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Combining pyrethroid-piperonyl butoxide (PBO) nets and clothianidin-based indoor residual spraying improves control of pyrethroid-resistant malaria vectors: a series of experimental hut trials in southern Benin

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Abstract

Background: A recent experimental hut trial demonstrated an antagonistic interaction between pirimiphos-methyl indoor residual spraying (IRS) and pyrethroid-piperonyl butoxide (PBO) nets, attributed to PBO-mediated inhibition of cytochrome P450 monooxygenase enzymes responsible for activation of pirimiphos-methyl. Clothianidin does not require activation by these enzymes, suggesting it may be less vulnerable to this negative interaction and thus a suitable partner IRS insecticide for pyrethroid-PBO nets.

Methods: We conducted three experimental hut trials over 6 months in Covè, southern Benin, to evaluate the entomological impact of combining different types of pyrethroid-PBO net (Olyset® Plus, PermaNet® 3.0) and clothianidin-based IRS (SumiShield® 50WG, Fludora® Fusion). In each trial, the impact of the combinations was compared to each method alone. Bioassays were performed with wild-collected mosquitoes to investigate insecticide susceptibility and interactions between PBO and clothianidin.

Results: Across all three trials, mortality was superior with the combinations (52–63%) compared to both nets (28–36%, $p<0.001$) and IRS (36–42%, $p<0.001$) alone. Blood-feeding inhibition with the combinations (64–84%) was higher than the IRS (1–4%, $p<0.001$) but similar to the nets (66–73%), except in trial 3, where it was modestly higher (84% vs 73%, $p=0.008$). The vector population was resistant to pyrethroids (<10% mortality at discriminating concentration) but susceptible to clothianidin (100% mortality at discriminating concentration). PBO pre-exposure improved clothianidin toxicity (59% vs 43%, $p<0.001$), although the effect was modest and dose-dependent.

Conclusions: Combining pyrethroid-PBO nets and clothianidin-based IRS shows potential to improve vector control by increasing mosquito mortality compared to nets alone, while enhancing mortality and blood-feeding protection relative to IRS alone. The improved

mortality likely reflects additive effects arising from the concurrent use of two tools with non-interfering modes of action, potentially augmented by synergistic interactions between PBO and clothianidin. The findings suggest that pyrethroid-PBO nets and clothianidin-based IRS are complementary interventions whose combined deployment may improve malaria vector control.

Keywords: Insecticide-treated nets, indoor residual spraying, malaria, vector control, insecticide resistance

Background

Increased control efforts are estimated to have averted 2.3 billion malaria cases and 14 million deaths since 2000 (1). Central to this progress was the large-scale deployment of vector control interventions, notably insecticide-treated nets (ITNs) and indoor residual spraying (IRS), which together accounted for approximately 78% of the case reduction observed in sub-Saharan Africa (2). Unfortunately, control progress has stalled globally since 2015, and the effectiveness of these tools is threatened on several fronts. Foremost among these challenges is the increasing resistance of malaria vector populations to the insecticides used in ITNs and IRS, especially the pyrethroids (3). In response, a new generation of vector control tools has been developed to improve control of malaria transmitted by insecticide-resistant mosquitoes. These innovations include ITNs combining a pyrethroid with a second insecticidal compound and IRS formulations containing new active ingredients with novel modes of action and prolonged residual activity.

One major type of next-generation ITN combines a pyrethroid with the synergist piperonyl butoxide (PBO), which partially restores pyrethroid efficacy by inhibiting cytochrome P450 monooxygenase (P450) enzymes associated with metabolic pyrethroid resistance in malaria vectors (4). The World Health Organisation (WHO) conditionally recommended the deployment of pyrethroid-PBO nets based on cluster-randomised controlled trials (cRCTs) in Tanzania (5) and Uganda (6) showing reduced malaria burden in communities using pyrethroid-PBO nets compared to those using pyrethroid-only nets. New IRS insecticides are also showing promise. Notably, formulations containing the neonicotinoid insecticide clothianidin have been added to the WHO list of prequalified vector control products (7) following evidence of entomological efficacy from experimental hut (8-10) and cRCTs (11, 12). Uptake of these new tools has increased substantially in recent years. Pyrethroid-PBO nets accounted for approximately 38% of all ITN shipments to sub-Saharan Africa since 2022

(13), while clothianidin-based IRS comprised 48% of all IRS sachets procured over the same period (14). Despite this, malaria transmission remains high in many regions, including those where next-generation tools have been deployed. This highlights the need to investigate strategies to address the gaps in protection left by these tools.

Some programmes with increased funding deploy ITNs and IRS concurrently in the same areas and households to achieve greater impact than is possible with either method alone. Evidence for the added benefit of this strategy over ITNs alone is, however, mixed, with some cRCTs reporting improved impact (5, 15-17) and others showing no additional benefit (5, 18-21). Given the substantial costs associated with co-deploying ITNs and IRS – which limits operational feasibility in most settings – the WHO has conditionally recommended against this strategy as a means of improving malaria control (22). Nevertheless, focal co-deployment of ITNs and IRS continues in some settings where resources permit, and research identifying the contexts and combinations most likely to provide additional benefit remains important for optimising deployment strategies in these areas.

Recent evidence from experimental hut trials has highlighted the importance of considering the biochemical characteristics of the compounds deployed through ITNs and IRS, and their potential interactions, when selecting suitable combinations of these tools for operational deployment. In Benin, combining pirimiphos-methyl IRS with pyrethroid-PBO nets reduced vector mortality compared to the IRS alone, providing evidence of an antagonism between these tools (23). This effect was attributed to a biochemical interaction between the compounds deployed through these interventions. Pirimiphos-methyl is a pro-insecticide requiring metabolic activation by P450s to become fully toxic (24), whereas PBO enhances pyrethroid potency by inhibiting these same enzymes. Consequently, prior exposure to PBO from sublethal contact with the pyrethroid-PBO net may inhibit the activation of pirimiphos-methyl in mosquitoes that subsequently rest on treated walls, resulting in reduced efficacy of

the IRS when these interventions are combined in the same household. These findings illustrate how insecticidal compounds deployed through separate interventions within the same household may interact biochemically, such that one component alters vector physiological responses to the other, thereby influencing the entomological impact of the combination relative to either method alone.

Unlike pirimiphos-methyl, clothianidin does not require metabolic activation by P450s, and may thus be less vulnerable to antagonistic interactions with pyrethroid-PBO nets. In fact, increasing evidence suggests that clothianidin can be detoxified by these same enzymes, creating the potential for a synergistic interaction with PBO. Indeed, biomolecular analyses have shown that alleles associated with P450 overexpression are linked to increased survival of malaria vectors exposed to clothianidin in experimental huts and susceptibility bioassays (25). Consistent with this, PBO pre-exposure has been shown to potentiate clothianidin toxicity against some vector populations (25-27). Thus, when pyrethroid-PBO nets and clothianidin-based IRS are combined in the same household, inhibition of detoxifying P450s by PBO on the net may enhance vector susceptibility to clothianidin on sprayed walls, resulting in greater vector control compared to either method alone. Given these complementary characteristics, studies investigating the impact of combining pyrethroid-PBO nets and clothianidin-based IRS are warranted. Experimental hut trials assessing the entomological efficacy of this combination compared to each method alone can provide insights into how these tools interact in a household setting and inform whether this strategy has the potential to improve malaria vector control.

In this study, we conducted a series of experimental hut trials to evaluate the impact of combining two major brands of pyrethroid-PBO net (Olyset® Plus and PermaNet® 3.0) with different types of clothianidin-based IRS against a pyrethroid-resistant vector population in Covè, southern Benin. Nets were tested in combination with clothianidin-only (SumiShield®

50WG) and clothianidin-deltamethrin mixture (Fludora® Fusion) IRS formulations. Susceptibility of the Covè vector population to the active ingredients in the hut treatments was investigated following standard WHO test procedures, and wall cone bioassays were performed inside huts to assess the residual efficacy of the IRS treatments. To complement the experimental hut results, laboratory bioassays were also performed to investigate interactions between PBO and clothianidin.

Methods

Experimental hut trial

Experimental hut trials are semi-field assays designed to evaluate the entomological efficacy of indoor vector control tools under controlled but ecologically realistic conditions. These studies simulate the natural conditions under which mosquitoes interact with vector control tools inside households in the presence of a human host, generating entomological endpoints such as vector mortality and blood-feeding inhibition, which can be used to parameterise transmission dynamics models that predict epidemiological impact at an operational scale (28, 29). Experimental hut trials are particularly valuable for assessing combinations of ITNs and IRS, as they allow for direct comparison of combined interventions against each method alone. This enables quantification of whether the combinations produce additional, redundant, or antagonistic effects on entomological outcomes, providing insights into how these tools interact with each other in a household setting and whether their combined deployment has the potential to provide greater vector control relative to either method alone. Ultimately, this provides a cost-effective means of identifying complementary ITN-IRS combinations suitable for larger-scale evaluation or operational deployment.

Study site and experimental huts

The trials were performed in experimental huts of standard West African design in the commune of Covè, Zou Department, southern Benin (7°14′N2°18′E). Before this study, the walls and ceilings of the huts were plastered with cement and left to cure for 1 month to prevent contamination from previous trials. The huts are surrounded by irrigated rice fields, which provide extensive breeding sites supporting a high year-round density of malaria vector mosquitoes. *Anopheles coluzzii* and *An. gambiae* sensu stricto (s.s.) occur sympatrically with the former predominating (30). Recent studies investigating insecticide resistance in the Covè vector population using standard WHO test procedures show that it is highly resistant to pyrethroids but remains largely susceptible to other insecticide classes, including clothianidin (31, 32). Genotyping and gene expression studies have revealed that pyrethroid resistance is mediated by a high frequency (>80%) of the knockdown resistance L1014F mutation and overexpression of genes encoding metabolic detoxification enzymes, particularly CYP6P3 (30, 31) – a P450 validated as an efficient metaboliser of pyrethroids (33).

Experimental hut treatments

Three parallel experimental hut trials were performed to evaluate the impact of combining different types of pyrethroid-PBO net and clothianidin-based IRS (Table 1). The first and second trials, respectively, tested Olyset® Plus (Sumitomo Chemical Co., Ltd.) – a permethrin-based pyrethroid-PBO net—and PermaNet® 3.0 (Vestergaard Sàrl) – a deltamethrin-based pyrethroid-PBO net – in combination with clothianidin-only IRS (SumiShield® 50WG, Sumitomo Chemical Co., Ltd.). Meanwhile, the third trial tested Olyset® Plus in combination with clothianidin-deltamethrin mixture IRS (Fludora® Fusion, Envu). In each trial, the impact of the combinations was compared to each method alone to assess the impact of layering one intervention on top of the other and inform decision-making across different programmatic scenarios.

Table 1 Treatment arms evaluated in the experimental hut trials. *SumiShield® 50WG IRS containing clothianidin alone was applied at a target application rate of 300 mg/m^2. Meanwhile, the Fludora® Fusion mixture IRS was applied at a target application rate of 225 mg/m^2, comprising 200 mg/m^2 of clothianidin and 25 mg/m^2 of deltamethrin.*

Treatment number	Trial 1	Trial 2	Trial 3
1	Untreated hut (control)	Untreated hut (control)	Untreated hut (control)
2	Untreated net (control)	Untreated net (control)	Untreated net (control)
3	Olyset® Plus	PermaNet® 3.0	Olyset® Plus
4	SumiShield® 50WG IRS	SumiShield® 50WG IRS	Fludora® Fusion IRS
5	Olyset Plus + SumiShield® 50WG IRS	PermaNet® 3.0 + SumiShield® 50WG IRS	Olyset Plus + Fludora® Fusion IRS

Treatments were randomly allocated to experimental huts to minimise selection bias. The cement-plastered walls and ceilings of huts were sprayed on 8th November, 2022, following standard WHO procedures (34) using Goizper compression sprayers (Goizper Group, IK) fitted with control flow valves and flat fan nozzles. Separate tank mixes were prepared for each hut, and the volume of insecticide solution was measured before and after spraying to calculate the actual volume applied and its deviation from the target. Spraying was accurate; the application volumes were within 10% of the target volume for all huts. Both types of IRS were applied at manufacturer-recommended application rates. For SumiShield® 50WG, which contains clothianidin alone, this was 300 mg/m^2. Meanwhile, for Fludora® Fusion, the application rate was 225 mg/m^2, comprising 200 mg/m^2 of clothianidin and 25 mg/m^2 of deltamethrin. Nets were hung over sleeping areas inside huts by threading nylon rope through the hanging loops on the top edges of the nets and tying the rope to nails fixed in the upper corners of the hut walls. Three replicate nets were used per treatment arm and rotated within the treatment every 2 days. To simulate wear and tear from routine field use, all nets were

also given six holes measuring 4 x 4 cm – one on each short side panel and two on each long side panel – as per WHO guidelines (35).

Experimental hut trial procedure

Healthy male volunteers aged 18–40 were recruited from the local community to sleep in experimental huts between 21:00 and 06:00, 6 days per week, to attract wild free-flying mosquitoes. The volunteers were rotated between experimental huts daily according to a Latin square design to minimise variation associated with differences in individual attractiveness to mosquitoes. Treatments were also rotated between huts weekly to reduce bias caused by differences in hut positional attractiveness. However, because logistical challenges preclude rotation of IRS, a split-plot design was used whereby nets were rotated between unsprayed and sprayed huts following separate Latin square designs.

Each morning, the volunteers collected all mosquitoes from the different compartments of the huts (under the net, room and veranda) using a torch and aspirator and placed them in labelled plastic cups. Mosquito collections were then transferred to the field laboratory for morphological identification, with all female *An. gambiae* s.l. set aside and scored for immediate mortality and blood-feeding status. Live mosquitoes were then provided access to cotton wool soaked in 10% (w/v) glucose solution, and delayed mortality was recorded every 24 h up to 120 h after collection to capture the delayed action of clothianidin. Mosquito collections were performed over 6 months between November 2022 and May 2023.

Experimental hut trial outcome measures

The raw data generated in experimental hut trials includes the total number of mosquitoes entering the huts, the number of dead mosquitoes, and the number of blood-fed mosquitoes, which can be stratified by hut compartment (room, under net, veranda). This is used to calculate various outcome measures that provide insights into the entomological efficacy of

the ITN-IRS combinations, compared to each other and to each method alone. Among these, mortality and blood-feeding inhibition are considered the primary outcomes because they relate directly to the most sensitive parameters comprising vectorial capacity: vector longevity and human-vector contact, respectively (36). Descriptions of the primary and secondary outcome measures selected for this trial are provided below.

The following primary outcome measures were used to express the entomological efficacy of the treatments:

i. **Delayed mortality (%)** – the percentage of dead mosquitoes after a 120 h holding period

ii. **Blood-feeding inhibition (%)** – the percentage reduction in blood-feeding with the treatment relative to the untreated control huts containing no treatment

$$Blood\ feeding\ inhibition\ (\%) = \frac{100\ (Bfu - Bft)}{Bfu}$$

Bfu is the percentage of blood-fed mosquitoes with the control, and *Bft* is the percentage of blood-fed mosquitoes with the treatment

The following secondary outcome measures were also used to express the entomological efficacy of the hut treatments and gain insights into their effects on vector behaviour:

i. **Entry (n)** – the total number of mosquitoes collected

ii. **Deterrence (%)** – the percentage reduction in the number of mosquitoes with the treatment relative to the untreated control huts containing no treatment, calculated as follows:

$$Deterrence\ (\%) = \frac{100\ (Tu - Tt)}{Tu}$$

Tu is the number of mosquitoes collected with the control, and *Tt* is the number of mosquitoes collected with the treatment

iii. **Exophily (%)** – the percentage of mosquitoes exiting into the veranda, providing a crude measure of potential irritant effects of the treatment

iv. **Overall killing effect (%)** – the number of additional mosquitoes The number of additional mosquitoes killed with the treatment over the untreated control expressed as a percentage of the total number of mosquitoes collected with the untreated control huts. This serves as an indicator of the mass killing effect of a treatment and thus its ability to provide community-wide protection, calculated as follows:

$$Overall\ killing\ effect\ (\%) = \frac{100\ (Kt - Ku)}{Tu}$$

Kt is the number of mosquitoes killed with the treatment, *Ku* is the number of mosquitoes killed with the control, and *Tu* is the number of mosquitoes collected with the control

Residual efficacy wall cone bioassays

Cone bioassays were performed on experimental hut walls 1 week after spraying to assess spray quality and at monthly intervals thereafter to assess residual efficacy according to WHO guidelines (37). The tests were performed with wild *An. gambiae* s.l. that were collected as larvae from breeding sites near the experimental huts in Covè and reared to adulthood. At each time-point, 5 cones were attached to the walls and ceiling in two randomly-selected huts for each IRS treatment. Around 10 unfed adult mosquitoes aged 3–5 days were transferred into each cone and exposed to the hut walls for 30 mins, providing a total of approximately 50 mosquitoes per hut and 100 per IRS type. At the end of exposure, mosquitoes were transferred to the field laboratory in untreated holding cups with access to cotton wool soaked in 10% (w/v) glucose solution. Knockdown was recorded 60 mins after exposure and delayed mortality was observed every 24 h up to 120 h after exposure to

capture the delayed action of clothianidin. Mosquitoes were concurrently exposed to untreated hut walls as a negative control.

Susceptibility bioassays

Tube tests and bottle bioassays were performed during the trial according to WHO guidelines (38) to investigate the susceptibility of the Covè vector population to the active ingredients in the hut treatments. *An. gambiae* s.l. were collected as larvae from breeding sites near the experimental huts and reared to adulthood for use in the tests. Mosquitoes were exposed in tube tests to filter papers impregnated with the discriminating concentrations of deltamethrin (0.05%) and permethrin (0.75%), and in bottle bioassays to the discriminating concentration of clothianidin (4 µg) to assess susceptibility to these insecticides. Tube tests were also performed with 5x and 10x the discriminating concentrations of deltamethrin and permethrin to assess pyrethroid resistance intensity. Last, to assess synergism and the contribution of P450s to pyrethroid resistance, mosquitoes were exposed to the discriminating concentrations of deltamethrin (0.05%) and permethrin (0.75%) with pre-exposure to PBO (4%). Clothianidin test bottles were prepared according to WHO guidelines using acetone as a solvent and the surfactant MERO® to prevent crystallisation (38). A total of 100 unfed mosquitoes aged 3–5 days were exposed to each treatment and corresponding controls for 60 mins in four replicates of approximately 25. Delayed mortality was recorded after 24 h for all exposures as per WHO recommendation (38).

PBO and clothianidin interaction bioassays

Previous studies have shown that pre-exposing some mosquito populations to the P450 inhibitor PBO can enhance the toxicity of clothianidin in laboratory bioassays (27). Such an interaction could synergise the effect of clothianidin and improve vector control impact when clothianidin-based IRS is combined with pyrethroid-PBO nets inside the same household. To

investigate whether pre-exposure to PBO could affect the toxicity of clothianidin-based IRS against the Covè vector population inside experimental huts, we conducted a modified version of the WHO tube test. Wild *An. gambiae* s.l. collected as larvae from breeding sites near the experimental huts were reared to adulthood and used for the tests. Filter papers were impregnated with formulated clothianidin (SumiShield® 50WG) using distilled water as solvent, following procedures described by Oxborough et al. (39), while 4% PBO-impregnated papers (4%) were obtained from University Sains Malaysia. To investigate the dose-response relationship, we tested a range of clothianidin concentrations: 0.025%, 0.05%, 0.125%, 0.25%, and 0.5% (w/v). These were selected based on preliminary range-finder assays indicating they would induce ≤90% mortality, minimising the risk of mortality saturation and enabling accurate assessment of the impact of PBO pre-exposure.

Separate cohorts of approximately 200 unfed mosquitoes aged 3–5 days were exposed to each concentration for 60 mins, with and without 60 mins pre-exposure to PBO, in 8 replicate tubes each containing 20–25 mosquitoes. Parallel exposures were conducted with filter papers treated with distilled water and PBO (4%) alone as controls. To control for potential mortality associated with transfer, mosquitoes in the control and clothianidin-only groups were also transferred from untreated tubes to their respective exposure tubes, mirroring the handling procedure of the PBO followed by clothianidin group. Knockdown was recorded at the end of exposure, and mosquitoes were transferred to untreated holding tubes with access to cotton wool soaked in 10% (w/v) glucose solution. Mortality was recorded every 24 h up to 168 h, with percentage mortality after 168 h serving as the primary endpoint based on delayed killing observed in previous studies (39).

Data analysis

Descriptive statistics were used to compute measures of central tendency (percentages) and dispersion (95% confidence intervals) for all outcome measures with each experimental hut treatment. Mortality results were also aggregated by month to calculate cumulative monthly percentage mortality and visualise trends over time. Binary outcomes expressed as percentages (mortality, blood-feeding, exophily, inside net) were analysed using a blocked logistic regression model, while count outcomes (entry) were assessed using negative binomial regression. In addition to treatment, each model fitted hut, sleeper and day as fixed effects to control for variation associated with these factors and improve precision. For each outcome measure, model-derived p-values were used to assign compact letter displays denoting the statistical significance of all pairwise comparisons at the 5% level using the 'multcompView' package in R version 4.4.2. To reinforce confidence in the robustness of the hut results, post-hoc simulation-based power analyses were also performed using the 'power_calculator_IRS' function in R version 4.4.2 (40). The analyses were performed separately for each trial and estimated the statistical power to detect a significant difference in mosquito mortality after 120 h at the 5% level between the ITN-IRS combinations and the corresponding ITN arms alone.

Residual efficacy of the IRS treatments in wall cone bioassays was assessed based on the number of months mosquito mortality was ≥80% as per WHO guidelines (37). The resistance status of the Covè vector population was also interpreted according to WHO criteria (38). A weak dose-response was observed with the different clothianidin concentrations tested in the PBO and clothianidin interaction bioassays, rendering the data unsuitable for traditional probit analysis. Percentage mortality after 168 h with clothianidin alone and clothianidin with pre-exposure to PBO was thus compared using logistic regression. We performed both a pooled analysis comparing mortality between exposure groups with all clothianidin concentrations tested and a stratified analysis comparing mortality between each

concentration. In addition to treatment and dose, testing day was fitted to the model as a fixed effect. Model-derived p-values and odds ratios were used to assess the impact of PBO pre-exposure on clothianidin toxicity. Test mortality in experimental huts and laboratory bioassays was adjusted using Abbot's formula (41) if control mortality was ≥5%. All analyses and data visualisations were performed in Stata version 18.

Results

Experimental hut results

Full experimental hut results, including the total number of mosquitoes collected, blood-feeding and dying in the different hut compartments with each treatment arm, are provided as supplementary material (Table S1).

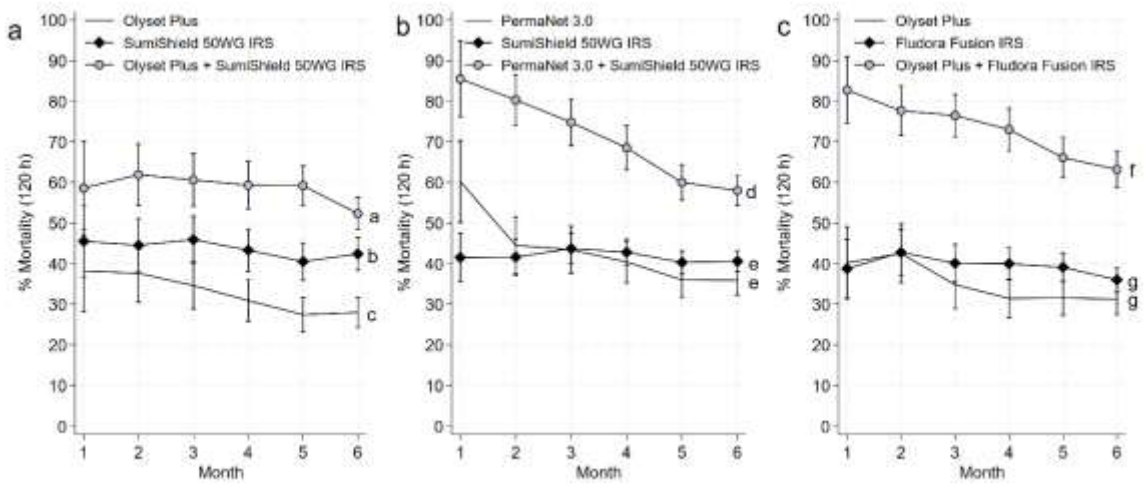
Combining pyrethroid-PBO nets and clothianidin-based IRS improved mortality of pyrethroid-resistant malaria vectors

Moderate mortality of pyrethroid-resistant malaria vectors was observed with the pyrethroid-PBO nets and clothianidin-based IRS alone. Pyrethroid-PBO nets induced the lowest mortality of any insecticidal treatment in each trial (28% and 31% with Olyset® Plus in trials 1 and 3, respectively, and 36% with PermaNet® 3.0 in trial 2) (Table 2). Mortality was slightly higher with clothianidin-based IRS insecticides across all trials; however, this difference was only statistically significant in trial 1 between Olyset® Plus and SumiShield® 50WG IRS (42% vs 28%, p=0.026).

Although pyrethroid-PBO nets and clothianidin-based IRS had moderate killing effects individually, combining these tools significantly improved vector mortality compared to each method alone across all trials (Fig. 1). In trial 1, the combination of Olyset® Plus + SumiShield® 50WG IRS induced significantly higher mortality (52%) than Olyset® Plus

(28%, $p<0.001$) and SumiShield® 50WG IRS (42%, $p=0.003$). Likewise, mortality was superior with PermaNet® 3.0 + SumiShield® 50WG IRS (58%) compared to PermaNet® 3.0 (36%, $p<0.001$) and SumiShield® 50WG IRS (39%, $p=0.005$) in trial 2. Finally, in trial 3, the Olyset® Plus + Fludora® Fusion IRS combination, which achieved the highest mortality of any treatment across the three trials (63%), also outperformed Olyset® Plus (31%, $p<0.001$) and Fludora® Fusion IRS (36%, $p=0.011$). Mortality with the untreated controls was low (<2%) across all trials.

Although overall killing effect values were not formally analysed, indicative patterns were observed across trials. In trials 1 and 2, where the clothianidin-only IRS formulation SumiShield® 50WG was evaluated, the combinations produced higher overall killing effects relative to both the ITN and the IRS alone, suggesting improved mass killing potential of the combinations over either method alone. In contrast, in trial 3, where the clothianidin-deltamethrin mixture IRS Fludora® Fusion was used, the overall killing effect was greater with the combination than the ITN alone but lower than the IRS alone, indicating reduced mass vectorial killing potential compared to the IRS deployed alone. These differences should be interpreted cautiously, however, as the imbalanced rotation design and use of different huts across trials may have influenced mosquito entry and numbers collected, potentially biasing overall killing effect estimates.



391

392 **Fig. 1** Cumulative monthly mortality after 120 h (%) of wild, pyrethroid-resistant *Anopheles*
393 *gambiae* sensu lato entering experimental huts containing pyrethroid-piperonyl butoxide nets
394 and clothianidin-based indoor residual spraying, applied alone and in combination, in Covè,
395 southern Benin. *Panel a shows results from trial 1 with Olyset® Plus and SumiShield®*
396 *50WG; panel b, results from trial 2 with PermaNet® 3.0 and SumiShield® 50WG; and panel*
397 *c, results from trial 3 with Olyset® Plus and Fludora® Fusion. For each trial, treatments*
398 *sharing a superscript letter did not differ significantly at the 5% level (i.e. p>0.05) after 6*
399 *months according to logistic regression analysis. Error bars represent 95% confidence*
400 *intervals.*

401 **Table 2** Overall mortality results (120 h) of wild, pyrethroid-resistant *Anopheles gambiae*
402 sensu lato entering experimental huts containing pyrethroid-piperonyl butoxide nets and
403 clothianidin-based indoor residual spraying, applied alone and in combination, in Covè,
404 southern Benin. **For each trial, values in the same column sharing a superscript letter do*
405 *not differ at the 5% level (i.e. p>0.05) according to regression analysis.*

Experimental hut treatment	*N* collected*	*N* dead 120 h	% Mortality 120 h* [95% CIs]	% Overall killing effect [95% CIs]
Trial 1				
Untreated hut	1607[a]	17	1.1[a] [0.6, 1.6]	–
Untreated net	1327[a]	17	1.3[a] [0.7, 1.9]	0.0 [-0.7, 0.7]

Olyset Plus	583[b]	163	28.0[b] [24.4, 31.6]	9.1 [7.5, 10.7]
SumiShield 50WG IRS	568[b]	241	42.4[c] [38.3, 46.5]	13.9 [11.9, 15.9]
Olyset Plus + SumiShield 50WG IRS	583[b]	305	52.3[d] [48.2, 56.4]	17.9 [15.7, 20.1]
Trial 2				
Untreated hut	1258[c]	21	1.7[e] [1.0, 2.4]	–
Untreated net	1139[c]	21	1.8[e] [1.0, 2.6]	0.0 [-1.0, 1.0]
PermaNet 3.0	713[d]	256	35.9[f] [32.4, 39.4]	18.7 [16.1, 21.3]
SumiShield 50WG IRS	842[d]	331	39.3[f] [36.0, 42.6]	24.6 [21.7, 27.5]
PermaNet 3.0 + SumiShield 50WG IRS	722[d]	419	58.0[g] [54.4, 61.6]	31.6 [28.3, 34.9]
Trial 3				
Untreated hut	1630[e]	23	1.4[h] [0.8, 2.0]	–
Untreated net	1487[e]	23	1.5[h] [0.9, 2.1]	0.0 [-0.8, 0.8]
Olyset Plus	552[fg]	173	31.3[i] [27.4, 35.2]	9.2 [7.5, 10.9]
Fludora Fusion IRS	956[f]	344	36.0[i] [33.0, 39.0]	19.7 [17.4, 22.0]
Olyset Plus + Fludora Fusion IRS	459[g]	290	63.2[j] [58.8, 67.6]	16.4 [14.3, 18.5]

Combining pyrethroid-PBO nets and clothianidin-based IRS enhanced blood-feeding protection compared to IRS alone

Blood-feeding with the IRS-only arms was high (95–98%) and similar to the untreated control hut in all trials (98–99%, $p>0.05$) (Table 3). In contrast, pyrethroid-PBO nets provided high levels of blood-feeding inhibition, which were consistently superior to the IRS-only arms ($p<0.01$), ranging from 66% with PermaNet® 3.0 in trial 2 to 73% with Olyset® Plus in trial 3.

Given the substantial blood-feeding inhibition with the pyrethroid-PBO nets and lack thereof with the IRS, combining these tools predictably enhanced blood-feeding protection compared to the IRS alone (Fig. 2). Indeed, blood-feeding inhibition was significantly higher with the combinations of Olyset® Plus + SumiShield® 50WG IRS and PermaNet® 3.0 + SumiShield® 50WG compared to SumiShield® 50WG IRS in trial 1 (66% vs 1%, $p<0.001$) and trial 2 (64% vs 4%, $p<0.001$), respectively. A similar improvement in blood-feeding

inhibition was observed in trial 3 with Olyset® Plus + Fludora® Fusion IRS relative to Olyset® Plus (84% vs 2%, $p<0.001$).

Blood-feeding with the ITN-IRS combinations was generally similar to the corresponding ITN-only arms, confirming that their personal protective benefit was largely attributable to the ITN. Indeed, blood-feeding inhibition was similar with Olyset® Plus + SumiShield® 50WG IRS compared to Olyset® Plus in trial 1 (66% vs 69%, $p=0.966$), and PermaNet® 3.0 + SumiShield® 50WG IRS compared to PermaNet® 3.0 in trial 2 (64% vs 66%, $p=0.265$). In trial 3, however, a modest but statistically significant improvement in blood-feeding inhibition was detected with the Olyset® Plus + Fludora® Fusion IRS combination relative to Olyset® Plus (84% vs 73%, $p=0.008$), indicating an additional personal protective benefit attributable to the IRS.

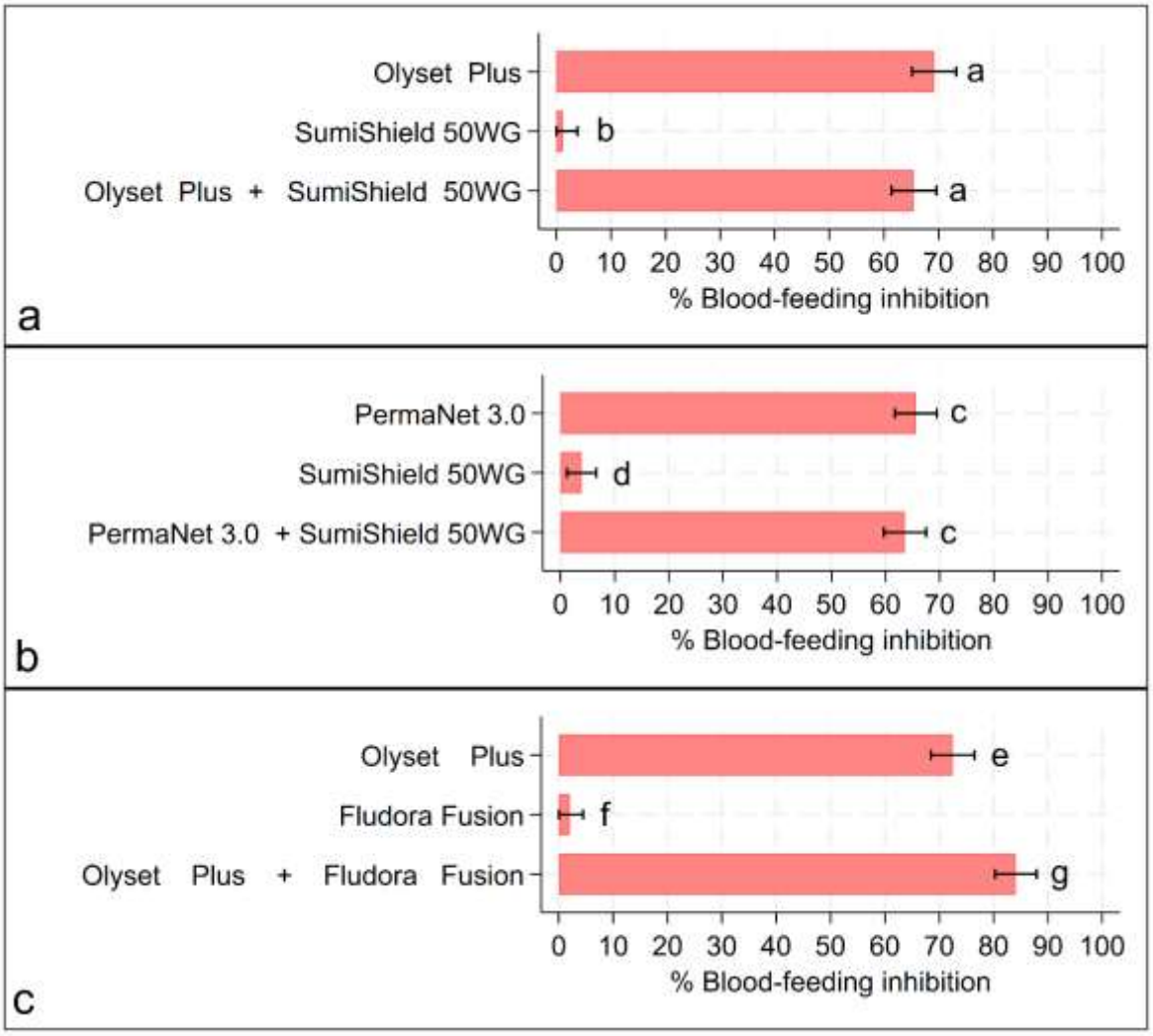


Fig. 2 Blood-feeding inhibition of wild, pyrethroid-resistant *Anopheles gambiae* sensu lato entering experimental huts containing pyrethroid-piperonyl butoxide nets and clothianidin-based indoor residual spraying, applied alone and in combination, in Covè, southern Benin. *Panel a shows results from trial 1 with Olyset® Plus and SumiShield® 50WG; panel b, results from trial 2 with PermaNet® 3.0 and SumiShield® 50WG; and panel c, results from trial 3 with Olyset® Plus and Fludora® Fusion. For each trial, treatments sharing a superscript letter did not differ significantly at the 5% level (i.e. p>0.05) according to logistic regression analysis. Error bars represent 95% confidence intervals.*

Table 3 Blood-feeding results of wild, pyrethroid-resistant *Anopheles gambiae* sensu lato entering experimental huts containing pyrethroid-piperonyl butoxide nets and clothianidin-based indoor residual spraying, applied alone and in combination, in Covè, southern Benin. **For each trial, values in the same column sharing a superscript letter do not differ at the 5% level (i.e. p>0.05) according to regression analysis.*

Experimental hut treatment	*N* collected*	*N* blood-fed	% Blood-feeding* [95% CIs]	% Blood-feeding inhibition* [95% CIs]
Trial 1				
Untreated hut	1607[a]	1586	98.7[a] [98.1, 99.3]	–
Untreated net	1327[a]	1083	81.6[b] [79.5, 83.7]	17.3[a] [14.1, 20.5]
Olyset Plus	583[b]	177	30.4[c] [26.7, 34.1]	69.2[b] [65.1, 73.3]
SumiShield 50WG IRS	568[b]	554	97.5[a] [96.2, 98.8]	1.2[c] [0.0, 3.9]
Olyset Plus + SumiShield 50WG IRS	583[b]	199	34.1[c] [30.3, 37.9]	65.5[b] [61.4, 69.6]
Trial 2				
Untreated hut	1258[c]	1249	99.3[d] [98.8, 99.8]	–
Untreated net	1139[c]	951	83.5[e] [81.3, 85.7]	15.9[d] [12.7, 19.1]
PermaNet 3.0	713[d]	244	34.2[f] [30.7, 37.7]	65.6[e] [61.7, 19.1]
SumiShield 50WG IRS	842[d]	803	95.4[d] [90.4, 96.8]	3.9[f] [1.2, 6.6]
PermaNet 3.0 + SumiShield 50WG IRS	722[d]	261	36.1[f] [32.6, 39.6]	63.6[e] [59.7, 67.5]
Trial 3				
Untreated hut	1630[e]	1614	99.0[g] [98.5, 99.5]	–
Untreated net	1487[e]	1255	84.4[h] [82.6, 86.2]	14.7[g] [11.7, 17.7]
Olyset Plus	552[fg]	150	27.2[i] [23.5, 30.9]	72.5[h] [68.5, 76.5]
Fludora Fusion IRS	956[f]	927	97.0[g] [95.9, 98.1]	2.0[i] [0.0, 4.5]
Olyset Plus + Fludora Fusion IRS	459[g]	72	15.7[j] [12.4, 19.0]	84.1[j] [80.3, 87.9]

Combining pyrethroid-PBO nets and clothianidin IRS had limited impact on mosquito entry and exiting

In all trials, mosquito entry was highest in the untreated control arms. All pyrethroid-PBO nets and clothianidin-based IRS applied alone reduced mosquito entry relative to the controls, indicating a deterrent effect. However, combining pyrethroid-PBO nets and clothianidin-

based IRS generally did not further reduce mosquito entry compared to the corresponding ITN or IRS alone, except the combination of Olyset® Plus + Fludora® Fusion IRS, which demonstrated significantly greater deterrence than Fludora® Fusion IRS alone (n=459 vs n=956, p<0.001). Differences in hut entry should, however, be interpreted with caution, as the imbalanced split-plot design used for treatment rotation across huts makes it difficult to distinguish between treatment and hut-position effects.

When applied alone, the pyrethroid-PBO nets and clothianidin-based IRS induced significantly higher exiting than the untreated controls across all trials, indicating an irritant effect. While the impact of the ITN-IRS combinations on exiting relative to the corresponding ITNs and IRS alone was broadly consistent across the trials, the absolute differences between study arms were small and varied in terms of their statistical significance (Table 4). Exiting was slightly lower with the ITN-IRS combinations compared to the IRS alone across all trials, although this effect was only significant in trial 1 between Olyset Plus + SumiShield® 50WG and SumiShield® 50WG IRS (89% vs 96%, p=0.001). Conversely, the combinations induced higher exiting than the ITN-only arms, although the effects were modest and statistical significance was not observed in trial 2 with PermaNet® 3.0 + SumiShield® 50WG IRS relative to PermaNet® 3.0 (88% vs 77%, p=0.137).

Mosquito sample sizes and power analysis results

A total of 14,426 female *An. gambiae* s.l. were collected across the three experimental hut trials, comprising 4,688 in trial 1, 4,674 in trial 2 and 5,084 in trial 3 (Table 4). Mosquito densities were thus broadly consistent across the three trials. These large sample sizes, combined with the large effect size observed with the ITN-IRS combinations, provided high levels of statistical power. The estimated power to detect a significant difference in mosquito mortality between the ITN-IRS combination and ITN-only arms was: 94.3% [91.1, 96.7] in

trial 1, 89.7% [85.7, 92.9] in trial 2 and 93.0% in trial 3 [89.5, 95.6]. This reinforces confidence in the robustness of the results, highlighting the superior impact of the ITN-IRS combinations on mosquito mortality.

Table 4 Entry and exiting results of wild, pyrethroid-resistant *Anopheles gambiae* sensu lato entering experimental huts containing pyrethroid-piperonyl butoxide nets and clothianidin-based indoor residual spraying, applied alone and in combination, in Covè, southern Benin. **For each trial, values in the same column sharing a superscript letter do not differ at the 5% level (i.e. p>0.05) according to regression analysis.*

Experimental hut treatment	*N* collected*	% Deterrence [95% CIs]	*N* exiting	% Exophily [95% CIs]*
Trial 1				
Untreated hut	1607[a]	–	757	47.1[a] [44.7, 49.5]
Untreated net	1327[a]	17.4 [10.8, 24.0]	571	43.0[a] [40.3, 45.7]
Olyset Plus	583[b]	63.7 [58.0, 69.4]	474	81.3[b] [78.1, 84.5]
SumiShield 50WG IRS	568[b]	64.7 [59.0, 70.4]	539	94.9[c] [93.1, 96.7]
Olyset Plus + SumiShield 50WG IRS	583[b]	63.7 [58.0, 69.4]	534	91.6[c] [89.3, 93.9]
Trial 2				
Untreated hut	1258[c]	–	567	45.1[d] [42.4, 47.8]
Untreated net	1139[c]	9.5 [1.9, 17.1]	490	43.0[d] [40.1, 45.9]
PermaNet 3.0	713[d]	43.3 [36.4, 50.2]	547	76.7[e] [73.6, 79.8]
SumiShield 50WG IRS	842[d]	33.1 [26.0, 40.2]	776	92.2[e] [90.4, 94.0]
PermaNet 3.0 + SumiShield 50WG IRS	722[d]	42.6 [35.7, 49.5]	635	88.0[e] [85.6, 90.4]
Trial 3				
Untreated hut	1630[e]	–	743	45.6[f] [43.2, 48.0]
Untreated net	1487[e]	8.8 [2.1, 15.5]	684	46.0[f] [43.5, 48.5]
Olyset Plus	552[fg]	66.1 [60.5, 71.7]	429	77.7[g] [74.2, 81.2]
Fludora Fusion IRS	956[f]	41.3 [35.2, 47.4]	916	95.8[h] [94.5, 97.1]
Olyset Plus + Fludora Fusion IRS	459[g]	71.8 [66.3, 77.3]	407	88.7[g] [85.8, 91.6]

Susceptibility bioassay results:

Covè vector population exhibits high-intensity pyrethroid resistance but susceptibility to clothianidin

Mortality of wild *An. gambiae* s.l. collected near the experimental hut site was very low following exposure to the discriminating concentrations of deltamethrin (5%) and permethrin (3%), confirming the high frequency of pyrethroid resistance in the Covè vector population (Fig. 3). Mortality increased progressively with 5x and 10x the discriminating doses of deltamethrin (5x: 34%, 10x: 67%) and permethrin (5x: 45%, 10x: 67%) but failed to surpass the WHO susceptibility threshold (≥98%) indicating high-intensity pyrethroid resistance. PBO pre-exposure improved the mortality response to the discriminating concentration of deltamethrin (from 5% to 63%) but not permethrin (from 3% to 5%), suggesting that P450s may play a more important role in resistance to type II pyrethroids (deltamethrin, alpha-cypermethrin) than type I (permethrin). In contrast, the discriminating concentration of clothianidin induced 100% mortality, providing evidence that the vector population remained susceptible to neonicotinoids. Mortality was negligible (≤4%) in the controls. Full summary tube and bottle bioassay results are provided as supplementary material (Table S2).

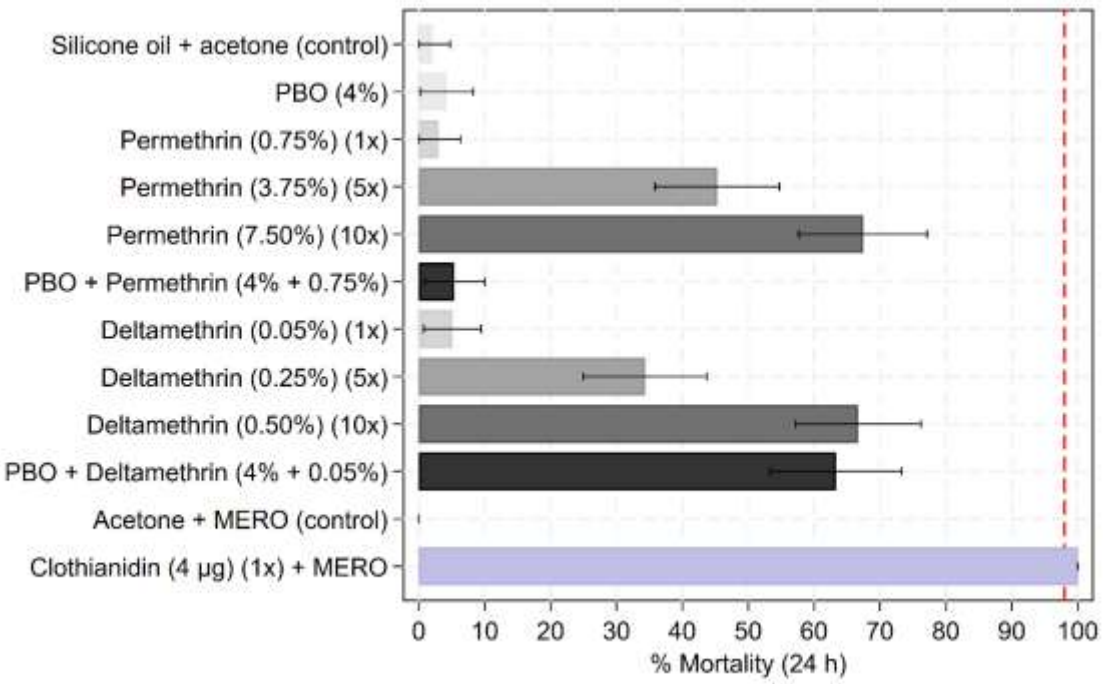


Fig. 3 Mortality after 24 h (%) of adult *Anopheles gambiae* sensu lato collected as larvae from breeding sites near experimental huts in Covè following exposure to standard discriminating concentrations of pyrethroids and clothianidin in World Health Organisation tube tests and bottle bioassays. *The red dashed line represents the 98% mortality threshold used to assess susceptibility and resistance intensity. Error bars represent 95% confidence intervals.*

Wall cone bioassay results

SumiShield® 50WG and Fludora® Fusion IRS exhibited strong residual performance

SumiShield® 50WG and Fludora® Fusion induced 100% cone bioassay mortality 1 week after spraying, confirming the quality of the spray application. Mortality remained very high (≥98%) throughout the trial with both products, demonstrating the strong residual performance of these next-generation IRS formulations (Fig. 4). Mortality of mosquitoes exposed to untreated control walls was very low (~2%), thus ruling out contamination as a

source of bias and validating the test. Full summary wall cone bioassay results are provided as supplementary material (Table S3).

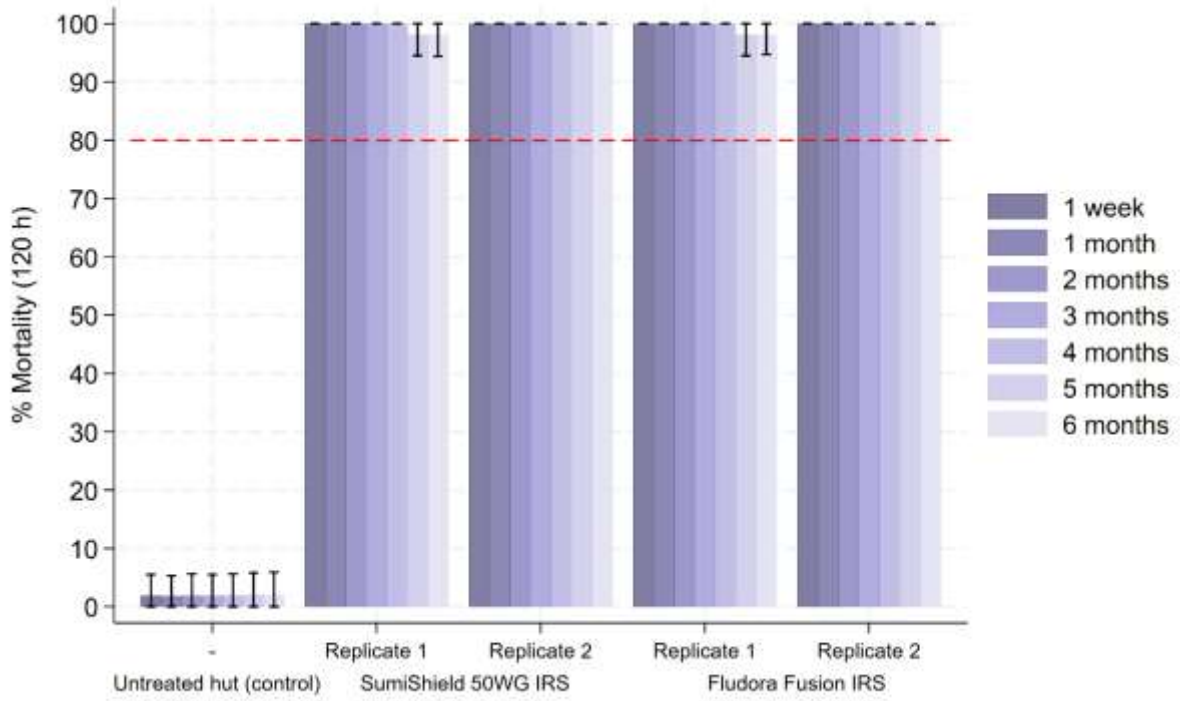


Fig. 4 Mortality after 120 h (%) of adult *Anopheles gambiae* sensu lato collected as larvae from breeding sites near experimental huts in Covè following exposure to hut walls in residual efficacy wall cone bioassays. *Bioassays were performed initially 1 week after spraying to assess spray quality and at monthly intervals thereafter to assess residual efficacy. The red dashed line represents the 80% mortality threshold recommended by the World Health Organisation to assess residual efficacy. Error bars represent 95% confidence intervals.*

PBO and clothianidin interaction bioassay results

PBO pre-exposure enhanced clothianidin toxicity

Mortality after 168 h pooled for all clothianidin concentrations tested was higher with PBO followed by clothianidin than clothianidin alone (43.0% vs 58.5%, p<0.001, OR=1.71 [95%

CIs: 1.43–2.05]). There was, however, variation in the mortality responses observed between tubes (Fig. 5), and the stratified analysis comparing mortality at each concentration showed that the synergistic effect of PBO was less pronounced at higher concentrations of clothianidin. PBO pre-exposure caused significant increases in mortality at concentrations of 0.025% (39.2% vs 51.7%, p=0.038, OR=1.53 [1.02–2.28]), 0.05% (31.3% vs 59.4, p<0.001, OR=2.62 [1.33–2.97]) and 0.125% (32.2% vs 53.5%, p<0.001, OR=2.06 [1.37–3.07]) while statistical significance was not observed at the 0.25% (45.5% vs 57.0%, p=0.055, OR=1.48 [0.99–2.22]) and 0.5% concentrations (66.7% vs 70.6%, p=0.458, OR=1.19 [0.76–1.86]). Mortality with filter papers treated with distilled water and PBO alone were 17% and 15%, respectively. Full summary PBO and clothianidin interaction bioassay results are provided as supplementary material (Table S4).

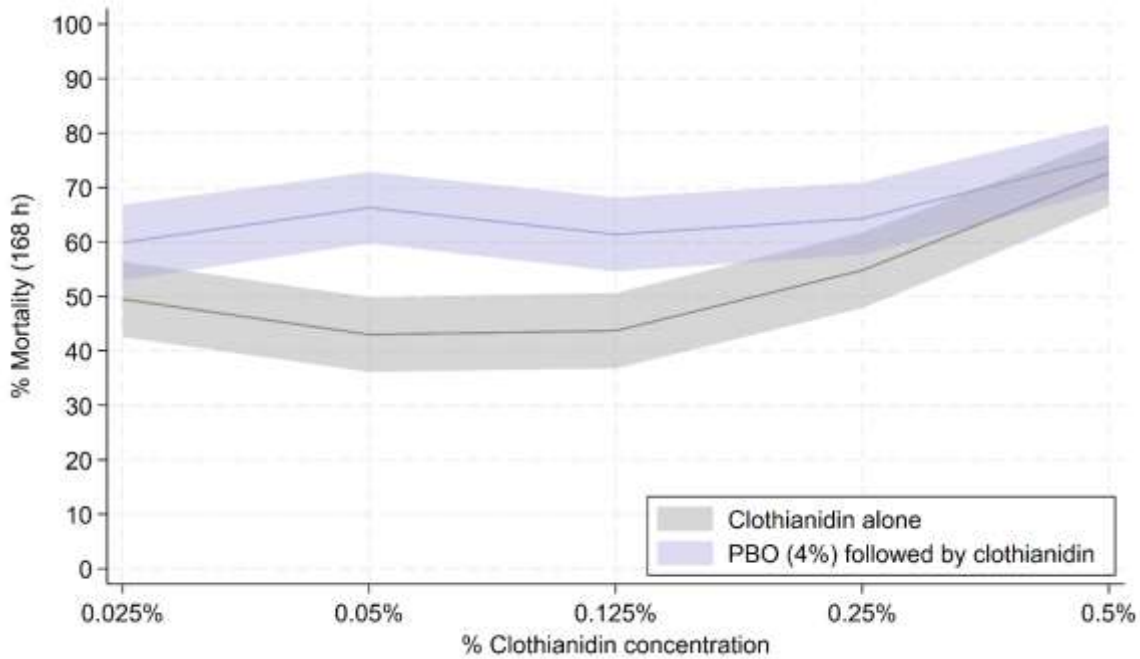


Fig. 5 Mortality after 168 h (%) of adult *Anopheles gambiae* sensu lato collected as larvae from breeding sites near experimental huts in Covè following exposure to filter papers impregnated with clothianidin formulation (SumiShield® 50WG) alone and with pre-

exposure to the cytochrome P450 monooxygenase inhibitor, piperonyl butoxide, in World Health Organisation tube tests. *Shaded areas represent 95% confidence intervals.*

Discussion

Where resources permit, ITNs and IRS can be co-deployed in the same areas to achieve greater malaria control than is possible with either method alone. However, a previous trial has shown reduced efficacy of pirimiphos-methyl IRS when combined with pyrethroid-PBO nets, likely due to PBO-mediated inhibition of P450s responsible for the bioactivation of this pro-insecticide (23). Clothianidin does not rely on activation by P450s and may instead be detoxified by these enzymes (25-27), reducing the risk of antagonism with pyrethroid-PBO nets. Pyrethroid-PBO nets and clothianidin-based IRS may therefore represent complementary tools for combined deployment, with greater potential for improved malaria control. Our findings support this hypothesis, showing that combining pyrethroid-PBO nets and clothianidin-based IRS in experimental huts produced greater entomological impact against pyrethroid-resistant vectors than either method alone.

Pyrethroid-PBO nets applied alone provided high levels of blood-feeding inhibition (66–73%), but modest vector mortality (28–36%). These findings are broadly consistent with experimental hut evaluations of pyrethroid-PBO nets in West Africa, where these nets provide strong blood-feeding protection but moderate killing effects, often only marginally superior to pyrethroid-only nets (42-46). Pyrethroid-PBO nets are designed to improve vector control by exploiting the inhibitory action of PBO against P450s, whose overexpression constitutes a major pyrethroid resistance mechanism. By inhibiting this pathway, PBO typically helps restore pyrethroid efficacy against resistant vectors. However, in some settings – more commonly in West Africa – sustained selection pressure has selected for high-intensity, multifactorial resistance which renders PBO less effective in restoring pyrethroid potency.

This is reflected in studies across the region showing a loss in PBO synergy in susceptibility bioassays (23, 47-50), corroborated in the present trial. The low mortality observed with pyrethroid-PBO nets in this trial thus reflects high-intensity resistance which attenuates the toxicity of the synergised pyrethroid. These findings reaffirm the limitations of pyrethroid-PBO nets in such settings and underscore the need both for stronger evidence of their epidemiological efficacy in West Africa (51) and for other interventions to complement or replace these nets in settings where their efficacy is compromised by resistance.

Clothianidin-based IRS applied alone did not significantly inhibit blood-feeding and induced 36–42% vector mortality over 6 months. The low blood-feeding inhibition is expected, as IRS lacks a physical barrier and acts primarily by killing mosquitoes resting on treated surfaces after blood-feeding. However, mortality was lower than reported with clothianidin-based IRS in earlier hut trials conducted in Benin (70–92%) and other sites (9, 52, 53). Suboptimal spray quality is an unlikely explanation for this reduced performance given the high mosquito mortality (≥98%) in wall cone bioassays and the fact that all post-spray tank volumes were within 10% of target values. Nevertheless, the absence of filter paper analysis, owing to funding constraints, means this cannot be ruled out. Reduced vector susceptibility is an alternative explanation, given that several studies have reported decreased neonicotinoid susceptibility associated with elevated P450 activity at multiple sites (25, 26, 54, 55), including in Benin (56). However, although some P450s are overexpressed in the Covè vector population (31, 57) and PBO pre-exposure modestly synergised clothianidin in interaction bioassays, bottle bioassays indicated that the vector population remained largely susceptible to clothianidin. The evidence is thus insufficient to attribute the lower-than-expected mortality with clothianidin-based IRS to reduced insecticide susceptibility, and the reasons for the suboptimal efficacy remain unclear. Further research is needed to understand the factors underlying the variable performance of clothianidin-based IRS.

The combinations of pyrethroid-PBO nets and clothianidin-based IRS induced higher vector mortality than both the ITNs and IRS alone. The additional killing effect over ITNs alone is consistent with previous evidence demonstrating the potential for improved vector control when combining ITNs with a non-pyrethroid IRS insecticide with an unrelated mode of action. Experimental hut trials have consistently shown that adding these IRS insecticides onto pyrethroid-only or pyrethroid-PBO nets substantially increases mortality relative to the nets alone (23, 58-63). This is primarily because high levels of pyrethroid resistance in the local vector populations had compromised the efficacy of the nets in these studies, while vector populations remained largely susceptible to the IRS insecticides. The addition of IRS to which vectors remained susceptible therefore compensates for the reduced efficacy of the net by killing mosquitoes which survived exposure to the net. Mortality was consequently restored to levels unachievable with the nets alone, as observed in the present trial. Importantly, several cRCTs have further demonstrated that co-deploying pyrethroid-only nets and non-pyrethroid IRS reduces malaria prevalence compared to nets alone (64), suggesting that the additional mortality observed here could translate into meaningful epidemiological impact when clothianidin-based IRS is deployed to complement pyrethroid-PBO nets under operational conditions.

While additional mortality over ITNs alone was expected based on previous studies, the improved killing effect compared to the IRS alone is noteworthy given findings from a recent hut trial conducted at the same site showing reduced mortality when pirimiphos-methyl IRS was combined with pyrethroid-PBO nets compared to the IRS alone (23). This effect may be attributable to the fact that pirimiphos-methyl requires metabolic activation by P450 enzymes within the mosquito to become fully toxic, whereas PBO inhibits these enzymes. Prior uptake of PBO in mosquitoes contacting the net may therefore have blocked this activation process, reducing the killing effect of pirimiphos-methyl IRS. Unlike pirimiphos-methyl, clothianidin

does not rely on P450-mediated bioactivation, and is therefore not subject to the same antagonistic interaction with PBO. The improved mortality observed here may consequently reflect an additive effect of deploying two tools with distinct and non-interfering modes of action. However, recent studies suggest that P450s may also contribute to clothianidin detoxification, meaning that inhibition of these enzymes by PBO could reduce its metabolic breakdown, creating the potential for a synergistic interaction. This is supported by previous laboratory studies (25-27) and the interaction bioassays presented here which showed potentiation of clothianidin toxicity following PBO pre-exposure. It is thus plausible that mosquitoes encountering the pyrethroid-PBO net before resting on a sprayed wall were rendered more susceptible to clothianidin, augmenting the killing effect of the IRS. Nevertheless, the potentiating effect of PBO on clothianidin in the interaction bioassays was modest and dose-dependent and the relative contribution of simple additivity versus biochemical synergism cannot be disentangled from the present data. Regardless of the mechanism through which the combinations improved mortality, the findings reaffirm the importance of considering the biochemical characteristics of, and potential interactions between, the compounds deployed through ITNs and IRS when selecting suitable combinations of these tools for operational use. Overall, the absence of antagonism and potential for synergism between these tools suggests that pyrethroid-PBO nets and clothianidin-based IRS are complementary vector control tools whose combined deployment has the potential to provide greater vector control than either method alone. However, given the additional costs of the combined strategy, further studies monitoring the impact on clinical malaria in areas where pyrethroid-PBO nets and clothianidin-based IRS have been co-deployed would be valuable to support these findings and better inform programmatic decision-making.

The improvement in blood-feeding inhibition with the combinations over IRS alone, alongside the broadly similar protection to ITNs alone is consistent with previous studies and reflects the contrasting modes of action of these tools (59-63, 65-67). ITNs provide a barrier that prevents blood-feeding while IRS lacks this barrier and acts primarily by killing mosquitoes after blood-feeding. Consequently, adding an ITN to IRS reduces blood-feeding, while adding IRS to ITNs generally offers limited additional blood-feeding protection. This pattern was observed in trials 1 and 2, using clothianidin-only IRS. In trial 3, however, where clothianidin-deltamethrin mixture IRS was used, the combination provided a modest but statistically significant improvement in blood-feeding protection over ITNs alone. Most hut trials report limited additional blood-feeding protection with ITN-IRS combinations over ITNs alone (59, 62, 63, 65-67), although marginal improvements have been observed when IRS is conducted with fast-acting, excitorepellent insecticides which repel or kill mosquitoes before they can blood-feed (58, 60). Unlike clothianidin, which exhibits delayed action and lacks excitorepellent properties, deltamethrin is fast-acting and induces marked irritancy responses including increased take-offs on treated blocks (68) and exiting in experimental huts (8, 69). The improved blood-feeding protection over ITNs alone in trial 3 is thus attributable to the irritant properties of the deltamethrin component, suggesting that clothianidin-deltamethrin mixtures may offer greater personal protection when combined with pyrethroid-PBO nets. However, any such benefit will likely be modest and could be offset if shorter mosquito contact with treated surfaces results in reduced insecticidal killing as evidenced by the lower overall killing effect with the combination over the IRS alone in trial 3.

The vector control landscape has shifted significantly in recent years, with several new product types becoming available for operational use. Most notably, pyrethroid-chlorfenapyr nets have emerged as the new standard-of-care for malaria vector control, accounting for

more than 70% of all ITN shipments to sub-Saharan Africa (13). In parallel, IRS products containing new active ingredients – the meta-diamide broflanilide (70) and the isoxazoline isocycloseram (71) – have become available, further expanding the vector control toolbox and improving prospects for rotational insecticide use. Against this backdrop, a more pressing operational question is how pyrethroid-chlorfenapyr nets interact with different IRS classes, and whether such combinations offer epidemiological and resistance management benefits beyond that achievable with nets alone. Furthermore, given increasing evidence for the suboptimal durability of next-generation ITNs (43, 72-75), studies assessing the impact of deploying IRS to complement nets in the second or third year post-distribution – when net usage and bioefficacy declines – are also warranted.

Conclusions

In a setting where pyrethroid-PBO nets and clothianidin-based IRS alone each showed moderate entomological efficacy, our findings indicate that combining these interventions has the potential to provide greater vector control impact. The combinations induced higher vector mortality than pyrethroid-PBO nets alone, while also providing greater mortality and blood-feeding protection compared to clothianidin-based IRS alone. The additional mortality observed with the combinations likely reflects an additive effect arising from the concurrent use of two insecticidal tools with distinct and non-interfering modes of action. However, biochemical synergism between PBO and clothianidin, as evidenced in the interaction bioassays, may have also contributed. These findings indicate that pyrethroid-PBO nets and clothianidin-based IRS are complementary tools whose combined deployment may represent an effective strategy for improving malaria control. Further studies monitoring the impact on clinical malaria in areas where pyrethroid-PBO nets and clothianidin-based IRS have been co-deployed would be valuable to support these findings and better inform decision-making.

List of abbreviations

cRCT – Cluster-randomised controlled trial

IRS – Indoor residual spraying

ITN – Insecticide-treated net

PBO – Piperonyl butoxide

P450 – Cytochrome P450 monooxygenase

WHO – World Health Organisation

Declarations

Ethical approval and consent to participate

Approval for conduct of the experimental hut trials was granted by the Research Ethics Committees of the Benin Ministry of Health (Ref: n° 21, 20/03/2023) and London School of Hygiene & Tropical Medicine (Ref: 26429). Written informed consent was obtained from all volunteers sleepers. Chemoprophylaxis was offered to mitigate the risk of malaria infection, and a stand-by nurse was available to provide free medical assistance to those presenting with febrile symptoms or other adverse events. Approval for using guinea pigs for blood-feeding of mosquito colonies was granted by the Animal Welfare Ethics Review Board (AWERB) of the London School of Hygiene & Tropical Medicine (2020-01B).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TS supervised the supervised experiments, analysed the data and prepared the manuscript. JN performed the experimental hut trials with support from TS and CN. BN and TS performed the laboratory bioassays. CN acquired funding, supervised the project and provided feedback on the final manuscript. All authors reviewed the manuscript.

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