Combining unrelated insecticides for improved control and management of insecticide resistant African malaria vectors

Corine Awonja Ngufor

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

University of London

JANUARY 2015

Department of Disease Control
Faculty of Infectious and Tropical Diseases
London School of Hygiene and Tropical Medicine

Funded by:
European Union through the African Vector Control: New Tools (AvecNet) project and B&M Foundation through Innovative Vector Control Consortium (IVCC)
Research group affiliation(s): Pan African Malaria Vector Consortium (PAMVERC)
This work was supervised by:

Professor Mark Rowland
Department of Disease Control, Faculty of Infectious and Tropical Diseases,
London School of Hygiene and Tropical Medicine

Declaration:

I, Corine Awonja Ngufor, 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: January 2015
ABSTRACT

It is now generally accepted that if nothing is done and insecticide resistance in malaria vectors especially to pyrethroids eventually led to widespread failure of current vector control strategies, the progress achieved so far in reducing the burden of malaria could be reversed. Interventions and operational tactics capable of controlling insecticide resistant malaria vector populations and delaying the evolution of resistance need to be urgently identified and properly investigated. One important insecticide resistance management strategy is to expose vector populations to a combination of unrelated insecticides.

In this study I investigated the potential of this combination concept to control and manage the spread of indoor resting insecticide resistant African malaria vectors. A series of field evaluations were performed in experimental huts in selected malaria endemic sites to investigate; 1. the impact of combining non-pyrethroid IRS or wall linings with pyrethroid LLINs against malaria vector populations with different levels of insecticide resistance and 2. The efficacy of LLINs treated with a pyrethroid and an alternative compound against pyrethroid resistant mosquitoes. The capacity of the combined intervention approach to delay the spread of insecticide resistance genes was investigated via genotyping studies.

I demonstrate that the use of combined interventions and mixture net with unrelated insecticides is an effective way to improve the control of pyrethroid resistance malaria vectors. However, the performance of these combinations will undoubtedly depend on the levels and type of resistance encountered. Where resistance to both insecticides exists, improved control is unlikely. While the use of single interventions would likely exacerbate resistance the combinations would be less beneficial for preventing selection of insecticide resistance when resistance genes are already well established. The impact of these findings on malaria vector control and resistance management is discussed.
Acknowledgements

In performing the work contained in this thesis, I have received help from many individuals and collaborating institutions that I would like to sincerely thank and recognise:

**Prof Mark Rowland:** I must thank my supervisor Mark for his tireless efforts in guiding and advising me from the conception of the study through the design, implementation and write up. I appreciate all the time he took to critically comment on the manuscripts and thesis. I am particularly grateful for the faith he demonstrated in my potentials; giving me opportunities to explore my abilities and setting me up on a pathway towards excellence in my career.

**Dr Raphael N'Guessan:** Many thanks to Raphael for the enormous support he provided throughout this study. He believed in me and never hesitated to provide his expertise and counsel whenever I needed it.

**Prof Hilary Ranson:** I am grateful to Hilary for the very important intellectual contributions to the design of this study and for fully supporting my candidature for the PhD as the coordinator of AvecNet project – it would not have been possible without this. I am also thankful for the opportunity she granted me to perform the genotyping studies in her laboratory.

**Terri O'halloran:** I am very grateful to Terri for all the administrative and moral support she provided throughout the study. I appreciate the warmth she always demonstrated towards me and her sincere commitment to facilitate my field activities.
**AvecNet Collaborators:** Field activities for this project were carried out at Centre National de Recherche et de Formation sur le Paludisme (CNRFP) and Centre Muraz (CM), Burkina Faso, Centre Suisse de Recherche Scientifique (CSRS), Cote D'Ivoire, Amani Centre of National Institute for Medical Research (NIMR), Tanzania and Centre de Recherche Scientifique Cotonou (CREC), Benin. The contributions of the field collaborators at these centres; Dr Benjamin Koudou (CSRS), Dr Sagnon N’Fale (CNRFP), Dr Roch Dabire (CM), Dr. Matthew Kirby (LSHTM/NIMR) and Mr. Patrick Tungu (NIMR) are well appreciated.

**Innovative Vector Control Consortium (IVCC):** I am grateful to the IVCC for supporting this work especially Mr David Malone for his intellectual contributions. I also appreciate the assistance I received from industrial partners of the IVCC: Syngenta and SUMITOMO.

**Centre de Recherches Entomologique de Cotonou (CREC):** A large part of this work was performed at the CREC/LSHTM field site in Benin where I have been based for the past 2.5 years. I will like to thank the Director, Prof Martin Akogbeto for his support and the entire laboratory and field staff (Josais Fagbohun, Abibath Odjo, Estelle Vigninou, Laurette Kiki, Edwige Paknou and all others) for their enormous assistance.

**Family and friends:** I will like most importantly to thank my family members and friends for their encouragement. I could not have done this without their moral support especially from my precious Dad, Mr. Ngufor Sammy.

**Funding:** The research in this thesis was funded by the following:
- A grant from the FP7 of the EU through the African Malaria Vector Control: New Tools Consortium (AvecNet)
- A grant from the Bill and Melinda Gates foundation through the Innovative Vector Control Consortium (IVCC).

**Contributions of other co-authors:** The thesis presented here is in the form of a collection of research paper; hence a lot of the work was collaborative in nature. I would like to acknowledge the contributions of my co-authors in the papers making up different chapters:

Chapters 1, 2 and 9: My estimated contribution in these chapters was 100%.

Chapter 3: The study was initially designed by Raphael N’Guessan and Mark Rowland (my supervisor). I supervised /performed the study, analysed the data, co-interpreted the results and drafted the manuscript which was revised by my supervisor.

Chapters 4, 5 and 6: These studies were co-designed by me, my supervisor and Hilary Ranson. I co-supervised/performed the field activities with research collaborators in the different field sites; Patrick Tungu (Tanzania, chapter 4), Emile Tchicaya (Burkina Faso and Cote d’Ivoire, chapters 5 and 6) and Mohammadou Chouaibou (Cote d’Ivoire, Chapter 6). I analysed the data with guidance from Paul Johnson, performed the genotyping studies, interpreted the findings and wrote the papers which received comments and some inputs from my supervisor prior to submission.
Chapter 7: This study was designed by Raphael N'Guessan and my supervisor. The study was performed by staff of the CREC/LSHTM laboratory. I assisted in data analysis, co-interpreted the results and co-wrote the paper with Raphael N'Guessan and my supervisor.

Chapter 8: This study was designed by me, Raphael N'Guessan, David Malone and my supervisor. I supervised/performed the study, analysed the data, co-interpreted the findings and wrote the paper. My supervisor made comments on the manuscript prior to submission.
Content

Declaration.................................................................................................................. 2
Abstract...................................................................................................................... 3
Acknowledgements.................................................................................................. 4
Glossary...................................................................................................................... 10

PART ONE.................................................................................................................. 12
Chapter 1: Introduction and Literature review............................................................ 13
  1.1 Malaria vector control ....................................................................................... 14
  1.2 The threats of insecticide resistance................................................................. 18
  1.3 Development of new insecticide products and delivery systems.................. 26
  1.4 Insecticide resistance management in malaria vectors................................... 29
  1.5 Conclusions and justification........................................................................... 37
  1.6 Study objectives............................................................................................... 38
  1.7 References........................................................................................................ 39
Chapter 2: Basic methodology.................................................................................... 49
  2.1 Experimental huts ............................................................................................ 50
  2.2 Susceptibility studies......................................................................................... 52
  2.3 Residual efficacy............................................................................................... 53
  2.4 Tunnel tests....................................................................................................... 53
  2.5 Molecular genotyping....................................................................................... 54

PART TWO.................................................................................................................. 55
Chapter 3: Combining indoor residual spraying with chlorfenapyr and long-lasting insecticidal bed nets .......................................................... 58
Chapter 4: Insecticide treated net wall hangings for malaria vector control: an experimental hut study in North-eastern Tanzania............................................ 78
Chapter 5: Combining organophosphate treated wall linings with long lasting insecticidal nets for improved control of pyrethroid resistant An gambiae................................. 97
Chapter 6: Combining organophosphate treated wall linings and long-lasting insecticidal nets fails to provide additional control over LLIN alone against multiple insecticide resistant Anopheles gambiae in Côte D'Ivoire: an experimental hut trial. 130
PART THREE .................................................................................................................. 155
Chapter 7: Mosquito Nets Treated with a Mixture of Chlorfenapyr and Alphacypermethrin Control Pyrethroid Resistant Anopheles gambiae and Culex quinquefasciatus Mosquitoes in West Africa ........................................................................ 156
Chapter 8: Olyset Duo (a Pyriproxyfen and Permethrin Mixture Net): An Experimental Hut Trial against Pyrethroid Resistant Anopheles gambiae and Culex quinquefasciatus in Southern Benin .................................................................................. 176

PART FOUR ....................................................................................................................... 206
Chapter 9: Discussion ....................................................................................................... 207
Annexes .......................................................................................................................... 234
Annex 1: WHO recommended longlasting insecticidal nets ........................................ 234
Annex 2: WHO recommended insecticides for IRS .......................................................... 235
Annex 3 Classes of insecticide used for vector control ...................................................... 236
Annex 4: Insect enzyme families involved in metabolic resistance .................................. 237
Annex 5: Cross resistance pattern for insecticides .............................................................. 239
Annex 6: Experimental hut design ................................................................................... 240
Annex 7: Insecticide treated wall linings ........................................................................... 241
## Glossary

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace-1&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Insensitive Acetyl cholinesterase gene</td>
</tr>
<tr>
<td>ACT</td>
<td>Artemisinin-based Combination Treatment</td>
</tr>
<tr>
<td>ALPHA</td>
<td>Alphacypermethrin</td>
</tr>
<tr>
<td>CCE</td>
<td>Carboxyl Choline Esterases</td>
</tr>
<tr>
<td>CDC</td>
<td>Centres for Disease Control</td>
</tr>
<tr>
<td>CFP</td>
<td>Chlorfenapyr</td>
</tr>
<tr>
<td>CS</td>
<td>Microencapsulated formulation</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DDT</td>
<td>Dicloro-diphenyl-tricloroethane</td>
</tr>
<tr>
<td>DL</td>
<td>Durable Lining</td>
</tr>
<tr>
<td>DNA</td>
<td>De-oxyribonucleic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Emulsifiable Concentrate formulation</td>
</tr>
<tr>
<td>EIR</td>
<td>Entomologic Inoculation Rate</td>
</tr>
<tr>
<td>GLMM</td>
<td>Generalised Linear Mixed Model</td>
</tr>
<tr>
<td>GPIIRM</td>
<td>Global Programme for Insecticide Resistance Management</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HDPE</td>
<td>High density polyethylene</td>
</tr>
<tr>
<td>IGR</td>
<td>Insect Growth Regulator</td>
</tr>
<tr>
<td>IRM</td>
<td>Insecticide Resistance Management</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Insecticide Spraying</td>
</tr>
<tr>
<td>ITN</td>
<td>Insecticide Treated Nets</td>
</tr>
<tr>
<td>ITWL</td>
<td>Insecticide treated wall lining</td>
</tr>
<tr>
<td>IVCC</td>
<td>Innovative Vector Control Consortium</td>
</tr>
<tr>
<td>kdr</td>
<td>Knock-Down Resistance</td>
</tr>
<tr>
<td>LLIN</td>
<td>Long Lasting Insecticide Treated Nets</td>
</tr>
<tr>
<td>LN</td>
<td>Long Lasting Insecticide Treated Nets</td>
</tr>
<tr>
<td>LSHTM</td>
<td>London School of Hygiene and Tropical Medicine</td>
</tr>
<tr>
<td>OP</td>
<td>Organophosphate</td>
</tr>
<tr>
<td>PBO</td>
<td>Piperonyl Butoxide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PPF</td>
<td>Pyriproxifen</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>RBM</td>
<td>Roll Back Malaria</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised Control Trial</td>
</tr>
<tr>
<td>VGSC</td>
<td>Voltage Gated Sodium Channel</td>
</tr>
<tr>
<td>WP</td>
<td>Wettable Powder</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WHOPES</td>
<td>World Health Organization Pesticide Evaluation Scheme</td>
</tr>
</tbody>
</table>
PART ONE

Chapter 1: This chapter is a review of current knowledge of malaria vector control, the threats and impact of insecticide resistance and the different recommended strategies for improving vector control and managing resistance. It also discusses the justification and objectives of the thesis.

Chapter 2: This is a short chapter which briefly summarises the basic methods used to answer the research questions.
PART ONE

CHAPTER 1: Introduction and Literature review
Chapter 1: Introduction and Literature review

1.1 Malaria vector control

Vector control has always been a critical facet of malaria prevention and control. Malaria parasite transmission can be effectively interrupted by targeting the vectorial capacity of the vector population via reducing vector abundance, vector longevity and preventing human-vector contact [1]. The methods used for malaria vector control can be broadly classified as non-chemical such as environmental management and biological control or chemical such as larviciding, insecticide treated nets (ITNs), indoor residual spraying (IRS) and space spraying. Until the 1940s, malaria vector control depended mostly on environmental management, improved housing improved sanitation, biological control, and the use of larvicides [2, 3]. The use of insecticides against adult mosquitoes became popular after the Second World War following the introduction of dichlorodiphenyltrichloroethane (DDT) in large vector control campaigns in Europe and Latin America [4].

Vector control today still relies primarily on killing adult mosquitoes with chemical insecticides deployed as either long-lasting insecticidal nets (LLINs) or indoor residual spraying (IRS). These two interventions have each brought about significant reductions in malaria incidence and child mortality in trials and pooled observational studies [5, 6]. Because they are highly effective, relatively low in cost and easier to rapidly scale up, LLINs and IRS have become the mainstay of most malaria vector control programmes in Africa [7]. As part of a drive towards universal coverage of all populations at risk, the use of these two interventions has increased substantially in the last decade contributing significantly to the recent reductions in malaria and saving hundreds of thousands of lives [8]. The older vector control methods are also
effective but unlike ITNs and IRS, most of them can only be used under certain conditions due to logistic and operational limitations.

1.1.2 Insecticide treated nets

The use of mosquito nets as a protection against nuisance insects is an old human practice [9]. Untreated bed nets through the physical barrier they offer are able to provide some protection against infective mosquitoes but once they become holed, which they do with time, protection is lost [10]. The idea of hand-treating mosquito bed nets with insecticides became popular in the early 1980s and was later supported by remarkable field evidence [6, 11, 12]. The long-lasting insecticidal net (LLIN) technology was eventually developed to address the technical and logistical constraints associated with re-impregnation of mosquito bed nets. LLINs resist the loss of insecticide during washing and extend the residual efficacy of the insecticide.

Insecticide treated nets (ITNs) serve both as a physical barrier and a chemical barrier providing a repellent and a toxic effect. They are capable of providing significant personal protection to the user, household protection and community protection when used on a community wide scale [13]. Even when holed with as many as 80 holes, ITNs still provide more protection to the sleeper compared to an intact untreated net [10, 14]. Pooled evidence from cluster-randomised control trials (RCTs) have associated community wide ITN use in areas with stable malaria transmission with significant reductions in child mortality (up to 24%) [15, 16], parasitaemia (up to 23%) [6, 16] and incidence of uncomplicated malarial episodes (by 50% compared to no nets) [6]. Because ITNs are effective, cheap and easy to deliver, without them as a vehicle for insecticides, it is unlikely that the goals of vector control coverage can be achieved and sustained in the most difficult to reach
communities. As a result, national malaria control programmes in endemic countries have in recent years prioritised universal coverage of populations at risk with LLINs. Household ITN ownership has thus increased significantly in the last decade contributing immensely to the recent reductions in malaria morbidity and mortality [8]. However, to sustain the gains in health outcomes with ITNs, efforts must continuously be made to improve net integrity, ensure behavioural compliance as to guarantee proper usage and maintain high coverage [11].

The pesticide Evaluation Scheme of the World Health Organisation (WHOPES) has so far fully approved 7 different brands of LLINs (see Annex 1) all of which are treated with pyrethroids [17]. Pyrethroids have remained the insecticide of choice for LLINs owing to their safety to humans, irritant effect which provides protection against biting and their relatively low cost [18].

1.1.2 Indoor residual spraying

Indoor residual spraying (IRS) involves spraying an effective dose of an insecticide typically once or twice a year on indoor surfaces where malaria vectors are likely to rest after biting. The aim is to reduce malaria transmission by reducing the survival of mosquitoes that enter human homes or dwelling units. While LLINs and IRS provide comparable levels of community protection when used on a large scale, IRS usually requires less dependence on behavioural compliance [21]. It has a distinguished historical role in the control of malaria and has been one of the main interventions leading to the elimination of malaria in half of the world’s regions, like in much of Southern Europe, North America, Japan, Central Asia and Latin America and it is still being widely used [5, 6]. Following interests awakened by the US President’s Malaria Initiative (PMI) the proportion of people protected by IRS in the WHO African Region
has increased significantly in recent years [19]. For example, WHO estimates show
that coverage with IRS has increased from less than 5% in 2005 to 11% in 2010 [8]
thus contributing to the recent reductions in malaria morbidity and mortality.

Twelve insecticides belonging to four classes of compounds (pyrethroids, DDT, organophosphates and carbamates) can be used for IRS [20] (see Annexes 2 and 3). However, most of these insecticides are very short-lived (2-6 months) when applied on most wall substrates; hence multiple rounds of IRS are often required for effective control in hyperendemic areas which are characterised by long transmission seasons. This is often challenged by the complex operational systems required for the implementation of IRS and the need to overcome user-fatigue [21].
1.2 The threats of insecticide resistance

1.2.1 The current situation

The efficacy of LLINs and IRS relies on the continued susceptibility of local vectors to the insecticides that are delivered through these interventions. Unfortunately resistance to all four classes of insecticides approved for vector control (pyrethrins, carbamates organophosphates and organochlorines; see Annex 3) has been reported in malaria vectors and is threatening to undermine the efficacy of LLINs and IRS [22]. The sub-Saharan African region is of critical concern since it also has a high malaria burden; a reduction in the effectiveness of vector control tools in the region could have severe consequences.

Resistance to DDT in malaria vectors dates as far back as the 1950s and is incriminated as the major cause of failure of the first Global Malaria Eradication campaign which was launched by the WHO in 1955 [4, 23]. DDT Resistance is currently widespread across West, Central and Eastern Africa [24]. Resistance to pyrethroids is particularly worrisome since malaria vector control currently depends heavily on this class of insecticides; it has unfortunately been identified in 64 countries with on-going malaria transmission [7]. Pyrethroid resistance in Africa was first found in An gambiae in Ivory Coast in 1993 [25]. It is spreading rapidly and has now been reported in over 27 countries in sub-Saharan Africa (Figure 1) [24]. High levels of pyrethroid resistance has for several years been reported in An gambiae sl from West and Central Africa including Benin [26], Burkina Faso[27], Republic of Guinea [28], Ghana [29], Mali [30], Niger [31], Nigeria [32] and Cote d’Ivoire [33], Cameroon [34, 35], Chad [36], Gabon [37], Equatorial Guinea [38]. In Eastern and Southern Africa, pyrethroid resistance is also high in Uganda [39], Ethiopia [40],
Kenya [41], Zambia [42] Zimbabwe [43], South Africa [44]. Insecticide resistance has recently been identified in countries like Tanzania [45, 46] and Mozambique [47] where malaria vectors had been reported to be largely susceptible just a few years before [24] thus demonstrating the speed at which it is spreading. Resistance to carbamates and organophosphates is increasingly being reported from West Africa. It has been identified in Ivory Coast [48-50], Ghana [51] and Burkina Faso [52]. The spread of carbamate and organophosphate resistance poses a major threat given that these insecticides due to their alternative mode of action are currently the only classes which can be used for IRS in the place of pyrethroids.

Figure 1: Status of pyrethroid resistance in Africa [24].
1.2.2 Mechanisms of insecticide resistance

Insecticide resistance can be defined as the ability of an insect to withstand the toxic effects of an insecticide by means of natural selection and mutations. It is an evolutionary phenomenon which entails the selection of a heritable characteristic in an insect population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended [53]. It results when random genetic mutations occur in certain individuals in a vector population in the presence of an insecticide enabling them to resist and survive the effects of the insecticide. By maintaining the insecticide in the environment of the vector population, selection pressure continues leading to an increase in the number of individuals carrying the resistant gene and eventually conferring phenotypic resistance to the insecticide.

Two major insecticide resistance mechanisms are responsible for most of resistance reported in *Anopheles* species; 1. target site resistance where the action site of the insecticide is modified such that the insecticide no longer binds effectively and 2. metabolic resistance where an increase in metabolism of the insecticide by the insect’s enzyme system prevents it from reaching its target site.

**Target site Resistance**

Target site resistance found in malaria vectors are of two types; mutations in the amino acid sequence of the voltage-gated sodium channel (VGSC) which confer resistance to pyrethroids and DDT and a mutation in the synaptic acetylcholinesterase enzyme (AChE1) (a critical enzyme in nerve transmission) which confers resistance to carbamates and organophosphates. The VGSC mutation usually enables the mosquito to withstand prolonged exposure to the
insecticide without being knocked down hence the mechanism is often referred to as knockdown resistance (kdr). Two distinct mutations in position 1014 of the amino acid sequence of the voltage-gated sodium channel have been associated with kdr in the major African malaria vector An gambiae; a substitution of the leucine residue with a phenyl alanine (L1014F) or a serine (L1014S) [54, 55]. A new kdr mutation (N1575Y) has been recently identified within domains III – IV of the VGSC [56]. The N1575Y was found to be strongly associated with the L1014F and has been identified in An gambiae in West and Central Africa. The synaptic acetylcholinesterase enzyme (AChE1) mutation is commonly called Ace-1R and it results from the substitution of a glycine with a serine (G119S) in the amino acid sequence leading to the production of an insensitive form of the enzyme hence conferring resistance to organophosphates and carbamates which normally target the enzyme [57, 58].

Metabolic resistance

Compared to target site resistance, metabolic resistance is a more dynamic process which results in increased biodegradation of the insecticide usually through the overproduction of detoxification enzymes [59]. A sufficient proportion of insecticide molecules are metabolized before reaching their target in the mosquito nervous system. Detoxification enzymes typically associated with insecticide resistance belong to 3 major gene families: the cytochrome P450 monooxygenases (P450s or CYPs), the carboxyl/choline esterases (CCEs) and the glutathione S-transferases (GSTs). The roles of each of these enzyme families in conferring metabolic resistance in malaria vectors are described in Annex 4. The P450s are the primary enzyme family associated with resistance to most insecticides including the pyrethroids, the most widely used insecticide for vector control. Compared to target
site resistance, metabolic resistance is usually more challenging to monitor owing to the lack of adequate genomic resources and technical limitations. Nevertheless, considerable progress has been recently made following the development of the Anopheles detox-chip [60]; several P450s in An. gambiae associated with pyrethroid resistance have now been identified via microarray studies [61]. Two of these (CYP6P3 and CYP6M2) have been confirmed as metabolisers of pyrethroids [61]. While these are now considered diagnostic markers for pyrethroid resistance, the picture is far from complete and further studies are needed.

Other mechanisms of resistance

Two other forms of resistance which have been less studied are; 1. cuticular resistance which is characterised by a modification of the insect cuticle that prevents or slows down the uptake of insecticides and 2. behavioural resistance which occurs when a modification in insect behaviour enables it to avoid contact and/or lethal effects of insecticides. Cuticular resistance was demonstrated in a study on An. funestus which suggested a correlation between cuticle thickness and pyrethroid resistance [62]. The mechanism has been further supported by the identification of two genes encoding cuticular proteins that were over-transcribed in pyrethroid-resistant Anopheles strains [63, 64]. Though this form of resistance has not been adequately studied in malaria vectors it could have a great impact on vector control given its ability to confer resistance to a broad range of insecticides which are absorbed through the cuticular membrane of the insect. Behavioural resistance has been demonstrated by an increase in outdoor host seeking behaviour following increased use of indoor malaria vector control interventions [65-67]. Other authors have reported a shift in peak biting times from later in the night (23:00–03:00) to late
morning (5:00-6:00) [68] or to a diurnal feeding pattern when humans are not under the net [69]. Nevertheless, all behavioural traits, may not be negative, as they could lead mosquitoes to feed on non-human animals. It is also possible to initially mistake the decline of a vector species as behavioural resistance; for example, studies in East Africa have documented a shift in major vector species from the indoor feeding *An gambiae* ss to the outdoor feeding vector *An arabiensis* following high reductions in *An gambiae* after high LLIN coverage [70].

**Multiple insecticide resistance**

Because the modes of actions of current insecticides are shared, a single target site mutation can result in a mosquito being resistant to pyrethroids and DDT (kdr) or to organophosphates and carbamates (*Ace*-1R). *Kdr* mutations have also been found in conjunction with *Ace*-1R resistance in several populations of the major malaria vector in Africa, *Anopheles gambiae* s.l [26, 71]. In addition, some metabolic enzymes which can be up regulated to confer resistance are able to metabolise more than one insecticide. Multiple insecticide resistance mechanisms can therefore be expressed in the same mosquito (see cross resistance patterns in Annex 5). This situation is particularly worrisome given the reliance on such a limited number of classes of insecticides. Vector control programmes which are confronted with this type of multiple insecticide resistance may be left with no other option until a new insecticide is identified and made available.
1.2.3 Impact of insecticide resistance on malaria control

While considerable effort has been put into diagnosing and identifying the different types of insecticide resistance in malaria vectors, the impact of insecticide resistance on malaria control is yet to be demonstrated unequivocally. This is mostly because the development and rapid spread of insecticide resistance in Africa has not been accompanied by a conspicuous failure in malaria control. Moreover some vector populations in West Africa with high frequencies of kdr have been successfully controlled with ITNs [72]. Nevertheless, given the poor state of routine epidemiological surveillance in most endemic areas, a decline in effectiveness may not be detected; hence the actual situation remains unclear [22]. The paucity of information on the epidemiological impact of insecticide resistance on current malaria control has also been attributed to the fact that resistance cannot be randomly allocated to communities and withheld from others as to separate its impact from confounding factors [22]. Other possible reasons which have been raised are; 1. the need for a higher threshold of resistance for malaria cases to increase, 2. multiple exposures of resistant vectors to an insecticide leading to higher mortality, 3. the higher susceptibility of older vectors and 4. the lower capacity of resistant vectors to transmit disease [7, 73, 74]; studies are however needed to properly investigate these hypotheses.

Notwithstanding the challenges in demonstrating the impact of insecticide resistance on malaria control, a small but increasing number of reports have indicated that insecticide resistance can impact malaria control negatively and could lead to malaria control failure. A classic example occurred in South Africa where malaria control failure with IRS followed the appearance of both metabolic resistance in An
funestus and drug resistance in the parasite [75]. Experimental hut studies in Benin showed reduced efficacy of LLINs and pyrethroid IRS in Southern Benin owing to multiple mechanisms of resistance to pyrethroids [76]. Due to high pyrethroid resistance, malaria vector populations in the Island of Bioko could only be successfully controlled after a change of IRS insecticide from a pyrethroid to a carbamate [38]. In a more recent longitudinal study, increased levels of pyrethroid resistance in Dielmo village, Senegal may have contributed to a rebound of malaria morbidity [77]. There is also evidence that LLINs can lose their protectiveness under field conditions when faced with high level pyrethroid resistant mosquitoes as found in Southern Benin [78].

Modelling studies have shown that if universal coverage is achieved, the failure of pyrethroids could result in about 259 000 additional annual deaths among children and 55 million additional malaria cases in the African region [7]. It is now generally accepted that if nothing is done and resistance especially to pyrethroids eventually led to widespread failure of LLINs and IRS, the progress achieved so far in reducing the burden of malaria could be lost [22]. The World Health Organisation (WHO) therefore calls for an immediate pro-active response to resistance to sustain the effectiveness of malaria vector control [22]. Resistance management would need to be built into vector control programmes without necessarily waiting for evidence of resistance or indisputable proof of control failure due to insecticide resistance. This requires proper monitoring for insecticide resistance, the identification of new products capable of fully controlling vector populations that are resistant to current insecticides and the application of resistance management strategies aimed at delaying the evolution of resistance.
1.3 Development of new insecticide products and new delivery systems

The need to develop a broader range of insecticides with novel modes of action that can circumvent resistance to current insecticides used for IRS and LLINs, has become more critical than ever before [79]. There is some concerted effort to identify new insecticidal compounds. The Innovative Vector Control Consortium (IVCC), a recently developed product development partnership is actively engaging with industry partners to leverage existing technology assets with the aim of finding and developing new products [80]. Two major tracks of the IVCC are the development of new insecticides and the reformulation of WHO approved insecticides from the existing portfolio of insecticides. The current IVCC pipeline for new products is somewhat promising; three new insecticides with no cross resistance to current insecticides are anticipated by 2023 [81].

A small number of products from the agricultural portfolio with novel modes of action such as chlofenapyr – a pyrrole [82], indoxocarb - an oxadiaxine [83] and neonicotinoids [84] have shown some potential for IRS. Chlorfenapyr for example provided full control of kdr and Ace-1 R resistant *An gambiae* strains in laboratory studies [85] and induced 82.9% and 45.6% mortality (at 1g/m²) in pyrethroid resistant *An gambiae* and *Cx quinquefasciatus* populations respectively in experimental huts in Southern Benin [82]. Though these insecticides induce considerable mortality rates in pyrethroid resistant mosquitoes when applied on bed nets, they are non-irritant and thus provide very low bloodfeeding inhibition; hence they are unsuitable for treating bed nets. The development of alternative active ingredients to pyrethroids for use on bed nets has unfortunately been very challenging. There is however prospects that some of these new non-irritant insecticides like chlorfenapyr could be
mixed with pyrethroids on bed nets to reinforce the protectiveness of the bed net through the irritant property of the pyrethroid.

Microencapsulation of existing insecticides can extend their residual life when applied to cement or mud plastered rooms as to cover entire transmission seasons reducing the need for costly repeated applications [86, 87]. This is particularly essential for some carbamate and organophosphate insecticides which when applied as IRS are very effective against pyrethroid resistant mosquitoes but also very short-lived (2-4 months) as opposed to pyrethroids and DDT which could last up to 6 months (see annex 2) [20]. One insecticide under reformulation is pirimiphos-methyl, a WHO-approved organophosphate insecticide. Field trials of an emulsifiable concentrate formulation (EC) demonstrated high level but short lived activity against anophelines and culicines [88-90]. A new microencapsulated formulation of the insecticide (Pirimiphos methyl CS) has been developed. Pirimiphos methyl CS applied at 1g/m2 as IRS on cement walls shows great promise for providing prolonged control of pyrethroid-resistant mosquitoes; mortality rates of pyrethroid resistant *An gambiae* in p-methyl CS treated experimental huts in Southern Benin was ~90% over 12 months [91].

The efficacy of an insecticide against a vector population is not just intrinsic to the insecticide but also depends on the technique used to deliver the insecticide. Indoor insecticide delivery systems are the most appropriate for most of the sub-Saharan African region where malaria parasite transmission occurs largely indoors. Unfortunately, the number of tools available for delivering insecticides indoors against malaria vectors is very limited, relying almost entirely on LLINs and IRS. New
or improved indoor delivery systems are urgently needed in order to diversify the “tool-box” for malaria vector control and to enhance capacity to effectively interrupt malaria transmission in holo-endemic areas in sub-Saharan Africa. The idea of covering the interior of home walls with insecticidal wall lining material is a new approach that simulates IRS. Insecticide treated wall linings (ITWL) can be produced via the long-lasting net technology which incorporates the insecticide into the fibres before yarn extrusion. Long-lasting pyrethroid treated plastic sheeting also known as durable lining (DL) have been developed using this technique and when used on interior walls they showed high acceptability and little or no decline in bioefficacy [92, 93]. Due to the long-lasting technology, it is hoped that DL may only need to be replaced on home walls after 3-4 years which is a major advantage over IRS which usually requires repeated treatments to cover the entire transmission season. DL also has the advantage of providing a more uniform covering of the wall with insecticide compared to IRS and of improving the interior appearance of traditional dwellings especially in rural areas [93]. Considering the current situation of widespread resistance to pyrethroids [22, 24], non-pyrethroid versions of this tool are more desirable.

While most wall linings which have been tested against malaria vectors are made of high density polyethylene plastic sheeting some issues have also been raised over the time required to install the plastic sheetings on home walls and the durability of the material on the wall [93]. In rural Africa, householders often cover their walls with wall hangings made from netting material to improve interior aesthetic appearance. Insecticide treated net wall hangings (NWH) could function in a manner which is
similar to DL and could be a more acceptable, practical and innovative means for delivering insecticides indoors.

1.4 Insecticide Resistance Management (IRM) in malaria vectors

Insecticide resistance management aims to protect and extend the useful life of current insecticides and any insecticides which become available in the future. In response to requests from both the World Health Assembly and the Board of the Roll Back Malaria Partnership, the Global Programme for Insecticide Resistance Management (GPIRM) was recently created and given the task to trigger coordinated action from all stakeholders and to lay the foundations for integrated practices for managing insecticide resistance in all malaria endemic countries [7]. In 2012, WHO and the Roll Back Malaria Partnership released the Global Plan for Insecticide Resistance Management in malaria vectors. Several strategies have been proposed within this plan for managing resistance to insecticides used for vector control and these include; rotations of insecticides, combination of interventions, mosaic and use of mixtures. These strategies work based on two major concepts: 1) removing selection pressure from an insecticide to allow vectors to revert to susceptibility (rotations and mosaics) or 2) overpowering resistance by continuing to kill more resistant vectors (mixtures and combined interventions) [94].

1.4.1 Removing selection pressure to allow vectors to revert to susceptibility.

The concept of removing selection pressure depends on the fitness costs often associated with resistance alleles causing the resistant individuals to be out-competed by susceptible rivals in the absence of selection pressure [53, 95]. Here
the aim is to enhance the survival of susceptible homozygotes relative to the resistant genotypes. As a result, the concept is most likely to succeed when resistant individuals are still rare in the vector population and if fitness costs associated with resistance are large [22]. The use of rotations and mosaics depend on this theory.

**Rotations**

Rotations involve the use over time of two or preferably more insecticide classes with different modes of action. Any resistance developed to the first insecticide is expected to decline over time when the second insecticide is introduced provided the resistance gene is not yet stable in the population and there is a fitness cost associated with it. The aim is to encourage or preserve susceptibility. Rotations like most other IRM strategies must, however, be implemented before a resistance gene becomes common and stable in a population. At that point, there may be no or limited fitness cost to the vector, and therefore the resistance gene may remain even if use of the insecticide that is causing the selection pressure is discontinued.

Rotations have been successful in many applications in agriculture and are considered to be effective in slowing the evolution of resistance and preserving susceptibility [22]; it is therefore recommended for vector control. A classic example demonstrating the effectiveness of rotations in vector control is the case of the Onchocerciasis Control Programme which was launched in 1975 in 11 West African countries [7, 96]. At the onset of the programme, organophosphates were applied weekly by aerial spraying unto breeding sites of the black fly vector. Resistance of blackflies to organophosphates was detected five years later. A rotational strategy involving the use of three chemical insecticide classes and one biological insecticide
was then adopted to counteract the resistance [96]. By the mid-1980s, the programme achieved full-scale rotations, which considerably reduced organophosphate resistance. It has been possible to reintroduce organophosphates in 90% of the Programme area in rotation with other insecticides, and the susceptibility of the black fly population to the other insecticide classes remained unchanged [7]. Rotations are unfortunately not currently possible with LLINs for malaria vector control as there are presently no alternatives to pyrethroids for treating bed nets. While annual rotation of insecticides used for IRS is recommended by the GPIRM [7], cross resistance between the existing limited classes of insecticides restricts the practical options for IRS rotations. This underscores the urgent need for novel insecticides with novel modes of action.

**Mosaics**

In the mosaic strategy two or more insecticides of different classes are applied in a spatially separated manner. One insecticide is used in one area and the other in an adjacent area. The effect is to reduce the proportion of the population exposed to one insecticide at a given time. Over time, insects susceptible to the insecticide in one sector will migrate to the adjacent sector and vice versa. This will create a ‘dilution effect’ which will slow the rate of selection of resistance to either insecticide. Some immigrating mosquitoes resistant to one insecticide will be killed by the other insecticide. The mosaic strategy has been successfully used in agriculture and can also be used for IRS and LLINs.

Mosaics can be applied for IRS by spraying different insecticides in different houses within a community (fine scale mosaic) or in adjacent communities (broad scale mosaic). A large scale trial was undertaken between 1996 and 2002 to evaluate
mosaics and rotations in managing insecticide resistance in the *An albimanus* in the coastal plain of Chilapas, Mexico [97]. Bioassay results showed that continuous use of a pyrethroid gradually increased pyrethroid resistance while with the mosaic and rotations, pyrethroid and organophosphatate resistance were selected at much lower levels. Results from biochemical assays showed highly varied enzyme activity nonetheless, the chances of high levels of resistance developing using the rotations or mosaic were significantly reduced relative to the regime with pyrethroid alone. One major conclusion from the study was that while both rotation and mosaic regimes performed very well in managing resistance in this study, the practicalities of operating and IRS mosaic scheme may pose too many logistical difficulties in a real control programme.

Mosaic can also be used on LLINs; popularly known as the two-in-one bed net. Such nets are treated on the sides with one chemical (usually a pyrethroid) and on the roof with another unrelated chemical (usually a non-pyrethroid insecticide or synergist). The two-in-one approach for LLINs is based on the idea that host-seeking mosquitoes would likely contact the roof of the net first in response to odour plumes or concentration gradients from the host [98]. The first studies on two-in-one nets evaluated bed nets treated with carbamates and organophosphates in experimental hut studies [99, 100]. The nets however, did not clearly show improved control of pyrethroid resistant *An gambiae* compared to pyrethroid only bed nets. In addition both insecticides used (organophosphates and carbamates) are unsafe for bed nets. A two-in-one LLIN treated on the roof with PBO (piperonyl butoxide) synergist and on the sides with deltamethrin (Permanet 3.0) was recently developed. PBO is an oxidase inhibitor which acts as a synergist by enhancing the toxicity of pyrethroids to mosquitoes which have oxidase-based forms of resistance to pyrethroids.
Experimental hut studies in Tanzania showed that Permanet 3.0 (washed and unwashed) induced comparable levels of mortality to Permanet 2.0 (treated only with deltamethrin) against malaria vectors which were largely susceptible to pyrethroids [101]. Unfortunately, Permanet 3.0 failed to provide an encouraging improvement in control of free-flying pyrethroid resistant An gambiae in Benin [102] and in Cote D'Ivoire [103] compared to Permanet 2.0. For such a mosaic net to be effective against pyrethroid-resistant mosquitoes, insects must contact both compounds (PBO on the roof first then deltamethrin on the sides) when seeking a blood meal. Unfortunately, with the mosaic design, this is not certain owing to the spatial separation of both chemicals. Nevertheless, mosaics can be useful for LLINs where there is a need to reduce human exposure to a more toxic compound by limiting it to the roof of the net.

1.4.2 Overpowering resistance by continuing to kill more resistant vectors.

The aim of this concept is to manage resistance by killing or reducing the proportion of resistance carriers by the simultaneous or near simultaneous use of alternative classes of insecticides. The idea is to expose the vector population to two or more unrelated insecticides at the same time and in the same place such that insect genotypes which develop resistance to one insecticide are killed by the other insecticide provided they are not resistant to both. The principle is similar to that of the combination therapy policy which is being used successfully to preserve the efficacy of anti-malarial drugs. As suggested by simulation models, strategies based on this concept can greatly reduce selection for resistance compared to rotations and mosaics which are only effective if fitness disadvantages associated with resistance are very large [94]. Unlike with rotations and mosaics, effectiveness is not
related to the degree of resistance fitness cost; rather the aim is to overpower resistance. In addition, certain insecticides when used together in this approach may also be synergistic and enhance kill through biochemical interaction or through behavioural interaction that enhances pick up of insecticide [104]. The concept therefore has potential benefit in 1. areas of susceptibility to prevent the emergence of resistance, and 2. areas of resistance to overpower resistance hence controlling the resistant vector population and preventing further selection. It can be applied for malaria vector control through the use of mixtures of insecticides (for IRS and LLINs) and through combining interventions such as by using IRS and LLINs (with unrelated insecticides) together in the same house.

**Mixtures**

Mixtures are products in which two insecticides of different classes (different modes of action) are co-formulated so that insects will be exposed to both insecticides at the same time. Previous research and modelling studies indicate that mixtures may be one of the best ways of delaying the evolution of insecticide resistance compared to rotations, broad scale mosaics and combination interventions since insect exposure to both insecticides at the same time is guaranteed [22]. Insecticide mixture formulations can be prepared for both IRS and LLINs interventions. To maximise the IRM potential for IRS mixtures, it is often recommended that both insecticides should have broadly similar rates of decay and should be used at their full operational target dose. As a result, questions have often been raised over the cost-effectiveness of mixtures for IRS [22]. However, because IRS contacts the insect for longer periods at a stage it is blood-fed and resting on walls, mixtures for IRS may be very efficacious for IRM and need to be fully explored for malaria vector control. An
insecticidal paint incorporating a mixture of two organophosphates and pyriproxyfen (INESFLY®) is being evaluated against malaria vectors. INESFLY® showed potential to provide prolonged control (up to 12 months) of susceptible and resistant strains of mosquitoes and sterilise surviving females in laboratory [105] and semi-field studies [106]. Nevertheless the efficacy of INESFLY® depended on the porosity of the substrate showing significantly reduced residual efficacy on porous surfaces like cement.

Some mixtures are currently available for LLINs. In contrast to the mosaic (two-in-one) net, the entire surface of the mixture LLIN is treated with both insecticides. This ensures that insects contact both insecticides at the same time. Most non-pyrethroids lack the excito-repellent property of the pyrethroids, an important characteristic for ensuring personal protection to the user. Bed nets prepared with a mixture of pyrethroid and non-pyrethroid compound (to which vectors are largely susceptible) provide opportunity to maximise insecticidal efficacy against pyrethroid resistant mosquitoes through the non-pyrethroid component while maintaining the excito-repellency properties through the pyrethroid component. A newly developed LLIN treated with a mixture of PBO and permethrin (pyrethroids) has shown better performance against pyrethroid resistant mosquitoes compared to a LLIN treated with permethrin only in experimental hut studies [107]. Such a performance was not achieved with a two-in-one net prepared with PBO (on the roof) and deltamethrin (on the sides) (Permanet 3.0) [102, 103]. Mixture LLINs involving other relatively safe insecticides to which vectors are largely susceptible such as chlorfenapyr, need to be developed. There are also prospects for mixing pyrethroids with insect growth regulators (IGR) like pyriproxyfen on bed nets. Laboratory studies have shown
reduced fertility of eggs laid by *An. stephensi* mosquitoes [108] and complete sterilization and life shortening effects in adult female *An. gambiae* [109] exposed to pyriproxyfen treated nets. A LLIN in which pyriproxyfen and a pyrethroid are combined could provide a combination of personal protection through the pyrethroid component and mass impact on vector populations through the sterilizing effect of the pyriproxyfen component. Such a combination LLIN is thus expected to be effective against multiple resistant strains of mosquito populations and to delay the spread of insecticide resistance genes if resistant mosquitoes which survive the net are sterilized.

**Combining interventions**

Increased resources for malaria control has provided opportunity for combining vector control interventions in the same house hence some national malaria control programmes now deploy LLINs and IRS together for improved impact [110, 111]. The main rationale is the additional chances to target the vector. Moreover, the excito-repellency of the pyrethroid in the LLIN and the physical barrier of the net may improve personal protection compared to the IRS alone. Such combinations however should as much as possible not involve the same insecticide as this increases selection pressure on the vector population [22]. As suggested by modelling studies, combining non-pyrethroid IRS or IRS like interventions with LLINs in the same house could synergistically improve mortality in that the repellent property of the pyrethroid in the LLIN may enhance pick up of the non-pyrethroid insecticide on the wall [104]. This strategy could thus provide improved control of pyrethroid resistant vector populations and prevent the spread of insecticide resistant genotypes. Combining carbamate treated plastic wall linings with LLINs in experimental huts in a pyrethroid
resistant area in Southern Burkina Faso, provided improved vector control and showed potential to prevent the selection of mosquitoes bearing the *Ace-1R* gene which confers resistance to carbamates and cross resistance to organophosphates [112]. The impact of combining LLINs with different non-pyrethroid IRS or wall linings needs to be investigated.

Because insecticide resistance is highly heterogeneous differing greatly in strength and mechanisms from one village or region to another, the performance of any vector control interventions whether applied alone or in combination, will likely vary from one location to another. This needs to be assessed by experimentation to guide control programmes into choosing the most appropriate techniques for improving the control of resistant mosquitoes in their localities.

### 1.5 Conclusion and Justification

IRS and ITNs are the cornerstone for malaria vector control in Africa. However, their efficacy is largely threatened by the development and spread of insecticide resistance in major vector species across Africa. While the impact of IR on malaria control is yet to be demonstrated unequivocally, a small but increasing number of reports indicate that IR is well-capable of undermining malaria control. In response to the situation several methods have been proposed for improving the control of insecticide resistant vector populations and for IRM. Nevertheless, their relevance in the management of insecticide resistance in malaria vectors is largely theoretical and the empirical basis needs to be fully explored. More experiential evidence is needed to strengthen confidence that the IRM strategies of using rotations, mosaics, mixtures and combination interventions do work. Based on modelling studies, IRM strategies which are based on the simultaneous use of unrelated insecticides are
likely to be very useful and effective as they have potential to improve vector control and delay the spread of insecticide resistance by preventing the selection of insecticide resistant genotypes. I therefore proposed to explore the potential of novel vector control tools and operational approaches involving two unrelated insecticides to improve the control of insecticide resistant African malaria vectors and to manage insecticide resistance by preventing the selection of resistant genes.

1.6 Study Objectives

**Overall Objective**

The main objective of this thesis was to investigate whether insecticide resistant African malaria vectors can better controlled and whether the selection of insecticide resistant genes can be prevented by using combinations of unrelated insecticides.

**Specific objectives**

1. To evaluate the efficacy of combining IRS with a non-pyrethroid insecticide (chlorfenapyr) and pyrethroid LLINs against pyrethroid resistant *An gambiae*.

2. To investigate the impact of combining organophosphate treated wall linings with pyrethroid LLINs against malaria vectors which are either resistant only to pyrethroids or to both insecticides.

3. To investigate the capacity of the combined interventions approach to prevent the spread of insecticide resistant *An gambiae*.

4. To evaluate the efficacy of LLINs treated with a mixture of a pyrethroid and an alternative insecticide against pyrethroid resistant *An gambiae*. 
References


Chapter 2: Basic Methodology

This chapter summarises the methods used in the studies reported in this thesis
Chapter 2: Basic Methodology

A series of field evaluations were carried out in experimental huts according to WHOPES guidelines in selected malaria endemic sites across East and West Africa [1, 2]. At each study site, WHO susceptibility tests were carried out to relate the performance of the treatments in the experimental huts to the level of insecticide resistance in the local vector population. The residual activity of the treatments in the experimental huts was monitored via cone bioassays. The differential selection of insecticide resistance genes were investigated via genotyping studies. Where necessary, laboratory experiments were also carried out to evaluate the efficacy of the combinations/mixtures against susceptible and resistant mosquitoes under controlled conditions using tunnel tests.

2.1 Experimental huts

Verandah-trap experimental huts provide the best means of evaluating, under controlled field conditions, the efficacy of novel indoor interventions or combinations of intervention against free-flying, wild populations of malaria vectors [1]. They are made of brick plastered with mud or cement on the inside, with a corrugated iron roof and are built on concrete plinths surrounded by water-filled moats to prevent entry of scavenging ants. The East African hut has a roof lined with palm thatch and an eave gap below the roof around the hut through which mosquitoes can enter (Annex 6). The West African hut has a ceiling of thick polyethylene sheeting lined with palm thatch on the interior surface with no eave gaps but with four window slits (1cm gap) through which mosquitoes enter (Annex 6). Both designs have verandah traps to capture the exiting mosquitoes. Prior to each trial, huts are refurbished to reduce any possibility of contamination from previous trials.
**Processing of mosquitoes and Outcome measures**

Each morning mosquitoes are collected from the floor, walls, window and verandah traps and brought into the laboratory where they are identified morphologically to species, scored for mortality and blood-feeding status. Live mosquitoes are held in netted plastic cups for 24 hours during which they are be provided 10% honey solution and delayed mortality recorded after this holding period. Emphasis is laid on malaria vectors (*An gambiae*, *An funestus* or *An arabiensis*) and the nuisance mosquito *Cx quinquefasciatus*. Live and dead *An gambiae* collected from the respective treatments are preserved for identification of insecticide resistance alleles where possible.

The entomological impact of each treatment in the experimental huts is expressed relative to the control in terms of the following:

1. Deterrence: percentage reduction in the number of mosquitoes caught in treated hut relative to the number caught in the control hut
2. Exiting rates: due to potential irritant effect of treatments expressed as percentage of the mosquitoes collected from the veranda trap
3. Inhibition of blood-feeding: reduction in blood-feeding rate relative to the control. Blood feeding inhibition (%) was calculated as follows:

\[
\frac{100(Bfu - Bft)}{Bfu}
\]

Where \(Bfu\) is the proportion of blood-fed mosquitoes in the untreated control huts and \(Bft\) is the proportion of blood-fed mosquitoes in the huts with a specific insecticide treatment.
4. Mortality: percentage of dead mosquitoes in treated hut at the time of collection and after a 24 h holding period corrected for control mortality.

5. The personal protective effect of the treatments which is described by a reduction in the number of blood-fed mosquitoes relative to the control hut. Personal protection (%) was calculated as follows:

\[
\frac{100(Bu - Bt)}{Bu}
\]

Where \( Bu \) is the number of blood-fed mosquitoes in the untreated control huts and \( Bt \) is the number of blood-fed mosquitoes in the huts with insecticide treatments.

6. The overall insecticidal effect of a treatment relative to the number of mosquitoes that would ordinarily enter an untreated control hut. Overall insecticidal effect (%) was estimated by using the following formula:

\[
\frac{100(Kt - Ku)}{Tu}
\]

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.

2.2 Susceptibility tests

For each site and each vector species, samples of adult mosquitoes which emerged from larvae collected from the experimental hut stations were tested in WHO test kits for susceptibility to the insecticides being tested in the experimental huts. Mosquitoes were exposed in batches of 100s to insecticide treated papers. Knock
down was recorded after 1 hour and mortality after 24h following WHOPES guidelines.

2.3 Residual efficacy

Through the course of the experimental hut trials, cone bioassays were performed on the treatments in the huts at fixed intervals to investigate the residual efficacy of the treatments in the experimental huts. *An gambiae* Kisumu a laboratory reared susceptible strain was exposed to the experimental hut treatments in WHO cones for 3 minutes on nets and 30 minutes on treated walls in situ following WHOPES guidelines [1,2]. Knockdown was recorded after 1 hour and mortality after holding for 24 hours.

2.4 Tunnel tests

To gain further insight, laboratory tunnel tests were undertaken on netting samples taken from the mixture LNs tested in experimental huts. The tunnel test allows expression of the behavioural interactions that occur between free-flying mosquitoes and LNs during host seeking [1].

The tunnel test apparatus is comprised of a glass cuboid tube, 25 cm high, 21 cm wide, 60 cm long, divided into two chambers by a transverse netting insert fitted onto a frame which slots across the tunnel. Nine 1 cm diameter holes are cut into the netting to allow passage of mosquitoes. In the bait chamber, a guinea pig is housed unconstrained in a wire meshed cage and in the other chamber 100 unfed female mosquitoes 3-5 days old were released at dusk and left overnight in the dark as per WHO guidelines. The following morning the numbers of mosquitoes found alive or dead, fed or unfed in each compartment are recorded. Live mosquitoes are
transferred to paper cups and supplied with a pad of cotton wool soaked in 10% glucose solution. Delayed mortality is observed after 24 h.

2.5 Molecular genotyping

The capacity of the combination/mixture approaches to prevent the selection of insecticide resistant genotypes was investigated by molecular genotyping. Samples of live and dead *An gambiae* mosquitoes collected from the experimental huts were analysed for resistance genes to investigate differential survival after exposure to the hut treatments. Genomic DNA was extracted using the Livak procedure [3]. Samples were identified to species and molecular form of *An gambiae* using SINE-PCR and molecular detection of the kdr (L1014F) and ace-1R (G119S) mutation alleles was carried out by real-time Taqman PCR as described by Bass et al.

References

PART TWO

Research question: Can improved vector control and insecticide resistance management be achieved when non-pyrethroid IRS or wall linings are combined with pyrethroid LLINs against pyrethroid resistant malaria vector population?

Chapter 3: Combining indoor residual spraying with chlorfenapyr and long-lasting insecticidal bed nets for improved control of pyrethroid-resistant *Anopheles gambiae*: an experimental hut trial in Benin

Chapter 4: Insecticide treated net wall hangings for malaria vector control: an experimental hut study in North-eastern Tanzania

Chapter 5: Combining organophosphate treated wall linings with long lasting insecticidal nets for improved control of pyrethroid resistant *An gambiae*.

Chapter 6: Combining organophosphate treated wall linings and long-lasting insecticidal nets fails to provide additional control over LLIN alone against multiple insecticide resistant *Anopheles gambiae* in Côte D'Ivoire: an experimental hut trial.
Chapter 3

Combining indoor residual spraying with chlorfenapyr and long-lasting insecticidal bed nets for improved control of pyrethroid-resistant Anopheles gambiae: an experimental hut trial in Benin

The material presented in this chapter has been published as:

Ngufor, CA; N'Guessan, R; Boko, P; Odjo, A; Vigninou, E; Asidi, A; Akogbeto, M; Rowland, M; (2011) Combining indoor residual spraying with chlorfenapyr and long-lasting insecticidal bed nets for improved control of pyrethroid-resistant Anopheles gambiae: an experimental hut trial in Benin. Malar J, 10. p. 343. ISSN 1475-2875 DOI: 10.1186/1475-2875-10-343
Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
1.1. Where was the work published? Malaria Journal
1.2. When was the work published? November 2011
1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion N/A

1.3. Was the work subject to academic peer review? YES
1.4. Have you retained the copyright for the work? OPEN ACCESS

If yes, attach evidence of retention

If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published. N/A
2.1. Where is the work intended to be published?
2.2. List the paper's authors in the intended authorship order
2.3. Stage of publication - Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)
I supervised/performed the study, analysed the data, co-interpreted the findings and drafted the manuscript.

Candidate's signature ____________________________________________

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

___________________________________________
Combining indoor residual spraying with chlorfenapyr and long-lasting insecticidal bed nets for improved control of pyrethroid-resistant *Anopheles gambiae*: an experimental hut trial in Benin

**Abstract**

**Background:** Neither indoor residual spraying (IRS) nor long-lasting insecticidal nets (LLINs) are able to fully interrupt transmission in holoendemic Africa as single interventions. The combining of IRS and LLINs presents an opportunity for improved control and management of pyrethroid resistance through the simultaneous presentation of unrelated insecticides.

**Method:** Chlorfenapyr IRS and a pyrethroid-impregnated polyester LLIN (WHO approved) were tested separately and together in experimental huts in southern Benin against pyrethroid resistant *Anopheles gambiae* and *Culex quinquefasciatus*. The bed nets were deliberately holed with either six or 80 holes to examine the effect of increasing wear and tear on protectiveness. *Anopheles gambiae* were genotyped for the kdr gene to assess the combination’s potential to prevent the selection of pyrethroid resistance.

**Results:** The frequency of kdr was 84%. The overall mortality rates of *An. gambiae* were 37% and 49% with the sixhole and 80-hole LLINs, respectively, and reached 57% with chlorfenapyr IRS. Overall mortality rates were significantly higher with the combination treatments (82-83%) than with the LLIN or IRS individual treatments. Blood feeding (mosquito biting) rates were lowest with the 6-hole LLIN (12%), intermediate with the 80-hole LLIN (32%) and highest with untreated nets (56% with the 6-hole and 54% with the 80-hole nets). Blood feeding (biting) rates and repellency of mosquitoes with the combination of LLIN and chlorfenapyr IRS showed significant improvement compared to the IRS treatment but did not differ from the LLIN treatments indicating that the LLINs were the primary agents of personal protection. The combination killed significantly higher proportions of Cx.quinquefasciatus (51%, 41%) than the LLIN (15%, 13%) or IRS (32%) treatments.

**Conclusion:** The chlorfenapyr IRS component was largely responsible for controlling pyrethroid-resistant mosquitoes and the LLIN component was largely responsible for blood feeding inhibition and personal protection. Together, the combination shows potential to provide additional levels of transmission control and personal protection against pyrethroid-resistant mosquitoes, thereby justifying the additional resources required. Chlorfenapyr has potential to manage pyrethroid resistance in the context of an expanding LLIN/IRS strategy.
**Background**

Long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) are the most widely implemented methods of malaria vector control [1]. Owing to operational and logistic constraints associated with running recurrent IRS campaigns, Insecticide-treated nets (ITNs) and LLINs have, until recently, been the more widely applied interventions in sub-Saharan Africa [2]. However, neither IRS nor LLINs are sufficient to achieve interruption of transmission in holoendemic areas of Africa when applied as single interventions [1]. As more resources are made available for malaria control through the Global Fund and President’s Malaria Initiative, there is growing opportunity for deploying LLINs and IRS as a combination intervention [1,3]. A recent analysis of malaria control programmes which deploy both interventions together gives evidence in a range of settings of added protection among those who sleep under LLINs in IRS-treated houses [4]. The added opportunity to target malaria vectors may justify the extra cost of combination interventions. Others have raised a concern that twinned interventions may increase the selection pressure for resistance if both LLIN and IRS deploy the same insecticide [5].

The efficiency of IRS and LLINs, whether deployed singly or in combination, depends on the continued susceptibility of the vectors to the insecticides delivered through these means. Resistance to the four classes of insecticides (pyrethroids, organophosphates, organochlorines and carbamates) approved for vector control has been found in a number of *Anopheles gambiae* populations [5-8]. Pyrethroids are the ideal insecticides for treating mosquito nets owing to their knockdown effect, excito-repellent properties and low mammalian toxicity [9]. Unfortunately, pyrethroid resistance due to the kdr mutation is now widespread particularly in West Africa.
Reduced efficacy of LLINs and IRS due to multiple pyrethroid resistance mechanisms has been reported in Benin [6,8,12]. One of the methods used for managing insecticide resistance is to expose insect vectors to a combination of insecticides which have different modes of action. Combining of IRS and LLINs as a twinned intervention provides opportunities for resistance management as two insecticides with contrasting modes of action can be delivered at the same time and place. On a previous occasion when LLINs were combined with wall linings made of pyrethroid-treated plastic sheeting (simulating IRS) in a pyrethroid resistance area of Burkina Faso, no improvement on mortality of An. gambiae was observed over LLIN alone [13]. However, when LLINs were combined with carbamate-treated plastic sheeting on the walls, the combination proved more effective against pyrethroid resistant An. gambiae than LLIN alone [14, 15].

Resistance to conventional insecticides and the threat of malaria control failure are the catalysts driving the development of alternative insecticides [9]. One new alternative being evaluated for vector control is chlorfenapyr, a pyrole insecticide [16,17]. Chlorfenapyr acts by targeting the oxidative pathway in insect mitochondria and shows no cross-resistance to DDT or pyrethroids [16]. The novel mode of action makes chlorfenapyr an ideal insecticide to complement the pyrethroids for the management of pyrethroid-resistant mosquitoes. Applied as IRS in experimental huts in southern Benin, chlorfenapyr at 1 g/m2 induced 82.9% and 45.6% mortality among pyrethroid-resistant An. gambiae and Culex quinquefasciatus populations respectively [17]. If chlorfenapyr IRS is combined with LLINs, then mosquito vectors which fail to be killed by the pyrethroid on the LLINs, owing to resistance, can then be targeted by the chlorfenapyr treatment on the wall. A greater impact on the vector
population and on transmission control would, therefore, be expected when such a combination was deployed in areas where pyrethroid-resistant \textit{An. gambiae} or multiple vector species abound.

To test this strategy, the combination of pyrethroid LLINs and chlorfenapyr IRS was examined under experimental hut conditions in the pyrethroid-resistant area of southern Benin. The relationship between the physical integrity of the bed net material (indicated by the number of holes in the bed net) and its impact against resistant mosquitoes was also examined.

\section*{Methods}

\textbf{Study site and experimental huts}

The study was carried out in experimental huts situated in Akron, a village on the periphery of Porto Novo, the administrative capital of the Republic of Benin. This is a crop production area with marshes that provide prolific breeding sites for mosquitoes over long seasons. The local \textit{An. gambiae} is resistant to pyrethroids and DDT [12]. The nuisance mosquito \textit{Cx. quinquefasciatus} is present year round and is resistant to pyrethroids, carbamates and organophosphate insecticides [8]. Seven experimental huts were selected for the study. These huts are typical of the West African region and are made from concrete bricks, with roofs of corrugated iron, ceilings of thick polyethylene sheeting covered with palm thatch on the interior surface and walls plastered with an unpainted cement/sand plaster. Each hut stands on a concrete base surrounded by a water-filled moat to exclude ants. Entry of mosquitoes occurs via four window slits, which are 1 cm wide and located on three sides of the hut.
Insecticide treatments

Seven treatments were compared in the experimental huts:

1. Unsprayed hut with 6-hole, untreated bed nets

2. Unsprayed hut with 80-hole, untreated bed nets

3. LLIN with 6 holes

4. LLIN with 80 holes

5. Chlorfenapyr IRS 500 mg/m² and LLIN with 6 holes

6. Chlorfenapyr IRS 500 mg/m² and LLIN with 80 holes

7. Chlorfenapyr IRS 500 mg/m²

The LLIN was WHOPES approved, made of multifilament polyester fibres, factory-coated with a wash-resistant formulation of deltamethrin at a target dose of 55 mg/m². The untreated bed nets were made of white 100-denier polyester multifilament net (Siam-Dutch Mosquito Netting Co., Bangkok, Thailand). To simulate badly worn nets, 80 holes of 2 cm² diameter were cut along each side and end panels. Nets with six holes, each measuring 4 cm², two on each side and one at each end to simulate less damaged nets were also tested. Chlorfenapyr SC (BASF, ‘Phantom 240SC’ with 240 g chlorfenapyr/litre) was sprayed onto interior walls and plastic sheeting using a Hudson compression sprayer equipped with a flat fan nozzle. The evaluation started one week after treatment and ran for two complete rotations between June and September 2010. Sleepers and mosquito collection Treatments were randomly allocated to the experimental huts. LLINs were rotated weekly between huts while the huts dedicated to the IRS treatments were fixed
throughout the study as these IRS treatments could not be rotated. Seven adult men served as volunteer sleepers and were rotated between treatments on successive nights to adjust for any variation in individual attractiveness to mosquitoes. Sleepers gave informed consent and were provided with chemoprophylaxis prior to the trial. They slept in the huts from 20:00 to 05:00 each night. Mosquitoes were collected each morning at 05:00 from under bed nets, floors, walls, ceilings and verandas using aspirators and torches. The collections were transported to the laboratory where mosquitoes were identified to species and scored as blood fed or unfed and live or dead. Live mosquitoes were held in netted plastic cups and supplied with 10% honey solution. Delayed mortality was recorded at 24 h, 48 h and 72 h. Male mosquitoes were not scored.

The entomological impact of each treatment was expressed relative to the control in terms of the following:

1. Deterrence: percentage reduction in the number of mosquitoes caught in treated hut relative to the number caught in the control hut;

2. Repellency (induced exiting) due to potential irritant effect of treatments expressed as percentage of the mosquitoes collected from the veranda trap of treated hut relative to percentage caught in veranda trap of control hut;

3. Inhibition of blood feeding: reduction in blood feeding rate relative to the control. This was calculated using the following model:

$$100 \frac{(Bfu - Bft)}{Bfu}$$

where Bfu is the proportion of blood-fed mosquitoes in the untreated control huts and Bft is the proportion of blood-fed mosquitoes in the huts with insecticide treatments;
4. Induced mortality: percentage of dead mosquitoes in treated hut at the time of collection and after a 72 h holding period relative to control hut.

5. The personal protective effect of the treatments which is described by a reduction in the number of blood-fed mosquitoes relative to the control hut was calculated as follows:

% Personal Protection = 100(Bu - Bt)/Bu

Where Bu is the number of blood-fed mosquitoes in the untreated control huts and Bt is the number of blood-fed mosquitoes in the huts with insecticide treatments.

**Ethical clearance**

Approval was obtained from the ethics committees of the London School of Hygiene and Tropical Medicine and the Benin Ministry of Health. Each trial participant gave written informed consent and was offered chemo-prophylaxis during and for one month after the experimental hut trial.

**Molecular assays**

To examine the potential for the combination treatment to prevent selection for resistance the dead and surviving *An. gambiae* were genotyped using PCR to assess the kdr frequency according to the method of Martinez-Torez et al. [18]. The resistance allele frequency at the kdr locus was analysed using Genepop software (version3.3) [19].
Data analysis

Data were entered in Excel and transferred to STATA 11.0 for further analysis. The numbers of mosquitoes collected each night were compared between treatments using a negative binomial regression model. Proportional data (exiting rate, blood feeding, and mortality) were analysed using logistic regression after adjusting for the effects of sleeper attractiveness and hut position.

Results

Over the three-month trial, 865 An. gambiae sl, 7,296 Cx. quinquefasciatus and over 1,000 Mansonia spp. females were caught in the huts. Only the data for An. gambiae and Cx. quinquefasciatus were analysed further.

Anopheles gambiae

The summary results of treatment efficacy against An. gambiae are presented in Table 1. There was a significant difference in the number of mosquitoes collected between the seven huts (P = 0.017). These differences could be due to differences in positional attractiveness to mosquitoes or to an effect of the IRS treatments or to both. Because the IRS treatments could not be rotated it was not possible to separate the effect of hut position from IRS treatment on mosquito entry or deterrence.

The untreated, holed nets provided only limited protection against biting An. gambiae, with the proportion blood-fed reaching 54% in huts with the 80-hole nets and 56% in huts with the 6-hole nets. The difference in mosquito feeding rate between untreated 80-hole nets and untreated 6-hole nets was not significant (P =
The untreated holed nets were, however, more protective than no net at all because the proportion blood feeding in the only hut that lacked nets (chlorfenapyr IRS) was 89.4%, a value which was significantly higher than the proportion blood feeding in huts with untreated nets (P = 0.0001). The holed LLINs were more protective than holed untreated nets (P = 0.0001) due to the pyrethroid on the LLIN providing protection through its excito-repellent and knockdown effects. The LLIN with 6 holes was significantly more protective (BFI = 78%) than the LLIN with 80 holes (BFI = 42%) (P = 0.001).

The addition of IRS with chlorfenapyr to a hut with LLIN did not alter the level of protection conferred by the LLIN, i.e. the proportions blood feeding with the combination were similar to that of the LLIN alone (P = 0.247 for 6-hole nets, P = 0.468 for 8-hole nets). The reduction in feeding rate with the combination was therefore attributable to the LLIN component rather than to the IRS component. The personal protection attributable to pyrethroid on the holed LLINs, relative to untreated nets, ranged from 56% to 75% protection. The overall mortality rates with the LLIN alone ranged from 37.3% for the 80-hole to 49.5% for the 6-hole nets; the number of holes made no significant difference to the level of mortality conferred by the pyrethroid treatment (P = 0.083). The overall mortality rate with chlorfenapyr IRS treatment was 56.7%. The combination of IRS and LLIN induced overall mortality rates of 82% and 83% and this was significantly greater than the mortalities induced by LLINs alone (P = 0.0001) or by chlorfenapyr alone (P = 0.0001). Whereas the majority of mosquitoes killed by the IRS when no LLIN was present had already blood fed (i.e. 87.2% [130/148] of the dead mosquitoes had blood-fed beforehand), only a minority of dead mosquitoes had managed to blood feed when a LLIN was present in the IRS treated hut (i.e. 9.2% [8/87] of the dead mosquitoes had blood fed...
beforehand through the 6-hole nets and 26.3% [17/65] through the 80-hole nets).

This indicates that in the absence of a LLIN the mosquitoes will blood feed on the sleeper before alighting on the insecticidal wall. But in the presence of an LLIN many mosquitoes alight on the wall and are killed before managing to feed.

Table 1: Summary of results obtained for *Anopheles gambiae* in the experimental huts

<table>
<thead>
<tr>
<th>Hut Treatment</th>
<th>Untreated Net with 6 holes</th>
<th>Untreated Net with 80 holes</th>
<th>LLIN with 6 holes</th>
<th>LLIN with 80 holes</th>
<th>Chlorfenapyr IRS</th>
<th>Chlorfenapyr IRS + LLIN with 6 holes</th>
<th>Chlorfenapyr IRS + LLIN with 80 holes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total females caught</td>
<td>78</td>
<td>147</td>
<td>91</td>
<td>110</td>
<td>263</td>
<td>105</td>
<td>71</td>
</tr>
<tr>
<td>Total females dead</td>
<td>4</td>
<td>5</td>
<td>45</td>
<td>41</td>
<td>149</td>
<td>87</td>
<td>65</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>5.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>37.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83&lt;sup&gt;d&lt;/sup&gt;</td>
<td>82.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>95% Confidence limits</td>
<td>0.2-10.5</td>
<td>0.5-6.3</td>
<td>39.2-59.7</td>
<td>28.2-46.3</td>
<td>50.7-62.60</td>
<td>75.7-90.1</td>
<td>71.4-93.3</td>
</tr>
<tr>
<td>Total females blood fed</td>
<td>48</td>
<td>80</td>
<td>11</td>
<td>35</td>
<td>235</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Blood feeding (%)</td>
<td>56.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>89.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>26.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>95% Confidence limits</td>
<td>45.4-67.4</td>
<td>45.4-62.5</td>
<td>5.4-18.8</td>
<td>23.1-40.5</td>
<td>85.6-93.1</td>
<td>10.7-25.5</td>
<td>16.5-37.1</td>
</tr>
<tr>
<td>Blood feeding Inhibition (%)</td>
<td>0</td>
<td>0</td>
<td>78</td>
<td>42</td>
<td>0</td>
<td>68</td>
<td>51</td>
</tr>
<tr>
<td>Personal protection (%)</td>
<td>0</td>
<td>0</td>
<td>75</td>
<td>56</td>
<td>0</td>
<td>57</td>
<td>76</td>
</tr>
<tr>
<td>Total females in verandah trap exiting (%)</td>
<td>37</td>
<td>58</td>
<td>64</td>
<td>75</td>
<td>109</td>
<td>85</td>
<td>49</td>
</tr>
<tr>
<td>95% Confidence limits</td>
<td>36.4-58.5</td>
<td>3.5-47.4</td>
<td>61-79.7</td>
<td>59.5-76.9</td>
<td>35.5-47.4</td>
<td>73.4-88.5</td>
<td>52.7-73.9</td>
</tr>
</tbody>
</table>

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

*Culex quinquefasciatus*

The effects of the treatments on *Cx. quinquefasciatus* are presented in Table 2. Untreated nets with 80 holes were less protective against *Cx. quinquefasciatus* than untreated nets with six holes (P = 0.0001). In the absence of any net a higher proportion of mosquitoes were able to blood feed (77% managed to blood feed in the huts with chlorfenapyr IRS and no net), indicating that the holed net was a partial barrier to *Culex* biting and feeding. The pyrethroid on the LLIN was highly protective.
and the level of blood feeding inhibition by the insecticide was higher for *Cx. quinquefasciatus* than for *An. gambiae*. However, only a small proportion of *Cx. quinquefasciatus* were killed by the pyrethroid (~15%), and this proportion was smaller than the proportion of *An. gambiae* killed by the same treatment. Chlorfenapyr IRS killed 32.3% of *Cx. quinquefasciatus*. The combination of IRS and LLIN was additive, killing in the range of 40-50%. As was observed with *An. gambiae*, the combination was protective against *Cx. quinquefasciatus* (mostly due to the LLIN component) and also succeeded in killing many *Cx. quinquefasciatus* (mostly due to the IRS component). As with *An. gambiae*, the proportion of *Cx. quinquefasciatus* blood feeding was dependent on the number of holes in the LLIN, irrespective of whether chlorfenapyr IRS was present or not.

Table 2: Summary of results obtained for *Culex quinquefasciatus* in the experimental huts

<table>
<thead>
<tr>
<th>Hut Treatment</th>
<th>Untreated Net with 6 holes</th>
<th>Untreated Net with 80 holes</th>
<th>LLIN with 6 holes</th>
<th>LLIN with 80 holes</th>
<th>Chlorfenapyr IRS</th>
<th>Chlorfenapyr IRS + LLIN with 6 holes</th>
<th>Chlorfenapyr IRS + LLIN with 80 holes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total females caught</td>
<td>533</td>
<td>1370</td>
<td>1014</td>
<td>1018</td>
<td>1507</td>
<td>1260</td>
<td>1083</td>
</tr>
<tr>
<td>Total females dead</td>
<td>9</td>
<td>27</td>
<td>152</td>
<td>133</td>
<td>487</td>
<td>642</td>
<td>455</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>1.7</td>
<td>2</td>
<td>15</td>
<td>13.1</td>
<td>32.3</td>
<td>51</td>
<td>42</td>
</tr>
<tr>
<td>95% Confidence limits</td>
<td>0.6-2.8</td>
<td>1.2-2.7</td>
<td>12.8-17.2</td>
<td>11.0-15.1</td>
<td>30.0-34.7</td>
<td>48.2-53.7</td>
<td>39.1-45.0</td>
</tr>
<tr>
<td>Total females blood fed</td>
<td>180</td>
<td>666</td>
<td>55</td>
<td>124</td>
<td>1157</td>
<td>52</td>
<td>107</td>
</tr>
<tr>
<td>Blood feeding (%)</td>
<td>33.8</td>
<td>48.6</td>
<td>5.4</td>
<td>12.2</td>
<td>76.8</td>
<td>4.1</td>
<td>9.9</td>
</tr>
<tr>
<td>95% Confidence limits</td>
<td>29.8-37.8</td>
<td>46.0-51.3</td>
<td>4.0-6.8</td>
<td>10.2-14.2</td>
<td>74.6-78.9</td>
<td>3.0-5.2</td>
<td>8.1-11.7</td>
</tr>
<tr>
<td>Blood feeding Inhibition (%)</td>
<td>0</td>
<td>0</td>
<td>84</td>
<td>75</td>
<td>0</td>
<td>88</td>
<td>80</td>
</tr>
<tr>
<td>Total females in verandah trap</td>
<td>225</td>
<td>389</td>
<td>677</td>
<td>603</td>
<td>638</td>
<td>784</td>
<td>687</td>
</tr>
<tr>
<td>Exiting (%)</td>
<td>42.2</td>
<td>28.4</td>
<td>66.8</td>
<td>59.2</td>
<td>42.3</td>
<td>62.2</td>
<td>63.4</td>
</tr>
<tr>
<td>95% Confidence limits</td>
<td>38.46.4</td>
<td>26-30.8</td>
<td>63.9-70</td>
<td>56.2-62.2</td>
<td>40.45</td>
<td>60-65</td>
<td>68-66.3</td>
</tr>
</tbody>
</table>

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

The results for the molecular studies are presented in Table 3. For each treatment, the frequency of the kdr allele did not differ between the survivor and dead collections of *An. gambiae* at the 5% significance level. These results did not show any selective advantage to kdr in the presence of the LLIN. Nor did it show any selective neutrality or disadvantage to kdr in the presence of the combination treatment. Initial genotyping of 100 adult *An. gambiae*, which emerged from larvae collected at the field site (Akron), showed a kdr frequency of 0.91, a value consistent with the samples collected from the huts. This high frequency of kdr and the relatively small samples analysed made it impossible for the current study to demonstrate any differential selection of kdr between the LLIN and combination treatments.

Table 3 Kdr allelic frequency (kdr alleles/total kdr and susceptibility alleles) among live and dead *Anopheles gambiae*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Alive</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorfenapyr (500mg/m2) IRS + LLIN with 80 holes</td>
<td>0.77 (14/18)</td>
<td>0.84 (32/38)</td>
</tr>
<tr>
<td>Chlorfenapyr (500mg/m2) IRS</td>
<td>1.0 (25/25)</td>
<td>0.90 (18/20)</td>
</tr>
<tr>
<td>LLIN with 80 holes</td>
<td>0.82 (33/40)</td>
<td>0.86 (19/22)</td>
</tr>
<tr>
<td>Untreated LLIN with 80 holes</td>
<td>0.82 (41/50)</td>
<td>0.89 (16/18)</td>
</tr>
</tbody>
</table>

Discussion

A combination of IRS and LLINs can only be justified economically if it provides greater levels of protection or greater transmission control than is achievable by the single interventions. The present comparisons show that the combining of chlorfenapyr IRS and pyrethroid LLINs in the same hut provides personal protection
(mostly attributable to the LLIN component) and transmission control potential (mostly but not wholly attributable to mortality induced by IRS) over and above what the individual components are able to achieve. With the massive injection of international aid by the Global Fund and President’s Malaria Initiative for universal coverage of LLINs and IRS transmission control, the roll-out of such a combination intervention appears well justified on the basis of the present suggestive small scale study. The spectre of resistance haunts our capacity to control malaria in the future. Pyrethroid resistance due to kdr or metabolic mechanisms is springing up in countries across sub-Saharan Africa [5]. While clear demonstration that such resistance is impacting negatively on control has yet to be made, there is growing evidence that pyrethroid- treated ITNs and LLINs provide less protection in areas such as southern Benin where multiple pyrethroid resistance has become prevalent [6,8]. Confronted with resistance, LLINs start to lose their protectiveness once they become holed and the more holes they accrue the less protective they become, as demonstrated in the present study. Previously it was shown that chlorfenapyr is capable of controlling pyrethroid-resistant An. gambiae and Cx. quinquefasciatus when applied as IRS [17]. The most important new findings from the current study were the additive levels of mortality and the reduced levels of blood feeding that can be achieved when chlorfenapyr IRS is combined with pyrethroid LLIN. A mortality of ~80% is similar to what can be achieved with pyrethroid IRS in an area of full susceptibility [6]. Taken together with the partial protection still to be had from LLINs, the combination of LLINs and chlorfenapyr IRS may prove to be a route out of the predicament presented by pyrethroid resistance.

These findings stand in contrast to the experimental hut studies in Burkina Faso in which a combination of pyrethroid-treated wall linings and ITN failed to induce any
increase in mortality of malaria vectors over that of ITN alone [13]. This difference can be attributed to the fact that the vector population in the current study is fully susceptible to the active component (chlorfenapyr) in the IRS treatment. These results corroborate previous experimental hut studies in Benin and in Burkina Faso where pyrethroid-treated nets were combined with carbamate-treated plastic wall sheetings to which there was also little or no resistance [15]. The relatively high mortality of mosquitoes in chlorfenapyr IRS-treated huts confirms the potential of the pyr-ole insecticide as an alternative IRS treatment [17]. Hut trials of IRS, whether with pyrethroid, carbamate, OP or pyrole [6,17,20], show little or no evidence of blood feeding inhibition among the mosquitoes collected from the huts. The inference is that hut-entering mosquitoes approach, contact and feed upon the host before resting on the insecticide-treated walls where they then pick up a lethal dose. The current trial supports that inference. But when faced with a barrier presented by the LLIN, some mosquitoes succeed in penetrating the holes and feed, while others fly away unfed from the net, not to leave the hut but to alight on the walls where they then pick up a lethal dose of chlorfenapyr before resuming host-seeking flights. This train of events could explain the higher proportion of unfed, dead mosquitoes in the combination LLIN/IRS huts than in the single intervention LLIN or IRS huts. It is perhaps the to-ing and fro-ing between bed net and wall that results in higher pick-up of insecticide and higher mortality rates than is achieved by blood-fed mosquitoes that after feeding on the host simply alight on the treated wall and remain stationary. Cx. quinquefasciatus, the nuisance mosquito in urban West Africa and filariasis vector in East Africa, is strongly resistant to pyrethroids and consistently records low mortality rates (less than 15%) in the presence of LLINs in experimental huts studies [21]. This is due to multiple resistance mechanisms to pyrethroids and
organophosphates [8]. In the current study, mortality of Cx. quinquefasciatus increased to 51% when the LLINs were combined with chlorfenapyr IRS. The fact that the combination killed up to three times more Cx. quinquefasciatus mosquitoes than LLINs alone enhances the acceptability and compliance of this combined strategy for malaria control in peri-urban settings.

Conclusion

Combining chlorfenapyr IRS and LLINs has an additive effect on the mortality of pyrethroid-resistant mosquitoes. In areas of high pyrethroid resistance or high transmission intensity, control programmes with sufficient resources should consider implementing a combination intervention of LLIN plus non pyrethroid-based IRS. Chlorfenapyr IRS is shown to be an ideal supplement to pyrethroid LLIN for improving the control of pyrethroid-resistant mosquitoes.

Acknowledgements

This project was financially supported by the Innovative Vector Control Consortium (IVCC). The authors are grateful to Dr Robert Sloss and David Malone of IVCC for advice and support. Without the encouragement, drive and commitment of Dr Robert Farlow, Dr Susanne Stutz and Egon Weinmuller of BASF, this project would not have been possible. LSHTM and CREC are members of the Pan African Malaria Vector Research Consortium http://www.pamverc.org. Corine Ngufor, Raphael N’Guessan, Alex Asidi and Mark Rowland are supported by the Malaria Centre of the London School of Hygiene & Tropical Medicine http://malaria.lshtm.ac.uk/.

Authors’ contributions

CN: Project supervision, data entry, data analysis and interpretation, drafted the manuscript. RN: Project design and supervision, data interpretation, manuscript
revision. PB, AO, EV: Conducted the field trials, molecular analyses, bioassay testing. AA: Co-supervision, data processing, manuscript revision. MA: Co-design of the project, manuscript review and revision. MR: Project design, coordination with IVCC and BASF, interpretation of the results, revision of the manuscript. All authors read and approved the final manuscript.

References

1. WHO: Global malaria programme; indoor residual spraying; use of indoor residual spraying for scaling up global malaria control and elimination World Health Organization, Geneva; 2006.


5. WHO: Technical basis for action against insecticide resistance: preserving the effectiveness of modern malaria vector control; Meeting report 2011.


12. Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, Strode C: Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from Southern Benin and Nigeria. BMC Genomics 2008, 9:538.


Chapter 4

Insecticide treated net wall hangings for malaria vector control: an experimental hut study in North-eastern Tanzania

The material presented in this chapter has been published as:

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

1. For a 'research paper' already published
1.1. Where was the work published? Malaria Journal
1.2. When was the work published? 17/09/2014
1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion N/A

1.3. Was the work subject to academic peer review? Yes
1.4. Have you retained the copyright for the work?
   If yes, attach evidence of retention
   If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published.
2.1. Where is the work intended to be published?
2.2. List the paper's authors in the intended authorship order
2.3. Stage of publication - Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press:

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)
   I co-designed and supervised/performed the study, analysed the data, co-interpreted the findings and drafted the manuscript. I am corresponding author for the submission process and for enquiries.
   
   Candidate's signature ____________________________

   Supervisor or senior author's signature to confirm role as stated in (3)
   ____________________________
Insecticide treated net wall hangings for malaria vector control: an experimental hut study in North-eastern Tanzania

Abstract

Background
Alternative long-lasting, practical and effective tools for applying insecticides on home walls against malaria vectors need to be developed. The use of wall hangings made from netting on interior walls for aesthetic purposes is a common practice in rural communities. Insecticide-treated net wall hangings can be produced in a long-lasting format and used in an approach that simulates indoor residual spraying (IRS).

Methods
The efficacy of net wall hangings (NWH) treated with the residual organophosphate insecticide, pirimiphos methyl (1g/sq m), was evaluated in experimental huts against malaria vectors in Muheza, Tanzania. To determine the optimum level of wall coverage required, NWH were tested on ceiling only, two walls, four walls, or four walls plus ceiling. Comparison was made with deltamethrin-treated NWH on two walls.

Results
Pirimiphos methyl (p-methyl)-treated NWH (on two walls) killed significantly higher proportions of anophelines (92% of Anopheles gambiae and 79% of Anopheles funestus) than the deltamethrin-treated NWH (15% of An. gambiae and 17% of An. funestus) (P<0.001). WHO susceptibility tests showed that the local vector population was susceptible to the organophosphates but resistant to pyrethroids. Mortality rates were significantly higher in huts with p-methyl NWH on two walls (92% for An. gambiae and 79% for An. funestus) than on ceiling only (61% for An. gambiae and 62% for An. funestus, P<0.05). There was no improvement in mortality when wall coverage with p-methyl NWH increased beyond two walls. Blood-feeding rates with p-methyl NWH were generally high across all the treatments (52-77%) and did not differ significantly from the control (64-67%). There was no evidence of reduced blood-feeding or increased exiting with increase in wall coverage with p-methyl NWH.
Conclusions

Net wall hangings are an effective means of delivering insecticide in the domestic environment against malaria vectors. They could be more practical and acceptable than IRS thus showing enormous potential for malaria vector control. Appropriate binding or incorporation technology needs to be developed to enable the production of p-methyl NWH with residual activity lasting over a number of years.
**Background**

Indoor residual spraying (IRS) has a distinguished historical role in the control of malaria. It has been one of the main interventions leading to the elimination of malaria in half of the world’s regions, such as in much of Southern Europe, North America, Japan, Central and South Asia and Latin America [1, 2]. In recent years, IRS has been scaled up significantly in Africa, contributing to the recent reductions in malaria morbidity and mortality [3, 4]. However, sustaining user compliance and overcoming the operational challenges associated with the implementation of IRS remains a major challenge [5] especially in holo-endemic areas in sub-Saharan Africa.

Insecticide-treated materials can be applied on home walls in a novel approach that simulates IRS. Long-lasting, pyrethroid-treated, plastic sheeting, which was originally developed for malaria control in refugee situations [6], has also been produced for use on the interior of home walls [7, 8]. This tool is popularly referred to as durable lining (DL). Pyrethroid-treated DL is manufactured using binding technology, which allows the insecticide to diffuse slowly to the surface in a controlled fashion, making it a long-lasting alternative to IRS. In a recent multicentre study, pyrethroid DL showed potential to overcome user-fatigue and the operational challenges associated with the use of recurrent IRS treatments in holo-endemic areas [8]. However, there are some concerns over the time required to install DL in homes and the durability of the plastic sheeting on home walls. More practical and flexible versions of this approach need to be developed.
The use of hangings made from different sorts of materials on interior home walls for the purpose of decoration is a common human practice. Home-owners in rural Africa sometimes cover their walls with wall hangings made from netting or curtain material. Insecticide-treated net wall hangings could operate in a similar manner to IRS if mosquitoes that enter the home rest on them. Because the netting material is widely available and much lighter in weight than plastic sheeting, net wall hangings (NWH) could be a more practical and acceptable means of delivering insecticides in the domestic environment.

Pyrethroids remain the most suitable insecticides for treating long-lasting, insecticide-treated bed nets (LLINs) owing to their rapid knockdown effect, low cost and low mammalian toxicity. To reduce selection pressure for pyrethroid resistance and help preserve this class of insecticides, the WHO recommends that pyrethroids be reserved for LLINs since LLINs will remain the most important public health intervention [9, 10]. Hence non-pyrethroid versions of DL and NWH are more desirable. They could be used on their own or in combination with LLINs for improved control of pyrethroid-resistant malaria vectors and for managing insecticide resistance. The current study, investigated the efficacy of NWH treated with pirimiphos methyl (p-methyl) CS (Actellic®300 CS), a WHO approved organophosphate insecticide, in experimental huts in Muheza, northeastern Tanzania. Comparison was made to pyrethroid (deltamethrin)-treated NWH. To determine the level of wall coverage required for optimum impact, NWH were tested on ceiling only, two walls, four walls, or four walls plus ceiling. WHO susceptibility tests were performed to investigate the existence of resistance to a range of insecticides recommended for IRS.
Methods

Study sites and experimental huts
The study was carried out in six experimental huts of East African design in Zeneti village in Muheza District, northern Tanzania (5°13’S and 38°39’E, altitude 193 m). *Anopheles gambiae s.l.* is the predominant vector in the wet season while *Anopheles funestus* is predominant in the dry season [11]. The trial ran between June and July of 2011 during the months that both species co-exist. The experimental huts conformed to the WHOPES-approved design [12] with some minor adjustments as described by Malima *et al.* [13]. The huts are made of brick plastered with cement on the inside with a corrugated iron roof, which is lined with palm thatch and has an eave gap below. There are veranda and window traps on each side of the hut. Two of the verandas were left open to allow mosquitoes to enter the huts through the eaves while the other two were screened to capture any mosquitoes that exited via the eaves.

Treatment and hanging of net wall hangings
Netting material used was 100-denier polyester netted fabric purchased from the local market. These were treated by dipping in either pirimiphos methyl CS (Actellic® 300CS, Syngenta, Basle, Switzerland) at 1 g/sq m or deltamethrin SC (K-Othrine 10SC, Bayer, Monnheim, Germany) at 55 mg/sq m. Treated NWH were left to dry in the shade for 24 hours before being hung onto the hut walls. In order to avoid contamination of the walls when rotating the treatments between the huts, an underlay of untreated plastic sheeting was used to separate the walls from the treated materials and these were rotated along with the respective treatments.
Treated nettings were simply hung onto nails that had been fitted at the top edge of the hut walls. Areas of the treated NWH covering the windows were then cut out to allow exit of mosquitoes to window traps.

**Sleepers and treatments**

Six adult men served as volunteer sleepers and were rotated between huts on successive nights to adjust for any variation in individual attractiveness to mosquitoes. Sleepers gave informed consent and were provided with chemoprophylaxis prior to the trial. They slept in the huts from 20:00 to 05:00 each night. White sheets were laid over the veranda and room floors to ease the collections of knocked down mosquitoes. Mosquitoes were collected each morning at 05:00 from under bed nets, floors, walls, ceilings, verandas, and window traps using aspirators and torches. The collections were transported to the laboratory where mosquitoes were identified to species and scored as blood fed or unfed and live or dead. Live mosquitoes were held in netted plastic cups and supplied with 10% glucose solution and delayed mortality was recorded after 24 hours. Male mosquitoes were not scored. Data were collected for 36 nights.

Ethical approval for the study was obtained from the Ethics Review Boards of the London School of Hygiene and Tropical Medicine and the Tanzanian National Institute of Medical Research.

The following six treatments were compared in the experimental huts:

1. Untreated control hut
2. Deltamethrin NWH on two walls
3. P-methyl NWH on ceiling
4. P-methyl NWH on two walls
5. P-methyl NWH on four walls
6. P-methyl NWH on four walls and ceiling (full coverage)

The treatments were rotated through the huts on a weekly basis following a Latin Square design to account for positional differences in attractiveness between the huts.

**Entomological outcomes**

The impact of each treatment was expressed in terms of the following entomological outcomes;

7. Deterrence: percentage reduction in the number of mosquitoes caught in treated hut relative to the number caught in the control hut
8. Exiting rates: due to potential irritant effect of treatments expressed as percentage of the mosquitoes collected from the veranda trap.
10. Blood feeding inhibition which is the reduction in blood-feeding rate relative to the control. Blood feeding inhibition (%) was calculated as follows:

\[
\frac{100(Bfu - Bft)}{Bfu} \times 100
\]

Where Bfu is the proportion of blood-fed mosquitoes in the untreated control huts and Bft is the proportion of blood-fed mosquitoes in the huts with a specific insecticide treatment.

11. Mortality rates: percentage of dead mosquitoes in hut at the time of collection and after a 24-hour holding period.
Residual activity
To determine the residual activity of the treated NWH, WHO cone bioassays were performed in situ at the beginning and the end of the trial. A total of 100 mosquitoes of the laboratory susceptible An. gambiae Kisumu strain were tested on each type of treated NWH in the experimental huts. The mosquitoes were exposed for 30 minutes following WHOPES guidelines [12]. Mortality was recorded after a 24-hour holding period.

Susceptibility testing
To test for the existence of resistance to a range of insecticides recommended for IRS, WHO susceptibility tests were performed on adult An. gambiae mosquitoes which emerged from larvae collected from the study area. Mosquitoes at three to five days old were exposed for one hour to filter papers treated to the recommended diagnostic dose of each insecticide in cylinder bioassays [14]. For pirimiphos methyl, a range of concentrations (0.025-0.25%) was tested and comparison was made to the laboratory-susceptible An. gambiae Kisumu strain. For each insecticide and each concentration of p-methyl, a total of 95-100 adult female mosquitoes were tested and the proportion dead recorded after 24 hours.

Knock down resistance (kdr) genotype testing
To investigate the presence of the kdr (L1014S) gene in the An. gambiae vector population in Muheza, genomic DNA was extracted from a random sample of mosquitoes collected from the experimental huts using the Livak procedure [15].
Molecular detection of the L1014S mutation alleles was carried out by real-time Taqman PCR as described by Bass et al. [16]

Data analysis
The numbers of mosquitoes entering the huts with the different treatments was analysed by negative binomial regression. The effects of the treatments on each of the main proportional entomological outcomes (exophily, blood feeding and mortality) were assessed using binomial generalized linear mixed models (GLMMs) with a logit link function, fitted using the ‘lme4’ package for R. A separate model was fitted for each outcome and for each mosquito species. In addition to the fixed effect of each treatment, each model included random effects to account for the following sources of variation: between the six huts used in the studies; between the six sleepers who slept in the huts; between the six weeks of the trial; and finally an observation-level random effect to account for variation not explained by the other terms in the model (over dispersion).

Results

Susceptibility tests
The WHO susceptibility tests showed that the local vector population was resistant to pyrethroids but susceptible to organophosphates (Figure 1). Mortality rates of wild anopheline mosquitoes from the study site were 100% across all the concentrations of p-methyl tested, confirming susceptibility to the organophosphate. The genotyping results revealed a kdr allele (L1014S) frequency of 0.22 in a random sample of 47 An. gambiae s.l. mosquitoes collected from the experimental huts.
Experimental hut trial

The numbers of wild anopheline mosquitoes entering, feeding and dying in the experimental huts during the trial are presented in Table 1 for *An. gambiae* and Table 2 for *An. funestus*. The exiting, blood-feeding and mortality rates are presented in Figures 2-4 respectively. A total of 423 *An. gambiae* and 277 *An. funestus* were collected from the experimental huts during the trial (Tables 1 and 2). The highest numbers were collected in the control hut. For both species, the average catch per night did not differ significantly between the p-methyl NWH on two walls and the pyrethroid DL on two walls. The level of deterrence with p-methyl NWH showed an increase with increasing wall coverage.

Hut exiting rates

Exiting rates were significantly higher in the huts with the treated NWH than the control (Figure 2) (for both species, P<0.05 for each treatment relative to control). The proportion exiting did not differ between the deltamethrin NWH (two walls) hut and the p-methyl NWH (two walls) hut for either species (P= 0.71 for *An. gambiae*, P= 0.85 for *An. funestus*). There was no evidence of a relationship between treatment-induced exiting and the level of wall coverage with p-methyl NWH.

Blood feeding

Blood-feeding rates were very high across all the treatments (Figure 3) hence the treated NWH provided very little or no blood-feeding inhibition relative to the control (Tables 1 and 2). The proportions blood-fed in huts with p-methyl NWH for both anopheline species (range of 52-75%) were not significantly different from the control hut (64% of *An. gambiae* and 67% of *An. funestus*, P>0.05) (Figure 3). The
proportion feeding in the hut with deltamethrin NWH on two walls (51% of *An. gambiae* and 61% of *An. funestus*) was also not significantly different from the proportion feeding in the hut with p-methyl NWH on two walls (62% of *An. gambiae* and 67% of *An. funestus*) (P= 0.07 for *An. gambiae*, P= 0.1 for *An. funestus*). As with exophily, the data showed no evidence of a relationship between the level of wall coverage with p-methyl NWH and blood-feeding rate by *Anopheles* species.

**Mortality**

Figure 4 presents the mortality rates in the different experimental huts. The treated NWH generally killed significantly larger proportions of mosquitoes than the control. Mortality of both anopheline species was much higher with p-methyl NWH on two walls (92% of *An. gambiae* and 77% of *An. funestus*) compared to deltamethrin NWH on two walls (15% of *An. gambiae* and 17% of *An. funestus*) (P<0.001 for both species) (Figure 4). The proportion dead also increased significantly in the hut with p-methyl NWH on two walls (92% for *An. gambiae* and 79% for *An. funestus*) compared to the huts with p-methyl NWH on ceiling only (61% for *An. gambiae* and 62% for *An. funestus*) (P= 0.004 for *An. gambiae* and P= 0.01 for *An. funestus*). Mortality rates in huts with p-methyl NWH on four walls and four walls plus ceiling were 87% and 90% respectively for *An. gambiae* and 75% and 77% respectively for *An. funestus* but these values did not differ significantly from that with p-methyl NWH on two walls (P>0.05 for both species) (Figure 4). Hence, the results did not show an improvement in mortality of either species when wall coverage with p-methyl NWH increased beyond two walls.
Residual activity

At the beginning of the trial both deltamethrin and p-methyl-treated NWH induced 100% mortality with the laboratory-susceptible An. gambiae Kisumu strain in WHO cone bioassays. By the end of the trial (after six weeks), mortality with p-methyl-treated NWH declined to 60% but remained >80% with deltamethrin-treated NWH (P<0.01) (Figure 5). Deltamethrin (at 55 mg/sq m) therefore showed a longer residual activity on the nylon netting material than p-methyl (at 1 g/sq m). No mortality was recorded in the control hut.

Discussion

New or improved practical and adaptable tools for delivering insecticides against malaria vectors are urgently needed. The current study was designed to investigate the potential of insecticide-treated NWH as a novel system for delivering insecticides indoors. The results indicate that mosquitoes will readily rest on them and be killed in the process.

P-methyl-treated NWH (on two walls) induced much higher mortality rates than deltamethrin-treated NWH (on two walls). The vector population was susceptible to organophosphates but resistant to pyrethroids as demonstrated in the WHO susceptibility bioassays. Insecticide resistance could have combined with pyrethroid excitorepellency to reduce the overall level of mortality in the partially treated rooms by causing the re-distribution of the surviving resistant mosquitoes on the untreated walls where they settle and evade any toxic effect of the insecticide. A previous
survey carried out in 2009/2010 in the study area (Muheza) showed full susceptibility to pyrethroids [17]. The present study therefore demonstrates a rapid development of resistance in this vector population between 2010 and 2011 and the impact that this may have on pyrethroid-based vector control tools. This rapid change from susceptibility to resistance could be due to high selection pressure posed by the massive distribution of LLINs in the Muheza district in 2010 following the Tanzanian government’s catch-up campaign to distribute nine million LLINs to children aged less than five years [18, 19]. Though the kdr gene was detected, further studies need to be performed to investigate the presence of other mechanisms of resistance to pyrethroids, which, in addition to the kdr, may have contributed to the level resistance observed. The impact of this shift in resistance status on the efficacy of the LLINs being used in the area also needs to be assessed.

Increasing wall coverage with p-methyl NWH from two walls to four walls or four walls plus ceiling (full coverage) did not improve on mortality. In contrast, a previous study with pyrethroid DL in an area with much higher levels pyrethroid resistance showed that it was necessary to cover all four walls before a significant level of mortality could be achieved [7]. However, people do not always cover all their walls with wall hangings since it may be aesthetically more appealing and more practical to cover a few walls. The results of the current study therefore suggest that p-methyl NWH could be a more scalable and cost-effective intervention than pyrethroid-treated NWH or pyrethroid DL. Nevertheless, the vector population was fully susceptible to the organophosphate but resistant to the pyrethroid. The performance of p-methyl NWH and the level of wall coverage required may depend on the resistance status of the targeted vector population.
Although mortality rates were high, blood-feeding rates with the treated NWH were generally high. This provides evidence that NWH act like IRS rather than insecticide-treated nets. With IRS-like treatments mosquitoes would normally first feed on the person sleeping in a hut or house before resting on the IRS-treated wall where they pick up the insecticide, unless there is an additional tool to prevent blood feeding. In a parallel study, combining p-methyl NWH with LLINs improved blood-feeding inhibition significantly (due to the LLIN component) [20]. Such combinations have also shown potential for insecticide resistance management whereby insect genotypes which are resistant to the insecticide in one intervention can be killed by the other insecticide if they are not resistant to both insecticides [21-23].

Although blood feeding rates with p-methyl NWH were high, mosquitoes were deterred from entering the treated huts compared to the control hut and this deterrent effect increased with increasing wall coverage with p-methyl NWH. Previous studies also demonstrated an increase in hut deterrence as wall coverage with pyrethroid DL increased [7]. Deterrence of mosquitoes from insecticide treated experimental huts is usually induced by the irritant or repellent effect of the insecticide. While this effect has been mostly associated with pyrethroids, some studies have also shown reduced mosquito entry in experimental huts treated with microencapsulated p-methyl IRS [24, 25]. By deterring mosquitoes from treated homes, p-methyl NWH shows potential to significantly reduce human-vector contact which could contribute substantially to reducing malaria transmission.
P-methyl showed a lower residual activity on NWH than deltamethrin. Studies with this slow-release microencapsulated formulation of the insecticide have shown prolonged residual activity on cement walls [24]. The insecticide particles probably scaled off the treated netting material over time due to movements during the rotations. Nevertheless, because the study was designed as a proof of concept to demonstrate the relevance of NWH, the nettings used were hand-dipped, so the short residual activity is not unexpected.

The netting material is a very benign substrate and as observed with ITNs, many kinds of insecticides can be readily applied to netting. They can then be delivered on walls through this NWH approach. The mortality rates observed in the current study show that p-methyl-treated NWH have potential to control indoor resting malaria vectors. It took less than 10 minutes for a team of two individuals to set up NWH on the four walls of an experimental hut whereas a previous study reported 60-75 minutes for three individuals to install pyrethroid DL in a house [8]. NWH is also lighter in weight and can be simply hung onto nails fitted at the edges of the ceiling by home owners. The DL plastic sheeting on the other hand is heavier and its installation usually requires a skilled team of individuals to ensure that it is well fitted as to reduce the risk of it falling off the wall. Hence NWH may be more practical or popular than DL or IRS. However, to guarantee added benefit from wide scale use of p-methyl NWH over standard IRS, the residual activity will need to last for years rather than months. Advanced binding technology therefore needs to be applied to develop long-lasting versions of NWH. In the meantime, hand-treated NWH can be used in the place of IRS in transitory house structures and in houses with mud walls which usually show very low residual activity with IRS applications [24]. NWH could
also be used to cover eave gaps and cracks and crevices on walls as to reduce mosquito entry into homes.

**Study limitations**

The numbers of mosquitoes collected in some of the huts were few especially the huts with p-methyl NWH on four walls and four wall plus ceiling. However, this effect could be attributed to the low density of mosquitoes in the study area and increased deterrence of mosquitoes from these huts posed by the higher levels of wall coverage with p-methyl NWH. Nevertheless, the trends observed were clear showing significantly higher mortality rates in huts with p-methyl NWH than huts with the untreated control and the pyrethroid NWH.

**Conclusion**

The results demonstrate that NWH are an effective means of delivering insecticides in the domestic environment since mosquitoes rested on them and were killed in the process. They could be more practical and acceptable than IRS or DL showing potential for malaria vector control. Appropriate binding or incorporation technology needs to be developed to enable the production of p-methyl NWH with residual activity lasting over a number of years.

**Competing interests**

The authors declare that they have no competing interests.
Author contributions

CN co-designed the study, supervised the project, analyzed the data and drafted the manuscript. PT performed the hut trial, bioassays, molecular analysis and commented on the manuscript. RM, MK and WK co-supervised the field work. MR designed the study and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Andy Bywater of Syngenta for providing the insecticide, Alex Wright of London School of Hygiene and Topical Medicine (LSHTM) for coordinating the molecular analysis and Prof. Hilary Ranson of Liverpool School of Tropical Medicine (LSTM) for supporting the study. Special thanks go to the volunteer sleepers and field team in Muheza for participating in the study. CN, MK and MR are supported by the Malaria Centre of the London School of Hygiene and Tropical Medicine: [http://malaria.lshtm.ac.uk/](http://malaria.lshtm.ac.uk/). The research leading to these results has received funding from the European Union Seventh Framework Programme FP7 (2007–2013) under grant agreement no. 265660 AvecNet. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Table 1 Numbers of *An. gambiae* entering, feeding and dying in p-methyl NWH treated experimental huts in Muheza, Tanzania

<table>
<thead>
<tr>
<th>Hut Treatment</th>
<th>Control (untreated DL)</th>
<th>Pyrethroid NWH on 2 walls</th>
<th>P-methyl NWH on 2 walls</th>
<th>P-methyl NWH on Ceiling</th>
<th>P-methyl NWH on 4walls</th>
<th>P-methyl NWH on 4 walls and ceiling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total females caught</td>
<td>171</td>
<td>86</td>
<td>57</td>
<td>60</td>
<td>35</td>
<td>14</td>
</tr>
<tr>
<td>Average catch per night</td>
<td>4.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Deterrence (%)</td>
<td>0</td>
<td>51</td>
<td>68</td>
<td>66</td>
<td>80</td>
<td>92</td>
</tr>
<tr>
<td>Total females blood fed</td>
<td>109</td>
<td>44</td>
<td>42</td>
<td>37</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Blood feeding Inhibition (%)</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>5</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Total dead</td>
<td>7</td>
<td>13</td>
<td>35</td>
<td>55</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>Corrected mortality (%)</td>
<td>0</td>
<td>11</td>
<td>59</td>
<td>92</td>
<td>90</td>
<td>86</td>
</tr>
</tbody>
</table>

Values along a row sharing the same letter superscript are not significantly different at the 5% level.

Table 2 Numbers of *An. funestus* entering, feeding and dying in p-methyl NWH treated experimental huts in Muheza, Tanzania

<table>
<thead>
<tr>
<th>Hut Treatment</th>
<th>Control (untreated DL)</th>
<th>Pyrethroid NWH on 2 walls</th>
<th>P-methyl NWH on Ceiling</th>
<th>P-methyl NWH on 2 walls</th>
<th>P-methyl NWH on 4walls</th>
<th>P-methyl NWH on 4 walls and ceiling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total females caught</td>
<td>136</td>
<td>60</td>
<td>28</td>
<td>31</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Average catch per night</td>
<td>3.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Deterrence (%)</td>
<td>0</td>
<td>56</td>
<td>79</td>
<td>77</td>
<td>89</td>
<td>95</td>
</tr>
<tr>
<td>Total females blood fed</td>
<td>91</td>
<td>37</td>
<td>19</td>
<td>21</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Blood feeding Inhibition (%)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total dead</td>
<td>7</td>
<td>7</td>
<td>12</td>
<td>25</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Corrected mortality (%)</td>
<td>0</td>
<td>6</td>
<td>39</td>
<td>78</td>
<td>76</td>
<td>74</td>
</tr>
</tbody>
</table>

Values along each row sharing the same letter superscript are not significantly different at the 5% level.
Figure 1: Susceptibility of *An. gambie* from Muheza, Tanzania to insecticides; Mortality (%) in WHO cylinder bioassays during hut trials.

Figure 2: Exiting rates of Anopheline mosquitoes in experimental huts with insecticide treated net wall hangings. For each species, histograms bearing the same letter label are not significantly different at the 5% level.
Figure 3: Blood feeding rates of Anopheline mosquitoes in experimental huts with insecticide treated net wall hangings. For each species, histograms bearing the same letter label are not significantly different at the 5% level.

Figure 4: Mortality of Anopheline mosquitoes in experimental huts with insecticide treated net wall hangings. For each species, histograms bearing the same letter label are not significantly different at the 5% level.
Figure 5: Mortality (%) of laboratory susceptible *An gambiae* kisumu exposed to treated NWH in cone bioassays before and after the experimental hut trial.
References


15. Livak K: Organization and mapping of a sequence on the Drosophila melanogaster X and Y chromosomes that is transcribed during spermatogenesis. *Genetics* 1984, **107**: 611-634.


Chapter 5

Combining organophosphate treated wall linings with long lasting insecticidal nets for improved control of pyrethroid resistant *An gambiae*.

The material presented in this chapter has been published as:

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

1. For a 'research paper' already published
1.1. Where was the work published? PLoSOne
1.2. When was the work published? January 2014
1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion N/A

___________________________________________________________________
___________________________________________________________________
1.3. Was the work subject to academic peer review? YES
1.4. Have you retained the copyright for the work? OPEN ACCESS
If yes, attach evidence of retention
If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published. N/A
2.1. Where is the work intended to be published?
2.2. List the paper's authors in the intended authorship order
2.3. Stage of publication - Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)
I co-designed the study, supervised/performed the field activities, performed the molecular analysis, analysed the data, co-interpreted the findings and wrote the manuscript.

___________________________________________________________________

Candidate's signature

Supervisor or senior author’s signature to confirm role as stated in (3)
Abstract

**Background:** New approaches to delivering insecticides need to be developed to improve malaria vector control. Insecticidal durable wall lining (DL) and net wall hangings (NWH) are novel alternatives to indoor residual spraying which can be produced in a long-lasting format. Non-pyrethroid versions could be used in combination with long-lasting insecticidal nets for improved control and management of insecticide resistant vector populations.

**Method:** Experimental hut trials were carried out in Valley du Kou, Burkina Faso to evaluate the efficacy of pirimiphos methyl treated DL and NWH either alone or in combination with LLINs against pyrethroid resistant *Anopheles gambiae ss*. Comparison was made with pyrethroid DL. Mosquitoes were genotyped for *kdr* and *ace-1R* resistant genes to investigate the insecticide resistance management potential of the combination.

**Results:** The overall *kdr* and *ace-1R* allele frequencies were 0.95 and 0.01 respectively. Mortality with p-methyl DL and NWH alone was higher than with pyrethroid DL alone (>95% vs 40%; P<0.001). Combining pyrethroid DL with LLINs did not show improvement in mortality (48%) compared to the LLIN alone (44%) (P>0.1). Combining p-methyl DL or NWH with LLINs reduced biting rates significantly (8-9%) compared to p-methyl DL and NWH alone (>40%) and killed all *An gambiae* that entered the huts. Mosquitoes bearing the *ace-1R* gene were more likely to survive in huts with p-methyl DL alone (p<0.03) whereas all resistant and susceptible genotypes were killed by the combination.

**Conclusion:** P-methyl DL and NWH outperformed pyrethroid DL. Combining p-methyl DL and NWH with LLINs could provide significant epidemiological benefits against a vector population which is resistant to pyrethroids but susceptible to organophosphates. There was evidence that the single intervention would select *kdr* and *ace-1R* resistance genes and the combination intervention might select less strongly. Technology to bind organophosphates to plastic wall lining would be worth developing.
Introduction

Malaria vector control largely depends on a limited collection of tools. Long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) have contributed significantly to the recent reductions in malaria morbidity and mortality burdens [1], and these interventions are reliable and effective in a wide range of situations. LLINs are easy to deliver even in the most remote communities and hence have been more widely deployed in malaria endemic countries in sub-Saharan Africa. IRS requires more complex operational delivery systems; it is thus mostly used in a targeted approach. Alternative efficacious and practical tools for delivering insecticides indoors need to be urgently developed in order to diversify the “tool-box” for malaria vector control and to enhance capacity to effectively interrupt malaria transmission in holo-endemic areas in sub-Saharan Africa.

The covering of home walls with insecticidal materials is a novel approach that simulates IRS. Insecticide treated plastic wall linings also known as durable lining (DL) can be produced via the long-lasting net technology which incorporates the insecticide into the fibres before yarn extrusion. Long-lasting pyrethroid DL when used on interior walls, showed high acceptability and little or no decline in bioefficacy after 12-15 months with minimal loss of insecticide [2,3]. Due to the long-lasting technology, it is hoped that pyrethroid DL may only need to be replaced on walls after 3-4 years. It can therefore be regarded as a long-lasting alternative to IRS which would be vital for high malaria transmission areas where recurrent IRS treatments are normally required for interruption of transmission. DL also has the advantage of providing a more uniform covering of the wall with insecticide.
compared to IRS and of improving the interior appearance of traditional dwellings especially in rural areas [3]. However, in the current era of pyrethroid resistance [4,5], the future of pyrethroid DL is rather questionable. Studies on pyrethroid DL in West Africa revealed relatively low mortality rates of 37-47% against pyrethroid resistant mosquitoes in experimental hut trials [6,7]. Mortality rates >70% have been recorded with pyrethroid IRS in a pyrethroid susceptible area in West Africa [8]. To reduce selection pressure for pyrethroid resistance on malaria vectors, the WHO recommends that pyrethroids should be reserved only for treating LLINs since they remain the most appropriate class of insecticides for this purpose [9]. This requires that DL treated with alternative insecticides should be urgently investigated and developed. One potential candidate insecticide is the WHO-approved organophosphate insecticide pirimiphos methyl (p-methyl). A new micro-encapsulated formulation of p-methyl (Actellic CS) shows residual activity for up to 9 months as an IRS treatment on cement walls and has been shown to control pyrethroid resistant An gambiae [10].

In rural Africa, householders often cover their walls with wall hangings made from netting material to improve interior aesthetic appearance. Insecticide treated net wall hangings (NWH) could function in a manner which is similar to DL and could be a more acceptable, practical and innovative means for delivering insecticides indoors. Curtains treated with pyrethroids have been shown to be effective against vectors of dengue in South America [11,12]. The potential of such materials to control malaria vectors is yet to be fully explored.

It is now clear that the development and rapid spread of insecticide resistance in An gambiae populations across Africa [4,13] is well capable of undermining vector
control [8,14-16]. The World Health Organisation (WHO) calls for an immediate pro-active response to insecticide resistance to sustain the effectiveness of malaria vector control [9,17]. This requires investigating ways in which insecticide resistance management can be applied for vector control. One available strategy is to combine interventions which deliver unrelated insecticides in the same place and at the same time [17]. This approach has potential to improve the control of the insect vector population and manage the spread of insecticide resistant insect genotypes [18,19]. The latter is based on the concept that insect genotypes which are resistant to the insecticide in one intervention can be killed by the insecticide in the other intervention [20].

The aim of the current study was to investigate via a series of experimental hut trials whether DL or NWH treated with pirimiphos methyl (p-methyl) CS applied alone or in combination with LLINs has the potential to control malaria transmitted by pyrethroid resistant *Anopheles gambiae* s.s. in Burkina Faso. Comparison was made to currently available pyrethroid DL. Using molecular genotyping studies, the capacity of the combination to potentially manage insecticide resistance by preventing the selection of organophosphate and pyrethroid resistant genotypes was also investigated.

**Materials and Methods**

*Experimental huts*

The trials were carried out at the Centre Muraz experimental hut station in Valley du Kou 5 (4°25’ W, 11°24’ N) situated near Bobo-Dioulasso, in South-western Burkina Faso. The station is surrounded by a huge rice growing valley. The rainy season extends from June to October and the dry season from November to May. The rice
paddies provide extensive breeding sites for mosquitoes throughout the year. The two molecular forms M and S of *An. gambiae s.s.* occur in sympatry notably at the end of the rainy season [21]. The study was performed in 6 experimental huts of the WHOPES approved West African design between August and November of 2011. Permission to use the hut station was obtained from Centre Muraz. The experimental huts are built on concrete plinths surrounded by water-filled moats to prevent entry of scavenging ants. Veranda traps capture the exiting mosquitoes. The huts are made of brick plastered with cement on the inside, with a corrugated iron roof. The ceiling is made of thick polyethylene sheeting and the walls have four window slits (1cm gap) through which mosquitoes enter. Prior to the study, huts were refurbished to reduce any possibility of contamination from previous trials.

**Susceptibility tests**

During the trials, samples of adult *An. gambiae which emerged from larvae* collected from the experimental hut site (Valley du Kou 5) were tested in WHO test kits for susceptibility to pyrethroids using deltamethrin 0.05% treated papers and to organophosphates using p-methyl 0.25% treated papers. 0.25% was used as a diagnostic dose for p-methyl based on preliminary studies which showed a concentration of ~0.1% induced 100% mortality in the *An. gambiae* kisumu laboratory susceptible strain (Ranson et al, unpublished data).

**Experimental hut treatments**

Three experimental hut trials were carried out. The first two trials lasted 6 weeks and the third lasted 4 weeks. The first trial aimed to evaluate the efficacy of p-methyl treated DL and NWH against pyrethroid resistant *An. gambiae*, comparing them to currently available pyrethroid DL (ZeroVector®, Vestergaard Frandsen, Switzerland).
The level of interior coverage required for optimum impact (walls only versus walls and ceiling) was also investigated. The following six single treatments were tested in the first trial:

1. Untreated Control (untreated plastic sheeting)
2. Pyrethroid treated durable lining (ZeroVector®, Vestergaard Frandsen, Switzerland) on walls
3. P-methyl CS treated durable lining (p-methyl DL) on walls
4. P-methyl CS treated net wall hangings (p-methyl NWH) on walls
5. P-methyl DL on walls and ceilings
6. P-methyl NWH on walls and ceilings

In the second experimental hut trial, the p-methyl DL and NWH were combined with LLINs and compared to LLINs alone and p-methyl DL and NWH alone. The following six interventions were tested:

1. Untreated Net with 6 holes
2. Pyrethroid LLIN (Permanet® 2.0 Vestergaard Frandsen, Switzerland), with 6 holes
3. P-methyl DL on walls and ceilings
4. P-methyl NWH on walls and ceilings
5. P-methyl DL on walls and ceilings + Pyrethroid LLIN with 6 holes
6. P-methyl NWH on walls and ceilings + Pyrethroid LLIN with 6 holes

In the third trial we compared the combination of pyrethroid DL and LLIN to the combination of p-methyl DL and LLINs. The aim of this trial was to explore the advantage of p-methyl DL over currently available pyrethroid DL to see whether there was any benefit to using the organononophosphate over the pyrethroid on the
lining material in a situation of high pyrethroid resistance frequency. The following treatments were tested:

1. Untreated Net with 6 holes
2. Pyrethroid LLIN (Permanet® 2.0 Vestergaard Frandsen, Switzerland), with 6 holes
3. Pyrethroid DL (ZeroVector®, Vestergaard Frandsen, Switzerland) on walls and ceilings + Pyrethroid LLIN with 6 holes
4. P-methyl NWH on walls and ceilings + Pyrethroid LLIN with 6 holes

**Treatment of materials**

The DL was 50% shade cloth made of woven high density polyethylene (HDPE) plastic (Capatex Ltd, UK). The NWH was 100 denier nylon netted fabric purchased from the local market. These materials were treated at 1g/m² with micro-encapsulated primiphos methyl (p-methyl) CS (Actellic® 300CS [PP511 CS]) provided by Syngenta. The insecticide was applied onto the plastic sheets by spraying with a Hudson Xpert knapsack sprayer and to nettings by hand dipping. Treated materials were left to dry for 24 hours in the shade before being set up in the experimental huts. Pyrethroid treated DL used in the study was HDPE woven fibre sheet factory treated with deltamethrin at 175mg/m². The LLIN (Permanet® 2.0, Vestergaard Frandsen, Switzerland) was WHOPES approved, made of polyester fibres, factory-coated with a wash-resistant formulation of deltamethrin at a target dose of 55 mg/m². To simulate wear and tear, bednets were intentionally holed with six 16cm² diameter holes (4 at the sides and 2 at the ends) according to WHOPES guidelines.
Setting up treated materials to walls

In order to minimise contamination of the hut walls when rotating the treatments, a removable underlying layer of untreated material (plastic lining) was used to separate the walls from the treated materials and these were rotated along with the respective treatments. Treated plastic sheeting were pinned to small battens that had been nailed unto the walls while treated netting were hung unto nails fitted at the edges of the ceiling. These methods of fixing the treated materials unto the walls also allowed the treatments to be easily rotated between huts on a weekly basis.

Rotation of sleepers and treatments

Treatments were allocated to the six experimental huts and rotated each week using a randomised Latin square design to adjust for any differential positional attractiveness of the huts. Weekly rotation with one day for cleaning between rotations minimised any carry over effect between the treatments. Six adult men served as volunteer sleepers to attract mosquitoes into the huts. They were rotated between huts on successive nights to adjust for any variation in individual attractiveness to mosquitoes. They slept in the huts from 20:00 to 05:00 each night. Mosquitoes were collected each morning at 05:00 from under bed nets, floors, walls, ceilings and verandas using collection tubes and torches. The collections were transported to the laboratory where mosquitoes were identified to species and scored as blood fed or unfed and live or dead. Live mosquitoes were held in netted plastic cups and supplied with 10% glucose solution and delayed mortality was recorded after 24h. Male mosquitoes were not scored.
Entomological Outcomes

The entomological impact of each treatment in this study was expressed in terms of the following entomological outcomes;

1. Deterrence: percentage reduction in the number of mosquitoes caught in treated hut relative to the number caught in the control hut

2. Exiting rates due to potential irritant effect of treatments expressed as percentage of the mosquitoes collected from the veranda trap

3. Inhibition of blood feeding: reduction in blood feeding rate relative to the control. This was as follows:

\[
\% \text{ Blood-feeding inhibition} = \frac{100 (Bfu - Bft)}{Bfu} \]

Where Bfu is the proportion of blood-fed mosquitoes in the untreated control huts and Bft is the proportion of blood-fed mosquitoes in the huts with a specific insecticide treatment;

4. Mortality: percentage of dead mosquitoes in treated hut at the time of collection and after a 24 h holding period corrected for control mortality.

5. The personal protective effect of the treatments which is described by a reduction in the number of blood-fed mosquitoes relative to the control hut was calculated as follows:
\[
% \text{Personal Protection} = \frac{100(Bu - Bt)}{Bu}
\]

Where \( Bu \) = is the number of blood-fed mosquitoes in the untreated control huts and \( Bt \) is the number of blood-fed mosquitoes in the huts with insecticide treatments.

6. The overall insecticidal effect of a treatment relative to the number of mosquitoes that would ordinarily enter an untreated control hut was estimated by using the following formula and expressed as a percentage:

\[
\text{Overall insecticidal effect (\%)} = \frac{100(K_t - K_u)}{(T_u - K_u)}
\]

where \( K_t \) is the number killed in the treated hut, \( K_u \) is the number dying in the untreated control hut, and \( T_u \) is the total number collected from the control hut.

**Residual activity of insecticide treatments**

To measure residual activity, WHO cone bioassays were undertaken on treated materials in situ using the laboratory-susceptible \( An.\ gambiae \) s.s. Kisumu strain. Adult females 3–5 days old were introduced into cones fixed to treated plastic sheeting/net wall hangings (for 30 minutes) and LLINs (for 3 minutes) according to WHO guidelines [22]. For each trial, approximately 40-50 adult females were tested in batches of 10 mosquitoes on each type of treated material each week. These were held in netted plastic cups, provided 10% glucose solution and mortality recorded after 24 hours.
**Studies on selection of insecticide resistance genes**

Samples of *An gambiae* (dead and alive) collected from the respective experimental hut treatments through the course of the trials were preserved for molecular analysis. Only samples from the first and second experimental hut trial were analysed. These mosquitoes were systematically selected from the alive and dead collections of the first trial to cover the entire period of the trial and to include equal numbers of bloodfed and unfed mosquitoes. For the second trial, because the numbers entering the huts had reduced, we analysed all samples collected. Genomic DNA was extracted using the Livak procedure [23]. Samples were identified to species and molecular form of *An gambiae* using SINE-PCR. Molecular detection of the *kdr* (L1014F) and *ace-1* (G119S) mutation alleles was carried out by real-time Taqman PCR as described by Bass et al [24].

**Statistical analysis**

The effects of the different experimental hut treatments on each of the main entomological outcomes (bloodfeeding, exophily and mortality) were assessed using binomial generalised linear mixed models (GLMMs) with a logit link function, fitted using the ‘lme4’ package for R. A separate model was fitted for each outcome. In addition to the fixed effect of each treatment, each model included random effects to account for the following sources of variation: between the 6 huts used in the studies; between the 6 sleepers who slept in the huts; between the 6 weeks of the trial; and finally an observation-level random effect to account for variation not explained by the other terms in the model (overdispersion). In comparing fixed effects between treatments, the binomial GLMM cannot estimate mortalities of exactly 0 or 100%, because the logits of 0 and 1 are undefined. Some treatments caused 100% mortality during the second hut trial; hence it was not possible to fit a
valid GLMM to this data. To overcome this problem, a small constant (0.5) was added to rows contributing to zero cell counts in this data before modelling the GLMM, allowing conservative estimates of treatment effects and p-values to be derived [25]. The numbers entering the huts were analysed using negative binomial regression.

For genotyping data, differences in survival of resistant genotypes for each treatment was analysed by Chi square and Fisher’s exact test. All analyses were performed using R version 2.12.2 for Windows [26].

**Ethics Statement**

Ethical approval for the study was obtained from the Ethics Review Board of the London School of Hygiene and Tropical Medicine (Approval No. 5872) and from the ‘Comite d’Ethique pour la Recherches en Sante’ (Approval No. 2011-6-33) of the Ministry of Scientific Research of Burkina Faso. Permission to use the experimental hut station was obtained from Centre Muraz. Written informed consent was obtained from the volunteers who slept in the experimental huts to attract mosquitoes.
Results

Susceptibility tests

*An gambiae* from Valley du Kou 5 was very resistant to pyrethroids recording 2% mortality with deltamethrin (0.5%) treated papers (Table 1). In contrast, mortality with pirimiphos methyl (0.25%) treated papers was 100% showing that the vector population was largely susceptible to the organophosphate (Table 2).

Experimental hut trials

Over 5000 *An gambiae* ss were collected from the experimental huts during the trials. The numbers of *Culex quinquefasciatus* collected were too few to permit further analysis.

1. Single intervention trial

A total of 3933 *An gambiae* ss were collected from the experimental huts during the trial. The results obtained are presented in Table 3 and Figure 1. Blood-feeding rates were generally high (70-83%) with the DL and NWH alone (Figure 1) since mosquitoes would normally feed on the person sleeping in the hut before resting on the wall. Hence the treatments provided limited blood-feeding inhibition (4-20%) and personal protection (29-56%) (Table 3). Mortality with pyrethroid DL was 40% (Figure 1). P-methyl treated DL and NWH induced much higher mortality rates (>95%) than pyrethroid DL (P<0.001). With only walls covered, p-methyl DL and NWH showed a similar performance. Highest mortality was attained when all hut surfaces (walls and ceiling) were covered with p-methyl NWH (99%) and hence for the follow on trials, the p-methyl DL and NWH treatments tested were applied on walls and ceiling.
2. **First combined intervention trial**

A total of 320 *An gambiae* ss were collected from the experimental huts during the second trial, far fewer than in the first trial. By this time, the rice in the fields had grown significantly and covered the breeding sites leading to lower numbers of mosquitoes entering the huts compared to the first trial. The results obtained in this trial are presented in Table 4 and Figure 2. The holed LLIN was more protective (23% bloodfed and 0% found inside the net) than the untreated holed net (81% bloodfed and 36% found inside the net) (Table 4 and Figure 2). Combining p-methyl DL and NWH with LLINs reduced bloodfeeding rates significantly (8-9% bloodfed) compared to p-methyl DL and NWH alone (Figure 2) (P<0.001). The combination therefore provided more bloodfeeding inhibition (90-91%) and personal protection (94-95%) than the p-methyl treatments alone (50% bloodfeeding inhibition and 51-70% personal protection) (Table 4). Mortality with the LLIN alone was 60% (Figure 2). Mortality was 100% when p-methyl DL and NWH where used whether alone or in combination with LLINs.

3. **Second combined intervention trial**

The results are presented in Table 5 and Figure 3. A total of 490 *An gambiae* ss were collected from the experimental huts during this trial (Table 5). Combining LLINs with pyrethroid DL did not show any improvement in mortality (48%) compared to the LLIN alone (44%) and pyrethroid DL alone (40%) (P>0.1) (Figure 3). Mortality was much higher (95%) when p-methyl DL was combined with LLINs.
**Residual activity of insecticide**

Mortality of laboratory reared susceptible *An gambiae* (Kisumu) tested in WHO cone bioassay on p-methyl DL and NWH, was 100% for the first 3-4 weeks of each of the trial but declined to 60-70% by the end of the trial. With pyrethroid DL, mortality remained 100% throughout the trial owing to the fact that the pyrethroid DL was factory coated using long-lasting technology.

**Selection of resistance alleles and genotypes**

The *An gambiae* population was predominantly of the M-molecular form. Of 559 *An gambiae* samples which were randomly selected from weekly collections from the experimental huts during the trials, 98% were identified as belonging to the M-form of *An gambiae ss*.

A total of 732 and 656 *An gambiae* samples collected from the first two experimental hut trials were analysed for *kdr* and *ace 1<sup>R</sup>* respectively. The summary results on allele frequencies in live and dead collections are presented in Table 6. Genotype survival rates are presented in Table 7. The overall *kdr* allele frequency was 0.95 (n=535) in the first trial and 0.86 (n=197) in the second trial while the overall *ace-1<sup>R</sup>* allele frequency was 0.01 (n=429) in the first trial and 0.03 (n=228) in the second trial. There was no difference in the frequency of *kdr* alleles between live and dead collections from any of the treatments (P>0.05) (Tables 6). Analysis of genotype frequency (Table 7) showed that survival of the *kdr* heterozygotes (47%) was no different from that of *kdr* homogygotes for resistance (52%) in the presence of LLIN (1<sup>st</sup> trial: P=0.71, 2<sup>nd</sup> trial: P=0.54).
While the $ace^{-1R}$ was low, there was generally a greater tendency for mosquitoes bearing the $ace^{-1R}$ allele to survive in huts with the p-methyl treatments alone in the single intervention trial. The $ace^{-1R}$ allele frequency was significantly higher in mosquitoes which survived in huts in which p-methyl treated DL and NWH were applied alone on walls and ceiling ($P \leq 0.03$) (Table 6). Analysis of genotype frequency showed that 100% (9/9) of $ace^{-1R}$ heterozygotes survived the p-methyl treatments but only 32% (105/323) of $ace^{-1}$ susceptibles survived ($P = 0.001$), indicating strong selection for the $ace^{-1R}$ resistance with the p-methyl interventions. In the second trial, all mosquitoes which entered the huts with p-methyl treatments whether applied alone or in combination with LLINs were killed (100% mortality). It was thus not possible to clearly demonstrate whether the combination prevents selection of the $ace^{-1R}$ gene compared to the single intervention of p-methyl (Tables 6 and 7). The low survival of $kdr$ with the combination indicates that the p-methyl component might prevent the further selection of $kdr$ resistance (Table 7).

**Discussion**

In the current study, p-methyl treated DL and NWH outperformed pyrethroid treated DL by killing almost all malaria vectors which entered the huts. This superior performance was due to the fact that the vector population was very resistant to pyrethroids but susceptible to organophosphates. As pyrethroid resistance continues to spread, the use of non-pyrethroids like organophosphates and carbamates for IRS is increasing. With the exception of the newly developed micro-encapsulated formulation of p-methyl which lasts up to 9 months on cement walls [10], most organophosphate and carbamate insecticides though very toxic to mosquitoes are
unfortunately short-lived when applied as IRS (2-4 months) compared to pyrethroids (up to 6 months) [17]. The development of long-lasting versions of p-methyl DL and NWH with residual activity over a number of years could significantly improve the usefulness of organophosphates in malaria vector control and enhance capacity to interrupt malaria transmission.

Increasing the level of wall coverage with p-methyl DL and NWH from walls only to walls plus ceiling did not have a major effect on the performance of these treatments in the experimental huts. Similar findings have been previously reported with pyrethroid DL [7]. This has positive implications for the scalability of these interventions since covering only walls as opposed to covering walls and ceilings is likely to be easier owing to the additional costs and practical difficulty of having to cover ceilings too. Pyrethroid DL was however found to induce significantly lower mortality when applied to two walls (20%) compared to all four walls (45%) [7]. It will be useful to investigate the performance of p-methyl DL and NWH when lower levels of wall coverage are achieved.

LLINs are capable of inducing high levels of mortality and providing significant personal protection to the user against a fully susceptible vector population. However, when faced with pyrethroid resistance, the insecticidal efficacy of the LLIN is significantly reduced, and the strength of the intervention may be compromised [8]. Nevertheless, with limited holes, LLINs may still provide partial protection against pyrethroid resistant vectors as shown in this study partly due to the physical barrier of the bed net and partly to the repellent property of the pyrethroid in the LLIN, and are thus much better than untreated nets or no nets at all. The current study demonstrates that the combining of p-methyl DL and NWH with LLINs induced high levels of mortality in a pyrethroid resistant population of malaria vectors and thus
should restore transmission control to levels which cannot be achieved by the LLIN alone due to pyrethroid resistance. Mosquitoes would normally enter the room and feed on the sleeper before landing on the walls where they pick up the insecticide. The combination therefore showed potential to control transmission, largely due to the p-methyl DL and NWH components, and provide personal protection mainly due to the LLIN component. As with most IRS and IRS-like treatments, significant personal protection cannot be expected with p-methyl DL and NWH alone if only individual households are lined. However, if entire villages are covered, community protection should arise from the control of mosquito populations as occurs with IRS campaigns.

In contrast to p-methyl DL, combining pyrethroid DL and LLIN in the same hut did not show any improvement in mortality when compared to the LLIN alone. This can be attributed to the high level of pyrethroid resistance in the vector population and served as a positive control to demonstrate the importance of a non-resisted insecticide in the durable lining or NWH intervention. The present study confirms the fact that combining pyrethroid DL with pyrethroid LLIN for improved control of malaria transmission by a vector population which is resistant to pyrethroids may be a futile attempt and might not warrant the resources invested. Theoretical models suggest that the increased repellency posed by the additional pyrethroid wall treatment in the combination hut may also have decreased the chances of insect contact with insecticide [27]. The combining of pyrethroid IRS or IRS-like treatments with pyrethroid LLINs is generally not encouraged mostly because it exposes local vector populations to more intense selection pressure for pyrethroid resistance genes [9]. However some vector control programmes may continue to deploy pyrethroid IRS together with LLINs in the hope of improving transmission control.
The performance of such a combination is likely to diminish if pyrethroid resistance exists in the targeted vector population and the threat of stronger resistance developing is more probable.

The frequency of the $kdr$ (L1014F) mutation in *An gambiae* in Vallee du Kou 5 as observed in the current study was very high (0.89) and had increased remarkably from 0.28 in 2005 [28]. This confirms the rapid spread of the $kdr$ among *An gambiae* populations across sub-Saharan Africa. Population genetic models suggest that the benefits of insecticide resistance management can be best achieved while resistance is still rare compared to when it is well established [20,29,30]. The high $kdr$ allele frequency in the vector population could not permit a robust investigation into selection for $kdr$ with the treatments tested. Nevertheless there was some evidence that selection of heterozygotes for $kdr$ was no greater than selection of homozygotes for $kdr$ and that selection of both genotypes would be delayed by the addition of p-methyl to an existing LLIN intervention. Meanwhile, mosquitoes bearing the $ace-1^R$ mutation were more likely to survive in huts when p-methyl DL and NWH were applied on walls and ceilings and no LLIN was in use. Because no live mosquitoes were collected from huts in the trial where p-methyl DL and NWH applied alone were compared with the combination of p-methyl DL/NWH and LLINs, it was not possible to demonstrate unequivocally the selective advantage or neutrality of resistance genes in the combination. But on the other hand there was similarly no evidence to indicate that any of the resistance alleles would be differentially selected by the combination, which is fair argument for applying the combination. There could also have been metabolic mechanisms of insecticide resistance in the vector population which in addition to the $kdr$ may have contributed to the levels of phenotypic resistance to pyrethroids that was observed.
Unfortunately, the absence of reliable DNA markers for the collection genes than can be up-regulated in metabolic resistance could not permit a realistic investigation into their selection in the current study. Apart from the resistance management potential, the study clearly shows that the combination would be a better option for controlling and providing protection against a vector population which is mostly resistant to pyrethroids but mostly susceptible to organophosphates than the single treatments alone. Considering the increasing reports of organophosphate resistance in malaria vectors in West Africa [31-33], there is opportunity to monitor what happens when the combination is deployed against a vector population which is partially resistant to both insecticides.

Residual activity with p-methyl treated DL and NWH declined over the course of the six weeks trials. This decline was faster than expected given the slow-release micro-encapsulated formulation of the insecticide used. The insecticide particles may have flaked off the treated materials during the course of the study. The study was designed as a proof of concept and the observed effect of p-methyl DL and NWH on mortality during these short term trials showed that mosquitoes will readily rest on p-methyl treated plastic wall linings and net wall hangings and be killed in the process. To maximise the benefits of these tools over IRS, the final product will need to have a residual activity that lasts for years rather than months. Advanced binding or incorporation technology needs to be developed to enable the development of a long lasting version of these tools.

Net wall hangings probably due to their light weight were much easier to hang on the walls than fixing of DL. Thus net wall hangings are potentially a more practical means of delivering insecticides indoors. Netting material is cheap and widely available. Treated NWH can be readily used in homes where IRS is short lived on
mud walls. Treated wall netting can also be used to cover eave gaps as to reduce mosquito entry into the home. Small scale randomised trials are desirable to further assess the efficacy, acceptability and practicability of treated NWH in homes.

**Conclusion**

Pirimiphos methyl treated DL and NWH show potential to provide improved control of pyrethroid resistant malaria vectors compared to currently available pyrethroid DL or IRS. Combining p-methyl DL/NWH with LLINs provides transmission control due mainly to the p-methyl DL/NWH component and personal protection due mainly to the LLIN component. Community wide protection and epidemiological impact are expected if p-methyl DL/ NWH are deployed in combination with LLINs against vector populations which are partly or mostly resistant to pyrethroids but mostly susceptible to organophosphates. There was clear evidence from the hut trial that the single intervention would select for resistance to \textit{kdr} and \textit{ace-1R} and some evidence that the combination intervention would not select so strongly for resistance. NWH are a practical means of delivering insecticides indoors and need to be further explored. Advanced binding or incorporation technology is required to develop genuine long-lasting p-methyl DL or NWH and produce benefits over IRS.

**Acknowledgements**

We thank Andy Bywater of Syngenta for providing the insecticide. We are grateful to Nestor Kesse and Benard Loukou for technical support and to Dr Christopher Jones (LSTM) and Dr Raphael N’Guessan (LSHTM/CREC) for their support and encouragements. Corine Ngufor and Mark Rowland are supported by the Malaria Centre of LSHTM.
Figure 1. Mortality and bloodfeeding rates of pyrethroid resistant *An gambiae* in experimental huts with single interventions. Percentage mortality (dark shade) and bloodfeeding (lighter shade) of pyrethroid resistant *An gambiae* in experimental huts in Valley du Kou with the indicated single treatments. P-methyl DL and NWH are compared to pyrethroid DL and an untreated control. For each response parameter (mortality or bloodfeeding), values for histograms sharing the same letter label are not significantly different (P>0.05).
Figure 2. Mortality and bloodfeeding rates of pyrethroid resistant *An. gambiae* in experimental huts with combined interventions. Percentage mortality (dark shade) and bloodfeeding (lighter shade) of pyrethroid resistant *An. gambiae* in experimental huts in Valley du Kou with the combined p-methyl wall treatment + LLINs versus single treatments alone. For each response parameter (mortality or bloodfeeding), values for histograms sharing the same letter label are not significantly different (P>0.05).
Figure 3: Mortality and bloodfeeding rates of pyrethroid resistant *An gambiae* in experimental huts (third trial). Percentage mortality (dark shade) and bloodfeeding (lighter shade) of pyrethroid resistant *An gambiae* in experimental huts in Valley du Kou with combination of p-methyl DL and LLIN versus combination of pyrethroid DL + LLIN. For each response parameter (mortality or bloodfeeding), values for histograms sharing the same letter label are not significantly different (P>0.05).

Table 1: Susceptibility of wild *An gambiae ss* from Valley du Kou 5 (VK5) to deltamethrin (0.05%) in WHO cylinder bioassays.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. tested</th>
<th>% KD (95% CI)</th>
<th>% KD (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An gambiae s.s.</em> (Kisumu)</td>
<td>100</td>
<td>100 (96 – 100)</td>
<td>100 (96 – 100)</td>
</tr>
<tr>
<td><em>An gambiae s.s.</em> (VK5)</td>
<td>100</td>
<td>5 (0 – 12)</td>
<td>2 (0 – 7)</td>
</tr>
</tbody>
</table>
Table 2: Susceptibility of wild *An gambiae* ss from Valley du Kou 5 (VK5) to pirimiphos methyl (0.25%) in WHO cylinder bioassays.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. tested</th>
<th>% KD (95% CI)</th>
<th>24h % mortality (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An gambiae</em> s.s. (Kisumu)</td>
<td>100</td>
<td>87 (80 – 94)</td>
<td>100 (96 – 100)</td>
</tr>
<tr>
<td><em>An gambiae</em> s.s. (VK5)</td>
<td>102</td>
<td>86 (79 - 92)</td>
<td>100 (96 -100)</td>
</tr>
</tbody>
</table>

Table 3: Personal protection and killing effect of p-methyl DL and NWH against pyrethroid resistant *An gambiae* in Valley du Kou, Burkina Faso (single intervention trial)

<table>
<thead>
<tr>
<th></th>
<th>Control (untreated DL on walls)</th>
<th>pyrethroid treated on walls DL</th>
<th>p-methyl DL on walls</th>
<th>p-methyl NWH on walls</th>
<th>p-methyl DL on walls and ceiling</th>
<th>p-methyl NWH on walls and ceiling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total females caught</td>
<td>995&lt;sup&gt;a&lt;/sup&gt;</td>
<td>464&lt;sup&gt;b&lt;/sup&gt;</td>
<td>523&lt;sup&gt;b&lt;/sup&gt;</td>
<td>841&lt;sup&gt;a&lt;/sup&gt;</td>
<td>615&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>490&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Deterrence (%)</td>
<td>-</td>
<td>53</td>
<td>47</td>
<td>15</td>
<td>38</td>
<td>51</td>
</tr>
<tr>
<td>Total females blood fed</td>
<td>781</td>
<td>282</td>
<td>417</td>
<td>557</td>
<td>483</td>
<td>345</td>
</tr>
<tr>
<td>Blood feeding Inhibition (%)</td>
<td>-</td>
<td>20</td>
<td>4</td>
<td>19</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>Personal Protection (%)</td>
<td>-</td>
<td>64</td>
<td>47</td>
<td>29</td>
<td>38</td>
<td>56</td>
</tr>
<tr>
<td>Exiting rates (%)</td>
<td>18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total dead</td>
<td>81</td>
<td>236</td>
<td>471</td>
<td>764</td>
<td>554</td>
<td>479</td>
</tr>
<tr>
<td>Corrected mortality</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>96&lt;sup&gt;c&lt;/sup&gt;</td>
<td>99&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Overall killing effect (%)</td>
<td>-</td>
<td>16</td>
<td>43</td>
<td>75</td>
<td>52</td>
<td>43</td>
</tr>
</tbody>
</table>

Values along each row sharing the same letter superscript are not significantly different at the 5% level
Table 4: Personal protection and killing effect of combining p-methyl DL and NWH with LLINs against pyrethroid resistant *An gambiae* in Valley du Kou, Burkina Faso (first combined intervention trial).

<table>
<thead>
<tr>
<th></th>
<th>Control (untreated net)</th>
<th>LLIN</th>
<th>p-methyl DL</th>
<th>p-methyl NWH</th>
<th>p-methyl DL + LLIN</th>
<th>p-methyl NWH + LLIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total females caught</td>
<td>81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>45&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Deterrence (%)</td>
<td>-</td>
<td>48</td>
<td>16</td>
<td>44</td>
<td>53</td>
<td>43</td>
</tr>
<tr>
<td>Total females blood fed</td>
<td>64</td>
<td>11</td>
<td>28</td>
<td>18</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Blood feeding Inhibition (%)</td>
<td>70</td>
<td>50</td>
<td>50</td>
<td>91</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td><strong>Personal Protection (%)</strong></td>
<td>-</td>
<td>83</td>
<td>56</td>
<td>72</td>
<td>95</td>
<td>94</td>
</tr>
<tr>
<td>Total inside net (%)</td>
<td>36</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Exiting rates (%)</td>
<td>33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>40&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>54&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total dead</td>
<td>12</td>
<td>25</td>
<td>68</td>
<td>38</td>
<td>45</td>
<td>46</td>
</tr>
<tr>
<td>Corrected mortality</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Overall killing effect (%)</strong></td>
<td>-</td>
<td>19</td>
<td>81</td>
<td>48</td>
<td>38</td>
<td>49</td>
</tr>
</tbody>
</table>

Values along each row sharing the same letter superscript are not significantly different at the 5% level

Table 5: Personal protection and killing effect of combining p-methyl DL vs pyrethroid DL with LLINs against pyrethroid resistant *An gambiae* in Valley du Kou, Burkina Faso (second combined intervention trial).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LLIN</th>
<th>pyrethroid DL + LLIN</th>
<th>p-methyl DL + LLIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total females caught</td>
<td>255&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Deterrence (%)</td>
<td>-</td>
<td>72</td>
<td>74</td>
<td>62</td>
</tr>
<tr>
<td>Total females blood fed</td>
<td>114</td>
<td>15</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Blood feeding Inhibition (%)</td>
<td>-</td>
<td>53</td>
<td>80</td>
<td>74</td>
</tr>
<tr>
<td><strong>Personal protection (%)</strong></td>
<td>-</td>
<td>87</td>
<td>95</td>
<td>90</td>
</tr>
<tr>
<td>Total inside net (%)</td>
<td>29</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Exiting rates (%)</td>
<td>31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total dead</td>
<td>24</td>
<td>32</td>
<td>32</td>
<td>94</td>
</tr>
<tr>
<td>Corrected Mortality (%)</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Overall killing effect (%)</strong></td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>32</td>
</tr>
</tbody>
</table>

Values along each row sharing the same letter superscript are not significantly different at the 5% level
Table 6: Comparative kdr and ace 1<sup>R</sup> allele frequencies in live and dead An gambiae ss collected from the experimental huts trials

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Kdr allele freq (n)</th>
<th>ace 1&lt;sup&gt;R&lt;/sup&gt; allele freq (n)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live</td>
<td>Dead</td>
<td>P</td>
<td>Live</td>
<td>Dead</td>
<td>P</td>
</tr>
<tr>
<td>Single intervention trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Control (untreated DL)</td>
<td>0.95 (140)</td>
<td>--</td>
<td>--</td>
<td>0.01 (97)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2 Pyrethroid DL</td>
<td>0.91 (51)</td>
<td>0.90 (51)</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3 P-methyl DL (walls only)</td>
<td>1.0 (9)</td>
<td>0.97 (58)</td>
<td>1</td>
<td>0.05 (21)</td>
<td>0.00 (28)</td>
<td>0.18</td>
</tr>
<tr>
<td>4 P-methyl NWH (walls only)</td>
<td>1.0 (18)</td>
<td>0.95 (58)</td>
<td>0.34</td>
<td>0.00 (36)</td>
<td>0.00 (60)</td>
<td>1</td>
</tr>
<tr>
<td>5 P-methyl DL (walls and ceiling)</td>
<td>1.0 (18)</td>
<td>0.94 (61)</td>
<td>0.35</td>
<td>0.07 (28)</td>
<td>0.00 (64)</td>
<td>0.01</td>
</tr>
<tr>
<td>6 P-methyl NWH (walls and ceiling)</td>
<td>1.0 (3)</td>
<td>0.96 (68)</td>
<td>1</td>
<td>0.05 (29)</td>
<td>0.00 (66)</td>
<td>0.03</td>
</tr>
<tr>
<td>Combined intervention trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Control (untreated DL)</td>
<td>0.87 (63)</td>
<td>--</td>
<td>--</td>
<td>0.04 (81)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2 LLIN</td>
<td>0.83 (9)</td>
<td>0.85 (27)</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3 P-methyl DL*</td>
<td>--</td>
<td>0.82 (31)</td>
<td>--</td>
<td>--</td>
<td>0.02 (34)</td>
<td>--</td>
</tr>
<tr>
<td>4 P-methyl NWH*</td>
<td>--</td>
<td>0.84 (19)</td>
<td>--</td>
<td>--</td>
<td>0.02 (31)</td>
<td>--</td>
</tr>
<tr>
<td>5 P-methyl DL + LLIN*</td>
<td>--</td>
<td>0.87 (23)</td>
<td>--</td>
<td>--</td>
<td>0.00 (42)</td>
<td>--</td>
</tr>
<tr>
<td>6 P-methyl NWH + LLIN*</td>
<td>--</td>
<td>0.94 (25)</td>
<td>--</td>
<td>--</td>
<td>0.07 (40)</td>
<td>--</td>
</tr>
</tbody>
</table>

*no live mosquitoes were collected from huts with these treatments

Table 7: Genotype selection by the single and combination treatments: percentage survival of An gambiae kdr and ace 1<sup>R</sup> genotypes collected from the experimental huts.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>kdr: % alive (live/total)</th>
<th>ace-1&lt;sup&gt;R&lt;/sup&gt;: % alive (live/total)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>RS</td>
<td>RR</td>
<td>SS</td>
<td>RS</td>
<td>RR</td>
</tr>
<tr>
<td>Single intervention trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Control (untreated DL)</td>
<td>100 (11/11)</td>
<td>100 (129/129)</td>
<td>100(96/96)</td>
<td>100(1/1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Pyrethroid DL</td>
<td>47 (7/15)</td>
<td>52 (44/85)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 P-methyl DL (walls only)</td>
<td>0 (0/4)</td>
<td>14 (9/63)</td>
<td>40 (19/47)</td>
<td>100 (2/2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 P-methyl NWH (walls only)</td>
<td>0 (0/6)</td>
<td>25 (18/70)</td>
<td>37 (36/96)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 P-methyl DL (walls and ceiling)</td>
<td>0 (0/7)</td>
<td>22 (18/82)</td>
<td>27 (24/88)</td>
<td>100 (4/4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 P-methyl NWH (walls and ceiling)</td>
<td>0 (0/6)</td>
<td>5 (3/62)</td>
<td>28 (26/92)</td>
<td>100 (3/3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined intervention trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Control (untreated DL)</td>
<td>100 (2/2)</td>
<td>100 (13/13)</td>
<td>100 (48/48)</td>
<td>100(75/75)</td>
<td>100(6/6)</td>
<td></td>
</tr>
<tr>
<td>2 LLIN</td>
<td>0(0/1)</td>
<td>33 (3/9)</td>
<td>23 (6/26)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 P-methyl DL*</td>
<td>0 (0/3)</td>
<td>0 (0/5)</td>
<td>0 (0/23)</td>
<td>0 (0/33)</td>
<td>0 (0/1)</td>
<td></td>
</tr>
<tr>
<td>4 P-methyl NWH *</td>
<td>0 (0/2)</td>
<td>0 (0/2)</td>
<td>0 (0/15)</td>
<td>0 (0/30)</td>
<td>0 (0/1)</td>
<td></td>
</tr>
<tr>
<td>5 P-methyl DL + LLIN*</td>
<td>0 (0/1)</td>
<td>0 (0/4)</td>
<td>0 (0/18)</td>
<td>0 (0/42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 P-methyl NWH + LLIN*</td>
<td>0 (0/3)</td>
<td>0 (0/22)</td>
<td>0 (0/35)</td>
<td>0 (0/5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*no live mosquitoes were collected from huts with these treatments, SS=Homozygous susceptible, RS=Heterozygous, RR=Homozygous resistant.
References


Chapter 6

Combining organophosphate treated wall linings and long-lasting insecticidal nets fails to provide additional control over LLIN alone against multiple insecticide resistant *Anopheles gambiae* in Côte D’Ivoire: an experimental hut trial.

The material presented in this chapter has been published as:

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

1. For a 'research paper' already published

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

1.2. When was the work published? **October 2014**

1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion **NA**

1.3. Was the work subject to academic peer review? **Yes**

1.4. Have you retained the copyright for the work? **Open Access**

If yes, attach evidence of retention

If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published.

2.1. Where is the work intended to be published?

2.2. List the paper's authors in the intended authorship order

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

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

**I co-designed the study, supervised/ performed the field activities, performed the molecular analysis, analysed the data, co-interpreted the findings and wrote the manuscript.**

Candidate's signature ________________________________________________

Supervisor or senior author's signature to confirm role as stated In (3) ______________
Combining organophosphate treated wall linings and long-lasting insecticidal nets fails to provide additional control over LLIN alone against multiple insecticide resistant Anopheles gambiae in Côte D'Ivoire: an experimental hut trial.

Abstract

Background

Insecticide-treated wall lining (ITWL) is a new concept in malaria vector control. Some Anopheles gambiae populations in West Africa have developed resistance to all the main classes of insecticides. It needs to be demonstrated whether vector control can be improved or resistance managed when non-pyrethroid ITWL is used alone or together with long-lasting insecticidal nets (LLINs) against multiple insecticide-resistant vector populations.

Methods

Two experimental hut trials were carried out as proofs of concept to evaluate pirimiphos methyl (p-methyl)-treated plastic wall lining (WL) and net wall hangings (NWH) used alone and in combination with LLINs against multiple insecticide-resistant An. gambiae in Tiassalé, Côte d’Ivoire. Comparison was made to commercial deltamethrin WL and genotypes for kdr and Ace-1R resistance were monitored.

Results

The kdr and Ace-1R allele frequencies were 0.83 and 0.44, respectively. Anopheles gambiae surviving discriminating concentrations of deltamethrin and p-methyl in WHO resistance tests were 57 and 96%, respectively. Mortality of free-flying An. gambiae in huts with p-methyl WL and NWH (66 and 50%, respectively) was higher than with pyrethroid WL (32%; P<0.001). Mortality with LLIN was 63%. Mortality with the combination of LLIN plus p-methyl NWH (61%) or LLIN plus p-methyl WL (73%) did not significantly improve upon the LLIN alone or p-methyl WL or NWH alone. Mosquitoes bearing the Ace-1R were more likely to survive exposure to p-methyl WL and NWH. Selection of heterozygote and homozygote Ace-1R or kdr genotypes was not less likely after exposure to combined LLIN and p-methyl treatments than to
single p-methyl treatment. Blood-feeding rates were lower in huts with the pyrethroid LLIN (19%) than with p-methyl WL (72%) or NWH (76%); only LLIN contributed to personal protection.

Conclusions

Combining p-methyl WL or NWH with LLINs provided no improvement in *An. gambiae* control or personal protection over LLIN alone in southern Côte d'Ivoire; neither did the combination manage resistance. Additional resistance mechanisms to *kdr* and *Ace-1R* probably contributed to the survival of pyrethroid and organophosphate-resistant mosquitoes. The study demonstrates the challenge that malaria control programmes will face if resistance to multiple insecticides continues to spread.
Background

Long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) are the most effective and widely used methods for controlling malaria vectors. The recent reductions in malaria morbidity and mortality across Africa has been attributed to a scale-up of these interventions and to better access to diagnostic testing and artemisinin combination therapy (ACT) to treat malaria [1]. Most national malaria control programmes have prioritized universal coverage of LLINs to populations at risk [2]. Campaigns of IRS are particularly appropriate for rapid transmission control. Both approaches require good organization and receptive communities [3]. LLIN effectiveness relies on people regularly sleeping under their nets. IRS is sometimes challenged by the complex organization required and by user-fatigue sometimes associated with recurrent rounds of spraying [3].

The recent development of insecticide-treated wall lining technology [4] offers the prospect of a novel system of insecticide delivery which is more residual than IRS and requires limited behavioural change. Interior walls can be lined with polymer sheeting (wall lining) or net wall hangings impregnated with insecticide. Using advances in binder technology, these tools can be prepared in a long-lasting format that allows the insecticide to diffuse to the surface in a controlled fashion, making them a long-lasting alternative to IRS. Pyrethroid-treated durable wall lining has been manufactured commercially using this technique and its use on interior wall surfaces has shown potential to improve user compliance and overcome the operational constraints associated with IRS [4,5]. Durable wall lining has the potential to remain efficacious on home walls for three to four years. However, with the increasing
problem of pyrethroid resistance on malaria vector control [6,7], non-pyrethroid forms of durable lining which can be used against pyrethroid-resistant malaria vectors are urgently needed [7,8]. Such materials could significantly reduce reliance on pyrethroids and enhance capacity to interrupt malaria transmission whilst living with pyrethroid resistance.

Organophosphates and carbamates, having a differing mode of action to pyrethroids, are potential alternative classes of insecticide which could be used on wall linings [9]. These classes are effective against pyrethroid-resistant mosquitoes when used as IRS or wall linings [10,11]. However, resistance to organophosphates and carbamates due to insensitive acetylcholinesterase (Ace-1R) has been reported in some pyrethroid-resistant malaria vector populations in West Africa [12-14]. Malaria vector control programmes, confronted by such multiple resistance, may be left with no option than resort to using these classes until new types of insecticide with novel modes of action are identified and made available.

The combining of non-pyrethroid IRS and pyrethroid LLIN has been recommended for resistance management and for improving control of insecticide-resistant malaria vectors [7]. This resistance management tactic relies on insect genotypes resistant to the insecticide in one intervention being killed by the insecticide in the other intervention provided they are not resistant to both insecticides [15]. Population genetics modelling indicates that combinations are less likely to provide this advantage when resistance to both insecticides is already present at detectable frequencies in the targeted vector population [8,15]. However, reality is often more complex than the prediction of models. Some combinations may still improve
personal protection or enhance kill through biochemical or behavioural interactions [7,15]. With limited alternatives available for malaria control, empirical studies are needed to demonstrate whether improved vector control can be expected when non-pyrethroid IRS or wall linings are combined with pyrethroid LLINs against a multiple insecticide-resistant vector population.

In the current study, the efficacy of organophosphate-treated wall linings (WL) and net wall hangings (NWH) applied alone and in combination with LLINs was compared with pyrethroid-treated WL against an Anopheles gambiae population of Tiassalé, southern Côte d’Ivoire, which is resistant to the main classes of insecticide used in adult vector control [13]. Differential selection of insecticide-resistant genotypes was investigated to assess the potential for resistance management.

**Methods**

**Susceptibility tests**

The local An. gambiae mosquito population in Tiassalé has shown strong phenotypic resistance to the main classes of insecticides used for vector control: the resistance ratio was previously reported as 138-fold for the pyrethroid, deltamethrin and 24-fold for the carbamate, bendiocarb [13]. The Tiassalé population has the broadest resistance profile documented to date, with resistance being mediated by target site and metabolic mechanisms [13,16,17]. To assess the current levels of resistance to 0.05% deltamethrin and 0.25% p-methyl WHO susceptibility tests were performed on samples of adult An. gambiae that had emerged from larvae collected from the
experimental hut site. A dosage of 0.25% was established as the diagnostic dosage for p-methyl using laboratory susceptible strains (H Ranson, pers comm).

**Experimental huts and study site**

The trials were carried out in six experimental huts available in a rice field in Tiassalé (5°54’ N, 4°50’W), in southern Côte d’Ivoire. The rice paddies provide extensive breeding sites for mosquitoes throughout the year. The experimental huts were of the WHOPES-approved West African design [18,19]. They were built on concrete plinths and surrounded by water-filled moats to prevent entry of scavenging ants. Veranda traps captured exiting mosquitoes. The huts were made of brick, plastered with cement, with corrugated iron roofs. The ceilings were made of high-density polyethylene sheeting and the walls had four window slits (with 1-cm gaps) through which mosquitoes could enter.

**Experimental hut treatments**

Two experimental hut trials each lasting six weeks and involving six treatments were carried out against pyrethroid-resistant *An. gambiae* in Tiassalé. In the first trial, the efficacy of p-methyl-treated WL and NWH was evaluated, alongside the currently available deltamethrin WL (ZeroVector®, VestergaardFrandsen, Switzerland). Comparison of walls only and walls plus ceiling coverage were investigated:

7. Control (untreated plastic sheeting)
8. Pyrethroid (deltamethrin)-treated WL (ZeroVector®, VestergaardFrandsen, Switzerland) on walls
9. P-methyl-treated WL on walls
10. P-methyl-treated NWH on walls
11. P-methyl WL on walls and ceilings
12. P-methyl NWH on walls and ceilings.

In the second hut trial, the p-methyl WL and NWH were combined with LLINs and compared to LLINs alone and p-methyl WL and NWH alone. The following six interventions were compared:

7. Untreated net with six holes
8. Pyrethroid LLIN (Permanet® 2.0 Vestergaard Frandsen, Switzerland), with six holes
9. P-methyl WL on walls and ceilings
10. P-methyl NWH on walls and ceilings
11. P-methyl WL on walls and ceilings + pyrethroid LLIN with six holes
12. P-methyl NWH on walls and ceilings + pyrethroid LLIN with six holes.

**Treatment of materials**

The WL was 50% shade cloth made of woven high-density polyethylene (Capatex Ltd, UK). The NWH was made of 100-denier nylon netting fabric. These materials were treated at 1 g/sq m with a micro-encapsulated formulation of pirimiphos methyl (Actellic® 300CS Syngenta, Switzerland). The WL was treated by spraying with a Hudson Xpert sprayer, while the netting fabric was treated by hand dipping. Pyrethroid-treated WL was factory-made, high-density polyethylene fibre sheeting impregnated with deltamethrin at 175 mg/sq m (Zerovector®, Vestergaard Frandsen, Switzerland). The LLIN (PermaNet® 2.0, Vestergaard Frandsen, Switzerland) was WHOPES-approved, made of 100-denier polyester, factory-coated with a wash-resistant formulation of deltamethrin at a target dosage of 55 mg/sq m. To simulate
wear and tear, the nets were intentionally holed with six 4-sq cm diameter holes (two on each side and one on each end) according to WHOPES guidelines [18]. The WL was fixed to the walls with nails while the NWH were hung from the top edge of the walls.

**Rotation of sleepers and treatments**

Treatments were rotated weekly using a Latin square design to adjust for any differences in positional attractiveness of the huts. To prevent contamination between treatments during rotations, an underlay of untreated material was used to separate the treated materials from the walls and these were rotated with the treatments. The huts were also thoroughly washed before each rotation. Six adult men served as volunteer sleepers to attract mosquitoes into the huts, and were rotated between huts on successive nights to adjust for any variation in individual attractiveness to mosquitoes. The volunteers slept in the huts from 20:00 to 05:00 each night. Mosquitoes were collected each morning at 05:00 from under bed nets, floors, walls, ceilings, and verandas using aspirators and torches. The collections were identified to species and scored as blood fed or unfed and live or dead. Live mosquitoes were supplied with 10% glucose solution and delayed mortality was recorded after 24 hours.

**Main entomological outcomes**

The entomological impact of each treatment in this study was expressed in terms of the following entomological outcomes:

12. **Deterrence**: percentage reduction in the number of mosquitoes caught in treated hut relative to the number caught in the control hut
13. Exiting rates: due to potential irritant effect of treatments expressed as percentage of the mosquitoes collected from the veranda trap

14. Inhibition of blood feeding: reduction in blood-feeding rate relative to the control:

\[
\% \text{ Blood-feeding inhibition} = \frac{100(Bfu - Bft)}{Bfu}
\]

where \( Bfu \) is the proportion of blood-fed mosquitoes in the untreated control huts and \( Bft \) is the proportion of blood-fed mosquitoes in the huts with a specific insecticide treatment

15. Mortality: percentage of dead mosquitoes in treated hut at the time of collection and after a 24-hour holding period corrected for control mortality

16. Personal protection: the proportional reduction in the number of blood-fed mosquitoes relative to blood-fed mosquitoes in the untreated control:

\[
\% \text{ Personal protection} = \frac{100(Bu - Bt)}{Bu}
\]

where \( Bu \) is the number of blood-fed mosquitoes in the untreated control huts and \( Bt \) is the number of blood-fed mosquitoes in the huts with a specific insecticide treatment.

**Residual activity of insecticide treatments**

To measure residual activity, WHO cone bioassays were undertaken on treated materials *in situ* using the laboratory-susceptible *An. gambiae s.s.* Kisumu strain. Adult females three to five days old were exposed in cones fixed to plastic sheeting/NWHs for 30 minutes in accordance with WHO IRS guidelines [23]. Knockdown was recorded after one hour and mortality was recorded after 24 hours.
Selection of insecticide resistance genes

Samples of *An. gambiae* collected from the respective experimental hut treatments through the course of the trials were preserved for molecular analysis. Genomic DNA was extracted using the Livak procedure [20]. Molecular detection of the *kdr* (L1014F) and *Ace-1*<sup>R</sup> (G119S) mutation alleles in live and dead samples from the hut treatments was carried out by real-time Taqman PCR as described by Bass *et al.* [21].

Statistical analysis

The effects of each treatment on entomological outcomes (net penetration, blood-feeding, exiting, and mortality) were assessed using binomial generalized linear mixed models (GLMMs) with a logit link function fitted using the ‘lme4’ package of R version 2.12.2 for Windows [27]. A separate model was fitted for each outcome. In addition to the fixed effects, each model included random effects to account for variation between the six huts, between the six sleepers, between the six weeks of the trial, and finally an observation-level random effect was included to account for variation not explained by the other terms in the model (over-dispersion). Differences in deterrence, personal protection and exiting rates between the treatments was analysed using negative binomial regression with adjustment for variation between huts and sleepers, based on numbers entering, killed, and blood feeding, respectively.

Analysis of differential survival of genotypes for *Ace-1*<sup>R</sup> and *kdr* resistance by treatment was analysed using the Mantel-Haenszel Chi-squared test.
Ethics statement

Ethical approval for the study was obtained from the Ethics Committee of the London School of Hygiene and Tropical Medicine (Approval No. 5872) and from the Ministry of Public Health of Côte d'Ivoire. Written informed consent was obtained from the sleeper volunteers.

Results

Susceptibility tests

The susceptibility tests confirmed that the *An. gambiae* population in Tiassalé were resistant to both deltamethrin and p-methyl. Mortality rates in WHO cylinder tests were 43% with deltamethrin 0.05% papers and 4% with p-methyl 0.25% papers (Table 1).

Experimental hut trials

Single intervention trial

A total of 466 *An. gambiae* were collected in the six experimental huts during the single intervention trial. The results obtained are presented in Table 2 and Figure 1. As expected with such IRS-type treatments, overall blood-feeding rates were very high across all single WL and NWH treatments (range between treatments: 82 and 94%) and none of these differed significantly from the control (95%). Mortality rates were higher with p-methyl WL (66%) than with pyrethroid WL (32%) (Figure 1). The performance of p-methyl WL did not differ consistently from p-methyl NWH.
Increasing the interior coverage with p-methyl WL and NWH from walls only to walls and ceilings showed, at best, only a small increase in mortality.

**Combined intervention trial**

A total of 557 *An. gambiae* were collected from the experimental huts during the combination trial. The results are presented in Table 3 and Figure 2. Blood feeding with the LLIN was significantly lower than with the untreated net (19 vs 57%; P<0.001). Blood-feeding rates were higher with the p-methyl WL (76%) and NWH treatments (72%) when applied alone. When p-methyl WL and NWH were combined with LLINs blood-feeding rates reduced significantly to 9 and 13%, respectively; these rates were not significantly different from those with the LLIN treatment. Thus the lower feeding rates associated with the combinations can be attributed to the LLIN component. The combination treatments conferred significantly more personal protection than the p-methyl WL or NWH alone (93 vs 0% and 92 vs 4%, respectively) (P<0.001) (Table 3).

Mortality of *An. gambiae* with the LLIN (63%) was significantly higher than with the untreated net (15%) (P<0.001) but did not differ significantly from the p-methyl WL alone (63 vs 61%; P = 0.68) or p-methyl NWH alone (63 vs 53%; P = 0.07). Mortality rates with the combinations were 72% for p-methyl WL plus LLIN and 61% for p-methyl NWH plus LLIN and neither of these values differed significantly from the LLIN alone (P>0.05), p-methyl WL (72 vs 61%, P=0.06) or NWH single treatments (61 vs 53%, P=0.78) (Figure 2). Thus the two combination treatments failed to induce significantly higher levels of mortality than the respective single treatments.
Resistance selection studies with *Anopheles gambiae ss*

The overall *kdr* and *Ace-1R* allele frequencies were, respectively, 0.83 and 0.44 during the trials. Tables 4 and 5 present the allele and genotype frequencies for *kdr* and *Ace-1R*. The frequency of the *kdr* allele did not differ between the live and dead collections of any of the treatments during either the single (M-H Chi sq=0.2, P=0.65) or combined intervention trial (M-H Chi sq=1.6, P=0.21) (Table 4). The *Ace-1R* allele frequency during the single intervention trial was generally higher in the live collections of the p-methyl WL and NWH interventions than in the dead collections (M-H Chi square = 12.9, df=1, P=0.0003); this indicates that *Ace-1R* bearing mosquitoes were more likely to survive in huts with p-methyl treatments. However, during the combined intervention trial, the *Ace-1R* allele frequencies in the single p-methyl interventions did not differ significantly between the live and dead collections (M-H Chi sq = 1.8, P=0.18). In the combination interventions the *Ace-1R* allele frequency was actually higher in live than in the dead collections but numbers collected were low and the difference was not significant between the live and dead samples (M-H Chi sq = 1.0, P=0.32).

Analysis by genotype reveals further trends (Table 5). There were very few mosquitoes bearing no *kdr* allele. There was no significant difference in the percentage survival of homozygotes for *kdr* (40%) over heterozygotes for *kdr* (43%) during exposure to the LLIN treatment despite *kdr* resistance being supposedly recessive. The addition of p-methyl NWH or WL to the LLIN in the combination interventions did not affect the survival of heterozygotes for *kdr* relative to homozygotes for *kdr* but did increase the proportions of these genotypes killed. With respect to the *Ace-1R*, heterozygotes (*RS*) and homozygotes for *Ace-1R* (*RR*)
showed higher percentage survival than susceptible homozygotes (SS) on exposure to p-methyl WL or NWH single treatments, both in the first trial (M-H Chi sq = 16.6, P<0.001) and in the second (M-H Chi sq = 5.1, P=0.02). This indicated selection for $Ace-1^R$ and shows the importance of analysis by genotype. With the combination intervention of LLIN and p-methyl NWH the trend remained in this direction, with selection of $Ace-1^R$ genotypes. With the combination of LLIN and p-methyl WL there was, on this occasion, no trend that favoured survival of $Ace-1^R$ genotypes ($RR$ and $RS$) over susceptible homozygotes (SS). Overall there was no clear evidence to indicate that the addition of LLIN to p-methyl-treated WL or NWH would prevent the selection of $Ace-1^R$ homozygotes and heterozygotes ($RR$ and $RS$) relative to the susceptible homozygotes (SS). All three genotypes showed quite high levels of survival against single p-methyl and combination interventions. There were many more resistant heterozygotes ($RS$) and far fewer resistant homozygotes ($RR$) collected than would be expected from Hardy-Weinberg ratios.

**Residual efficacy**

The residual efficacy of the p-methyl WL and NWH as determined by cone bioassays using *An. gambiae* Kisumu declined from 100% during the first two to three weeks of the trial to 60-70% by the end of the trial.
Discussion

The aim of the study was to evaluate the efficacy and selection of resistance by p-methyl-treated WL when either applied alone or in combination with LLINs against an *An. gambiae* population in southern Côte d’Ivoire which was resistant to pyrethroids and organophosphates [13]. The reported trial was part of a multicentre trial designed to demonstrate as a proof of concept whether non-pyrethroid wall liners could provide benefits for control when combined with LLINs against malaria vector populations with differing levels of insecticide resistance. It was also hoped to assess their potential for resistance management. In the trial of similar interventions conducted in Burkina Faso where vectors were also resistant to pyrethroids but largely susceptible to organophosphates, the p-methyl WL and NWH were far more effective, killing almost all mosquitoes (>95%) that entered the huts even without the addition of LLIN [22]. The lower mortality rates achieved with p-methyl-treated WL in the Côte d’Ivoire study (50-65%) can therefore be attributed to the high levels of phenotypic resistance to organophosphates. Despite the poorer levels of control relative to the Burkina Faso study, p-methyl WL and NWH, proved to be a better option against this multiple insecticide-resistant vector population than commercial pyrethroid WL, which killed only 30% of mosquitoes entering the huts.

High vector mortality and personal protection against biting mosquitoes are the desired outcomes of any vector control tool or combination of tools. LLINs are very efficacious in areas of full susceptibility to pyrethroids, where they can induce high mortality rates in mosquito populations and provide personal protection to net users [23]. Although the insecticidal efficacy of LLINs may be compromised when
confronted with moderate to high pyrethroid resistance, LLINs can still be protective as shown in both the present Côte d’Ivoire and previous Burkina Faso studies [22] owing to the barrier effect of the net and the residual killing effect of the pyrethroid. Hence, LLINs can remain an important public health intervention even against malaria vector populations which have moderate levels of resistance to pyrethroids [8]. Against vector populations which are resistant to pyrethroids but largely susceptible to the insecticide applied on the walls, the combining of pyrethroid LLINs with non-pyrethroid IRS has shown, in small scale hut trials, improved levels of mortality (mostly due to the wall treatment) and improved personal protection (due to the LLIN) [10,24]. Under such circumstances the combination appears to restore mortality rates to levels comparable to that achieved with LLINs alone in areas where vectors are susceptible to pyrethroids [23-25]. In the present study, the combination failed to provide improved mortality over the LLIN alone against a multiple insecticide-resistant malaria vector population. This is a very disturbing finding considering the limited classes of insecticides currently available for malaria vector control. Until a class of insecticide with a novel mode of action is developed for vector control, malaria programmes faced with such multiple insecticide resistance may have no suitable alternatives to complement or provide a boost to failing LLINs. The study demonstrates the threats and challenges that the malaria vector control community will face if such resistance to multiple insecticides is left unchecked and continues to spread.

In other parts of West Africa, the Ace-1R gene has often been reported in pyrethroid-resistant An. gambiae populations at low frequencies [12,22,26,27]. While heterozygotes for Ace-1R did show some selective advantage over homozygotes for
susceptibility in the Burkina Faso study [22], the Côte d’Ivoire Tiassalé population had a far higher frequency of $Ace^{-1^R}$ and the use of organophosphate WL clearly demonstrated the survival and selection of $Ace^{-1^R}$ genotypes. A parallel mechanistic investigation on the Tiassalé population has demonstrated gene duplication at the $Ace^{-1^R}$ locus [16]; the duplication may account for the dominance and survival advantage of $Ace^{-1^R}$ genotypes and would also explain the departure from Hardy-Weinberg expectation and the surplus of heterozygotes. While the number of mosquitoes collected and analysed for $Ace^{-1^R}$ in the second (combination) trial was not huge, there was no convincing evidence that $Ace^{-1^R}$ heterozygotes or homozygotes were less likely to survive exposure to the combination relative to the single p-methyl interventions or that the combination would manage resistance. This, together with the quite high survival rates among mosquitoes that bore no $Ace^{-1^R}$ alleles, suggests the presence of another mechanism, independent of $Ace^{-1^R}$, going undetected in survivors, which was partly responsible for organophosphate resistance. Recent studies showed improved mortality of $An.~gambiae$ from Tiassalé exposed to bendiocarb, pyrethroids and an organophosphates (fenitrothion) with different synergists, thus implicating enhanced P450s and esterases in the resistance to all three classes of insecticide [16,17,28,29]. An investigation of the genetic basis of resistance in the Tiassalé population has associated genes from the CYP6 subfamily with resistance to pyrethroids and carbamates [16]. It is important to identify the specific enzyme families, which in association with the $Ace^{-1^R}$ mechanism, combine to increase resistance to p-methyl in this vector population.

While no large-scale community trial has been published on the combined effects of pyrethroid LLIN and organophosphate IRS compared to LLIN alone, two community
randomized trials of LLIN and carbamate IRS have been published recently: one in Tanzania [30] and one in Benin [31]. Both were in areas of high-frequency pyrethroid resistance and low-frequency carbamate resistance. The Tanzanian trial showed an added effect of the combination over LLIN alone, and this result was therefore consistent with the outcome of the Burkina Faso experimental hut trial of LLIN and OP wall liners (and local susceptibility status). The contrasting findings from the two multicentre hut trials in Burkina Faso and Côte d’Ivoire illustrate the uncertainty of outcome when faced with resistance to multiple insecticides rather than single insecticides. From the outcome of the Côte d’Ivoire trial, there can be no doubt that selection of multiple insecticide resistance will only make it harder to control malaria.

**Conclusion**

P-methyl WL and NWH performed better than pyrethroid WL against multiple pyrethroid and organophosphate-resistant *An. gambiae*. Combining p-methyl WL and NWH with LLINs provided no improvement in mortality and personal protection compared to the LLIN alone. There was no evidence that the combination of pyrethroid LLIN and organophosphate WL would prevent the selection of either *kdr* or *Ace-1*R resistance when both are present at detectable or moderate frequencies. The study demonstrates the challenge that malaria vector control programmes are faced with when confronted with such high levels of phenotypic resistance to multiple insecticides. Strategies of insecticide deployment or rotation to delay the rapid spread of the *Ace-1*R gene in Africa and the further development of multiple insecticide-resistant vector populations are urgently required.
Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

The trials were designed by CN, BK and MR. The field studies were performed by CN, MC, ET, BL, and NK and the activities were supervised by CN, MC, RN, and BK. Molecular analysis was performed by CN. Data were processed and analysed by CN, PJ and MR and the results interpreted by CN, RN, BK, and MR. The paper was written by CN and MR. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Andy Bywater of Syngenta for providing the insecticide. We are grateful to the entire field team and volunteer sleepers in Tiassalé, Côte d’Ivoire for their participation. Special thanks to Prof Hilary Ranson and Dr Christopher Jones of Liverpool School of Tropical Medicine (LSTM) for supporting the genotyping studies. CN, RN and MR are supported by the Malaria Centre of the London School of Hygiene and Tropical Medicine: http://malaria.lshtm.ac.uk/. The research leading to these results has received funding from the European Union Seventh Framework Programme FP7 (2007–2013) under grant agreement no. 265660 AvecNet. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
<table>
<thead>
<tr>
<th>Species</th>
<th>Insecticide-treated papers</th>
<th>Number tested</th>
<th>24-hr % mortality (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. gambiae</em> Tiassalé (wild resistant)</td>
<td>deltamethrin 0.05%</td>
<td>99</td>
<td>43 (33-56)</td>
</tr>
<tr>
<td></td>
<td>p-methyl 0.25%</td>
<td>99</td>
<td>4 (1-10)</td>
</tr>
<tr>
<td><em>An. gambiae</em> Kisumu (susceptible lab strain)</td>
<td>deltamethrin 0.05%</td>
<td>100</td>
<td>100 (96-100)</td>
</tr>
<tr>
<td></td>
<td>p-methyl 0.25%</td>
<td>99</td>
<td>100 (96-100)</td>
</tr>
</tbody>
</table>
Table 2  Efficacy of p-methyl wall lining and net wall hanging against pyrethroid-resistant *Anopheles gambiae* in Tiassalé, Côte d’Ivoire (single intervention trial)

<table>
<thead>
<tr>
<th>Hut treatment</th>
<th>Control (untreated WL)</th>
<th>Pyrethroid WL on walls</th>
<th>P-methyl WL on walls</th>
<th>P-methyl NWH on walls</th>
<th>P-methyl WL on walls and ceiling</th>
<th>P-methyl NWH on walls and ceiling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total females caught</td>
<td>53</td>
<td>114</td>
<td>98</td>
<td>70</td>
<td>54</td>
<td>77</td>
</tr>
<tr>
<td>Deterrence (%)</td>
<td>-</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total females blood fed</td>
<td>50</td>
<td>95</td>
<td>90</td>
<td>57</td>
<td>47</td>
<td>69</td>
</tr>
<tr>
<td>Blood-feeding inhibition (%)</td>
<td>-</td>
<td>12</td>
<td>1</td>
<td>14</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Personal protection (%)</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Exiting rates (%)</td>
<td>45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total dead</td>
<td>9</td>
<td>37</td>
<td>65</td>
<td>34</td>
<td>30</td>
<td>53</td>
</tr>
<tr>
<td>Corrected mortality</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39&lt;sup&gt;d&lt;/sup&gt;</td>
<td>47&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>63&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values along each row sharing the same letter superscript are not significantly different at the 5% level.
Table 3 Efficacy p-methyl wall lining and net wall hanging combined with long-lasting insecticidal nets against multiple insecticide-resistant *Anopheles gambiae* in Tiassalé, Côte d’Ivoire (combined intervention trial)

<table>
<thead>
<tr>
<th>Hut treatment</th>
<th>Control (untreated net)</th>
<th>LLIN</th>
<th>P-methyl WL</th>
<th>P-methyl NWH</th>
<th>P-methyl WL + LLIN</th>
<th>P-methyl NWH + LLIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total females caught</td>
<td>130</td>
<td>108</td>
<td>94</td>
<td>126</td>
<td>53</td>
<td>46</td>
</tr>
<tr>
<td>Deterrence (%)</td>
<td>-</td>
<td>17\textsuperscript{a}</td>
<td>28\textsuperscript{a}</td>
<td>3\textsuperscript{a}</td>
<td>59\textsuperscript{b}</td>
<td>65\textsuperscript{b}</td>
</tr>
<tr>
<td>Total females blood fed</td>
<td>74</td>
<td>20</td>
<td>71</td>
<td>91</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Blood-feeding inhibition (%)</td>
<td>-</td>
<td>67</td>
<td>0</td>
<td>0</td>
<td>84</td>
<td>77</td>
</tr>
<tr>
<td>Personal protection (%)</td>
<td>0\textsuperscript{a}</td>
<td>73\textsuperscript{b}</td>
<td>4\textsuperscript{c}</td>
<td>0\textsuperscript{c}</td>
<td>93\textsuperscript{b}</td>
<td>92\textsuperscript{b}</td>
</tr>
<tr>
<td>Total inside net (%)</td>
<td>54\textsuperscript{a}</td>
<td>15\textsuperscript{b}</td>
<td>-</td>
<td>-</td>
<td>6\textsuperscript{a}</td>
<td>10\textsuperscript{b}</td>
</tr>
<tr>
<td>Exiting rates (%)</td>
<td>29\textsuperscript{a}</td>
<td>51\textsuperscript{b}</td>
<td>53\textsuperscript{b}</td>
<td>38\textsuperscript{ac}</td>
<td>33\textsuperscript{a}</td>
<td>59\textsuperscript{b}</td>
</tr>
<tr>
<td>Total dead</td>
<td>20</td>
<td>68</td>
<td>57</td>
<td>67</td>
<td>38</td>
<td>28</td>
</tr>
<tr>
<td>Corrected mortality (%)</td>
<td>0\textsuperscript{a}</td>
<td>56\textsuperscript{bc}</td>
<td>54\textsuperscript{bc}</td>
<td>45\textsuperscript{b}</td>
<td>67\textsuperscript{c}</td>
<td>54\textsuperscript{bc}</td>
</tr>
</tbody>
</table>

Values along each row sharing the same letter superscript are not significantly different at the 5% level
Table 4  Comparative kdr and Ace-1R allele frequencies in live and dead Anopheles gambiae collected from experimental huts in Tiassalé

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Kdr allele frequency (n)</th>
<th>Ace-1R allele frequency (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live</td>
<td>Dead</td>
</tr>
<tr>
<td><strong>First trial (single intervention)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Pyrethroid WL</td>
<td>0.87</td>
<td>0.89</td>
</tr>
<tr>
<td>2 P-methyl WL (walls only)</td>
<td>0.81</td>
<td>0.83</td>
</tr>
<tr>
<td>3 P-methyl NWH (walls only)</td>
<td>0.81</td>
<td>0.88</td>
</tr>
<tr>
<td>4 P-methyl WL (walls and ceiling)</td>
<td>0.91</td>
<td>0.89</td>
</tr>
<tr>
<td>5 P-methyl NWH (walls and ceiling)</td>
<td>0.75</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>Second trial (combined intervention)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Control (untreated net)</td>
<td>0.91</td>
<td>0.91</td>
</tr>
<tr>
<td>2 LLIN</td>
<td>0.88</td>
<td>0.86</td>
</tr>
<tr>
<td>3 P-methyl WL</td>
<td>0.88</td>
<td>0.79</td>
</tr>
<tr>
<td>4 P-methyl NWH</td>
<td>0.79</td>
<td>0.83</td>
</tr>
<tr>
<td>5 P-methyl WL + LLIN</td>
<td>0.95</td>
<td>0.89</td>
</tr>
<tr>
<td>6 P-methyl NWH + LLIN</td>
<td>0.84</td>
<td>0.81</td>
</tr>
</tbody>
</table>
Table 5  Genotype selection by the single and combination treatments: percentage survival of *Anopheles gambiae kdr* and *Ace-I^r* genotypes collected from the experimental huts in Tiassalé

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SS (% alive)</th>
<th>RS (% alive)</th>
<th>RR (% alive)</th>
<th>SS (% alive)</th>
<th>RS (% alive)</th>
<th>RR (% alive)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First trial (single intervention)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1  Pyrethroid WL</td>
<td>33 (1/3)</td>
<td>73 (16/22)</td>
<td>65 (57/88)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2  P-methyl WL (walls only)</td>
<td>0 (0/3)</td>
<td>55 (6/11)</td>
<td>29 (10/34)</td>
<td>15 (3/20)</td>
<td>28 (22/78)</td>
<td>-</td>
</tr>
<tr>
<td>3  P-methyl NWH (walls only)</td>
<td>75 (3/4)</td>
<td>0 (0/2)</td>
<td>50 (13/26)</td>
<td>25 (3/12)</td>
<td>59 (30/51)</td>
<td>0 (0/4)</td>
</tr>
<tr>
<td>4  P-methyl WL (walls and ceiling)</td>
<td>0 (0/2)</td>
<td>75 (3/4)</td>
<td>41 (13/32)</td>
<td>15 (2/13)</td>
<td>51 (22/43)</td>
<td>-</td>
</tr>
<tr>
<td>5  P-methyl NWH (walls and ceiling)</td>
<td>0 (0/1)</td>
<td>55 (6/11)</td>
<td>30 (6/20)</td>
<td>0 (0/9)</td>
<td>43 (24/56)</td>
<td>25 (1/4)</td>
</tr>
<tr>
<td><strong>Second trial (combined intervention)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1  Control (untreated net)</td>
<td>100 (2/2)</td>
<td>77 (10/13)</td>
<td>83 (64/77)</td>
<td>70 (7/10)</td>
<td>85 (66/78)</td>
<td>100 (4/4)</td>
</tr>
<tr>
<td>2  LLIN</td>
<td>0 (0/2)</td>
<td>43 (10/23)</td>
<td>40 (31/78)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3  P-methyl WL</td>
<td>0 (0/2)</td>
<td>50 (6/12)</td>
<td>53 (18/34)</td>
<td>17 (1/6)</td>
<td>70 (23/33)</td>
<td>40 (2/5)</td>
</tr>
<tr>
<td>4  P-methyl NWH</td>
<td>0 (0/1)</td>
<td>67 (10/15)</td>
<td>50 (14/28)</td>
<td>43 (3/7)</td>
<td>56 (19/34)</td>
<td>50 (2/4)</td>
</tr>
<tr>
<td>5  P-methyl WL + LLIN</td>
<td>0 (0/2)</td>
<td>20 (1/5)</td>
<td>23 (10/44)</td>
<td>20 (2/10)</td>
<td>18 (7/39)</td>
<td>50 (2/4)</td>
</tr>
<tr>
<td>6  P-methyl NWH + LLIN</td>
<td>0 (0/2)</td>
<td>67 (6/9)</td>
<td>50 (13/26)</td>
<td>17 (1/6)</td>
<td>56 (15/27)</td>
<td>75 (3/4)</td>
</tr>
</tbody>
</table>

SS = susceptible homozygotes, RS = resistant heterozygotes, RR = resistant homozygotes
Figure 1: Mortality and blood feeding rates of multiple insecticide resistant *An gambiae* (Tiassalé) in experimental huts (single interventions trial). For each outcome (mortality or bloodfeeding), histograms bearing the same letter label are not significantly different at the 5% level.

Figure 2: Mortality and blood feeding rates of multiple insecticide resistant *An gambiae* (Tiassalé) in experimental huts (combined interventions trial). For each outcome (mortality or bloodfeeding), histograms bearing the same letter label are not significantly different at the 5% level.
Authors' contributions

The trials were designed by CN BK and MR, The field studies were performed by CN, MC, ET, BL and NK and the activities were supervised by CN, MC, RN and BK. Molecular analysis was performed by CN. Data was processed and analysed by CN, PJ and MR and the results interpreted by CN, RN, BK and MR. The paper was written by CN and MR. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Andy Bywater of Syngenta for providing the insecticide. We are grateful to the entire field team and volunteer sleepers in Tiassalé, Côte d'Ivoire for their participation. Special thanks to Prof Hilary Ranson and Dr. Christopher Jones of LSTM for supporting the genotyping studies. Corine Ngufor, Raphael N’Guessan and Mark Rowland are supported by the Malaria Centre of the London School of Hygiene and Tropical Medicine: http://malaria.lshtm.ac.uk/. The research leading to these results has received funding from the European Union Seventh Framework Programme FP7 (2007–2013) under grant agreement no. 265660 AvecNet. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

3. Rowland M: Malaria control: bednets or spraying? Malaria control in the Afghan 

Traore AS, Diallo B, Konate M, Guindo A: Multicentre studies of insecticide-treated 
durable wall lining in Africa and South-East Asia: entomological efficacy and household 

evaluation of ZeroFly® - an insecticide incorporated plastic sheeting against malaria 
vectors and its impact on malaria transmission in tribal areas of northern Orissa. Ind J 

African anopheline mosquitoes: what are the implications for malaria control? Trends 

7. WHO: Global plan for insecticide resistance management. World Health Organization, 

8. WHO: The technical basis for coordinated action against insecticide resistance: preserving 
the effectiveness of modern malaria vector control. World Health Organization, Geneva, 
2010.

9. WHO: Pesticides and their application for the control of vectors and pest of public health 

V: Indoor use of plastic sheeting impregnated with carbamate combined with long-
lasting insecticidal mosquito nets for the control of pyrethroid-resistant malaria vectors. 

indoor residual formulation of the organophosphate insecticide pirimiphos methyl for 
prolonged control of pyrethroid-resistant mosquitoes: an experimental hut trial in 

12. Alou LPA, Koffi AA, Adja MA, Tia E, Kouassi PK, Koné M, Chandre F: Distribution of 
ace-1 R and resistance to carbamates and organophosphates in Anopheles gambiae s.s. 
populations from Côte d’Ivoire. Malar J 2010, 9:167


PART THREE

Research question: Can the efficacy of LLINs against pyrethroid resistant malaria vectors be improved when treated with a mixture of pyrethroids and an alternative compounds to which vectors are susceptible?

Chapter 7: Mosquito nets treated with a mixture of chlorfenapyr and alphacypermethrin control pyrethroid resistant *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes in West Africa

Chapter 8: Olyset Duo® (a pyriproxyfen and permethrin mixture net): an experimental hut trial against pyrethroid resistant *Anopheles gambiae* and *Culex quinquefasciatus* in Southern Benin.
Chapter 7

Mosquito nets treated with a mixture of chlorfenapyr and alphacypermethrin control pyrethroid resistant *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes in West Africa

The work in this chapter has been published as:

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

1. For a 'research paper' already published
1.1. Where was the work published? PLoSOne
1.2. When was the work published? February 2014
1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion N/A

1.3. Was the work subject to academic peer review? YES
1.4. Have you retained the copyright for the work? OPEN ACCESS
   If yes, attach evidence of retention
   If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published. N/A
2.1. Where is the work intended to be published?
2.2. List the paper's authors in the intended authorship order
2.3. Stage of publication - Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press

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

Candidate's signature ________________________________________________
Supervisor or senior author's signature to confirm role as stated in (3) ____________________________________________
Abstract

Background: The effectiveness of insecticide treated nets is under threat across Africa south of the Sahara from the selection of pyrethroid resistance in *Anopheles gambiae* mosquitoes. To maintain progress against malaria it is necessary to identify alternative residual insecticides for mosquito nets. Mixtures of pyrethroid and insecticides with novel mode of action provide scope for both improved control and management of resistance through concurrent exposure to unrelated insecticides.

Methods: The pyrrole chlorfenapyr and the pyrethroid alphacypermethrin were tested individually and as a mixture on mosquito nets in an experimental hut trial in southern Benin against pyrethroid resistant *An. gambiae* and *Culex quinquefasciatus* mosquitoes. The nets were deliberately holed to simulate the effect of wear and tear.

Results: The nets treated with the mixture of chlorfenapyr 200mg/m² and alphacypermethrin 25mg/m² killed a proportion of *An. gambiae* (77%, 95%CI: 66-86%) significantly greater than nets treated with alphacypermethrin 25mg/m² (30%, 95%CI: 21-41%) but not significantly different from nets treated with chlorfenapyr 200mg/m² (69%, 95%CI: 57-78%). The nets treated with the mixtures procured personal protection against *An. gambiae* biting (58-62%) by a greater margin than the alphacypermethrin treated net (39%), whereas the chlorfenapyr treated net was not protective. A similar trend in mortality and blood feeding inhibition between treatments was observed in *Cx quinquefasciatus* to that seen in *An. gambiae*, although the effects were lower. A mixture of alphacypermethrin with chlorfenapyr applied at 100mg/m² had an effect similar to the mixture with chlorfenapyr at 200mg/m².

Conclusion: The effectiveness of ITNs against pyrethroid resistant mosquitoes was restored by the mixture: the alphacypermethrin component reduced human-vector contact while the chlorfenapyr controlled pyrethroid-resistant mosquitoes. The complementary action of these unrelated insecticides demonstrates that the combination on nets has potential for preventing malaria transmission in areas compromised by the spread of pyrethroid resistance.
Introduction

Long lasting insecticidal nets (LLINs) are considered best practice for malaria vector control because they are effective, reliable, robust and relatively simple to deliver even in remote regions [1]. The recent reductions in malaria-associated morbidity and mortality across sub Saharan Africa is largely attributed to the massive roll out of LLINs during the last decade [1-2]. Owing to their low cost, longer residual activity and safety, the pyrethroids remain the ideal insecticides for treating LLINs [1, 3]. Unfortunately, resistance to pyrethroids is spreading fast across Africa south of the Sahara and has now been reported in malaria vectors in 27 countries [4].

While a negative epidemiological impact of pyrethroid resistance on malaria control has yet to be demonstrated unequivocally, an increasing number of reports have indicated that pyrethroid resistance is capable of undermining the effectiveness of LLINs [5-6]. The situation in southern Benin is particularly grave since the premier brands of LLIN give only limited personal protection and kill fewer mosquitoes in that region than in regions of susceptibility [5]. It is now recognised that if nothing is done and further selection of resistance to pyrethroids leads to failure of LLINs, the progress achieved so far in reducing the burden of malaria could be reversed [7].

The need to identify alternative insecticides that can circumvent resistance and preserve the effectiveness of LLINs has become critical. Some alternative insecticides (such as the organophosphates and carbamates) have been tested on nets [8-9]. These candidates show toxicity but generally lack the irritancy of pyrethroids, an important property for reducing mosquito biting rates and providing
personal protection to users of insecticide treated nets (ITNs). Mosquito nets can be treated with a mixture of pyrethroid and non-pyrethroid insecticides to maximise insecticidal efficacy against pyrethroid resistant mosquitoes while providing a degree of excito-repellency. Such mixtures may also provide opportunity for insecticide resistance management because insect phenotypes which are not killed by one component due to resistance will be controlled by the other provided they are not resistant to both insecticides [10-11].

An alternative insecticide to pyrethroids that is presently under development is chlorfenapyr, a pyrrole which owing to its novel mode of action is active against both pyrethroid resistant and susceptible anophelines and culicines [12-13]. When evaluated on mosquito nets against wild mosquitoes in experimental huts, chlorfenapyr was found to be more effective than pyrethroids at killing resistant *Anopheles* and *Culex* [14-15]. The use of this pyrrole by itself on mosquito nets neither deters nor repels and so needs to be combined with an excito-repellent insecticide [15]. Chlorfenapyr has already shown great promise when applied as IRS in conjunction with LLINs for improved transmission control of pyrethroid resistant mosquitoes [16]. Preliminary studies with a mixture of chlorfenapyr and alphacypermethrin on ITNs has shown good potential for control of *An arabiensis* [17].

The objective of the trial reported here was to determine in experimental huts the potential of mosquito nets treated with a mixture of chlorfenapyr and the pyrethroid alphacypermethrin to protect against and control host-seeking *An gambiae* and *Cx quinquefasciatus* that are strongly resistant to pyrethroids.
Materials and methods

Study site and experimental huts

The study was conducted on a private land at Akron, a village on the outskirts of Porto Novo, Benin. The owner of the land gave permission to conduct the study on this site. The site supported breeding of *An gambiae* M form that are pyrethroid-resistant due to high frequency of *kdr* (>90%) and increased activity of cytochrome P450s [18]. The nuisance mosquito *Cx quinquefasciatus* is present year round and shows resistance to pyrethroids, carbamates and organophosphates [19]. Five experimental huts were selected for the trial. The hut design was the West African type recommended by WHO [20].

Mosquito net treatments and trial procedure

The nets were made of white 100-denier polyester (SiamDutch Mosquito Netting Co., Bangkok, Thailand). To simulate damaged nets, six standardized holes (each measuring 4cm x 4cm) were cut into the sides and ends of each net as recommended by WHO [20]. The nets were treated with SC formulations of alphacypermethrin (Fendona 60SC, BASF, Germany) and chlorfenapyr (Phantom 240SC, BASF, Germany) in aqueous solution either separately or mixed together. The treatment arms were: (i) untreated control net, (ii) chlorfenapyr 200mg/m² treated net, (iii) alphacypermethrin 25mg/m² treated net, (iv) chlorfenapyr 100mg/m² and alphacypermethrin 25mg/m² mixture treated net, and (v) chlorfenapyr 200mg/m² and alphacypermethrin 25mg/m² mixture treated net.
The 5 treatments were randomly allocated to the 5 experimental huts and rotated weekly between huts. Adult volunteers slept in the huts from 20.00 - 05.00 and were rotated between huts on consecutive nights. Informed consent to participate was given before recruitment and daily chemoprophylaxis was provided until 4 weeks after the trial. Each morning the volunteers helped to collect mosquitoes from inside the rooms, nets and verandah traps. Mosquito collections were made on 36 nights over six weeks between April and June 2010. The identification to species, mortality counts and determination of feeding status and gonotrophic condition were made in the laboratory. Live mosquitoes were held in netted plastic cups and provided with 10% honey solution; mortality was recorded after 72h. Climatic information for each day of the trial was collected from the weather station based in Porto Novo.

The principal aim was to assess the efficacy of treatments relative to untreated control nets in terms of (i) deterrenacy: the proportional reduction in the number of mosquitoes entering huts with treated nets, (ii) insecticide induced exiting rates: estimated from the proportions of mosquitoes collected from the verandahs of treatment and control huts, (iii) blood-feeding inhibition rate: the reduction in the proportion of blood fed mosquitoes in huts with treated nets compared to huts with untreated nets = (1 - proportion bloodfed in treatment/proportion bloodfed in control) x 100; (iv) mortality rate: the proportion of mosquitoes dying within 72h, corrected for control mortality; (v) personal protection.

**Data analysis**

The analysis of numbers of mosquitoes collected within huts (overall total, blood-fed and dead totals) was carried out using negative binomial regression after adjusting
for the effects of hut and sleeper. The analysis of treatment effects on the proportions of mosquitoes blood-feeding, exiting or killed was carried out using logistic regression, with treatments as fixed effects and huts, sleepers as random effects. All statistical analysis was conducted by using STATA 9 software (STATA Corp., College Station, USA).

Species and resistance characterisation

Samples of An. gambiae s.l. reared from larval collections near the trial site were identified to species using PCR [21] and to molecular form using PCR RFLP [22]. WHO test kits, lined with test papers treated with alphacypermethrin in silicon oil were used to determine susceptibility of An. gambiae and Cx. quinquefasciatus females reared from larval collections.

PCR diagnostic test for detection of kdr mutations was carried out on An. gambiae and Cx. quinquefasciatus mosquitoes as described by Martinez-Torres et al. [23].

Toxicology

Chlorfenapyr has a WHO toxicological classification III, an LD50 oral toxicity in rats of >400 mg /kg body weight and acute dermal toxicity >2000 mg /kg; a category of similar to many insecticides used in public health [24]. A risk assessment of the use of chlorfenapyr on nets was undertaken by BASF toxicologists using assumptions, parameters and default values defined in the WHO generic risk assessment model [25]. The calculated exposure levels to chlorfenapyr were all below the relevant dermal and systemic acceptable exposure levels for repeated exposure. Exposure
was deemed acceptable based on conservative scenarios from the WHO model, indicating safe use of the chlorfenapyr-treated nets for the intended use.

Ethical clearance

Written informed consent was obtained from all volunteers recruited to the experimental hut studies. The study was approved by ethics committees of the London School of Hygiene and Tropical Medicine and the Ministry of Health in Benin.

Results

Experimental Hut Trial

Over the six week trial, 515 *Anopheles gambiae* s.l., 3764 *Culex quinquefasciatus* and 453 *Manson*ia females were caught in the huts. Only the data on *An. gambiae* s.l. and *Cx. quinquefasciatus* were analysed further (Table 1&2).

Compared to huts containing untreated nets the entry rates of both species into huts with ITNs were up to 40% less (p<0.001) (Table 1&2). Hut entry rates did not differ significantly between huts with alphacypermethrin, chlorfenapyr or mixture treated nets.

In huts where nets were treated with alphacypermethrin or alphacypermethrin- chlorfenapyr mixtures the proportions of *An. gambiae* and *Cx. quinquefasciatus* that exited into verandahs were significantly greater than huts where nets were untreated or treated with chlorfenapyr (p<0.0001) (Table 1&2). For *An. gambiae* the proportions that exited into verandahs did not differ between huts with chlorfenapyr treated or untreated nets (Table 1), whereas for *Cx. quinquefasciatus* the proportion
that exited into verandahs was greater for chlorfenapyr treated nets (p<0.001) (Table 2).

Blood-feeding inhibition of *An. gambiae* was not evident where nets were treated with chlorfenapyr alone (figure 1A). There was some blood-feeding inhibition of *An. gambiae* where nets were treated with alphacypermethrin alone (22%, P= 0.042). and further inhibition where nets were treated with the mixtures (35%, 51%, P<0.01). The difference in bloodfeeding inhibition between the mixture with chlorfenapyr 200mg/m2 and alphacypermethrin alone was not significant (P=0.061).

Blood-feeding inhibition rates were generally higher for *Cx. quinquefasciatus* than for *An. gambiae*, reaching 28% inhibition for the chlorfenapyr net, 81% for the alphacypermethrin net and 78% and 82% for the mixtures (Figure 1). For *Cx. quinquefasciatus* the inhibition of blood-feeding was attributable more to the pyrethroid component than to the chlorfenapyr component.

Personal protection against the biting of *An gambiae* ranged from 58% to 62% with the insecticide mixtures, higher than with either insecticide when applied to nets alone (Table 1). Personal protection from the mixtures was higher still against *Cx. quinquefasciatus* (86% to 90%) but was not significantly different from alphacypermethrin net (Table 2).

Mortality rates of *An. gambiae* and *Cx. quinquefasciatus* where nets were treated with alphacypermethrin were less than 30%, and were presumably due to the high level of pyrethroid resistance in the two species (Figure 2). Nets treated with the insecticide mixtures induced three times higher mortality of *An. gambiae* (75%) and *Cx quinquefasciatus* (47-50%) than the alphacypermethrin treated nets (P<0.01). Mortalities of *An. gambiae* with the mixtures were not significantly different to that
induced by chlorfenapyr alone (P=0.082 for the mixture with chlorfenapyr 100mg/m² and P=0.065 for chlorfenapyr 200mg/m²) and hence the mixture’s toxicity is attributable mainly to the chlorfenapyr component (Figure 2). The difference in mortality of An. gambiae between mixtures with low and high dosages of chlorfenapyr was not significant (P=0.917).

A similar trend in mortality to that seen in An. gambiae was observed in Cx quinquefasciatus, although the effect of each treatment on mortality was 20-50% lower.

The average daily temperature (minimum, minimum) during the first week of the trial was 31.5 degree (27.7, 34.3). Temperature gradually decreased each week and by the 12th week was 27.8 (25.4, 30.2).

**Residual activity**

Cone bioassays conducted each month with the An. gambiae (Kisumu) susceptible strain on nets treated with the mixture or alphacypermethrin induced 87-100% mortality (N=50 per test) throughout the 3 months. Bioassays on nets treated with chlorfenapyr killed 77% initially and 65% by the end of the trial.

**Species characterization and resistance status**

Only An. gambiae s.s. M form was found in the trial area. In WHO susceptibility tests with pyrethroid test papers percentage mortality was 20% for An gambiae and 17% for Cx quinquefasciatus. In molecular assays on An. gambiae the frequency of kdr was 0.86. No molecular assays were conducted on contemporary samples of Cx quinquefasciatus but in an earlier characterization in the area the frequency of kdr was 0.63 [19].
Discussion

The aim of the study was to determine whether ITNs treated with a mixture of alphacypermethrin and chlorfenapyr have the potential to provide individual protection against mosquito biting and control of mosquitoes in regions of West Africa where the development of pyrethroid resistance in An. gambiae is undermining the effectiveness of pyrethroid treated nets and threatening malaria control. In experimental huts nets treated with the insecticide mixture reduced survival of An. gambiae by 75% and prevented host-vector contact by 40-50%, an effect similar to pyrethroid treated nets and LLINs in areas that have not yet developed pyrethroid resistance [5, 26]. The loss of efficacy of pyrethroid treated nets when encountering with pyrethroid resistance at the level that occurs in Southern Benin [6, 27] was therefore restored by the mixture.

By combining a pyrethroid and a pyrrole on the same net, it was possible to benefit from the properties of each insecticide: the protective (excito-repellent) effect of the pyrethroid and the killing effect of the pyrrole against pyrethroid resistant Anopheline and Culicine mosquitoes. The low rates of mosquito mortality and blood feeding inhibition shown by the alphacypermethrin ITN being compared were typical of pyrethroid ITN trials in this area against An. gambiae and Cx. quinquefasciatus [5, 27]. Transmission control through mosquito mortality and personal protection from mosquito biting are important attributes of any vector control tool as these work together to reduce vectorial capacity. By both procuring individual protection and
killing mosquitoes the chlorfenapyr-alphacypermethrin mixture shows greater potential for malaria control in areas with high level pyrethroid resistance than could be achieved by pyrethroid LLINs.

Chlorfenapyr toxicity to mosquitoes in bioassay is positively temperature dependent (N’Guessan and Rowland, unpublished data). At the ambient temperatures recorded outdoors during the trial the lowest minimum daily temperature was 25.4 and the highest minimum daily temperature was 28.7 degrees. Chlorfenapyr was very effective within this range.

Previously, mixtures of a carbamate (carbosulfan) and a pyrethroid were evaluated on mosquito nets but were not taken forward due to mammalian toxicity issues associated with the carbamate [28]. Chlorfenapyr has a non neurological mode of action and would be a more appropriate companion insecticide due to its safety [24], effectiveness against mosquitoes, and absence of cross resistance to any existing class of insecticide [13, 29]. Reducing the concentration of chlorfenapyr by half (to 100mg/m²) did not reduce the effectiveness of the mixture, and has obvious cost benefits.

Quite apart from the restoration of control which mixtures promise, empirical research and modelling predict that mixtures are the most efficient tactic for managing insecticide resistance [7]. The attributes required of mixtures in order to prevent the selection of resistance alleles are rigorous, and include the maintenance of effective concentrations of both insecticide components over the lifetime of the net [10-11]. This is the challenge which the next generation of bi-treated LLINs and the
formulators of mixtures on nets must meet. In any event, insecticide mixtures for malaria control are a modern day reality. LLIN coverage is going universal and IRS with non-pyrethroid insecticides is being applied concurrently with LLINs as malaria control policy in many areas of high malaria transmission [30-31]. Chlorfenapyr is already showing potential as an IRS treatment in combination with pyrethroid LLIN [16]. A long lasting mixture of chlorfenapyr and alphacypermethrin on nets, if realised, will make an essential contribution to the next generation of LLINs and to prevention of malaria.

**Acknowledgements:** The authors thank Drs Susanne Stutz, Wolfgang Paulus (BASF Corp., Ludwigshafen, Germany), James Austin (BASF Corp., NC, USA) and Robert Farlow for supplying samples and for technical input; Robert Sloss for technical programme support (IVCC, Liverpool, UK). We are grateful to E. Vigninou and A. Foungnikin for field and laboratory assistance. LSHTM and KCMC are members of the Pan African Malaria Vector Research Consortium (http://www.pamverc.or.tz). RN, CN and MR are members of the Malaria Centre of the London School of Hygiene & Tropical Medicine (http://malaria.lshtm.ac.uk).
Figure 1. Blood-feeding inhibition rates of *Anopheles gambiae* and *Culex quinquefasciatus* in experimental huts.

Figure 2. Control corrected mortality of *Anopheles gambiae* and *Culex quinquefasciatus* in experimental huts.
Table 1. Experimental hut trial results against pyrethroid resistant *Anopheles gambiae*.

<table>
<thead>
<tr>
<th></th>
<th>Untreated net</th>
<th>Chlorfenapyr 200mg/m²</th>
<th>Alphacypermethrin 25mg/m²</th>
<th>Mixture 1 (Chlorfenapyr 100 + Alpha 25)</th>
<th>Mixture 2 (Chlorfenapyr 200 + Alpha 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total females caught</strong></td>
<td>102ᵃ</td>
<td>76ᵇ</td>
<td>81ᵇ</td>
<td>80ᵇ</td>
<td>66ᵇ</td>
</tr>
<tr>
<td><strong>Deterrence %</strong></td>
<td>-</td>
<td>26</td>
<td>21</td>
<td>22</td>
<td>35</td>
</tr>
<tr>
<td><strong>Total females in verandah trap</strong></td>
<td>36</td>
<td>31</td>
<td>56</td>
<td>59</td>
<td>50</td>
</tr>
<tr>
<td><strong>Exiting % (95% CI)</strong></td>
<td>35 (26-45)ᵃ</td>
<td>41 (30-52)ᵃ</td>
<td>69 (59-79)ᵇ</td>
<td>74 (64-83)ᵇ</td>
<td>76 (65-86)ᵇ</td>
</tr>
<tr>
<td><strong>Total females blood fed</strong></td>
<td>77</td>
<td>59</td>
<td>47</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td><strong>Blood-feeding % (95% CI)</strong></td>
<td>75 (66-83)ᵃ</td>
<td>78 (68-87)ᵃ</td>
<td>58 (47-69)ᵇ</td>
<td>36 (26-47)ᶜ</td>
<td>49 (36-61)bc</td>
</tr>
<tr>
<td><strong>Personal protection %</strong></td>
<td>-</td>
<td>23</td>
<td>39</td>
<td>62</td>
<td>58</td>
</tr>
</tbody>
</table>

Mixture 1 and 2 applied alphacypermethrin at 25mg/m² and chlorfenapyr at 100 and 200 mg/m² respectively. Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).

Table 2. Experimental hut trial results against pyrethroid resistant *Culex quinquefasciatus*.

<table>
<thead>
<tr>
<th></th>
<th>Untreated net</th>
<th>Chlorfenapyr 200mg/m²</th>
<th>Alphacypermethrin 25mg/m²</th>
<th>Mixture 1 (Chlorfenapyr 100 + Alpha 25)</th>
<th>Mixture 2 (Chlorfenapyr 200 + Alpha 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total females caught</strong></td>
<td>811ᵃ</td>
<td>527ᵇ</td>
<td>567ᵇ</td>
<td>487ᵇ</td>
<td>492ᵇ</td>
</tr>
<tr>
<td><strong>Deterrence %</strong></td>
<td>-</td>
<td>35</td>
<td>30</td>
<td>40</td>
<td>39</td>
</tr>
<tr>
<td><strong>Total females in verandah trap</strong></td>
<td>227</td>
<td>279</td>
<td>408</td>
<td>370</td>
<td>354</td>
</tr>
<tr>
<td><strong>Exiting % (95% CI)</strong></td>
<td>28 (25-31)ᵃ</td>
<td>53 (49-57)ᵇ</td>
<td>72 (68-75)ᶜ</td>
<td>76 (72-79)ᶜ</td>
<td>72 (68-76)ᶜ</td>
</tr>
<tr>
<td><strong>Total females blood fed</strong></td>
<td>430</td>
<td>200</td>
<td>57</td>
<td>44</td>
<td>59</td>
</tr>
<tr>
<td><strong>Blood-feeding % (95% CI)</strong></td>
<td>53 (49-56)ᵃ</td>
<td>38 (34-42)ᵇ</td>
<td>10 (7-12)ᶜ</td>
<td>9 (7-12)ᶜ</td>
<td>12 (7-15)ᶜ</td>
</tr>
<tr>
<td><strong>Personal protection %</strong></td>
<td>-</td>
<td>53</td>
<td>87</td>
<td>90</td>
<td>86</td>
</tr>
</tbody>
</table>

Mixture 1 and 2 applied alphacypermethrin at 25mg/m² and chlorfenapyr at 100 and 200 mg/m² respectively. Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).
References


17. Oxborough R, Kitau J, Matowo J. Feston E, Mndeme R, et al. (2013). ITN mixtures of chlorfenapyr (pyrrole) and alphacypermethrin (pyrethroid) for improved control of
pyrethroid resistant *Anopheles arabiensis* and *Culex quinquefasciatus*. PLoS ONE 8: e55781.

18. Djouaka RF, Bakare AA, Coulibaly ON,, Akogbeto MC, Ranson H, et al. (2008) Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from Southern Benin and Nigeria. BMC Genomics 9:53


Chapter 8

Olyset Duo® (a pyriproxyfen and permethrin mixture net): an experimental hut trial against pyrethroid resistant *Anopheles gambiae* and *Culex quinquefasciatus* in Southern Benin.

The research presented in this section has been published as:

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

1. For a 'research paper' already published
1.1. Where was the work published? PLoSOne
1.2. When was the work published? April 2014
1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion N/A

1.3. Was the work subject to academic peer review? YES
1.4. Have you retained the copyright for the work? OPEN ACCESS
   If yes, attach evidence of retention
   If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published. N/A
2.1. Where is the work intended to be published?
2.2. List the paper's authors in the intended authorship order
2.3. Stage of publication - Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)
   I co-designed the study, supervised/performed the field activities, analysed the data, co-interpreted the findings and wrote the manuscript.

Candidate's signature ________________________________
Supervisor or senior author's signature to confirm role as stated In (3) ________________________________
Abstract

Background
Alternative compounds which can complement pyrethroids on long-lasting insecticidal nets (LN) in the control of pyrethroid resistant malaria vectors are urgently needed. Pyriproxyfen (PPF), an insect growth regulator, reduces the fecundity and fertility of adult female mosquitoes. LNs containing a mixture of pyriproxyfen and pyrethroid could provide personal protection through the pyrethroid component and reduce vector abundance in the next generation through the sterilizing effect of pyriproxyfen.

Method
The efficacy of Olyset Duo®, a newly developed mixture LN containing pyriproxyfen and permethrin, was evaluated in experimental huts in southern Benin against pyrethroid resistant Anopheles gambiae and Culex quinquefasciatus. Comparison was made with Olyset Net® (permethrin alone) and a LN with pyriproxyfen alone (PPF LN). Laboratory tunnel tests were performed to substantiate the findings in the experimental huts.

Results
Overall mortality of wild pyrethroid resistant An. gambiae s.s. was significantly higher with Olyset Duo than with Olyset Net (50% vs. 27%, P=0.01). Olyset DUO was more protective than Olyset Net (71% vs. 3%, P<0.001). The oviposition rate of surviving blood-fed An. gambiae from the control hut was 37% whereas none of those from Olyset Duo and PPF LN huts laid eggs. The tunnel test results were consistent with the experimental hut results. Olyset Duo was more protective than Olyset Net in the huts against wild pyrethroid resistant Cx. quinquefasciatus although mortality rates of this species did not differ significantly between Olyset Net and Olyset Duo. There was no sterilizing effect on surviving blood-fed Cx. quinquefasciatus with the PPF-treated nets.

Conclusion
Olyset Duo was superior to Olyset Net in terms of personal protection and killing of pyrethroid resistant An. gambiae, and sterilized surviving blood-fed mosquitoes. Mixing pyrethroid and pyriproxyfen on a LN shows potential for malaria control and management of pyrethroid resistant vectors by preventing further selection of pyrethroid resistant phenotypes.
Background

Malaria vector control relies primarily on two interventions: long lasting insecticidal nets (LNs) and indoor residual spraying (IRS). Both interventions have contributed significantly to the recent reductions in malaria morbidity and mortality observed across sub-Saharan Africa [1]. While several classes of insecticide can be used for IRS, the pyrethroids are currently the only class of insecticide recommended by the World Health Organisation (WHO) for treating LNs owing to their safety, excito-repellency and rapid knock down effect. Pyrethroid resistance has become widespread in malaria vectors in several malaria endemic parts of the world [2]. Recent reports across Africa have shown that pyrethroid resistance threatens to undermine the effectiveness of LNs and without prompt action, the benefits so far achieved in the control of malaria could be reversed [3,4].

The prospects for identifying alternative insecticides to pyrethroids for treating mosquito bed-nets are limited [3]. Most alternatives tested on mosquito nets are too toxic to mammals and lack the excito-repellent property inherent in pyrethroids; hence they provide little or no direct personal protection to users [5-8]. However, without LNs as a vehicle for insecticide, it is unlikely that the goal of universal coverage with personal protection can be achieved or sustained in most malaria endemic communities [3]. Strategies to preserve the efficacy of LNs in the era of pyrethroid resistance are therefore paramount. Mosquito nets can be treated with a combination of pyrethroid and non pyrethroid insecticide to which vectors are susceptible. This approach provides an opportunity to preserve the protectiveness of the net through the excito-repellent properties of the pyrethroid while enhancing toxicity through the non-pyrethroid alternative [9]. Use of mixtures on nets has the
potential to manage insecticide resistance if insects resistant to one insecticide are susceptible to and killed by the other [4,10,11].

Pyriproxyfen is an insect juvenile hormone mimic, recommended for larval control by WHO [12,13]. It is safe to humans and shows no cross resistance to other classes of insecticides used for vector control [14]. The primary use of pyriproxyfen is as an insect growth regulator to inhibit adult emergence hence its use for mosquito control has been limited to larval stages [12,15,16]. However, pyriproxyfen has also been reported to inhibit oogenesis and sterilize adult mosquito vectors [17]. Studies with adult *Aedes aegypti* have demonstrated reduced fecundity in females which have tarsal contact with pyriproxyfen treated substrates [18,19]. Earlier studies on Anophelines demonstrated reduced fertility in the eggs oviposited by *Anopheles stephensi* females exposed to pyriproxyfen treated netting [20]. More recent studies have shown complete sterilization of *An. gambiae* females exposed to pyriproxyfen treated netting [17] and *An. arabiensis* females exposed one day after feeding to pyriproxyfen in CDC bottle bioassays [21].

Mixing pyriproxyfen with pyrethroids on mosquito nets could provide a combination of personal protection through the pyrethroid component and mass population effect on the next generation of vectors through the sterilizing effect of the pyriproxyfen component on parental females. Such a mixture LN is expected to be effective against a wide range of mosquito species including those with multiple mechanisms of resistance to current insecticides. It could also slow the spread of pyrethroid resistance genes if deployed in areas where pyrethroid resistance is still rare. In the current study, we evaluated the efficacy of Olyset Duo (Sumitomo Chemical Company Ltd); a newly developed pyriproxyfen and permethrin incorporated
polyethylene LN in experimental huts against wild, free flying pyrethroid resistant *An. gambiae* and *Cx. quinquefasciatus* in Southern Benin where both mosquito species are highly resistant to pyrethroids. Comparison was made to a WHOPES-recommended LN treated with permethrin alone (Olyset Net; Sumitomo Chemical Company Ltd) and a LN treated with pyriproxyfen alone, which was formulated to the same technical specifications as Olyset Duo. Studies with resistant strains were also carried out using laboratory tunnel tests to corroborate the findings in the experimental huts.

**Materials and methods**

*Study site and experimental huts*

The study was carried out at the CREC experimental hut station in Akron, a village on the outskirts of Porto Novo, Benin. The site supports breeding of *An. gambiae* M form that are pyrethroid-resistant due to high frequency of *kdr* (>90%) and increased activity of cytochrome P450s [22]. The nuisance mosquito *Cx.. quinquefasciatus* is present year round and shows resistance to pyrethroids, carbamates and organophosphates [22]. Four experimental huts of the West African design as recommended by WHO were used for the study. The huts are built on concrete plinths surrounded by water-filled moats to prevent entry of scavenging ants. Mosquitoes exiting the huts are captured by veranda traps. The huts are made of brick plastered with cement on the inside, with a corrugated iron roof and have a ceiling of palm thatch and four window slits (1cm gap) on their walls through which mosquitoes enter.
**Treatments and trial procedure**

The following four treatments were tested in the experimental huts.

13. Untreated control mosquito net (polyethylene net),
14. Pyriproxyfen (PPF) LN (Sumitomo Chemical Co. Ltd., Tokyo, Japan),
15. Olyset Net® (Sumitomo Chemical Co. Ltd., Tokyo, Japan) – a WHOPES-recommended standard permethrin incorporated LN,
16. Olyset Duo® (Sumitomo Chemical Co. Ltd., Tokyo, Japan) – a newly developed 1% w/w pyriproxyfen and 2% w/w permethrin incorporated LN.

Olyset DUO and Olyset Net have the same concentration of permethrin. Olyset DUO however has a faster permethrin bleed rate (rate of release from the net fibres to the surface) than Olyset Net. Preliminary laboratory studies revealed a shorter regeneration time of permethrin in Olyset DUO (3days) than Olyset Net (7days) confirming the faster rate. PPF LN does not contain permethrin but has a similar pyriproxyfen bleed rate as Olyset DUO.

To simulate wear and tear, the bed nets were intentionally holed with six 16cm² holes (two holes on each side and one on each end) according to WHOPES guidelines [23]. Treatments were allocated to the experimental huts on a weekly basis following a Latin square design to adjust for any variation in site attractiveness of the huts. Four adult human volunteers were offered chemoprophylaxis and slept in the huts from 20:00 to 05:00 each night of the study; they were rotated between huts on successive nights to adjust for any variation in individual attractiveness to mosquitoes.
**Outcome measures**

Mosquitoes were collected each morning at 05:00 from under bed nets, floors, walls, ceilings and verandas using aspirators and torches. The collections were transported to the laboratory where the mosquitoes were morphologically identified to genus/species using taxonomic keys and samples of *An gambiae* were confirmed as M form [24]. They were then scored as blood fed or unfed and live or dead. Live mosquitoes were held in netted plastic cups and supplied with 10% glucose solution and delayed mortality was recorded after 24h. Male mosquitoes were not scored.

Because pyriproxyfen acts by sterilizing the adult female mosquito, the impact of the treatments on the reproduction of surviving blood-fed mosquitoes was investigated by detecting whether there was a reduction in the fecundity (number of eggs per female) and fertility (proportion of laid eggs hatching) of these mosquitoes compared to the control. After scoring for mortality (24h post-collection from the experimental huts), the live blood-fed mosquitoes of each treatment were kept in separate cages and provided access to a second blood meal. Once gravid (within 2-3 days), individual mosquitoes were chambered separately in their own netted plastic cups containing approximately 50ml of fresh water. The chambers were monitored daily for eggs and the number of eggs laid by each female mosquito was recorded for up to 9 days. A pinch of larval food was added to any chamber which contained eggs and the numbers of larvae (L2) which hatched were recorded after another 4-6 days.

For each type of LN, the efficacy in experimental huts and the sterilizing effect on mosquitoes which survived the hut treatments were studied using the following outcome measures.
Direct effects on adult females in experimental huts:

17. Deterrence: percentage reduction in the number of mosquitoes caught in treated hut relative to the number caught in the control hut.

18. Exiting rates: due to potential irritant effect of treatments expressed as percentage of the mosquitoes collected from the veranda trap.

19. Inhibition of blood-feeding: reduction in blood-feeding rate relative to the control. Blood feeding inhibition (%) was calculated as follows:

\[
100 \frac{(Bfu - Bft)}{Bfu}
\]

Where \(Bfu\) is the proportion of blood-fed mosquitoes in the untreated control huts and \(Bft\) is the proportion of blood-fed mosquitoes in the huts with a specific insecticide treatment.

20. Mortality: percentage of dead mosquitoes in treated hut at the time of collection and after a 24 h holding period corrected for control mortality.

21. The personal protective effect of the treatments which is described by a reduction in the number of blood-fed mosquitoes relative to the control hut.

Personal protection (%) was calculated as follows:

\[
100 \frac{(Bu - Bt)}{Bu}
\]

Where \(Bu\) is the number of blood-fed mosquitoes in the untreated control huts and \(Bt\) is the number of blood-fed mosquitoes in the huts with insecticide treatments.

22. The overall insecticidal effect of a treatment relative to the number of mosquitoes that would ordinarily enter an untreated control hut. Overall insecticidal effect (%) was estimated by using the following formula:

\[
100 \frac{(Kt - Ku)}{Tu}
\]
where $K_t$ is the number killed in the treated hut, $K_u$ is the number dying in the untreated control hut, and $T_u$ is the total number collected from the control hut.

Effects on sterility and reproduction of surviving blood-fed females:

1. The proportion of females ovipositing: proportion of blood-fed females which laid eggs.
2. Fecundity: the number of eggs per blood fed female observed.
3. Reproductive rate: the number of larvae per blood fed female observed.
5. Reduction in fecundity: the percentage reduction in number of eggs per surviving blood fed female observed for a given treatment relative to the control. This was calculated as follows:

$$\frac{100(Ec - Et)}{Ec}$$

Where $Ec$ is the mean number of eggs per surviving blood-fed female observed in the control while $Et$ is the mean number of eggs per surviving blood-fed female observed in a given treatment.

6. Reduction in reproductive rate: the percentage reduction in number of larvae per surviving blood fed female observed for a given treatment relative to the control. This was calculated as follows:

$$\frac{100(Lc - Lt)}{Lc}$$
Where \( L_c \) is the mean number of larvae per surviving blood-fed female observed in the control while \( L_t \) is the mean number of larvae per surviving blood-fed female observed in a given treatment.

**Tunnel tests**

To gain further insight, laboratory tunnel tests were undertaken on netting samples taken from the hut LNs using the *An. gambiae* VKPER strain which was fixed for the pyrethroid knockdown resistance (*kdr*) gene. The tunnel test allows expression of the behavioural interactions that occur between free-flying mosquitoes and LNs during host seeking. It consists of a square glass cylinder (25 cm high, 25 cm wide, 60 cm in length) divided into two sections by means of a netting frame fitted into a slot across the tunnel [23]. In one of the sections, a guinea pig was housed unconstrained in a small cage, and in the other section 50 unfed female mosquitoes aged 5–8 days were released at dusk and left overnight. The net samples measured 25cm x 25cm and were deliberately holed with nine 1-cm holes to give opportunity for mosquitoes to penetrate into the animal baited chamber for a blood meal; an untreated net sample served as the control. The tests were performed at 25–27\(^\circ\)C and 75–85% RH. The next morning, the numbers of mosquitoes found alive or dead, fed or unfed, in each section were scored. Live mosquitoes were provided with 10% glucose solution and delayed mortality recorded after 24 hours. Approximately 100 adult females in 2 replicate tunnel tests were tested on each type of netting. Blood-fed mosquitoes which remained alive after 24hrs were assessed for sterilizing effects of pyriproxyfen as described above.
**Susceptibility testing**

WHO resistance test kits lined with 0.75% permethrin-treated papers were used to determine the frequency and the strength of resistance to permethrin in *An. gambiae* mosquitoes of the VKPER strain and wild Akron strain relative to the susceptible Kisumu strain. A range of exposure times (1-120 minutes) were tested on batches of 20 unfed *An. gambiae* female 2–5 day old Akron and Kisumu strains. Eighty mosquitoes per exposure period were tested. Deaths were scored 24 h later. Log-time mortality curves were generated using probit analysis and estimates of the time required to kill 50% (LT50) of each strain and the resistance ratios relative to the susceptible laboratory strain (PoloPlus version 1.0).

**Statistical analysis**

The effects of the experimental hut treatments on each of the proportional outcomes (net penetration, blood-feeding, exiting and mortality) were assessed using binomial generalised linear mixed models (GLMMs) with a logit link function, fitted using the ‘lme4’ package for R. A separate model was fitted for each outcome. In addition to the fixed effect of each treatment, each model included random effects to account for the following sources of variation: between the 4 huts; between the 4 sleepers; between the weeks of the trial; and finally an observation-level random effect to account for variation not explained by the other terms in the model (over dispersion). Differences in deterrence, personal protection and mass killing effect between the treatments was analysed using negative binomial regression with adjustment for the abovementioned covariates.
The proportions of surviving blood-fed females from the different treatments that laid eggs was analysed using Chi-square. The proportions of eggs that hatched to larvae was analysed using logistic regression while the numbers of eggs laid and the numbers of larvae per surviving female were analysed using the Kruskal Wallis test. These analyses were performed using STATA version 11.1 Texas USA.

**Ethics Statement**

Ethical approval for the study was obtained from the Ethics Review Board of the London School of Hygiene and Tropical Medicine and from the Ministry of Health of Benin. Permission to use the experimental hut station was obtained from ‘Centre de Recherches Entomologique de Cotonou’. Written informed consent was obtained from the volunteers who slept in the experimental huts to attract mosquitoes.

**Results**

**Susceptibility tests**

The summary results of the exposure time mortality bioassays with permethrin-treated papers in WHO cylinder kits are shown in Table 1. An accurate LT50 value could not be determined for the laboratory susceptible *An. gambiae* Kisumu strain since mortality rates >90% were achieved within 1 minute of exposure. LT50 values were 6.92 minutes for the *An. gambiae* VKPER strain and 19.48 minutes for wild *An. gambiae* from Akron. The results thus showed that the *An. gambiae* VKPER strain and the wild *An. gambiae* from Akron were at least 6.9 and 19.4 fold more resistant to permethrin than the laboratory susceptible *An. gambiae* Kisumu strain (Table 1). The wild *An. gambiae* from Akron was 2.8 times more resistant to permethrin than the *An. gambiae* VKPER strain.
**Experimental hut trial**

1. *Anopheles gambiae*

Blood feeding and mortality: A total of 303 *An. gambiae* were collected from the experimental huts during the trial. The numbers entering each of the treated huts per night were higher than in the control, hence there was no evidence of a deterrent effect on *An. gambiae* with any of the treatments (Table 2). The proportion exiting from huts with control nets (31%) did not differ significantly from that with PPF LN (29%; P=0.72) (Table 2). Exiting rates were much higher from huts with Olyset Duo (52%) and Olyset Net (56%) which might be attributed to the excito-repellent property of permethrin in both nets. Percentage blood-fed with the PPF LN (59%) did not differ significantly from the control net (53%, P=0.44) or Olyset Net (45%, P=0.07) (Figure 1). The lowest blood-feeding rate was achieved with Olyset Duo (13%). Olyset Duo also provided significantly higher levels personal protection (71%) than Olyset Net (3%, P<0.001) and PPF LN (0%, P<0.001) (Table 3). Lower proportions of mosquitoes were collected from inside the permethrin treated nets (Olyset Net: 11% and Olyset Duo: 4%) than from the PPF LN (35%, P<0.001) or control nets (39%, P<0.001) (Table 3). The proportion collected from inside Olyset Net (11%) did not differ significantly from that from Olyset Duo (4%, P=0.07).

Mortality with PPF LN (21%) was higher than the control net (8%, P=0.03) but did not differ significantly from Olyset Net (27%, P=0.24) (Figure 1). Much higher mortality rates were achieved with Olyset Duo than with Olyset Net (50% vs 27%; P=0.01) and PPF LN (50% vs 21% P<0.001). Olyset Duo induced a higher overall killing effect on *An. gambiae* than did Olyset Net (48% vs 27%, P<0.05) (Table 4).

Reproductive effects: The impact of the different LNs on the fecundity and reproductive rate of surviving blood fed female *An. gambiae* from the experimental
huts (alive after 24h) are presented in Table 5. The numbers of blood-fed pyrethroid resistant mosquitoes surviving the hut treatments and the numbers observed for sterilizing effects were both very small. Nevertheless the sterilizing effect of the pyriproxyfen-treated nets on *An. gambiae* was very obvious. The proportions from the control hut which laid eggs was 37% resulting in an average of 37 eggs per female observed with 98% hatching to larvae (Table 5). The numbers of blood-fed mosquitoes from the Olyset Net hut which laid eggs and the number of eggs and larvae per female were higher but not significantly higher than with the control. None of the surviving blood fed females from the huts with PPF LN or Olyset Duo laid eggs. Hence the pyriproxyfen-treated nets (PPF LN and Olyset Duo) completely sterilized the surviving blood-fed mosquitoes resulting in 100% reductions in the fecundity and reproductive rate of these mosquitoes relative to the control (Table 5).

2. *Culex quinquefasciatus*

Blood feeding and mortality: A total of 5889 *Cx. quinquefasciatus* were collected from the experimental huts during the trial. There was no evidence of a deterrent effect on this species with any of the treatments (Table 2). The proportions dead and blood-fed are presented in Figure 2. Blood feeding rates with PPF LN (36%) did not differ significantly from the control (43%, P=0.09). The proportion blood-fed with the permethrin treated nets (Olyset Net=12% and Olyset Duo =2%) was significantly lower than with the control or PPF LN (P<0.05). The proportion collected from inside the LN was significantly lower with Olyset Duo (4%) than Olyset Net (9%, P<0.001). Olyset Duo also provided more personal protection (92%) than Olyset Net (53%, P<0.001) and PPF LN (0%, P<0.001) (Table 3). Exiting rates were higher with Olyset Duo (66%) than with Olyset Net (59%, P=0.001) and PPF LN (32%, P<0.001) (Table 2). Mortality with Olyset Net (12%) was higher than with PPF LN (8%, P=0.01) and
both were significantly higher than the control (3%, P<0.001). However, unlike with *An. gambiae*, mortality of *Cx. quinquefasciatus* with Olyset Duo (13%) did not differ significantly from that with Olyset Net (12%, P=0.27) (Figure 2 and Table 4). The overall killing effect did not differ between the LN s either (12% vs 13%, P=0.35).

Reproductive effects: Table 6 presents the effects of the different LN types on the fecundity and fertility of random samples of blood-fed *Cx. quinquefasciatus* mosquitoes which survived the experimental hut treatments (alive after 24h). The proportion that laid eggs and the number of eggs per female did not differ significantly between any of the treatments and the control (P>0.05). In contrast to *An. gambiae*, there was little or no reduction in fecundity of live blood-fed *Cx. quinquefasciatus* from huts with PPF LN (3%) or Olyset Duo (0%). The hatch rates of eggs laid by mosquitoes from huts with PPF LN (72%) and Olyset Duo (98%) did not differ significantly from the control (85%, P>0.05). There was a small reduction in offspring per live blood-fed female *Cx. quinquefasciatus* observed from the PPF LN (20%). No reduction in fecundity or offspring was detected with samples from the Olyset Duo (0%) (Table 6).

**Tunnel test**

The tunnel test results with the *An. gambiae* VKPER laboratory strain are presented in Table 7. The proportion penetrating the net was 95% with the control and 100% with PPF LN. Net penetration rates were significantly reduced with the two permethrin treated nets and the difference was greater with Olyset Duo (16%) than with Olyset Net (63%, P<0.05). The proportion feeding on the bait showed a pattern consistent with penetration. None of the mosquitoes in the tunnel with Olyset Duo
succeeded in feeding (0% blood-fed). Blood feeding inhibition was higher with Olyset Duo (100%) than with Olyset Net (68%) or PPF LN (0%). The trend of blood feeding inhibition was very similar to what was observed in the experimental huts (Table 3).

Mortality was 0% in the control tunnel and 3% in the PPF LN tunnel. Mortality increased significantly with the permethrin treatments and as in the hut trial was significantly higher with Olyset Duo (100%) than with Olyset Net (91%, P<0.05) (Figure 1). However, the mortality rates recorded in the tunnel tests were much higher than the rates observed in the experimental huts and this might be attributable to the weaker resistance in the VKPER strain compared to the wild mosquitoes.

The effects on the reproduction of blood-fed mosquitoes which survived the tunnel test treatments are presented in Table 8. Because Olyset Duo tunnel test killed all the mosquitoes it was not possible to assess the sterilizing effect of Olyset Duo on *An. gambiae* VKPER in the tunnel bioassays. The proportion from the control tunnel which laid eggs was 34% with each laying female producing an average 106 eggs. With PPF LN, the proportion which laid eggs was 4% and none of these eggs hatched to larvae. This resulted in a 99% reduction in fecundity and a 100% reduction in reproductive rate with PPF LN relative to the control. The tunnel tests therefore corroborated the experimental hut trials by also showing an improved killing and protective effect with Olyset Duo compared to Olyset Net and the complete sterilization of *An. gambiae* VKPER exposed to PPF LN.
**Discussion**

Providing universal coverage of LNs to populations at risk has become a priority for national malaria control programmes in recent years [11]. In areas where vectors are largely susceptible to pyrethroids, LNs are highly effective and the levels of mortality and personal protection achieved in experimental hut trials against such vector populations usually exceed 80% [25,26]. In the current study, mortality rates and personal protection with the WHOPES-recommended LN (Olyset Net) were very much lower (27% and 3% respectively). This serves to confirm the poor performance of standard LNs reported in several studies in Southern Benin which is due to the presence of multiple mechanisms of pyrethroid resistance in *An. gambiae* in this region [22,26-28]. Olyset Duo demonstrated superior performance to Olyset Net in the experimental huts against this resistant population in terms of higher levels of mortality and personal protection. Although both LNs contain the same concentrations of permethrin, the bleed rate of the insecticide is higher in Olyset Duo than Olyset Net. The surface concentration of permethrin is therefore likely to be higher in Olyset Duo and this may potentially account for the higher mortality rates and personal protection observed with Olyset Duo. Nevertheless, the PPF LN did cause some mortality by itself both in the huts and laboratory studies [20] which may mean there could be an additive effect of the two active ingredients in Olyset Duo. Bioassay studies with the two AIs alone and together in dipped nets are the simplest approach to distinguish between the possibilities of faster bleed rate inducing additional mortality of resistant mosquitoes and interaction between independently acting insecticides.
While it is encouraging that Olyset Duo provided additional mortality of *An. gambiae* and greater personal protection compared to Olyset Net, the main rationale behind incorporating pyriproxyfen was to reduce the size of the first filial generation by reducing the reproductive rate of the parental generation through sterilization. While the number of surviving mosquitoes collected from the Olyset Duo treatment arm was limited, the trial did provide encouraging support for that expectation. The results show that pyrethroid resistant *An. gambiae* that contact the net in the course of feeding and which fail to be killed by a pyrethroid-only LN treatment owing to their resistant status can be sterilized if the LN also contains pyriproxyfen. This would predict that greater reductions in the abundance of pyrethroid resistant malaria vectors would be achieved with community wide use of Olyset Duo than with LNs treated only with pyrethroids. In effect Olyset Duo acts rather like a larvicide – acting to reduce the number of F1 progeny reaching adulthood in the next generation. However, owing to the small numbers of surviving blood-fed mosquitoes collected and observed for reproductive effects - a clear limitation of the study - care should be taken not to over interpret these encouraging results. Proof that better reductions in transmission can be achieved with Olyset DUO than Olyset Net will require a fully-powered, large scale community randomised trial in discrete clusters with their own breeding sites.

By selectively sterilizing surviving pyrethroid-resistant *An. gambiae*, Olyset Duo also shows potential to slow down or prevent further selection of pyrethroid resistance. However, because the benefits of a resistance management approach are less likely to be attained in areas where resistance is well established [10], the nets will need to be deployed in areas where resistance is still rare in order to fully test such a resistance management strategy. In the first instance further hut trials involving
mixed susceptible and resistant populations are needed to investigate the potential
capacity of Olyset Duo to prevent selection of the pyrethroid resistance.

In contrast to *An. gambiae*, mortality rates of wild pyrethroid resistant *Cx. quinquefasciatus* in the huts with Olyset Duo did not differ significantly from that with Olyset Net. The pyriproxyfen-treated nets (Olyset Duo and PPF LN) similarly failed to sterilize surviving blood-fed *Cx. quinquefasciatus* mosquitoes. *Cx. quinquefasciatus* from West Africa are difficult to control with pyrethroids due to resistance involving multiple mechanisms [22,29]; hence the low mortality rates in this species with either LN was not unexpected. There could be inherent differences in the physiology, behaviour, contact or up-take of pyriproxyfen between *Cx. quinquefasciatus* and *An. gambiae* that might have lessened the chances of blood-fed *Cx. quinquefasciatus* mosquitoes being sterilized by the pyriproxyfen-treated nets. Blood feeding inhibition was significantly higher against *Cx. quinquefasciatus* than *An. gambiae* across all treatments hence the surviving *Cx. quinquefasciatus* mosquitoes may not have contacted the nets long enough to pick up doses of pyriproxyfen sufficient to sterilize them. The possibilities of cross resistance to pyriproxyfen in this strongly pyrethroid resistant *Cx. quinquefasciatus* population also cannot be ruled out. Further studies need to be performed to investigate these hypotheses under controlled laboratory conditions.

Notwithstanding the lack of sterilization, Olyset Duo provided better personal protection against *Cx. quinquefasciatus* than Olyset Net (53% vs. 92%). This suggests that even though a significant reduction in the abundance of *Cx. quinquefasciatus* might not be expected from community-wide use of Olyset Duo, the mixture LN may still provide better protection against this species than the pyrethroid-only LN. While the impact on malaria vectors is of primary interest, the
capacity of Olyset Duo to improve personal protection against *Cx. quinquefasciatus*, may improve acceptability to LN users [30].

**Conclusion**

By killing more pyrethroid resistant *An. gambiae* and sterilizing surviving blood-fed females through the pyriproxyfen component, Olyset Duo has potential to provide better control of malaria transmission than pyrethroid only LNs in areas where pyrethroid resistance is compromising the efficacy of current LNs. The apparent lack of impact of pyriproxyfen on *Culex quinquefasciatus* mosquitoes requires further investigation. A community randomised trial is necessary to demonstrate whether the sterilizing effect of Olyset Duo will provide additional malaria transmission control over Olyset Net.

**Acknowledgements**

The authors thank John Lucas, John Invest, Takao Ishiwatari and Yoshinori Shono (Sumitomo Chemical Company), for supplying samples and for technical input, and Robert Sloss for technical programme support (IVCC, Liverpool, UK). We are grateful to Estelle Vigninou for field and laboratory assistance. LSHTM and CREC are members of the Pan African Malaria Vector Research Consortium (http://www.pamverc.or.tz). CN, RN and MR are members of the Malaria Centre of the London School of Hygiene & Tropical Medicine (http://malaria.lshtm.ac.uk)
Table 1: Susceptibility of mosquito strains to permethrin-treated papers (0.75%) in WHO cylinder bioassays.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Slope</th>
<th>LT50$^\text{5}$ (minutes)</th>
<th>(95% CI)</th>
<th>LT50 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. gambiae Kisumu</td>
<td>0.68</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>An. gambiae VKPER</td>
<td>1.58</td>
<td>6.92</td>
<td>4.95-9.39</td>
<td>~7</td>
</tr>
<tr>
<td>An. gambiae Akron (wild)*</td>
<td>3.73</td>
<td>19.48</td>
<td>17.05-22.17</td>
<td>~20</td>
</tr>
</tbody>
</table>

*Samples were collected as larvae from breeding sites close to the experimental huts in Akron during the trial,
$^\text{5}LT50$ = time taken for 50% of mosquitoes to be killed.

Table 2: Entry and exiting rates of wild mosquitoes in experimental huts during the trial

<table>
<thead>
<tr>
<th></th>
<th>Untreated net</th>
<th>PPF LN</th>
<th>Olyset Net</th>
<th>Olyset Duo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anopheles gambiae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total females caught</td>
<td>64</td>
<td>91</td>
<td>76</td>
<td>72</td>
</tr>
<tr>
<td>Average catch per night</td>
<td>1.1$^a$</td>
<td>1.6$^a$</td>
<td>1.3$^a$</td>
<td>1.3$^a$</td>
</tr>
<tr>
<td>% Deterrence</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total females exiting</td>
<td>20</td>
<td>26</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>% Exiting</td>
<td>31$^a$</td>
<td>29$^a$</td>
<td>53$^b$</td>
<td>56$^b$</td>
</tr>
</tbody>
</table>

| **Culex quinquefasciatus** |               |        |            |            |
| Total females caught     | 1331          | 1456   | 1597       | 1505       |
| Average catch per night  | 23.4$^a$      | 25.5$^a$ | 28.0$^c$ | 26.4$^a$ |
| % Deterrence             | -             | 0      | 0          | 0          |
| Total females exiting    | 375           | 488    | 908        | 943        |
| % Exiting                | 29$^a$        | 32$^b$ | 59$^c$     | 66$^d$     |

$^a,b,c,d$ Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).
Figure 1. Mortality and bloodfeeding rates of pyrethroid resistant *Anopheles gambiae* in experimental huts. Percentage mortality (lighter shade) and bloodfeeding (darker shade) of pyrethroid resistant *An. gambiae* in experimental huts in Akron. For each response parameter (mortality or bloodfeeding), values for histograms sharing the same letter label are not significantly different (P > 0.05). Error bars represent 95% confidence intervals.

Table 3: Blood-feeding inhibition and personal protection rates in the experimental huts

<table>
<thead>
<tr>
<th></th>
<th>Untreated net</th>
<th>PPF LN</th>
<th>Olyset Net</th>
<th>Olyset Duo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anopheles gambiae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total blood fed</td>
<td>35</td>
<td>54</td>
<td>34</td>
<td>10</td>
</tr>
<tr>
<td>% Blood fed</td>
<td>53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Blood feeding inhibition</td>
<td>-</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Personal Protection</td>
<td>-</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Inside net</td>
<td>39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Culex quinquefasciatus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total blood fed</td>
<td>480</td>
<td>626</td>
<td>175</td>
<td>30</td>
</tr>
<tr>
<td>% Blood fed</td>
<td>36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Blood feeding inhibition</td>
<td>-</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Personal Protection</td>
<td>-</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>92&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Inside net</td>
<td>39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d</sup> Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).
Table 4: Overall killing effect in the experimental huts

<table>
<thead>
<tr>
<th></th>
<th>Untreated net</th>
<th>PPF LN</th>
<th>Olyset Net</th>
<th>Olyset Duo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anopheles gambiae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total females dead</td>
<td>4</td>
<td>19</td>
<td>21</td>
<td>36</td>
</tr>
<tr>
<td>Corrected mortality</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Overall killing effect</td>
<td>-</td>
<td>23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Culex quinquefasciatus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total females dead</td>
<td>50</td>
<td>152</td>
<td>212</td>
<td>228</td>
</tr>
<tr>
<td>Corrected mortality</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Overall killing effect</td>
<td>-</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Numbers in the same row sharing a letter superscript do not differ significantly (P > 0.05).

Figure 2. Mortality and bloodfeeding rates of pyrethroid resistant *Culex quinquefasciatus* in experimental huts. Percentage mortality (lighter shade) and bloodfeeding (darker shade) of pyrethroid resistant *Culex quinquefasciatus* in experimental huts in Akron. For each response parameter (mortality or bloodfeeding), values for histograms sharing the same letter label are not significantly different (P>0.05). Error bars represent 95% confidence intervals.
Table 5: Fecundity and Fertility of blood-fed *An. gambiae* females alive after 24h from experimental huts

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PPF LN</th>
<th>Olyset Net</th>
<th>Olyset Duo</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of blood fed females observed</td>
<td>27</td>
<td>19</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>% of females that oviposited (95% CI)</td>
<td>37(17-57)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47(20-74)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total number of eggs laid</td>
<td>1003</td>
<td>0</td>
<td>850</td>
<td>0</td>
</tr>
<tr>
<td>Eggs per female laying eggs (95% CI)</td>
<td>100</td>
<td>-</td>
<td>121</td>
<td>-</td>
</tr>
<tr>
<td>Fecundity: eggs per blood fed female observed (95% CI)</td>
<td>37(15-58)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57(30-74)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>% reduction in fecundity per female observed</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Total number of larvae</td>
<td>981</td>
<td>0</td>
<td>782</td>
<td>0</td>
</tr>
<tr>
<td>Hatch rate %, (95% CI)</td>
<td>98 (97-99)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>92 (90-94)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Larvae per female laying eggs (95% CI)</td>
<td>98</td>
<td>-</td>
<td>112</td>
<td>-</td>
</tr>
<tr>
<td>Larvae per female observed (95% CI)</td>
<td>36(14-57)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52(39-71)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>% reduction in reproductive rate per blood fed female observed</td>
<td>-</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>**Values along each row sharing the same letter superscript are not significantly different at the 5% level.**</sup>
Table 6: Fecundity and Fertility of blood-fed *Cx. quinquefasciatus* alive after 24h from experimental huts

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PPF LN</th>
<th>Olyset Net</th>
<th>Olyset Duo</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of blood fed females observed</td>
<td>102</td>
<td>108</td>
<td>83</td>
<td>36</td>
</tr>
<tr>
<td>% of females that oviposited (95% CI)</td>
<td>34(22-44)a</td>
<td>31(22-40)a</td>
<td>30(21-41)a</td>
<td>44 (28-62)a</td>
</tr>
<tr>
<td>Total number of eggs</td>
<td>4287</td>
<td>4398</td>
<td>3239</td>
<td>2159</td>
</tr>
<tr>
<td>Eggs per female laying eggs</td>
<td>122</td>
<td>129</td>
<td>130</td>
<td>135</td>
</tr>
<tr>
<td>Fecundity: eggs per blood fed female observed (95% CI)</td>
<td>42(30-52)a</td>
<td>41(29-53)a</td>
<td>39(26-52)a</td>
<td>58(33-84)a</td>
</tr>
<tr>
<td>% reduction in fecundity per female observed</td>
<td>-</td>
<td>3</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Total number of larvae</td>
<td>3634</td>
<td>3171</td>
<td>2753</td>
<td>2116</td>
</tr>
<tr>
<td>Hatch rate (%) (95% CI)</td>
<td>85 (84-86)a</td>
<td>72(71-74)b</td>
<td>85(84-86)a</td>
<td>98(97-99)c</td>
</tr>
<tr>
<td>Larvae per female laying eggs</td>
<td>104</td>
<td>96</td>
<td>109</td>
<td>132</td>
</tr>
<tr>
<td>Larvae per female observed</td>
<td>36 (24-47)a</td>
<td>29 (19-40)a</td>
<td>35(21-48)a</td>
<td>58(32-83)a</td>
</tr>
<tr>
<td>% reduction in reproductive rate per blood fed female observed</td>
<td>-</td>
<td>20</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

*a,b,c* Values along each row sharing the same letter superscript are not significantly different at the 5% level

Table 7: Tunnel test results with *An. gambiae* VKPER

<table>
<thead>
<tr>
<th>Net Sample</th>
<th>N</th>
<th>Mortality (%)</th>
<th>Penetration (%)</th>
<th>Blood-fed (%)</th>
<th>Blood feeding inhibition (%)</th>
<th>% Blood-fed and alive (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>112</td>
<td>0a</td>
<td>95a</td>
<td>93a</td>
<td>-</td>
<td>93a (104)</td>
</tr>
<tr>
<td>95% CI</td>
<td>0-5</td>
<td>89-98</td>
<td>86-97</td>
<td></td>
<td></td>
<td>86-97</td>
</tr>
<tr>
<td>PPF LN</td>
<td>114</td>
<td>5a</td>
<td>100a</td>
<td>95a</td>
<td>0a</td>
<td>91a (104)</td>
</tr>
<tr>
<td>95% CI</td>
<td>2-8</td>
<td>96-100</td>
<td>89-98</td>
<td></td>
<td></td>
<td>84-96</td>
</tr>
<tr>
<td>Olyset Net</td>
<td>92</td>
<td>91b</td>
<td>63b</td>
<td>30b</td>
<td>68b</td>
<td>9b (8)</td>
</tr>
<tr>
<td>95% CI</td>
<td>84-96</td>
<td>52-73</td>
<td>21-41</td>
<td></td>
<td></td>
<td>4-16</td>
</tr>
<tr>
<td>Olyset Duo</td>
<td>110</td>
<td>100c</td>
<td>16c</td>
<td>0c</td>
<td>100c</td>
<td>0c (0)</td>
</tr>
<tr>
<td>95% CI</td>
<td>97-100</td>
<td>10-25</td>
<td>0-3</td>
<td></td>
<td></td>
<td>0-3</td>
</tr>
</tbody>
</table>

*a,b,c* Values along each column sharing the same letter superscript are not significantly different at the 5% level
Table 8: Fecundity and fertility of *An. gambiae* VKPER alive after exposure to LN samples in tunnel tests

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PPF LN</th>
<th>Olyset Net</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of blood fed females observed</td>
<td>104</td>
<td>104</td>
<td>8</td>
</tr>
<tr>
<td>% laid (95% CI)</td>
<td>34 (25-44)^a</td>
<td>4 (1-10)^b</td>
<td>38 (9-75)^a</td>
</tr>
<tr>
<td>Total number of eggs</td>
<td>3720</td>
<td>24</td>
<td>230</td>
</tr>
<tr>
<td>Eggs per female laying eggs</td>
<td>106</td>
<td>6</td>
<td>77</td>
</tr>
<tr>
<td>Fecundity: eggs per blood fed female observed (95% CI)</td>
<td>32 (20-54)^a</td>
<td>0.2^b</td>
<td>29 (2-53)^a</td>
</tr>
<tr>
<td>% reduction in fecundity per female observed</td>
<td>-</td>
<td>99</td>
<td>0</td>
</tr>
<tr>
<td>Total number of larvae</td>
<td>1740</td>
<td>0</td>
<td>190</td>
</tr>
<tr>
<td>Hatch rate (%) (95% CI)</td>
<td>47 (46-49)^a</td>
<td>0^b</td>
<td>83 (77-87)^c</td>
</tr>
<tr>
<td>Larvae per female laying eggs</td>
<td>50</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>Larvae per bloodfed female observed</td>
<td>17 (11-30)^a</td>
<td>0^b</td>
<td>24 (1-50)^a</td>
</tr>
<tr>
<td>% reduction in reproductive rate per blood fed female observed</td>
<td>-</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

^a,b,c Values along each row sharing the same letter superscript are not significantly different at the 5% level
References


PART FOUR

Chapter 9: Discussion

This chapter focuses on highlighting the similarities and differences between the outcomes and conclusions from the previous chapters and discusses their significance in relation to malaria vector control and insecticide resistance management.
Chapter 9: Discussion

As insecticide resistance genes continue to spread in malaria parasite vectors, the threat on malaria control is increasing. There is an overriding need to identify reliable strategies which can improve the control of insecticide resistant vectors and manage resistance [1]. The different recommended chemical based strategies for resistance management have been discussed in detail in chapter 1 of this thesis and they include the use of rotations, mosaics, mixtures and combined interventions. The simultaneous use of a combination of unrelated insecticides in the same place and time is considered a reliable approach as it has potential to overpower resistance killing a greater proportion of mosquitoes and prevent further selection of the resistance genes [2]. However, the current understanding of this strategy is largely theoretical and empirical studies validating its relevance for insecticide resistant malaria vectors are lacking. The work in this thesis was designed to investigate under semi-field conditions, the potential of different tools and tactics based on the combined use of unrelated insecticides for improving the control of insecticide resistant malaria vectors and for insecticide resistance management. The results of the various studies performed have been discussed separately in the previous chapters; hence the current chapter focuses on highlighting the similarities and differences between the outcomes and conclusions from each chapter and discussing their significance in relation to malaria vector control and insecticide resistance management.
1. **Impact of pyrethroid resistance on the efficacy of pyrethroid LLINs**

ITNs are effective, relatively cheap and easy to deliver even in the most remote communities; hence the World Health Organisation (WHO) recommends full coverage with ITNs in malaria endemic areas in Africa [3]. It is however clear from these studies that the use of LLINs on its own provides limited control of pyrethroid resistant mosquitoes. Reduced mortality rates were recorded in experimental huts in which LLINs were applied alone in Akron, Benin (Chapter 3, 7 and 8), Valley du Kou, Burkina Faso (Chapter 5) and Tiassales, Cote D’Ivoire (chapter 6) which were all characterised by high levels of resistance to pyrethroids. In areas where malaria vectors are susceptible to pyrethroids, significantly higher mortality rates have been achieved with most pyrethroid LLINs in experimental huts [4, 5]. The results from this thesis therefore confirm findings from several studies reporting the poor performance of ITNs in experimental huts in pyrethroid resistant areas in West Africa [6-9].

While the reduced efficacy of LLINs in experimental huts in pyrethroid resistant areas could be regarded as a proxy, the impact of pyrethroid resistance on the efficacy of LLINs should ideally be measured from an epidemiological perspective. Evidence from community randomised control trials on the epidemiological impact of pyrethroid resistance on the efficacy of LLINs is necessary as it is the most reliable basis for decision-making in public health. However, as discussed in chapter 1 of this thesis, establishing such a link between resistance and the epidemiology of malaria is complicated by the fact that resistance cannot be randomly allocated to some communities and withheld from others as to control confounding factors. The nearest alternative is to investigate if changes in the observed effectiveness of pyrethroid LLINs in communities are associated with variations in resistance. Another option is
to investigate whether there are any associations between sporozoite infection rate in malaria vectors with changes in insecticide resistance and operational impact [1, 10].

Notwithstanding the reduced performance of LLINs when confronted with pyrethroid resistant vectors, the results from this thesis did show that LLINs were consistently more protective against pyrethroid resistant mosquitoes than the untreated nets and wall treatments in all the study sites. A recent meta-analysis on the efficacy of insecticide treated bed nets against African Anophelines in experimental huts also found that ITNs are more effective than untreated nets regardless of resistance [11]. This shows that the irritant effects of pyrethroids in LLINs persist to an extent when pyrethroid resistance is detectable in the target vector population. Pyrethroid LLINs may thus remain a relevant public health intervention for providing personal protection even against mosquitoes which show some resistance to pyrethroids until an effective and protective alternative insecticide for treating LLINs or a more reliable intervention is identified. However, because the continued use of LLINs will certainly lead to further selection, pyrethroid resistance and any associated operational impact must be continuously monitored. Moreover, studies have also shown that LLINs lose their residual protective effect against pyrethroid resistant mosquitoes when they become significantly holed [12-14]. Data from Equatorial Guinea and Malawi showed evidence of a linear increase in infection with *P. falciparum* per category increase in the deterioration of bed nets [15]. While WHO guidelines assume that LLINs remain effective for 3-5 years, there is a growing body of evidence from the field which suggests that LLINs may become significantly holed and even unusable within 1-3 years of field use [16, 17]. This calls for the production of more durable nets with improved textile integrity and for proper monitoring to ensure that LLINs deployed in
pyrethroid resistant areas are repaired or replaced in time before they become significantly holed and lose their residual protective effect against pyrethroid resistant mosquitoes.

2. Combined use of unrelated insecticides for improved control of pyrethroid resistant malaria vectors

The concept of combining insecticides with unrelated modes of action can be used to boost the control of pyrethroid resistant malaria vectors with LLINs and to manage insecticide resistance. Two major ways of achieving this were studied in this thesis 1. combining pyrethroid LLINs with non pyrethroid IRS or wall linings and 2. mixing pyrethroids with non-pyrethroid compounds on bed nets. The studies were performed in sites with differing levels of resistance to investigate how the performance of combinations could be affected by the type and level of resistance encountered in the target vector population. Table 1 below summarises the findings with An gambiae in the different experimental huts studies reported in this thesis.
Table 1: Summary of results with *An gambiae* in experimental hut studies investigating combinations of insecticides

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Experimental hut station</th>
<th>Resistance status</th>
<th>Main Treatments tested</th>
<th>Mortality rates</th>
<th>Bloodfeeding inhibition (BFI)</th>
<th>Remark</th>
</tr>
</thead>
</table>
| 3       | Akron, Benin             | -Resistant to pyrethroids  
-Susceptible to Chlorfenapyr | 1. Chlorfenapyr IRS  
2. Pyrethroid ITN (6 holes)  
3. Pyrethroid ITN (80 holes)  
4. Chlorfenpyr IRS + Pyrethroid LLIN | 57%  
50%  
38%  
82-83% | 0%  
78%  
42%  
51-68% | Combination provided improved mortality mostly due to CFP IRS and BFI mostly due to LLIN |
| 5       | Valley du Kou, Burkina Faso | -Resistant to pyrethroids  
-Susceptible to organophosphates | 1. Pirimiphos methyl WL  
2. Pyrethroid LLIN (Permanet 2.0)  
3. P-methyl WL + Pyrethroid LLIN | 100%  
60%  
100% | 50%  
70%  
90% | Combination provided improved mortality mostly due to Pmethyl WLs and improved BFI mostly due to LLIN |
| 6       | Tiassales, Cote D’Ivoire | -Resistant to pyrethroids  
-Resistant to organophosphates | 1. Pirimiphos methyl WL  
2. Pyrethroid LLIN (Permanet 2.0)  
3. P-methyl WL + pyrethroid LLIN | 55-60%  
65%  
65-70% | 0%  
67%  
77-84% | No improvement in mortality with combination due to multiple-insecticide resistance |
| 7       | Akron, Benin             | -Resistant to pyrethroids  
-Susceptible to Chlorfenapyr | 1. Pyrethroid ITN  
3. CFP+pyrethroid net | 30%  
~80% | 35%  
40-45% | Significantly higher mortality rate with mixture net compared to pyrethroid net |
| 8       | Akron, Benin             | -Resistant to pyrethroids | 1. Pyrethroid LLIN (Olyset Net)  
2. Pyriproxyfen+pyrethroid mixture LLIN (Olyset Duo) | 28%  
50% | 15%  
75% | Olyset Duo provided improved mortality and, improved BFI and sterilised surviving bloodfed females |
2.1 Combining non-pyrethroid IRS or wall linings with LLINs for improved control of pyrethroid resistant mosquitoes

When pyrethroid LLINs were combined with chlorfenapyr IRS in Benin (chapter 3) and with p-methyl wall linings in Burkina Faso (chapter 5) the control of pyrethroid resistant malaria vectors improved significantly compared to LLINs. Against these vector populations, the combinations provided personal protection (mostly due to the LLIN) and improved mortality rates (mostly due to the non-pyrethroid wall treatment) thus demonstrating the relevance of the combined intervention approach. Conversely, compared to pyrethroid LLINs alone, no improvement in mortality was achieved when the p-methyl wall linings and LLIN combination was tested against the Tiassales Cote D'Ivoire vector population which was resistant to the insecticides used in both interventions [18] (chapter 4). The improved performance of the combination in Benin and Burkina Faso is thus attributable to the fact that the local vector populations though resistant to the pyrethroid in the LLIN were largely susceptible to the insecticide applied on the wall (chlorfenapyr in Benin and p-methyl in Burkina Faso).

The difference in outcome between the Burkina Faso and Cote D'Ivoire studies demonstrates that insecticide resistance is undoubtedly a factor that may determine whether the combined use of vector control interventions will provide additional protection against malaria. This will mean that before a combination of interventions is deployed, the levels and mechanisms of resistance to the insecticides involved in the combination must be properly investigated in the targeted vector population; improved vector control may not be achieved when there is resistance to both insecticides. Reports of resistance to organophosphates and carbamates in malaria vector populations that are already largely resistant to pyrethroids are increasing in
West Africa [19-21]. Unfortunately, these insecticides are currently the only class of insecticides which can be used for wall treatments in lieu of pyrethroids. These findings therefore highlight the urgent need for new insecticide classes with no cross resistance to current insecticides. The pyrole chlorfenapyr through its novel mode of action has shown potential to improve the control of pyrethroid resistant mosquitoes when applied as IRS [22, 23] and studies are underway to develop a long-lasting formulation of the insecticide. Moreover, chlorfenapyr is a slower acting insecticide which unlike the current fast acting vector control insecticides, imposes less intense selection for resistance [24, 25]. Chlorfenapyr can therefore be included in a rotational IRM programme and/or used in combination with LLINs for improved vector control as shown in this study while we await the other new classes of insecticides envisaged by the innovative vector control consortium (IVCC) by 2023 [26]. These insecticides will be the first new insecticide classes coming into the market in over 30 years. While it is essential that attempts should be made to shorten the development time of these new insecticides, efforts to maintain the effectiveness of LLINs and IRS with current insecticides should also be intensified. This requires good stewardship of existing vector control insecticides ideally within a rotational IRM strategy to prevent the spread of the type of resistance to multiple insecticides encountered in Cote d’Ivoire.

While improved vector mortality was achieved when non-pyrethroid wall treatments were combined with deltamethrin LLINs in Benin and Burkina Faso, combining commercial deltamethrin DL with deltamethrin LLINs in Burkina Faso provided no improvement in mortality of pyrethroid resistant mosquitoes compared to the single treatments alone (Chapter 3). Similar results were obtained in a previous study in experimental huts in Burkina Faso investigating the combination of permethrin DL
with permethrin LLINs compared to the single interventions alone [27]. A recent phase III trial assessing the impact of deltamethrin DL applied alone or in combination with deltamethrin LLINs in villages in Western Angola also failed to demonstrate a significant improvement in the reduction of Anopheles densities and parasite infected blood smears in villages with the combination relative to villages with deltamethrin DL alone [28]. Analysis of human antibody markers showed significantly reduced human-vector contact with the combination, nevertheless, there was no study arm with LLINs alone to assess whether this effect was only due to the LLIN component.

Owing to the longer residual activity of pyrethroid DL relative to pyrethroid IRS [29, 30], plans have been made to scale up commercial pyrethroid DL across Africa. However, as coverage with pyrethroid LLINs continues to rise, most wall treatments (IRS or wall linings) will eventually be deployed against a background of high coverage with LLINs in most malaria endemic areas. This suggests that pyrethroid versions of insecticide treated wall linings may be unsuitable for improving malaria vector control in most endemic areas and huge investments into this product may be imprudent. Combinations involving the same class of insecticides are also not recommended as selection pressure would likely increase leading to even more resistance [31, 32]. It has been suggested that the use of pyrethroids should be discontinued for other vector control purposes except on mosquito nets as to preserve susceptibility until a safer and more effective alternative for treating bed nets is identified [31]. The results obtained here uphold these recommendations especially for pyrethroid resistant areas and highlight the need for wall linings treated with non-pyrethroid insecticides. P-methyl has also been identified as a suitable
alternative insecticide to treat wall linings. Advanced binding technology could be applied to develop long-lasting versions of p-methyl wall linings.

2.2 Mixtures on bed nets for improved control of pyrethroid resistant mosquitoes

In chapters 7 and 8 of this thesis, studies were performed to evaluate the efficacy of mosquito nets treated with a mixture of non-pyrethroid and a pyrethroid for improved vector control with LLINs. The rationale for mixing pyrethroids with non pyrethroid insecticide on bed nets was to enhance toxicity to pyrethroid resistant mosquitoes through the non-pyrethroid compound while maintaining the protective excito-repellent property of the bed net through the pyrethroid. Chlorfenapyr (chapter 7) and pyriproxyfen (chapter 8) have been identified as alternative compounds which can be combined with pyrethroids on bed nets for improved control of pyrethroid resistant mosquitoes.

The chlorfenapyr and alphacypermethrin mixture net provided improved levels of mortality of pyrethroid resistant mosquitoes through the chlorfenapyr component while maintaining significant levels of personal protection through the alphacypermethrin component. Just like with the combined intervention approach, improved mortality was achieved because the vector population was susceptible to the chlorfenapyr component. These results were consistent with studies which demonstrated improved control of laboratory reared resistant and susceptible strains of *Cx quinquefasciatus* with ITNs treated with a mixture of chlorfenapyr and alphacypermethrin in tunnel tests compared to nets treated with the single insecticides [33]. Unlike most other non-pyrethroid insecticides which have been
tested on bed nets, toxicology studies have indicated that chlorfenapyr is safe for use on bed nets based on WHO guidelines [34, 35]. Because there is currently no record of resistance to chlorfenapyr, this mixture net shows potential to be effective against a wide range of malaria vectors including those with multiple resistant mechanisms to current insecticides. The net therefore provides hope for preventing malaria transmission in areas where pyrethroid resistance is compromising the efficacy of current LLINs. Further studies are ongoing to develop a long-lasting and wash resistant formulation of this mixture net.

Unlike chlorfenapyr which acts primarily by killing mosquitoes, pyriproxyfen is an insect growth regulator which acts by sterilising adult female mosquitoes and the rationale for mixing it with a pyrethroid on a net (Olyset Duo) was to sterilize pyrethroid resistant female mosquitoes which survived the pyrethroid treatment of the net owing to their resistance status. The study provided evidence of this expectation in both laboratory and field situations, thus predicting significant reductions in vector abundance if Olyset Duo is used on a large scale. In addition to this sterilising effect, recent studies on Anophelines have shown inhibition of adult emergence from immature stages introduced into oviposition sites which had been previously visited by adult females exposed to pyriproxyfen [36, 37]. This will suggest that mosquitoes exposed to Olyset Duo could autodisseminate the IGR to breeding sites leading to massive reductions in the vector populations. This hypothesis will however be challenged by the low density of anophelines in relation to the surface area of aquatic habitats and the tendency for mosquitoes to not visit oviposition sites when they have been sterilised.

In contrast to *An gambiae*, the study showed no sterilizing effect by Olyset Duo on *Cx quinquefasciatus*. The possible reasons for this observation were discussed in
detail in chapter 8 of this thesis and one reason which could be of major concern is the likelihood of cross resistance to pyriproxyfen in this multiple resistant *Cx quinquefasciatus* population. While this is yet to be demonstrated in Culicine mosquitoes, resistance to pyriproxyfen mediated by a significant over-expression of P450 enzymes was previously reported in white flies [38, 39]. It therefore needs to be demonstrated whether P450s which have been identified as over-expressed in pyrethroid resistant *An gambiae* and *Cx quinquefasciatus* populations would metabolise pyriproxyfen as to monitor the possibilities of cross resistance. Nevertheless, some studies have shown a sterilizing impact of pyriproxyfen on susceptible *Cx quinquefasciatus* mosquitoes when used in insecticidal wall paint [40] and in controlled laboratory bioassays [36]. While this sterilising effect with *Cx quinquefasciatus* was reduced compared to what was achieved with *An gambiae* [36], the findings suggest that delivery systems that ensure longer contact time such as wall treatments and applications to larval breeding sites may be more appropriate for pyriproxyfen on this species.

Although it is not clear how much the addition of a second active ingredient will add to the total cost of manufacturing of the mixture LLINs, the cost of deploying IRS and LLINs together would likely be greater and more demanding operationally than for the mixture LLIN alone. In addition, mixtures whether on bed nets or for IRS may be more effective for insecticide resistance management since contact with both insecticides is more certain compared to the combined intervention approach [1, 31, 41]. Therefore mixture nets could be more desirable than the combined intervention approach if comparable levels of impact can be demonstrated in terms of their ability to improve vector control in pyrethroid resistant areas and to manage resistance. The levels of mortality achieved when chlorfenapyr IRS was combined with
pyrethroid LLIN (chapter 3) were similar to that for the chlorfenapyr and pyrethroid mixture bed nets (chapter 7) (both >80%). Both studies were performed against the same pyrethroid resistant *An gambiae* vector population in Akron, Southern Benin showing that the two approaches may provide parallel levels of improved the control against pyrethroid resistant mosquitoes. Further studies comparing the insecticide resistance management potential of the two strategies are needed. For such interventions to be effective in IRM, the active ingredients should ideally be applied at the full operational dose and should decay at the same rate [1]. This could be easier to achieve with mixture LLIN through the long-lasting net technology compared to the combined IRS and LLIN approach since IRS with most current insecticide are short-lived (<6months). This suggests that mixture LLINs may be more effective for IRM than combined interventions. Nevertheless, long-lasting non-pyrethroid ITWL if developed could also be used in the place of non-pyrethroid IRS to make the most of the resistance management potential of the combined intervention approach.

3. **Combined use of unrelated insecticides for managing insecticide resistance**

Insecticide resistance genes protect an insect from the toxic effect of a particular insecticide allowing it to survive in the presence of that insecticide leading to further selection of the insecticide resistant gene [42]. By adding another insecticide with an alternative mode of action to which vectors are susceptible through the combined intervention or mixture strategy, it should be possible to kill more of the resistant genotypes and slow down selection of the insecticide resistant gene over time [2]. Genotyping studies were performed with samples of live and dead *An gambiae* from
each of the hut trials (chapters 3, 5 and 6) in an attempt to assess the capacity of the combined interventions approach to slow down this selection process. The selection studies focused only on target site resistance (*kdr* and *Ace1-R*) owing to the lack of reliable markers and high throughput techniques for monitoring other resistance mechanisms.

Unfortunately, none of these studies were able to demonstrate a selective advantage or neutrality of the *kdr* with any of the treatments owing to the high *kdr* allele frequency (>85%) in all three sites (Benin, Burkina Faso and Cote d'Ivoire). Population genetic modelling studies have shown that the benefits of most insecticide resistance management strategies are less likely to be achieved where the resistance gene is already well established [2, 31], hence these results with kdr are not unexpected. While it is unclear whether the presence of the *kdr* gene alone is sufficient to cause operationally significant levels of phenotypic resistance [10, 43, 44], *kdr* usually combines with other resistance mechanisms to exacerbate phenotypic resistance to pyrethroids and DDT. Further studies in sites with low to moderate frequencies of the gene should therefore be performed to assess the impact of IRM on the selection of the *kdr* gene.

In the Burkina Faso (Chapter 5) and Cote D'Ivoire (Chapter 6) studies, the genotyping results showed a clear survival advantage of *Ace1-R* bearing mosquitoes in huts in which p-methyl wall linings were applied alone and no LLIN was in use. Previous studies also demonstrated a survival advantage of *An gambiae* mosquitoes bearing the *Ace1-R* gene when exposed to bendiocarb (a carbamate) IRS and treated wall linings in experimental huts in Burkina Faso [45]. The *Ace1-R* gene has been strongly associated with resistance to organophosphates and carbamates [20, 46, 47]. Molecular studies have suggested that this survival advantage of *Ace1-R*
bearing mosquitoes may be due to a duplication of the gene [46, 48]. The Ace-1R gene has been detected at very low to moderate frequencies in some vector populations in West Africa which have not shown phenotypic resistance to organophosphates and carbamates in WHO resistance bioassays [19, 49, 50]. With increasing threats of pyrethroid resistance most malaria control programmes have turned to organophosphates and carbamate for IRS. Even though effective vector control can be achieved in the short-term when these insecticides are applied alone against vector populations with low Ace1-R frequencies as was the case in the Burkina Faso study, the findings from the selection studies suggest that continuous use could quickly lead to high frequencies of the Ace1-R gene eventually making the insecticides redundant in these areas.

In the Burkina Faso study, there was full susceptibility to the organophosphate leaving no survivors in the huts with the combinations (100% mortality); hence it was also not possible to demonstrate unequivocally the selective advantage or neutrality of the combination on the Ace1-R genes in this site. In Tiassale, Cote d'Ivoire where the Ace1-R allele frequency was moderately high (0.44), the combination failed to prevent selection of mosquitoes bearing the Ace1-R gene. These findings make evident the fact that the use of mixtures/combinations is less likely to manage resistance in areas where resistance to both insecticides being used is already detectable. Under such circumstances of resistance to multiple insecticides, the most appropriate strategy as recommended by the GPIRM is to rotate insecticides over short periods of time beginning with the insecticides to which local vectors show the least resistance [1]. However, rotations are particularly effective only if the resistance gene has an associated fitness cost. Unfortunately, the high fitness cost of the homozygous ace 1R in An gambiae [47, 51] can be compensated for by its
duplication in the heterozygous *Ace1-R* [46, 48], hence it is also unlikely that rotations would successfully manage insecticide resistance posed by the *Ace1-R* gene. The results therefore demonstrate that the effective application of insecticide resistance management strategies will inevitably require new insecticides with new modes of action. It also highlights the need to implement resistance management early enough as to prevent the development of resistance to multiple insecticides when control and/or management become difficult to achieve with the existing portfolio of insecticides.

Just like the combined intervention approach, the use of mixtures on mosquito nets also provides opportunity for insecticide resistance management since two unrelated insecticides are presented to the vector population at the same time. Because pyrethroid resistance is already well-established in the Akron vector population where the mixture nets were tested, further genotyping studies were not performed to assess the potential of the mixture nets to prevent selection of pyrethroid resistance. In addition, there are currently no records of resistance mechanisms in *An gambiae* to the alternative compounds (chlorfenapyr and pyriproxyfen) in the mixture nets which could be monitored for selection. However, as discussed in Chapter 1 of this thesis, mixtures applied on LLINs may be very effective for IRM strategy since insect contact with both insecticides is more guaranteed. It is therefore important to evaluate these mixture nets in areas where pyrethroid resistance is still rare in order to assess their capacity to manage pyrethroid resistance by preventing the selection of pyrethroid resistant genotypes.
1.5 Future considerations on combining interventions and mixture nets

Even though experimental hut studies are a good simulation of human occupied houses and they provide useful information on the interactions between the treatments, humans and mosquitoes, results from large scale field trials are needed to confirm whether improved control with the combined interventions and mixture nets tested in this thesis will translate to additional protection against malaria. Data from cross-sectional studies on programmes deploying IRS and LLIN interventions together have demonstrated evidence in a range of settings of added protection among those who sleep under LLINs in IRS treated houses [52]. However such non-experimental studies have inherent limitations; hence there is a need for fully-powered rigorous randomised control studies in which confounding factors are effectively controlled. A few community randomised control trials of LLINs and IRS combinations have been completed in Benin, Tanzania, The Gambia and one is ongoing in Sudan [32]. The trials in Tanzania [53] and Benin [54] have been published and both evaluated the combined use of pyrethroid LLINs and carbamate IRS in high transmission settings with high resistance to pyrethroids but low resistance to carbamates. The Tanzanian trial showed that the combination provided significant additional protection over the LLIN alone while the Benin trial showed no evidence of added protection with the combination. The result from Tanzania was consistent with the outcome of the experimental hut trials of LLIN and p-methyl wall linings in Burkina Faso where vectors were resistant to pyrethroids but susceptible to the organophosphate (chapter 5). While questions have been raised over the design of the Benin study and the lack of sufficient clusters [32, 55], the authors did argue that the lack of improved impact with the combination may have been due to the low
residual life of the carbamate insecticide which was only applied once throughout the trial unlike in the Tanzanian trial where two applications were made to cover the entire transmission season. Data from Bioko Island, a malaria endemic area in Equatorial Guinea with year round transmission demonstrated an increase in the prevalence of malaria infection in children over time associated with the low residual life of bendiocarb IRS [56]. This underscores the need for long-lasting forms of IRS treatments and for alternative insecticide delivery systems on home walls like ITWL which have been demonstrated in this thesis as useful substitutes to IRS (chapters 4-6) if they can be developed in a long-lasting format using advanced binding technology. Long-lasting non-pyrethroid wall linings when used in combination with LLINs could optimize the advantages of the combination approach.

The different insecticides used for IRS and the different recommended LLIN brands induce different effects on mosquitoes conferring varying levels of protection which could affect the outcome of the combined intervention approach. Studies by Okumu et al compared several combinations of WHOPES approved IRS insecticides and LLIN brands in experimental huts against pyrethroid susceptible An. arabiensis [57]. The results demonstrated improved levels of mortality in huts in which LLINs were combined with less irritant IRS insecticides like pirimiphos methyl and higher deterrent effect in huts with more irritant IRS insecticides like DDT. Modelling studies also suggested the possibilities of antagonisms between some insecticide combinations via interference of their modes of action [58]. Studies also need to be performed against susceptible and resistant populations of other vector species like An. gambiae in both semi-field and field situations in order to guide malaria vector control programmes in such settings into choosing the most appropriate combinations to use for improving transmission control.
While the resistance gene selection studies focused on target site resistance, there are metabolic forms of resistance in the vector populations in these studies which in addition to the kdr and Ace-1R gene, would have contributed to the levels of resistance observed in each of the study sites. In Cote D'Ivoire, this was indicated by the survival of some mosquitoes which did not bear the Ace1-R gene in huts with the combinations. Microarray studies have identified P450 genes associated with resistance significantly over-expressed in the Valley du Kou (CYP6P3 and CYP6Z2) [59], Tiassale (CYP6M2) [46] and Akron (CYP6P3 and CYP6M2) [60] vector populations which were studied. Some of these genes (CYP6P3, CYP6M2) have validated as insecticide metabolizers [61]. Monitoring metabolic resistance is complex and requires that mosquitoes should be standardised by their physiological status which may be very challenging for such experimental hut selection studies. This constitutes some of the difficulties associated with developing reliable evidence for IRM strategies. However, metabolic resistance particularly the mono-oxygenases whether on its own or in combination with target site resistance has potential to cause control failure; this was the case in South Africa in 2000 with An funestus [62]. Reliable DNA markers for the array of metabolic genes that can be up-regulated in vector populations resistant to current insecticides should be urgently identified and appropriate techniques and strategies should be developed to permit more rigorous assessment of the impact of IRM strategies on the evolution of a wider range of insecticide resistance genes.

1.6 The place of non-chemical vector control interventions

While chemical-based vector control interventions have had a critical role in reducing the burden of malaria, it is very evident that their continuous efficacy is threatened by the evolution and spread of insecticide resistance in malaria vectors. The results
obtained in this thesis have shown that the proposed methods for managing insecticide resistance are not necessarily the panacea for sustaining vector control with chemical based methods especially in areas where resistance is already established. Therefore, while efforts are underway to contain resistance and preserve the efficacy of LLINs and IRS, the most widely implemented vector control tools, it will be equally useful to develop and invest in other non-chemical vector control methods. These could be implemented along with LLINs and IRS within an integrated framework as to reduce our dependence on insecticides for malaria vector control and enhance capacity to further reduce malaria transmission. In addition, current WHO guidelines on integrated vector management encourage the use of multiple tools to improve impact on vector borne diseases and reduce the pressures of insecticide resistance [63].

Other non-chemical methods which have been used or proposed for malaria vector control include environmental management, house screening, the use of odour baited traps, the release of sterile insects, biological control methods and use of repellents. While some of these methods like the release of sterile insects and odour baited traps are still under development and are yet to be demonstrated as effective wide scale malaria vector control strategies, others like environmental management and house screening have shown enormous potential. Environmental management for example was the mainstay of vector control in the pre-DDT era and is considered a major contributing factor to the elimination of malaria in some parts of the world [64]. House improvement and screening prevents mosquito entry into homes thus reducing exposure to malaria transmitting mosquitoes. It has the added benefit of protecting everyone in the home thus preventing issues of equity within the household [65]. A randomised control study in The Gambia demonstrated
significantly reduced mosquito entry in screened houses resulting in a substantial reduction in anaemia in children [66]. Nevertheless, environmental management and house screening have not been widely implemented as a vector control strategy in malaria endemic areas in Africa owing to the high costs and challenges associated with scaling them up. However, given the threats posed by insecticide resistance, it becomes necessary to invest in such non-chemical interventions and find ways of making them cost-effective for malaria vector control.

4. Conclusions
The current thesis was designed to generate some evidence on the use of a combination of insecticides for improving the control of pyrethroid resistant malaria vectors and for insecticide resistance management. While a few limitations to the studies performed such as the lack of sufficient mosquito sample size in some cases could be identified, some conclusions can be readily drawn:

a) The efficacy of pyrethroid LLINs is significantly reduced when confronted with pyrethroid resistant malaria vectors. Nevertheless, pyrethroid LLINs are still better against partially resistant malaria vectors than untreated nets or no intervention at all.

b) The control of pyrethroid resistant malaria vectors can be significantly improved if pyrethroid LLINs are combined with IRS or wall linings which deploy an insecticide with an alternative mode of action to which the local vector population is largely susceptible. The combination can provide personal protection mostly due to the pyrethroid LLIN and improved mortality mostly due to the alternative insecticide on the wall.
c) Insecticide resistance is undoubtedly an important factor when considering combinations; improved vector control is unlikely when there is resistance to both insecticides.

d) Combining pyrethroid LLINs with pyrethroid IRS or wall linings provided no improvement in control of pyrethroid mosquitoes thus upholding WHO recommendations against the use of combinations with insecticides of similar classes.

e) Genotyping studies are unlikely to demonstrate a selective advantage or neutrality of a resistance gene with resistance management strategies when gene frequencies are already high and the resistance mechanism is well established. Studies on resistance management should be performed in sites where the frequency of the targeted gene is low-moderate.

f) $Ace1-R$ resistance genes will be selected when organophosphate wall treatments are used on their own for malaria vector control. When combined with pyrethroid LLINs against vectors that are largely susceptible to the organophosphate, selection may reduce but when resistance to both insecticides is already detectable in the target vector population the combinations are unlikely to prevent selection of insecticide resistance genes.

g) Reliable DNA markers for the array of genes for metabolic enzymes which can be up-regulated in metabolic resistance to current insecticides should be urgently identified to allow proper investigation of IRM strategies.

h) Chlorfenapyr and pyriproxyfen are suitable compounds which could be combined with pyrethroids on LLINs for improved control of pyrethroid resistant malaria vectors.
i) The levels of improved control of pyrethroid resistant malaria provided with mixture nets may be comparable to what is achievable when pyrethroid LLINs are combined with non pyrethroid IRS (if similar insecticides are involved).

j) Community randomised trials are needed to assess whether the combination approaches tested in this thesis will provide additional protection against malaria and manage resistance under field situations.
References


27. Chandre F, Dabire RK, Hougard JM, Djogbenou LS, Irish SR, Rowland M, N'Guessan R: **Field efficacy of pyrethroid treated plastic sheeting (durable lining) in combination with long lasting insecticidal nets against malaria vectors.** *Parasit Vectors* 2010, **3**:65.


33. Oxborough RM, Kitau J, Matowo J, Feston E, Mndeme R ea, Mosha F, Rowland M: **ITN Mixtures of Chlorfenapyr (Pyrrole) and Alphacypermethrin (Pyrethroid) for Control of Pyrethroid Resistant Anopheles arabiensis and Culex quinquefasciatus.** *PLoS ONE* 2013, **8**:e55781. doi:55710.51371/journal.pone.0055781.


60. Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, Strode C: Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from Southern Benin and Nigeria. BMC Genomics 2008, 9:538.


Annexes

Annex 1

**WHO recommended long-lasting insecticidal nets**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Product type</th>
<th>Status of WHO recommendation</th>
<th>Status of Publication of WHO specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>DawaPlus® 2.0</td>
<td>Deltamethrin coated on polyester</td>
<td>Interim</td>
<td>Published</td>
</tr>
<tr>
<td>Duranet®</td>
<td>Alpha-cypermethrin incorporated into polyethylene</td>
<td>Full</td>
<td>Published</td>
</tr>
<tr>
<td>Interceptor®</td>
<td>Alpha-cypermethrin coated on polyester</td>
<td>Full</td>
<td>Published</td>
</tr>
<tr>
<td>LifeNet®</td>
<td>Deltamethrin incorporated into polypropylene</td>
<td>Interim</td>
<td>Published</td>
</tr>
<tr>
<td>MAGNetTM</td>
<td>Alpha-cypermethrin incorporated into polyethylene</td>
<td>Full</td>
<td>Published</td>
</tr>
<tr>
<td>Olyset Net®</td>
<td>Permethrin incorporated into polyethylene</td>
<td>Full</td>
<td>Published</td>
</tr>
<tr>
<td>Olyset® Plus</td>
<td>Permethrin and PBO incorporated into polyethylene</td>
<td>Interim</td>
<td>Published</td>
</tr>
<tr>
<td>PermaNet® 2.0</td>
<td>Deltamethrin coated on polyester</td>
<td>Full</td>
<td>Published</td>
</tr>
<tr>
<td>PermaNet® 3.0</td>
<td>Combination of deltamethrin coated on polyester strengthened border (side panels) and deltamethrin and PBO incorporated into polyethylene (roof)</td>
<td>Interim</td>
<td>Published</td>
</tr>
<tr>
<td>Royal Sentry®</td>
<td>Alpha-cypermethrin incorporated into polyethylene</td>
<td>Full</td>
<td>Published</td>
</tr>
<tr>
<td>Yorkool® LN</td>
<td>Deltamethrin coated on polyester</td>
<td>Full</td>
<td>Published</td>
</tr>
</tbody>
</table>

*adapted from http://www.who.int/whopes/Long_lasting_insecticidal_nets_06_Feb_2014.pdf?ua=1
Annex 2

WHO recommended insecticides for indoor residual spraying against malaria vectors *

<table>
<thead>
<tr>
<th>Insecticide and formulation¹</th>
<th>Class group²</th>
<th>Dosage (g a.i/m²)</th>
<th>Mode of action</th>
<th>Duration of effect (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DDT WP</strong></td>
<td>OC</td>
<td>1-2</td>
<td>contact</td>
<td>&gt;6</td>
</tr>
<tr>
<td><strong>Malathion</strong></td>
<td>OP</td>
<td>1-2</td>
<td>contact</td>
<td>2-3</td>
</tr>
<tr>
<td><strong>Fenitrothion WP</strong></td>
<td>OP</td>
<td>2</td>
<td>contact &amp; airborne</td>
<td>3-6</td>
</tr>
<tr>
<td><strong>Pirimiphos-methyl WP &amp; EC</strong></td>
<td>OP</td>
<td>1-2</td>
<td>contact &amp; airborne</td>
<td>2-3</td>
</tr>
<tr>
<td><strong>Pirimiphos-methyl CS</strong></td>
<td>OP</td>
<td>1</td>
<td>contact &amp; airborne</td>
<td>4-6</td>
</tr>
<tr>
<td><strong>Bendiocarb WP</strong></td>
<td>C</td>
<td>0.1-0.4</td>
<td>contact &amp; airborne</td>
<td>2-4</td>
</tr>
<tr>
<td><strong>Propoxur WP</strong></td>
<td>C</td>
<td>1-2</td>
<td>contact &amp; airborne</td>
<td>3-6</td>
</tr>
<tr>
<td><strong>Alpha-cypermethrin WP &amp; SC</strong></td>
<td>PY</td>
<td>0.02-0.03</td>
<td>contact</td>
<td>4-6</td>
</tr>
<tr>
<td><strong>Bifenthrin WP</strong></td>
<td>PY</td>
<td>0.025-0.05</td>
<td>contact</td>
<td>3-6</td>
</tr>
<tr>
<td><strong>Cyfluthrin WP</strong></td>
<td>PY</td>
<td>0.02-0.05</td>
<td>contact</td>
<td>3-6</td>
</tr>
<tr>
<td><strong>Deltamethrin SC-PE</strong></td>
<td>PY</td>
<td>0.1-0.3</td>
<td>contact</td>
<td>3-6</td>
</tr>
<tr>
<td><strong>Deltamethrin WP, WG</strong></td>
<td>PY</td>
<td>0.02-0.03</td>
<td>contact</td>
<td>3-6</td>
</tr>
</tbody>
</table>


¹CS = capsule suspension; EC = emulsifiable concentrate; SC = suspension concentrate; SC-PE = polymer enhanced suspension concentrate; WG = water dispersible granule; WP = wettable powder.

²OC = organochlorines; OP = organophosphates; C = carbamates; PY = pyrethroids.
Annex 3

Classes of insecticides used for vector control

### Pyrethroid

Pyrethroids are synthetic versions of pyrethrins which are naturally occurring compounds derived from members of the chrysanthemum family. Pyrethroids have been the chemicals of choice in public health for the past few decades because of their relatively low toxicity to humans, rapid knockdown effect, relative longevity (3–6 months when used for IRS) and low cost. They act on the sodium channels of the insect vector, preventing them from closing, leading to continuous nerve excitation, paralysis and death. They also have an irritant effect, causing an excitorepellency response, resulting in hyperactivity, rapid knock-down, feeding inhibition, shorter landing times and undirected flight, all of which reduce the ability of vectors to bite. Pyrethroids are used for both IRS and LLINs in the form of α-cypermethrin, bifenthrin, cyfluthrin, deltamethrin, permethrin, λ-cyhalothrin and etofenprox. Bifenthrin is only used for IRS. They are the only class of insecticides used on WHOPES-approved LLINs and the most commonly used for IRS. In 2009 for example, pyrethroids were estimated to account for 75% of IRS coverage. Malaria control is therefore considered to be very dependent on this class of insecticides.

### Organochlorines

Organochlorines are used in IRS in the form of dichlorodiphenyltrichloro-ethane (DDT), which was the insecticide used predominantly in the malaria eradication campaigns of the 1950s. Other members of this class which have been used for malaria control are lindane and dieldrin. Both insecticides are no longer recommended due to widespread resistance (lindane) and toxicity to humans (dieldrin). DDT has a relatively long residual activity (6–12 months) when used for IRS depending on the dose and the substrate making it a suitable insecticide for holoendemic areas with long transmission seasons. Like pyrethroids, DDT has a spatial repellency and an irritant effect on malaria vectors that strongly limit human-vector contact. This obliges surviving mosquitoes to feed and rest outdoors which contributes to effective disease-transmission control.

At the Stockholm Convention on Persistent Organic Pollutants in 2001, the use of DDT was banned for all applications except disease control, because of its environmental effects when used in large volumes in agriculture. In 2006, the WHO released a position statement reasserting the public health value of DDT for IRS since the number of equally effective, efficient, alternative insecticides for public health is limited. The use of DDT was thus permitted until "locally safe, effective, and affordable alternatives are available for a sustainable transition from DDT".

### Organophosphates

Organophosphates which can be used for IRS are fenitrothion, malathion and pirimiphos-methyl. They act on the mosquito vector by inhibiting cholinesterase, preventing breakdown of the neurotransmitter acetylcholine, resulting in neuromuscular overstimulation and death of the vector. Organophosphates are very toxic but do not induce an excitorepellency response from the vector. Organophosphates currently used for malaria control are significantly more expensive than other insecticides. For some compounds, toxicological monitoring is required for accidental overexposure during IRS. The organophosphates in the existing port-folio of WHOPES approved insecticides are also short-lived (2–3 months) when used for IRS and may require 2-3 rounds of spraying per year in long transmission seasons which is usually very difficult (if not impossible) to achieve and sustain in most settings. However, advanced formulation technology has recently been used to develop a new microencapsulated formulation of pirimiphos-methyl which has shown potential to provide prolonged malaria vector control in small scale trials.

### Carbamates

Carbamates are used for IRS in the form of propoxur and bendiocarb. Like organophosphates, these compounds are highly effective and induce little or no excitorepellency response from the vector. They also have short residual activity (2–6 months when used for IRS) and are more expensive than pyrethroids and DDT. The mode of action of carbamates is similar to that of organophosphates.
Annex 4

Insect enzyme families involved in metabolic resistance to insecticides

<table>
<thead>
<tr>
<th>Cytochrome P450 (CYP) monooxygenases:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 detoxification enzymes are best known for their monooxygenase activity, reactive or polar groups into xenobiotics or endogenous compounds. They are the primary enzyme family responsible for metabolising pyrethroids in insects. Genome studies have revealed 111 P450 enzymes in An gambiae and as in other insects, only a small proportion of these enzymes are capable of detoxifying insecticides. Several mosquito CYPs have been found over-transcribed in resistant field populations or laboratory colonies [1] of malaria vectors [2]. However, higher activity of enzymes and/or over expression of these genes does not necessarily correlate with insecticide resistance. CYPs which have been validated as pyrethroid metabolizers include; An. gambiae CYP6M2 and CYP6P3 [3,4], Anopheles minimus CYP6A3 and CYP6P7 [5] and An funestus CYP6P9 [6]. Recent studies showed that the An gambiae CYP6M2 is also capable of metabolising the organochlorine DDT hence demonstrating the possibilities of metabolic cross resistance [7].</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carboxyl choline esterases (CCEs):</th>
</tr>
</thead>
<tbody>
<tr>
<td>introducing Esterases act by rapidly binding and slowly turning over the insecticide, hence they sequester rather than rapidly metabolise the insecticide. Esterase based resistance has been extensively studied in Culex mosquitoes [8]. Over-production of CCEs has been mostly associated with organophosphate resistance in mosquitoes. Their ability to metabolise pyrethroids has been suggested [9]. However, no specific CCE has yet been validated as a pyrethroid metabolizer.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glutathione S-transferase (GSTs):</th>
</tr>
</thead>
<tbody>
<tr>
<td>The primary role of GSTs in mosquito insecticide resistance is in the metabolism of DDT to DDE, although they also have a secondary role in resistance to organophosphates. GST-based DDT resistance is common in a number of Anopheline species resulting from the intense use of this insecticide for malaria control over several decades[10]. Their potential role in the sequestration of pyrethroids has been suggested [11]; nevertheless further investigation is required to understand the underlying mechanism.</td>
</tr>
</tbody>
</table>

References:


11. Kostaropoulos I, Papadopoulos AI, Metaxakis A, Boukouvala E, Papadopoulou-Mourkidou E: Glutathione S-transferase in the
Cross resistance patterns of different classes of insecticide

<table>
<thead>
<tr>
<th>Class</th>
<th>Metabolic</th>
<th>Target Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Esterases</td>
<td>Mono-oxygenases</td>
</tr>
<tr>
<td>Pyrethroids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbamates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organophosphates</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GSH, glutathione; AchE, acetylcholinesterase; circle size reflects relative impact of mechanism of resistance
Annex 6

Experimental huts designs

West African experimental hut  East African experimental hut
Annex 7

Insecticide treated wall lining materials

P-methyl treated DL  P-methyl treated NWH