Onchocerciasis Control Programme

PBO: piperonyl butoxide

PCR: Polymerase Chain Reaction

PMI: U.S. President's Malaria Initiative

PPF: Pyriproxifen

PVC: Polyvinyl Chloride

RBM: Roll Back Malaria

RNA: Ribonucleic acid

SAPs: Sensory Appendage Proteins

SET: Screening plus EaveTubes

USAID: United States Agency for International Development

UV: Ultra-violet

VCAG: Vector Control Advisory Group

VGSC: Voltage Gated Sodium Channel

WHO: World Health Organization

WHOPES: World Health Organization Pesticide Evaluation Scheme

Part One

Chapter 1: Introduction and literature review

1. Introduction and literature review

1.1. Malaria control in Africa: progress and challenges

Malaria is a life-threatening disease caused by protozoan parasites of the genus *Plasmodium*. Human malaria is mostly mediated by four malaria parasites species including *Plasmodium vivax*, *P. malaria*, *P. ovale* and *P. falciparum*, which is the most prevalent on the African continent. In addition to these parasites known to exclusively affect humans, a fifth malaria parasite, *Plasmodium knowlesi*, which was originally described as malaria parasite of monkeys [1] was also shown to occasionally infect humans. Malaria is transmitted by female *Anopheles* mosquitoes with about 70 species of these having demonstrable malaria transmission potential [2]. The most important mosquito vector populations driving malaria transmission in Africa are *Anopheles gambiae*, *Anopheles coluzzii*, *Anopheles arabiensis* and *Anopheles funestus* [3]. The disease disproportionately affects children and pregnant women, with the highest burden occurring in sub-Saharan Africa [4].

Although the first global malaria eradication attempt launched in the 1950s was successful in a range of settings, no major success occurred in equatorial Africa. After a long period of neglect, partly due to the economic and financial crisis in the 70s and 80s, the Roll Back Malaria initiative was established in 1998 as part of a global plan to reduce malaria burden. This initiative culminated in the Abuja declaration in 2000 with recognition of country-level leadership as a major requisite for the reduction and eventual elimination of malaria in Africa. This renewed political commitment with increased international and domestic financing has resulted in the scale up of effective malaria control interventions. The wide scale implementation of these control measures has contributed to an unprecedented decline in malaria burden over recent years [5]. Indeed, between 2000 and 2015 worldwide malaria mortality rates declined by 47%, corresponding to an estimated 4.3 million malaria deaths averted, and by 54% in the World Health Organization Africa region.

To build on the headway achieved over the past 15 years and sustain progress, the World Health Organization (WHO) has developed the Global Technical Strategy for Malaria 2016-2030 [6]. However, recent estimates suggest that progress has stalled for the first time in over a decade between 2015 and 2017, with evidence of increased cases of malaria in some areas [7]. To meet the milestones set forth in the WHO strategic document, there is a need to address a range of challenges threatening further progress. The emergence of malaria parasite resistance to

frontline antimalarial medicines in the Greater Mekong sub-region [8], reports of increasingly high levels of vector resistance [9–11] and transmission occurring at times and places not targeted by existing tools are significant challenges [12–14]. Additionally, financial resources currently allocated to malaria control are far less than what is required for malaria control and elimination [15]. Achieving the 2030 goal is therefore contingent on development of innovative control tools and sustained investment in malaria.

1.2. Malaria vector control

Vector control is an important facet of the global malaria prevention and control efforts, preventing malaria transmission by reducing mosquito daily survival, human-vector contact and vector abundance [16]. Prior to World War II, the focus of vector control measures was mainly on environmental management, biological control, house improvement and larviciding [17,18]. However, the discovery of the insecticidal property of DDT in 1939 triggered a shift in control strategies toward insecticide based interventions [19]. Current vector control practice still relies on the use of insecticides deployed on mosquito nets and on mosquito resting surfaces inside houses. Interestingly, the recent reduction in malaria burden, which is attributed mostly to the wide scale roll-out of insecticide based interventions, either in the form of long lasting insecticidal nets (LLINs) or indoor residual spraying (IRS), provides tangible evidence that vector control is central to the elimination goal.

1.2.1 Insecticide treated nets

Mosquito nets are the most widely used form of vector control method and an important vehicle of insecticide due to their robustness and scalability even in hard-to-reach settings. Although all mosquito nets currently in use are treated with insecticides, mosquito nets were originally non-insecticidal [20]. Intact, untreated mosquito nets provide some level of protection as a physical barrier against blood-seeking mosquitoes [21]. However, protection from untreated nets is reduced when the nets acquires holes during routine household use. In the early 2000s, insecticide treated nets (ITNs) were introduced to preserve net efficacy even when nets become moderately damaged. The efficacy of ITNs was demonstrated in a meta-regression analysis, showing up to 41% reduction in malaria incidence among net users compared to non-users [22]. While ITNs have proven effective in controlling malaria, there were operational challenges associated with the rapid decay of insecticide in the net, which required regular re-

treatment to maintain efficacy [23]. Re-treatment of mosquito nets is cumbersome for the community. Inevitably, user-fatigue resulting from recurrent net treatment led to low rates of re-treatment. With the advent of innovative net manufacturing technologies, the short serviceable life of ITNs was addressed through the development of long lasting insecticidal nets (LLINs) [24]. Insecticide in LLINs is either coated or incorporated in the net fibres. To receive a recommendation from the World Health Organization (WHO), LLINs are expected to demonstrate resistance to washing (up to 20 washes) and retain efficacy under routine household use for three years [25]. Evidence from field studies suggest that LLINs can retain efficacy for three years or more under field use [24]. All currently available LLINs are treated with pyrethroids, which provides personal protection to net-user through excito-repellency; the protective efficacy is extended to non-users when nets are used on a large scale due to mosquito population reduction creating a community mass-effect.

The efficacy of vector control interventions including LLIN was originally evaluated through the World Health Organization Pesticide Evaluation Scheme (WHOPES). However, this process was revised in 2017 in order to: (i) speed up the evaluation process, (ii) support wide-scale distribution of frontline vector control tools, (ii) improve control of neglected tropical disease and (iii) address some of the challenges facing vector control programs including insecticide resistance in malaria vector mosquitoes. Twenty-two different brands of LLINs have so far been PQ listed (https://www.who.int/pq-vector-control/prequalified-lists/en/) (Annex 1).

With increased financial support from the Global Fund to Fight AIDS, Tuberculosis and Malaria (Global Fund) and the President's Malaria Initiative (PMI), coupled with renewed political will, the number of people owning and using an insecticide treated nets have increased substantially over the past years. For instance, ITN use rose from a low of < 2% in 2000 to 50% in 2018 [4]. This increase in net use has resulted in a substantial reduction in malaria burden, with protection from ITNs accounting for most of the reduction in disease burden (68% of 663 million clinical malaria averted) between 2000 and 2015 [5].

1.2.2 Indoor residual spraying

Targeting indoor resting mosquitoes with residual insecticide is the second most important form of vector control. Spraying house walls with insecticide interrupts malaria transmission by killing female mosquitoes that enter houses and rest on walls. Indoor residual spraying (IRS) with DDT was the backbone of the 1955-1979 malaria elimination campaign. The campaign

was successful in different parts of the world and contributed to the elimination of malaria in parts of Europe, North America, Latin America, Japan and Central Asia. The elimination campaign was also deployed in a number of African countries including Namibia, Mozambique, Botswana, South Africa, and Swaziland [26]. Despite the successful implementation of DDT-based IRS, spray campaigns with DDT have been discontinued in many endemic countries because of the potential hazardous effect [27] of the insecticide. Nevertheless, DDT was reintroduced in some countries and deployed in restricted areas with high malaria transmission intensity following the "Stockholm Convention on Persistent Organic Pollutants" in 2007 [28]. Spraying with pyrethroids, on the other hand, is considered safer and was shown to reduce malaria prevalence when applied on house walls [29,30]. There are currently eight insecticides and one insecticide mixture prequalified by WHO as IRS insecticides (Annex 2). These chemicals belong to four classes of public health adulticides including pyrethroids, carbamates, organophosphates and neonicotinoids. With resistance to pyrethroids increasing in strength and prevalence [31], many spray programmes have switched to non-pyrethroids to improve control and reduce selection pressure on mosquito vectors. Even though WHO advocates for a shift in insecticide policy toward non-pyrethroid chemicals [32], IRS operations using insecticides from alternative classes of chemistry including carbamate and organophosphate are now under threat from the rapid spread of resistance to these actives in malaria vectors [10,33,34]. Moreover, these insecticides are short-lived especially on mud walls which are the type of wall commonly found in rural areas. The short residual life of alternative compounds leading to multiple sprays round in year-round malaria transmission areas together with logistic challenges associated with recurrent campaigns is resulting in fewer people being protected by IRS. For example, recent estimates from the WHO world malaria report show that global IRS protection have declined from 5% in 2010 to less than 3% in 2018 [4]. This low IRS coverage rate could be further reduced if a proposed cut of 44% for PMI-funding on IRS campaign takes effect [35].

In addition to the low IRS coverage rate, the effectiveness of this vector control strategy could be undermined by changes in mosquito resting behavior inside houses. Although spray operations mainly target house walls and occasionally ceilings, this is based on historical evidence showing resting behavior of mosquitoes on these surfaces. However, data from recent investigations showed that a signification proportion of mosquitoes, up to 50% in metal-roofed houses [36], were found resting on surfaces not routinely targeted by IRS. While the findings

that IRS does not cover all resting surfaces are a concern for vector control, additional studies are needed to determine whether observed change in mosquito resting behavior result in reduced IRS effectiveness.

House modification following house spraying is another factor that decrease IRS impact [37]. A few studies conducted in South Africa [38] and India [39] report between 50-80% decline in IRS coverage rate within two to three months post-spray. The sudden decrease in IRS protected properties was due to the insecticide being removed by house owners through wall washing/brushing, wall plastering and painting. This change in house design often goes undetected as there is currently no follow up monitoring of sprayed houses. However, given the proven effectiveness of IRS in vector control and its already low coverage rate, guidelines on IRS monitoring are required to capture the actual IRS coverage rate and inform decision making by national malaria control programmes.

1.3. The challenge of insecticide resistance

1.3.1. Emergence of insecticide resistance in Africa

Insecticide resistance is an inherited genetic trait which provides individual mosquitoes the ability to survive exposure to a toxic dose of insecticide that would be lethal to most individuals of the same species [40]. As effort to increase ownership and use of insecticide based vector control tools have intensified, so has the spread of resistance in African malaria vectors [10]. Between 2010 and 2018, resistance to at least one insecticide class has been reported in malaria vector species in 73 countries with ongoing malaria transmission [7]. The resistance situation in sub-Saharan Africa is concerning as this part of the world carries the highest burden of malaria. Resistance to pyrethroids, the main insecticide class used on bed nets, has increased in prevalence and intensity over recent years [31,41]. The first case of resistance to this insecticide was reported in Africa in *An. gambiae* s.l. from Côte d'Ivoire [42] and has now been found in all major African malaria vectors in West [43–49], Central [50–54], East [55–58] and Southern Africa [59]. Surprisingly, no pyrethroid resistance has been reported in *Anopheles* mosquitoes from Namibia [60] and Botswana [61] in south-western Africa despite presence of resistance in neighbouring countries. The increase in the prevalence of pyrethroid resistance is a major concern for vector control efforts as all currently available LLINs rely on pyrethroids. Resistance to DDT was first reported following the wide-scale deployment of DDT-based IRS

during the first malaria elimination programme and was considered the main factor that contributed to the failure of the eradication campaign [19,62]. DDT resistance has spread across Africa with a distribution pattern similar to that of pyrethroids. Malaria mosquitoes have also developed resistance to insecticides from other classes of insecticide. Resistance to carbamates, for example the commonly used insecticide bendiocarb, is widespread, with prevalence of resistance being highest in countries located in West and Southern Africa (irmapper.com, accessed September 2020). Unlike the above-mentioned insecticide classes, resistance to organophosphates is less common. Perhaps as a result of the increasing use of the organophosphate insecticide pirimiphos methyl in IRS campaigns and in agriculture, cases of resistance to this insecticide has been reported in *An. gambiae* and *An. arabiensis* in West [63]and East Africa [64], but no resistance has so far been detected in *An. funestus*.

Multiple insecticide resistance phenotypes have been reported in various African countries including Côte d'Ivoire [44], Mali [65]and Kenya [66]. Malaria vector control may prove challenging in these countries given the limited insecticide options currently available.

1.3.2. Insecticide resistance mechanisms

To survive exposure to otherwise lethal doses of insecticide, mosquitoes have developed a range of defensive mechanisms. Mosquitoes develop resistance to insecticides mainly through modification of the target site of the insecticide (target site resistance) and through increased breakdown or sequestration of the insecticide (metabolic resistance). In addition to these two major types of insecticide resistance mechanisms, less well-studied resistance mechanisms have been reported. These include: (i) reduced penetration or absorption of the insecticide through modification of mosquito's cuticle (cuticular resistance), (ii) sequestration of chemosensory proteins and (iii) behavioural avoidance of insecticide-treated surfaces. Although these mechanisms of resistance differ in the way the insecticide is prevented from reaching its target site, they all provide a survival advantage to mosquitoes in the presence of insecticide.

1.3.2.1. Major types of insecticide resistance mechanisms

1.3.2.1.1. Target-site resistance

Target site resistance is one of the major and well-characterized mechanisms of resistance. It entails changes in insecticide-binding target site and this alteration results in a reduction of the amount of insecticide reaching the site. For example, point mutations in the *para* voltage-gated sodium channel (VGSC) ─ the target site for pyrethroids and the organochlorine DDT [67–69] ─ alter the binding affinity of these insecticides. This type of resistance is also referred to as knockdown resistance (*kdr*), since it enables mosquitoes to withstand longer exposure to insecticides without being knocked down. Knock down resistance stems from the substitution of the amino acid leucine (Leu) to either phenylalanine (Phe) or serine (Ser) at codon 1014. Due to their original disparate geographical distribution, the Peu-Phe substitution is referred to as *kdr* West because it was originally found mostly in West Africa whereas the Leu-Ser was limited to East Africa and termed as *kdr* East. However, there is now evidence of an overlap in the distribution of these mutations, with both occurring across most of the African continent – [70–73][68–71]. The N1575Y (substitution of asparagine to tyrosine), originally identified in Burkina Faso, is another VGSC mutation [69] which, in combination with 1014F, enhances strength of resistance to pyrethroids and DDT [74]. Although the frequency of the N1575Y is relatively low, the mutation is spreading rapidly and has been reported in several West African countries (Benin [69,75], Burkina Faso [69], Côte d'Ivoire [76], Ghana [69]) and two countries in central Africa (Democratic republic of Congo [54] and Cameroon [77]). Despite their widespread distribution, *kdr* mutations alone have not been associated with any control failure in *Anopheles* mosquitoes. Similar to knock down resistance, a point mutation in the gene coding for the neuro-transmitter acetyl-cholinesterase (*Ace-1*), which is the target site for carbamate and organophosphate insecticides, leads to a mutant form of the enzyme (*Ace-1R*) that does not bind with these insecticides. This resistance mechanism results from the substitution of the amino acid glycine to serine at codon 119. Resistance to carbamate and organophosphate was initially reported in *Culex pipiens* but is now widespread in *Anopheles* mosquitoes. A fitness cost associated with the *G119S* mutation in the absence of insecticides has been documented in *Culex* and *Anopheles* mosquitoes [78,79]. However, the deleterious effect linked with the *G119S* mutation was shown to be offset by duplications which pair resistant and susceptible alleles on the same chromosome [80–82][78–80].

1.3.2.1.2. Metabolic resistance

Metabolic resistance is the other major type of resistance mechanism with demonstrable epidemiological significance [83]. This form of insecticide resistance arises from enhanced detoxification/sequestration of insecticides. The role of a range of metabolic enzymes including carboxylesterases (COEs), Glutathione S-Transferases (GSTs) and cytochrome P450 mono-oxygenases (P450s) in insecticide resistance have been demonstrated in various species of mosquitoes. The latter type of metabolic enzymes, the cytochrome P450s, is involved in the majority of metabolism based detoxification of insecticides, mainly pyrethroids and carbamates. The most important P450 enzymes, known to detoxify most insecticides used in public health, belong mainly to the Cyp6 subfamily [61,82–84]. Some of these enzymes have been linked to pyrethroid resistance in a number of *Anopheles* species, including Cyp6P3, Cyp6P4, Cyp6M2, Cyp6Z1, Cyp6Z2, Cyp4J5 and CYP9K1 in *Anopheles gambiae* s.l. [53,87–93], and Cyp6P9a,b, Cyp6P4a,b, Cyp6Z1, Cyp6AA1 and Cyp6M7 in *Anopheles funestus* [85,86,94–96][83,84,92–94]. CYP6M2 and CYP6P3 have been validated as pyrethroid metabolizers, and the latter has also been shown to detoxify carbamates [63,97], providing evidence of the ability of these genes to metabolize insecticides from unrelated classes. This is a serious concern for malaria control since insecticide resistance management strategies involving rotation or combination of these insecticides may be compromised. Interestingly, the juvenile hormone analogue pyriproxyfen (PPF) can be metabolized by a broad range of P450 genes including *Cyp6P2*, *Cyp6Z2*, *Cyp6M2* and *Cyp9J5* [98]. PPF is combined with permethrin on Olyset DUO net to sterilize insecticide resistant mosquitoes for improved control, thus metabolic resistance potentially threatens the future efficacy of this new class of net.

While an increasing number of countries are reporting data on nation-wide distribution of target site resistance mechanisms *kdr* and *Ace-1R*, detection and tracking of metabolic resistance genes has lagged. This is mostly due to the lack of DNA marker associated with metabolic resistance in African malaria vector mosquitoes [99]. However, some markers have recently been identified that could support the surveillance of the occurrence and spread of metabolic-based resistance mechanisms in *Anopheles funestus* [100].

1.3.2.2. Additional insecticide resistance mechanisms

The role of target site mutation and overexpression of detoxification enzymes in conferring resistance in malaria vector populations is well established. However, the emergence of resistant mosquitoes displaying strong and multiple-resistance phenotype suggest the occurrence of additional broad-spectrum resistance mechanisms.

1.3.2.2.1. Cuticular and Sequestration of chemosensory proteins

Cuticular resistance is changes in the mosquito cuticle ─ the outermost part of the mosquito body ─ which delays or prevents the uptake of external compounds, including insecticides, either through changes in cuticle thickness or composition [101]. The cuticle thickening phenotype is conferred by the over-expression of cytochrome P450 genes (mainly *Cyp4G16* and *Cyp4G17*) [102] and a number of cuticle proteins (CPs) [103]. This mechanism of resistance has been documented in major African *Anopheles* populations including *An. gambiae* [103], *An. funestus* [104] and *An. arabiensis* [89]. While cuticle thickening results in slow insecticide uptake, alteration in cuticle composition leads to a complete inhibition of insecticide penetration. Altered cuticle composition is associated with hardening of the mosquito cuticle through the over-expression of two oxidases (a laccase and a tyrosinase) and the translocation of cuticular component toward the cuticle, which is driven by over-transcribed ABC transporters [101]. Since the mechanism underlying decreased insecticide penetration is not insecticide-specific, its spread in malaria vectors might pose a serious threat to insecticide-based vector control measures because of its potential to render a large spectrum of unrelated insecticides ineffective.

Sequestration of chemosensory proteins has been described only recently as an insecticide resistance mechanism in multiple insecticide resistant strains of *An. gambiae* mosquitoes from Burkina Faso and Côte d'Ivoire [105]. It results from an over-expression of a member of the chemosensory proteins (CPs) ─ sensory appendage protein SAP2─ which is enriched in the mosquito leg and was shown to specifically target pyrethroids. Although this additional mechanism of resistance is a concern for malaria vector control, SAP2 silencing was shown to substantially restore susceptibility to pyrethroids [105]. This could pave the way for the identification of SAP2 neutralizing compounds which could be incorporated in bed nets to inhibit expression of this protein and thus improve control of pyrethroid resistant vector species.

1.3.2.2.2. Behavioural resistance

Changes in mosquito behaviour that results in the avoidance of insecticide treated surfaces is referred to as behavioural resistance [13,106]. Indoor-evading vectors are difficult to control as they mediate transmission at places (outdoor) [107–109] and times (when people are indoor but not under bed nets) [110,111] not targeted by current core interventions. Behavioural resistance differs from a shift in species composition, which typically occurs after a successful elimination of the dominant vector species in a given area [112]. Although there is good evidence that changes in mosquito behaviour are a direct consequence of the extensive use of indoor-targeted vector control measures, the mechanisms underpinning this form of resistance remain sparse [113].

1.3.3. Impact of insecticide resistance on current frontline control tools

Although the decreased susceptibility of *Anopheles* mosquitoes to major adulticides used in public health has been demonstrated, the impact of resistance on malaria transmission remains inconclusive. Data from observational studies indicate that pyrethroid-based vector control interventions are still protective even in areas with pyrethroid resistance. For instance, the use of pyrethroid ITNs in various malaria endemic countries was associated with a substantial reduction in malaria incidence in children despite moderate to high pyrethroid resistance in the local *Anopheles* malaria vectors [114–117]. A further example is the impact of deltamethrin based IRS in a pyrethroid resistance setting on Bioko Island. Malaria indicator surveys conducted during the deltamethrin spray campaign showed a significant decline in malaria prevalence in children [118]. Some of the factors potentially contributing to the continued efficacy in areas of insecticide resistance include: (i) impaired development of malaria parasite in mosquitoes surviving exposure to insecticides [119], (ii) increased susceptibility to insecticides of older, potentially infectious mosquitoes [120,121] and (iii) reduced longevity of mosquitoes following exposure to insecticide treated surfaces [122]. Although these studies provide evidence that pyrethroid ITNs and IRS retain efficacy in areas with pyrethroid resistance, these investigations are observational and were not designed to rigorously evaluate the potential impact of pyrethroid resistance. A WHO-coordinated study involving five countries (Benin, Sudan, Kenya, Cameroon and India) was designed to investigate whether the efficacy of pyrethroid LLINs was being compromised in areas of pyrethroid resistance [123]. Although findings from Benin showed no association between pyrethroid resistance and malaria indicators [124], confounding effects including difference in malaria transmission and

resistance intensity between study arms may have masked the impact of resistance. Ultimately, randomized controlled trials to assess the public health impact of resistance cannot be conducted given that random allocation of resistance in the field to account for confounding factors is impossible.

Despite the challenge in demonstrating the epidemiological impact of insecticide resistance, several lines of evidence provide insight into the negative effect that insecticide resistance may be having on malaria control. Experimental hut studies conducted in Benin showed significantly reduced entomological efficacy (personal protection and mosquito mortality) of pyrethroid ITN in a high pyrethroid resistance area (Ladji) in the south of the country compared to the north (Malanville) where *Anopheles* mosquitoes were susceptible to pyrethroids [125]. These findings were confirmed in subsequent household studies reporting no added protective benefit of pyrethroid ITN relative to untreated net in areas with high pyrethroid resistance [126]. Moreover, a systematic review and meta-analysis of experimental hut studies evaluating the performance of pyrethroid ITN demonstrated a significant impact of pyrethroid resistance on entomological indicators [127]. Mathematical modelling using experimental hut data predicted up to 40% fewer malaria cases averted by ITN deployed in areas with resistance compared to settings with fully susceptible vector populations [128]. Further evidence that resistance may be compromising the efficacy of control tools is provided in a recent randomized controlled trial in an area with pyrethroid resistance in Muleba, Tanzania [129]. Data from the trial showed a significant reduction in malaria prevalence in children sleeping under Olyset Plus LLIN compared to those using standard pyrethroid net. Olyset Plus is a synergist net which incorporates a mixture of deltamethrin and piperonyl butoxide (PBO) on all net panels. PBO is a synergist that neutralizes the action of metabolic enzymes and partially restore the efficacy of pyrethroid insecticides. The reduced efficacy of standard pyrethroid nets compared to the PBO LLINs in the community trial suggests that pyrethroid resistance may have an epidemiological impact. The most cited example of the impact of resistance comes from a study in an area bordering Mozambique and South Africa (KwaZulu Natal) where the emergence of metabolic resistance in *An. funestus* led to an increase in malaria cases [130]. However, it should be noted that development of drug resistance in malaria parasites could have also contributed to the observed control failure. A similar finding was reported from Dielmo village in Senegal where insecticide resistance was considered the likely cause of reduced LLIN efficacy and a rebound in malaria-specific morbidity [131]. Improvement in malaria control following a change in IRS insecticide, either in response to resistance or

increased malaria cases, has been documented and provide indirect evidence that insecticide resistance is adversely impacting pyrethroid based control measures. For example, reduction in malaria transmission as a result of a switch from pyrethroids to carbamate or organophosphate insecticides has been reported in several countries such as Bioko Island [132] and Uganda [133].

Although there is as yet no unequivocal evidence that insecticide resistance is undermining the efficacy of pyrethroid LLINs, it is now generally accepted that if resistance continue to spread, the gains made so far will be lost. Indeed, modelling simulations predict that the loss of pyrethroid efficacy in a context of universal coverage will translate into an increase in malaria burden [134]. The threat of insecticide resistance prompted the release of the Global Plan for Insecticide Resistance Management (GPIRM) in malaria vectors [134]. The GPIRM is a five-pillar strategy developed by the World Health Organization in 2012 with the aim of slowing the spread of resistance and preserving the efficacy of current control methods. However, insecticide resistance has increased in prevalence and intensity since the introduction of this resistance mitigating plan, with the lack of alternative tools required to implement the range of strategies laid out in GPIRM being the likely contributing factor. The low uptake of the plan is also driven by the lack of clear-cut evidence of the epidemiological impact of resistance, the limited availability and high cost of insecticides with new mode of action and a shortage of human, infrastructural and financial resources [135].

1.3.4. Insecticide resistance management

Insecticide resistance management is one of the five pillars in the GPIRM, which aims to delay the occurrence of insecticide resistance, preserve the efficacy of current insecticides and thus reduce the need to switch to cost-prohibitive alternative products. Insecticide resistance strategies should ideally be deployed when resistance-linked mutations are very rare and barely detectable in the insect population. Unfortunately, resistance to the most commonly used insecticides is widespread with evidence of some resistance alleles reaching fixation. However, the implementation of currently proposed resistance management activities may still reduce resistance gene frequency, particularly for emerging resistance mechanisms and those that come with a cost for the mosquitoes. WHO recommended insecticide resistance management strategies are based on current core vector control interventions (LLINs and IRS) and include: (i) rotation of insecticides, (ii) combination of interventions, (iii) mosaic and (iv) mixtures. These strategies are designed to manage resistance either through reducing selection pressure

(rotation and mosaics) or killing of resistant mosquitoes using new classes of insecticides (combined interventions and mixtures) [136].

1.3.4.1. Rotation and mosaics

These strategies work through suppression of the insecticide selection pressure and are mostly effective when the resistance mechanism is associated with a fitness cost. Individual mosquitoes carrying costly resistance mechanisms thrive only in the presence of insecticide. Consequently, the removal of the selection pressure provides a survival advantage to susceptible mosquitoes and reduces the proportion of resistant vectors. This is most effective when resistance-associated fitness cost is high.

Rotation entails the deployment over time of two or more insecticides with different mode of action. The rationale behind this technique is to reduce mosquito exposure to a particular insecticide. Under the scenario of low resistance frequency and high fitness cost, rotation of different insecticides results in a decline of resistance to the first insecticide while the second chemical is being introduced, thus preserving susceptibility. The most cited example of a successful implementation of rotational strategy is the case of the West African Onchocerciasis Control Program (OCP) [137]. Also known as river blindness, Onchocerciasis is caused by a parasitic worm *Onchocerca volvulus* and is transmitted by blackflies. In addition to the use of Mectizan to clear the parasite reservoir in human host, the programme targeted the blackfly larvae through weekly application of the organophosphate temephos in breeding habitats. Following emergence of resistance to the larvicide, the programme subsequently initiated a rotational strategy involving the use of six insecticides from three chemical classes (a pyrethroid, a carbamate and three organophosphate) and a biological insecticide (*Bacillus thuringiensis*) to successfully mitigate the resistance problem. Implementation of a similar resistance management approach is not currently feasible in malaria vector control. First, unlike the blackfly which breeds only in a particular type of water (river water with 2m/s current), rotational use of insecticides is not feasible in malaria control programmes targeting immature stages of mosquitoes as these vectors breed in various types of breeding habitats that can be temporary. Second, there is currently very limited insecticide options available for malaria control. However, with funding from UNITAID, the NgenIRS partnership led by the Innovative Vector Control Consortium (IVCC) has been set up to accelerate the introduction of third generation IRS insecticides and support insecticide resistance management through rotation of different insecticides. This may expand the lifespan of these new insecticides and

delay the rise of insecticide resistance (http://www.ngenirs.org). Rotational use of insecticides from unrelated classes is in use in Bioko [118] Island and Southern Africa [138] and is now part of the PMI (President's Malaria Initiative) plans for countries across sub-Saharan African.

Mosaics can be used to avoid the build-up of resistant mosquito populations. The aim of this approach is to reduce the potential of the mosquitoes being exposed to a single compound over their lifetime, which otherwise would impose selection pressure and lead to resistance. Deployment of unrelated chemicals in neighbouring areas offers the opportunity for the mosquitoes to be exposed to both insecticides through migration between areas and be killed, provided that genes conferring resistance to both insecticides are rare or absent in the mosquito population. Mosaics can be used in IRS campaigns either at broad scale in neighbouring communities or at fine small scale within village with different types of insecticides deployed.

1.3.4.1. Combined interventions and mixtures

The aim of these approaches is to kill or reduce the population of resistant mosquitoes by simultaneously exposing them to two or more unrelated insecticides. The insecticides are deployed in the same place and at the same time such that mosquitoes surviving exposure to one insecticide due to resistance would be killed by the other insecticide provided multiple resistance is absent or rare in the vector population. Unlike rotation and mosaics, this resistance management concept does not rely on fitness cost and is designed to overpower resistance rather than preserving susceptibility. Evidence from modelling work indicate that the use of mixture and combined interventions are more effective at delaying the emergence of insecticide resistance than rotation and mosaics [136].

Ideally, the two interventions should contain insecticides with a different mode of action to reduce selection pressure and kill resistant mosquitoes. Hut studies demonstrated improved control with pyrethroid LLIN combined with non-pyrethroid IRS compared to LLIN alone in pyrethroid resistance settings where the local malaria vectors were susceptible to the IRS insecticide [140,141]. However, given that resistance to classes of insecticides being considered for IRS in spreading, such an approach may not be effective in area with multiple resistance. For instance, combining pyrethroid LLIN with pirimiphos methyl IRS failed to improve control in an area of Côte d'Ivoire where *Anopheles* mosquito populations were resistant to the insecticides in both interventions [142]. This underscores the need for new insecticides to make combinations a more practical resistance management strategy.

Mixing insecticide into a single product reduce the chance of resistance developing as mosquitoes not killed by the first active ingredient will be killed by the partner insecticide provided the target vector population is not resistant to both insecticides. Mixtures are more effective than the previously described resistance management approaches, as mosquito exposure to the unrelated insecticides is guaranteed [143]. For mixtures to be effective, the two co-formulated insecticides should possess similar decay rate and be used at their full application rate. LLIN and IRS mixtures are currently available for vector control and were shown to improve control of resistant mosquito populations [144,145].

1.4. Addressing the insecticide resistance challenge: improved versions of current mainstay control strategies

In response to the increasing threat of pyrethroid resistance, considerable efforts are being made to preserve the efficacy of current control tools. New generation nets incorporating different compounds deployed in a mosaic or mixture style have been introduced to improve control of pyrethroid resistant mosquitoes. Mixture nets are treated either with two unrelated insecticides (alpha-cypermethrin-chlorfenapyr mixture net: Interceptor G2 net) [146,147] or an insecticide mixed with an insect growth regulator (permethrin-pyriproxyfen mixture nets: Olyset DUO LN) [148] or the synergist PBO (piperonyl butoxide-treated insecticidal net: PBO LN such as Olyset Plus [149]).. PBO inhibits the action of metabolic enzyme of the P450 family and enhance the toxicity of pyrethroid insecticide in the net. In some synergist LLINs, the two compounds (the PBO and the pyrethroid insecticide) are spatially separated with different compounds applied on the top and the side panel of the net (PermaNet 3.0 [150]). The disease control potential of mixture and synergist nets relative to pyrethroid-only nets have been confirmed in randomized controlled trials in areas of high pyrethroid resistance [129,139,151]. Drawing on the evidence that most mosquito host seeking activity occurs on a bed net roof, a mosquito net with an insecticidal barrier net placed above the top panel of the net has recently been designed to target insecticide resistant malaria vectors with alternative resistance breaking insecticides [152]. The location of the barrier net means that insecticides not currently recommended for net treatment due to safety concern could be used for effective vector control. This new type of net holds significant vector control potential and was shown to kill a greater proportion of pyrethroid resistant mosquitoes compared to standard net in experimental huts in

a pyrethroid resistant area in Burkina Faso [152]. This simple innovative modification to net design is predicted to reduce malaria incidence to level similar to that with new generation LLINs and may be favoured over these nets as small quantity of insecticide is required for net treatment. However, community field trials demonstrating impact on malaria metrics are required before this new net is considered for use in public health.

The significant contribution of IRS to reducing the malaria burden over the past years makes it an important tool within the global malaria elimination effort. In light of the value of IRS, new classes of insecticides such as pyrroles and neonicotinoids are being tested as indoor residual sprays to address current constraints on IRS including vector resistance and the short residual life of currently available chemistries [145].

While development efforts to improve the efficacy of current core interventions (i.e. LLINs and IRS) should be sustained, additional control strategies are obviously needed to supplement these tools and drive transmission to level required for malaria elimination, especially in areas with high insecticide resistance and transmission intensity [153].

1.5. House improvement as a malaria control strategy

Traditional malaria control methods target exclusively mosquito behaviour that occurs inside houses: mosquito nets are designed to prevent insects from feeding on humans sleeping beneath them whilst IRS kills female mosquitoes once they have successfully fed on unprotected humans. However, a careful scrutiny of the life cycle of the adult mosquito indicates that there is a range of vulnerable stages that can be targeted to further reduce malaria transmission [154–160]. A key phase is the behaviour of host-seeking female mosquitoes around residential houses. There is a strong body of evidence that major African malaria vectors primarily enter houses via the "eave space" which is the gap between the edge of walls and the roof of houses [16–1631]. This has been confirmed in a recent behavioural study by means of video tracking techniques [164]. The observation that mosquitoes enter houses via eave gaps has been exploited to reduce indoor mosquito density and malaria transmission by physically blocking the eave space [165] or screening this space using an insecticide treated curtain [166] or eave baffles [167]. Protection can be enhanced by screening windows, doors and filling up cracks in house walls which make the house mosquito-proof. There is also empirical evidence that improvement to houses not only protects against mosquitoes but also reduces anaemia [168].

More recently, a systematic review on housing and malaria provides evidence that residents of houses with features that restrict mosquito entry had lower odds of malaria infection and lower incidence of clinical malaria [169]. In some settings, protection derived from better housing is on par with that from insecticide treated nets [170].

House modification as a protective measure against disease-spreading insects is not a new practice. The vector control potential of mosquito-proofed houses was first demonstrated in the pioneering work of Angelo Celli [171] over a century ago. Although there is evidence that incremental improvement to housing has contributed to malaria reduction and elimination in developed countries [172–174], its full disease reduction potential remain largely underexploited in the developing world. However, with the rapid economic growth in Africa and the need for additional houses to meet its expanding population, there is an opportunity to deploy improved housing as a complementary measure to existing control tools. As now being supported by the Roll Back Malaria and the United Nations Development programmes, a cross-sector collaboration, especially with the housing sector to promote incorporation of protective designs into housing in areas at risk of malaria is key to the successful implementation of this strategy [175]. The reason for the increasing momentum for better housing as a sustainable malaria control approach is threefold. First, improved dwellings have few or no openings and are thus less prone to invasion by disease-carrying vectors. Second, although there are increasing reports of outdoor biting, the bulk of malaria transmission still occurs indoors [176], making house-tailored interventions an important measure within the global malaria elimination strategy. Third, there is empirical evidence that development of the malaria parasite and survival of females mosquitoes is compromised when temperature exceeds an optimal threshold [177,178]. This occurs mostly in modern iron-roofed houses which have higher maximum indoor temperature compared to cooler thatched-roofed residences [179]. Together, these factors make better housing a promising control approach which could be integrated into existing method for improved malaria transmission control.

1.6. The EaveTubes strategy

The observation that host-seeking African malaria vectors predominantly enter human dwellings through eaves – an important source of host attractant cues – motivated the initial development of the EaveTubes technology [180]. EaveTubes are pieces of PVC pipe that can be inserted into small cylindrical holes which are drilled into filled eave spaces to maintain

airflow and to attract mosquitoes. When mosquitoes are drawn in the tubes by the human odours emanating through the openings, they contact a piece of netting laden with powdered insecticides. The netting is treated with an electrostatic coating that uses polarity to bind insecticides onto the netting. This innovative delivery system, originally used for various purposes including control of cotton pests [181], enables the transfer of a high dose of insecticide capable of killing highly pyrethroid resistant *Anopheles* mosquitoes, even when pyrethroids are used [182]. In addition to the improved insecticide bioavailability and the resistance breaking potential of the electrostatic netting, insecticides that are prohibitively costly for use in IRS could be used in EaveTubes because only small quantities of active ingredient per house are required. Moreover, since insecticide treated tubes are placed at eave level, there is potentially a lower risk of exposure to house occupants. As a result, products considered unsuitable for use on nets could be acceptable for use in EaveTubes. EaveTubes are combined with "mosquito proofing" of houses, which involves screening of windows and blocking of gaps in houses. By attracting and killing blood-seeking mosquitoes, the EaveTubes are comparable to a "lure and kill" bait. This may have led to the recent classification of this type of control method as a "lethal house lure" approach by the WHO Vector Control Advisory (VCAG).(https://apps.who.int/iris/bitstream/handle/10665/274451/WHO-CDS-VCAG-2018.03-eng.pdf)

Although house improvement does not require insecticide treatment, the addition of an insecticidal component in the EaveTubes approach could have community benefit by killing mosquitoes and reducing local populations. A modelling study using data from small-scale investigations of this control method has predicted a 70% reduction in malaria transmission potential, even when only one-third of houses in a community receive EaveTubes [183]. The insecticide component of the strategy could address the concern of mosquito being deflected from EaveTubes protected houses to neighbouring unprotected houses. Indeed, results from a recent study suggest that deployment of EaveTubes in houses does not result in an increased risk of mosquito bites among people living in adjacent unprotected houses [184]. This is a positive outcome since coverage is unlikely to be 100% either due to houses that are not amenable to the EaveTubes installation or poor community adherence. However, findings from this study should be interpreted with caution given that deflection was investigated under controlled conditions in experimental houses and potential for a different outcome with real houses cannot be ruled out. As with any potential complementary tools, EaveTubes will be deployed against a background of existing interventions. Predictive modelling, however,

suggest that the benefit of integrating EaveTubes into traditional control measures depend on how the technology is implemented with respect to LLIN and IRS. According to the model simulation, the benefit of combining EaveTubes with LLIN or IRS is additive [183].

The potential of the EaveTubes approach to reduce malaria transmission has been explored in a number of initial studies conducted in experimental huts within large enclosures. The first series of experiments were designed to optimize the technology by examining various aspects of EaveTubes that impact mosquito entry including diameter of the tubes, optimal height above ground and angle of the tube [185]. Further experiments were performed and served as a proof of concept, demonstrating that EaveTubes plus screening reduce mosquito house entry and kill host-seeking female mosquitoes as they make contact with the insecticide treated tubes. There is also evidence that female mosquitoes that manage to enter houses e.g. through open doors, could be killed by the insecticide treated insert when trying to exit houses via the eaves either to carry on searching for blood meals or to find an oviposition site [184]. The most interesting part of this set of initial studies was conducted in a complex ecosystem simulating a Tanzanian village environment inside a large, screened field cage to assess potential of the technology on a self-sustaining colonies of *An. arabiensis* mosquitoes. Findings from these semi-field studies demonstrated the greater impact of EaveTubes on mosquito population suppression and indoor biting relative to LLIN alone [185].

The promising results from these preliminary studies on EaveTubes have led to a cluster randomized controlled trial (CRT) designed to investigate whether the technology reduces malaria transmission, compared to current best practice of LLINs [186].

1.7. Rationale of the study

LLINs and IRS have been instrumental in reducing the global malaria burden and have contributed to the elimination of the disease in a number of countries. While these tools will continue to play a major role in malaria control, their continued effectiveness may be undermined by insecticide resistance in vectors, as evidenced by the recent rise in malaria cases in some endemic African countries [4]. To sustain the considerable advances in malaria control, there is a need to expand the current “vector control tool-box”. Most malaria transmission occurs indoors [176], with evidence that simple modification to house design have considerable

disease reduction potential. The EaveTubes is a type of house improvement that could be integrated with current control tools for a more effective control of malaria [6]. This vector control strategy reduces mosquito vectorial capacity by limiting human-vector contact (house modification) and reducing vector longevity through deployment of insecticide in EaveTubes. As described above, a series of studies have provided evidence for the potential of this control measure to reduce malaria transmission, but a number of important questions remain related to the functioning of the technology. Research studies making up this PhD thesis were conducted in the context of the CRT investigating whether EaveTubes plus screening (SET) deployed in combination with LLIN provide additional protection against malaria transmission compared to current standard of care in a high pyrethroid resistance area in central Côte d'Ivoire [186].

The electrostatic netting, which is the insecticide delivery system and an essential component of the EaveTubes strategy, has resistance breaking potential [182]. However, evidence of this has, to date, been mostly obtained using laboratory colonies. Field studies investigating this claim need to be conducted in areas where insecticide resistance has been characterized in detail (i.e. prevalence, intensity and associated underlying mechanisms). The potential impact of resistance on the entomological efficacy of pyrethroid-only LLIN in these areas also need to be tested to understand what is possible in the absence of EaveTubes.

Current insecticide resistance management strategies work by removing the insecticide selection pressure or by bypassing resistance through the use of insecticides with a novel mode of action [134]. The EaveTubes intervention overpowers resistance through increased bioavailability of insecticide on the surface of the electrostatic netting. Although existing insecticides were found to be more effective when applied on the electrostatic netting, whether the community deployment of these insecticides would select for resistance needs testing. This would require monitoring of changes in the prevalence, intensity and genetic mechanisms of insecticide resistance over time following wide-scale use of this new control measure in a high pyrethroid resistance setting.

The electrostatic netting within the EaveTubes was shown to hold powder formulation of insecticide for effective control of resistant mosquitoes. However, only chemicals from a handful of classes including pyrethroids have been previously tested against *Anopheles gambiae* mosquitoes [182]. Whether active ingredients from other classes could be adapted for

use against insecticide resistant *Anopheles* mosquitoes warrants investigation. In addition, given that the nature of the electrostatic netting differs from that of common substrates such as house walls and bed nets, the residual activity of a range of insecticides belonging to various classes need to be explored.

Although only a small amount of powder formulated insecticide (1g) is deployed on electrostatic netting for high-level control of resistant malaria vectors [182], deployment of the insert has logistical costs including heavy machinery and regular washing of the netting (insert) before re-treatment. This could be a major obstacle to the implementation of the strategy, especially in resource-poor settings. There is therefore a need to investigate alternatives means of delivering insecticide in EaveTubes that could provide a more scalable and practical insecticide delivery system for use in the "lethal house lure" approach for malaria control.

1.8. Study objectives

My thesis aims to improve our understanding of how EaveTubes control malaria transmission in pyrethroid resistant area using entomological endpoints and explore ways to optimise the intervention.

Specific objectives

1: Characterise insecticide resistance and its impact on pyrethroid-only LLIN in central Côte d'Ivoire

a) To investigate the prevalence, intensity and genetic mechanisms of insecticide resistance in *Anopheles gambiae* in a selection of study clusters

b) To evaluate in experimental huts the efficacy of standard pyrethroid-only LNs in a highly pyrethroid-resistant site adjacent to the CRT area

2: Optimise and evaluate EaveTubes

a) To screen multiple insecticides and select the one with highest residual activity for application on eave tubes inserts

b) To evaluate the efficacy of the selected insecticide in experimental huts within enclosure

c) Investigate whether the “resistance breaking” powder formulation selects for resistance in wild *Anopheles gambiae* mosquito population

3. Explore alternatives to powders, including the use of next generation LLIN material and IRS formulations

a) To evaluate the efficacy of synergist LLINs against pyrethroid resistant *An. gambiae* in experimental huts prior to testing in EaveTubes

b) To assess as a proof of concept whether netting pieces of new generation LLINs and dipping of tube plus netting in insecticide formulation could be used as long lasting alternatives to powder formulations

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PART TWO: Characterisation of insecticide resistance and its impact on the entomological efficacy of pyrethroid-only LLIN in central Côte d'Ivoire

Chapter 2: Fine scale spatial investigation of multiple insecticide resistance and underlying target-site and metabolic mechanisms in *Anopheles gambiae* in central Côte d'Ivoire

Chapter 3: Evaluation of standard pyrethroid based LNs (MiraNet and MagNet) in experimental huts against pyrethroid resistant *Anopheles gambiae* M'bé, Côte d'Ivoire: potential for impact on vectorial capacity.

Chapter 2

Fine scale spatial investigation of multiple insecticide resistance and underlying target-site and metabolic mechanisms in *Anopheles gambiae* in central Côte d'Ivoire

The work in this chapter has been published as:

Welbeck A. Oumbouke, Patricia Pignatelli, Antoine M.G. Barreaux, Innocent Z. Tia, Alphonsine A. Koffi, Ludovic P. Ahoua Alou, Eleanore D. Sternberg, Matthew B. Thomas, David Weetman and Raphael N'Guessan. Fine scale spatial invetigation of multiple insecticide resistance and underlying target-site and metabolic mechanisms in *Anopheles gambiae* in central Côte d'Ivoire. **Sci Rep** 10, 15066 (2020). https://doi.org/10.1038/s41598-020-71933-8

Fine scale spatial investigation of multiple insecticide resistance and underlying target-site and metabolic mechanisms in *Anopheles gambiae* in central Côte d'Ivoire

Abstract

Routine monitoring of occurrence, levels and mechanisms of insecticide resistance informs effective management strategies, and should be used to assess the effect of new tools on resistance. As part of a cluster randomised control trial evaluating a novel insecticide-based intervention in central Côte d'Ivoire, we assessed resistance and its underlying mechanisms in *Anopheles gambiae* populations from a subset of trial villages. Resistance to multiple insecticides in *An. gambiae s.s.* and *An. coluzzii* was detected across villages, with dose-response assays demonstrating extremely high resistance intensity to the pyrethroid

deltamethrin (>1500-fold), and mortality following exposure to pyrethroid-treated bednets was low (<30% mortality in cone bioassays). The 1014F *kdr* mutation was almost fixed (≥90%) in all villages but the 1575Y *kdr*-amplifying mutation was relatively rare (<15%). The carbamate and organophosphate resistance-associated *Ace-1* G119S mutation was also detected at moderate frequencies (22-43%). Transcriptome analysis identified overexpression of P450 enzymes known to metabolise pyrethroids (CYP9K1, CYP6P3, and CYP6M2), and also a carboxylesterase (COEAE1F) as major candidates. CYP6P3 expression was high but variable (up to 33-fold) and correlated positively with deltamethrin resistance intensity across villages (r^2=0.78, P = 0.02). Tools and strategies to mitigate the extreme and multiple resistance provided by these mechanisms are required in this area to avoid future control failures.

Background

Insecticide-based control methods continue to play a crucial role in reducing vector-borne diseases. Insecticides are deployed against malaria mosquitoes most commonly via long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS). The significant increase in coverage with LLINs over the past 20 years has been associated with a marked reduction in malaria burden [1]. However, recent estimates suggest that progress has stalled, with insecticide resistance likely one of the major contributing factors. Whilst selection from other sources, especially agriculture [2], may be important in some areas, there is evidence that the wide scale use of IRS and particularly LLINs is contributing to selection for pyrethroid resistance in major African vectors of malaria [3]. Resistance to pyrethroids is likely to increase

further over the coming years, given that pyrethroids remain an important component of all currently available bednets, including newer dual-action LLINs [4–6].

Until recently, only four classes of insecticides (pyrethroids, organochlorines, carbamates and organophosphates) were licenced for use to control adult mosquito vectors. The pyrrole insecticide, chlorfenapyr, and the neonicotinoid, clothianidin, have recently been added to this list and are deployed either alone or in combination with pyrethroids [7–9]. Except for these new insecticide classes, resistance to all currently available insecticides has been documented in *Anopheles* mosquito species across much of sub-Saharan Africa [10–14]. The best known mechanisms conferring resistance to insecticides are target site modification and increased detoxification. Substitutions in the para voltage-gated sodium channel (VSGC) ─ the target site for pyrethroids and DDT [15–17] (L1014F and L1014S) ─ are widespread in *An. gambiae* and confer knock down resistance (*kdr*), with a third variant (N1575Y)[17] capable of amplifying resistance where present[18]. A further mutation (G119S) in acetylcholinesterase (*Ace-1*) causes resistance to organophosphate and carbamate insecticides, which target this enzyme [19–21]. The G119S mutation is associated with a fitness cost in the absence of insecticides [22] but *Ace-1* gene duplication, coupling resistant and susceptible alleles, or multiple resistant alleles on the same chromosome, has emerged in *Anopheles gambiae* mosquitoes to offset deleterious effects [23].

Metabolic resistance arises from enhanced detoxification of insecticides. Three classes of metabolic enzymes, carboxylesterases (COEs), glutathione-S-transferases (GSTs) and cytochrome P450s have been linked with resistance in various species of mosquitoes, with the latter most frequently implicated in metabolism of pyrethroids and carbamates [10,24–26]. Overexpression of several P450s has been associated with insecticide resistance, but relatively few have been validated as metabolizers *in vitro*, and thus only these can be regarded definitively as candidates capable of causing resistance. Notably, CYP6M2, CYP6P3 and CYP9K1 have all been validated not only as pyrethroid-metabolizers but also of unrelated insecticides (DDT, bendiocarb and pyriproxyfen, respectively) demonstrating how the substrate flexibility of some P450s can cause cross-resistance by metabolizing insecticides from diverse classes [10,27,28].

Here we report on a study aimed at evaluating the current status of insecticide resistance in malaria vectors in central Côte d'Ivoire. Previous research has shown that *Anopheles* malaria vectors in Côte d'Ivoire have developed resistance to all of the four traditional classes of approved adulticides [21,29,30]. Resistance mechanisms detected in Côte d'Ivoire to date include *kdr* and *Ace-1* (mutation and duplication) [29] and, in *An. coluzzii* from the southern part of the country, overexpression of P450 genes, especially *Cyp6M2* and *Cyp6P3* [10]. However, information on resistance intensity and a comprehensive assessment of the genetic mechanisms driving resistance in *An. gambiae* is lacking (and especially for central Côte d'Ivoire). The present study was thus conducted prior to the onset of a cluster randomized controlled trial (CRT) of the In2Care EaveTubes [31], to characterize insecticide resistance across a subset of villages and provide a baseline against which future changes may be measured through the course of the CRT.

Results

Insecticide resistance and LLIN efficacy

Mortality rates of *An. gambiae* s.l. exposed to WHO diagnostic doses of deltamethrin, cyfluthrin, and bendiocarb were generally quite low with most villages below 50% (Fig. 2.1), and lower still for DDT (<15%). Mortality results for the two pyrethroids were strongly correlated across villages (Spearman's $\rho = 0.98$, $n = 8$, $P < 0.001$), and each was also positively correlated with bendiocarb mortalities, though neither significantly (maximum $\rho = 0.64$, minimum $P = 0.09$). There was significant variation among villages in bioassay mortalities for each insecticide, though there was no difference between groups of villages comprising the study arms for any insecticide (Table 2.1). For pirimiphos methyl, there was only one survivor out of over 800 females tested. However, the 1% dose used is four times the standard recommended diagnostic concentration, and results are best interpreted as evidence that higher intensity resistance is absent, rather than the population being fully susceptible.

The intensity of resistance to deltamethrin measured using adapted CDC bottle assays was extremely high in all villages (RR_{50} range 1441 to 2405) (Table 2.2 and Table 2.S1A&B). There was no difference between villages (overlapping 95% confidence intervals of LD 50 values in Table 2.2).

Exposure to a pyrethroid-only LLIN (PermaNet 2.0) killed 100% of the susceptible *An. gambiae* Kisumu strain but fewer than 30% from any study village (Fig. 2.2). Though the correlation between net-induced mortality and resistance intensity to deltamethrin was not significant ($\rho = 0.41$, n = 8, P = 0.32), the generally poor performance of the pyrethroid-only LLIN tested is consistent with the very high pyrethroid resistance in the villages. Mortality rates of mosquitoes exposed to LLIN material differed significantly between villages (Table 2.3).

Species identification and target-site resistance

Overall, 975 randomly selected *An. gambiae* s.l., which comprised of unexposed and pyrethroid bioassay survivors, were identified to species by PCR. A subset of these mosquitoes were screened for common resistance-linked *kdr* mutations in the voltage-gated sodium channel. The predominant malaria vector species in seven of the villages was *An. gambiae* (84-98%) with a single village (Kouakro) in which *An. coluzzii* and *An. gambiae* were found in comparable proportions (50%) (Table 2.4).

The 1014S mutation was not detected in any of the 367 mosquito samples screened. The 1014F mutation was found at very high frequency (>0.9) whereas the 1575Y allele was present at low frequency (<0.15) in mosquito populations across villages. Allele frequencies of the 1014F mutation did not differ among villages ($\chi^2_7 = 12.2$, P = 0.59) (Table 2.5). Likewise, allele frequencies of the 1575Y mutation were very similar across villages ($\chi^2_7 = 1.1$, P = 0.99) (Table 2.5). The frequency of 1575Y also did not differ between unexposed mosquitoes and bioassay survivors ($\chi^2_1 = 0.05$, P = 0.82). In each village, neither locus showed significant deviation from Hardy-Weinberg equilibrium (Table 2.5).

There was significant variation in allelic frequency of the G119S polymorphism across villages (22% to 43%; $\chi^2_7 = 22.75$, P = 0.002), which essentially reflected variation in heterozygote vs susceptible homozygotes because resistant homozygotes were extremely rare (Fig. 2.3). Analysis of the qPCR dye balance ratio in heterozygotes, which can indicate variation in the relative number of duplicated serine alleles, showed no significant variation among villages in serine/glycine ratios ($F_{1,7} = 0.94$, P = 0.47), suggesting a similar copy number profile of serine alleles. There was no evidence that the frequency of G119S differed between *An. coluzzii* and *An. gambiae* in the mixed-species village of Kouakro ($\chi^2_1 = 1.2$, P = 0.27).

Genome-wide transcription analysis

Whole genome microarray experiments were conducted to identify candidate genes potentially involved in insecticide resistance in the dominant species *An. gambiae* collected from two of the study villages (N'Guessan Pokoukro and Sessenouan), in comparison with two susceptible strains, using a strict criterion for significance based on replicated fold change and multiple-testing corrected P-value thresholds.

Out of a total of 14,914 probes screened, 616 corresponding to 525 genes were significant according to the above filtering criteria (Fig. 2.4, Table 2.S2A). Of the 267 genes (with 340 transcripts) over-expressed in all comparisons, we focused on those with known or putative links to detoxification or resistance more broadly, which comprised of 18 genes, including 11 cytochrome P450s, 3 glutathione S-transferases (GSTs), 2 carboxylesterases, an alcohol dehydrogenase, and peroxidase, a redox gene and transporters and cuticular genes (Table 2.S2A). The three detoxification genes within the top 20 most over-expressed genes were cytochrome P450s (Table 2.S2B) of which *Cyp6P3* and *Cyp9K1* exhibited >10-fold change and *Cyp6M2* with ≥8 fold-change, but more variability across comparisons, relative to susceptible lab strains (Table 2.S2B). Other highly over-expressed genes (within top 20) lack current description or have no putative link to insecticide resistance, based on current knowledge, such as the most highly expressed gene (h+ transporting atp synthase subunit: fold change >60). It is interesting to note that one of the two overexpressed esterases is the target site gene *Ace-1* with average overexpression of almost 3-fold, consistent with the expected presence of duplicated resistance alleles.

Quantitative RT-PCR expression of candidate genes in selected villages

Candidate genes chosen for further analysis using qRT-PCR included the most over-expressed detoxification genes (*Cyp6P3*, *Cyp9K1*, and *Cyp6M2*), the most overexpressed esterase *COEA1F*, and the redox partner gene cytochrome P450 reductase. A further P450, *Cyp6Z3*, was chosen because it was significant in 3 out of four comparisons and we wished to examine whether the stringency of our filtering might be excluding potential valid detoxification candidates. The validation also included two under-expressed genes; one meeting the significant threshold across all comparisons (*GSTD11*) and one that was strongly

underexpressed in one population (*Cyp9J5*), providing additional variation for qPCR vs microarray validation.

There was good agreement between qPCR and microarray estimates of gene expression (r^2 = 0.73, P = 0.001) (Fig. 2.S1). Fold change was generally higher in microarray results except for *Cyp6P3* and *Cyp9K1*, which showed higher expression in qPCR analysis.

The expression levels of the eight chosen candidate genes were assessed for variation across the eight villages. There was significant variation in the level of expression of all genes among villages (Kruskal Wallis tests, maximum P < 0.01) (Fig. 2.5 & Table 2.S4A). The highest general level of expression was for *Cyp6P3*; with a particular peak in the N'Guessan Pokoukro village (33-fold change) but much lower levels in some other villages. Interestingly, there was a significant correlation between fold change in *Cyp6P3* and the intensity of resistance to deltamethrin (r^2 = 0.78, P = 0.023) (Fig. 2.S2). Expression level of all screened genes did not differ between unexposed mosquitoes and those that survived exposures to deltamethrin and cyfluthrin (Fig. 2.S3 & Table 2.S4B).

Discussion

Insecticide resistance in African malaria vectors is one of the major challenges facing malaria control programmes. A better understanding of the prevalence, intensity and mechanisms of resistance could inform the development of resistance management strategies. Results from the present study, the first of its kind on *An. gambiae s.s.* from Côte d'Ivoire, demonstrate phenotypic variation at a small spatial scale likely underpinned by variation in resistance mechanisms, notably P450 expression level and variation in *Ace-1* genotypic frequencies.

Phenotypic resistance

High prevalence of resistance was evident for all insecticides tested, with the exception of pirimiphos methyl which was tested at a higher than diagnostic dose. These results are consistent with findings from previous studies conducted in the same area [21,32]. Multiple insecticide resistance has been previously documented in *An. coluzzii* from the southern part of the country (Tiassale) [10,21]. This observation is of significant concern for vector control, as resistance to non-pyrethroids limits the options for pyrethroid resistance management.

The intensity of deltamethrin resistance detected in the present study is among the highest reported to date in *Anopheles* mosquitoes. While quantitative measure of resistance enables detection of potential changes in resistance level in mosquito populations [33], intensity level associated with operational control failure has yet to be defined. Nevertheless, the poor performance of LLINs in WHO cone assays against the local *Anopheles* mosquitoes is consistent with the high resistance intensity recorded and is suggestive of a potential loss of community protection from pyrethroid-only LLINs in this area.

Resistance mechanisms

The molecular basis of the multiple insecticide resistance phenotype was investigated using microarray experiments performed on *An. gambiae* from two villages (one from each study arm). Analysis focused on overexpression of potential resistance-linked gene, but it should be noted that many genes of unknown function or no putative link to insecticide resistance were also significantly over-expressed in field mosquitoes compared to susceptible lab colonies. If this observation is reproducible, it could merit further investigation. Of the most highly overexpressed genes, *Cyp6P3* and *Cy6PM2* have been implicated repeatedly in pyrethroid resistance and also in resistance to carbamates in *An. gambiae* and/or *An. coluzzii* [10,34] and are known to metabolize pyrethroids [35]. Overexpression of *Cyp9K1* has been linked to pyrethroid resistance in *An. gambiae* s.l. from Cameroon [12], Benin [36] and Bioko Island [28], and has also recently been validated as a pyrethroid and pyriproxyfen metabolizer [28]. This is the first report of significant over-expression of *Cyp9K1* in Côte d'Ivoire, and the fold change in expression in mosquitoes from our study area is much higher than expression reported in previous studies [28,36]. The over-transcription of this set of P450s, coupled with the near fixation of *Vgsc* 1014F and the presence of the 1575Y mutations in the local malaria vectors, likely underpins the extreme resistance to pyrethroids and DDT in this part of Côte d'Ivoire. The carboxylesterase COEAE1F and the cytochrome P450 reductase (CPR) were among the significantly over-expressed detoxification candidates. Carboxylesterases can play a role in pyrethroid metabolism, for example when paired secondarily with P450s such as CYP6Z2 [37] (to which the candidate CYP6Z3 is extremely similar) and CPR is a redox partner for P450s and might also link with resistance [38]. These over-transcribed genes could have contributed to the high pyrethroid resistance observed. Although pyrethroid resistance in this population of mosquitoes is associated with both target site insensitivity and metabolic mechanisms, evidence from a recent study suggests that the latter resistance type is likely to

account for the most extreme pyrethroid resistance intensity detected [11]. DDT resistance is often mediated by over-expression of Glutathione S transferases (GST) and *kdr*-based mechanisms. The absence of over-expressed GST indicates that the high DDT resistance might have been primarily due to 1014F and in some cases also 1575Y *kdr* mutations, perhaps coupled with overexpression of some genes less commonly associated with DDT resistance such as *Cyp6M2* [39]. The resistance intensifying mutation 1575Y was detected at relatively low frequency (<15%) and found only in mosquitoes with the phenylalanine allele, confirming that this mutation only occurs on a 1014 haplotype background [17]. Originally identified in Burkina Faso, the 1575Y mutation is spreading across the continent and has been reported in West and Central Africa [13,40]. Understanding the key determinants behind the rapid increase in the prevalence of the 1575Y *kdr* allele could help slow or even stop the spread of this mutation. Further investigation is also needed to determine if the survival advantage associated with the co-occurrence of the 1575Y and 1014F [34] mutations could negatively impact control efforts. The allelic frequency of this emerging gene should be closely monitored in areas where novel tools incorporating pyrethroids are deployed.

Carbamate resistance is primarily mediated by acetylcholinesterase insensitivity (G119S) and elevated expression of certain P450s [10]. The high survival to bendiocarb is consistent with the high frequency of *Ace-1* heterozygotes, which as shown by elevated *Ace-1* expression are likely present in higher copy numbers which raises carbamate resistance [10]. *Cyp6P3* was also over-expressed and has been shown to generate a moderately bendiocarb-resistant phenotype via transgenic expression and to metabolize bendiocarb, albeit with low catalytic efficiency. Indeed, susceptibility to bendiocarb in *An. gambiae* mosquitoes from Bioko has been reported despite over-expression of *Cyp6P3* [28], and it may be that this is a mechanism of lesser importance. The role of *Cyp6M2*, which generates a much stronger resistance phenotype than *Cyp6P3* via transgenic expression but does not metabolise bendiocarb remains unclear, but it is certainly plausible that both combine with *Ace-1* copy number variation of resistant alleles to generate resistance phenotypes as observed in *An. coluzzii* from southern Côte d'Ivoire [10].

Overall the resistance mechanisms detected in the study area are similar to those of *An. coluzzii* from southern Côte d'Ivoire [10]. These vector populations are from the same country and potentially exposed to the same insecticide selection pressure; mainly from the use of pyrethroid treated nets and insecticides for crop protection [2]. However, the elevated expression of the pyrethroid and pyriproxyfen metabolizing enzyme CYP9K1 in this study was

not reported in the Tiassale mosquitoes. It could be that the frequency of this gene was low and undetectable at the time the Tiassale mosquito was characterised (in 2014) and might have increased only recently.

Fine scale variation

The villages were all within 50 km radius away from the town of Bouaké and varied between a few km and a few tens of km apart. Yet, there was significant variation in both phenotypic data for all insecticides to which resistance was detected and in expression of all genes studied across villages. Monitoring of insecticide resistance in malaria vectors is often performed at large geographical scale. However, as seen in the present and previous studies [41,42], variation in insecticide resistance can occur at small spatial scales. This result indicates the need to account for potential micro geographic variation during resistance surveys, rather than assuming broad-scale homogeneity for which single sites can act as reliable sentinels. Although wide-ranging phenotypic testing programmes incorporating fine-scale testing are unlikely to be realistic for most programmes, variation detected by molecular marker-based surveillance could aid in identifying sites of interest which could be prioritised for phenotypic testing. Interestingly, *Cyp6P3,* which showed the highest expression and high variation among villages correlated positively with resistance intensity suggesting a useful gene expression assay to predict resistance intensity.

Conclusion

Results from this study are concerning given that *Anopheles* mosquitoes from this part of Côte d'Ivoire have developed strong resistance to the main insecticides currently being used for malaria control. Metabolic genes that were found to be over-expressed in this study have previously been shown to metabolize some of the compounds being incorporated in new classes of bed nets. For example, a range of P450s, including those identified in the present study (*Cyp6P3*, *Cyp6M2* and *Cyp9K1*) metabolize pyriproxifen - an insect growth regulator deployed in nets to sterilise pyrethroid resistant mosquitoes [43]. This is consistent with the poor performance of Olyset Duo, a permethrin plus pyriproxyfen mixture LLIN in experimental huts in these areas of Côte d'Ivoire [44] and in a randomised controlled trial in Burkina Faso [45] where these pyriproxifen metabolizing genes were also found [11]. Use of PBO co-treated

LLINs could be a more promising option in this area, given the apparent importance of P450 overexpression, though careful evaluation of efficacy and durability will be required.

The insecticide selected for use in the lethal house lure CRT is the pyrethroid beta-cyfluthrin [46]. This is because a previous study showed that the EaveTubes technology delivers an overwhelming dose of insecticide causing high levels of mortality of even resistant mosquitoes [46]. The data from the current study provides baseline information to track whether this additional use of pyrethroids on top of LLINs in the trial area will lead to changes in phenotypic resistance and associated molecular mechanisms.

Methods

Study area and collection of mosquitoes

This study was performed as part of a two-armed cluster randomized controlled trial (CRT) evaluating the impact of an intervention defined by the WHO Vector Control Advisory Group as a "Lethal House Lure", which combines household screening (S) with a novel insecticide delivery system called In2Care EaveTubes (ET). The trial, which ran between May 2017 and May 2019 in central Côte d'Ivoire in the Gbèkè district, aimed to investigate whether the use of screening plus EaveTubes (SET) on top of universal coverage of LLINs (PermaNet 2.0), provides greater protection against malaria than LLINs alone. The design of the trial is described in Sternberg *et al* [31] and involves 40 villages, half assigned to SET plus LLINs, and the other half allocated to LLINs alone. The study area is a pre-forest zone with a humid tropical climate and covers an area of 9,136 km^2 with a population of over one million people. Rice farming is the dominant form of subsistence agriculture and the presence of rice growing valleys across the region provides extensive breeding sites for *Anopheles* mosquitoes. Malaria transmission is year-round with a peak during the rainy season (from May to October) [47,48].

Eight study villages (four in each treatment arm) were selected for insecticide resistance monitoring, based on the availability of mosquito breeding sites for sampling (Fig. 2.6). A description of each sampling site is provided in Table 2.6. Mosquitoes were collected from each village using the dipping method from September 2016 to November 2016. Whenever possible, mosquito larvae were collected from at least two breeding sites spread out over the village, and collections from the same village were subsequently pooled. Larvae were transported to the insectary at the Institut Pierre Richet (IPR), fed on ground Tetramin fish food

and reared to adulthood under ambient temperature. Emerging adult mosquitoes were kept in netted cages and maintained on 10% honey solution. All adult female mosquitoes were morphologically identified as *An. gambiae* s.l. using taxonomic keys.

Insecticide susceptibility assays

To assess the prevalence of resistance in *Anopheles* mosquitoes from the CRT area (central Côte d'Ivoire), WHO susceptibility tests were performed between September and November 2016 using adult *An. gambiae s.l.* mosquitoes emerged from larvae collected in eight selected CRT villages. The pyrethroid insecticide, beta-cyfluthrin is the active deployed in the EaveTubes [46] whereas deltamethrin is the insecticide in the LLIN (PermaNet 2.0). Bioassays were conducted using papers treated with diagnostic concentration of these two insecticides: 0.05% deltamethrin and 0.15% cyfluthrin. Additionally, susceptibility tests using paper impregnated with 4% DDT, 0.1% bendiocarb and 1% pirimiphos methyl were performed to assess the level of resistance to all four classes of WHO approved neurotoxic insecticides. The mosquitoes tested were 2-3 day-old adult female mosquitoes, emerged from larvae collected from study villages and reared in the insectary at IPR. Approximately 100 mosquitoes, in batch of 25, were exposed for 1h to insecticide-treated papers, and mortality was recorded 24h later. The same number of mosquitoes were exposed to untreated papers and served as control. Mosquitoes that survived exposure to either of the pyrethroids were monitored for an additional 24h, after which the survivors were preserved in RNA later for subsequent molecular testing.

Resistance intensity assays

To determine the intensity of resistance to pyrethroids in the local *Anopheles* mosquitoes, adapted CDC bottle assays were performed. Since both interventions (LLIN and EaveTubes) are treated with the same type of pyrethroids (pyrethroid type II), the intensity of pyrethroid resistance was determined using pyrethroid from one of these interventions. Bottles were coated with a range of deltamethrin concentrations (7.81µg/mL to 1000µg/mL), producing a range of mortality rates between 0% and 100% in mosquitoes from the study villages. Each bioassay included a control bottle treated with only acetone. The susceptible *An. gambiae* Kisumu strain (SS) served as reference and was tested against dosage range 0.001µg/mL-

0.5µg/mL. Two to three days old adult female mosquitoes were exposed for 1h at each concentration in four replicates of 25.

WHO cone bioassay

To determine the impact of resistance on susceptibility to the bed nets (PermaNet 2.0) deployed in the study area, standard cone bioassays were performed according to WHO procedures using adult female mosquitoes emerged from larvae collected from the eight study villages and the susceptible Kisumu strain. Approximately 60 mosquitoes were exposed to netting sample for 3 min and the mortality rate was determined 24h later. Control mosquitoes (~60) were exposed to an untreated net and served as control.

Species identification and target site resistance mechanisms

To type mosquitoes to species and identify target site resistance mechanisms in *Anopheles* mosquitoes from study villages, genomic DNA was extracted from a pair of legs taken from field mosquitoes that survived exposure to deltamethrin and cyfluthrin in WHO cylinder assays, and from a subset of unexposed female mosquitoes. The legs were boiled in 20µL of buffer solution for 90 min at 95°C. The member of the *An. gambiae* complex were identified to species using SINE-PCR [49].

TaqMan PCR assays were used to screen mosquito samples for mutations in the voltage gated sodium channel, including the 1014S, 1014F and 1575Y [17,50], and for the ace-1 G119S [51] resistance mutation in acetylcholinesterase. Heterozygotes for *An. gambiae and An. coluzzii* are all expected to include duplications in some combination of (1) G and S alleles are paired on a single chromosome - a heterogeneous duplication (2), an unduplicated G allele, and (3) a multicopy S allele [52]. Variation in composition of G and duplicated S alleles can be detected quantitatively as a difference in dye balance in heterozygotes in TaqMan qPCR [53].

Whole genome microarray

A genome-wide transcription profiling was performed to identify genes differentially expressed in mosquitoes from two CRT villages (one from each study arm) relative to

susceptible lab strains. All of the villages involved in the CRT were at least 2km apart; however, to capture the whole range of over/under expressed genes in mosquitoes from the study area, two villages much further away from each other were selected for microarray analysis. Mosquitoes used in microarray studies were confirmed as *An. gambiae* using SINE-PCR.

Gene expression profiles of unexposed, female *An. gambiae* mosquitoes from one control village (N'Guessan Pokoukro) and the survivors of deltamethrin exposure from one intervention village (Sessenouan) were compared to those of two susceptible lab strains, *Anopheles gambiae* Kisumu and *Anopheles gambiae* Ngousso, using an interwoven loop design (Fig. 2.S4). Inclusion of survivors from one village and unexposed from another, with the highest prevalence of pyrethroid resistance maximised chances of identifying resistance-associated candidate genes, whilst ensuring that overexpression induced primarily by exposure (i.e. gene induction) was precluded. Field-collected mosquitoes included in the microarrays analysis were solely the most predominant species, *Anopheles gambiae*. Significant differential expression between field mosquitoes from the two villages and the two insecticide susceptible lab strains was identified using a filtering approach. This was based on a $P < 0.05$ (after Bonferroni correction), a fold change in expression > 2 or <-2 and directionality i.e. the same direction of differential expression (upregulated or down-regulated) in the 4 comparisons (N'guessan Pokoukro vs Kisumu, N'guessan Pokoukro vs Ngousso, Sessenouan vs Kisumu, Sessenouan vs Ngousso). Total RNA was extracted from batches of ten female *An. gambiae* mosquitoes using a PicoPure RNA isolation kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Total RNA extracted from mosquitoes was treated using DNase (RNase free DNase set, Qiagen Hilden Germany). Before further use, the concentration and quality of the extracted RNA were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and a 2100 Bioanalyzer (Agilent Technologies). Four biological replicate extractions of total RNA for each mosquito population or colony were amplified and labelled using the Low Input Quick Amp Labeling Kit (Agilent Technologies). The Agilent Agam15k array was used for dual-color hybridizations (N'guessan Pokoukro vs Kisumu, N'guessan Pokoukro vs Ngoussou, Sessenouan vs Kisumu, Sessenouan vs Ngoussou) [54]. The labelled samples were hybridized using a Gene Expression Hybridization Kit (Agilent Technologies). Washing, scanning and feature extraction were performed according to the manufacturer's

recommendations. The design of the microarray experiment was optimized through comparison of the above strains across four microarray slides.

Quantitative reverse transcriptase PCR for candidate gene expression in field mosquitoes

The expression of a subset of genes from microarray known to play a role in insecticide resistance in *Anopheles gambiae* mosquitoes was taken forward for validation and measurement in field mosquitoes from the eight villages using reverse-transcription quantitative PCR (RT-qPCR). For each village, the expression for each gene of interest was measured in three cohorts of mosquitoes: non-exposed, deltamethrin and cyfluthrin survivors. Prior to qPCR experiments, RNA was extracted from field mosquitoes and quantified using the Nanodrop spectrophometer. cDNA was subsequently synthesized from 11ng of RNA using oligo(dT) 20 (50 μM) and SuperScript III (200U) (Invitrogen) and purified through a DNA-binding column (Qiagen). Three pairs of primers of each target gene were designed using Primer-BLAST tool (NCBI: http://www.ncbi.nhi.gov/tools/primers-blat/). The primer pair with the highest efficiency value (~100%), determined by running standard qPCR using serial dilution of a single cDNA sample, was selected for subsequent qPCR (details of the primers are given in Table 2.S3). For each qPCR reaction, four biological replicates of each treatment group and two technical replicates were used. QPCR was performed using an Agilent Mx3005P QPCR System and the cycling condition was as follow: 95°C for 3 min, 40 cycles of 95°C for 10 s and 60°C for 10 s. Expression of the genes was normalized using references genes (Ribosomal S7 and Elongation Factor).

Statistical analysis

Mosquito mortality rates were compared using Generalized linear models with a binary link function in SPSS v23. WHO assessments of mortality rates are: less than 90% indicates resistance; higher than 98% indicates susceptibility: between 90 and 98% requires further testing to confirm resistance status [55]. The intensity of resistance (Resistance Ratio, or RR50) was estimated using the R statistical software version 2.15.0 to compare the LD50 of the wild population relative to that of the susceptible lab strain. The variation in bioassay mortality rates of *An. gambiae* mosquitoes between villages was tested using Generalised Linear Model (GLM). The spearman test was used to test the correlation between resistance intensity to deltamethrin and bioassay mortality rates. The frequencies of target site resistance mutations in field *Anopheles* mosquito populations were compared between study villages using a χ^2-

square test with Yates continuity correction. Concordance with Hardy-Weinberg equilibrium was assessed for each resistance marker in each village using the permutation-based probability test in Genepop [56,57], with Bonferroni correction applied for multiple testing.

A MAANOVA model was used to analyse microarray data using previously described custom R-scripts[54]. Differentially expressed genes (over/under expressed) were those with a fold change consistently greater than 2 or less than -2 across the four comparisons (N'guessan Pokoukro vs Kisumu, N'guessan Pokoukro vs Ngousso, Sessenouan vs Kisumu, Sessenouan vs Ngousso) and with a significant Bonferroni-corrected p value in all four comparisons.

Outliers were identified and excluded from the qPCR dataset prior to analysis. The ΔΔCt method incorporating PCR efficiency was used to compare expression of each target gene between field mosquitoes and the lab strain [58]. Significant difference in fold change between field samples and the reference lab colony was estimated using a t-test ($P < 0.05$). Kruskal Wallis test was used to compare the level of expression of candidate genes across the three groups of field mosquitoes (unexposed group and mosquitoes surviving exposure to the two different pyrethroids in WHO cylinder assays).

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files)

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Acknowledgments

This study was funded by the Bill and Melinda Gates foundation. Grant Number: OPP1131603.

Author contributions

WAO, RN, DW, MBT and EDS designed the study. WAO, IZT, AAK, AMGB and LPAA collected the samples and performed the phenotypic testing. WAO and PP conducted the

molecular work. WAO and DW did the data analysis. WAO, RN and DW wrote the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors declare no competing interests

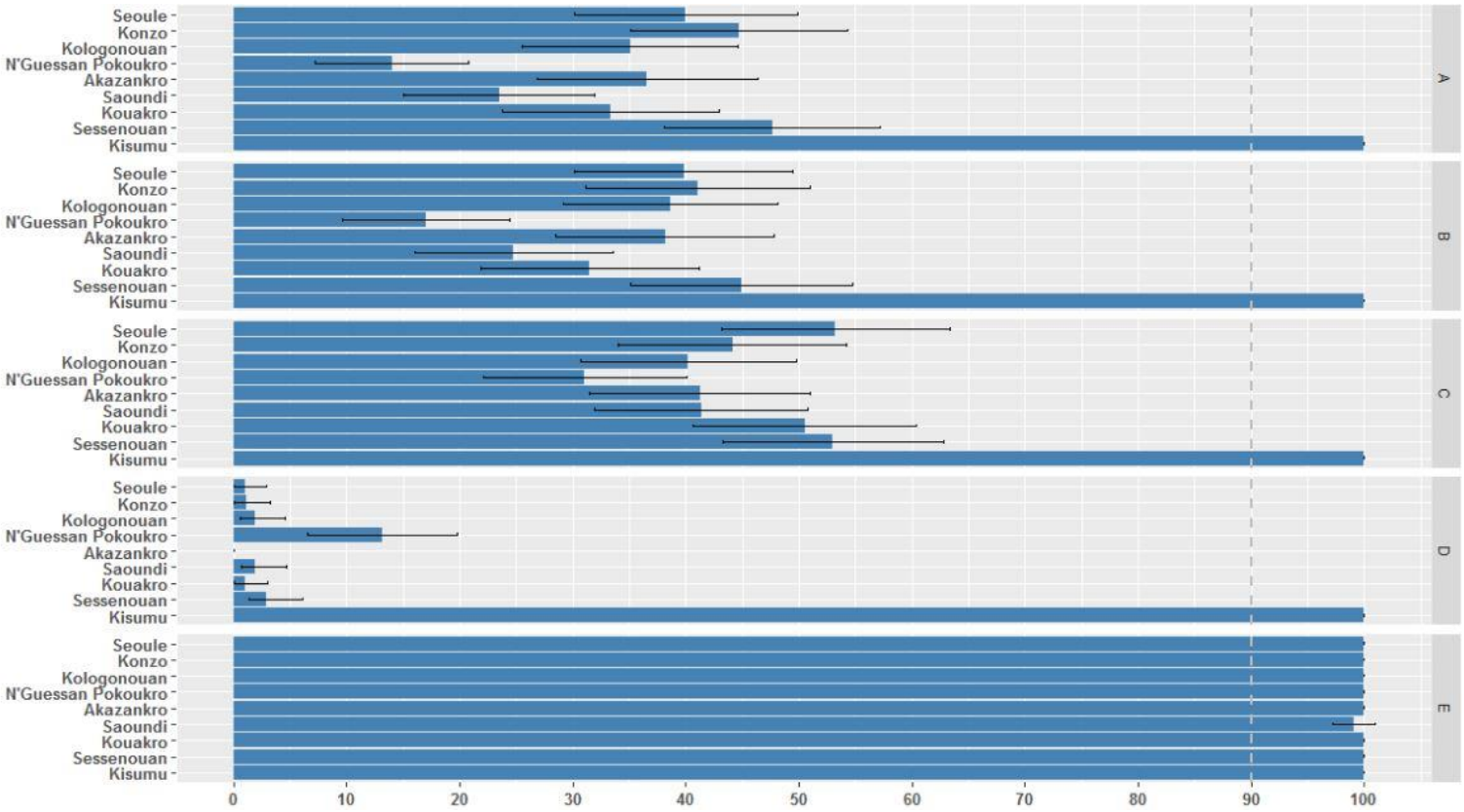

Fig. 2.1 Twenty-four-hour percentage mortality of *An. gambiae* from each village exposed in diagnostic bioassays to A) 0.05% deltamethrin, B) 0.15% cyfluthrin, C) 0.1% bendiocarb, D) 4% DDT and E) 1% pirimiphos methyl. Error bars represent 95% Cis and the dotted line indicates WHO resistance threshold.

Table 2.1: Generalised linear model testing the effects village on bioassay mortality for each insecticide

	Village (arm)		
	Wald $\chi 2$	df	P
deltamethrin	35.245	6	0.000004
cyfluthrin	25.53	6	0.0003
bendiocarb	14.52	6	0.024
DDT	18.03	6	0.006
pirimiphos methyl	not calculated because mortality near 100%		

Table 2.2: Intensity of resistance to deltamethrin in *An. gambiae* from different villages in the study area prior to the study.

Strain	Slope (SE)	LD 50 (95% CI)	LD 95 (95% CI)	RR50
Kisumu*	1.3 (0.18)	0.015 (0.009-0.022)	0.261 (0.136-0.767)	-
Akanzakro	1.7 (0.2)	27.2 (20.3-35.2)	250.1 (166.7-451.-0)	1873
Kologonouan	1.5 (0.1)	21.9 (15.8-28.5)	289.3 (190.0-534.4)	1504
Konzo	1.6 (0.1)	23.5 (19.1-28.3)	237.4 (173.7-358.2)	1617
Kouakro	1.7 (0.17)	22.4 (17.3-28.0)	213.5 (145.0-376.6)	1542
N'Guessan Pokoukro	2.1 (0.2)	33.7 (25.7-43.2)	207.0 (139.6-377.6)	2314
Saoundi	1.7 (0.1)	35.0 (28.9-41.9)	322.4 (237.8-477.3)	2405
Seoule	1.7 (0.1)	21.0 (17.2-25.0)	183.0 (139.1-261.3)	1441
Sessenouan	1.4 (0.1)	27.4 (20.8-34-8)	390.3 (256.3-708.0)	1883

*Susceptible reference strain;

LD: lethal doses expressed in μg/mL;

RR50: Resistance ratio, calculated by dividing the LD50 of the field mosquito population by that of the susceptible reference strain

Table 2.3: Generalised linear model testing the effects of village on net induced mortality

	Village (arm)		
	Wald $\chi 2$	df	P
PermaNet 2.0	20.87	7	0.004

Table 2.4: Species composition in the study villages

Study villages	*Anopheles gambiae* (N)	*Anopheles coluzzii* (N)	Hybrids (N)
Akanzakro	117	0	2
Kologonouan	86	0	0
Konzo	99	2	0
Kouakro	53	53	0
N'Guessan Pokoukro	160	12	1
Saoundi	116	2	0
Seoule	99	1	1
Sessenouan	158	12	1

N: number of *Anopheles gambiae* mosquitoes identified to species by SINE-PCR

Table 2.5: Frequencies of 1014F and 1575Y *kdr* alleles in *Anopheles gambiae* from study villages.

Study villages	N tested	L1014F					N1575Y				
		LL	LF	FF	F (1014F)	P value	NN	NY	YY	F (1575Y)	P value
Akanzakro	47	0	0	47	1	0.59	36	11	0	0.12	0.32
Kologonouan	46	0	1	45	0.99		38	6	2	0.11	
Konzo	48	0	4	44	0.96		35	13	0	0.14	
Kouakro	45	0	9	36	0.90		36	9	0	0.10	
N'Guessan Pokoukro	47	0	5	42	0.95		37	10	0	0.11	
Saoundi	41	1	4	36	0.93		34	6	1	0.11	
Seoule	40	1	1	38	0.96		31	8	1	0.13	
Sessenouan	53	0	0	53	1		43	9	1	0.10	

N: number of samples, L: leucine, F: phenylalanine, N: asparagine, Y: tyrosine. P values are from χ^2-squared tests

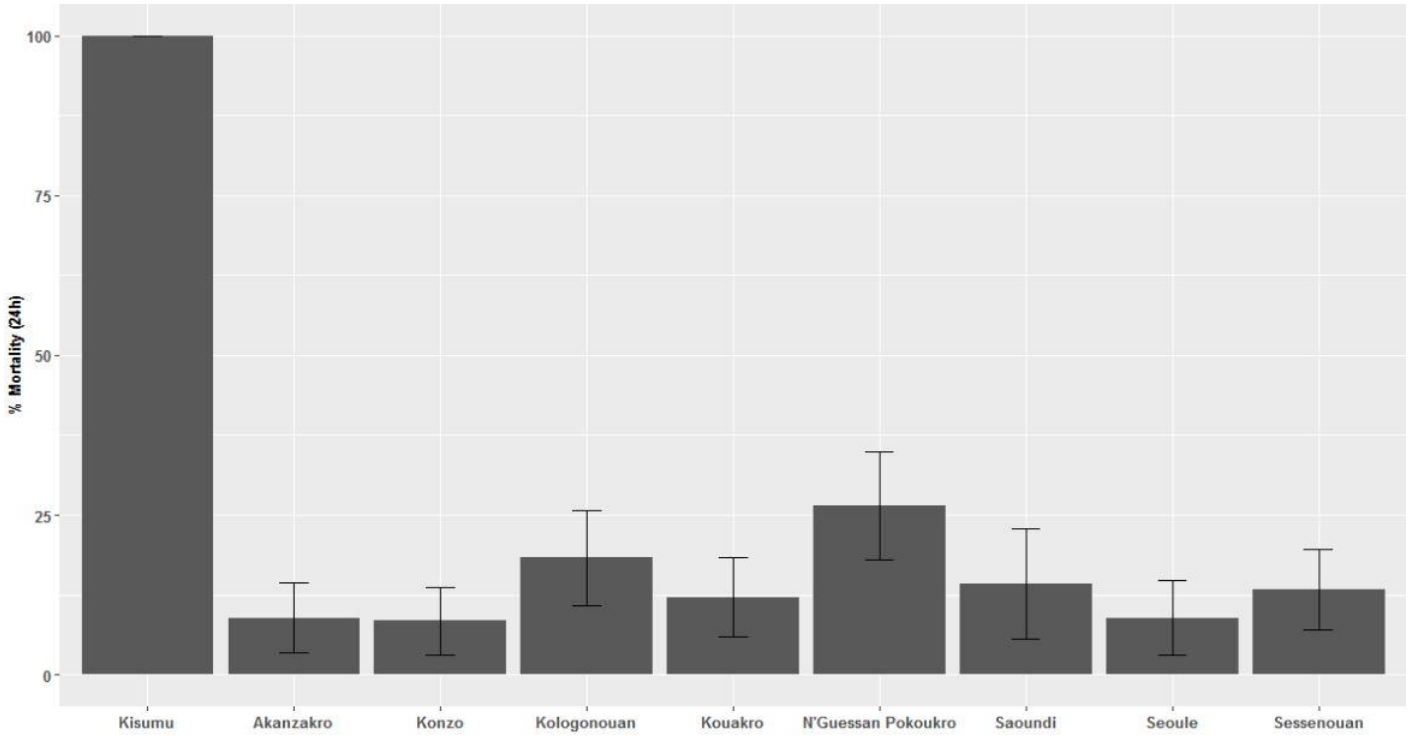

Fig. 2.2 Percentage mortality of susceptible Kisumu and resistant *Anopheles gambiae* exposed to LLIN material in WHO cone bioassays. Error bars indicate 95% Cis.

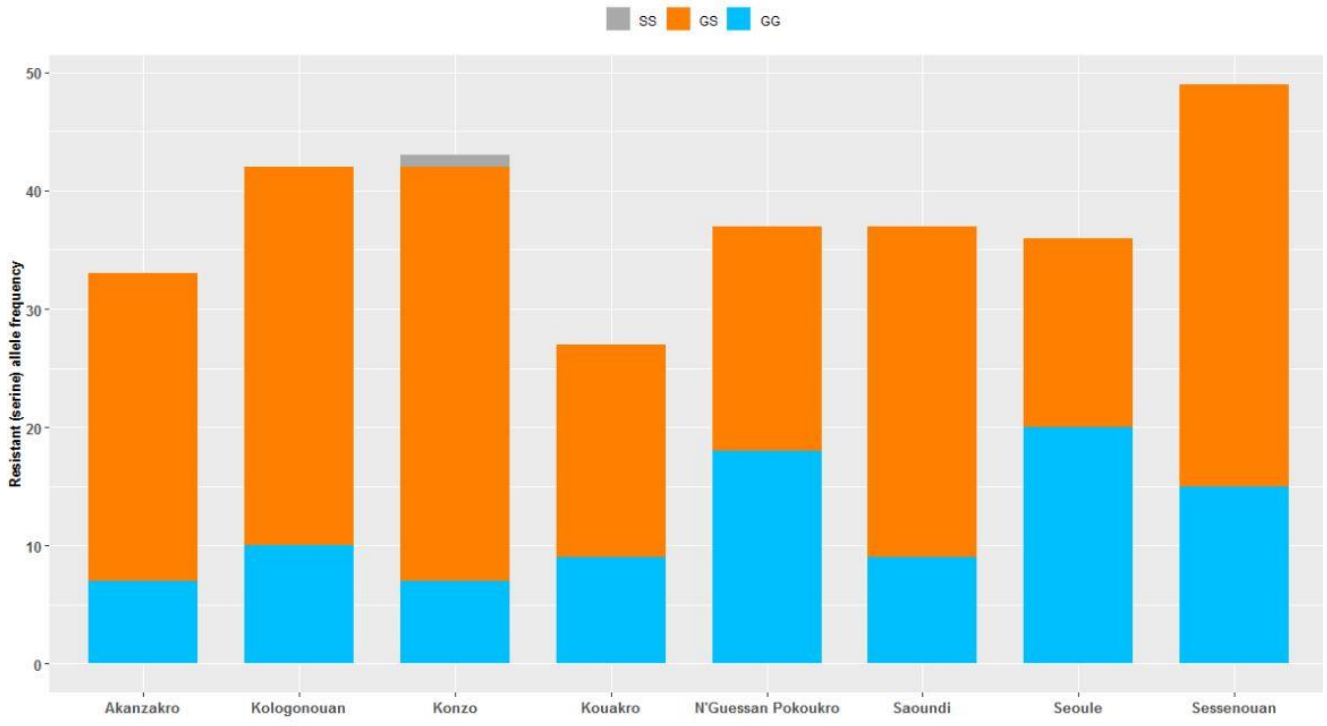

Fig. 2.3 Genotypic frequencies of the *Ace-1* G119S mutation in *Anopheles gambiae* mosquitoes. GG: Homozygote wild type; GS: Heterozygote resistant, SS: homozygote resistant

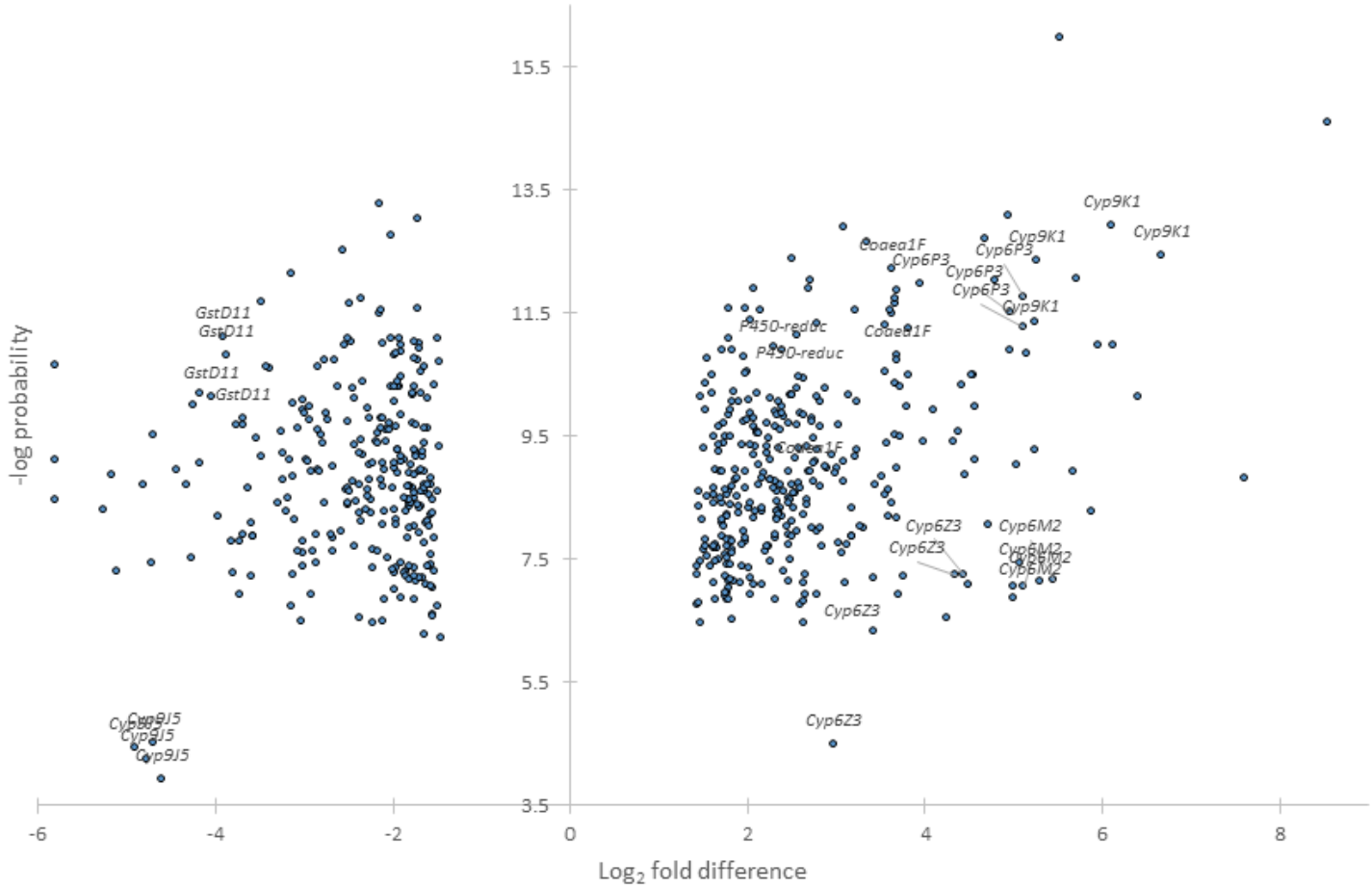

Fig. 2.4 Differentially expressed probes in *Anopheles gambiae* s.s. from two villages compared to two susceptible lab colonies. Average log_2-transformed fold-differences are plotted against average negative log probabilities. Probes from genes chosen for qPCR validation are labelled.

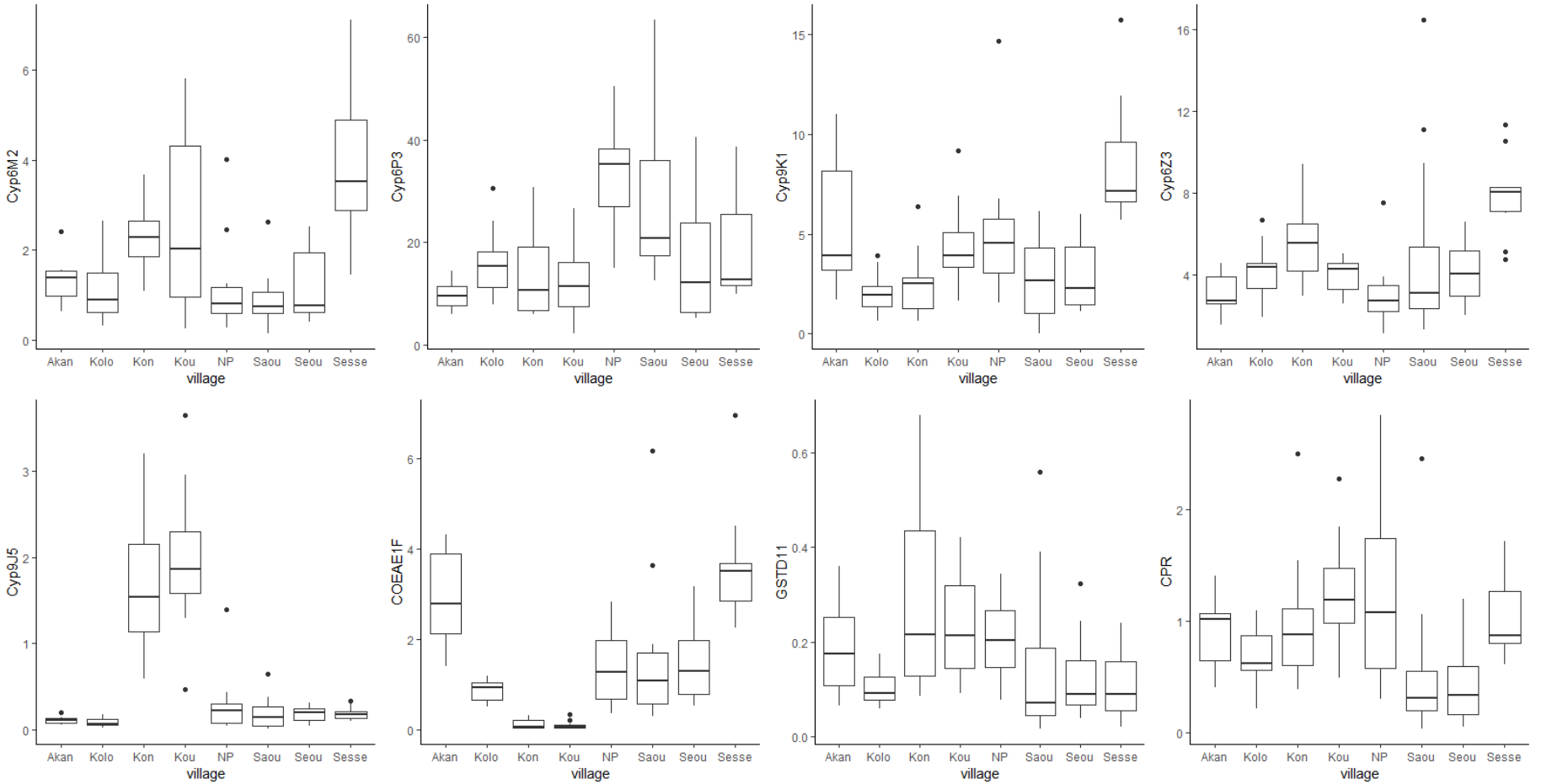

Fig. 2.5 Box-whisker plots show mean fold difference in expression of candidate genes (relative to susceptible colony samples) across villages. The boxes represent the 25% and 75% quartiles and the whiskers indicate 5% - 95% quartile ranges. The horizontal line within each box represents the mean fold difference in gene expression, and the dots denote outliers

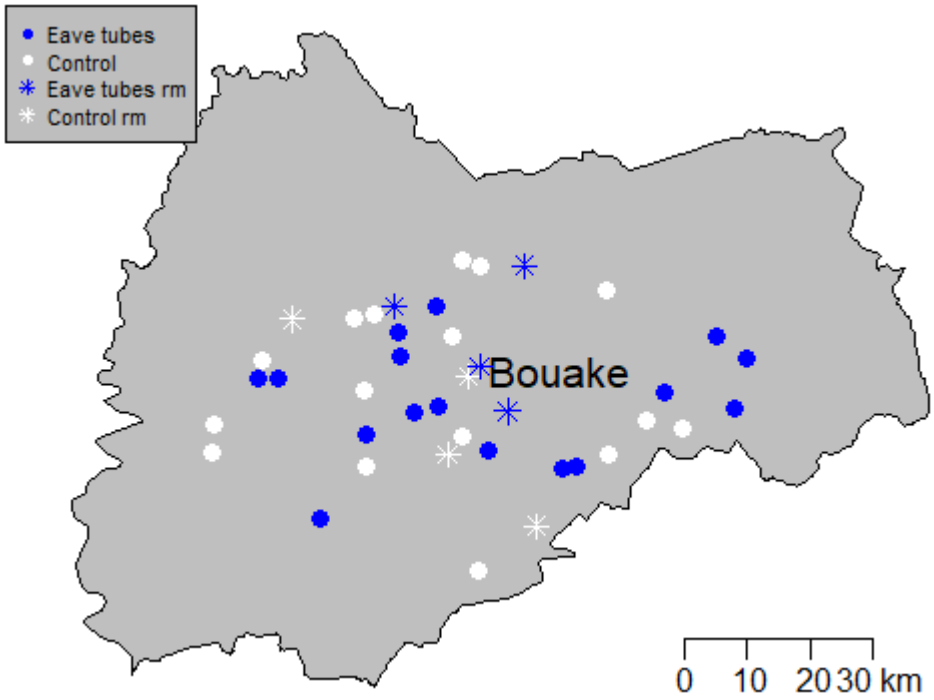

Fig. 2.6 Map showing study villages involved in insecticide resistance monitoring (rm)

Table 2.6: Location of study villages and description of mosquito breeding habitats.

Study village	Geographic coordinates		Arm	Type of breeding habitats
	Longitude	Latitude		
N'Guessan Pokoukro (NP)	7°56'N	5°20'W	Control (LLIN)	Water puddle
Kologonouan (Kolo)	7°66'N	5°17'W	Control (LLIN)	Water puddle
Konzo (Kon)	7°46'N	5°07'W	Control (LLIN)	Vegetable farm + rice field
Seoule Ahounzè (Seou)	7°76'N	5°42'W	Control (LLIN)	Rice field
Sessenouan (Sesse)	7°69'N	5°17'W	SET and LLIN	Vegetable farm + rice field
Kouakro (Koua)	7°83'N	5°08'W	SET and LLIN	Rice field + water puddle
Saoundi (Saou)	7°78'N	5°26'W	SET and LLIN	Rice field
Akazankro (Akan)	7°62'N	5°09'W	SET and LLIN	Vegetable farm + rice field

SET: Screening plus EaveTubes, LLIN: long-lasting insecticidal net.

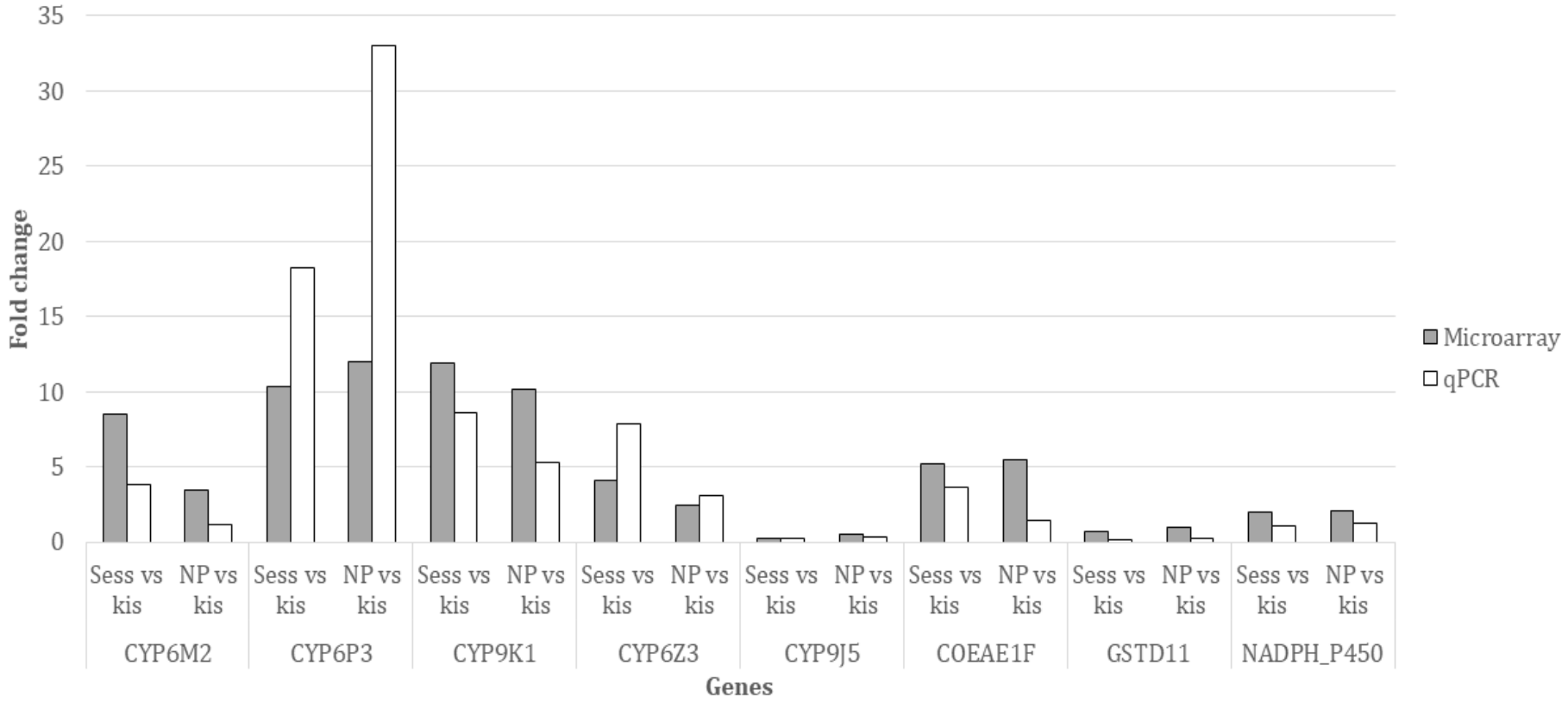

Fig. 2.S1: Side-by-side fold change in gene expression measured by microarrays and qRT-PCR for selected candidate genes. The overall correlation is r^2= 0.73.

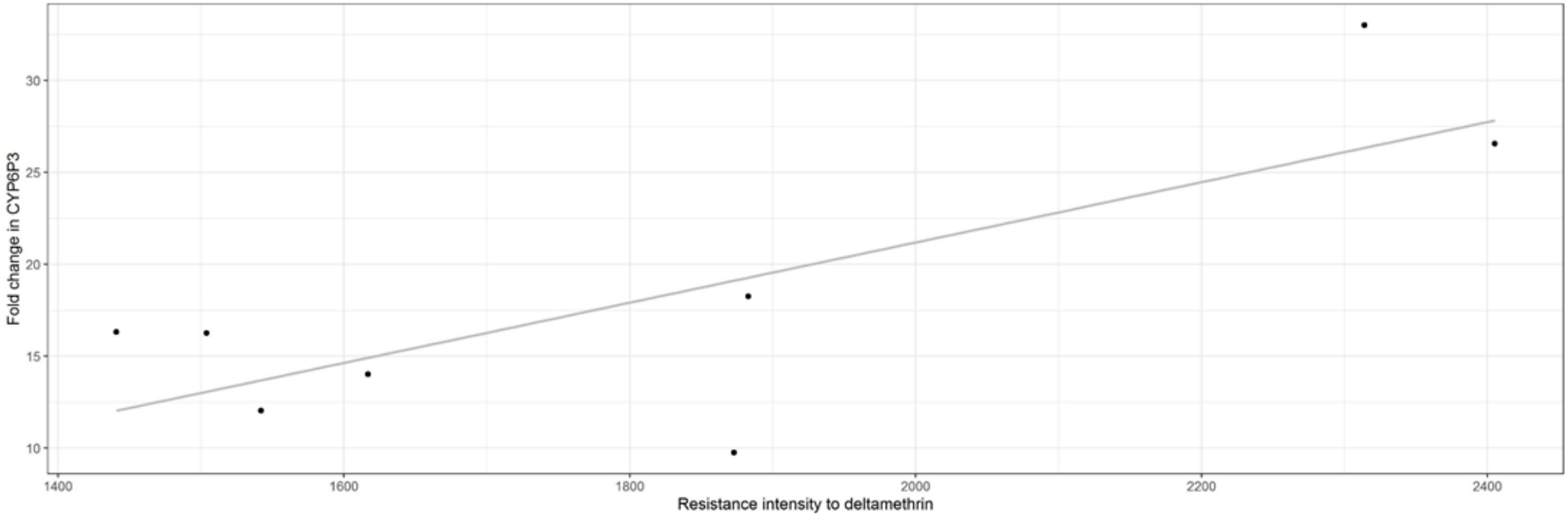

Fig. 2.S2: Association between fold change in *Cyp6P3* and resistance intensity to deltamethrin

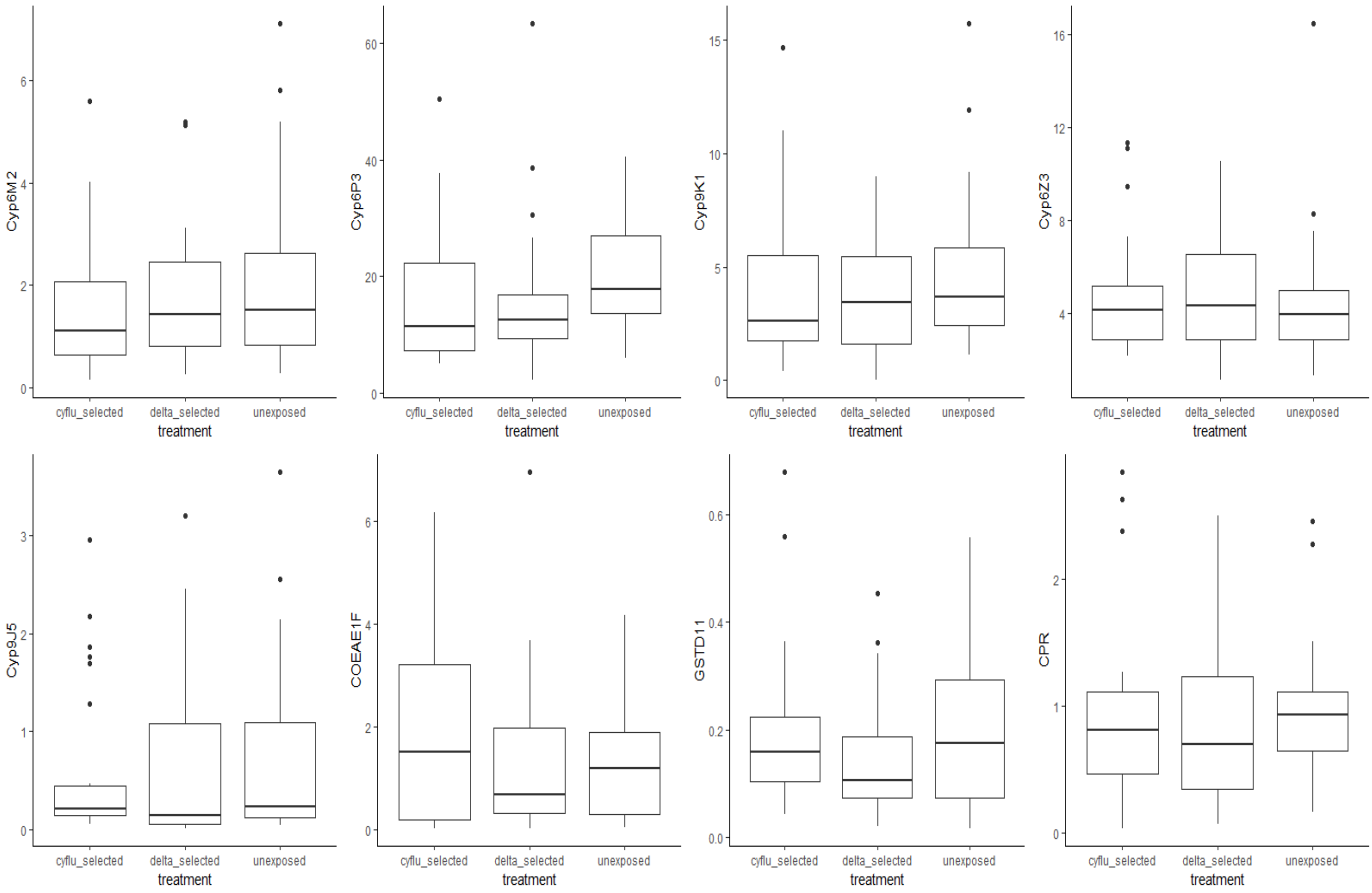

Fig. 2.S3: Boxplots show mean fold change in expression of candidate genes across treatments. The boxes represent the 25% and 75% quartiles and the whiskers indicate 5% - 95% quartile ranges. The horizontal line within each box represents the mean fold difference in gene expression and the dots denote outliers.

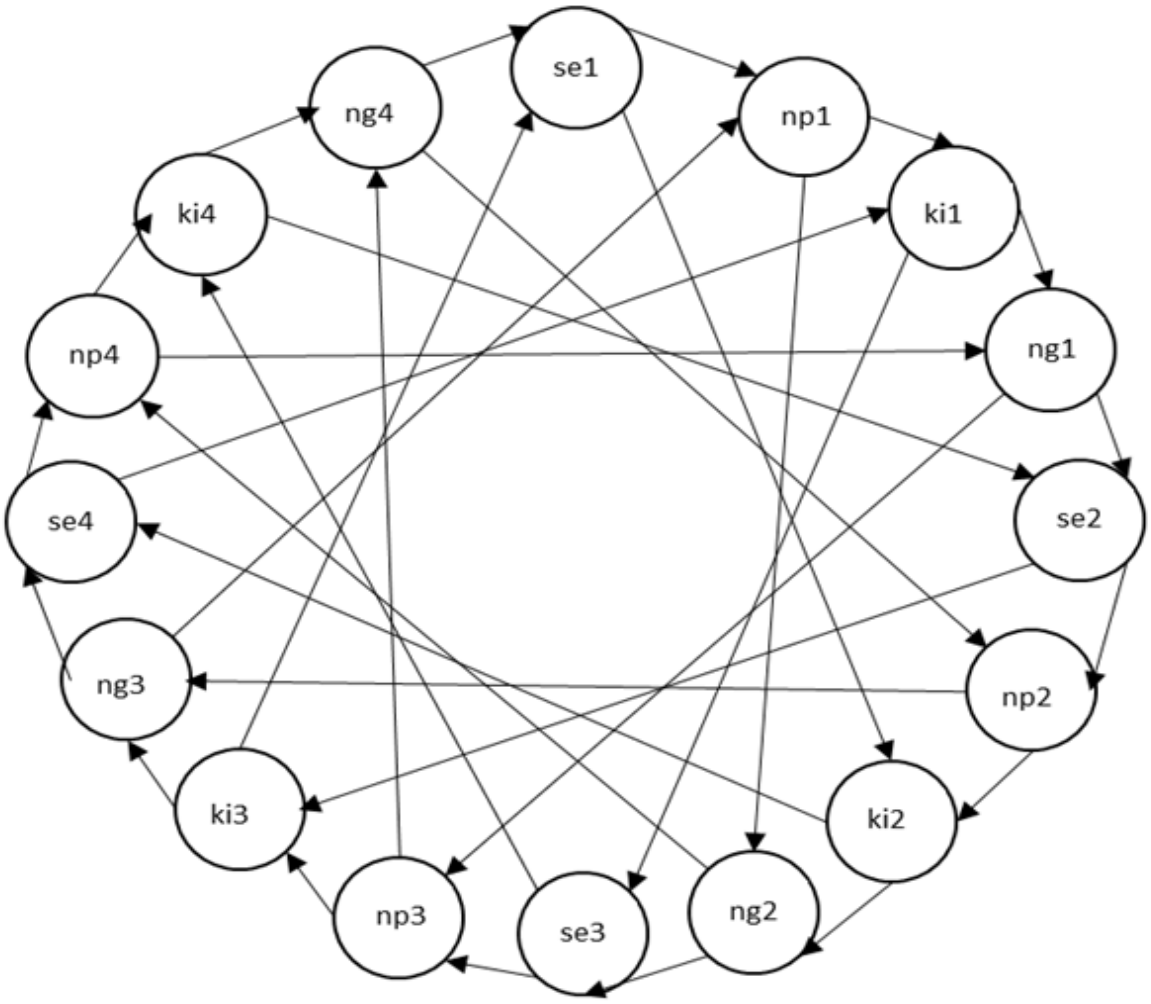

Fig. 2.S4: Interwoven microarray loop design comparing field mosquito samples from two CRT villages (one control cluster: np=N'guessan Pokoukro and one intervention cluster: se=Sessenouan) and two lab colonies (kis= *An. gambiae* Kisumu and ng= *An. gambiae* N'goussou). Each circle represents mRNA extracted from a pool of 10 female *An. gambiae* s.s. Individuals microarrays are represented by arrows (32 in total). The direction of the arrows indicates dye labelling.

Table 2.S1: Twenty-four-hour mortality of A) *Anopheles gambiae* Kisumu and B) *Anopheles gambiae* from each study village after exposure to a range of deltamethrin concentration in adapted CDC bottle assay.

Table 2.S2: Microarray results (.xlsx). Table 2.S2A: Microarray results for all probes. Table 2.S2B: Subset of microarray results showing genes significantly overexpressed and sorted by average pairwise fold change

Table 2.S3: Details of primers used in qRT-PCR analysis (.xlsx)

Table 2.S4: Statistical results on comparison of fold change in gene expression among chosen CRT villages (Table 2.S4A) and between treatments (Table 2.S4B)

Chapter 3

Evaluation of standard pyrethroid based LNs (MiraNet and MagNet) in experimental huts against pyrethroid resistant *Anopheles gambiae* M'bé, Côte d'Ivoire: potential for impact on vectorial capacity

The work in this chapter has been published as:

Welbeck A. Oumbouke, Alphonsine A. Koffi, Ludovic P. Ahoua Alou, Mark Rowland, Raphael N'Guessan. Evaluation of standard pyrethroid based LNs (MiraNet and MagNet) in experimental huts against pyrethroid resistant *Anopheles gambiae* M'bé, Côte d'Ivoire: potential for impact on vectorialcapacity. **PLoS ONE 2018** 14(4): e0215074. https://doi.org/10.1371/journal.pone.0215074

Evaluation of standard pyrethroid based LNs (MiraNet and MagNet) in experimental huts against pyrethroid resistant *Anopheles gambiae* M'bé, Côte d'Ivoire: potential for impact on vectorial capacity.

Abstract

Background

There is evidence from experimental hut and household studies that the entomological efficacy of long lasting pyrethroid treated nets (LLINs) is compromised in areas of pyrethroid resistance. The rapid increase in resistance intensity in African malaria vectors could further undermine the performance of these nets. The pyrethroid resistance intensity in *Anopheles gambiae* s.l. M'bé from central Côte d'Ivoire is reported to be high (> 1700 fold). Whether this translates into an increase in entomological indicators of malaria transmission needs investigation.

Method

The efficacy of two long lasting insecticidal nets (LN) MiraNet and MagNet, both alpha-cypermethrin based was evaluated in experimental huts against pyrethroid resistant *Anopheles gambiae* in M'bé, central Côte d'Ivoire. All nets were deliberately holed to simulate wear-and-tear and were tested unwashed and after 20 standardized washes.

Results

The entry rates of *An. gambiae* s.l. into huts with insecticide treated nets were 62-84% lower than entry into huts with untreated nets ($p < 0.001$). Exit rates of *An. gambiae* s.l. with unwashed MiraNet and MagNet LNs were significantly greater than with untreated nets (50-60% vs 26%) and this effect after washing 20 times nets did not decrease. Blood-feeding with both nets was significantly inhibited relative to the untreated reference net (31-55%) ($p < 0.001$). Washing MiraNet LN 20 times had no significant impact on protection against *An. gambiae* s.l. bites but it did cause a significant fall by 40% in protection with MagNet LN ($p < 0.001$). All insecticide

treated nets induced higher mortality of *An. gambiae* s.l. than the untreated net ($p < 0.05$). The impact though significant was limited (14-30%). The personal protection against *An. gambiae* s.l. bites derived from all treatments was high (75-90%). The overall insecticidal effect was compromised by pyrethroid resistance and was not detectable in some treatments.

Conclusion

In this area of high pyrethroid resistance intensity (over 1700 fold), both MiraNet and MagNet LNs still conferred appreciable personal protection against mosquito bites despite inducing only slightly greater mortality of pyrethroid resistant *Anopheles* mosquitoes than untreated nets. The impact is comparable to moderately intense Benin resistance area (207 fold) and Burkina Faso (over 1000 fold). This preserved level of protection plus the small but sensitive killing of mosquitoes may continue to impact vectorial capacity despite high intensity of resistance. Nevertheless, there is an obvious need for strategies and nets with novel mode of action to enhance vector control.

Background

Insecticide treated mosquito nets and indoor residual spraying of insecticide remain the cornerstones of public health strategies for preventing malaria. These core vector control methods have contributed to the decline in malaria burden, accounting for over three-quarters of the 663 million clinical cases of malaria averted over the past 15 years in Sub-Saharan Africa [1]. Long Lasting Insecticidal Nets (LLINs) made the major contribution due to the increased ownership and use of these nets in malaria endemic areas. The estimated proportion of households in areas at risk with at least one LLIN has increased from only 2% in 2000 to 79% in 2015 [2]. Control measures based on house spraying, on the other hand, have declined in coverage. The high cost of alternative non-pyrethroid chemicals might explain the recent decline in IRS coverage from 5 to just 3% [2]. While a range of insecticides is available for use in IRS, although effectively limited by cost, there is a few classes of insecticides (pyrethroids and pyrrole) and recently an insect growth inhibitor (pyriproxyfen) approved for net treatment [3–5]. Resistance to pyrethroids is now widespread in major malaria vectors [6], thus threatening the continued effectiveness of pyrethroid-based interventions. While a resistance mitigating plan has been developed [7], options are presently limited but momentum for the development of new classes of chemistry is growing and new products may become available in the near future [8].

Although there is extensive evidence from experimental hut and household studies showing reduced entomological efficacy of insecticide treated nets against insecticide resistant vectors [9,10], there is as yet no definitive evidence of a correlation between insecticide resistance and malaria metrics. A recent study across five countries (Benin, Soudan, Kenya, Cameroon and India) has attempted to address the question of whether insecticide resistance can undermine the protective efficacy of insecticide treated nets [11] and results from one of the study sites (Benin) showed that LLINs continue to provide some protection against malaria even with highly pyrethroid resistant *Anopheles* mosquitoes [12]. However, determination of resistance levels (high versus low) in the study was based on WHO susceptibility assay mortality and such resistance prevalence assays may give an incomplete picture of resistance [13]. It is therefore plausible that,

the absence of resistance impact on bed net efficacy seen in southern Benin could be due to the fact that the resistance intensity profiles did not differ between both study arms.

The impact of resistance is difficult to demonstrate because resistance cannot be randomized [7]. Although there is no empirical data linking experimental hut data to malaria transmission indicators, mathematical models do predict an impact on transmission [14]. Hut data furthermore provides the opportunity to assess the impact of insecticide resistance on the potential for LLINs to provide individual (blood feeding inhibition) and community level protection (killing effect).

Measuring resistance intensity in *Anopheles* mosquitoes across experimental hut stations could help link the strength of resistance with the efficacy of interventions being evaluated. So far resistance intensity using adapted CDC bottle assays has been determined in local *Anopheles* mosquitoes from only two Western African countries (Benin and Burkina Faso). In Benin areas with moderate intensity of resistance to alpha-cypermethrin (207 fold), Interceptor 1 LN, an alpha-cypermethrin based LN washed 20 times continued to inhibit blood feeding by 47% in experimental huts [15] while in Burkina Faso with higher resistance intensity to deltamethrin (over 1000 fold) it reduced feeding by 15% [16]. Mortality rates of *An. gambiae* in the two scenarios were low (around 20%) but greater than that with untreated nets. This suggests that LLINs would continue to provide some level of protection even when resistance is as high as that reported in Burkina Faso. Whether such limited level of control and protection is maintained across settings with similar or higher resistance intensity needs investigation.

The intensity of pyrethroid resistance in *An. gambiae* s.l. from the M'bé field station in central Côte d'Ivoire is among the highest ever reported (>1700 fold) [17]. The present experimental hut study was designed to investigate whether the extremely high level of resistance observed in the local *Anopheles* mosquito population from M'bé translates into an increase in entomological indicators of malaria transmission such as mosquito survival and blood feeding rates. The performance of two LNs (MiraNet and MagNet), both alpha-cypermethrin based was therefore evaluated in M'bé experimental huts against pyrethroid resistant *An. gambiae* s.l. in central Côte d'Ivoire.

Methods

Study area

The M'bé field site is located in central Côte d'Ivoire, 40km south of Bouaké. The station is surrounded by a large rice growing valley producing year round *An. gambiae* s.l., mainly M form. The resistance profile of the M'bé mosquito population appears multifactorial involving target site insensitivity and increased expression of metabolic enzymes. A recent study conducted in 2016 at the M'bé field site showed over 1700 fold resistance to deltamethrin in the local *Anopheles* mosquitoes [17]. This level of resistance intensity is among the highest ever reported in African malaria vectors.

Susceptibility tests

Bioassays were conducted using papers treated with diagnostic concentration of 0.05% alpha-cypermethrin (insecticide on the LNs). Two to three-day old adult female mosquitoes, emerged from larvae collected at M'bé field station and reared in the insectary at the Institut Pierre Richet were used for the susceptibility tests. Approximately 100 mosquitoes in batch of 25 were exposed for 1h to insecticide-treated papers and mortality was recorded 24h later.

Experimental huts

A field trial was carried out at M'bé in experimental huts constructed to WHOPES-approved West African design [18]. The hut trial took 5 weeks (from October to November 2014), corresponding to 25 night collections per hut. The huts were made of bricks, plastered with cement, with a corrugated iron roof. The ceilings were lined with plastic sheeting and the walls were supplied with four 1-cm window slits which serve as mosquito entry points. The huts were built on a concrete pillar surrounded by water-filled moats to prevent entry of predators. Exiting mosquitoes were captured in verandah trap.

LLINs and washing procedure

MiraNet LN is a Long Lasting net manufactured by A to Z Textile Mills, Tanzania. Alpha-cypermethrin is incorporated into 135-denier, monofilament, high-density polyethylene (HDPE) fibres, with the target dose of 4.5g/kg alpha-cypermethrin. MiraNet LN was a prototype under evaluation by WHO for recommendation at the time of the trial.

MagNet LN is a warp knitted fabric netting material containing 5.8 g/kg alpha-cypermethrin incorporated in monofilament HDPE, 150-denier manufactured by V.K.A. Polymers. MagNet LN received full WHOPES recommendation in 2011[19].

LNs were washed individually in accordance with standardized WHO Phase II washing protocols [20]. Nets were washed in 10 litres of tap water containing 2g/litre of soap ("savon de Marseille"). Each net was agitated for 3 min, left to soak for 4 min and further agitated for 3 min totalling 10 min for one washing cycle. Agitation was done by stirring the net with a wooden pole at 20 rotations per minute. Nets were rinsed using clean water and dried horizontally in the shade and subsequently stored at ambient temperature (27°C± 2°C). The regeneration interval between washes was 2 days for MiraNet LN and 1 day for MagNet LN [18].

Experimental hut study design

The following five treatment arms were tested in experimental huts: (i) unwashed MiraNet LN, (ii) MiraNet LN washed 20 times, (iii) unwashed MagNet LN, (iv) MagNet LN washed 20 times (v) untreated 100 denier polyester net.

Treatments were randomly assigned to five experimental huts and rotated on a weekly basis according to a randomized Latin square design to account for potential bias resulting from differential hut attractiveness. Prior to the trial, the nets were artificially holed with 16cm^2 holes (2 on each side and 1 on each end) to simulate the physical condition of damaged net in the field. At the end of a five-night rotation, the huts were thoroughly cleaned and aired for one day to prevent cross-contamination of huts from the different treatment arms. Five adult men took part in the hut trial as volunteer sleepers after informed consent. Human volunteers slept in the huts from 20.00 to 05.00 and were rotated between huts on successive nights to minimize any bias resulting from difference in individual attractiveness to host-seeking mosquitoes. Each morning, dead and live mosquitoes were collected from inside the room, under bed nets and traps using mouth-suction

aspirators and torches. Mosquito collections were done on 25 nights over 5 weeks. Upon transportation to the laboratory, mosquitoes were identified to species using taxonomic keys and gonotrophic status was scored as unfed, blood fed, semi-gravid or gravid. Live female mosquitoes were held in plastic cups covered with netting and provided with 10% honey solution; mortality was recorded after 24h.

The efficacy of MiraNet and MagNet LNs was evaluated using the following entomological parameters as per WHO guidelines [21]: (i) deterrency: the percent reduction in the number of mosquitoes in treatment hut relative to control hut with untreated net; (ii) exit rate (iii) blood feeding inhibition rate: the percentage reduction in blood feeding in hut with treated net compared to hut with untreated net; (iv) percentage mortality of adult females; (v) overall insecticidal effect (as described in N'Guessan et al [9]) = 100 (Kt-Ku)/Tu where Kt is the number killed in the treated hut, Ku is the number dying in the untreated control hut, and Tu is the total number collected from the control hut [9,22,23]; (vi): personal protection: percentage reduction in mosquito biting in hut with treated net compared to hut with untreated net = [1-(number bloodfed in treatment/number bloodfed in control) x100].

Chemical assays

The alpha-cypermethrin content of the LNs (washed and unwashed) from the five treatment arms was assessed before, after washing and after field trial based on WHO guidelines [20]. A piece of netting measuring 30cm x 30cm was cut from each of the five locations of each net. Extraction of alpha-cypermethrin was performed using the CIPAC method [24]. Alpha-cypermethrin was extracted by refluxing with xylene for 30 minutes in presence of dioctyl phthalate as internal standard and citric acid. Concentration of the insecticide was subsequently quantified by Gas Chromatography with Flame Ionization Detection (GC-FID).

Cone bioassays on nets

Bio-efficacy of LNs (washed and unwashed) was assessed using WHO cone bioassays at two different time points: before and after field trial. Five insectary-reared *An. gambiae* Kisumu

females aged 2-5 days were tested in four replicate cone assays on five sections of each net as per WHO guidelines at 25± 2°C and 75± 10% humidity. Knocked down mosquitoes were scored 60 min post-exposure and mortality recorded after 24 h observation period.

Ethical permission

Ethical approval for the study was granted by the Ministry of Health in Côte d'Ivoire and the Ethical committee of the London School of Hygiene and Tropical Medicine. Written informed consent was obtained from all trial participants. Study volunteers were monitored for potential intervention-related side effects and were provided with antimalarial drug (ACTs) when tested positive for malaria. In the event that volunteers fell sick from any disease, including malaria, they were replaced until they recover and take over.

Statistical analysis

Data were analysed using the R statistical software version 2.15.0. Proportional outcomes from the bioassays (mortality) and the hut trial (exophily, blood feeding and mortality) were analysed using generalised linear mixed models (GLMMs) with a binomial distribution and a logit link function was fitted to the data using the "lme4" package [25]. For the hut data, net type and hut were included as fixed effects and sleepers, day of mosquito collection were treated as random effects. Interactions between bednet type and washes were also included in the models. Numeric outcomes (number entering each hut, feeding and dying) were analysed using generalised linear models with a Poisson distribution. Pairwise comparisons were performed using the "multcomp" package in R [26].

Results

Susceptibility tests

Prior to the experimental hut trial, WHO susceptibility assays on female *An. gambiae* s.l. mosquitoes from M'bé to 0.05% alpha-cypermethrin-treated papers resulted in 32% mortality (n tested = 104), indicating a high frequency of resistance to pyrethroids in the study area.

Experimental hut trial

Overall, 3614 *An. gambiae* s.l. females were caught in huts over the 5-week trial at M'bé (Table 3.1). The entry rates of *An. gambiae* s.l. into huts with insecticide treated nets were 62-84% lower than entry into huts with untreated nets ($p < 0.001$) (Table 3.1).

Exit rates of *An. gambiae* s.l. with unwashed MiraNet and MagNet LNs were significantly greater than untreated net (50-60% vs 26%) and washing 20 times these nets did not decrease the effect (Table 3.1).

Blood-feeding was inhibited in every hut relative to control but the levels of inhibition though significant were moderate (31-55%) ($p < 0.001$). Washing MiraNet LN 20 times had no significant impact on protection against *An. gambiae* s.l. bites, but washing MagNet LN 20 times resulted in a 40% decrease in protection, with evidence for significant interaction between net type and wash treatment ($p = 0.005$) (Table 3.1, Fig. 3.1 A).

The mortality before and after washing the LNs mirrored that of the blood-feeding. All insecticide treated nets induced higher mortality of *An. gambiae* s.l. than the untreated net ($p < 0.05$). The mortality rates across all treatment types were limited (range 14%-30%) (Fig. 3.1 B). There was evidence for significant loss of activity with MagNet LN after 20 washes but not with MiraNet LN (significant interaction between net type and wash treatment; $p < 0.05$).

The level of personal protection against *An. gambiae* s.l. bites that derived from all treatments was high (75-90%). The overall insecticidal effect on mosquitoes was compromised by pyrethroid resistance at this site and was marginal (< 4%) across all treatments (Table 3.1).

Cone bioassays

Before and after field trial, knock down and mortality rates of susceptible *An. gambiae* s.l. were nearly 100% (> 99%) with all treated nets (data not shown).

Chemical assays

The mean alpha-cypermethrin content in nets before and after field testing is shown in Table 3.2. Chemical analysis showed that initial concentrations of alpha-cypermethrin in both LNs were close

to the target dose of 4.5 g/kg±25% for MiraNet LN and 5.8g/kg±25% for MagNet LN, with a within-net variation of less than 10%. After 20 washes, the alpha-cypermethrin content was 4.13 with MiraNet LN and 5.35 with MagNet LN, corresponding to an overall retention rate of about 85% for both LNs. The drop in insecticide content did not differ between MiraNet LN (14%) and MagNet LN (15%) (Table 3.2). While the loss in alpha-cypermethrin content after washing did not impact the efficacy of MiraNet LN (Fig. 3.1A & B), the same magnitude of decline in chemical content resulted in a significant decrease in the effect size (blood feeding inhibition and mortality) with MagNet LN. After 5 weeks of use in experimental huts, there was a marginal decrease (< 10%) in alpha-cypermethrin content.

Discussion

The present study was designed to investigate in experimental huts the performance of two pyrethroid LNs (MiraNet and MagNet) against *An. gambiae* s.l. in an area of high resistance intensity to deltamethrin (over 1700-fold resistance) in Bouaké, Côte d'Ivoire. We observed appreciable levels of protection against mosquito bites (blood feeding inhibition) in the order of 31-55% despite high resistance intensity. These are within the protection range seen in Burkina Faso (15-25%) [27] with comparable resistance strength and in areas of lower intensity in Benin (47-57%) [15].

MiraNet and MagNet LNs in our present trial induced marginal mortality of *An. gambiae* s.l. (14-30%) albeit greater than the untreated nets. The trend is consistent with the hut trials from Burkina Faso and Benin and there is no evidence to suggest that increasing intensity of resistance worsens control of *An. gambiae* mosquitoes. However, one potential limitation of the study is that the intensity study by Glunt et al. was conducted at the same site as the current trial but at different time period: E.g. the intensity data was collected in October 2016 whereas the hut trial fell two years behind, i. e. October to November 2014. Considering that insecticide resistance is dynamic, one cannot rule out the fact that intensity might have been different at the time of the hut trial. It is plausible that with an intensity of 1700 fold in that year 2014, the corresponding effect size might have been different. Nevertheless, in the same paper by Glunt et al. PermaNet 2.0 LN, another pyrethroid-only LN, evaluated at the same site and period as the 1700-fold resistance intensity bioassays showed an impact against *An. gambiae* s.l. similar to that in the current trial

(60% blood feeding inhibition vs 31-55%; 20% mortality vs 14-30%).

Before washing, mosquito mortality and blood feeding inhibition rates were significantly higher with MagNet LN compared to MiraNet LN. The difference in efficacy could be due to the difference in concentration of alpha-cypermethrin in both LNs (6.43 g/kg AI for MagNet LN versus 4.5 g/kg AI for MiraNet LN). While washing both nets 20 times decreased blood feeding inhibition and mortality rates, the reduction in effect size was significant only for MagNet LN, indicating that MiraNet LN was more wash resistant than MagNet LN.

Although the overall insecticidal effect of pyrethroid-treated nets is lost in the presence of resistance, a substantial protection can still be afforded to net users as evidenced in this study. Vectorial capacity as expressed by MacDonald [28] is sensitive to the reduction in vector host contact and more so to the mortality of the malaria vector. The significant level of protection that holed nets continue to offer plus the small but sensitive killing of pyrethroid resistant *Anopheles* population in the present study and in neighboring Benin and Burkina Faso would suggest that LLINs could still reduce malaria transmission despite resistance. This supports the WHO continuous advocacy of universal coverage with pyrethroid LNs, despite increasing level of insecticide resistance. Recent observational cohort studies conducted in Benin and Malawi demonstrated a reduction in incidence of malaria infection in LLINs users compared to bed net non-users in settings with moderate to high pyrethroid resistance [12,29]. However, this level of protection could be lost not only when resistance strength increases further [27] but also with declining bed net physical integrity [30].

To preserve the efficacy of LLINs, a range of new generation LNs have been developed. The design of these nets is generally based on the combination of unrelated insecticides (alpha-cypermethrin-chlorfenapyr mixture net: Interceptor G2 net) [16,31] or mixture of one insecticide with either a synergist (piperonyl butoxide-treated insecticidal net: PBO LN) [32,33] or an insect juvenile hormone mimic (permethrin-pyriproxyfen mixture net: PPF LN) [34]. Combination of insecticides with contrasting mode of action is one of the WHO recommended tactics for insecticide resistance management [7]. In a recent experimental hut trial in M'bé, Interceptor G2 LN killed very high proportion of *An. gambiae* s.l. (82-87%) that entered huts [35]. This effect

size (high mortality) with the G2 LN demonstrates the impact of resistance on pyrethroid LNs and indicates what pyrethroids would have achieved in the absence of resistance. It also stresses the need for alternative tools or strategies to overcome insecticide resistance.

The design of new brand of bed net treated with pyrethroids only seems to be driven by the availability of commercially sustainable market further supported by the WHO policy for universal coverage with LLINs. However, with clear-cut evidence from a number of observational studies that elimination of malaria will require additional measures beyond current best practice of pyrethroid-only LNs, control efforts should be devoted to the development of new and effective insecticides and strategies to counter resistance and sustain progress toward elimination.

Conclusion

Despite high resistance intensity (over 1700 fold) found in M'bé, both MiraNet and MagNet LNs still confer appreciable protection against mosquito bites and induce slightly greater mortality of pyrethroid resistant *Anopheles* mosquitoes than untreated nets. The impact is comparable to moderately intense Benin resistance area (207 fold) and Burkina Faso (over 1000 fold). The significant level of protection that holed nets continue to offer plus the small but sensitive killing of pyrethroid resistant *Anopheles* population would suggest that LLINs may still reduce malaria transmission despite high intensity of resistance. However, the data suggests that the community protection arising from the overall insecticidal effect of LLINs could be compromised in this area of Côte d'Ivoire with high vector resistance. There is an urgent need for development of novel strategies or LLIN with novel mode of action to enhance vector control.

Acknowledgments

The authors thank the technical staff at IPR and the volunteer sleepers for their participation in the experimental hut trial.

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Table 3.1: Experimental hut trial results against pyrethroid resistant *An. gambiae* s.l.

	Untreated net	MiraNet LN 0w	MiraNet LN 20w	MagNet LN 0w	MagNet LN 20w
Total females caught	1594[a]	257[b]	582[c]	578[c]	603[c]
% Deterrency	–	83.9	63.5	63.7	62.2
Total females exiting	419	130	336	349	343
% Exiting (95% CI)	26.3 (24.1-28.4)[a]	50.6 (44.5 - 56.7)[b,c]	57.7 (53.7-61.7)[c]	60.4 (56.4-64.4)[c]	56.9 (52.9-60.8)[c]
Total females blood fed	983	100	249	159	246
% Blood feeding Inhibition	–	36.9	30.6	55.4	33.8
Personal protection %	–	89.8	74.7	83.8	75.0
Overall insecticidal effect (%)	–	-5.52	-2.89	3.07	-0.69

Values in the same row sharing a letter superscript do not differ significantly ($p > 0.05$, GLMMs)

Table 3.2: Chemical analysis of alpha-cypermethrin on LNs in the experimental hut trial in M'bé

Treatment	Concentration of alpha-cypermethrin (g/kg)		
	Before trial	After washing	After trial
MiraNet LN unwashed	4.50	–	4.62
MiraNet LN 20 washes	4.79	4.13	4.10
MagNet LN unwashed	6.43	–	5.95
MagNet LN 20 washes	6.33	5.35	4.87

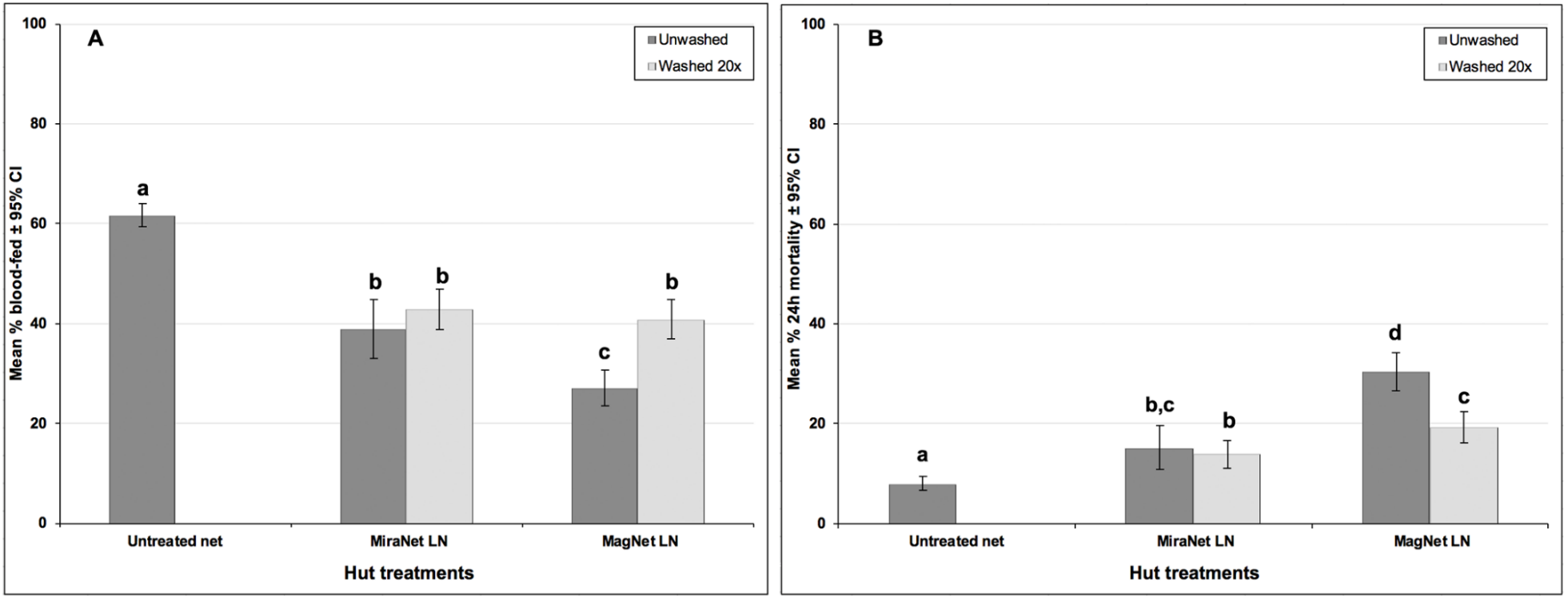

Fig.3.1 Experimental hut trial against wild free-flying pyrethroid resistant *An. gambiae* s.l. M'bé with MiraNet and MagNet LNs. (A) Percentage blood-feeding, (B) Percentage mortality. Bars bearing the same letter label are not significantly different at the 5% level ($p < 0.05$, GLMMs). Error bars represent 95% CIs.

PART THREE: Optimize and evaluate EaveTubes against pyrethroid resistant *Anopheles gambiae* mosquitoes

Chapter 4: Screening and field performance of powder-formulated insecticides on eave tube inserts against pyrethroid resistant *Anopheles gambiae*: an investigation into actives prior to a randomized controlled trial in Côte d'Ivoire.

Chapter 5: Spatio-temporal trend in insecticide resistance in *Anopheles gambiae* following wide-scale deployment of a "Lethal house Lure" in combination with standard LLIN in central Côte d'Ivoire

Chapter 4

Screening and field performance of powder-formulated insecticides on eave tube inserts against pyrethroid resistant *Anopheles gambiae*: an investigation into actives prior to a randomized controlled trial in Côte d'Ivoire

The work in this chapter has been published as:

Welbeck A. Oumbouke, Innocent T. Zran, Antoine M.G. Barreaux, Alphonsine Koffi, Eleanore D. Sternberg, Matthew B. Thomas and Raphael N'Guessan. Screening and field performance of powder-formulated insecticides on eave tube inserts against pyrethroid resistant *Anopheles gambiae*: an investigation into actives prior to a randomized controlled trial in Côte d'Ivoire**. Malar J** 17, 374 (2018). https://doi.org/10.1186/s12936-018-2517-9

Screening and field performance of powder-formulated insecticides on eave tube inserts against pyrethroid resistant *Anopheles gambiae*: an investigation into actives prior to a randomized controlled trial in Côte d'Ivoire.

Abstract

Background

The widespread emergence of insecticide resistance in African malaria vectors remains one of the main challenges facing control programmes. Electrostatic coating that uses polarity to bind insecticide particles is a new way of delivering insecticides to mosquitoes. Although previous tests demonstrated the resistance breaking potential of this application method, studies screening and investigating the residual efficacy of broader range insecticides are necessary.

Methods

Eleven insecticide powder formulations belonging to six insecticide classes (pyrethroid, carbamate, organophosphate, neonicotinoid, entomopathogenic fungus and boric acid) were initially screened for residual activity over 4 weeks against pyrethroid resistant *Anopheles gambiae* sensu lato (*s.l*). from the M'bé valley, central Côte d'Ivoire. Tests were performed using the eave tube assay that simulates the behavioural interaction between mosquitoes and insecticide-treated inserts. With the best performing insecticide, persistence was monitored over 12 months and the actual contact time lethal to mosquitoes was explored, using a range of transient exposure time (5s, 30s, 1min up to 2 min) in the tube assays in laboratory. The mortality data were calibrated against overnight release recapture data from enclosure around experimental huts incorporating treated inserts at the M'bé site. The natural recruitment rate of mosquitoes to the tube without insecticide treatment was assessed using fluorescent dust particles.

Results

Although most insecticides assayed during the initial screening induced significant mortality (45-100%) of pyrethroid resistant *An. gambiae* during the first two weeks, only 10% beta-cyfluthrin retained high residual efficacy, killing 100% of *An. gambiae* during the first month and >80% over 8 subsequent months. Transient exposure for 5 seconds of mosquitoes to 10% beta-cyfluthrin produced 56% mortality, with an increase to 98% when contact time was extended to 2min ($P = 0.001$). In the experimental hut enclosures, mortality of *An. gambiae* with 10% beta-cyfluthrin treated inserts was 55% compared to 44% of mosquitoes that

contacted the inserts treated with fluorescent dusts. This indicates that all host-seeking female mosquitoes that contacted beta-cyfluthrin treated inserts during host-seeking were killed.

Conclusion

The eave tube technology is a novel malaria control approach which combines house proofing and targeted control of *Anopheles* mosquitoes using insecticide treated inserts. Beta-cyfluthrin showed great promise for providing prolonged control of pyrethroid resistant *An. gambiae* and has potential to be deployed year-round in areas where malaria parasites are transmitted by highly pyrethroid resistant *An. gambiae* across sub-Saharan Africa.

Background

Wide scale use of insecticide-based interventions such as indoor residual sprays (IRS) and long lasting insecticide treated nets (LLINs) has contributed to a substantial reduction in the global malaria burden in recent years [1,2]. However, the sustainability of these approaches is now being threatened by the evolution of insecticide resistance [3,4], creating a need for more diverse vector control tools [5].

The eave tube is a recent innovation that offers a novel approach for delivering insecticides to malaria mosquitoes [6]. The approach involves blocking the eaves of houses (if open) and inserting pieces of PVC pipe to act as 'chimneys' to channel the human odours mosquitoes use as cues to locate hosts for blood feeding, out of the house. When host-seeking mosquitoes enter a tube, they encounter an insert treated with an insecticide. The current version of the eave tube inserts uses electrostatic netting to hold powder formulations of insecticides. Mosquito contact with the netting results in very efficient transfer of powder particles such that even highly pyrethroid resistant mosquitoes can be killed with pyrethroid insecticides due to the overwhelming doses[7]. When eave tubes are combined with screening of windows and doors to reduce mosquito entry via other routes, the approach provides both physical protection and a killing effect, much like an insecticide treated net but at the level of the household.

Semi-field and modelling studies indicate that screening plus eave tubes (SET) could reduce transmission of malaria at community level above and beyond universal coverage of LLINs [8–10]. Based on these promising results, a cluster randomized controlled trial (CRT) is now being conducted in central Côte d'Ivoire [11] to evaluate epidemiological impact at village level. The current paper reports on a series of initial studies to screen a range of candidate insecticides for use in this trial, together with an evaluation of potential residual activity of a smaller number of promising insecticides to select a final product and inform likely retreatment frequency for the CRT.

Materials and methods

Mosquitoes and insecticides

Experiments were performed with *Anopheles gambiae* mosquitoes collected from a rice growing area adjacent to the M'bé experimental hut station in central Côte d'Ivoire, approximately 40 km north of the city of Bouaké. These rice fields provide mosquito-breeding

habitat year-round. A comprehensive characterization of the local mosquito population showed that the M variant of the *An. gambiae* complex, now referred to as *Anopheles coluzzii*, is predominant in the area and exhibits high levels of resistance to pyrethroid and carbamate insecticides [12,13]. Recently, over 1700 fold resistance against deltamethrin was detected in the M'bé population of *An. coluzzii* compared to the Kisumu laboratory strain, using adapted CDC bottle assays [14]. The high resistance intensity exhibited by this vector population makes it a good strain for testing potential resistance breaking chemistry or novel insecticide delivery systems, such as the electrostatic coating technology. In the experiments described below, mosquitoes were collected as larvae and pupae from breeding sites around M'bé and reared to adult in the insectary of the Institut Pierre Richet (IPR) in Bouaké, under ambient climatic conditions. Five-day-old sugar-fed only female mosquitoes were used in all laboratory and semi-field assays.

The list of insecticides initially screened for residual performance is given in Table 4.1. Overall, 11 wettable powder formulation of insecticides including pyrethroids, carbamates, organophosphates, neonicotinoids, entomopathogenic fungus and boric acid were tested. The products were selected for testing based on, criteria such as toxicity, commercial availability as pest control products, however a handful of experimental formulations were also tested. All the insecticides evaluated were powder formulations.

Application of insecticide powders on eave tube inserts

Eave tube inserts that fit into locally produced PVC tubes have been designed with electrostatic netting attached to a polyethylene frame consisting of a plastic circle with six spokes and a central protruding node (see [9] for images of the insert design). The frame provides physical support to the netting and allows easy insertion inside eave tubes. This prototype was used in the present study to investigate the persistence of insecticide applied on eave tube insert.

Candidate active ingredients were applied on eave tube inserts manually; 5g of each "active" (powder-formulated insecticide) was weighed and poured evenly onto an eave tube insert placed in the middle of a 20 cm long PVC tube. To prevent active from falling through the tube, both ends of the pipe was sealed off with a plastic lid and the tube was then shaken by hand for 1 min. To allow for adequate distribution of the insecticide on the two sides of the insert, the tube was turned every 10 seconds. The tube was then put on a table for 2 minutes to allow the dust to settle and adhere to the insert, and then the treated insert was moved to clean tube and

shaken for 15 seconds to remove any excess of powder. After treatment, the insert was placed in a third, clean tube. Four to six inserts were treated for each insecticide; approximately 4g of powder were collected after treatment, leaving approximately 1 g of powder on the insert. An excess of powder was used during treatment to ensure thorough saturation of the inserts with the powders. Inserts were tested 1-day post-treatment (T0), then kept for subsequent monitoring of residual efficacy at regular intervals. To better approximate decay rates under realistic conditions, the inserts were kept individually in eave tubes inserted in holes drilled at eave level in an experimental house on the IPR campus. The inserts were stored in these tubes throughout the testing period and removed only for persistence monitoring.

The "eave tube" bioassay

This bioassay method used a 20 cm long piece of PVC tube with an insecticide-treated insert placed in the tube such that it is flush with one end of the pipe (Fig. 4.1a). The opposite end of the tube is fitted with untreated netting to keep mosquitoes inside of the tube, and mosquitoes are introduced into the tube on this clean end using mouth aspirators. The "eave tube" bioassay was performed during daytime. A host cue is placed behind the treated insert and the mosquitoes are allowed to recruit freely to the insert over a fixed period of time. This experimental set up was designed to simulate the interaction between mosquitoes and eave tube inserts in the field, where heat and odor cues draw host-seeking female mosquitoes into the tube where they then make contact with the insecticide-laden insert (see [15] for similar methodology).

Initial screening of powder insecticides

The aim of this set of experiments was to identify chemicals that retained efficacy against pyrethroid resistant mosquitoes for at least 4 weeks post-treatment. Persistence assays were performed on a fortnightly basis, and insecticides with significant declines in residual activity over the testing period were dropped from further testing. A total of ~ 60 unfed female mosquitoes aged 4-5 days were exposed in batch of 15 to each insert for 3 min using the eave tube bioassay. A hand was used as the attractive cue behind the treated insert. To eliminate any potential biases from differential attractiveness of volunteers, hand from the same individual was used in all assays. Exposure to an untreated insert served as the control. At the end of the exposure period, mosquitoes were released in netted cages with access to a 10% honey solution on cotton pads. Mortality was scored after a 24h holding period, except for the fungus-exposed group, which was scored 7 days later.

Persistence monitoring

The only insecticide that persisted for 1 month during the initial screening was 10% beta-cyfluthrin. New inserts were treated with 10% beta-cyfluthrin and residual activity was monitored at approximately monthly intervals for 12 months using the same eave tube bioassays, but with some refinement of the protocol. The three modifications were: (1) the host cue was changed from a hand to a bottle filled up with boiling water and wrapped in a worn sock (worn over night), to allow for more assays to be run in parallel, (2) female mosquitoes were deprived of sugar 6 h prior to the bioassay to maximize host-seeking behavior, and (3) the duration of the bioassay was extended from 3 min to 1 h. Although mosquitoes remained inside the tube for 1h, it is important to note that the actual contact time was still determined by the host-seeking response of each individual mosquito. Approximately 60 mosquitoes (four replicates of 15 mosquitoes per tube) were tested. At the end of the 1h behavioral assay, mosquitoes were transferred to observation cages, supplied with 10% sugar water solution, and mortality scored 24h.

Supplementary experiments

Results from residual efficacy assays show that 10% beta-cyfluthrin was the longest lasting chemical when applied on eave tube inserts. To further explore the vector control potential of this insecticide formulation, additional experiments were performed in a semi-field setting and in the laboratory using reduced contact times.

Field performance of insecticide treated insert

Experiments were conducted at the M'bé phase II experimental huts station between June and September 2017 using experimental huts constructed to the West African design [16]. The huts are 3.25 m long, 1.76 m wide and 2 m high. The interior walls of the huts are made of concrete brick, with a corrugated iron roof. A plastic cover was affixed onto the roofing as ceiling. Each hut was built on a concrete base with a water-filled moat, to protect against invertebrate predators. The huts were customized to allow evaluation of eave tube inserts; namely, six holes were drilled at eave level (1.7 m from the ground) on three sides of the hut (two holes on each side). Eave tubes were fitted into the holes and inserts freshly treated with 10% beta-cyfluthrin were placed in the tubes. To allow for the recapture of mosquitoes after contact with the eave tube inserts, the huts had to be in an enclosed structure (Fig. 4.1b). A wooden frame was erected on the concrete base, 50 cm from the exterior wall of the hut. Plastic sheeting was used as a

roof on the enclosure, and extended beyond the edge of the enclosure as an awning, to protect against rain entering the enclosure. The bottom half of the frame was made out of wooden panels and the top half was screened with polyethylene netting. White plastic sheeting was installed on the floor of the enclosure to facilitate the collection of dead mosquitoes. The door of the enclosure was positioned on the front side of the hut and closed with a zipper to prevent mosquitoes escaping.

Overnight release-recapture experiments were conducted in two modified experimental huts, situated 50 m apart. In the first experiment, six inserts treated with beta-cyfluthrin were installed in one experimental hut and six untreated inserts were placed in tubes in the second experimental house. Two adult volunteers were recruited from nearby villages to sleep in the huts. During the experiment, sleepers were rotated between the two huts. Before the start of the experiment, study participants slept in the experimental huts for a week to build up human odors and maximize mosquito host seeking response. At 20:00, volunteers entered the huts to sleep under intact, untreated net. A total of 100, 5 day-old female *An. gambiae* (M'bé strain) were released into each enclosure 15 min after volunteers retired to their respective huts. Mosquitoes were sugar-starved for 6 h prior to the release, but still provided with tap water to prevent desiccation. In the following morning, at 05:00, mosquitoes were recaptured both inside the experimental huts and within the enclosures using flashlights and aspirators. Live recaptured mosquitoes were subsequently held in netted plastic cups and supplied with 10% sugar solution. Survival was monitored for 24h.

Measurement of mosquito host-seeking response in the enclosure

To assess how many mosquitoes actually enter the eave tubes and came into contact with the inserts over the course of a night, a second experiment was conducted using fluorescent powder. The procedure of the experiment was similar to that described above, except that the inserts were treated with a non-toxic fluorescent dust instead of beta-cyfluthrin. The procedure for applying the fluorescent dust was similar to that used for hand-treating insert with powder insecticide as described in an earlier section. Again, the experimental huts were fitted with 6 eave tube inserts and 100 sugar-starved *An. gambiae* M'bé mosquitoes were released in each enclosure each study night. To prevent cross-contamination with the fluorescent powder, mosquitoes were caught individually using clean hemolysis tubes. Recaptured mosquitoes were killed with chloroform and their bodies subsequently checked for fluorescent particles,

indicative of contact with treated inserts, using a UV light microscope (Dino Lite Premier, USA). A third experiment was also conducted where eave tubes were simply left open overnight to estimate how many mosquitoes passed through the tubes. The following morning at 05:00, the volunteers blocked the eave tubes using untreated inserts and mosquitoes inside and outside the hut were collected and counted.

Short contact assays

Unlike house walls, where a mosquito might rest for a longer period of time, the time that vectors spend in contact with an eave tube insert could be relatively transient [17,18]. Overnight survival in the enclosures with insecticide-treated inserts could indicate either that the mosquito did not come into contact with a treated insert or that it did not stay in contact long enough to pick up a lethal dose.

Likewise, while the presence of coloured particles on a recaptured mosquito does indicate contact with the eave tube insert, the absence of fluorescent particles could indicate either no contact, or that the mosquito did not stay in contact long enough to be contaminated with a visible amount of particles.

To evaluate whether beta-cyfluthrin can kill even with brief contact, individual mosquitoes were exposed to freshly treated inserts using the same modified eave tube bioassay. A range of exposure time (5s, 30s, 1min and 2 min) was tested on 6 h sugar-starved 5-day-old female *An. gambiae* M'bé. A transparent tube was used instead of a standard PVC tube, to enable direct observation of mosquito behaviour within the tube and to allow measurement of contact duration using a stopwatch. A total of 52 mosquitoes was tested individually for each time period. Following exposure, mosquitoes were removed from the eave tube and housed in 150mL plastic cups and provided with sugar solution. Mortality was scored 24h post-exposure.

To test whether a contact time of only 5 seconds is sufficient for fluorescent particles to transfer from the insert to the mosquito, 50 female *An. gambiae* mosquitoes were exposed individually to inserts treated with fluorescent powder using the same modified eave tube assay. After 5 s of contact, the mosquito was removed and the body examined under UV light for the presence of coloured particles.

Statistical analysis

Data were entered into an excel spreadsheet and transferred into the R statistical software version 3.4.0 for analysis. The decline in efficacy over time across insecticides was analysed using Bayesian generalized linear models (BGLMs) with the "arm" package. Insecticide treatments were included in the model as explanatory variable and mosquito mortality as the outcome. Interactions between insecticides and persistence testing intervals (time since treatment) were also included in the model. Pairwise comparisons were performed with the final model using the "multcomp" package in R. For the release recapture experiments, generalized mixed effect models (GLMMs) with a binomial distribution and a logit link function was fitted to the data using the "lme4" package for R. Treatment and enclosure were included as fixed effects and sleepers were included as a random effect. Data from the short contact eave tube assay were analyzed using Bayesian generalized linear models with a binomial distribution.

Results

Initial screening of powder insecticides

Fig. 4.2 shows the results of the eave tube bioassay tests with the 11 initial candidate powder insecticides, tested at T0, 2 weeks and 1 month post-treatment against the pyrethroid resistant *An. gambiae* M'bé strain. Comparing the 11 insecticides at T0 and 2 weeks post-treatment, most killed a significant proportion (45 - 100%) of *An. gambiae* mosquitoes. However, there was a significant ($P < 0.05$) decline in activity 4 weeks after treatment, with mortality dropping below 25% for almost all of the insecticides ($P<0.001$). In contrast, beta-cyfluthrin retained full residual activity (>90% mortality) over the screening period of 1 month.

Persistence monitoring

Based on the initial screening, beta-cyfluthrin was selected for its persistence on inserts over 12 months; the results are summarized in Fig. 4.3. Beta-cyfluthrin was highly effective, continuing to kill >80% of *An. gambiae* up to 9 months post-treatment. Mortality of *An. gambiae* declined steadily over time down to 67% by month 11 and 20% by month 12.

Experimental hut evaluations

The proportions of *An. gambiae* mosquitoes recaptured in the experimental hut enclosures are presented in Table 4.2, both for the experiment using insecticide-treated inserts and for the one

using inserts treated with fluorescent dust. Table 4.2 also presents the proportions of mosquitoes found dead (insecticide treatment) or recaptured with fluorescent dust particles.

Mosquito recapture rate was consistently high in all experiments (more than 80%). It is possible that a few mosquitoes escaped through the door of the enclosure during release, thus accounting for the small difference in number between mosquitoes released and that recaptured.

Mortality rates with the untreated control and fluorescent powder treated inserts was <5%. When inserts treated with beta-cyfluthrin were used, about half of the mosquitoes tested died by the morning of collection (55% immediate mortality) and this increased to 64% within 24 h post-exposure, but the difference was not significant ($P > 0.05$).

Results from the experiment using the fluorescent powder showed that, on average 44% of mosquitoes released in the enclosure had coloured particles on their body after recapture. This suggests that slightly less than half of the released mosquitoes made contact with the inserts overnight. Given that this is broadly consistent with the mortality observed when beta-cyfluthrin was used in the experimental huts (44% with coloured particles versus 55% immediate mortality with beta-cyfluthrin), this suggests that all of the mosquitoes encountering the insecticide-treated inserts were killed. When eave tubes were left open, > 75% of mosquitoes were caught inside the experimental hut. This indicates that, in the absence of the inserts the majority of mosquitoes will pass through the tubes overnight.

Short contact assay

Fig. 4.4 shows the 24h mortality of *An. gambiae* mosquitoes after 5s, 30s, 1min or 2 min exposure to inserts freshly treated with beta-cyfluthrin. There was a positive relationship between exposure duration and mortality, i.e. the longer the exposure time the higher the mortality rate. Percent mortality was 56% with the shortest exposure time (5s), and increased significantly to 88.5% when contact time was increased to 1 minute ($P = 0.003$). A 2-min contact with a freshly treated insert was sufficient to produce almost 100% mortality in a pyrethroid resistant *An. gambiae* strain, But the difference in mortality between 1 min and 2min exposure was not significant ($P > 0.05$). There was no mortality in the control group. When mosquitoes were exposed for just 5 s on inserts treated with fluorescent dust, 100% of mosquitoes were contaminated with the coloured particles.

Discussion

Malaria elimination will require innovative vector control tools that are not compromised by insecticide resistance. The eave tube is part of a new mosquito control strategy that involves screening windows, closing eaves, and the targeted delivery of insecticide on eave tube inserts. The intervention will be trialed in Côte d'Ivoire to test whether it can impact malaria incidence. The study presented here was designed, in part, to identify a suitable insecticide for use in the trial, and to explore a diversity of insecticides that could potentially be used in the eave tubes for prolonged control of insecticide resistant *Anopheles* populations.

Results from residual efficacy bioassays show that the majority of insecticides tested in the present study produced significant mortality (45-100%) in the local M'bé strain of *An. gambiae* mosquitoes, when freshly applied on eave tube insert. This confirms that a wide range of actives from diverse insecticide classes could be successfully applied on electrostatic netting for effective control of insecticide resistant malaria vectors and provides further evidence of the potemtial of the technology to bypass resistance [7].

While most candidate actives were highly effective at killing mosquitoes immediately following treatment, only one (10% beta-cyfluthrin) retained efficacy beyond 1 month. Previous studies with some of the same insecticides have reported longer residual activity than what was observed in the present study but this could be due to the difference in the nature of the substrate (electrostatic netting versus walls). The rapid loss in efficacy observed with some actives could also be due to a number of factors that are known to degrade insecticides used during indoor residual spraying campaign, including temperature, humidity and UV-light [19]. The underlying mechanisms for the rapid decay that was observed with some actives should be evaluated in further studies. However, different formulations could help mitigate some of these factors. For example, the use of UV protection additive could prevent insecticide breakdown due to photolysis and prolong the effective lifespan of chemicals. Although candidate actives were exposed to environmental conditions similar to those in local villages, persistence could still differ for a number of reasons when the insecticides are deployed in the field. For example, exposure to smoke from cooking in real houses could impact the long-term insecticidal efficacy of chemicals deployed in the eave tube. This issue has also been reported with insecticide-treated durable wall lining, where the efficacy can be undermined by dirt accumulation [20].

This emphasizes the need for continued monitoring of persistence and timely re-treatment of inserts once efficacy starts to decline.

Although the focus of this study was on readily available formulations of insecticides, there is clearly an opportunity for reformulating or repurposing a number of active ingredients for use in eave tubes. This could be useful, for example, in resistance mitigation and management where one of the recommended strategy is the use of unrelated insecticidal compounds in rotations or mosaics to delay the spread of insecticide resistant genes [21,22]. Additionally, a diversity of active ingredients suited for deployment in eave tubes could be useful for addressing constraints on IRS. The relatively high cost of non-pyrethroid insecticide formulations coupled with a proposed reduction in IRS funding will result in much fewer houses being sprayed across sub-Saharan Africa [23] but only a small amount of insecticide is needed to protect a house with eave tubes. Moreover, most insecticides are short-lived when applied on mud wall, which is common in most rural endemic areas across sub-Saharan Africa. This may be less of a problem with the eave tube technology given that insecticides are deployed on substrate with standard characteristics.

In the experimental huts, beta-cyfluthrin produced 55% mortality of pyrethroid resistant *An. gambiae* mosquitoes. Although the mortality observed in the experimental huts is consistent with findings from previous studies [8,9], mortality was much higher in laboratory bioassay. This could be either due to a percentage of mosquitoes not entering the tubes over the course of the night or that contact with the treated inserts was too transient for the mosquito to pick up a lethal dose of insecticide. When inserts were treated with fluorescent powder and placed in the experimental huts, the proportion of mosquitoes that contacted the fluorescent dust (44%) was generally consistent withthe mortality (55%) induced by beta-cyfluthrin treated inserts. This suggests that not all female mosquitoes came into contact with the treated inserts but those females that contacted the tube died, and this would have happened within the first 2 minutes of exposure. In other words, overnight mortality is likely determined by the probability a mosquito will come into contact with the treated insert rather than the probability the probability the mosquito will die given it has contacted a treated insert (if the inserts are freshly treated with insecticides). Interestingly, the proportion of mosquitoes entering through open tubes (>75%) was higher than the contact rates estimate with beta-cyfluthrin and fluorescent powder. This difference in mosquito behavior could be due to a change in the flow of human odours

emanating from volunteer-occupied hut, which might be attenuated when tubes are screened with the inserts.

Overall, on the basis of its performance and residual activity, as well as commercial availability and existing regulatory approval in Côte d'Ivoire, beta-cyfluthrin was selected for the eave tube CRT. While having a pyrethroid insecticide in the eave tube might not seem an ideal option in an area of pyrethroid resistance, the resistance breaking properties of the electrostatic netting still enables use of a pyrethroid. Nonetheless, it will be important to monitor the potential for further selection for pyrethroid resistance. Moreover, screening for other active ingredients should be considered a priority in order to increase the scope for developing more sustainable resistance management strategies [24].

Abbreviations

SET: Screening plus EaveTubes; PVC: polyvinyl chloride; CDC: Centers for Disease Control and Prevention; UV: Ultra-violet; GLM: Generalized Linear Model; GLMM: Generalized Mixed Effect Model

Declarations

Author's contribution

WAO participated in the study design, conducted the data analysis and drafted the manuscript. WAO, ITZ and AMGB performed the experiments. RN, MBT and EDS contributed to the study design and edited the manuscript. AK provided administrative support. All author read and approved the final manuscript.

Acknowledgements

We would like to thank all technicians at IPR for their assistance. The authors also thank the volunteer sleepers for their participation in the experimental hut study.

Competing interests

All authors declare that they have no competing interests

Availability of data and materials

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

Ethics approval and consent to participate

The study was approved by the ethical review committee of the London School of Hygiene and Tropical Medicine (reference 11223) and the Ministry of Health in Côte d'Ivoire. The volunteers hut sleepers were > 18 old and provided written informed consent before taking part in the trial. The study participants were not given prophylaxis since mosquitoes used in the overnight release recapture were laboratory-reared and did not receive a blood meal.

Funding

This research is supported by a grant to the Pennsylvania State University from the Bill & Melinda Gates foundation.

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Table 4.1: List of insecticides initially screened for residual performance against pyrethroid resistant *An. gambiae* M'bé strain.

Commercial name (supplier)	Active ingredients (Dose)	Chemical classes
Actellic (Syngenta, Switzerland)	Pirimiphos methy (16g/kg); Thiamethoxam	Organophosphate; neonicotinoid
NA	Azametiphos (10%)	Organophosphate
NA	*Beauveria bassiana* (10%)	Fungus
Ficam D (Bayer, Germany)	Bendiocarb (1.25%)	Carbamate
BISTAR 10 WP (FMC, India)	Bifenthrin (10%)	Pyrethroid
BorActin (Rockwell labs Ltd, USA)	Orthoboric acid (99%)	Boric acid
Tempo Ultra (Bayer, Germany)	Beta-cyfluthrin (10%)	Pyrethroid
Spritex (Denka International BV, Barneveld, The Netherlands	Deltamethrin (0.25%)	Pyrethroid
Drione (Bayer, Germany)	Pyrethrin (1%) ; Piperonyl Butoxide (10%)	Pyrethroid; synergist
NA	Permethrin (25%)	Pyrethroid
Sevin (TechPac LLC, Atlanta)	Carbaryl (5%)	Carbamate

Commercial names are provided for insecticides that are available on the market; NA indicates the insecticide was experimental formulation and not a commercially available product

Table 4.2: Release-recapture (%) and response of pyrethroid resistant *An. gambiae s.l.* within enclosure at M'bé, Côte d'Ivoire.

Treatment	Total released	% recaptured (95% C.I.)	% Immediate mortality (95% C.I.)	% 24h mortality (95% C.I.)	% with fluorescent dust (95% C.I.)
Untreated insert	395	90.38 [87.5 −93.3]	1.12[a] [0.03 −2.21]	2.8[a] [1.1 −4.5]	−
10% beta-cyfluthrin treated insert	389	84.31 [80.7 −87.9]	55[b] [49.6 −60.4]	64[b] [58.8 −69.2]	−
Fluorescent dust-treated insert	790	87.6 [85.5 −89.7]	−	−	44.4 [40.7 −48.1]

*Values in the same column not sharing a letter superscript differ significantly (P<0.05, GLMM)

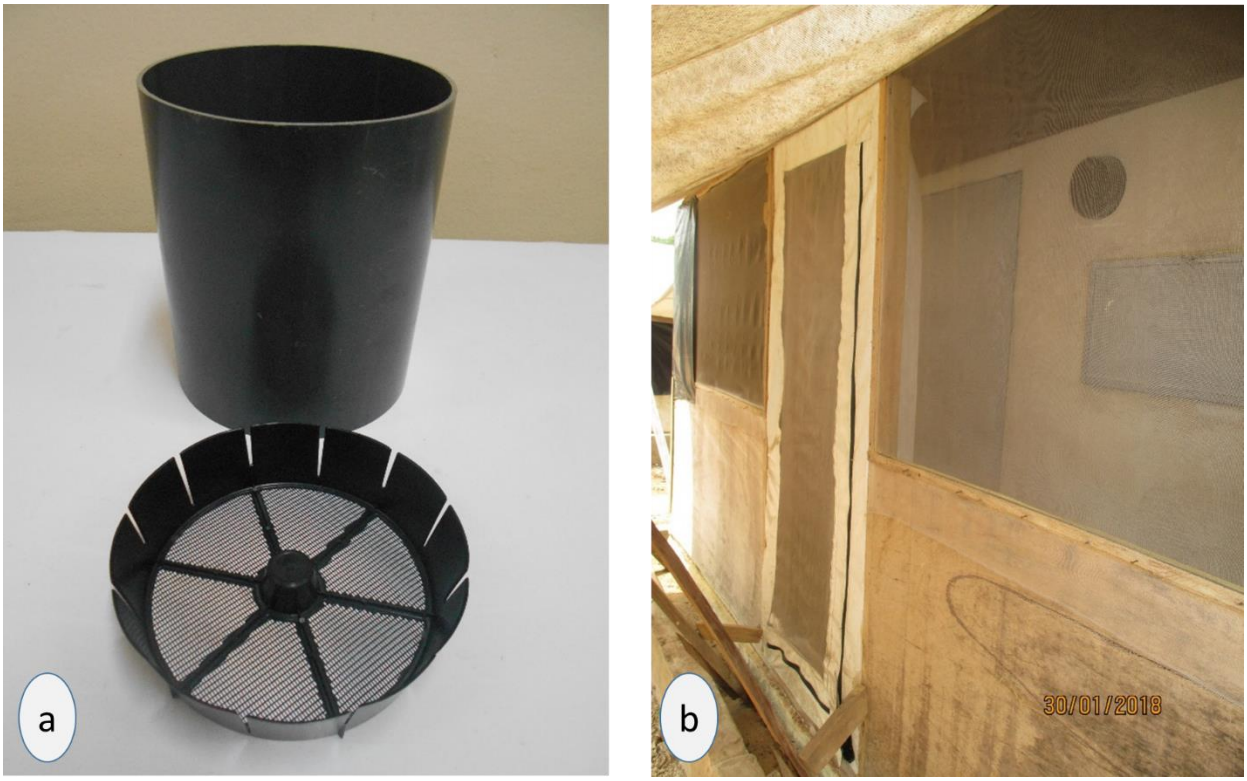

Fig. 4.1 a) Photo of the components of the eave tube assay; b) Picture of the experimental hut fitted with eave tubes.

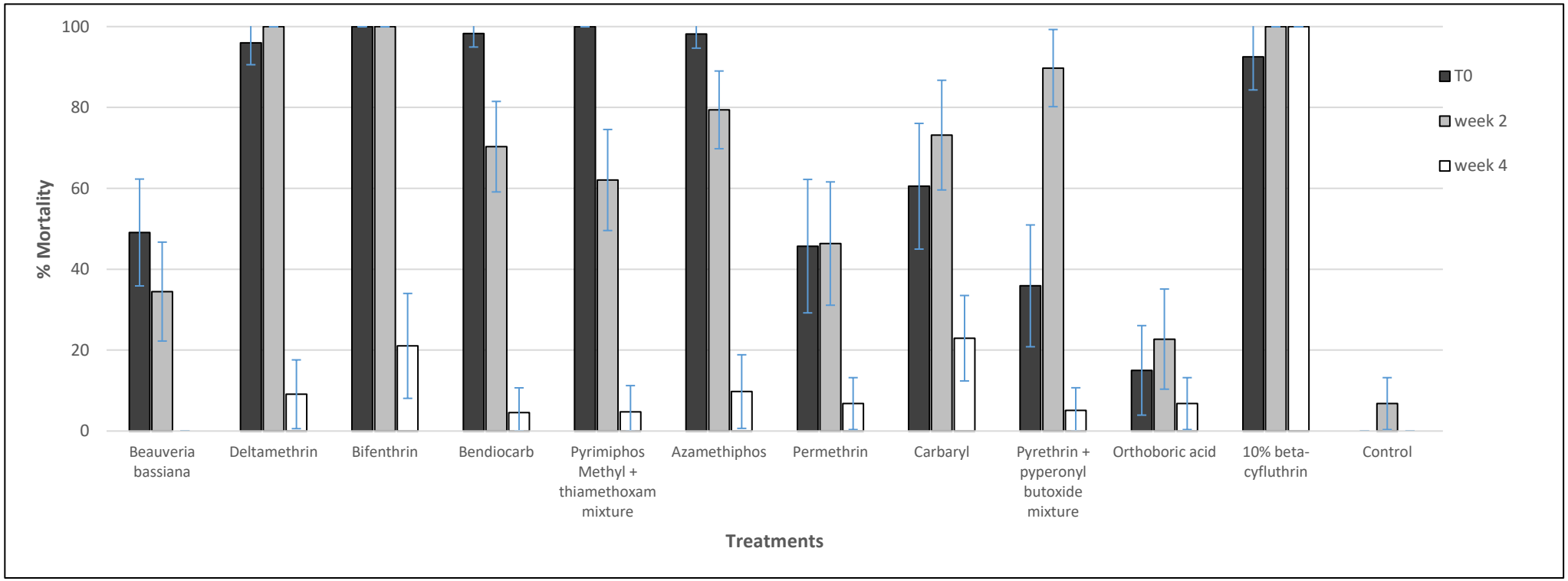

Fig. 4.2 Weekly mortality rates of pyrethroid resistant *An. gambiae s.l.* M'bé strain after exposure to insecticide treated insert using 3min eave tube assay

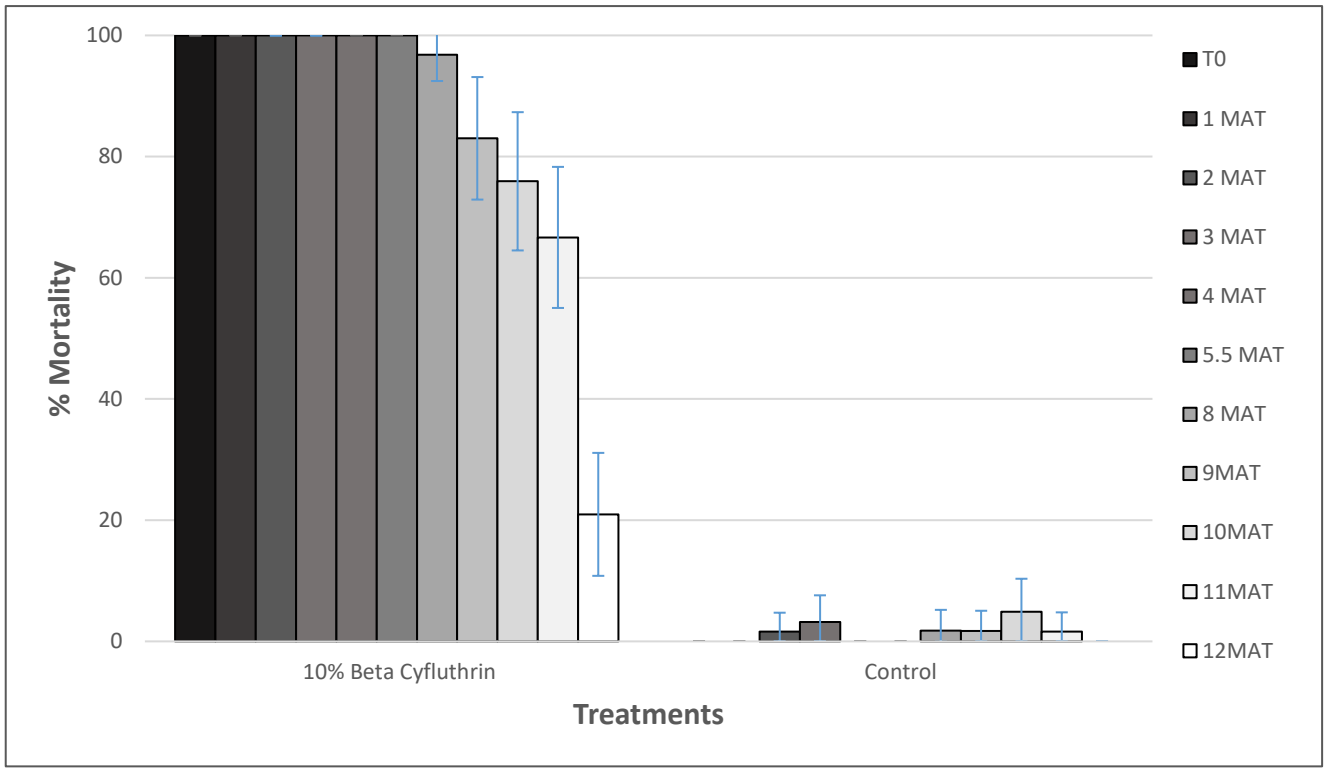

Fig. 4.3 Residual activity over twelve months of 10% beta-cyfluthrin (selected from initial screening) on insert against pyrethroid resistant *An. gambie s.l.* from M'bé

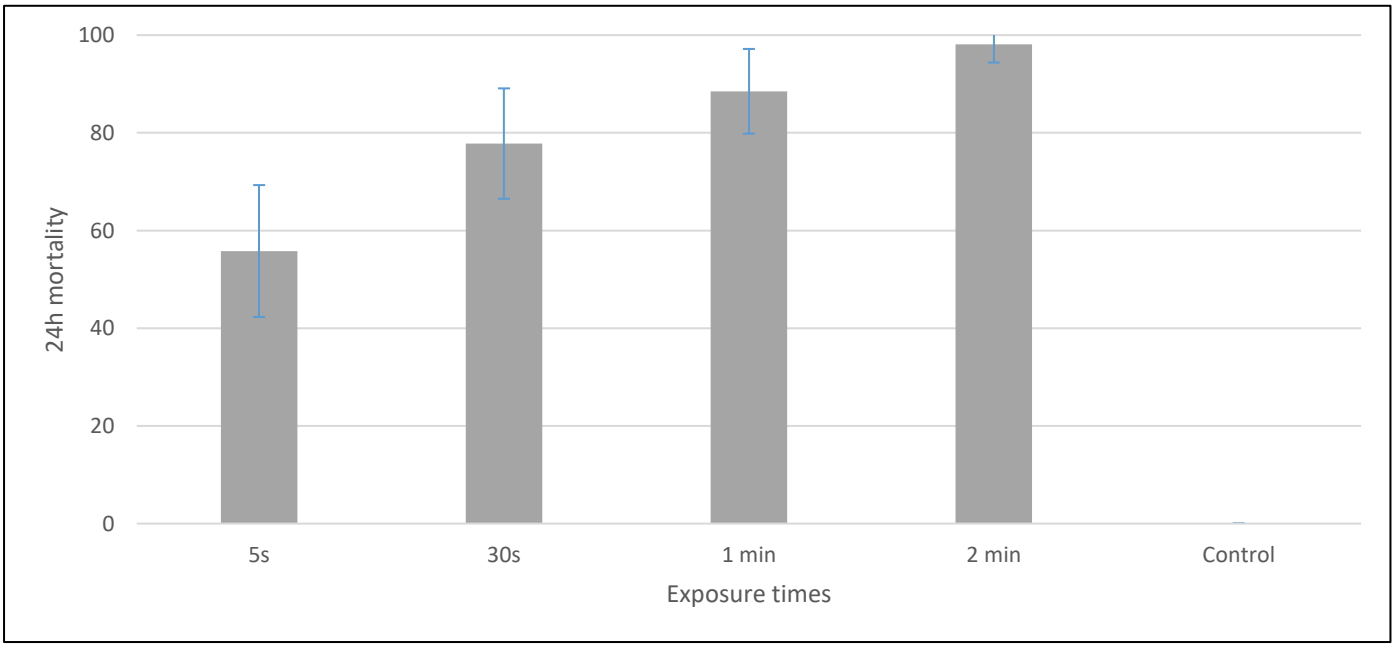

Fig. 4.4 Actual exposure time and induced mortality of individual pyrethroid resistant *An. gambiae s.l.* from M'bé with 10% beta-cyfluthrin treated insert

Chapter 5

Spatio-temporal trend in insecticide resistance in *Anopheles gambiae* following wide-scale deployment of a "Lethal house Lure" in combination with standard LLIN in central Côte d'Ivoire

The materials presented in this chapter will be submitted for publication in Emerging Infection Diseases as:

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Spatio-temporal trend in insecticide resistance in *Anopheles gambiae* following wide-scale deployment of a "lethal house lure" in combination with standard LLIN in central Côte d'Ivoire

Abstract

The In2Care EaveTubes is a novel house-based intervention which uses the electrostatic coating technology as insecticide delivery system. Although this insecticide application method has resistance breaking potential, it is necessary to evaluate whether community-level deployment of pyrethroid treated EaveTubes would increase selection pressure on malaria mosquitoes. In the context of a cluster randomised controlled trial (CRT) of screening plus EaveTubes (SET) in the presence of long lasting insecticidal net (LLIN), we assessed the spatio-temporal change in resistance to pyrethroids and additionally to other insecticide classes (carbamates, organophosphates and organochlorines) in *Anopheles gambiae* from a subset of trial villages. Pyrethroid resistance intensity increased significantly in study arms over the timeframe of the study and this was mostly associated with transcriptional change in the carboxylesterase *COEAE1F*. However, the difference between arms was not significant, which was consistent with the trend found with a range of metabolic resistance genes detected in the area. Analysis of the knockdown resistance mutations (1014F, 1014S and 1575Y) suggest no role of these genes in the observed change in pyrethroid resistance phenotype. There was no significant change in resistance pattern with insecticides from the other major adulticide classes tested except with pirimiphos methyl, against which resistance prevalence increased in some villages despite no temporal change in *Ace-1* allelic frequency. The increase in pyrethroid resistance level associated with the deployment of pyrethroid treated EaveTubes suggests that a non-pyrethroid version of the technology should be considered.

Background

Recent gains in reducing malaria burden have mostly been driven by the widespread roll-out of insecticide-based control measures, which have been associated with prevention of about 500 million clinical cases of malaria since 2000 [1]. While vector control remains a key component of the global malaria control strategy, the efficacy of existing control methods relies on continued vector susceptibility to insecticides. Unfortunately, insecticide resistance in African malaria vector populations of malaria is widespread with resistance to at least one of the four major classes of neurotoxic adulticides reported in 73 countries in 2018 [2]. The emergence and geographical distribution of insecticide resistance may have contributed to the recent stagnation of progress in malaria control with increased cases of malaria reported in 55 countries between 2015 and 2017 [3].

The rise in insecticide resistance in malaria vectors is ascribed with the extensive use of insecticide in agriculture for crop protection [4] and the wide-scale use of insecticide based vector control strategies, most notably long-lasting insecticidal net (LLINs) [5]. Target-site insensitivity and increased expression of metabolic genes are the two most studied types of insecticide resistance mechanisms. The former results in reduced affinity between the insecticide and its binding site. This occurs in the para voltage-gated sodium channel (vgsc), through a substitution from leucine to either phenylalanine (L1014F) or serine (L1014S), and confers knock down resistance (*kdr*) to pyrethroids, the insecticides deployed on all existing nets, and dichlorodiphenyltrichloroethane (DDT) [6,7]. Another variant of the *kdr* resistance mechanism occurring in vgsc is known as N1575Y [8] which, in conjunction with L1014F, enhances resistance to pyrethroids and DDT. Aside from these three well-described *kdr* resistance mutations, additional mutations in the sodium channel have recently been reported in *Anopheles gambiae* and *Anopheles coluzzii* mosquitoes [9]. Most of these previously unknown mutations were shown to occur on a 1014F haplotype background, and may increase resistance to pyrethroids. The occurrence of target site modifications has also been reported in acetylcholinesterase, which inhibits the neuro transmitter acetylcholine, and was shown to cause resistance to organophosphates and carbamates [10,11]. Metabolic resistance involves increased activity of three families of enzymes: carboxylesterases (COEs), Glutathione-S-transferases (GSTs) and cytochrome P450s [12]. Genes from the P450s family have increasingly been linked to the detoxification of pyrethroids and carbamates [13–16]. Apart from these major classes of insecticide resistance mechanisms, additional less well-studied

mechanisms of resistance may be contributing to the strong phenotype of resistance being reported in malaria vectors, especially across West Africa [17,18]. Reduced penetration of insecticide associated with the thickening of insects' cuticles have been linked with resistance in vector populations [19]. Recently, a new type of insecticide resistance mechanisms involving an over-expression of a family of chemosensory proteins, referred to as sensory appendage proteins (SAPs), was shown to mediate pyrethroid resistance in *Anopheles gambiae* mosquitoes [20]. All of these resistance mechanisms that confer greater survival benefit to mosquitoes in the presence of insecticides represent a substantial challenge to malaria control efforts.

Although vector resistance is widespread in much of sub-Saharan Africa, the situation in West African countries, including Côte d'Ivoire, is particularly worrisome with resistance to all but the newest licenced adulticides reported in the key vector species, *An. gambiae* and *An. coluzzii* [21]. Reported resistance mechanisms for southern Côte d'Ivoire include target site mutations (*kdr* and *Ace-1*), and overexpression of P450s genes, notably *Cyp6M2* and *Cyp6P3* [13]. More recently, a comprehensive investigation into the resistance profile of *An. gambiae* s.s. mosquitoes in central Côte d'Ivoire, conducted prior to a randomized controlled trial (CRT) of the In2Care EaveTubes strategy [22], reported resistance mechanisms broadly similar to that in *An. coluzzii* from Tiassalé, in southern Côte d'Ivoire [23]. However, additional important detoxification enzymes, most notably the pyrethroid and pyriproxyfen-metabolizing P450 enzyme, *Cyp9K1*[24], were found to be strongly over-expressed.

The EaveTubes trial is investigating whether house screening (S) plus EaveTubes (ET) in combination with LLINs provide greater protection against malaria transmission than LLINs alone in a pyrethroid resistance area in central Côte d'Ivoire. The In2Care EaveTubes is a novel house-based intervention which involves screening of houses to prevent mosquito house entry and killing of host-seeking mosquitoes as they make contact with an insecticide treated electrostatic netting placed in PVC tube inserted in eave gap. From several available insecticides tested, the pyrethroid beta-cyfluthrin, provided the best residual killing activity in the eave tubes against local mosquitoes and was selected for the trial [25]. Though effective against resistant mosquitoes, a clear concern is that additional use of a second pyrethroid in eave tubes with that used on LLINs could select for further resistance. The study reported here aimed to (i) investigate the temporal and spatial dynamic of insecticide resistance in *Anopheles* mosquitoes in trial arms (SET+LLIN and LLIN alone) over the course of the CRT and (ii) explore whether the dual source of selection pressure from pyrethroids deployed in both

PermaNet 2.0 (deltamethrin) and EaveTubes (beta-cyfluthrin), translates into an increase in phenotypic resistance and associated molecular mechanisms in the local *Anopheles* vector populations.

Methods

Study area

The study was conducted concurrent with the CRT in the Gbèkè region in central Côte d'Ivoire. The trial involves 40 villages located within 50 km radius of the city of Bouaké, with 20 villages assigned to each study arm. The area is characterized by a humid tropical climate with an average annual temperature of 18.9 °C and average relative humidity of 66.6%. Rice cultivation is the main agricultural practice, along with some vegetable farming. Malaria transmission occurs year-round and peaks during the wet season, between May and October [26,27].

The CRT spanned 2 years, between May 2017 and May 2019. To assess change in resistance prevalence, intensity and gene frequency in *Anopheles* mosquitoes from the study area, insecticide resistance monitoring was performed at four different time points: before the trial (2016), at two points during the course of the trial (2017-2019) and after completion of the trial (2019).

Mosquito collection and rearing

Longitudinal resistance monitoring surveys were performed in eight villages (four per study arm) once in 2016, 2017, 2018, and 2019. Village selection for resistance monitoring was mainly driven by the presence of productive mosquito-breeding habitats. Mosquito larvae were collected from each village using the dipping method. This was performed in the same time period each year (September to November) except for the 2019 monitoring survey where collection was done between June and August. *Anopheles* larvae and pupae were collected from a variety of habitats spanning small puddles to rice fields. To avoid collection of larvae hatched from eggs laid by one or a few female mosquitoes, which might not give an accurate profile of resistance, mosquito larvae were sampled, if possible, from multiple breeding sites. Collection from various breeding habitats from the same village were then pooled and transported to the insectary at the Institut Pierre Richet (IPR), Bouaké for rearing. Mosquito larvae were fed on

ground Tetramin fish food and reared to adulthood under controlled conditions. Emerging adult mosquitoes were kept in netted cages and maintained on 10% honey solution until susceptibility testing. All adult female mosquitoes were morphologically identified as *An. gambiae* s.l. using taxonomic keys.

WHO susceptibility tests

Insecticide resistance prevalence in the *Anopheles gambiae* s.l. mosquitoes from the trial area was assessed annually between 2016 and 2019 using standard WHO susceptibility assays. Mosquitoes were exposed to papers supplied by the Vector Control Research Centre of Universiti Sains Malaysia, impregnated with diagnostic concentrations of deltamethrin (0.05%), the insecticide in the LLIN (PermaNet 2.0), and cyfluthrin (0,15%), the insecticide deployed in the EaveTubes [25]. To track change in resistance to the four major classes of adulticides used in public health, the list of insecticides tested per year was expanded whenever possible to include bendiocarb (0.1%, tested once every year), DDT (4%, tested in 2016, 2017 and 2019), and pirimiphos methyl (0.25%, tested in 2018 and 2019). Two to three-day old adult female mosquitoes, emerged from larvae collected from study villages were used in WHO susceptibility assays. Four replicates of 20-25 adult female mosquitoes were exposed for 1h to insecticide-treated papers, alongside 20-25 exposed to untreated paper (control). Mosquito mortality was recorded 24h post-exposure. Mosquitoes that survived exposure to either pyrethroid were monitored for an additional 24h, after which the survivors were preserved in RNA later for subsequent molecular testing.

Resistance intensity assays

The level of resistance to deltamethrin was also monitored annually, using adapted Center for Disease Control and Prevention (CDC) resistance intensity bioassay. The cap and the inside of each 250 mL Wheaton bottle was coated with a range of deltamethrin concentrations (7.81µg/mL to 1000µg/mL), producing a range of mortality rates between 0% and 100% in mosquitoes from the study villages. Control bottles were treated with acetone only. The susceptible *An. gambiae* Kisumu strain (SS) served as a reference and was tested against dosage range 0.001µg/mL-0.5µg/mL. Two to three days old adult female mosquitoes were exposed for 1h at each concentration in four replicates of 25.

Mosquito genotyping

To determine mosquito species and genotype *Anopheles* mosquitoes from study villages for the presence of target site resistance mechanisms, genomic DNA was extracted from a pair of legs taken from field mosquitoes unexposed to insecticides. The legs were boiled in 20µL of buffer solution for 30 min at 95°C. Identification to species within the *An. gambiae* complex was performed using SINE-PCR [28].

Detection of mutations in the voltage gated sodium channel, 1014S, 1014F and 1575Y [8,29], and the *Ace-1* G119S [30] mutation in acetylcholinesterase was performed on unexposed mosquitoes using TaqMan® PCR assays.

Whole genome microarray

A first genome-wide transcription analysis was performed in 2016 prior to the start of the EaveTubes trial to identify genes differentially expressed in *Anopheles gambiae* populations from two study villages relative to susceptible laboratory colonies of *Anopheles* mosquitoes [22]. The second microarray analysis in 2019 was performed after completion of the trial to (i) identify any changes in gene expression between 2016 and 2019 and (ii) establish whether additional resistance-associated genes, which probably were absent or undetectable at baseline, have been selected over the duration of the CRT.

The microarray experimental design followed that used in 2016, with whole genome transcription analysis performed on *Anopheles* mosquitoes from the same villages (one from each study arm) using an interwoven loop design (Fig. 5.S1). However, in contrast to 2016 where *An. gambiae* s.s. was the most predominant species in the two villages studied, Sessenouan (SET+LLIN arm) and N'Guessan Pokoukro (LLIN alone arm), *An. coluzzii* became the most prevalent species in N'Guessan Pokoukro (NP) in 2019. Therefore, field mosquitoes included in the microarray analysis were *An. gambiae* from Sessenouan and *An. coluzzii* from N'Guessan Pokoukro.

Gene expression profiles of unexposed, female *An. coluzzii* mosquitoes from N'Guessan Pokoukro village and the survivors of deltamethrin exposure from Sessenouan village were compared to those of two susceptible lab strains, *Anopheles gambiae* Kisumu and *Anopheles gambiae* Ngousso. The use of unexposed mosquitoes from the control village and bioassay survivors from the intervention village was consistent with the 2016 microarray design, which

allows comparison of gene expression between years (2016 and 2019). Significant differential expression between field mosquitoes from the two villages and the two insecticide susceptible lab strains was identified using a filtering approach. This was based on a $P < 0.05$ (after Bonferroni correction), a fold change in expression > 2 or <-2 and directionality i.e. the same direction of differential expression (upregulated or down-regulated) in the 4 comparisons (N'Guessan Pokoukro vs Kisumu, N'Guessan Pokoukro vs Ngousso, Sessenouan vs Kisumu, Sessenouan vs Ngousso). Total RNA was extracted from batches of ten female *An. gambiae* s.l. mosquitoes using a PicoPure RNA isolation kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Total RNA extracted from mosquitoes was treated using DNase (RNase free DNase set, Qiagen Hilden Germany). Before further use, the concentration and quality of the extracted RNA were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and a 2100 Bioanalyzer (Agilent Technologies). Four biological replicate extractions of total RNA for each mosquito population or colony were amplified and labelled using the Low Input Quick Amp Labeling Kit (Agilent Technologies). The Agilent Agam15k array was used for dual-color hybridizations (N'Guessan Pokoukro vs Kisumu, N'Guessan Pokoukro vs Ngoussou, Sessenouan vs Kisumu, Sessenouan vs Ngoussou) [31]. The labelled samples were hybridized using a Gene Expression Hybridization Kit (Agilent Technologies). Washing, scanning and feature extraction were performed according to the manufacturer's recommendations. The design of the microarray experiment was optimized through comparison of the above strains across four microarray slides.

Quantitative reverse transcriptase PCR for candidate gene expression in field mosquitoes

The expression of (i) a subset of genes found over-expressed at baseline from microarray 2016 results and (ii) additional genes detected in the 2019 samples with known insecticide metabolism activity was investigated using reverse-transcription quantitative PCR (RT-qPCR). The qPCR analysis was performed only on unexposed mosquitoes from eight study villages because previous qPCR analyses of baseline candidate genes showed no difference in gene expression between bioassay survivors and unexposed mosquitoes [22]. Moreover, the focus of the present study was not on insecticide-induced genes, but rather temporal changes in expression of genes potentially associated with pyrethroid resistance following use of pyrethroid-based control interventions. Prior to qPCR experiments, RNA was extracted from field mosquitoes and quantified using the Nanodrop spectrophometer. cDNA was subsequently

synthesized from 11g of RNA using oligo (dT) 20 (50 μM) and SuperScript III (200U) (Invitrogen) and purified through a DNA-binding column (Qiagen). Three pairs of primers of each target gene were designed using Primer-BLAST tool (NCBI: http://www.ncbi.nhi.gov/tools/primers-blat/). The primer pair with the highest efficiency value (~100%), determined by running standard qPCR using serial dilution of a single cDNA sample, was selected for subsequent qPCR (details of the primers are given in Table 5.S1). For each qPCR reaction, four biological replicates of each treatment group and two technical replicates were used. QPCR was performed using an Agilent Mx3005P QPCR System and the cycling condition was as follows: 95°C for 3 min, 40 cycles of 95°C for 10 s and 60°C for 10 s. Expression of the genes was normalized using references genes (Ribosomal S7 and Elongation Factor).

Statistical analysis

WHO assessments of mortality rates are: less than 90% indicates resistance; higher than 98% indicates susceptibility: between 90 and 98% requires further testing to confirm resistance status [32]. The variation in bioassay mortality of *An. gambiae* mosquitoes and gene frequencies over time between villages, study arms and years was tested using generalized linear mixed effect models (GLMMs) with a binomial distribution and a logit link function using the "lme4" package. The models included bioassay mortality as the response variable, insecticide tested as the explanatory variable with villages and year treated as random effects. Analysis of gene frequency data was performed using also a generalised linear mixed models with allelic frequency as fixed effect and study arm, villages and year as random effects. The intensity of resistance (Resistance Ratio, or RR50) was estimated by comparing the LD50 of the wild population relative to that of the susceptible lab strain. A MAANOVA model was used to analyse microarray data using previously described custom R-scripts [31]. Differentially expressed genes (over/under expressed) were those with a fold change consistently greater than 2 or less than -2 across the four comparisons (N'Guessan Pokoukro vs Kisumu, N'Guessan Pokoukro vs Ngousso, Sessenouan vs Kisumu, Sessenouan vs Ngousso) and with a significant Bonferroni p value in all four comparisons.

Prior to analysis of the qPCR data, significant outliers were identified in SPSS v23 and excluded from the dataset. The ΔΔCt method incorporating PCR efficiency was used to compare expression of each target gene between field mosquitoes and the lab strain[33]. Significant difference in fold change between field samples and the reference lab colony was

estimated using a t-test ($P < 0.05$). A linear mixed model with study arm nested within village was used to compare the level of expression of candidate genes between study villages, intervention arms and years.

Results

Trends in phenotypic resistance in *An. gambiae* mosquitoes

The trend in bioassay mosquito mortality in trial villages over the study period is presented in Fig. 5.1 and Fig 5.S2. Over the four-year monitoring period, 13,641 female *Anopheles gambiae* s.l. mosquitoes were tested in bioassays with deltamethrin (0.05%), cyfluthrin (0.15%), DDT (4%), bendiocarb (0.1%) and pirimiphos methyl (0.25%). *Anopheles gambiae* populations from surveyed villages were resistant to all the insecticides tested (Fig. 5.1). Resistance prevalence was highest with DDT (<15% mortality) (Fig. 5.S2) with no significant difference in resistance frequencies between years ($P > 0.05$). The pattern of resistance with deltamethrin mirrors that of cyfluthrin, with mortality rates for both insecticides ranging between 12% and 52% (Fig. 5.1 A & B). Although there was some variation in mortality rates for both pyrethroids in study villages over the study period, temporal change in resistance prevalence was generally not significant ($P > 0.05$). Resistance prevalence with deltamethrin showed no significant temporal change in both intervention arms ($P > 0.05$, Fig. 5.2 A). In contrast, mosquito mortality with cyfluthrin decreased significantly over time in LLIN and SET+LLINs arms (Fig. 5.2 B). There was generally no evidence of a difference in temporal response between arms with either pyrethroid ($P > 0.05$). Resistance to bendiocarb persisted in all study villages between 2016-2019; however, there was generally no evidence of a significant increase in bendiocarb resistance prevalence over time ($P > 0.05$) (Fig. 5.1C); overall mean mortality rates ranged between 26.8-53.2%. While *An. gambiae* s.l. mosquito populations from surveyed villages were resistant to the organophosphate pirimiphos methyl (PM), the frequency of PM resistance was lower compared to that with insecticides from other classes tested; mortality rates ranged from 48.6-81.42%. (Fig. 5.1D). There was evidence of a significant increase in the prevalence of resistance to PM over time in most villages ($P < 0.001$).

The intensity of resistance to deltamethrin was high at baseline, in 2016, in all villages reaching over 1400 fold (ranging 1441-2405 fold) (Table 5.1, Fig. 5.3). The level of resistance to deltamethrin increased significantly over time in CRT villages ($P < 0.0001$) and both arms ($P = 0.004$), reaching up to 3000-fold by the end of the trial (Table 5.1,2&3, Fig. 5.3&4). The

change in resistance intensity in 2019 compared to baseline corresponds to an increase of 36% in study villages, 29% in the LLIN arm and 41% in SET+LLIN arm. Although the intensity of deltamethrin resistance was generally greater in the SET+LLIN arm than in the LLIN-only arm, the difference between arms was not significant in any year (P = 0.47). (Table 5.3).

Dynamics of *Anopheles* mosquito species

Overall, 3,735 *An. gambiae* s.l. mosquitoes from WHO susceptibility assays were identified to species level. *An gambiae* and *An. coluzzii* were found in sympatry in all villages over the study period. In 2016, *An. gambiae* was the dominant vector species in the study area (50-100%) with *An. coluzzii* making up only a small fraction of the mosquito population in most villages (< 10%) and study arms (3.2-13%) (Fig. 5.5 & 6). While the proportion of the latter species increased in the following years in surveyed villages (4.5-90%) and study arms (30.4-53.2%) compared to baseline, *An. gambiae* was found to be consistently more abundant throughout. The proportions of hybrids were very low, never exceeding 2% of the collection across monitoring years. In general, both malaria vector sibling species, *An. gambiae* and *An. coluzzii*, were found in comparable proportions between study arms from 2017 onwards (Fig. 5.6).

Temporal and spatial variation of target-site resistance genes

A total of 2,106 mosquitoes were screened for target-site mutations over the four consecutive years. The 1014S mutation was only detected in 2019 in one mosquito specimen, heterozygote for the mutation, from one village (Saoundi) in the intervention arm (SET+LLIN). The frequency of the *vgsc* 1575Y mutation was found to be consistently low throughout the course of the study (15%) (Fig. 5.7 A & C). Although there was a declining trend in allelic frequencies of the 1575Y mutation over time, this temporal change was neither significant across villages nor in study arms (P > 0.05). There was no evidence of a significant difference in allelic frequency of this mutation between arms at any time point (P > 0.05).

The 1014F mutation, on the other hand, was found at much higher frequencies (0.62-1%) and appeared to vary between years in study villages and intervention arms (Fig. 5.7 B & D). There was a decrease in allele frequency of the 1014F mutation in 2019 as compared to baseline in all villages, but the difference was only significant in the Kouakro village where this decreased significantly from 0.90 to 0.72% (P = 0.01) (Fig. 5.7B). In both study arms, allele frequencies of the 1014F mutation were significantly lower at all-time points compared to baseline (P <

0.01) (Fig. 5.7D). However, breaking down the data by species showed that the 1014F allele frequency increased in the most dominant vector species (*An. gambiae*) reaching almost fixation in 2019 (0.98 in LLIN arm, and 1 in SET+LLIN arm) (Table 5.4). Over the course of the study period, the 1014F mutation was found to be consistently higher in *An. gambiae* than in *An. coluzzii* ($P < 0.01$). Although allele frequencies of this mutation were comparable between arms in 2016 ($P = 0.7$) and in 2017 ($P = 0.2$), there was a significant difference in subsequent years with a higher frequency in the LLIN arm (0.91) compared to the SET+LLIN arm (0.87) in 2019 ($P = 0.008$). However, there was no evidence of a significant difference in allele frequency between LLIN (0.98) and SET+LLIN (1) in the major vector species *An. gambiae* in 2019 ($P > 0.05$).

The allele frequency of the insensitive acetylcholinesterase mutation, *Ace-1,* varied in all villages over years, ranging between 0.22 to 0.51 (Fig. 5.8). There was an increase in the frequency of the *Ace-1* mutation between 2016 and 2019 in most villages, but the rise in frequency was only significant in N'Guessan Pokoukro village (from 0.26 in 2016 to 0.51 in 2019, $P = 0.04$).

Genome-wide transcription analysis

Microarray experiments were conducted with mosquito samples collected in 2019 to explore whether the roll-out of pyrethroid based interventions (SET and LLINs) in the study area have selected for additional resistance genes and/or resulted in a significant increase in expression of the resistance-linked detoxification genes identified at baseline (2016). These molecular assays involved unexposed *An. coluzzii* mosquitoes from one control village and *An. gambiae* from one intervention village. Differentially expressed genes were those that exhibited a consistently higher/lower expression in resistant field mosquito population from both study villages versus susceptible lab strains with a significant multiple-testing corrected P-value. Based on this filtering approach, a total of 551 significant probes were identified, out of the 14,914 tested (Table 5.S2). 310 probes were over-transcribed corresponding to 241 genes (Table 5.S3). Over-expressed genes with demonstrable or likely link with resistance included 8 cytochrome P450s, 2 glutathione S-transferases (GSTs), one redox gene, one salivary protein (D7r2), and 13 cuticular genes. The composition of over-expressed genes was generally similar between 2016 and 2019; however, additional well-characterized P450 genes, not found significantly over-expressed in 2016, was identified in 2019 (*Cyp6P4* and *Cyp6Z3*) in addition to those detected at baseline (*Cyp6P3*, *Cyp9K1* and *Cyp6M2*). It is also worth noting that

cuticular genes were the most predominant over-expressed genes in 2019 with up to 13 genes compared to only one in 2016. Interestingly, four cuticular genes which were not differentially expressed in 2016, were within the top 20 overexpressed genes in the 2019 samples, of which *CPLCG4*, *CPCFC1* and *CPLCG5* exhibited >29-fold change and *CPAP3-C* with >11-fold change, relative to susceptible strains (Table 5.S3). Other highly over-expressed genes included the cytochrome P450s *Cyp6P3* (18-fold change compared with 14.2 in 2016) and *Cyp9K1* (16.3 fold-change compared with 20.1 in 2016). Additional most highly over-expressed genes comprised a salivary protein (D7r2, 37.2 fold-change), GSTS1 (FC = 20.5), Rps9 (FC = 17) and h+ transporting atp synthase subunit (FC = 85.9).

Potential changes in transcriptome expression were determined with *An. gambiae* mosquitoes from the intervention Sessenouan village based on estimated difference in gene fold change over time (Table 5.S4). Such investigation was not possible with the control N'Guessan Pokoukro village, because of the change in species composition in that village resulting in different *Anopheles* species (*An. gambiae* in 2016 and *An. coluzzii* in 2019) being used in microarray studies between years, which preclude any comparison. Out of the 310 probes over-expressed in all the field resistant mosquito populations compared to susceptible lab colonies, 62 probes corresponding to 46 genes had higher fold change in 2019 than in 2016 in *Anopheles* mosquitoes from the intervention Sessenouan village. The highest absolute fold change differences between 2019 and 2016 were with cuticular genes including *CPLCG5* (FC = 18.5), *CPLCG4* (four probes, average FC = 12.1), *CPCFC1* (FC = 9.7), *CPR* 59 (FC = 8.4) and *CPLCG3* (FC = 7).

Temporal and spatial change in expression of candidate genes

To investigate changes in expression of resistance-associated genes across selected study villages and intervention arms over time, several candidate genes from the 2016 and 2019 microarray results were selected. This included over-transcribed genes with putative or established association with insecticide resistance, which were over-expressed in both years (*Cyp6P3*, *Cyp6M2*, *CyP9K1*), in 2016 only (*COEAE1F*), and in 2019 only (*Cyp6P4*, *Cyp6Z3*), and genes that exhibited higher fold change in 2019 compared to level in 2016 (*CPLCG5* and *CPF3*). Gene expression was significantly higher in selected trial villages in 2019 compared to levels at baseline with all screened candidate genes except CPLCG5 and CPR 131 ($P < 0.05$, Fig. 5.9). Between 2016 and 2019, gene expression with *Cyp6P3* was consistently highest, peaking at 150-fold change in 2019 in the SET+LLIN Kouakro village. While fold change in

expression with candidate genes was generally higher in 2019 compared to 2016 in study arms, the difference in expression between years was significant for *COEAE1F* only (P = 0.005). Consistent with the resistance intensity data, gene expression levels with the most highly over-expressed genes were comparable between intervention arms (Fig. 5.10).

Discussion

Although early work provides evidence that pyrethroid treated EaveTubes can break resistance in African malaria mosquitoes, it was unclear whether this intervention, if taken to scale, would increase the level of pyrethroid resistance in mosquitoes. To address this, the current study was conducted in the context of a randomised controlled trial of the EaveTubes intervention to investigate the dynamic of insecticide resistance in *Anopheles* malaria mosquitoes from a subset of CRT villages over the course of the trial.

An. gambiae and *An. coluzzii* were the only *Anopheles* mosquitoes identified in the area. Although both vector species were found in sympatry throughout the trial, the former appeared as the predominant species, and occurred in much higher proportion over the three-year follow-up period. However, earlier studies investigating the diversity of mosquito species in the study area by means of human landing catches (HLC) documented the occurrence of additional malaria transmitting mosquitoes including *An. funestus* and *An. nili* [27]. Vector composition has been shown to vary according to mosquito sampling technique [34] and in the current study, mosquitoes used for resistance monitoring were obtained from larval collections rather than HLC. It is likely that the difference in mosquito sampling method (HLC versus larval collections) might account for the absence of *An. funestus* and *An. nili* in the mosquitoes that were collected.

At baseline, the local *An. gambiae* mosquito population exhibited high prevalence and intensity of resistance to pyrethroids, with deltamethrin resistance level as high as 2400 fold (ranging 1441-2405). This level of resistance increased significantly across study villages and intervention arms over the course of the CRT, reaching up to 3000 fold (ranging 1697-3061) by the end of the trial. The rise in the level of resistance to pyrethroids suggests that the deployment of the pyrethroid-based interventions (SET+LLIN and LLIN alone) had exerted a selection pressure on malaria vector mosquitoes. Evidence of an increase in pyrethroid resistance following deployment of pyrethroid based interventions, for example pyrethroid LLIN, was documented in a range of studies [35,36]. Although the focus of this study was on

the temporal change of resistance in response to vector control methods, additional sources of selection pressure, namely from agricultural and household use of insecticides [37,38], might have contributed to the observed increase in pyrethroid resistance intensity. It is worth emphasizing that, while there was clear-cut evidence of a significant increase of resistance intensity to deltamethrin, no such pattern was detected in the resistance prevalence data. This adds to the existing evidence that diagnostic dose assays, although useful in providing information on the spread of resistance, have less potential in detecting major changes in resistance intensity [39]. For example, findings from a three-year study in Burkina Faso similarly reported no change in pyrethroid resistance prevalence, despite evidence of a significant temporal increase in the intensity of pyrethroid resistance [17].

Results of the study revealed a decreasing trend of vgsc-1014F mutations, which was particularly evident in intervention arms. Although unexpected, these findings are consistent with a study in Sudan reporting a declining frequency of the vgsc-1014F mutation in *An. arabiensis* following distribution of pyrethroid LLIN [40]. The decrease in allele frequencies of *kdr* mutations, although not unique to this study, are in contrast to the rising level of pyrethroid resistance and suggests no contribution of this gene to the change in phenotypic resistance.

Allelic frequency of an emerging resistance gene, for example pyrethroid resistance strengthening vgsc-1575Y mutation, is expected to increase further in response to the wide-scale use of pyrethroid based control tools. However, as shown in this study and elsewhere [41], the frequency of the vgsc-1575Y mutation remained relatively low over the study period. This is probably due to the fact that this *kdr* gene co-occurs exclusively with the vgsc-1014F, which is almost fixed in most West African countries, thus limiting potential for further increase in allele frequency. Indeed, this was evidenced in a recent work showing no significant increase of this mutation over a two-year period in a setting where the vgsc-1014F mutation had reached fixation in the local populations of *An. gambiae* [8]. On the other hand, the vgsc-1014S mutation, formerly limited to East-Africa, is now co-occurring along with vgsc-1014F across West Africa. Over the course of the study, this mutation was detected in only one mosquito specimen, heterozygote for the mutation, and was found on a vgsc-1014F background. While this third variant of the *kdr* mutation is being reported in an increasing number of countries in West Africa [42–44], its allele frequency, as seen in this study, appears relatively low. However, the emergence of this resistance mutation in this part of Africa is

giving rise to individual mosquitoes bearing the double East (1014S) and West African *kdr* (1014F) mutations [44,45]. This underscores the need for studies exploring the contribution of the co-occurrence of these genes to pyrethroid resistance and potential impact on control efforts.

Microarray analysis were performed on mosquitoes sampled from study villages before and after the CRT to investigate the underlying molecular mechanisms contributing to the increasing level of pyrethroid resistance. Resistance to pyrethroid was initially common in the area and, was mainly driven by P450 genes: *Cyp6P3*, *Cyp6M2* and *Cyp9K1*. While these well-characterised genes were still up-regulated in the 2019 mosquito samples, additional resistance-associated genes including, most notably P450s (*Cyp6P4* and *Cyp6Z3*), cuticular genes, Glutathione-S-transferase (*GSTS1*) and salivary gland gene (*D7r2*) were among the most over-transcribed resistance-associated genes identified at the end of the trial. In a context of decreasing pattern of vgsc-*kdr* mutations, the significant increase in the intensity of pyrethroid resistance was probably due to the marked rise of this set of genes, especially those linked to pyrethroid resistance, for example P450s and cuticular genes. It should be noted that the top emerging resistance-linked enzymes were mostly cuticular proteins belonging to a range of protein families (CPLCG, CPCFC, CPR and CPAPn). This suggests that these selected cuticular proteins (CPs) may have played a role in the increased phenotypic resistance. However, this result should be interpreted with cautious as the microarray data were obtained from only two villages and the other villages may have different trends. CPs made up the bulk of the mosquito cuticle [46] and, when over-expressed, were shown to be associated with cuticle thickening that reduces insecticide penetration and confer resistance to insecticide from unrelated classes. The role of cuticular genes in insecticide resistance has, until recently, received little attention. However, given the rise of extremely high resistance phenotypes in mosquitoes, which are unlikely to be mediated by target-site and metabolic resistance mechanisms alone, there is increasing interest in understanding the molecular mechanisms underpinning cuticular resistance in *Anopheles* mosquitoes and its role in pyrethroid resistance [46,47].

The deployment of new PermaNet 2.0 LN in the study area resulted in a significant increase in pyrethroid resistance level over the course of the EaveTubes trial, and this was primarily attributable to the increasing level of detoxifying enzymes including the carboxylesterase

COEAE1F. This is consistent with previous studies [41,48] and suggests that, unless control methods incorporating insecticides with new mode of action are developed for vector control, pyrethroid resistance intensity may evolve further as coverage and net usage rates increase.

Although a significant temporal increase in pyrethroid resistance intensity was evident in both study arms, the level of resistance was comparable between arms over the course of the trial. This is in line with the gene expression data showing similar expression level of the most over-transcribed genes between SET+LLIN and LLIN-only arms at all time points. However, because of the dual source of selection pressure from the pyrethroids in the net (deltamethrin) and the EaveTubes intervention (beta-cyfluthrin), resistance intensity was expected to be significantly higher in the SET+LLIN arm. One possible reason explaining the lack of difference in resistance intensity between arms include proximity of control and intervention villages with potential for migration of mosquitoes between intervention arms. Indeed, study villages were a few kms apart and it is possible that mosquito migration between LLIN and SET+LLIN villages made the detection of the difference difficult. This is supported by highly variable resistance bioassay and gene expression data, between villages within arms. It is also possible that the two-year resistance monitoring period was too short for evidence of stronger selection pressure in SET+LLIN arm to be detected in bioassays and resistance genotyping analysis. Follow-up resistance monitoring studies in the trial area should be conducted to provide a definitive evidence on whether pyrethroid treated EaveTubes combined with pyrethroid LLIN exert stronger selection pressure on malaria mosquitoes compared to LLIN alone.

The pyrethroid treated EaveTubes intervention was shown to reduce malaria incidence by 38%, compared to LLIN alone in this high pyrethroid resistance setting (Sternberg et al, submitted) This indicates that despite the significant increase in pyrethroid resistance intensity and resistance mechanisms associated with the SET+LLIN intervention, the intervention did control malaria to some extent. Nevertheless, the increase in pyrethroid resistance, as shown in the present entomological study, is a concern and stresses the need for new product development of new active ingredients that show no cross-resistance to existing chemical classes that can be deployed in EaveTubes for improved vector control and effective insecticide resistance management.

Despite evidence of between-year variation, the resistance pattern remained relatively constant with the other insecticides tested, except for the organophosphate pirimiphos methyl (PM). The

prevalence of resistance to PM increased significantly in half of surveyed villages despite no on-going PM based IRS in the area. This could indicate selection pressures coming from agricultural use of the insecticide. A recent study in Cameroon reported resistance in malaria vectors against a new insecticide class for IRS (the neonicotinoid clothianidin), resulting from an unregulated use of this chemical for crop protection [49]. This emphasizes that cross-sectoral collaboration between agriculture and public health is of utmost importance to develop resistance management strategies and preserve the efficacy of existing and new insecticides. The increase in PM resistance could be due to the rise in the carboxylesterase COEAE1F and potentially cuticular proteins. Nevertheless, additional studies are required to uncover the resistance mechanisms driving PM resistance in this setting and elsewhere in Africa.

Conclusion

Deploying pyrethroid treated EaveTubes and LLIN in the same geographical setting was associated with a significant increase in pyrethroid resistance level mostly due to over-expressed P450 genes and potentially cuticular proteins. Although the pyrethroid-based lethal house lure was shown to be effective in reducing malaria transmission (Sternberg et al, submitted), its impact on pyrethroid resistance compels product development effort to identify insecticides that show no cross-resistance with pyrethroids for use in the EaveTubes. As previously demonstrated with IRS, deploying EaveTubes with non-pyrethroid insecticides against a background of LLIN may slow down the spread of pyrethroid resistance [40,50,51] and is the way forward. Given the rise of resistance-associated cuticular genes, there is an urgent need for functional genetic validation studies in this area to understand the potential contribution of these genes to increasing levels of resistance both to pyrethroids and other insecticides (pirimiphos methyl). With *kdr* mutation reaching fixation level in this part of Côte d'Ivoire and in most West African countries, the emergence of highly resistant mosquitoes, as demonstrated in this study and elsewhere [41,48], appeared mostly due to metabolic and additional resistance mechanism including cuticular genes.

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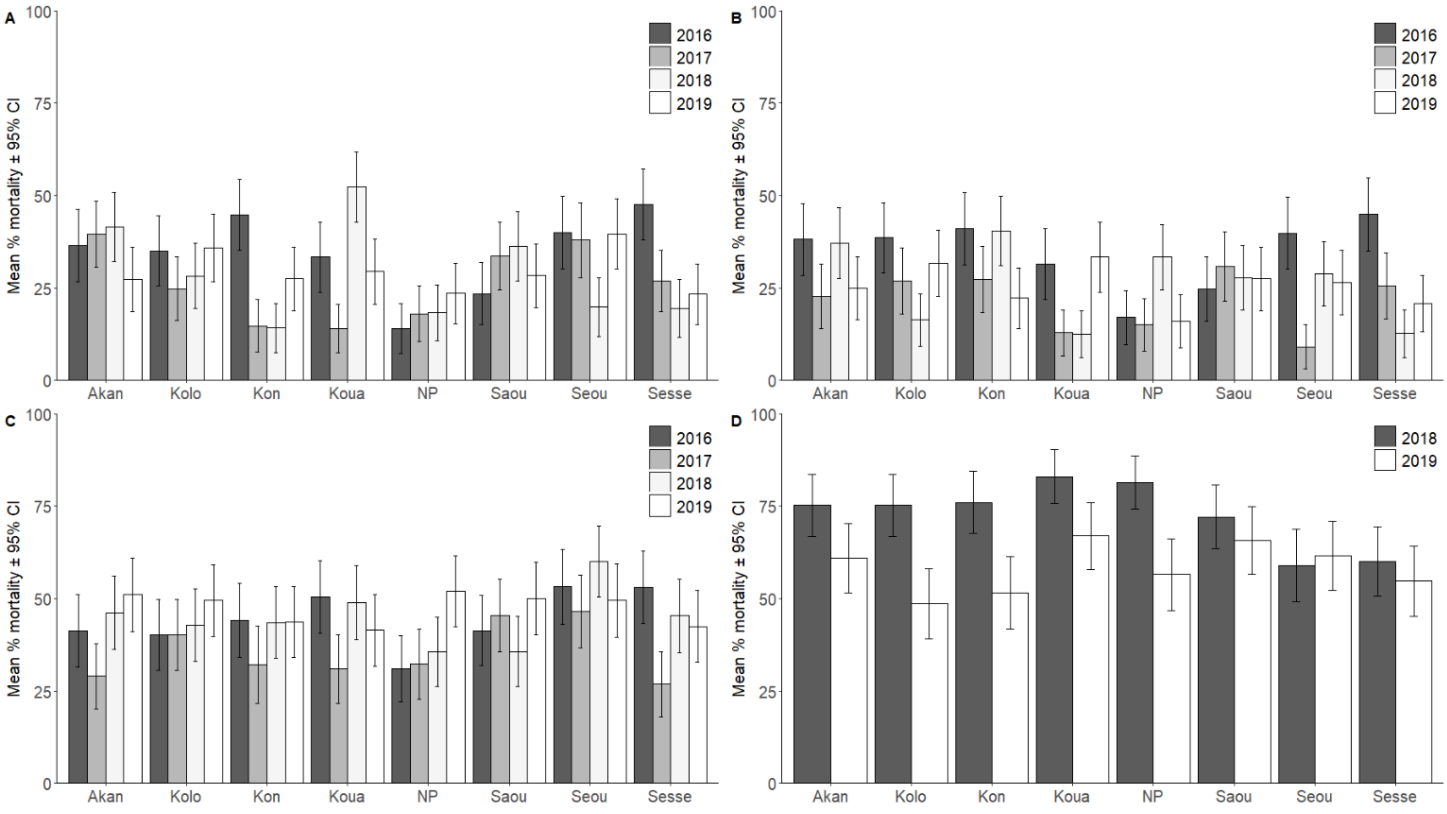

Fig. 5.1 Mean percentage mortalities of *Anopheles gambiae* s.l. mosquitoes from chosen CRT villages following exposure to diagnostic concentration of (A) deltamethrin, (B) cyfluthrin, (C) bendiocarb and (D) pirimiphos methyl in WHO cylinder assays between 2016-2019.

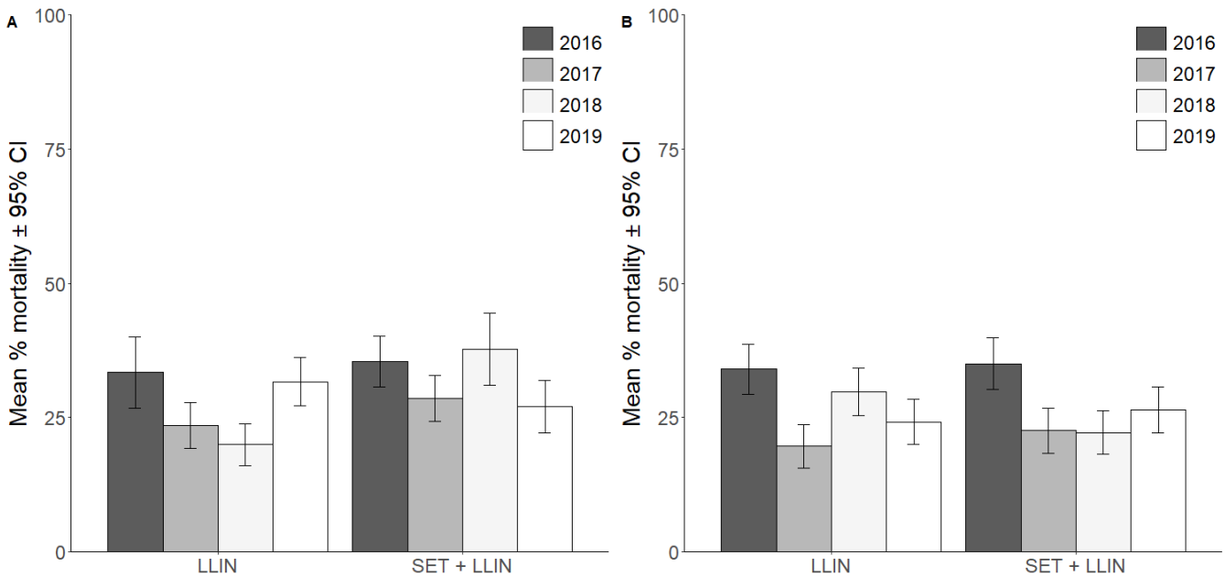

Fig. 5.2 Mean percentage mortalities of *Anopheles gambiae* s.l. mosquitoes from study arms following exposure to diagnostic concentration of (A) deltamethrin and (B) cyfluthrin in WHO cylinder assays between 2016-2019.

Table 5.1: Temporal variation in resistance intensity to deltamethrin in a subset of CRT villages between 2016 and 2019.

	2016			2017				2018				2019			
Strains	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Change in RR* (95%CI)**	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Change in RR* (95%CI)**	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Change in RR* (95%CI)**
Kisumu#	1.3 (0.18)	0.01 (0.009 - 0.02)	–	1.3 (0.18)	0.01 (0.009 - 0.02)	–	–	1.3 (0.18)	0.01 (0.009 - 0.02)	–	–	1.3 (0.18)	0.01 (0.009 - 0.02)	–	–
Akan	1.7 (0.18)	27.2 (20.3 - 35.2)	1873	1.6 (0.14)	30.6 (24.7 - 37.3)	2102	1.1 (0.8 - 1.5)	2.1 (0.21)	43.8 (34.9 - 54.8)	2954	1.6 (1.2 - 2.05)	1.7 (0.19)	39.2 (29.7 - 51.0)	2644	1.4 (1.1 - 1.8)
Kolo	1.5 (0.15)	21.9 (15.8 - 28.5)	1504	1.4 (0.14)	22.5 (16.6 - 29.2)	1549	1.03 (0.7 - 1.4)	1.4 (0.14)	35.3 (26.6 - 45.6)	2375	1.6 (1.2 - 2.1)	1.4 (0.16)	27.4 (19.0 - 37.2)	1884	1.2 (0.9 - 1.7)
Konzo	1.6 (0.13)	23.5 (19.1 - 28.3)	1617	1.8 (0.13)	26.7 (22.6 - 31.2)	1834	1.1 (0.8 - 1.5)	1.8 (0.19)	46.2 (35.3 - 59.7)	3178	2 (1.5 - 2.6)	1.6 (0.16)	40.8 (31.4 - 52.0)	2803	1.7 (1.3 - 2.3)
Koua	1.7 (0.17)	22.4 (17.4 - 28.0)	1542	1.6 (0.16)	26.5 (19.9 - 33.9)	1822	1.2 (0.9 - 1.6)	1.8 (0.19)	48.1 (37.4 - 61.1)	3305	2.1 (1.6 - 2.8)	2.0 (0.14)	45.4 (38.4 - 53.5)	3061	2 (1.5 - 2.6)
NP	2.1 (0.25)	33.7 (25.7 - 43.2)	2314	1.6 (0.13)	34.6 (27.9 - 42.3)	2380	1.03 (0.8 - 1.3)	1.7 (0.18)	39.5 (30.0 - 51.1)	2660	1.2 (0.9 - 1.5)	1.4 (0.14)	37.5 (28.5 - 48.1)	2528	1.1 (0.9 - 1.4)
Saou	1.7 (0.12)	35.0 (28.9 - 41.9)	2405	1.7 (0.18)	27.0 (20.4 - 34.6)	1855	1	1.9 (0.16)	42.1 (34.5 - 50.9)	2894	1.5 (1.2 - 2.0)	1.8 (0.14)	38.1 (31.6 - 45.7)	2621	1.4 (1.1 -1.8)
Seou	1.8 (0.14)	21.0 (17.2 - 25.0)	1441	1.5 (0.23)	24.8 (15.5 - 36)	1707	1.2 (0.9 - 1.6)	1.6 (0.19)	38.3 (27.7 - 51.4)	2631	1.8 (1.4 - 2.4)	1.6 (0.12)	25.2 (20.6 - 30.2)	1697	1.2 (0.9 - 1.6)
Sesse	1.4 (0.13)	27.4 (20.8 - 34.8)	1881	1.3 (0.12)	31.0 (22.4 - 41)	2133	1.1 (0.9 - 1.5)	1.7 (0.16)	36.4 (28.5 - 45.7)	2501	1.3 (1.03 - 1.7)	1.6 (0.15)	38.7 (30.0 - 49)	2606	1.4 (1.1 - 1.8)

#Susceptible reference strain;
LD: lethal doses expressed in µg/mL;
RR50: Resistance ratio, calculated by dividing the LD50 of the field mosquito population by that of the susceptible reference strain
* difference in resistance ratio in mosquito population from the same village over two consecutive years.

Table 5.2: Temporal variation in resistance intensity to deltamethrin in *Anopheles gambiae* s.l. from study arms

	2016*			2017				2018				2019			
Study arms	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Change in RR* (95%CI)**	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Change in RR* (95%CI)**	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Change in RR* (95%CI)**
LLIN	1.6 (0.09)	24.7 (21.7 - 28.0)	1701	1.6 (0.09)	27 (23.1 - 31.1)	1854	1.1 (0.9 - 1.3)	1.6 (0.1)	39 (33.2 - 45.5)	2665	1.6 (1.4 - 1.8)	1.5 (0.09)	31.4 (26.5 - 36.7)	2159	1.3 (1.1 - 1.5)
SET + LLIN	1.6 (0.1)	26 (22.3 - 30.1)	1790	1.5 (0.9)	30.9 (26.1 - 36.0)	2121	1.2 (1.03 - 1.35)	1.9 (0.1)	42.3 (37.2 - 41.9)	2908	1.6 (1.4 - 1.8)	1.8 (0.1)	41.7 (35.9 - 48.2)	2810	1.6 (1.4 - 1.8)

* difference in resistance ratio in mosquito population from the same study arm over two consecutive years.

Table 5.3: Relative increase of resistance intensity to deltamethrin in *Anopheles gambiae* s.l. from study arms over the course of the CRT

	2016*			2017			2018			2019		
Study arm	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Slope (SE)**	**LD50 (95%CI)**	**RR50**	**Slope (SE)**	**LD50 (95%CI)**	**RR50**
LLIN	1.6 (0.09)	24.7 (21.7 - 28.0)	–	1.6 (0.09)	27 (23.1 - 31.1)	–	1.6 (0.1)	39 (33.2 - 45.5)	–	1.5 (0.09)	31.4 (26.5 - 36.7)	–
SET + LLIN	1.6 (0.1)	26 (22.3 - 30.1)	1.1 (0.8 - 1.5)	1.5 (0.9)	30.9 (26.1 - 36.0)	1.1 (0.99 - 1.3)	1.9 (0.1)	42.3 (37.2 - 41.9)	1.1 (0.97 - 1.2)	1.8 (0.1)	41.7 (35.9 - 48.2)	1.3 (1.1 - 1.4)

Table 5.4: Variation in allele frequencies of vgsc *kdr* mutations (1014F and 1575Y) in *Anopheles gambiae* from study villages between 2016-2019

Study arms	Species	2016			2017			2018			2019		
		N tested	F(1014F)	F(1575Y)	N tested	F(1014F)	F(1575Y)	N tested	F(1014F)	F(1575Y)	N tested	F(1014F)	F(1575Y)
LLIN	*An. gambiae* (S)	178	0.97	0.12	178	0.96	0.09	184	0.95	0.11	192	0.98	0.08
	An. coluzzii (M)	3	0.5	0.00	133	0.68	0.10	108	0.61	0.11	78	0.76	0.08
	An. gambiae s.l. + M/S	181	0.96	0.12	314	0.84	0.10	293	0.82	0.11	271	0.91	0.08
SET+LLIN	*An. gambiae* (S)	167	0.97	0.11	126	0.99	0.08	177	1	0.08	181	1	0.06
	An. coluzzii (M)	19	0.87	0.08	188	0.67	0.07	95	0.68	0.09	90	0.6	0.09
	An. gambiae s.l. + M/S	186	0.96	0.10	315	0.8	0.08	272	0.88	0.08	274	0.87	0.07

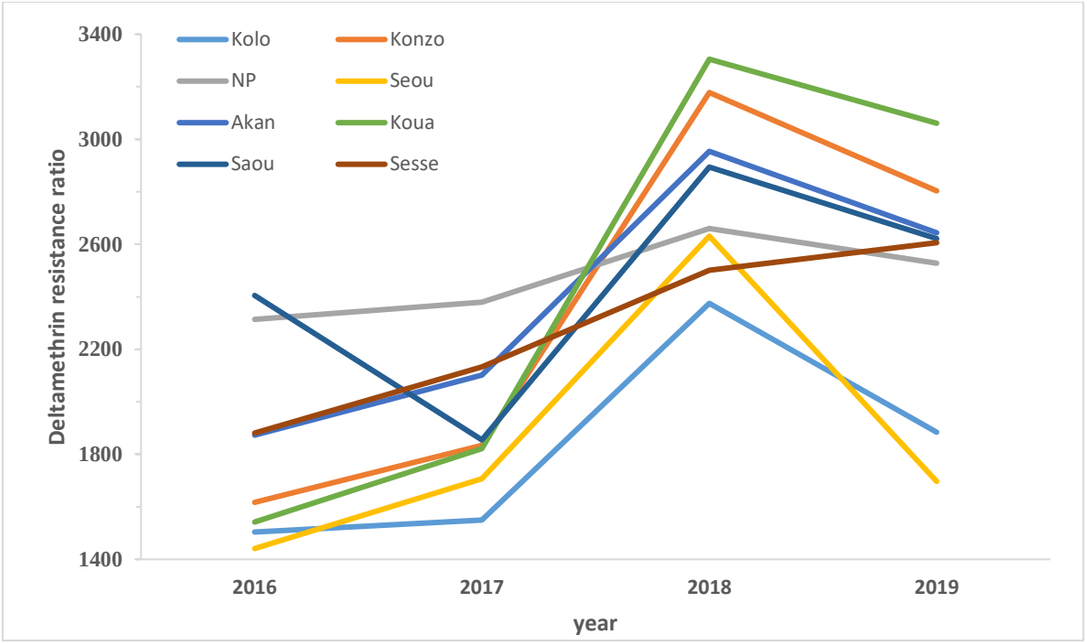

Fig. 5.3: Temporal variation of deltamethrin resistance ratio (RR50) in study villages between 2016-2019

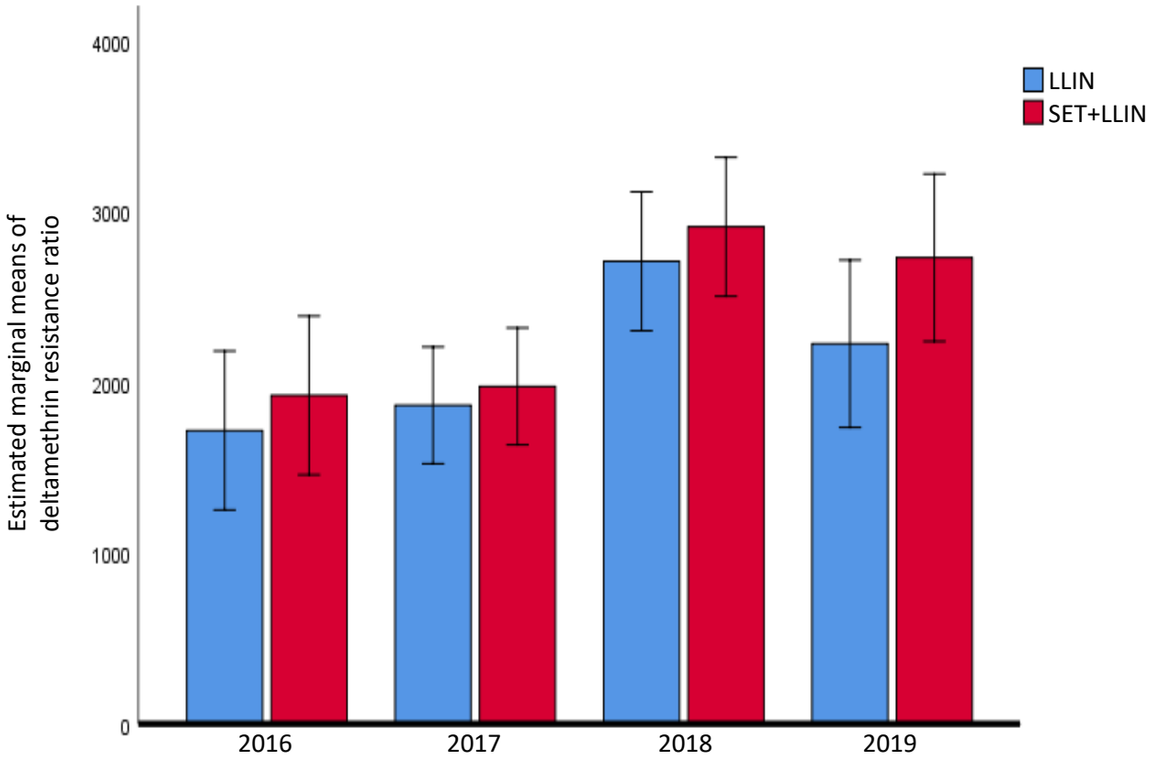

Fig. 5.4: Temporation variation of estimated marginal means of deltamethrin resistance ratio in intervention arms. Error bars indicate 95% confidence intervals (Cis)

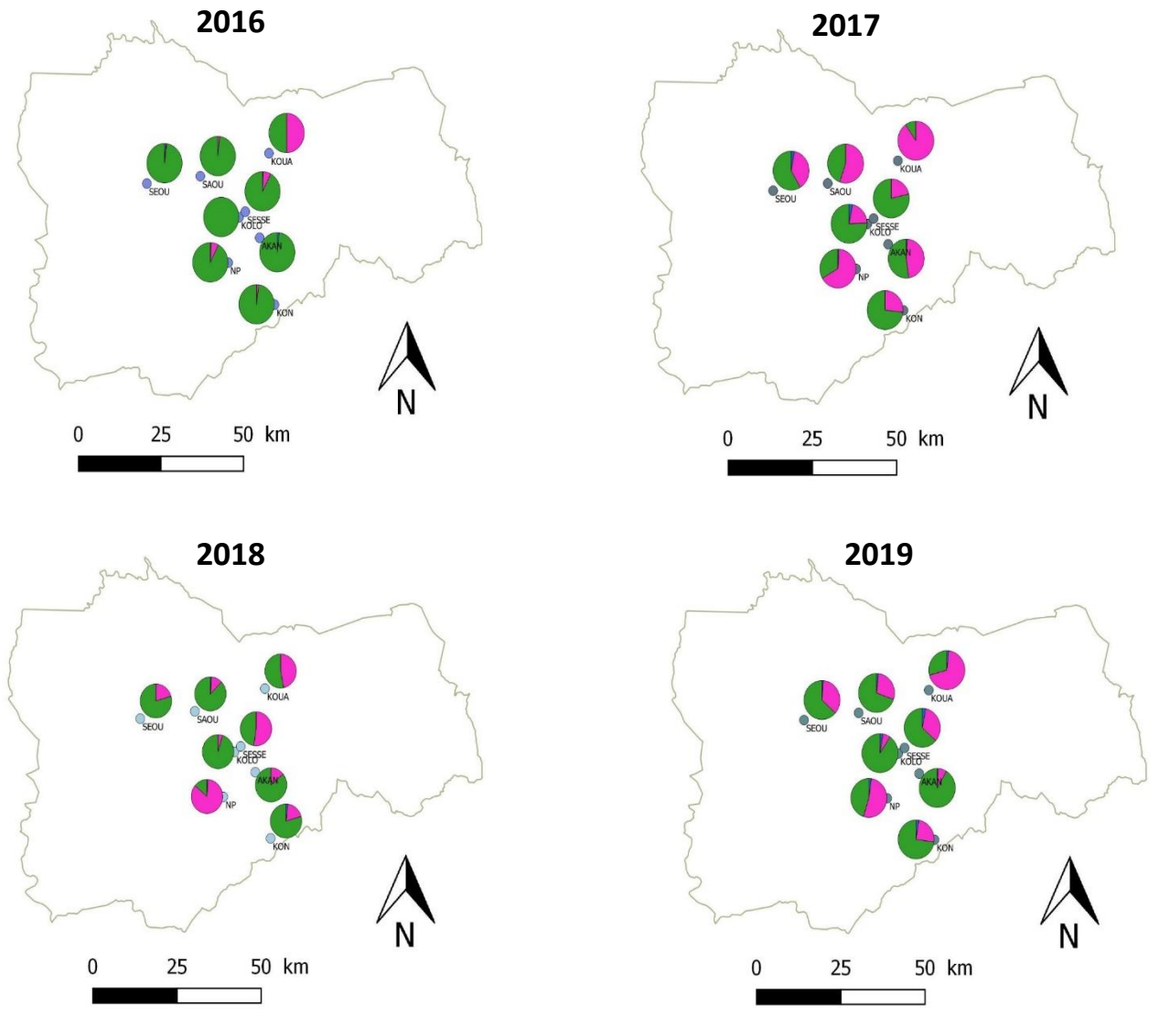

Fig. 5.5 Variation in species composition in chosen study villages between 2016-2019. The **green area in the pie charts represent the proportion of *An. gambiae*, the pink area represents the proportion of *An. coluzzii* and the blue area represent the proportion of hybrid (*An. gambiae*/*An. coluzzii*)**

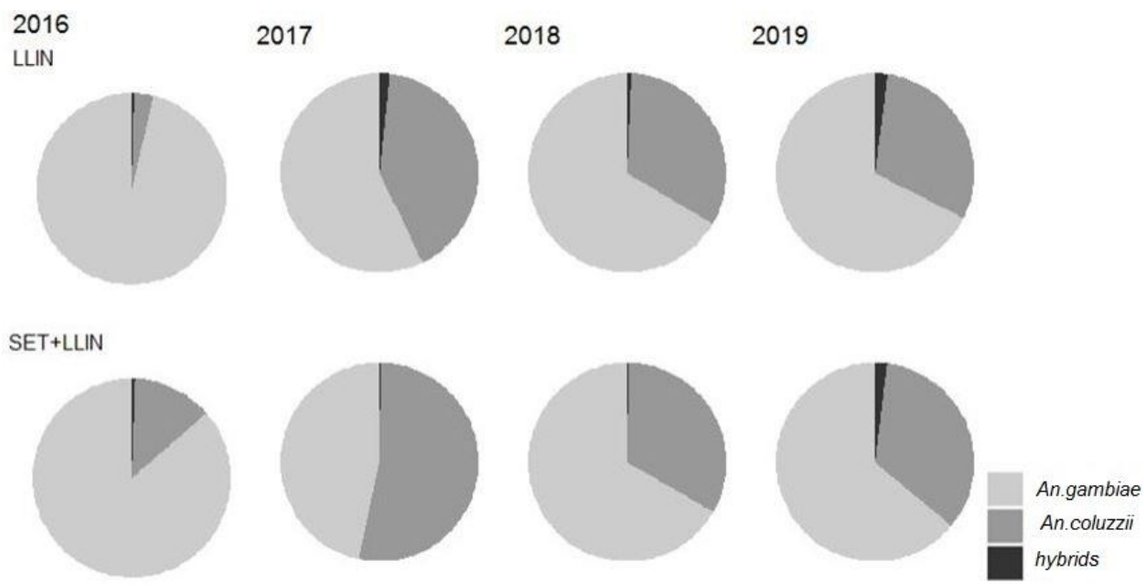

Fig. 5.6 Variation in species composition in study arms between 2016-2019

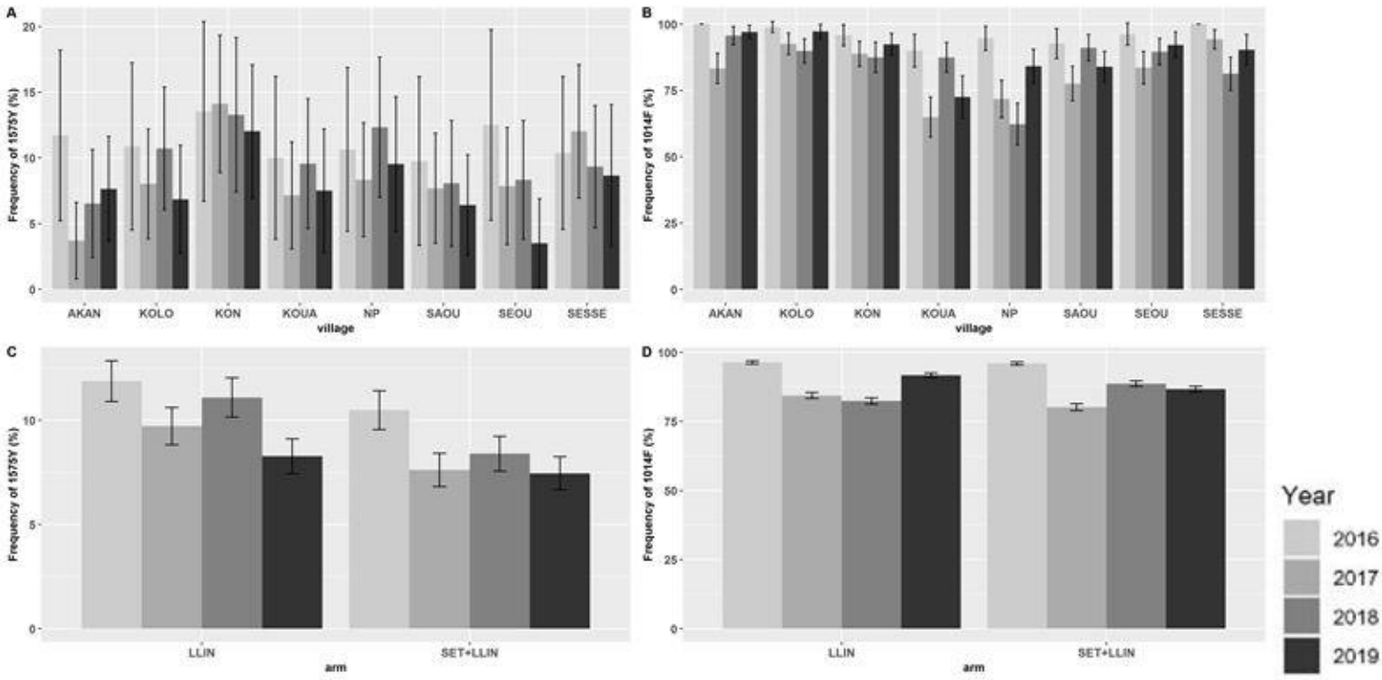

Fig. 5.7 Variation in allele frequencies of vgsc *kdr* (1014F and 1575Y) mutations in *Anopheles gambiae* from study villages (A-B) and intervention arms (C-D) between 2016-2019. Between 40-85 *Anopheles gambiae* mosquitoes were screened for 1014F and 1575 *kdr* mutation in each sampling year.

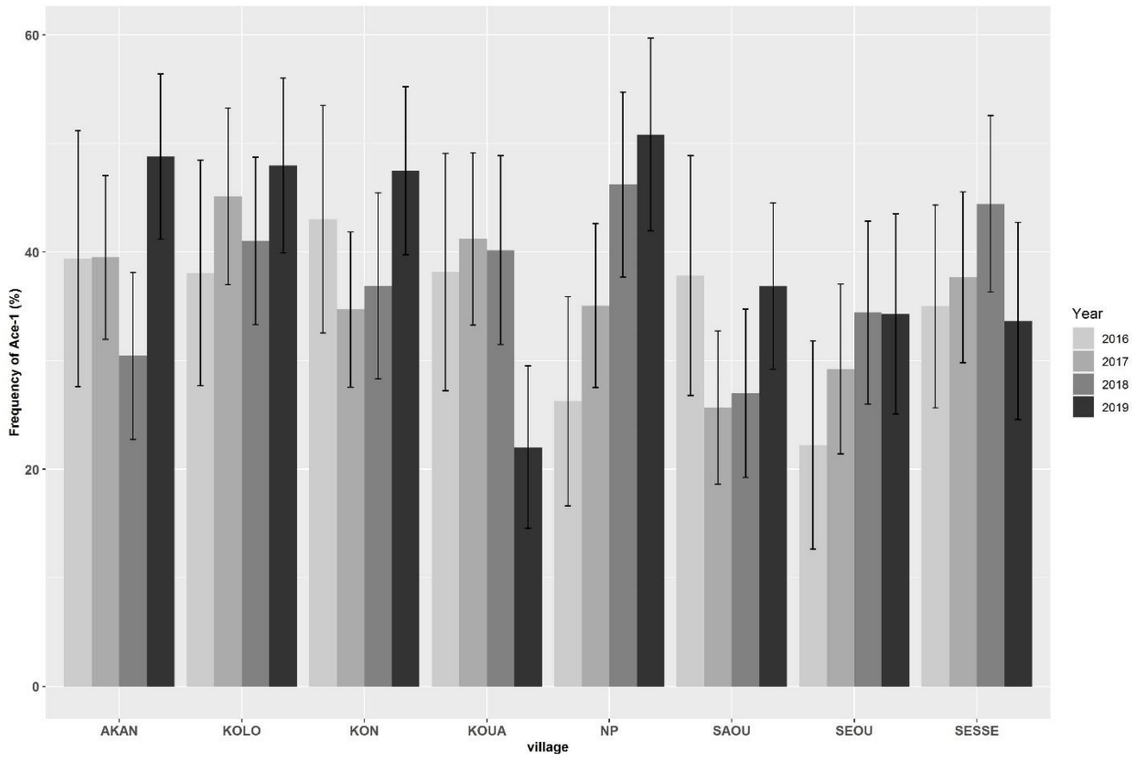

Fig. 5.8 Variation in allele frequencies of *Ace-1R* in *Anopheles gambiae* from study villages between 2016-2019.

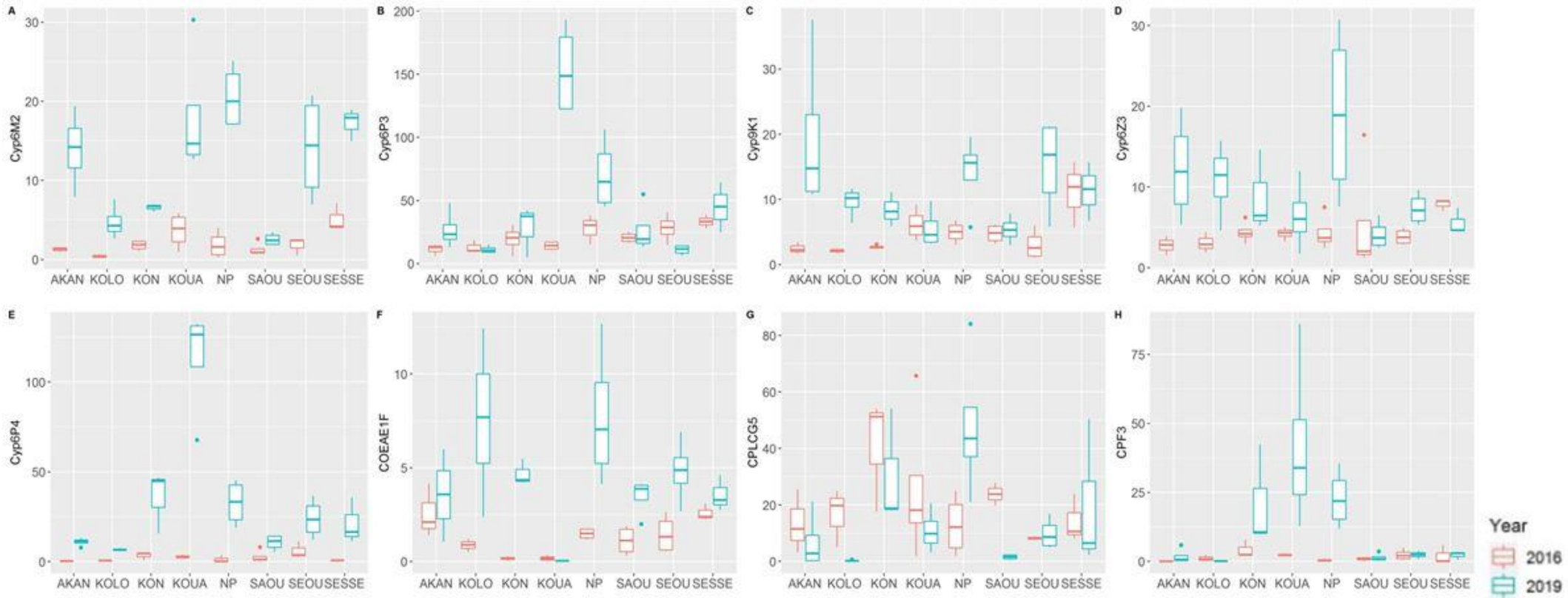

Fig. 5.9 Box-whisker plots show temporal change in mean fold difference in expression of candidate genes (relative to susceptible colony samples) across villages. The boxes represent the 25% and 75% quartiles and the whiskers indicate 5% - 95% quartile ranges. The horizontal line within each box represents the mean fold difference in gene expression, and the dots denote outliers

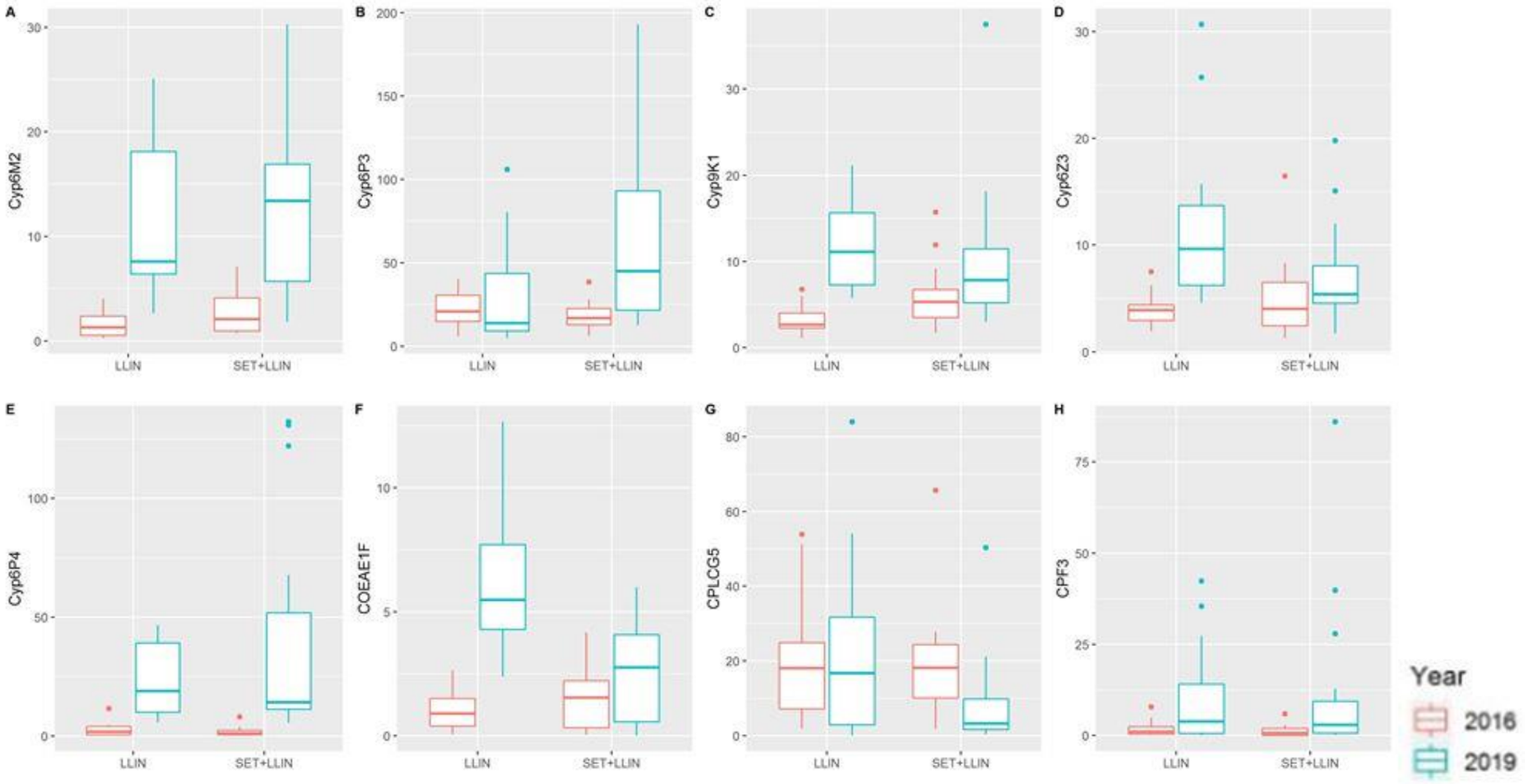

Fig. 5.10 Box-whisker plots show temporal change in mean fold difference in expression of candidate genes (relative to susceptible colony samples) across study arms. The boxes represent the 25% and 75% quartiles and the whiskers indicate 5% - 95% quartile ranges. The horizontal line within each box represents the mean fold difference in gene expression, and the dots denote outliers

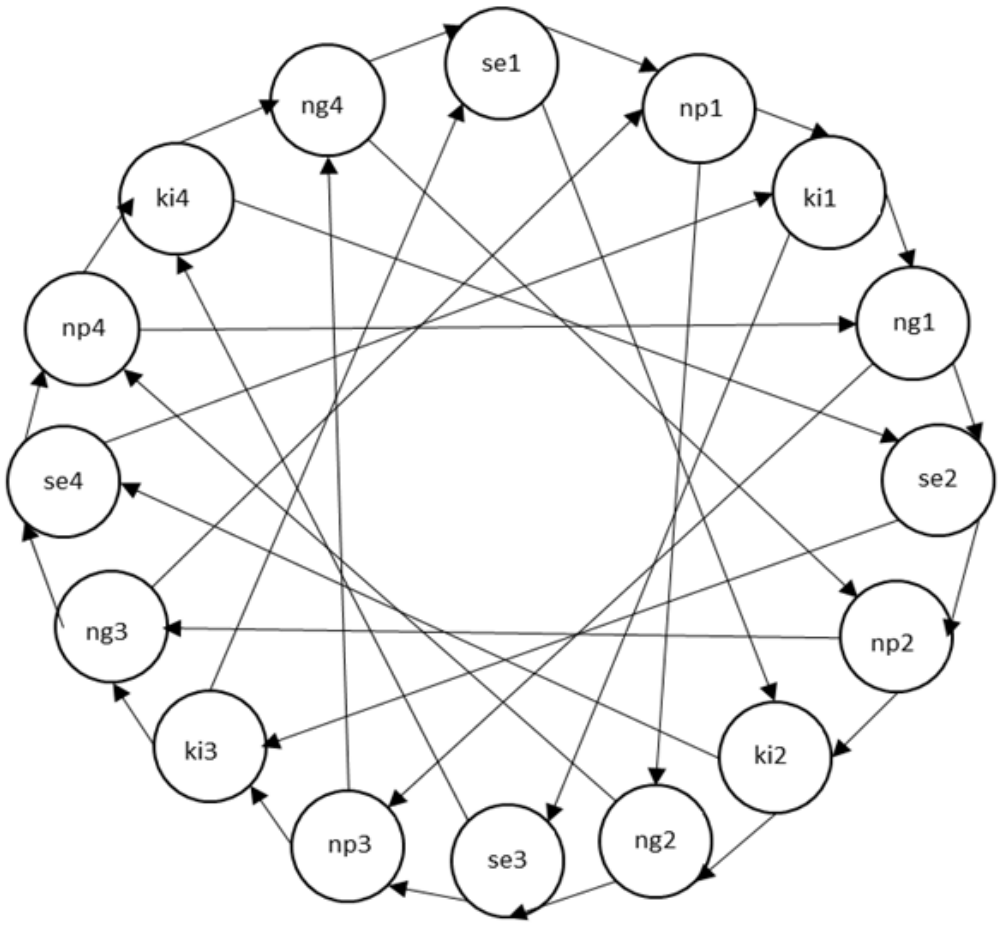

Fig. 5.S1: Interwoven microarray loop design comparing field mosquito samples from two CRT villages (one control cluster: np=N'Guessan Pokoukro and one intervention cluster: se=Sessenouan) and two lab colonies (kis= *An. gambiae* Kisumu and ng= *An. gambiae* N'goussou). Each circle represents mRNA extracted from a pool of 10 female *An. gambiae* s.s. Individuals microarrays are represented by arrows (32 in total). The direction of the arrows indicates dye labelling.

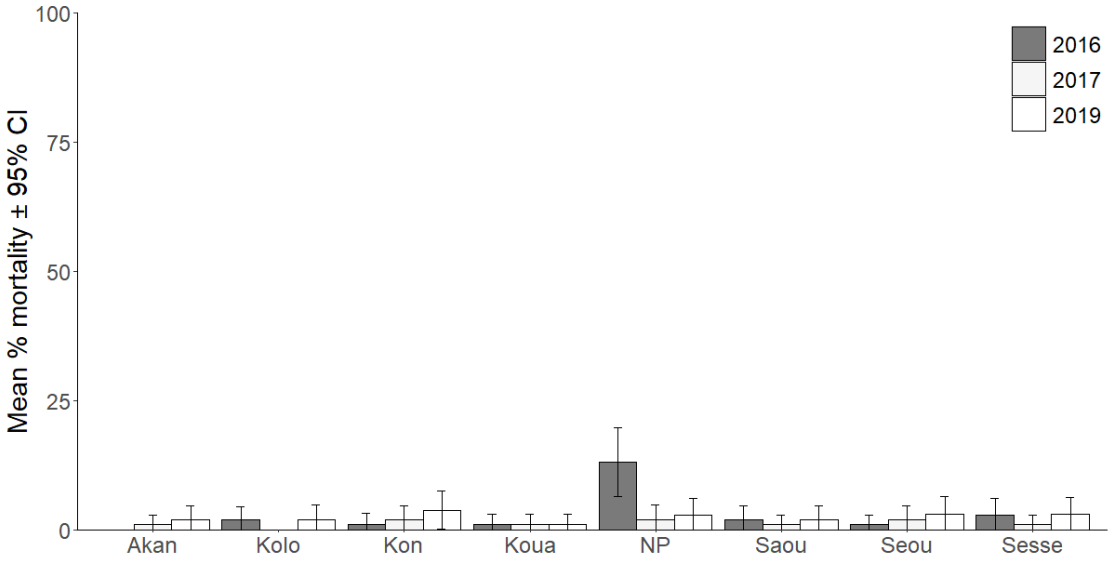

Fig. 5.S2 Mean percentage mortalities of *Anopheles gambiae* s.l. mosquitoes from chosen CRT villages following exposure to diagnostic concentration of DDT in WHO cylinder assays between 2016-2019

PART FOUR: Explore alternatives to powders, including next generation LN material and sprays

Chapter 6: Evaluation of an alpha-cypermethrin + PBO mixture long-lasting insecticidal net VEERALIN® LN against pyrethroid resistant *Anopheles gambiae*: an experimental hut trial in M'bé, central Côte d'Ivoire

Chapter 7: Exploring alternative insecticide delivery options in a "Lethal House Lure" for malaria control

Chapter 6

Evaluation of an alpha-cypermethrin + PBO mixture long-lasting insecticidal net VEERALIN® LN against pyrethroid resistant *Anopheles gambiae*: an experimental hut trial in M'bé, central Côte d'Ivoire

The work in this chapter has been published as:

Welbeck A. Oumbouke, Mark Rowland, Alphonsine A. Koffi, Ludovic P. Ahoua Alou, Camara Soromane and Raphael N'Guessan. Evaluation of an alpha-cypermethrin + PBO mixture long-lasting insecticidal net VEERALIN® LN against pyrethroid resistant *Anopheles gambiae*: an experimental hut trial in M'bé, central Côte d'Ivoire. **Parasites Vectors** 12, 544 (2019). https://doi.org/10.1186/s13071-019-3796-x

Evaluation of an alpha-cypermethrin + PBO mixture long-lasting insecticidal net VEERALIN® LN against pyrethroid resistant *Anopheles gambiae*: an experimental hut trial in M'bé, central Côte d'Ivoire

Abstract

Background: Long-lasting insecticidal nets (LLINs) are the primary method of malaria prevention. However, the widespread resistance to pyrethroids among major malaria vector species represents a significant threat to the continued efficacy of pyrethroid LLIN. Piperonyl butoxide (PBO) is a synergist that inhibits the activity of metabolic enzymes of the cytochrome P450 family known to detoxify insecticides including pyrethroids. Synergist LLIN incorporating PBO and a pyrethroid may provide improved control compared to pyrethroid-only LLIN.

Methods: The efficacy of VEERALIN® LN (VKA polymers Pvt Ltd, India), an alpha-cypermethrin PBO synergist net was evaluated in experimental huts in M'bé, central Côte d'Ivoire against wild pyrethroid resistant *Anopheles gambiae*. Comparison was made with a standard alpha-cypermethrin-treated net (MAGNet® LN, VKA polymers Pvt Ltd, India). Nets were tested unwashed and after 20 standardized washes.

Results: VEERALIN® LN demonstrated improved efficacy compared to MAGNet® LN against wild free-flying pyrethroid-resistant *An. gambiae*. Before washing, VEERALIN®LN produced mortality of *An. gambiae* (51%) significantly higher than the standard pyrethroid-only net (29%) ($P < 0.0001$). Although there was a significant reduction in mortality with both LLINs after 20 washes, VEERALIN® LN remained superior in efficacy to MAGNet® LN (38 *vs* 17%) ($P < 0.0001$). Blood-feeding was significantly inhibited with both types of insecticide-treated nets relative to the untreated control net ($P < 0.0001$). Unwashed VEERALIN® LN induced significantly higher blood-feeding inhibition of *An. gambiae* (62.6%) compared to MAGNet® LN (35.4%) ($P < 0.001$). The difference persisted after washing, as there was no indication that either LLIN lost protection against biting or blood-feeding. The level of personal protection derived from the use of VEERALIN® LN was high (87%) compared to MAGNet® LN (66–69%) whether unwashed or washed. The AI content of VEERALIN® LN after 20 washes decreased from 6.75 to 6.03 g/kg for alpha-cypermethrin and from 2.95 to 2.64 g/kg for PBO, corresponding to an overall retention of 89% for each compound.

Conclusions: The addition of the synergist PBO to a pyrethroid net greatly improved protection and control of pyrethroid-resistant *An. gambiae*. The pyrethroid-PBO VEERALIN® LN has the potential to reduce transmission in areas compromised by pyrethroid resistance.

Keywords: *Anopheles gambiae*, Experimental hut, Insecticide resistance, PBO, Long-lasting insecticidal net.

Background

Long-lasting insecticidal nets (LLINs) are considered best practice for malaria prevention in the majority of African countries. The estimated proportion of people sleeping under nets in sub-Saharan Africa rose to 53% in 2015 from a low of less than 2% in 2000. This increase in net use has resulted in about half a billion clinical malaria cases averted over the same time period [1]. This substantial reduction in malaria cases justifies ongoing efforts by National Malaria Control Programmes (NMCPs) to increase ownership and use of LLIN.

Despite the significant headway made, malaria remains a major public health problem in many countries. Recent estimates from the WHO World Malaria Report indicate that progress has stalled between 2015 and 2017, with some countries even reporting an increase in the number of cases [2]. One potential factor contributing to this is the rise in resistance to pyrethroid insecticides in malaria vectors across Africa. Although some malaria and health facility surveys in Benin have not provided evidence that resistance is adversely affecting malaria transmission or burden [3, 4], household and hut trials [5, 6] indicate that pyrethroid resistance can significantly reduce the efficacy of standard LLIN for vector control and personal protection. While findings from these malaria and health facility surveys in Benin suggested no association between pyrethroid resistance and malaria transmission, these are observational studies and therefore provide no conclusive evidence on the impact of resistance. Moreover, malaria prevalence remains high in many areas of Benin despite the widespread use of LLIN. This emphasises the need for additional control measures to improve control and reduce malaria transmission.

Although LLIN may provide some protection against insecticide-resistant *Anopheles* mosquitoes, this may depend on the frequency and strength of the resistance [7–9]. To meet the resistance challenge and restore malaria vector control, new active ingredients are being developed and tested. A new class of net combines two compounds: the pyrethroid and the synergist piperonyl butoxide (PBO) for improved control of pyrethroid-resistant *Anopheles* mosquitoes. PBO is an insecticide synergist which inhibits the action of resistance-associated metabolic enzymes of the cytochrome P450 family [10]. The inhibition of P450 enzymes by the PBO results in the pyrethroid on the net being available to induce excito-repellency and mortality. The role of these enzymes in the detoxification of insecticides including pyrethroids and to cause resistance is well documented [11–14]. The addition of PBO to pyrethroid net as a strategy to overcome resistance especially in areas where this is driven by overexpression of P450 enzymes known to metabolise pyrethroids has been demonstrated in a range of experimental hut trials across Africa [15–19]. Simulation modelling suggests that a switch in net policy toward pyrethroid-PBO net would result in up to 0.5

clinical malaria cases averted per 1000 people per year [20]. Pyrethroid-PBO net was given World Health Organization (WHO) policy recommendation as a new class in 2017 based on epidemiological data from a cluster randomized trial in Muleba, Tanzania [21], which showed that Olyset® Plus LN (Sumitomo Chemicals Co. Ltd, Tokyo, Japan) reduced malaria-infection prevalence by 33% over 21 months of use compared to the standard LLIN, Olyset® Net, under a scenario of high pyrethroid resistance and net use. A recent Cochrane review predicted that PBO-pyrethroid LLIN is expected to be more effective in areas of moderate to high resistance mediated by metabolic resistance than in settings of low or no insecticide resistance [22].

The recommendation of new product class applies to all pyrethroid-PBO nets prequalified by the WHO [23]. All of these products differ from Olyset® Plus in terms of their design/specifications, which in turn is likely to affect their field performance. Key differences between these products include the spatial location of the PBO (all net panels or just the top panel), PBO loading dose, type and concentration of pyrethroid and wash-fastness and bioavailability of PBO or partner pyrethroid. VEERALIN® LN (VKA polymers Ltd, Tamil Nadu, India) is a new PBO-alphacypermethrin synergist LLIN that contains PBO on all net panels and recently acquired WHO interim recommendation. The Vector Control Product Evaluation Centre (VCPEC) based within Institut Pierre Richet (IPR) in Bouaké, central Côte d'Ivoire was therefore commissioned by the WHO to undertake a phase-2 experimental hut study of VEERALIN® LN in an area of high pyrethroid resistance mostly mediated by cytochrome P450 metabolic mechanisms.

Methods

Study area and experimental huts

The hut trial was conducted at the M'bé field station in central Côte d'Ivoire, 40 km south of Bouaké city. The site is a large rice irrigated valley producing mostly *An. coluzzii* year-round. The mosquito population from the site has developed resistance to multiple insecticide classes. Resistance mechanisms include target site insensitivity (1014F and *Ace-1*) [24] and increased activities of insecticide-metabolizing enzymes (esterases, oxidases and GSTs) [25] including highly overexpressed CYP6P3 (Oumbouke & N'Guessan, in preparation). A recent investigation into the level of resistance to pyrethroids in *Anopheles*-mosquitoes from the study area reported over 1700-fold resistance to deltamethrin [26].

The West African style experimental huts were used for the field trial. [27]. They were made of concrete bricks, with roofs of corrugated iron, ceilings lined with plastic sheeting and the interior walls plastered with cement. Each hut was built on a concrete base surrounded by a water-filled

moat to prevent entry of mosquito predators. Mosquitoes enter the hut through four 1-cm wide window slits, located on three sides of the hut. Mosquitoes exiting the hut are caught in a veranda trap located on the fourth side.

WHO susceptibility assays

To determine the prevalence of resistance to pyrethroids, WHO cylinder assays were conducted using papers treated with diagnostic concentration of 0.05% alpha-cypermethrin, the same pyrethroid used in MAGNet® and VEERALIN® LLINs. WHO susceptibility tests were performed using 2–3 day-old adult female mosquitoes, collected as larvae from the M'bé field station. Four replicates of 25 female mosquitoes were tested in cylinder assays and mortality was scored 24 h after exposure. Exposure of the susceptible *An. gambiae* strain to alpha-cypermethrin treated paper in cylinder tube was conducted to check the quality of the insecticide-treated paper. Mosquitoes exposed to untreated paper served as control.

LLINs and washing procedure

MAGNet® LN is a long-lasting net containing 5.8 g/kg alpha-cypermethrin incorporated in monofilament, high-density polyethylene (HDPE), 150-denier manufactured by VKA polymers. MAGNet® LN received full WHOPES recommendation in 2011 [28].

VEERALIN® LN is a long-lasting net manufactured by VKA polymers Pvt Ltd, India. Alpha-cypermethrin is incorporated into 130-denier monofilament polyethylene fibres with a target dose of 6.0 g AI/kg (216 mg AI/m^2) alpha-cypermethrin and 2.2 g/kg (79.2 mg/m^2) PBO.

The nets were washed following the WHOPES-phase II washing protocol [29]. The time for regeneration of the active ingredients between washes was 1 day for MAGNet® LN and 5 days for VEERALIN® LN and therefore washing was done every 5 days using 2 g/litre soap solution ('savon de Marseille'). One complete washing cycle of each net ran for 10 min as follows: each net was first agitated for 3 min then left to soak for 4 min and again agitated for 3 min. Net agitation was performed by stirring each net with a wooden pole at 20× *rpm*. After washing, nets were rinsed twice in clean water (10 l per rinsing, i.e. 20 l per net). Nets were dried horizontally in the shade, then stored at ambient temperature (27 ± 2 °C) between washes.

Net treatments and experimental hut trial procedure

The following treatment arms were trialed in experimental huts: (i) VEERALIN® LN unwashed; (ii) VEERALIN® LN washed 20 times; (iii) MAGNet® LN unwashed; (iv) MAGNet® LN washed 20 times; and (v) untreated polyester net (100 denier).

These five treatment arms were randomly allocated to 5 experimental huts. To account for potential bias due to differential hut attractiveness, nets were rotated among huts every week according to a balanced Latin square scheme. Three nets were used per treatment arm and each net was tested within hut on 2 consecutive nights during the week. Before the hut trial, holes (16-cm^2 in diameter) were made in the nets (2 on each side and 1 on each end) to simulate moderately damaged net during field use. The huts were thoroughly cleaned and aired for a day at the end of each rotation.

The hut trial spanned 5 weeks (from June to July 2014) corresponding to 30 nights of collection per hut. Five local human volunteers gave informed consent and slept in the huts from 20:00 h to 05:00 h each night. To reduce bias resulting from the inherent difference in individual attractiveness to host-seeking mosquitoes, sleepers were rotated between huts on successive nights. Each morning, mosquitoes were collected from huts using mouth-operated aspirators from inside the room, nets and veranda traps and physiological status (live, dead, unfed, blood-fed, semi-gravid, gravid) recorded. Mosquitoes were transported to the laboratory at the Institut Pierre Richet (IPR), Bouaké, Côte d'Ivoire, and identified to the species level. Live female mosquitoes were provided with 10% honey solution and mortality recorded 24 h later.

Outcome measures

The following outcomes were used to assess the efficacy of the treatments as per WHO guidelines [30]: (i) deterrence: the percent reduction in the number of mosquitoes in treatment hut relative to control hut with untreated net; (ii) exit rate; (iii) blood-feeding inhibition rate: the percentage reduction in blood-feeding in a hut with treated net compared to a hut with untreated net; (iv) percentage mortality of adult females; (v) overall insecticidal effect = 100 (Kt–Ku)/Tu, where Kt is the number of mosquitoes killed in the treated hut, Ku is the number dying in the untreated control hut and Tu is the total number collected from the control hut [5]; (vi) personal protection; percentage reduction in mosquito biting in hut with treated net compared to hut with untreated net = [1 – (No. of mosquitoes blood-fed in treatment/No. of mosquitoes blood-fed in control) × 100].

Chemical analysis

Determination of alpha-cypermethrin content in unwashed and washed MAGNet® and VEERALIN® LLINs was performed before and after washing and post-trial in accordance with WHO guidelines [29]. PBO content was also assessed in VEERALIN® LN. A piece of netting measuring 30 × 30 cm was cut from each of the five locations of each net. Extraction of alpha-cypermethrin and PBO was performed using the CIPAC method [31]. These compounds were extracted by refluxing with xylene for 30 min in the presence of dioctyl-phthalate as an internal standard and citric acid. Concentrations of alpha-cypermethrin and PBO were subsequently measured by Gas Chromatography with Flame Ionization Detection (GC-FID).

Cone bioassays

The efficacy of VEERALIN® and MAGNet® LLINs was assessed by WHO cone bioassay using susceptible *An. gambiae* before and after washing and after field trial. One hundred 2–5 day-old female mosquitoes were subjected to 3 min exposure in replicates of 5 mosquitoes per cone at 25 ± 2 °C and a relative humidity of 75 ± 10% [30]. Mortality was scored 24 h after exposure.

Statistical analysis

Data were entered into an Excel database and subsequently imported into the R statistical software version 2.15.0. for analysis. Proportional outcomes from the bioassays (mortality) and the hut trial (exophily, blood-feeding and mortality) were analysed using generalised linear mixed models (GLMMs) with a binomial distribution and a logit link function was fitted to the data using the *lme4* package [32]. Net type and hut were included as fixed effects, and sleepers and day of mosquito collection were treated as random effects. Numeric outcomes (number entering each hut, feeding and dying) were analysed using generalised linear models with a Poisson distribution. Pairwise comparisons were performed using the *multcomp* package in R [33].

Results

WHO susceptibility assays

Mortality of the susceptible *An. gambiae* exposed to 0.05% alpha-cypermethrin in WHO susceptibility tests was 100%. Mortality of *An. gambiae* from M'bé exposed to the diagnostic dose of alpha-cypermethrin was 68% (n = 108), indicating frequency of resistance to pyrethroids of 32% at the study site.

Experimental hut trial

In the 5-week trial, 1054 *An. gambiae*-mosquitoes were collected from the control hut, representing a mean number of 29 females per night. Both MAGNet® and VEERALIN® LLINs reduced hut entry of *An. gambiae*; unwashed MAGNet® LN reduced entry by 52% and unwashed VEERALIN® LN by 65%. There was little reduction of deterrency after washing the two LLINs 20 times (Table 6.1).

Relative to the untreated control, the proportions of mosquitoes exiting into the verandas was significantly greater with each type of insecticide treated net by 47–65% (GLMMs, $P < 0.0001$) (Table 6.1, Table 6.S1 & Table 6.S2). Before washing VEERALIN® and MAGNet® LLINs induced similar level of exiting (55%) but after washing exiting was significantly greater for VEERALIN® LN (64.7%) than for MAGNet® LN (46.8%) (GLMMs, $P < 0.0001$).

Blood-feeding was significantly inhibited by insecticide-treated net treatment compared to the untreated control net (GLMMs, $P < 0.0001$). Unwashed VEERALIN® LN induced significantly greater blood-feeding inhibition (62.7%) than MAGNet® LN (35.5%) (GLMMs, $P < 0.0001$) (Table 6.1, Fig. 6.1). The difference persisted after washing, being no loss of protection with either LN.

All insecticide-treated nets induced greater mortality than the untreated net (GLMMs, $P < 0.0001$) (Fig. 6.2) (Table 6.S1 & Table 6.S2). The unwashed VEERALIN® LN produced mortality of 51%, although this was significantly greater than that induced by MAGNet® LN unwashed (29%) (GLMMs, $P < 0.0001$). After washing, mortality with the PBO-LLIN and pyrethroid-only LLIN decreased significantly to 38.2% for VEERALIN® LN and to 17.3% for MAGNet® LN (GLMMs, $P < 0.0001$); the decrease relative to the unwashed net was 24.8% for VEERALIN® LN and 40% for MAGNet® LN.

The level of personal protection derived from the use of VEERALIN® and MAGNet® LLINs (unwashed and washed) against *An. gambiae*-biting ranged between 86.6–87.1% with VEERALIN® LN and 66.2–69% for MAGNet® LN before and after washing. The Overall Killing Effect was low (< 16%) across all treatments (Table 6.1). Before washing, VEERALIN® LN induced significantly greater overall killing effect (15.5%) compared to MAGNet® LN (11.8%), but the difference was not significant (GLM, $P = 0.41$). Although there was a reduction in killing effect with VEERALIN® (11.5%) and MAGNet® (6.4%) LLINs after washing, the decrease in effect was only significant with MAGNet® LN (GLM, $P = 0.014$).

Cone bioassays

Mortality rates of the susceptible *An. gambiae* were 100% with all treated nets assayed in WHO cone at the three time points (before, after washing and after field trial).

Chemical analysis

The mean alpha-cypermethrin content in MAGNet® and VEERALIN® LLINs and the concentration of the synergist PBO in VEERALIN® LN are presented in Tables 6.2 and 3. The initial concentrations of alpha-cypermethrin in VEERALIN® LN (6.91 and 6.75 g/kg) and MAGNet® LN (6.39 and 5.95 g/kg) were close to the target dose of 6 g/kg ± 25% for VEERALIN® LN and 5.8 g/kg ± 25% for MAGNet® LN, with a within-net variation of less than 10%. After washing, the alpha-cypermethrin content was 6.03 g AI/kg for VEERALIN® LN and 5.65 g AI/kg for MAGNet® LN corresponding to an overall wash retention rate of 89% for VEERALIN® LN and 95% for MAGNet® LN. After the 5-week hut trial, there was marginal decline in alpha-cypermethrin content (< 15%) with either LLIN washed or unwashed. The initial concentration of PBO in the unwashed VEERALIN® LN (2.63 g/kg) was within the acceptable range of the target dose of 2.2 g/kg ± 25% but was slightly overdosed in the VEERALIN® LN that was destined to be washed 20 times (2.95 g/kg) (Table 6.3). After 20 washes, there was a decrease in PBO content from 2.95 to 2.64 g AI/kg, corresponding to an overall wash retention of 89%. After hut trial, there was a small decrease in PBO content (< 20%, Table 6.3).

The decrease in insecticide content after washing of VEERALIN® and MAGNet® LLINs was associated with a significant decrease in hut mortality; however, personal protection was maintained and blood-feeding rates did not differ between unwashed and 20 times washed LLINs (Tables 6.2, 3, Figs. 6.1& 2).

Discussion

Malaria control and pyrethroid-only nets are under threat from the increasing prevalence and intensity of pyrethroid resistance among malaria vectors [34]. To preserve insecticide mosquito net technology, the most widely used form of vector-control method, and continue progress toward elimination, a class of mosquito net incorporating the synergist piperonyl butoxide (PBO) has been developed to neutralise some forms of metabolic resistance to pyrethroids. On the basis of a cluster randomised trial of Olyset® Plus LN, which demonstrated epidemiological evidence of the greater effectiveness of pyrethroid-PBO nets in areas of resistance, the WHO has conditionally endorsed

pyrethroid-PBO nets as a new product class for malaria control in areas where resistance is conferred by monooxygenase-based resistance mechanisms. Apart from Olyset® Plus LN, there are several brands of PBO LLINs, which are being developed for approval by the WHO prequalification team. The purpose of the present study was to evaluate in experimental huts the efficacy of the pyrethroid-PBO net, VEERALIN® LN *versus* the pyrethroid-only net, MAGNet® LN, against pyrethroid-resistant populations of *An. gambiae* mosquitoes at the M'bé field station in Côte d'Ivoire.

In experimental huts, MAGNet® LN, an alpha-cypermethrin treated net reduced mosquito survival and blood-feeding by approximately 30% for both outcomes. This low effect size achieved by MAGNet® LN against pyrethroid-resistant *An. gambiae* s.l. mosquitoes is consistent with findings from previous experimental hut trials with pyrethroid-only LLINs performed at the same site [9, 26] and elsewhere [7, 8, 35]. This provides further evidence of the poor performance of pyrethroid LLIN in areas where malaria-vectors have developed multiple mechanisms of pyrethroid resistance [7, 36].

The addition of PBO to alpha-cypermethrin in the net was associated with a significant improvement in control and protection against mosquito bites. VEERALIN® LN killed significantly higher proportions (38–51%) of the highly resistant population of *An. gambiae* compared to MAGNet® LN (17–29%). In previous hut trials comparing pyrethroid-PBO net with pyrethroid-only nets, e.g. Olyset® Plus *versus* Olyset® LLINs or PermaNet® 3.0 *versus* PermaNet® 2.0 LLINs, the difference in induced mortality between PBO and standard LLIN could not be attributable to PBO conclusively because the original concentration of pyrethroid or the bleed rate of pyrethroid in the pyrethroid-PBO net differed from that in the pyrethroid-only LLIN [17, 18, 37]. In the present study, the loading dose of alpha-cypermethrin in VEERALIN® and MAGNet® LLINs were similar (6 and 5.8 g/kg, respectively) as was the wash retention of alpha-cypermethrin over 20 washes (89 and 95%, respectively). Therefore, the substantial increase in mortality observed with VEERALIN® LN was most likely due to the PBO component, which is known to inhibit the activity of key pyrethroid-detoxifying enzymes [10]. However, it should be noted that full control of pyrethroid-resistant mosquitoes was not achieved with VEERALIN® LN in experimental huts. This could be due to the presence of resistance mechanisms unaffected by the synergist PBO. Another plausible explanation could be that the dose of PBO (target dose of 2.2 g/kg) deployed in VEERALIN® LN and the bleed rate of PBO to the net surface (wash retention index = 98.9% per wash) was insufficient to inhibit the range of P450 enzymes associated with resistance in the local *An. gambiae*. For example, in an area of Benin with increased oxidase

activity, Olyset® Plus LN containing 5 times higher the loading concentration of PBO (10 g/kg) and a much higher bleed rate (wash retention index of 96% per wash) produced significantly higher mortality of the local *An. gambiae*-mosquitoes (67–81%) [18] compared to the effect size with VEERALIN® LN in the present study. Of course, the resistance situation in Benin [38] would not be directly comparable with the resistance situation in Côte d'Ivoire [24] and care should be taken not to overinterpret or compare trial data taken from different locations or times. Nevertheless, there is a significant variation in the loading dose and wash retention index of PBO in the current brands of pyrethroid-PBO nets pre-qualified by WHO. There is an urgent need for comparative trials of the different brands of pyrethroid-PBO LLINs in the same location and time in order to rank their efficacy or equivalence. The doses applied to the different brands should be informed by calibration studies designed to determine the dose and the optimal bleed rate of PBO required to fully inhibit oxidase-based resistance mechanisms in the target vector species.

Apart from the greater killing effect observed with VEERALIN® LN, there was a significant reduction in human-vector contact resulting from the high blood-feeding inhibition (60%), deterrence (> 64%), exiting of mosquitoes (55–64%) and personal protection (87%). The blood-feeding inhibition and personal protection against mosquito bites is arguably a more important attribute of pyrethroid-PBO LLIN than mortality. While the level of protection induced by VEERALIN® LN did not decrease with washing, there was a significant decrease in mortality after 20 standardized washes. Nevertheless, VEERALIN® LN remained superior in terms of mortality to MAGNet® LN washed to some extent. The significant loss in mortality and maintenance of personal protection observed with VEERALIN® LN after washing stresses the need for evaluating the durability of PBO net under operational household conditions. Reduction in mosquito mortality occurring after washing is a shortcoming common to all existing pyrethroid PBO nets. Hut trials with PermaNet® 3.0 LN performed in pyrethroid-resistant areas in Benin [15] and Côte d'Ivoire [17] showed a significant decrease in efficacy after washing both in terms of mortality and blood-feeding inhibition with the PBO net performing no better than the pyrethroid-only LLIN. A typical example is the community trial of Olyset® Plus LN in Tanzania: the PBO content under rural condition of use decreased by 83% compared to a decrease by only 42% for permethrin after 21 months. Despite this decrease in PBO content over this period, a 33% reduction in malaria-infection prevalence in children protected with Olyset® Plus LN was still observed compared to those living in area covered with Olyset® LN. The superior performance of the PBO net Olyset® Plus LN was sustained over 21 months of use in the Tanzanian study and efficacy is still being monitored to determine whether this effect is maintained over the assumed net lifespan of three years [21].

Most hut trials evaluating the efficacy of PBO nets were conducted in areas where *An. gambiae* (*s.l.*) is the predominant malaria-vector species [15, 17–19]. Hut efficacy data of PBO nets against other major malaria vectors including *An. funestus* and *An. arabiensis* is mainly confined to East Africa. In a recent WHOPES-commissioned hut trial carried out in Ifakara, Tanzania, VEERALIN® LN produced low mortality of *An. arabiensis* and *An. funestus*, which was not significantly different to MAGNet® LN [39]. This contrasts with findings from the present study and the difference in performance of VEERALIN® LN in both countries could be attributed to the inherent differences in behaviour between mosquito vector species, in the strength/mechanisms of resistance or to differences in hut design used [27].

Although the present study demonstrated the potential of VEERALIN® LN to enhance control and reduce transmission in areas compromised by pyrethroid resistance, proof of impact on malaria metrics would ideally require large scale cluster randomized trials in a West African setting. VEERALIN® LN belongs to the same class of net as Olyset® Plus LN. According to the latest WHO recommendation on deployment of PBO nets, a candidate PBO net belonging to the same class of a net for which epidemiological data are available does not need to be subjected to another CRT [40]. Instead, the effectiveness of the candidate PBO net is to be assessed using appropriate and relevant entomological endpoints as recently set forth by WHO [41]. Following the demonstration by the CRT in Muleba, Tanzania, of the benefit of PBO net over standard pyrethroid net on malaria metrics, all currently available PBO nets, have been endorsed by WHO [40]. Deployment of PBO net by National Malaria Control Programmes is now advocated for by WHO in areas where resistance is mostly driven by monooxygenase-based mechanisms. A second CRT currently underway in Uganda is evaluating two types of pyrethroid-PBO net (PermaNet® 3.0 and Olyset® Plus LLINs) [42]. This trial may provide evidence on whether the difference in dose and location of PBO between these nets under evaluation make any difference to the size of the effect on transmission. Given the recommendation to endemic countries to deploy PBO-based LLIN, it will be necessary to demonstrate that each type of pyrethroid PBO nets is efficacious against metabolic resistant *Anopheles* mosquitoes. WHO now requires that all second-in-class products need to demonstrate equivalence to the first-in-class in experimental hut conditions [40, 41]. Studies based on non-inferiority in experimental hut trials that will generate evidence on the relative entomological efficacy of all five pyrethroid PBO nets are essential to generate that knowledge and ensure impact.

Conclusions

The pyrethroid-PBO VEERALIN® LN was more efficacious than standard pyrethroid-only MAGNet® LN in experimental huts both in terms of mosquito mortality and protection against mosquito bites and therefore meets WHO interim approval. The study provides evidence on the potential of PBO nets to enhance control of pyrethroid-resistant *An. gambiae* mosquitoes and reduce transmission in West Africa.

Acknowledgments

The authors would like to thank the volunteer sleepers for participating in the experimental hut trial. We are also grateful to the technical staff of VCPEC for their contribution to the study. We thank Olivier Pigeon (Gembloux, Belgium) for performing the chemical analysis.

Declarations

Ethics approval and consent to participate

The study was approved by the Ministry of Health in Côte d'Ivoire and the Ethical Committee of the London School of Hygiene and Tropical Medicine. Adult study volunteers who slept in the huts gave written informed consent prior to their participation in the hut trial. Trial participants were examined regularly during the course of the study for malaria symptoms by a freelance nurse. Sleepers testing positive for malaria were replaced and provided with artemisinin-based combination therapy antimalarial drug.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

Financial support for the study was obtained from the World Health Organization Pesticide Evaluation Scheme (WHOPES) (grant awarded to RN).

Authors' contributions

RN and MR conceived the study. RN, WO, AK and LA designed the hut trial. WO and LA analysed the data. LA and SC supervised the field trial. WO and RN wrote the manuscript. MR revised the manuscript. All authors read and approved the final manuscript.

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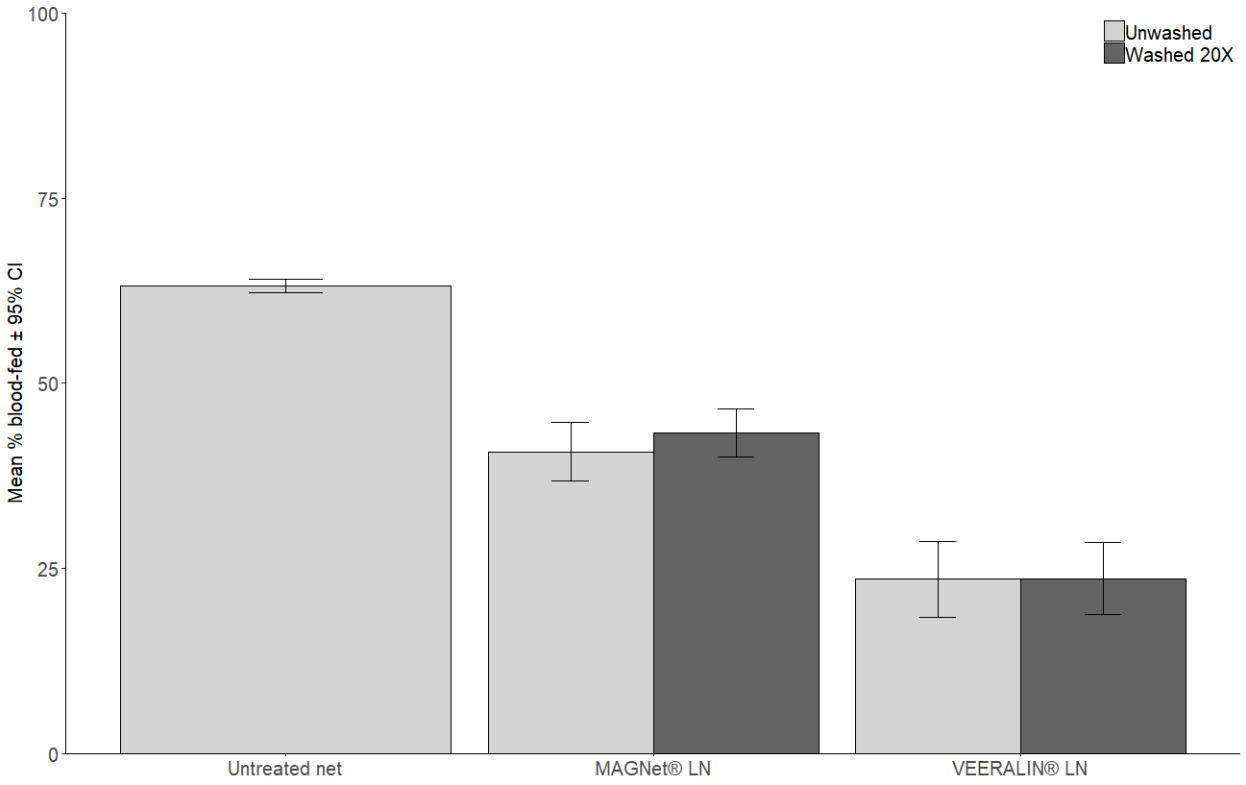

Fig. 6.1 Blood-feeding rates of wild pyrethroid resistant *An. gambiae* in experimental huts in M'bé, Côte d'Ivoire. Error bars represent 95% Cis

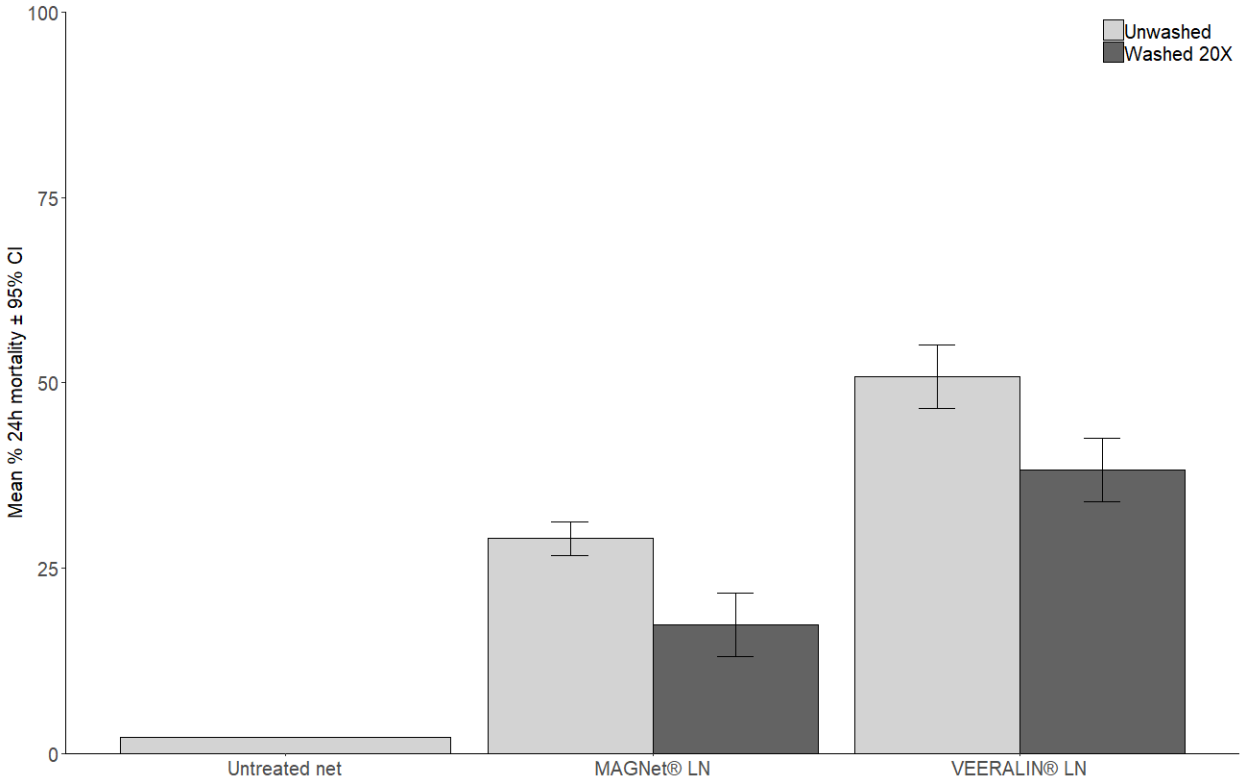

Fig. 6.2 Mortality rates of wild pyrethroid resistant *An. gambiae* in experimental huts in M'bé, Côte d'Ivoire. Error bars represent 95% CIs

Table 6.1 Experimental hut trial results of unwashed and 20-times washed pyrethroid-PBO and pyrethroid-only LLIN against pyrethroid resistant *Anopheles gambiae* in M'bé, Côte d'Ivoire

	Untreated net	MAGNet® LN 0w	MAGNet® LN 20w	VEERALIN® LN 0w	VEERALIN® LN 20w
Total no. of females caught	1054	506	519	366	377
Mean no. caught/night	29.2[a]	14.0[b]	14.4[b]	10.2[c]	10.5[c]
% Deterrence	–	52.0	50.7	65.3	64.2
Total no. of females in veranda	248	279	243	203	244
% Exiting (95% CI)	23.5 (21.0–26.1)[a]	55.1 (50.8–59.5)[b]	46.8 (42.5–51.1)[c]	55.5 (50.4–60.6)[b]	64.7 (59.9–69.5)[d]
Total no. of blood-fed females	665	206	225	86	89
% Blood-feeding inhibition	–	35.5 (31.3–39.7)	31.4 (27.4–35.4)	62.7 (57.7–67.6)	62.6 (57.7–67.5)
% Personal protection	–	69.0[a]	66.2[a]	87.1[b]	86.6[b]
Overall insecticidal effect (%)	–	11.8[a]	6.4[b]	15.5[a]	11.5[a]

Note: Values in the same row sharing a letter superscript do not differ significantly ($P > 0.05$, GLMMs)

Table 6.2 Content of alpha-cypermethrin in LLINs used in the experimental hut trial

Treatment	Concentration of alpha-cypermethrin (g/kg)		
	Before trial	After washing	After trial
MAGNet® LN unwashed	6.39	–	6.47
MAGNet® LN 20 washes	5.95	5.65	5.84
VEERALIN® LN unwashed	6.91	–	7.40
VEERALIN® LN 20 washed	6.75	6.03	5.78

Table 6.3 Content of piperonyl butoxide (PBO) in VEERALIN® LN used in hut trial

Treatment	Concentration of PBO (g/kg)		
	Before trial	After washing	After trial
VEERALIN® LN unwashed	2.63	–	3.90
VEERALIN® LN 20 washed	2.95	2.64	2.40

Chapter 7

Exploring alternative insecticide delivery options in a “Lethal House Lure” for malaria control

The materials presented in this chapter will be published in Malaria Journal as:

Welbeck A. Oumbouke, Antoine M.G. Barreaux, Innocent T. Zran, Rosine Wolie, Alphonsine A. Koffi, Yao N’Guessan, Ludovic P. Ahoua Alou, Eleanore D. Sternberg, Matthew B. Thomas and Raphael N’Guessan. Exploring alternative insecticide delivery options in a “Lethal House Lure” for malaria control.

Exploring alternative insecticide delivery options in a "Lethal House Lure" for malaria control

Abstract

Background: New malaria control strategies, in addition to long lasting insecticidal nets and indoor residual spraying, are required to further reduce malaria burden. The In2Care® EaveTubes is a house modification designed to block and kill malaria mosquitoes, attempting to enter houses, using an electrostatic netting treated with insecticide powder. A previous study demonstrated high residual efficacy of insecticide-treated electrostatic netting in semi-field setting, but persistence under village condition of use is still untested. The current study evaluated the residual bioefficacy of beta-cyfluthrin treated inserts deployed during a cluster randomized controlled trial (CRT) of EaveTubes in central Côte d'Ivoire. New generation LLINs and IRS insecticides with proven resistance breaking potential were also evaluated under semi-field conditions for potential use as alternative methods to deliver insecticides in the lethal house lure.

Methods: Using the previously described "eave tube assay", the residual efficacy of beta-cyfluthrin treated inserts deployed in trial villages was evaluated every month, using pyrethroid resistant *Anopheles gambiae* s.l. from central Côte d'Ivoire. Pieces of netting from new generation LLINs (PermaNet 3.0 roof, Olyset Plus, and Interceptor G2) were placed inside tubes and tested. PVC tubes coated with pirimiphos methyl were also tested as vehicles for insecticides in tubes. Performance of these potential alternatives to electrostatic netting, in comparison to beta-cyfluthrin treated inserts, was assessed in experimental huts using release-recapture experiments. Decay of insecticidal activity was monitored at monthly intervals.

Results: The efficacy of beta-cyfluthrin was relatively short-lived in the field during the CRT, with mortality of pyrethroid resistant *Anopheles gambiae* mosquitoes declining below 80% after 4 months. In the release-recapture experiment, mortality rates from the roof of PermaNet 3.0 (50.4%) and pirimiphos methyl coated PVC tube (66.8%) were in the same range as mortality rates from beta-cyfluthrin treated insert (62.8%). However, efficacy was significantly lower with both Olyset Plus (25.9%) and Interceptor G2 LNs (21.6%). Persistence of insecticides applied on the PVC tube and in the nets was low, with all active ingredients showing a significant decrease in activity (< 50% mortality) within 2 months.

Conclusion: Beta-cyfluthrin provided effective control of pyrethroid resistant *Anopheles* mosquitoes for only 4 months under field condition. This stresses the need for new formulation of insecticides or alternative vehicles of insecticide that give prolonged control of insecticide resistant mosquitoes. The level of efficacy with netting from PermaNet 3.0 and PVC tube coated with pirimiphos methyl was comparable to the beta-cyfluthrin treatment originally selected for use in the CRT. However, the short persistence of these alternative modes of insecticide delivery calls for further product development for EaveTubes.

Background

The primary methods of malaria vector control currently in use are long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS). These methods prevent disease transmission by targeting mosquito behaviours that occurs inside of houses, namely blood feeding and resting [1,2]. Even though these strategies have contributed to most of the recent reduction in malaria burden across sub-Saharan Africa [3], the disease remains an important public health problem, claiming about half a million lives annually [4]. New tools that target mosquitoes surviving exposure to insecticide treated surfaces [5] and those biting outside of sleeping hours and outdoor [6] are required to build on the recent gains, and meet the control target set forth in the World Health Organization (WHO) Global Technical Strategy [7].

An improved understanding of mosquito ecology and behaviour [8] could inform the design of new strategies of control. There is evidence that major African malaria vectors have a strong preference for using eave gaps (the space between the roof and the wall) found in many traditional African houses as an entry point. This behaviour offers vector control opportunities; for example host-seeking mosquitoes could be prevented from entering houses through the blocking of eave gap and other openings in house walls [9,10]. Evidence from a number of observational and randomized controlled trials suggest that house improvement that prevents mosquito entry is associated with reduction of indoor mosquito biting and transmission of malaria [11–13]. Although house modification has contributed to malaria elimination in developed countries, its potential as a vector control tool remains largely underexploited in Africa. However, there is now increasing interest in adding house improvement to the current malaria control arsenal [14].

While blocking eaves of houses prevent mosquito entry, the strong affinity that mosquitoes have for this opening means that it can be targeted for insecticide treatment. In2care EaveTubes is a house modification intervention classified generically as a "lethal house lure" (https://apps.who.int/iris/bitstream/handle/10665/274451/WHO-CDS-VCAG-2018.03-eng.pdf) by the WHO Vector Control Advisory Group (VCAG). This control method consists of taking sections of plastic pipe and fitting these with a screened insert and installing them into a closed eave space. Similarly to open eaves, human odours emanating from houses are channelled but through the tubes which takes advantage of the mosquito preference for entering through the eaves. The insert placed inside the tube is treated with insecticide powder formulation that delivers a lethal dose onto mosquitoes as they attempt to enter house to blood

feed. Thus, the lethal house lure, in this case, consists of a physical component comprised of netting insert (blocking mosquito entry) and a chemical component (insecticide) used to treat the netting. The potential for this approach to control malaria vectors and reduce transmission was demonstrated in a number of semi-field and modelling studies [12,13,15–17]. Whether the encouraging results from these preliminary small-scale evaluations will impact malaria metrics is under investigation in a cluster randomised controlled trial (CRT) in central Côte d'Ivoire [18].

The insert inside the In2Care EaveTubes has a special electrostatic coating which enhances the bioavailability of powder formulated insecticides on the netting surface [19]. Evidence from previous work show that various active ingredients and formulations with proven resistance breaking potential can be deployed on electrostatic netting to good effect when freshly applied [19], but only the pyrethroid beta-cyfluthrin was effective over a 9 month period [15]. Although the persistence observed with beta-cyfluthrin is encouraging, this high residual activity was obtained under controlled conditions, which might be different if evaluated under field conditions.

While electrostatic netting treated with insecticide holds potential for controlling insecticide resistant mosquitoes when deployed as a lethal house lure, there is scope for tapping into existing technologies including new generation LLINs and IRS insecticides to achieve a similar effect when inserted or applied in an eave tube. New LLINs are coming to market, treated with a mixture of a pyrethroid and either a synergist (piperonyl butoxide (PBO) [20–23]), an insect growth regulator (pyriproxyfen (PPF) [24–26]) or a pyrrole insecticide (chlorfenapyr [27–30]). Similarly, there are new IRS products formulated with the organophosphate insecticide pirimiphos methyl [31] or the neonicotinoid clothianidin [32]. These new products are effective against pyrethroid resistant mosquitoes and could be deployed as a lethal house lure in areas with pyrethroid resistant vectors.

The present study aimed to investigate: (i) the residual activity of pyrethroid treated insert under field conditions, and (ii) alternative ways of delivering insecticides in in a lethal house lure, either by using netting from new generation LLINs or dipping the tube in insecticide solutions.

Methods

Mosquitoes

Experiments were conducted with *Anopheles gambiae* sensu lato (s.l.) mosquitoes collected around Bouaké, central Côte d'Ivoire. This mosquito population has a high prevalence of resistance to the major classes of mosquito adulticides, including pyrethroids [33–35]. Mosquitoes were collected as larvae from breeding sites using the dipping method and reared to adult in insectary under controlled temperature and humidity conditions (27 ± 2 °C, 60 ± 20% RH). Larvae were fed on grounded Tetramin baby fish food. Adult mosquitoes emerging from pupae were placed in 30cm x 30cm netted cages and maintained on 10% honey solution until testing.

Residual activity of beta-cyfluthrin treated EaveTubes insert under field conditions

This assessment or experiment was done as part of a cluster randomised controlled trial (CRT) in central Côte d'Ivoire. Forty villages were selected for the CRT with half assigned to EaveTubes plus screening (SET) and LLIN and the other half allocated to LLIN alone [36]. The CRT was investigating whether SET provides any added protective benefit against malaria transmission on top of LLIN. Beta-cyfluthrin was selected for the CRT based on an earlier study that found long-lasting activity (>9 months) of this pyrethroid on eave tube insert under controlled, semi-field conditions [15].

Inserts fitted to houses in the 20 intervention villages were machine-treated by In2care with a wettable powder formulation of 10% beta-cyfluthrin (Tempo 10©, Bayer). The dose of insecticide applied was in the range 300–500 mg per insert.

To monitor the efficacy of treated insert under field conditions in real houses, residual activity was tested monthly using a subsample of inserts from study villages using the eave tube bioassay.

The procedure of this bioassay was described in detail in Oumbouke *et al*. [15]. In brief, the assay comprises of a 20-cm long plastic tube containing an insert such that it is flush with one end of the tube and mosquitoes are introduced into the tube through the opposite end, which is fitted with an untreated netting to keep mosquitoes inside the tube. A 1.5L plastic bottle filled with hot water and wrapped in stocks worn the previous night was placed behind the insert and served as a host cue. Mosquitoes attracted to the heat and odour cues then contact the insecticide-laden insert. The eave tube assay is similar to the previously described MCD bottle assay [37] in that both mimic the interaction between host-seeking mosquitoes and insecticide-treated surfaces. To increase host-seeking activity, mosquitoes were starved for 6h prior to testing. Approximately 100 mosquitoes in batches of 20-25 were exposed for 1 hour in the eave

tube bioassay. Following exposure, mosquitoes were released in netted cages and provided with 10% honey solution and mortality scored after 24h.

Four beta-cyfluthrin treated inserts were sampled from each eave tube village every month for this monitoring activity. The number of inserts tested was based on logistical constraints in the field. Bioefficacy testing was performed monthly until activity decreased below 80% mortality at which point all of the inserts in the villages were replaced with freshly treated inserts.

Semi-field evaluation of two alternative insecticide delivery approaches in EaveTubes

Insecticide treatments

Insecticide-treated electrostatic netting in tubes was shown to produce a significant reduction in overnight mosquito survival in previous semi-field studies [12,15–17]. The experiments described here explore alternatives to electrostatic netting for delivering insecticides in this system. The following new generation LLIN and IRS insecticides were tested in experimental hut surrounded by enclosure at the M'bé field station near Bouaké, central Côte d'Ivoire:

PermaNet 3.0 roof: PermaNet 3.0 is a long-lasting insecticidal net manufactured by Vestergaard S.A. (Switzerland). The top panel, which was tested in the present study, is made of monofilament polyethylene (100 denier) fabric and treated with a mixture of the pyrethroid deltamethrin at 4g/kg and the synergist piperonyl butoxide (PBO) at 25g/kg. The side panels (not tested here) are made of multi-filament polyester (75 denier) fabric with a strengthened lower part incorporated with deltamethrin at 2.8g/kg.

Olyset Plus is a long-lasting insecticidal net manufactured by Sumitomo Chemical (Japan). The net is made of 150 denier high-density mono-filament polyethylene yarn incorporating a mixture of the pyrethroid permethrin at 20g/kg and PBO at 10g/kg on all net panels.

Interceptor G2 is a long-lasting net manufactured by BASF (Germany). The net is a dual-active LLIN made up of knitted multi-filament polyester fibres incorporating a mixture of the pyrethroid alpha-cypermethrin at 2.4g/kg and the pyrrole insecticide chlorfenapyr at 4.8g/kg.

The organophosphate pirimiphos methyl is a WHO recommended insecticide used extensively in IRS campaigns. Capsule suspension formulation of pirimiphos methyl (Actellic CS, Basel, Switzerland) was tested in the present study.

All of these alternatives were compared against electrostatic netting treated with beta-cyfluthrin.

Semi-field enclosure

Semi-field performance of the alternative tube treatments was tested in two experimental huts at the M'bé field station, near Bouaké, central Côte d'Ivoire. The huts are of the West African design [38], 3.25 m long, 1.76 m wide and 2 m high. The interior walls of the huts are made of concrete brick, with a corrugated iron roof. A plastic cover was affixed onto the roofing as ceiling. Each hut was built on a concrete base with a water-filled moat, to prevent invertebrate predators from preying on dead or knocked down mosquitoes. A number of modifications were made to the huts for these experiments: (i) six holes were drilled at eave level (1.7 m from the ground) on three sides of the hut (two holes on each side), (ii) insecticide treated tubes were fitted into the holes, (iii) an enclosure was built around each hut to allow recapture of mosquitoes outside of the hut (Fig. 7.1). The semi-field enclosure consists of a wooden frame erected on the concrete base, 50 cm from the exterior wall of the hut. The roof was made of plastic sheeting which extended beyond the edge of the enclosure as an overhang to prevent rain from entering. The bottom half of the frame was made out of wooden panels and the top half was screened with polyethylene netting. White plastic sheeting was installed on the floor of the enclosure to facilitate the collection of dead mosquitoes. A zipper access door was positioned on the front side of the hut to allow entry into and exit from the enclosure.

Release-recapture experiments

In the first experiment, six 30 cm x 30 cm netting samples were cut from the LN and inserted in tubes in one experimental hut. Six pieces of untreated netting of the same size were placed in the second experimental hut, located 50m away. The netting samples were cut from Olyset Plus and Interceptor G2 and from the roof panel of PermaNet 3.0 and evaluated on different occasions.

In a second experiment, tubes were dipped in aqueous solution of pirimiphos methyl at $10g/m^2$. The tubes were treated by rolling one tube at a time in insecticide solution for 5 minutes and subsequently left to dry for 24h before testing. Tubes treated with pirimiphos methyl were screened with untreated netting. A control hut fitted with untreated tube containing untreated netting was tested in parallel. In the third experiment, six inserts freshly treated with beta-cyfluthrin were installed in one experimental hut (the intervention) and six untreated inserts were placed in tubes in a second experimental house (the control).

Two adult volunteers were recruited to sleep in the huts. Volunteers sleepers rotated between huts on consecutive nights to account for any potential difference in attractiveness to

mosquitoes. The volunteers entered the hut at 20:00h and slept under intact untreated nets. Approximately 100 non-bloodfed sugar starved 5-day old female *An. gambiae* mosquitoes were released into each enclosure every release night 15 min after sleepers entered their respective huts. Mosquitoes were recaptured the following day at 05:00 inside the enclosure. Mosquitoes collected were brought back to the laboratory at Institut Pierre Richet (IPR) in Bouake, Côte d'Ivoire. Dead mosquitoes were counted and discarded. Surviving mosquitoes were provided with 10% honey solution and any delayed mortality was scored up to 72h later.

Sample size calculations

Evidence from previous semi-field studies suggests that insecticidal tube produces about 50% reduction in overnight mosquito survival [12,13,15,16]. Based on this, the number of release night required to detect a 50% reduction in survival with 80% power and significance level of 5% was determined for each treatment in the R software using the "pwr" package. Eight replicates of release-recapture were performed for each treatment, which according to the sample size calculation was above the number required to demonstrate the expected effect size.

Insecticide susceptibility assays

Insecticide susceptibility assays were performed to measure susceptibility to the constituent actives in the LLINs and pirimiphos methyl in the local *An. gambiae* mosquito population. Diagnostic concentration of the pyrethroids deltamethrin (0.05%), permethrin (0.75%), alpha-cypermethrin (0.05%) and pirimiphos methyl (0.25%) were tested in WHO cylinders following WHO guidelines. A higher concentration of pirimiphos methyl (1%) was also tested in assays. Synergist assays were conducted by pre-exposing mosquitoes to PBO, which neutralises the activity of the cytochrome P450s involved in pyrethroid metabolism in mosquitoes. Because of stability issues with chlorfenapyr on filter paper, adapted Centre of Disease and Control (CDC) bottle assays were used to measure resistance to chlorfenapyr. Bottles were coated with chlorfenapyr at the diagnostic dose of 50µg/mL [39]. Four replicates of 25 female mosquitoes (sugar fed, aged 2-3 days) were exposed for 1h to insecticide treated papers or bottles. Mortality was recorded 24h (pyrethroids) and 72h (chlorfenapyr) post-exposure. Mosquitoes in the control batch were held for 72h before scoring mortality.

Residual activity of new generation LLINs and pirimiphos methyl treatment

The residual activity of the best performing alternative delivery methods (PermaNet 3.0 roof and pirimiphos methyl coated PVC tube) in the release-recapture experiments was assessed.

Four 30cm x 30cm pieces from PermaNet 3.0 netting and four PVC tubes treated with pirimiphos methyl at the dosages of $1g/m^2$ and $10g/m^2$ were tested using the previously described eave tube assays [15]. Testing was performed on the netting pieces and the treated tubes at monthly intervals. To evaluate AI decay under realistic ambient conditions, the pieces of the LN (installed in tubes) and the IRS treated tubes were stored between testing in holes drilled at eave level in an experimental hut at the institute. Four replicates of 25 non-blood fed 6h sugar-starved, 5-day old mosquitoes were tested for each bioassay. Intervention and control mosquitoes were monitored for up to 72h before scoring post-exposure mortality.

When mortality decreased below 50%, the netting samples were washed once and re-tested in the eave tube bioassays. Net washing was conducted following WHO guidelines [40]. Briefly, the pieces were washed individually for 10 min in a soap solution (savon de Marseille at 2g/L of deionised water) using a shaker bath set a 155 movements/min and 30°C. Samples were then rinsed twice in clean water for 10min and left to dry for 3-4 hours. Washed netting samples were tested only after full regeneration of the active ingredient (1 day) [41].

Chemical analysis

Content of deltamethrin and piperonyl butoxide was determined in the roof panel of unwashed PermaNet 3.0 netting at month 0, and the washed samples at month 2. Extraction of deltamethrin and PBO was performed using the CIPAC method [42]. Both compounds were extracted by refluxing with xylene for 30 minutes in presence of dioctyl phthalate as internal standard and citric acid. Concentrations deltamethrin and PBO was subsequently measured by Gas Chromatography with Flame Ionization Detection (GC-FID).

Data analysis

All statistical analysis was performed using the R software version 3.5.3. Residual efficacy data across treatments was analysed using generalized linear models (GLMs) with the “arm” package. The models included insecticide treatments as independent variable and mosquito mortality as the outcome. Interactions between insecticides and residual efficacy testing interval were also included in the models. Pairwise comparisons were performed with the final model using the “multcomp” package. For the release-recapture experiments, generalized

linear mixed models (GLMMs) with a binomial distribution and a logit link function was fitted to the data using the "lme4" package. The models included treatment as fixed effect. Enclosure, sleepers and release-recapture study nights were treated as random effects. Significance of the fixed effect in the model was tested using likelihood ratio test (LRT). Susceptibility bioassay data were analysed using a χ 2-square test with Yates continuity correction.

Ethical approval

Ethical clearance for the study was obtained from the ethics review committee of the London School of Hygiene and Tropical Medicine and the Côte d'Ivoire National Ethics Committee. Hut sleepers were all male and > 18 years old. Written informed consent was obtained from all volunteer sleepers taking part in the study prior to the release-recapture experiments.

Results

Bioefficacy and residual activity of beta-cyfluthrin treated inserts deployed in study villages

Bioefficacy and residual activity of the beta-cyfluthrin treated inserts collected from study villages are presented in Fig. 7.2. Five rounds of insert retreatments were done over the two years of the cluster randomized controlled trial. Mortality of pyrethroid resistant mosquitoes exposed to the beta-cyfluthrin treated inserts from the first two treatment rounds was generally < 80% within only three months post-treatment (Fig. 7.2). Beta-cyfluthrin was more durable in the subsequent rounds, killing over 82% of *An. gambiae* by the end of three months (Fig. 7.2). While mosquito mortality rates decreased significantly by four months for inserts from the second treatment round (<75% mortality, $P < 0.05$), the decrease in efficacy was marginal in subsequent rounds, with inserts producing >80 % mortality of pyrethroid resistant *An. gambiae* mosquitoes.

WHO susceptibility assays

The mortality rates of *An. gambiae* s.l. mosquitoes exposed to the diagnostic concentrations of the active ingredients in PermaNet 3.0, Interceptor G2, Olyset Plus, and to pirimiphos methyl are presented in Fig. 7.3. Mortality with the pyrethroid insecticides were less than 25%, indicating a high prevalence of resistance to this class of insecticide. Pre-exposure to PBO resulted in a significant increase in mortality in the pyrethroid resistant *An. gambiae* mosquitoes, from 17 to 38% with permethrin ($\chi^2_1 = 10.69$, $P = 0.001$) and from 23 to 95% with deltamethrin ($\chi^2_1 = 107.8$, $P < 0.001$). While *An. gambiae* s.l. mosquitoes exhibited high

resistance to the 0.25% pirimiphos methyl diagnostic concentration (54.7% mortality), effective susceptibility was restored (100% mortality) was recorded when the dose was increased four-fold to 1%. Chlorfenapyr produced 98% mortality confirming susceptibility to this non-neurotoxic insecticide.

Semi-field performance of new generation LLINs and pirimiphos methyl treatment deployed as part of a "Lethal House Lure"

Results from the overnight release-recapture experiments are summarised in Table 7.1. A total of 4774 female *An. gambiae* s.l. mosquitoes were released over the release-recapture study period. The proportion of mosquitoes recaptured was consistently high in all experiments (> 89% mosquito recapture rate).

Mortality of *An. gambiae* s.l. mosquitoes released was significantly higher with all insecticidal tubes (21.6-66.8%), compared to the untreated control tube (<5%) ($P < 0.001$).

Inserts treated with 10% beta-cyfluthrin killed a greater proportion of pyrethroid resistant *An. gambiae* s.l. (62.8%) than did any of the new generation nettings ($P < 0.001$). PermaNet 3.0 was the best performing netting, killing about half of the mosquitoes recaptured (50.4%) and the difference in kill rate compared to Olyset Plus (25.9%) and Interceptor G2 (21.6%) was significant ($P < 0.001$). Although mortality with Olyset Plus was higher than that with Interceptor G2, the difference in efficacy was not significant ($P = 0.35$).

Mortality with the 10% pirimiphos methyl treated tube (66.8%) was higher than all of the LLINs (21.6-50.4%), $P < 0.001$) but did not differ significantly from beta-cyfluthrin (62.8%, $P = 0.57$).

Residual activity

Based on results from the release-recapture experiments, only PermaNet 3.0 and pirimiphos methyl coated tube were assessed further for residual efficacy at different time points (month 0, month 1, month 2). (Fig. 7.4 & 5).

PermaNet 3.0 LN samples in EaveTubes killed a significantly higher proportion of *An. gambiae* s.l. mosquitoes compared to the control untreated net (<5% mortality, Fig. 7.4). Mortality with fresh PermaNet 3.0 netting was 98.1%; however, efficacy decreased significantly over time, down to 77.8 % by month 1 ($P = 0.005$) and 45.2% by month 2 ($P < 0.001$). Washing PermaNet

3.0 after month 2 resulted in a significant increase in mortality compared to the unwashed PermaNet 3.0 at month 2 (from 45.2 to 76.6, $P < 0.01$).

Both doses of pirimiphos methyl (0.25% and 1%) resulted in >98% mortality in pyrethroid resistant *An. gambiae* s.l. at month 0 ($P = 0.96$, Fig. 7.5). Although the higher dose was still effective at month 1 (>80% mortality), there was a significant decrease in efficacy by 75% ($P < 0.01$) with the lower dose. By month 2, efficacy with the 1% pirimiphos methyl declined by about 50% compared to month 0, but the reduction in activity was much greater with the 0.25% pirimiphos methyl (up to 86%). This indicates a dose-dependent persistence with the higher dose of pirimiphos methyl retaining significantly greater residual efficacy over the two month testing period.

Chemical analysis

The mean deltamethrin and PBO content in the pieces of PermaNet 3.0 netting are presented in Table 7.2. The initial concentrations of deltamethrin (4.09 g/kg) in PermaNet 3.0 was close to the target dose of 4g/kg±25%. Likewise, the dose of the synergist PBO (24.1g/kg) in unwashed PermaNet 3.0 was close to the target concentration of 25g/kg±25%. The mean deltamethrin content in the month 2 PermaNet 3.0 netting following one wash was 3.5 g/kg, which was still within the target concentration range (3-5g/kg) although the PBO content was halved (from 24.1 to 11.42 g/kg) (Table 7.2).

Discussion

With the international effort to identify new approaches for controlling malaria, there is increasing interest in house modification that could lead to reduced risk of malaria transmission. The In2Care EaveTubes is an example of such an intervention, designed to block mosquito entry points and kill mosquitoes as they attempt to enter the house, by insertion of insecticide-treated electrostatic netting in their path to the interior of the house via the eave gap. The present study builds on previous work on the resistance breaking potential of netting electrostatically treated with insecticide powders under laboratory and semi-field conditions. The aim of the current study was to 1) evaluate the residual efficacy of beta-cyfluthrin treated inserts placed in inhabited village houses, and to 2) further explore alternative technologies for delivering insecticides in tube using a combination of laboratory and semi-field experiments.

The bioefficacy and residual activity of beta-cyfluthrin on inserts deployed in trial villages showed mosquito mortality below 80% four months after treatment during the first two rounds despite higher impact (>80%) in subsequent rounds. Although freshly treated inserts were bio-effective against pyrethroid resistant mosquitoes, the residual activity recorded in the present study was much shorter than in a previous study which showed >80% mortality for over 9 months [15]. This disparity could be: due to differences in insecticide application method; inserts deployed in the trial villages were treated using an 'insecticide application machine' [18] developed by In2Care, while in the previous study, inserts were treated by hand [15]. It is possible that the amount of insecticide deposited by machine treatment was lower than that deposited by hand treatment. It could also be possible that the inserts in villages collected dust, which could result in reduced bioavailability of insecticide, rather than a decline in insecticide content. An effective lifespan of four months for beta-cyfluthrin treated inserts under field conditions means that multiple re-treatment rounds would be required to cover the long transmission season in holoendemic settings. Thus there is a need for long-lasting formulations to facilitate large-scale use of the lethal house lure strategy.

While female mosquitoes of endophilic malaria vectors are shown to rest on insecticide-treated house wall long enough to pick up a lethal dose of insecticide even when slow-acting chemistries are deployed [43,44], evidence from filming studies show that mosquitoes attempting to enter people's dwellings via eave gap in search for a blood meal spend on average <5 min on insecticide-treated insert [45]. This suggests that, in order to be effective, the insecticide in the tube should have the attributes of fast-killing and high toxicity with capacity to control insecticide resistant mosquitoes with an exposure time of just a few minutes. The current insecticide delivery system used in the EaveTubes strategy ─the electrostatic coating─ meets these criteria and was shown to break resistance even under scenario of transient contact time through enhanced bioavailability and high transfer of insecticide [19]. Although the electrostatic coating has demonstrative potential, the development of new insecticides and new formulations provides opportunities for alternative insecticide delivery methods in the lethal house lure. The semi-field performance of nettings from new generation LLINs and tubes coated with pirimiphos methyl was evaluated in experimental huts and compared to 10% beta-cyfluthrin treated insert. The kill rate with beta-cyfluthrin (63%) was in the same range as the mortality rates produced by the top of a PermaNet 3.0 or tubes treated with pirimiphos methyl (50-66.8%). The mortality observed was broadly consistent with results from previous studies of insecticide treated EaveTubes conducted at the same study site and in East Africa

[12,13,15,16]. It is worth noting that the ~50% mortality induced by these treatments corresponds to the actual proportion of female mosquitoes contacting the tube over a release-recapture study night (~44%) [15].

The level of efficacy achieved with the top side of PermaNet 3.0 netting and tube treated with pirimiphos methyl (> 50 % mortality) in release-recapture experiments is predicted to have significant impact on malaria transmission according to a mathematical modelling study [46]. This suggests that alternative mode of delivery of insecticides including pieces of netting from synergist LLIN and eave tube dipped in insecticide solution (pirimiphos methyl) could be used in "Lethal House Lure" approach for malaria control.

Although all of the new generation LLINs tested were efficacious against pyrethroid resistant female mosquitoes in the semi-field trial, the magnitude of the impact was significantly lower with Olyset Plus (permethrin and PBO) and Interceptor G2 (alpha-cypermethrin and chlorfenapyr) than with PermaNet 3.0 (deltamethrin and PBO). The difference between the roof of PermaNet 3.0 and Olyset Plus LLINs is likely due to the difference in the levels of toxicity of the pyrethroids in the net. PermaNet 3.0 is impregnated with type II pyrethroid deltamethrin, whereas Olyset Plus is treated with type I pyrethroid permethrin. There is evidence that type II pyrethroids, which contains an alpha cyano group, are more toxic than type I pyrethroids [47], and this is supported by the results of the WHO susceptibility assays where deltamethrin killed significantly higher proportion (95%) of pyrethroid resistant mosquitoes pre-exposed to PBO compared to permethrin (38%). In addition to the difference in the type of pyrethroid used in these nets, the dose of PBO in the roof of PermaNet 3.0 (25g/kg) is almost three times higher than that in Olyset Plus LN (10g/kg).

The poor performance of the dual-active Interceptor G2 was unexpected given prior evidence from experimental hut studies with human occupied IG2 LN nets demonstrating high efficacy against wild free-flying pyrethroid resistant mosquitoes [30,48]. Susceptibility to chlorfenapyr was confirmed in CDC bottle assay, however the efficacy of this non-neurotoxic insecticide depends on a number of factors including exposure duration and the mosquito's circadian activity [49]. Chlorfenapyr is a pro-insecticide and is converted by P450 enzymes into its potent form at night, when mosquitoes are active. Because the release-recapture studies were conducted overnight, it is unlikely that the low mortality observed was a result of chlorfenapyr not being metabolised to its toxic form. On the other hand, given that the interaction between host-seeking mosquitoes and tubes is relatively transient in EaveTubes [45,50], it is possible

that the exposure duration on the mixture net was not sufficiently long for the mosquitoes to pick up a lethal dose of chlorfenapyr which could account for the low mortality induced by Interceptor G2.

Persistence of the alternatives in the tubes was low, and no products show effective control of pyrethroid resistant mosquitoes beyond 2 months. Pirimiphos methyl was short-lived, even when 10 times higher the concentration of Actellic 300 CS was used. The low persistence of Actellic CS reported in the present study contrast with results from previous experimental huts and randomized controlled trials demonstrating much longer residual activity of pirimiphos methyl (≥75% mortality for ~one year) on wall substrates commonly found in rural African houses [31,51]. The low persistence was potentially due to the difference in substrate type (cement wall versus plastic tube). It could also be that environmental factors such as humidity, temperature and UV exposure might have contributed to the rapid decline in activity [52].

Persistence of active ingredients in the new generation LN, PermaNet 3.0 (roof), was also short with mortality rates decreasing below 50% within 2 months. Since the nettings were directly exposed to environmental conditions, it is likely that the same factors mentioned above might have combined to degrade the insecticide in the nets. Washing PermaNet 3.0 roof resulted in a partial recovery in efficacy, which was consistent with the chemical analysis results. Indeed, about half the initial concentration of PBO remained in the 2 month old PermaNet 3.0 netting after one wash, which appeared sufficient to neutralize metabolic enzymes and restore net efficacy to some extent. Nevertheless, the rapid decline in PBO content within 2 months could impact persistence in the eaves.

The nets tested in the present study are treated with low concentration of insecticides to reduce toxicity to net-users. However, since nets are deployed in tube placed at eave height, and therefore out of reach of house residents, higher than currently recommended dose of insecticides in net and chemistry not allowed on net due to safety concern could be considered to improve efficacy and persistence. Likewise, based on the dose-dependent efficacy and persistence pattern with pirimiphos methyl and the position of tubes at eave level, tubes could be treated with higher concentrations of insecticide to provide prolonged control of insecticide resistant mosquitoes while minimising exposure to house occupants.

Conclusion

Beta-cyfluthrin was short-lived on electrostatic netting under field condition when places in tubes at the eaves of houses, providing effective control of pyrethroid resistant *Anopheles*

gambiae mosquitoes for only four months. To improve the feasibility of the lethal house lure for malaria control, insecticide treatment options were evaluated for improved persistence. Coating PVC tubes with an insecticide solution (pirimiphos methyl) and screening tubes with netting from new generation LLINs, mainly the top panel of PermaNet 3.0, reduced overnight mosquito survival to levels consistent with beta-cyfluthrin treatment. This provides proof of principle that existing technologies could be used as alternative mode of insecticide delivery to broaden options for deploying insecticide in EaveTubes. However, the short persistence of the alternative options investigated calls for further product development for EaveTubes.

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Acknowledgments

This research is supported by a grant to the Pennsylvania State University from the Bill & Melinda Gates Foundation, grant number OPP1131603. The authors thank Melinda Hadi from Vestergaard for suppling PermaNet 3.0 net sample and helping with the chemical analysis.

Author contributions

WAO, RN, MBT and EDS designed the study. WAO, AMGB, IZT, NY, AAK, and LPAA conducted the lab and field experiments. WAO analysed the data and wrote the manuscript.

RN, MR, MBT, EDS and WAO edited the manuscript. All authors read and approved the final manuscript.

Competing interest

EDS currently holds a position funded by Vestergaard S.A.

Fig. 7.1 Picture of the experimental hut fitted with EaveTubes

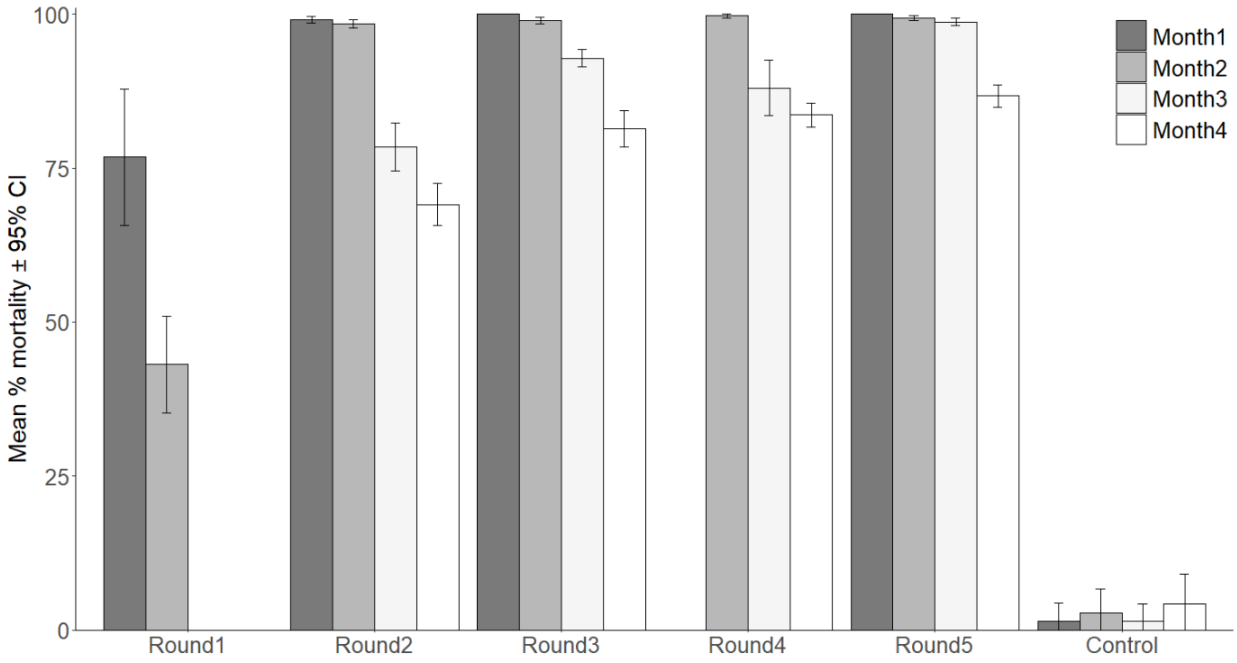

Fig. 7.2 Average mortality of pyrethroid resistant *An. gambiae* mosquitoes exposed to beta-cyfluthrin treated inserts retrieved from trial villages. Bars represent average mortality for the 20 EaveTubes villages. Round indicates insert re-treatment cycle performed during the EaveTubes trial; Round1: Mar 17-May 17, Round2: Jul 17-Aug 17; Round3: Dec 17-Jan18; Round4: Apr18-May18, Round5: Oct 18-Nov 18 . Error bars indicate 95% confidence intervals.

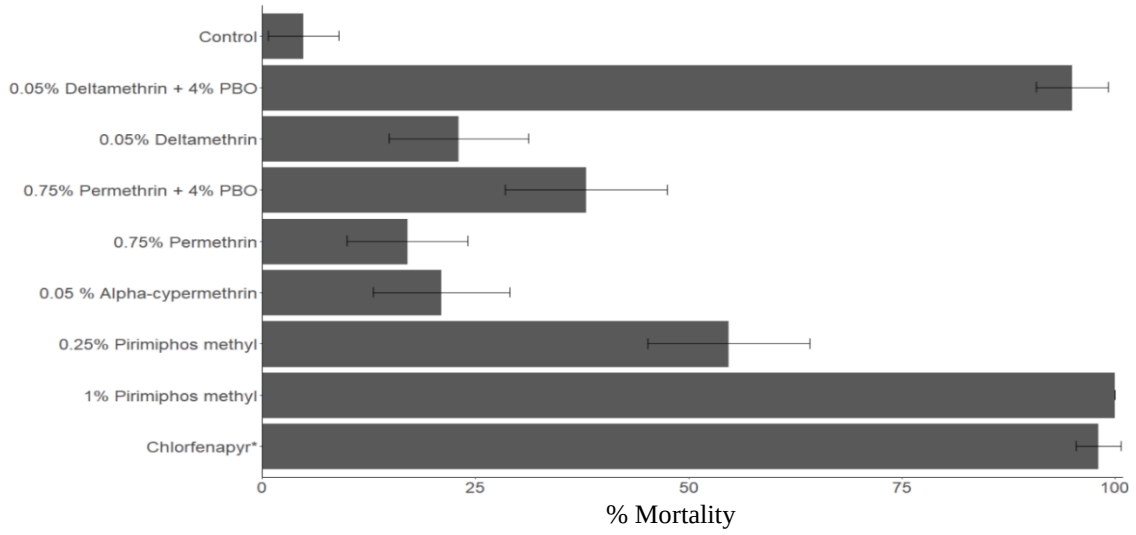

Fig. 7.3 Mortality (%) of wild *An. gambiae* s.l. from Bouaké, Côte d'Ivoire exposed to insecticides in WHO susceptibility bioassays. Error bars indicate 95% confidence intervals. *Susceptibility assays with the pyrrole insecticide chlorfenapyr were performed using CDC bottle assays.

Table 7.1: Semi-field release-recapture results of insecticidal eave tube against pyrethroid resistant *Anopheles gambiae* s.l. in enclosure.

	Untreated insert	**Beta-cyfluthrin treated insert**	**PermaNet 3.0 (deltamethrin + PBO)**	**Olyset Plus (permethrin + PBO)**	**Interceptor G2 (alphacypermethrin + chlorfenapyr)**	**Tubes treated with pirimiphos methyl at 10g/m^2**
Total released	759	811	754	809	796	807
% recaptured (95% C.I.)	93.5 (91.7 - 95.2)	91.5 (89.6 - 93.4)	89.5 (87.2 - 91.8)	92.6 (90.8 - 94.4)	94.8 (93.3 - 96.3)	94 (92.4 - 95.6)
% mortality (95% C.I.)	3.52^a (2.2 - 4.9)	62.8^b (59.3 - 66.3)	50.4^c (46.6 - 54.2)	25.9^d (22.8 - 29)	21.6^d (18.7 - 24.5)	66.8^b (63.4 - 70.1)

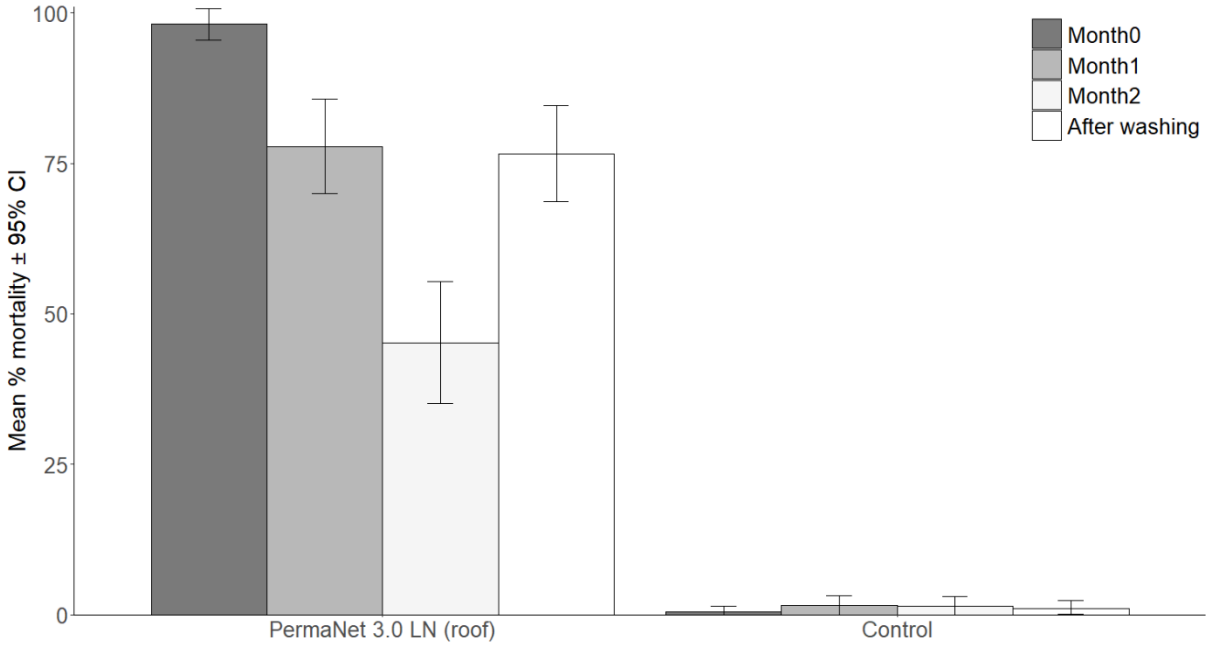

Fig. 7.4 Residual activity in ET bioassays of netting samples from PermaNet 3.0 (roof) LN tested against pyrethroid resistant *Anopheles gambiae* mosquitoes from Bouake with 1h exposure and 24h recovery. Error bars indicate the 95% confidence intervals. "After washing" corresponds to Month2 net samples washed 1X.

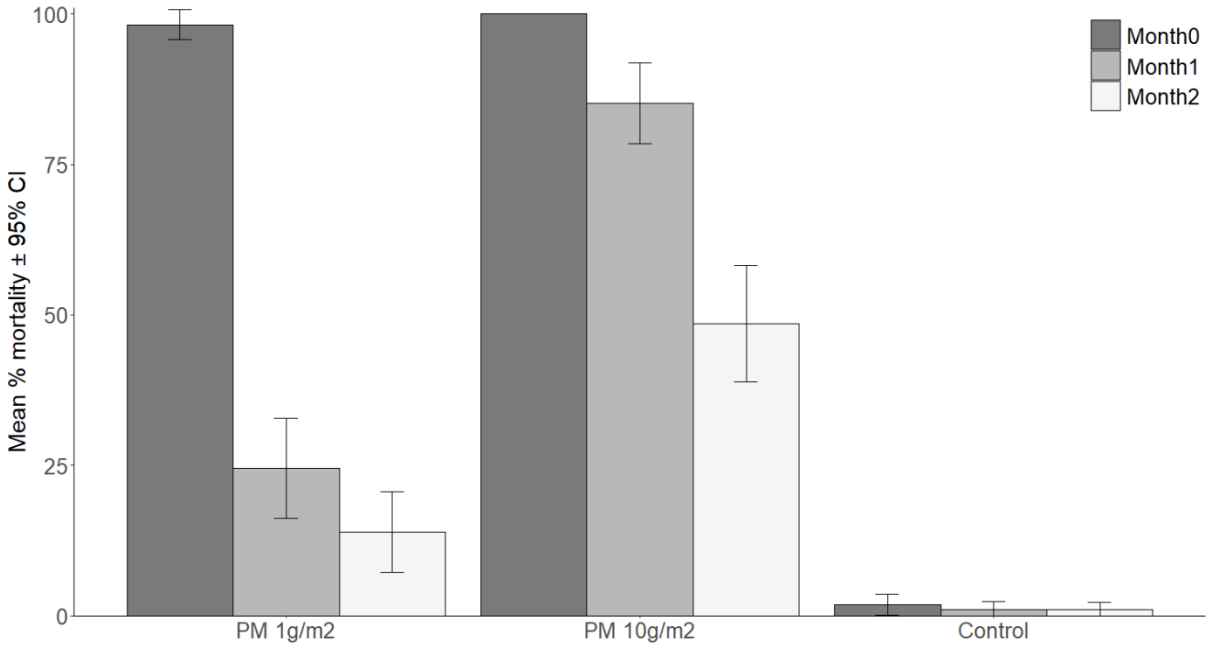

Fig. 7.5 Residual activity in ET bioassays over 2 months of PVC tube coated with pirimiphos methyl at 1g/m^2 and 10g/m^2 tested against pyrethroid resistant *Anopheles gambiae* mosquitoes from Bouaké with 1h exposure and 24h recovery. Error bars indicate the 95% confidence intervals.

Table 7.2: Content of deltamethrin and piperonyl butoxide (PBO) in the roof panel of PermaNet 3.0 LN used in release-recapture experiments

Treatment	Concentration of deltamethrin (g/kg)	Concentration of PBO (g/kg)
Unwashed PermaNet 3.0 LN (roof)	4.09	24.1
2-month old PermaNet 3.0 LN (roof) washed 1 X	3.5	11.42

Part FIVE

Chapter 8: Discussion

Chapter 8: Discussion

1. Overview of the key findings

The global push to expand coverage of core vector control tools, namely long lasting insecticidal nets and indoor residual spraying, has led to a halving of malaria burden across sub-Saharan Africa between 2000 and 2015 [1]. Although this success has resulted in a renewed interest to eliminate the disease, the recent stagnation in progress observed between 2015 and 2017 [2] suggests that additional strategies, unaffected by challenges such as insecticide resistance, are urgently needed to supplement existing interventions. The prospect of house improvement as a malaria control strategy has been discussed in detail in chapter 1 of this thesis. This form of vector control, which involves the blocking of mosquito entry routes, is known to have contributed to malaria elimination in developed countries, but remains largely underexploited in Africa. Based on the historical role of this intervention in malaria control and the expanding body of evidence that deliberate modification of houses can reduce malaria transmission, there is interest in exploring the disease control potential of this strategy in developing countries. The work described in this thesis has focused on a novel type of house modification referred to as screening plus In2care EaveTubes [3]. This house-based intervention has recently been described by WHO as a "lethal lure house" approach and combines house improvement and targeted delivery of insecticide in the eave space to reduce human-vector contact and kill insecticide resistant mosquitoes as they attempt to enter people's houses to search for blood meal. As with a bed net, the intervention has a physical component that blocks mosquitoes' entry routes and an insecticidal component (insecticide treated EaveTubes). The prospect for this novel control approach to reduce transmission of malaria was demonstrated in a range of modelling [4] and semi-field studies [5,6]. Yet, little is known about its functioning and potential contribution to malaria control in areas with high pyrethroid resistance. Consequently, the present PhD project was designed, as part of a large randomised controlled trial of screening plus EaveTubes (SET), to contribute to our understanding of how EaveTubes control malaria in pyrethroid resistant area and explore ways to optimise the intervention. This was addressed through three specific objectives.

The first part of this thesis investigated the resistance profile of the local *Anopheles* mosquito population prior to the SET trial (chapter 2) and evaluated the impact on the efficacy of pyrethroid-only LLINs against these vectors in experimental hut (chapters 3 & 6). Results from these studies reported multiple insecticide resistance in the local *An. gambiae* mosquitoes with

target-site mutations and over-expressed metabolic genes including P450s and carboxylesterases as the main underlying mechanisms. The intensity of pyrethroid resistance in the area was extremely high, reaching over 2500-fold change in most sentinel sites and, to our knowledge, exceeded the levels so far reported in the literature. As a consequence, control of the malaria vector with pyrethroid-only LLINs within experimental huts was severely compromised (<30% mortality) despite appreciable level of individual protection against mosquito bites (31-55% blood feeding inhibition).

The second part of this work investigated the residual efficacy of a wide range of insecticides on the electrostatic netting, which is the insecticide delivery method of the EaveTubes intervention (chapter 4). Whether the community-wide deployment of insecticide treated EaveTubes exert any selection pressure on mosquitoes was also explored (chapter 5). A range of powder-formulated insecticides belonging to various insecticide classes was tested on EaveTubes insert and the one with the highest residual efficacy was selected for subsequent testing under semi field conditions (chapter 4) and in the SET trial in study villages (chapter 5 & 7), against the local insecticide resistant mosquitoes. All candidate insecticides were effective against highly pyrethroid resistant *Anopheles gambiae* when freshly applied but only the pyrethroid beta-cyfluthrin was longer lasting. The duration of effective action with this pyrethroid was > 9 months under semi-field conditions, but did not exceed 4 months under village conditions. In experimental huts, EaveTubes treated with beta-cyfluthrin killed all mosquitoes that contacted the tube, with evidence that mosquitoes spent on average a minimum of 2 min on insecticide treated insert (chapter 4). On the other hand, the community-wide use of beta-cyfluthrin treated EaveTubes was associated with a significant increase in the intensity of resistance to pyrethroids (chapter 5). This was supported by a significant temporal increase in expression of metabolic genes identified prior to the trial (*COEAE1F*), and the selection of new resistance genes, including cuticular genes.

Based on the limited persistence of beta-cyfluthrin under village conditions, the third part of this study investigated alternative technologies for delivering insecticides in EaveTubes including LLIN material treated with a pyrethroid and a synergist, dual-active LLIN material and long-lasting IRS insecticide formulation. The synergist LLIN (top panel of PermaNet 3.0) and, and the pirimiphos methyl IRS insecticide showed high-level of efficacy against highly resistant Anopheles mosquitoes, with the later delivery sytems providing similar level of control as beta-cyfluthrin treated insert in EaveTubes (chapter 7). Although these technologies demonstrated potential as alternative methods for deploying insecticides in EaveTubes, none of

them was found to maintain insecticidal activity beyond two months. This underscores the need for additional studies to further optimize this novel vector control concept.

These results summarise the key findings of the thesis and have been discussed in detail in previous chapters. The present chapter, therefore, discusses the findings in relation to current knowledge on vector control and highlights potential future directions.

2. Potential contribution of pyrethroid-only LLIN to malaria control in a context of increasing level of pyrethroid resistance

Pyrethroid LLINs have been the major contributor to the recent decline in malaria burden, with about 68 % of the gains attributed to the wide-scale use of nets [1]. This progress in control has led to intensified efforts by National Malaria Control Programmes to increase net ownership and usage. Standard LLINs reduce malaria transmission by providing: (i) personal protection to net users via a combination of the physical barrier of the net and the irritant effect of pyrethroid, and (ii) community protection through the mass killing of mosquitoes. Given that the efficacy of these nets is reliant on the continued susceptibility of vector mosquitoes, the widespread emergence of pyrethroid resistance in major African malaria vectors poses a significant threat to the future efficacy of this control strategy. Indeed, as shown in chapters 3 & 6 of this thesis and in previous meta-analysis [7] and hut studies [8–10], the entomological efficacy of pyrethroid-only LLIN is being compromised in areas with high pyrethroid resistance, and this calls for additional control strategies to meet global control targets. The poor performance of these nets in the study area was anticipated, since a range of pyrethroid resistance associated mechanisms including knock down resistance mutations (1014F and 1575Y) and over-production of efficient pyrethroid metabolizing enzymes, mainly P450s (e.g. CYP6P3, CYP9K1 and CYP6M2) and the carboxylesterase COEAE1F was detected in the local *Anopheles* mosquito population (chapter 2).

Although reduced efficacy of pyrethroid LLINs was evident in the mortality recorded in experimental hut trials, this disregards the potential impact of pyrethroids on vector longevity and blood feeding propensity, which are key determinants of vectorial capacity. Further, age is a key factor influencing the susceptibility of mosquitoes to insecticides. The fraction of these pyrethroid resistant mosquitoes old enough to transmit malaria in these areas may still be suppressed as resistance-linked enzymes were shown to degrade with age [11,12]

A recent investigation of the long-term consequences of pyrethroid exposure on surviving pyrethroid resistant mosquitoes reported a delayed mortality effect [13], which was associated with a shortening of the mosquito lifespan. Interestingly, pyrethroid insecticides, which induce outright killing effects against susceptible mosquito populations, are now acting more like late-life acting products in the face of increasing resistance. This delayed effect following LLIN exposure was predicted to substantially reduce the malaria transmission potential of pyrethroid resistant mosquitoes [13], since 9-16 days [14–16] are required for malaria parasites to develop and mosquitoes to become infectious. In addition to the delayed effect of pyrethroid exposure on mosquito longevity, sub-lethal doses of pyrethroids were shown to impair the development of the *Plasmodium falciparum* parasite within pyrethroid resistant mosquitoes [17]. Together, evidence from these studies suggest that the limited level of control induced by pyrethroid LLINs in this thesis may still impact malaria transmission. The sub-lethal effect of pyrethroids on resistant mosquitoes may, at least partly, explain the disconnect between the continued effectiveness of LLIN and pyrethroid resistance. For example, in a recent randomised controlled trial in the Gambia, the use of pyrethroid LLINs was associated with a significant reduction in malaria prevalence in children sleeping under nets despite high pyrethroid resistance in the local *Anopheles* mosquitoes [18]. This suggests that conventional pyrethroid-only LLIN could still provide malaria control benefit and, therefore, supports on-going effort to increase access to bed net in malaria endemics areas. However, while the delayed mortality effect of pyrethroid may partly mitigate the impact of resistance and thus justify the continued use of standard LLIN for malaria control, there is evidence that this effect can be lost when pyrethroid resistance increases in intensity. For instance, although in a study by Viana *et al* [13], there was evidence of a sub-lethal effect from LLIN exposure, this effect appeared substantially reduced or absent against a population of mosquito with high pyrethroid resistance intensity. Moreover, a recent study reported no delayed effect in a lab colony carrying the pyrethroid resistance associated vgsc-1014F mutation [19]. These findings are concerning, given the low immediate mortality following exposure to pyrethroids LLINs reported in this thesis. The likely absence of impact on mosquito longevity following exposure could translate into a loss of community protection in this area with high level of pyrethroid resistance. Furthermore, given that insecticide concentration declines when nets age, this could exacerbate increasing pyrethroid resistance and further reduce LLIN efficacy under field condition.

The intensity of resistance in this area was extremely high, reaching over 2000 fold prior to the roll-out of new pyrethroid LLIN in the study area. How such high intensity of resistance

(measured using adapted CDC bottle assays) relates to the field performance of pyrethroid based intervention is unclear. Exposing mosquitoes to a range of higher than diagnostic dose (E.g. 1X, 5X and 10X) of insecticide is another common way of measuring the strength of resistance, and WHO states that "when resistance is confirmed at the 5× and especially at the 10× concentrations, operational failure is likely" [20]. Although these methods are routinely used to measure resistance intensity, reaching a consensus on how to quantitatively measure the strength of resistance has recently been stressed [21]. Because the long-term impact of pyrethroid diminishes with resistance intensity, studies to estimate the level of pyrethroid resistance that is functionally relevant to malaria transmission are crucial. Such data may help identify areas where LLINs might no longer be protective, and guide decision-making on whether to switch from pyrethroid LLINs to more effective vector control tools.

Even though LLIN efficacy may be compromised when confronted with resistance, reduction of human-vector contact, due to the physical barrier of the net and the irritant property of pyrethroids, could still contribute to disease control in the face of increasing resistance. Protection against mosquito bites was substantial in these studies (up to 50% blood feeding inhibition) and was significantly and consistently higher than that with untreated net. This is in line with findings from a meta-analysis of hut data suggesting that pyrethroid LLINs offer greater personal protection than untreated nets, irrespective of resistance [22]. In addition to the significant inhibition of blood feeding in experimental huts, studies investigating the sublethal effect of LLIN exposure reported a reduction in subsequent host-seeking propensity in pyrethroid resistant mosquitoes [19]. This significant level of protection from pyrethroid net in the presence of resistance provides good rationale for incorporating pyrethroids in the new generation of LLINs being introduced to mitigate pyrethroid resistance and improve malaria control.

The substantial contribution of standard LLIN to the recent achievement in malaria control means that this vector control tool will remain a core component of the global malaria control strategy. Unfortunately, evidence suggest that the scale-up of standard pyrethroid LLIN is exerting a selection pressure on malaria vectors. This has been confirmed in the current thesis with pyrethroid resistance increasing significantly over time following deployment of new PermaNet 2.0 net in the study area (chapter 7). This rise in the level of pyrethroid resistance was consistent with a significant increase of metabolic enzymes over time (COEAE1F) and the emergence of cuticular proteins known to confer high-level pyrethroid resistance. Increasing pyrethroid resistance subsequent to the scale up of standard LLIN has been reported in a range

of studies. For example, a study by Yahouedo et al [23], exploring the dynamic of pyrethroid resistance following the widespread distribution of pyrethroid LLIN in south-eastern Benin, reported a significant increase in the prevalence of pyrethroid resistance, driven by an increased transcription of metabolic detoxification genes [23]. Similar findings were reported from DRC, with increased prevalence and intensity of pyrethroid resistance coinciding with the scale-up of pyrethroid LLIN [24]. Further, the use of pyrethroid treated net was shown to be the driving factor for a genetic sweep, which occurred in a region of the genome that control cytochrome P450 mediated pyrethroid resistance in *Anopheles funestus* [25], one of the major malaria vectors in Africa.

The intensification of pyrethroid resistance, which partly results from the continued use of pyrethroid treated nets, could have significant control implications as this may further compromise existing and new interventions incorporating pyrethroids. In fact, although protection against bites appeared substantial against pyrethroid resistant mosquitoes in experimental huts, it is noteworthy that this level of protection (30-50% blood feeding inhibition) reported in this thesis is much lower compared to levels reported before the advent of pyrethroid resistance (>90 % reduction in blood feeding) [8]. This suggests that the potential of standard pyrethroid nets to reduce human-vector contact (personal protection), which is mostly ascribed to the irritant effect of pyrethroids, may be further undermined as pyrethroid resistance increase in intensity. This is a major concern as all the new generation LLINs designed to restore net efficacy are incorporated with pyrethroids [26–28]. Monitoring of the declining irritant effect of pyrethroids in these new generation LLINs is therefore of utmost importance. Pyrethroid insecticides are still used on nets despite increasing report of pyrethroid resistance across sub-Saharan Africa because no substitute insecticide with pyrethroid attributes is currently available. While new classes of insecticides have recently been approved for use in bednets to kill (chlorfenapyr) [26] or sterilize (pyriproxifen) [29] pyrethroid resistant *Anopheles* mosquitoes, these products do not prevent mosquito blood feeding, and therefore cannot be used as stand-alone insecticide on nets. There is a significant prospect that if pyrethroid resistance continue to increase in prevalence and, more importantly in intensity, the limited killing effect and moderate protection from standard nets would be lost. This emphasizes the need for insecticides with novel mode of action and innovative vector control strategies to improve control of insecticide resistant mosquitoes and reduce malaria burden in Africa south of the Sahara.

3. Deploying insecticides in "eave space" for malaria control: opportunities and challenges

There is increasing recognition that additional control measures would be needed to meet the malaria control target outlined in the Global technical strategy for malaria 2016-2030 (GTS)[30]. The potential contribution of house-based interventions to malaria control and elimination has been emphasized in the Roll Back Malaria (RBM) global framework for Action and Investment to Defeat Malaria 2016-2030 (AIM) [31]. Standard house improvement does not generally rely on the use of insecticides to reduce malaria transmission [32]. Nonetheless, a number of house modification strategies including In2care EaveTubes [3], insecticide treated eave baffles [33] and insecticide treated eave and window screening [34] have an insecticidal component which, according to modelling simulation [4], could contribute to a community-wide effect under high coverage scenario. The insecticide delivery system of the EaveTubes intervention investigated in the present study is the electrostatic netting. As discussed in chapter 1, this new insecticide application method holds insecticide particle via polarity and was shown to improve the efficacy of some of the WHO-licenced adulticides against insecticide resistant African malaria vectors through enhanced bioavailability [35]. Even though an early lab study has shown that insecticide could be applied on electrostatic netting to control susceptible and resistant *Anopheles* mosquitoes, only a couple of insecticides have been tested (deltamethrin and bendiocarb) with no evidence that a broader range of chemicals could be deployed to good effect [35]. This was addressed in chapter 4 of this thesis, with results showing that most freshly applied insecticides from a wide range of insecticide classes including pyrethroid, carbamate, organophosphate, neonicotinoid, entomopathogenic fungus and boric acid were effective (45-100% mortality) against a population of *An. gambiae* mosquitoes with extremely high pyrethroid resistance (>1700 fold resistance to deltamethrin) [36]. This high level of control was confirmed in experimental huts with hut mosquito mortality (50-60%) broadly consistent with previous semi-field studies conducted in Kenya [5] and Tanzania [6]. Data from lab and hut studies showed that about 50% of the mosquitoes released, which corresponds to the percentage mortality, came into contact with the electrostatic netting over a release-recapture study night with further evidence that mosquitoes spent a minimum of 2min on the netting. Although these results provide some new insight into the behavioural interaction between mosquitoes and electrostatic netting within EaveTubes, additional studies using video-recording approach are needed to investigate how interaction varies across different classes of insecticides and mosquito species. Such study may offer new insights for improving the EaveTubes intervention. While not all host-seeking mosquitoes make contact with the insecticide treated netting during a study night, presumably due to a reduced flow of human odour passing through the screened tube [5], a recent study showed that the proportion of female

mosquitoes encountering the netting increases significantly over subsequent study nights [37]. However, these studies were conducted under semi field condition and disregarded the potential impact of human behaviour. In fact, it is likely that the proportion of mosquitoes that come in contact with the netting might be lower than reported here, especially in village conditions where people spend time outdoors for various activities and represent readily accessible sources of a blood meal.

The finding that various classes of insecticides with different modes of action can be successfully deployed in EaveTubes is positive from a resistance management perspective, as it provides prospects for using multiple insecticides in rotation, mosaic or mixture. The rotational use of insecticides in EaveTubes can be implemented at national, sub-national, village or even household level with different classes of insecticides deployed such that mosquitoes are exposed to various insecticides with contrasting mode of action. This may delay or prevent emergence of resistance in the targeted mosquito population. The implementation of such a WHO recommended resistance management strategy is currently challenging with indoor residual spraying, given that only a limited number of insecticides are currently available for spray campaign- although considerable efforts are being made, mainly through the NgenIRS project and the Zero by 40 initiative, to bring new IRS insecticide formulations to market. Nevertheless, while insecticides with novel modes of action may become available in a near future for vector control, it is likely that these new or re-purposed chemicals will be cost-prohibitive especially for IRS. These insecticides might be best suited for the EaveTubes strategy or related interventions, as a comparatively smaller amount of insecticide is required per house protected. Another crucial opportunity with deploying insecticide in eave space is that the location of the treatment, which is generally out of reach from house occupants, allows the use of insecticides that are considered unsafe in existing mainstay vector control tools (LLINs and IRS). Although most of the active ingredients currently approved for use in vector control has been tested on the electrostatic netting in this thesis, future studies testing other promising WHO PQ listed vector control products, for example chlorfenapyr (pyrrole), clothianidin (neonicotinoid) and the insect growth regulator piriproxyfen, are needed.

The electrostatic technology provides significant prospect for diversifying the range of insecticides available for vector control. However, its application has been limited to the EaveTubes strategy, which is suitable only for a particular type of houses (brick-walled houses). As discussed above, eave baffles are a related mode of insecticide delivery in houses, which could be deployed in poorer-quality dwellings for malaria control. Previous studies have shown

that a broad range of insecticide could be applied on eave baffle to reduce human-vector contact and thus transmission [33,38]. Deploying a spatial repellent in eave ribbon wrapped around open eaves was also shown to expand the protection zone to include the immediate vicinity of the house and prevent transmission occurring in the peri-domestic area [39]. Data from another recent study suggests that this approach can be used to protect migratory farmers living in temporary (makeshift) houses [40]. The use of spatial repellents in eave spaces also provide a push-pull based control opportunity as mosquitoes repelled from houses could be subsequently caught up in a mosquito trap positioned outdoor [41]. These different modes of delivering insecticide in mosquito house entry area including electrostatic netting, eave baffles and eave ribbons provide a significant opportunity for extending the lethal house lure strategy to the wide range of house types found in malaria endemic settings. In addition to the above benefits associated with deploying insecticide in eave spaces, such an approach also holds potential to reduce outdoor transmission, though this is beyond the scope of this thesis. Recent evidence does indicate that outdoor biting mosquitoes will enter people's houses at some point during their lifetime [42] before they become infectious and, therefore, could be targeted at house entry point (eave of houses) with insecticide deployed either on electrostatic netting or alternative insecticide delivery systems. Given the increasing importance of outdoor malaria transmission and the significant challenge this poses for malaria control and elimination, the potential of house-based intervention to reduce transmission occurring outdoor should be further explored.

While mosquitoes might rest on indoor interventions (E.g. insecticide treated house wall) for a longer period of time, evidence from previous studies reveal that mosquito contact time around house entry point is comparatively shorter [43,44]. This is one of the main challenges associated with controlling mosquitoes in eave spaces and has implications for target product profile choices. The transient nature of mosquito contact with EaveTubes implies that insecticides with fast-acting effect may be preferred over deterrent or slow-acting products. As discussed above, the electrostatic coating technology was shown to kill resistant mosquitoes even when contact time is as short as a few seconds [35]. For example, in chapter 4 of this thesis, 5 seconds exposure of a highly pyrethroid resistant mosquitoes to pyrethroid treated electrostatic netting produced >50% mortality in lab assays. These results support the use of such modes of insecticide delivery system in eave spaces.

Although the location of EaveTubes provides opportunities for using insecticides considered unsuitable for use in traditional interventions (due to the close proximity with humans), products deployed in this area are directly exposed to environmental factors (temperature,

humidity and UV-light exposure) known to break down insecticides [45]. While these factors were shown to degrade insecticide in indoor interventions (LLINs and IRS) [45], the effect is likely much greater with EaveTubes due to the more direct exposure. This could explain the unexpectedly short residual activity with some of the insecticides (for example the organophosphate pirimiphos methyl) tested in EaveTubes, despite previous studies reporting higher residual activity with the same products applied as an IRS [46,47]. It is also worth noting that the electrostatic netting in the EaveTubes differs from traditional substrate and this might also account for the shorter duration of insecticidal activity on the electrostatic netting. Exposure to UV-light is likely the most significant factor involved in insecticide degradation. Therefore, development of insecticide formulations with UV protection additives that protect the active ingredient from the impact of sunlight could prolong the duration of effective action of chemicals deployed in eave of houses.

Of all the insecticides tested under semi-field condition, only the pyrethroid beta-cyfluthrin was found to be long-lasting (> 80% mortality for more than 9 months) with mosquito mortality with the other products decreasing below 80% within one month. Persistence with beta-cyfluthrin was lower still (< 4 months) under village conditions. These results have been discussed in detail in chapter 4 and 6. As reported with durable wall lining [48], dust accumulation either from cooking or other sources results in the insecticide on the electrostatic netting being covered with dirt which lead to a reduction in the amount of insecticide bioavailable to the mosquitoes. This suggests that the rapid decline in residual activity observed in this study with most insecticides may not necessarily equate to an actual decrease in insecticide content. Analysis of the data on monthly persistence bioassay and the chemical content of the electrostatic netting at each testing time point (not measured in this study) might have provided more insight into this.

The short persistence of insecticide on electrostatic netting under village conditions is a significant challenge which needs addressing before the EaveTubes intervention is considered for wider use in public health. Alternative insecticide delivery options were investigated in this thesis and included netting from new generation LLINs and an IRS insecticide formulation (Chapter 7). Results from these studies showed that substituting the electrostatic netting with a netting treated with pyrethroid and PBO mixture or dipping the tube in an aqueous solution of pirimiphos methyl produced level of control of pyrethroid resistant mosquitoes in experimental hut broadly similar to pyrethroid treated electrostatic netting. Although these alternative means of delivering insecticide in EaveTubes were short-lived, it should be noted that only standard

doses of insecticides that are recommended in IRS or LLINs have been tested. As discussed above, the location of the EaveTubes provides scope for using higher than recommended concentration of insecticides with prospect for boosting efficacy and residual activity.

4. Insecticide resistance management potential of insecticide treated EaveTubes

The resistance breaking impact of the electrostatic technology was confirmed in the present thesis (chapter 4), using a population of *Anopheles* mosquitoes that exhibits an extremely high intensity of pyrethroid resistance. Although there is an unequivocal evidence that such insecticide application method can improve the efficacy of existing WHO approved insecticides against resistant malaria vectors, little is known about its potential from a resistance management perspective. In chapter 5 of this thesis, resistance to a range of insecticides from major chemical classes including pyrethroid was monitored in a subset of CRT villages, to investigate any potential changes in pyrethroid resistance in response to the deployment of pyrethroid treated EaveTubes and standard pyrethroid LLIN in the study area. Results from this study showed a significant increase in the intensity of pyrethroid resistance in both study arms (SET + LLIN and LLIN only arm) over time, but this was significantly higher in the EaveTubes arm (SET+LLIN). The increase in the level of pyrethroid resistance was consistent with a significant increase in detoxification genes associated with pyrethroid resistance (P450s and potentially cuticular genes). It is possible that the pressure from the EaveTubes component might be playing a more important role in the increase in pyrethroid resistance reported. In fact, in the EaveTubes arm, mosquitoes are in theory less exposed to pyrethroid LLINs because houses are made mosquito proof. Therefore, the selection pressure will mostly come from the pyrethroid in the EaveTube. The selection of a pyrethroid (beta-cyfluthrin) for the CRT was based on its good residual activity under semi-field condition, commercial availability, safety and existing country regulatory approval. Interestingly, the community-wide use of pyrethroid EaveTubes was associated with a 38% reduction in malaria incidence (Sternberg et al, submitted), which provides proof of the malaria reduction potential of this novel vector control concept. However, the reported impact of this intervention on the selection of pyrethroid resistance suggests that pyrethroid version of this strategy may be unsuitable from a resistance management standpoint. Indeed, combination of interventions incorporating the same class of insecticides are not recommended, as this increases insecticide selection pressure [49]. Non-pyrethroid version of the EaveTubes strategy should therefore be considered for vector control.

5. Insecticide resistance in central Côte d'Ivoire and implication for future vector control

The spread of insecticide resistance in African malaria vectors is one of the most important challenges facing National Malaria Control Programmes (NMCPs) and may be a contributing factor to the recent increase in malaria burden in a number of countries. *Anopheles gambiae* mosquitoes from this part of Côte d'Ivoire have developed multiple insecticide resistance, which is mostly underpinned by target site mutation and metabolic genes of the P450 family. Standard pyrethroid LLIN is the only vector control strategy currently used in the country. As demonstrated in this thesis, community-wide use of pyrethroid based control strategies is associated with the escalation of pyrethroid resistance in *Anopheles gambiae* mosquitoes. This implies that pyrethroid resistance may further increase in prevalence and intensity in malaria endemic countries as efforts to increase standard bednet ownership and usage intensify.

Apart from the EaveTubes and screening strategy, which was found to be effective in this area, the significant role of metabolic resistance in the reported pyrethroid resistance suggests that synergist LLINs may provide improved control of the local pyrethroid resistant malaria vectors. Interestingly, the synergist LLIN VEERALIN tested in experimental hut in chapter 6 was found to be more effective in controlling this population of *Anopheles* mosquitoes compared to standard LLIN in terms of mosquito mortality and protection against mosquito biting. The chlorfenapyr plus alpha-cypermethrin mixture net (Interceptor G2 LLIN) could also be an alternative control option, as a previous hut study demonstrated a significant potential of this dual-active net to control malaria transmission in this area (> 80 % mortality, > 40% blood feeding inhibition) [10]. Whether this new class of net provides any additional protection against malaria transmission compared to a standard pyrethroid-only LLIN is currently under investigation in randomized controlled trials in Benin and Tanzania. In addition, implementation pilot studies are underway in selected African countries to assess the cost-effectiveness of this net under operational condition. Although the concept of deploying a pyrethroid plus an insect growth regulator (pyriproxyfen) on bednet showed promise as a vector control strategy against pyrethroid resistant mosquitoes in previous studies [27,50,51], the presence of P450s enzymes, which were shown to metabolise piriproxyfen, suggests that this new generation net may not improve control in this area. This is illustrated by the limited impact of Olyset Duo against malaria in Burkina Faso (incidence rate ratio 0·88 [95% CI 0·77-0·99; p=0·04) [52].

Indoor residual spraying with the organophosphate insecticide pirimiphos methyl is being deployed in a range of countries to improve control of pyrethroid resistant *Anopheles*

mosquitoes [53,54]. However, the report of organophosphate resistance in the study area suggests that insecticides with novel modes of action are needed. The neonicotinoid clothianidin which acts as an agonist on nicotinic acetyl choline receptors, is a new insecticide developed to combat pyrethroid resistance. A recent hut study in Benin has demonstrated the potential of this slow-acting insecticide to control pyrethroid resistant mosquitoes [55] and could be an effective control strategy in this setting with resistance to multiple insecticides. It is, however, worth noting that instances of suspected resistance to clothianidin have been previously reported in various African countries [56]. This underscores the need to investigate the status of resistance to this insecticide in the local *Anopheles* mosquitoes before this chemical could be considered for use in this area.

Although most of the alternative control strategies described above have demonstrable vector control potential and could be used in the study area to improve malaria control, they only target the feeding and resting behaviour of female mosquitoes. However, there is a range of promising control interventions that target different stages of the mosquito life cycle and holds significant potential against highly resistant malaria parasite vectors [57,58]. These additional measures could be deployed either alone or in combination with other effective interventions in an integrated fashion to improve control of insecticide resistant mosquitoes. For example, the natural feeding behaviour of both male and female mosquitoes offers control opportunities as sugar source could be targeted with insecticide to control resistant malaria mosquitoes. Such approach is referred to as Attractive Targeted Sugar Bait (ATSB) and has been successfully tested alone and in combination with current standard of care (LLIN) against highly pyrethroid resistant mosquitoes in semi-field [59,60]. A recent large-scale field trial evaluating the efficacy of ATSB in combination with standard LLINs in Mali demonstrated a significant reduction in mosquito density and entomological inoculation rate [61]. In addition to sugar feeding, adult mosquitoes were shown to aggregate into swarms for mating at specific sites where they could be targeted with an insecticide spray. In a recent study in Burkina Faso, targeting mosquito swarm with an aerosol insecticide was associated with a significant reduction in the size of the mosquito population and a change in male mosquito age structure towards younger male mosquitoes that are unable to mate [62]. Other interventions including larval source management (LSM), which involves the management of mosquito breeding sites to prevent the immatures stages of mosquito from reaching adulthood and has proven effective in a range of settings [63,64]. While challenges associated with the existence of multiple and inaccessible mosquito larval habitats have limited the potential of this intervention, there is now interest in

using drone technology [65], for example to map out mosquito breeding sites for a more efficient targeting of immature stages of mosquitoes. A randomised controlled trial evaluating LSM in combination with house improvement and standard net is currently underway in Malawi [66]. This CRT should provide evidence on whether an integrated vector management approach using these interventions provides any added protective benefit compared to current standard of care. Gene drive is one of the new contemporary control methods which is receiving increased attention and may be a game changer for malaria control. This novel control strategy involves the spread of genetic traits in the wild mosquito population to reduce mosquito vector competence (E.g. gene that impedes parasite development within mosquito) [67] and/or vectorial capacity (e.g. genetic traits which reduce mosquito survival or fertility) [68] which are both key determinants of malaria transmission. Although not exhaustive, this list of potential alternative control strategies, in addition to the EaveTubes intervention investigated in this thesis, might be effective against the multiple insecticide resistant mosquitoes from the study area and in settings with similar resistance profile. Nevertheless, fully powered randomised controlled trials are required to demonstrate the epidemiological impact of most of these promising control interventions in setting with differing level of resistance and transmission intensity before consideration for use in public health. Given that these trials are costly and lengthy to conduct, studies are currently underway to investigate whether key entomological indicators from experimental hut studies could be identified and used as a proxy to predict potential epidemiological impact of new control interventions

6. Limitations and Future perspective

The current thesis was designed to better understand the functioning of the EaveTubes intervention and how this strategy control malaria in an area with high pyrethroid resistance. While the resistance breaking potential of this novel control strategy was demonstrated against a highly resistant population of *Anopheles gambiae*, none of the new generation IRS insecticides including the recently PQ listed IRS insecticide clothianidin was evaluated for residual efficacy on electrostatic netting. The low residual activity of the insecticides screened in this thesis for bioefficacy and residual activity calls for further product development studies to identify longer lasting insecticide for use in EaveTubes. New insecticide formulation mixed with UV-resistant additives should be considered to prevent insecticide breakdown and thus prolong the duration of effective action of insecticide in EaveTubes. Further investigation of alternative insecticide delivery options including netting from new generation nets is equally important to broaden the scope for deploying insecticides in EaveTubes for malaria control.

Standard netting incorporating doses higher than those tested in the current thesis should also be evaluated. Additionally, filming studies to better understand mosquito behaviour around the eave of houses could inform further optimization of the intervention. Although the electrostatic technonology was efficacious against the local highly resistant *An. gambiae* mosquitoes, the impact of this new insecticide delivery system was not tested against *An. funestus*, the second major malaria vector species in the study area. This limitation was due to the fact that mosquitoes used in resistance monitoring studies were sampled from breeding sites typical for *Anopheles gambiae,* which were readily accessible in the study area unlike for *An. funestus.* Future studies should rely on F1 generation of mosquitoes obtained from blood fed, indoor resting females mosquitoes. This sampling method, in contrast to larval collection, capture all major malaria vectors mediating tramsmission in the area. Another limitation to the study is that information on the use of insecticides for crop protection in the area was not collected. This data would be useful to understand the potential contribution of additional sources of insecticide selection pressure to the reported increase in pyrethroid resistance in the area.

The positive and significant correlation between pyrethroid resistance intensity and gene expression provide evidence that reliable DNA-based resistance markers could provide a means for tracking the spread of insecticide resistance. The development of these markers are urgently needed for a more efficient monitoring and management of insecticide resistance in African malaria vectors. The deployment of the pyrethroid treated EaveTubes and standard LLIN in the study area have given rise to additional genes, especially several linked to cuticle formation. Understanding of the potential impact of cuticular resistance, which can cause cross resistance across insecticide classes is crucial. Functional genetic validation studies, for example RNAi-induced knockdown of these genes, should be performed to investigate their potential role in the reported temporal change in phenotypic resistance.

7. Conclusion

Although pyrethroid LLINs have contributed to most of the gains achieved over recent years, the recent stalling of progress means that new control interventions are urgently needed. Indeed, data from this study confirmed that pyrethroid resistance is undermining the entomological efficacy of standard LLIN in a setting where high allelic frequency of target site mutation and over-expression of pyrethroid metabolizing enzymes are reported in *Anopheles gambiae* mosquitoes. Targeting these mosquitoes using the electrostatic technology was shown to restore the efficacy of existing insecticides that are less effective when deployed through traditional delivery system (LLIN and IRS). Moreover, this work provides evidence that mosquitoes that

come into contact with the electrostatic netting are killed in about 2 min, which makes the electrostatic netting a suitable insecticide delivery system for the EaveTubes strategy or similar house-based intervention given the transient nature of mosquito behaviour around eave space.

While further studies are required to further optimize the EaveTubes intervention, its potential to reduce vectorial capacity of highly pyrethroid resistant *Anopheles gambiae* mosquitoes and thus malaria transmission has been demonstrated in this thesis. Notably, the CRT showed 38% reduction in malaria incidence associated with the use of the intervention compared to existing control method in an area with high transmission intensity and pyrethroid resistance. The expanding housing market in Africa provides an opportunity to incorporate protective features in house design to reduce malaria transmission. However, as with any intervention that targets the built environment, the lack of existing distribution pathways means that cross sectoral collaboration between public health and the housing sector will be crucial for deploying house based interventions in Africa for malaria control.

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Annexes

Annex 1: List of current WHO prequalified long lasting insecticidal net, as of September 2020

Net brand name	Manufactuerer name and address	Active ingredient
Olyset Net	Sumitomo Chemical Co., Ltd, Japan	Permethrin
Olyset Plus	Sumitomo Chemical Co., Ltd, Japan	Permethrin, Piperonyl butoxide
Interceptor	BASF SE, Germany	Alpha-cypermethrin
Interceptor G2	BASF SE, Germany	Alpha-cypermethrin, chlorfenapyr
Royal Sentry	Disease Control Technologies, LLC, USA	Alpha-cypermethrin
Royal Sentry 2.0	Disease Control Technologies, LLC, USA	Alpha-cypermethrin
Royal Guard	Disease Control Technologies, LLC, USA	Alpha-cypermethrin, pyriproxyfen
PermaNet 2.0	Vestergaard SA, Switzerland	Deltamethrin
PermaNet 3.0	Vestergaard SA, Switzerland	Deltamethrin, Pieronyl butoxide
Duranet LLIN	Shobikaa Impex Private Limited, India	Alpha-cypermethrin
Duranet Plus	Shobikaa Impex Private Limited	Alpha-cypermethrin, Piperonyl Butoxide
MiraNet	A to Z Textile Mills Ltd, Tanzania	Alpha
MAGNet	V.K.A. Polymers Pvt Ltd, India	Alpha-cypermethrin
VEERALIN LLIN	V.K.A. Polymers Pvt Ltd, India	Alpha-cypermethrin, Piperonyl Butoxide
Yahe LN	Fujian Yamei Industry & Trade Co Ltd, China	Deltamethrin
SafeNet	Mainpol GmbH, Germany	Alpha-cypermethrin
Yorkool LN	Tianjin Yorkool International Trading Co., Ltd, China	Deltamethrin
Panda Net 2.0 LLIN	LIFE IDEAS Biological Technology Co., Ltd, China	Deltamethrin
Tsara Boost	NRS Moon netting FZE, Dubai	Deltamethrin, Piperonyl butoxide
Tsara Plus	NRS Moon netting FZE, Dubai	Deltamethrin, Piperonyl butoxide
Tsara Soft	NRS Moon netting FZE, Dubai	Deltamethrin
Tsara	NRS Moon Netting FZE, Dubai	Deltamethrin

Annex 2: List of current WHO prequalified insecticides for indoor residual spraying, as of September 2020

Net brand name	Manufactuerer name and address	Active ingredient
SumiShield 50WG	Sumitomo Chemical Co., Ltd, Japan	Clothianidin
Fendona 10 SC	BASF SE, Germany	Alpha-cypermethrin
Fendona 6 SC	BASF SE, Germany	Alpha-cypermethrin
Fendona 5 WP	BASF SE, Germany	Alpha-cypermethrin
RUBI 50 WP	Tagros Chemicals India Pvt. Ltd	Alpha-cypermethrin
RUBI 100 WP	Tagros Chemicals India Pvt. Ltd	Alpha-cypermethrin
RUBI 50 SC	Tagros Chemicals India Pvt. Ltd	Alpha-cypermethrin
RUBI 100 SC	Tagros Chemicals India Pvt. Ltd	Alpha-cypermethrin
RUBI 250 WG-SB	Tagros Chemicals India Pvt. Ltd	Alpha-cypermethrin
PALI 250 WG	Tagros Chemicals India Pvt. Ltd	Deltamethrin
Revival 100 WP	Tagros Chemicals India Pvt. Ltd	Lambda-cyhalothrin
Revival 100 CS	Tagros Chemicals India Pvt. Ltd	Lambda-cyhalothrin
Pendulum 6 SC	Gharda Chemicals Limited	Alpha-cypermethrin
ICON WP	Syngenta Crop Protection AG, Switzerland	Lambda-cyhalothrin
K-Othrine WG250	Bayer S.A.S., France	Deltamethrin
K-Othrine Polyzone	Bayer S.A.S., France	Deltamethrin
Ficam	Bayer S.A.S., France	Bendiocab
Bayer S.A.S.	Bayer S.A.S., France	Clothianidin, Deltamethrin
Actellic 300CS	Syngenta Crop Protection AG, Switzerland	Pirimiphos-methyl
Actellic EC	Syngenta Crop Protection AG, Switzerland	Pirimiphos-methyl
ICON 10 CS - IRS	Syngenta Crop Protection AG, Switzerland	Lambda-cyhalothrin
Bistar 10 WP	FMC Corporation, Philadelphia, USA	Bifenthrin
Vectron20WP	Mitsui Chemicals Agro, Inc., Japan	Etofenprox
FastM	Saerfu (Henan) Agrochemical Co., Ltd., China	Bendiocarb