

VECTOR CONTROL

Efficacy of the Olyset Duo net against insecticide-resistant mosquito vectors of malaria

Corine Ngufor,^{1,2,3*} Raphael N'Guessan,^{1,2,3} Josias Fagbohoun,² Damien Todjinou,² Abibath Odjo,² David Malone,⁴ Hanafy Ismail,⁵ Martin Akogbeto,² Mark Rowland^{1,3}

Olyset Duo is a new long-lasting insecticidal net treated with permethrin (a pyrethroid) and pyriproxyfen, an insect growth regulator that disrupts the maturation of oocytes in mosquitoes exposed to the net. We tested the Olyset Duo net against pyrethroid-resistant *Anopheles gambiae* mosquitoes, which transmit malaria parasites, in laboratory bioassays and in a trial in Benin using experimental huts that closely resemble local habitations. Host-seeking mosquitoes that entered to feed were free to contact the occupied nets and were collected the next morning from exit traps. Surviving blood-fed mosquitoes were observed for effects on reproduction. Control nets were treated with pyrethroid only or pyriproxyfen only, and nets were tested unwashed and after 20 standardized washes. The Olyset Duo net showed improved efficacy and wash resistance relative to the pyrethroid-treated net in terms of mosquito mortality and prevention of blood feeding. The production of offspring among surviving blood-fed *A. gambiae* in the hut trial was reduced by the pyriproxyfen-treated net and the Olyset Duo net both before washing (90 and 71% reduction, respectively) and after washing (38 and 43% reduction, respectively). The degree of reproductive suppression in the hut trial was predicted by laboratory tunnel tests but not by cone bioassays. The overall reduction in reproductive rate of *A. gambiae* with the Olyset Duo net in the trial was 94% with no washing and 78% after 20 washes. The Olyset Duo net has the potential to provide community control of mosquito populations and reduce malaria transmission in areas of high insecticide resistance.

INTRODUCTION

Long-lasting insecticidal mosquito nets remain the most popular and widely used public health intervention for preventing malaria because they provide effective protection for individual users and are simple to deliver, even in remote communities (1). Pyrethroids have been the insecticide of choice for treating mosquito nets owing to their safety, effectiveness, rapid action, and capacity to repel mosquitoes (2). Unfortunately, the development and rapid spread of resistance to pyrethroids in mosquito vectors of the malaria parasite threaten to undermine the effectiveness of insecticidal mosquito nets (3, 4). In areas of high resistance, pyrethroid-treated nets have shown reduced effectiveness in several small-scale trials (4, 5), and this is driving the search for new insecticides that can maintain the utility of this once-effective means of malaria prevention (1, 4).

Research and development of alternative insecticides to pyrethroids for treating mosquito nets has been challenging because most nonpyrethroids tested lack the important property of excitorepellency, the characteristic responsible for stimulating mosquito movement away from the pyrethroid-treated surface and preventing blood feeding. To retain this attribute, the mosquito nets can be treated with a mixture of pyrethroid and nonpyrethroid insecticides, which maintains the personal protective effect mediated by the pyrethroid component while inducing mortality through the companion insecticide. This combined action should enable long-lasting insecticidal nets to kill

mosquitoes and maintain community protection when deployed on a large scale against mosquito populations that are resistant to pyrethroids (4). Combining mixtures of insecticides on mosquito nets has the potential to delay the selection of pyrethroid resistance in areas where resistance is still rare (6, 7).

Olyset Duo is a new type of mosquito net that is treated with a mixture of permethrin (a pyrethroid) and pyriproxyfen. Pyriproxyfen belongs to a category of insecticides known as insect growth regulators, which prevent mosquito larvae from molting and adult insects from producing fertile eggs (8–11). A preliminary assessment of the unwashed Olyset Duo net against pyrethroid-resistant *Anopheles gambiae* in southern Benin demonstrated the potential of this mixture-treated, long-lasting insecticidal mosquito net to kill adult mosquitoes and sterilize survivors (12). A sterile survivor makes no contribution to the next generation, and mosquito populations should decrease over time through the collective sterilizing action of pyriproxyfen on nets.

To ensure that new brands of mosquito nets are fit for malaria control, the World Health Organization (WHO), through the pesticide evaluation scheme (WHOPES), has set criteria that a new long-lasting insecticidal net must fulfill to attain recommendation for public health use. The WHO guidelines stipulate that a long-lasting insecticidal mosquito net must retain biological effectiveness after 20 standardized washes in defined laboratory bioassay tests and in experimental hut trials in malaria-endemic regions (13). The WHO Vector Control Action Group, which advises WHO on new vector control tools, goes one step further and stipulates that for use against pyrethroid-resistant vector populations, the mixture-treated, long-lasting insecticidal mosquito net must induce mosquito mortality or provide personal protection to a level significantly greater than a net treated with pyrethroid only (14).

¹London School of Hygiene and Tropical Medicine, London WC1E 7HT, U.K. ²Centre de Recherches Entomologiques de Cotonou, Ministry of Health, Cotonou 06 BP 2604, Benin. ³Pan African Malaria Vector Research Consortium, London WC1E 7HT, U.K. ⁴Innovative Vector Control Consortium, Liverpool L3 5QA, U.K. ⁵Liverpool School of Tropical Medicine, Liverpool L3 5QA, U.K.

*Corresponding author. Email: corine.ngufor@lshtm.ac.uk

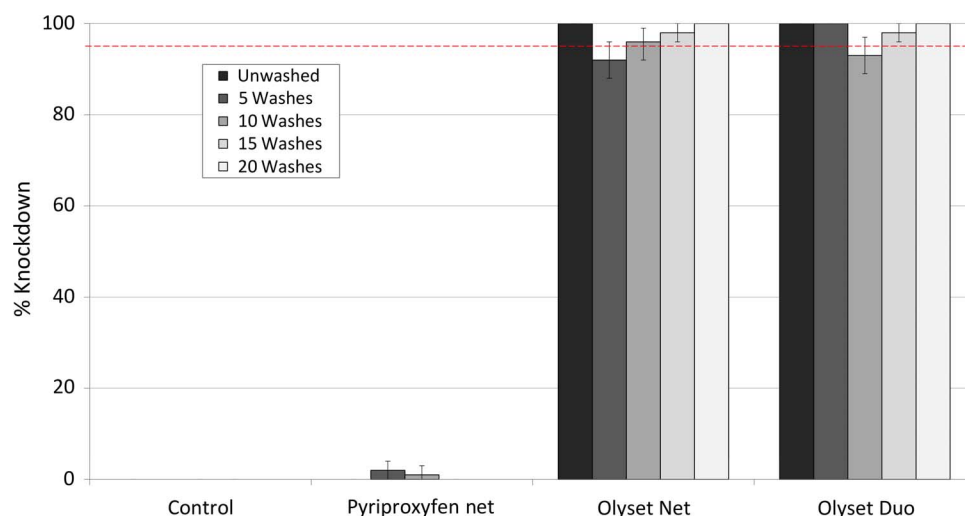


Fig. 1. Knockdown of susceptible mosquitoes of the *A. gambiae* Kisumu strain in cone bioassays. One hundred fifty unfed 3- to 5-day-old female mosquitoes of the susceptible *A. gambiae* Kisumu strain were tested in cone bioassays with each bed net and for each wash point for 3 min in replicates of five mosquitoes per cone. Knockdown is shown for pyriproxyfen-treated net, Olyset Net, and Olyset Duo net as percentage. A total of four replicate net samples were tested for each wash point (50 mosquitoes per replicate). Knockdown was recorded after 1 hour. Each bar represents percentage of knockdown at each wash point (0, 5, 10, 15, and 20 washes) for the different bed net types tested. The red dotted line indicates WHO cutoff for knockdown (95%) after 20 washes in cone bioassays. Error bars represent 95% confidence intervals (CI). Knockdown with Olyset Duo was >95% after 20 washes, demonstrating that Olyset Duo meets WHO criteria for efficacy in cone bioassays.

Here, we report on a phase 1 laboratory study and a phase 2 experimental hut study of the mixture-treated, long-lasting insecticidal mosquito net (Olyset Duo) conducted to meet WHO guidelines. The phase 2 study was performed at the collaborative trial site of the London School of Hygiene and Tropical Medicine and Centre de Recherches Entomologiques de Cotonou (CREC) in Benin. The Olyset Duo net was trialed against a standard WHO-recommended, long-lasting insecticidal mosquito net treated with pyrethroid alone (Olyset Net) or pyriproxyfen alone.

RESULTS

Phase 1 laboratory testing of the biological efficacy of mosquito nets after standardized washing

Olyset Duo fulfills WHO criteria for efficacy in cone bioassays.

We recorded the proportions of female mosquitoes of the pyrethroid-susceptible *A. gambiae* strain that were knocked to the floor after 1 hour or killed after 24 hours after a 3-min exposure to pieces of long-lasting insecticidal netting in WHO cone bioassays (Figs. 1 and 2). Netting was unwashed or washed 5, 10, 15, or 20 times before testing. To meet WHOPES efficacy criteria, a long-lasting net tested in cone bioassays should achieve >95% knockdown or >80% mortality after 20 washes (13). Knockdown rates after 1 hour were 0% with the untreated control and <3% with the pyriproxyfen-treated net. Knockdown with the nets containing permethrin (Olyset Net and Olyset Duo) was >90% for all of the wash points tested. No mortality was recorded with the control net after 24 hours, and mortality with the pyriproxyfen-treated net did not exceed 3% at any of the wash points tested. The unwashed Olyset Net and Olyset Duo both killed 100% of exposed female mosquitoes. Mortality with Olyset Net decreased to 70% after 5 washes and remained at that level after 20 washes. With Olyset Duo, mortality was more variable,

recording 58% at 10 washes but >80% at 20 washes. On the basis of the knockdown and mortality rates achieved with Olyset Duo after 20 washes (100 and 85%, respectively), the net fulfills the WHOPES cone test efficacy criteria (13).

The effect of the insecticide treatments on reproduction is based on the proportion of blood-fed females that survive to oviposit (lay egg batches), their fecundity (the number of eggs laid), and fertility (the proportion of eggs that hatch into larvae). None of the female mosquitoes that remained alive after exposure to the unwashed pyriproxyfen-treated net laid any eggs; hence, they were completely sterilized and their fecundity, fertility, and offspring were reduced by 100% (Tables 1 and 2). It was not possible to assess the reproductive effects with the unwashed Olyset Net and Olyset Duo netting pieces because no mosquitoes survived exposure to these nets. After five washes, there was no discernable reduction in oviposition rate, mean fecundity, or mean offspring of female

mosquitoes exposed to any of the net types in the cone bioassays relative to untreated control mosquito nets.

Olyset Duo outperforms Olyset Net in WHO tunnel tests. The tunnel test is a bioassay system comprising an animal bait positioned behind insecticide-treated netting that allows free expression of the behavioral interactions that occur between free-flying mosquitoes and treated nets during host-seeking behavior. Efficacy is measured in terms of the capacity of a treated net to kill or prevent mosquitoes from feeding on the animal bait. Tunnel tests were performed with pieces of netting of each type washed 0, 10, and 20 times using the pyrethroid-resistant *A. gambiae* VKPer laboratory strain that contains the *L1014F* knockdown resistance gene. The mortality and blood-feeding inhibition rates in the tunnel tests are presented in Figs. 3 and 4, respectively. WHOPES efficacy criteria for long-lasting insecticidal mosquito netting in the tunnel test are >80% mortality or >90% blood-feeding inhibition after 20 washes (13). Mortality with the pyriproxyfen-treated net was 30% when the net was unwashed and decreased further after 10 and 20 washes (Fig. 3). Mortality was higher with unwashed Olyset Duo (100%) than with unwashed Olyset Net (84%), and this trend was maintained after both 10 and 20 washes (Fig. 3). After 20 washes, mortality with Olyset Duo remained >80% but with Olyset Net mortality decreased to 65%. Mortality with the untreated control net was 9%.

Blood-feeding inhibition with Olyset Duo was 100% when Olyset Duo was unwashed and 94% after 20 washes (Fig. 4). Blood-feeding inhibition was significantly higher with Olyset Duo than with Olyset Net at all wash intervals ($P < 0.05$). The mortality and blood-feeding inhibition rates achieved in the tunnel test with Olyset Duo after 20 washes (83 and 94%, respectively) demonstrate that the net meets WHOPES criteria for efficacy in tunnel tests (13).

The reproductive effects on surviving blood-fed *A. gambiae* VKPer female mosquitoes are presented in Tables 1 and 2. There were no survivors

collected from tunnels containing unwashed Olyset Duo netting. The two survivors collected from tunnels with unwashed Olyset Net were too few for analysis. Only 6% of female mosquitoes exposed to unwashed pyriproxyfen-treated netting oviposited eggs, with each female laying an average of three eggs, resulting in a 99% reduction in the number of offspring relative to the untreated control netting (Table 2). The percentage of offspring decreased from 99 to ~40% reduction with pyriproxyfen-treated netting washed 10 or 20 times. A similar trend after washing was observed with Olyset Duo. As expected with a pyrethroid-only-treated net, there was little or no suppression of reproductive outcomes with Olyset Net washed 10 or 20 times.

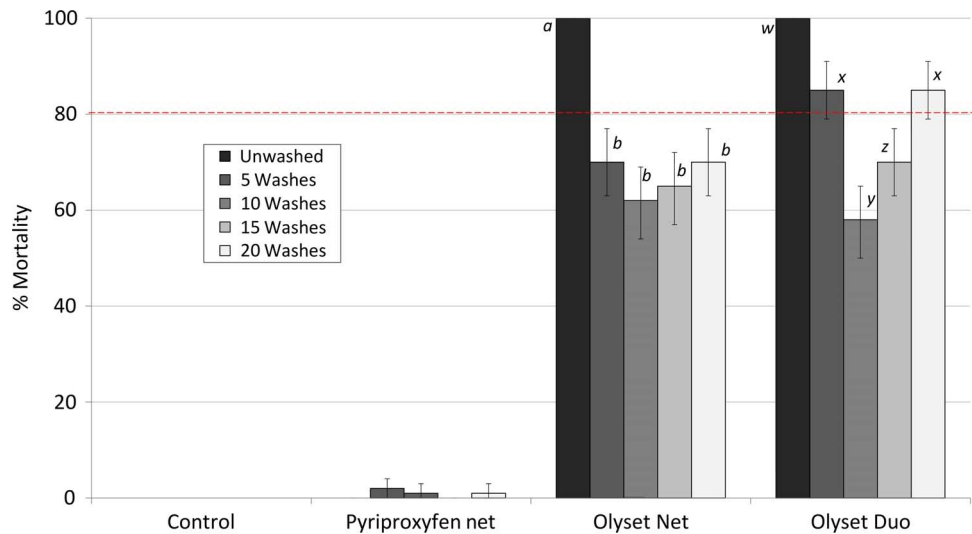


Fig. 2. Mortality of susceptible mosquitoes of the *A. gambiae* Kisumu strain in cone bioassays. One hundred fifty unfed 3- to 5-day-old susceptible *A. gambiae* Kisumu female mosquitoes were tested for 3 min per cone for each type of bed net (pyriproxyfen-treated net, Olyset Net, and Olyset Duo) at each wash point in replicates of five mosquitoes. A total of four replicate net samples were tested at each wash point (~40 mosquitoes per replicate). The number of dead mosquitoes was recorded after a 24-hour holding period. Each bar represents percent mortality at each wash point (0, 5, 10, 15, and 20 washes) for the different bed net types tested. For each net type, bars bearing the same letter label are not significantly different at the 5% level ($P > 0.05$) (generalized linear mixed models). Error bars represent 95% CIs. The red dotted line indicates WHO cutoff for mortality (80%) in cone bioassays after 20 washes. Mortality with Olyset Duo was >80% after 20 washes, showing that the net meets WHO criteria for efficacy in cone bioassays.

Phase 2 testing of the biological efficacy of mosquito nets under simulated household conditions

We assessed the efficacy and wash resistance of each type of long-lasting insecticidal net (pyriproxyfen-treated net, Olyset Net, and Olyset Duo) under carefully controlled household conditions. The nets (unwashed and washed 20 times) were tested for their ability to prevent biting by wild, host-seeking pyrethroid-resistant *A. gambiae* mosquitoes using volunteers sleeping overnight in experimental huts. These experimental hut studies enabled indoor vector control interventions to be evaluated according to their capacity to deter mosquito entry into a home, inhibit biting and blood feeding, and induce mortality of the mosquito vector (13).

Pyrethroid resistance in the local malaria vector population in Cove is high.

To determine the frequency and strength of resistance to permethrin in the wild *A. gambiae* mosquito population of Cove, Benin, we tested mosquitoes collected as larvae from breeding sites close to the experimental huts in WHO resistance kits lined with 0.75% permethrin-treated papers. After 1-hour exposure, mortality was 100% with the susceptible strain and 10% with the wild *A. gambiae* from Cove, indicating a resistance frequency of 90%. The time required to kill 50% of the mosquitoes (LT_{50}) was 1.2 min (95% CIs, 0.70 to 1.4) for the susceptible Kisumu strain and 163 min (95% CIs, 143 to 183) for the wild Cove strain, which thus showed 136-fold resistance to permethrin.

Olyset Duo is more efficacious than pyrethroid nets against pyrethroid-resistant mosquitoes in experimental hut studies. The experimental hut trial results are presented in Table 3 and Figs. 5 and 6. Mosquito entry rates were high, with average catch per hut ranging between 68 and 91 female *A. gambiae*

Table 1. Oviposition rate for surviving pyrethroid-resistant *A. gambiae* VKPer mosquitoes in tunnel tests.

Net type	No. of washes	No. of females observed (blood-fed alive at 24 hours)	No. of females laying eggs	% Blood-fed females laying eggs (95% CI)*	% Oviposition inhibition (95% CI)
Control	—	176	152	86 (81–91) ^a	—
Pyriproxyfen net	0	198	11	6 (3–9) ^b	94 (90–97)
	10	175	105	60 (51–69) ^{c,d}	30 (20–41)
	20	188	48	26 (20–32) ^e	70 (63–77)
Olyset Net	10	28	24	86 (73–98) ^a	0 (0–15)
	20	16	12	75 (54–96) ^{a,c}	13 (0–37)
Olyset Duo	10	21	9	43 (22–64) ^d	50 (26–74)
	20	12	9	75 (51–100) ^{a,c}	13 (0–41)

*Values bearing the same superscript are not significantly different at the 5% level ($P > 0.05$). Generalized linear mixed models.

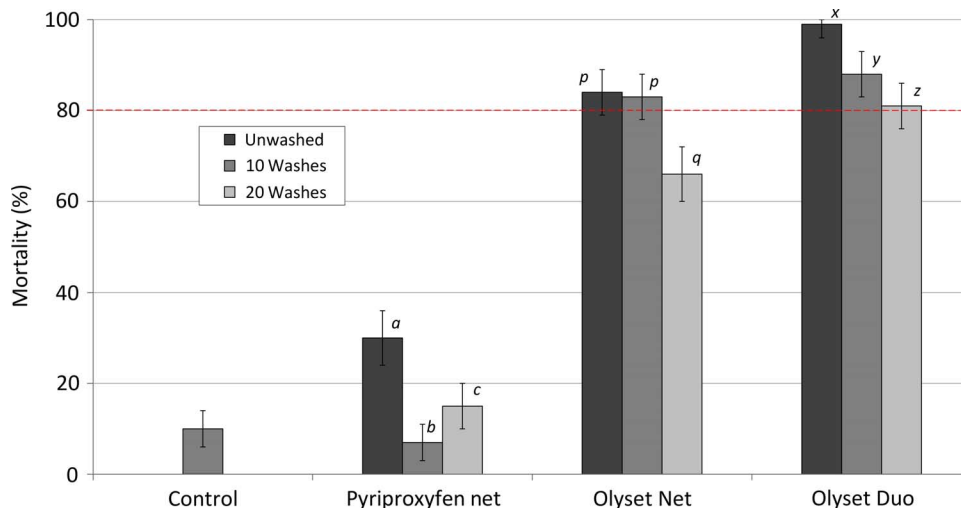


Fig. 3. Mortality of the pyrethroid-resistant *A. gambiae* VKPer strain in tunnel tests. About 200 adult female pyrethroid-resistant mosquitoes of the *A. gambiae* VKPer strain (aged 5 to 8 days) were tested in four replicate tunnel tests for each type of netting (control untreated, pyriproxyfen-treated net, Olyset Net, and Olyset Duo) at three washing intervals (0, 10, and 20 washes). Tunnels were kept overnight in a dark room at 25° to 27°C and 75 to 85% relative humidity. Mosquitoes were collected from the tunnel in the morning, and mortality was recorded after a 24-hour holding period. Each bar represents percentage of mortality at each wash point for the different bed net types. For each net type, bars bearing the same letter label are not significantly different at the 5% level ($P > 0.05$) (generalized linear mixed models). The red dotted line indicates WHO cutoff for mortality (80%) after 20 washes in tunnel tests. Error bars represent 95% CIs. Mortality with Olyset Duo was >80% after 20 washes, showing that the net meets WHO criteria for efficacy in tunnel tests.

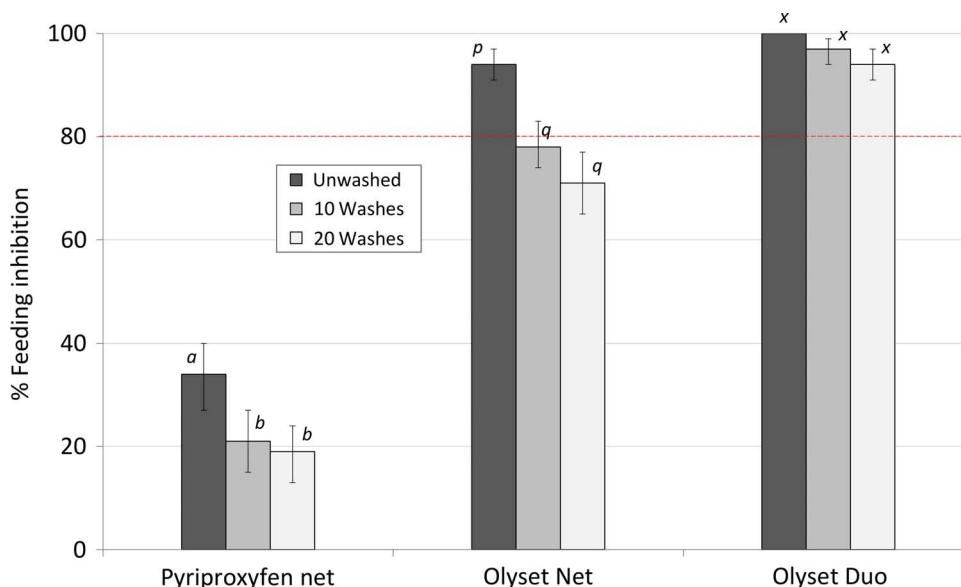


Fig. 4. Inhibition of blood feeding by the pyrethroid-resistant *A. gambiae* VKPer strain in tunnel tests. About 200 adult female pyrethroid-resistant mosquitoes of the *A. gambiae* VKPer strain (aged 5 to 8 days) were tested in four replicate tunnel tests for inhibition of blood feeding by each type of netting (pyriproxyfen-treated, Olyset Net, and Olyset Duo) at three washing intervals (0, 10, and 20 washes). Tunnels were kept overnight in a dark room at 25° to 27°C and 75 to 85% relative humidity. Mosquitoes were collected from the tunnel in the morning, and blood-feeding rates were recorded. Each bar represents percentage of blood-feeding inhibition (relative to the control untreated net) at each wash point for the different bed net types. For each net type, bars bearing the same letter label are not significantly different at the 5% level ($P > 0.05$) (generalized linear mixed models). The red dotted line indicates WHO cutoff for blood-feeding inhibition (80%) after 20 washes in tunnel tests. Error bars represent 95% CIs. Blood-feeding inhibition with Olyset Duo was >80% after 20 washes, showing that the net meets WHO criteria for efficacy in tunnel tests.

per night, and no differences were found between any of the nets (Table 3). Exit rates were generally higher with the permethrin-treated nets (Olyset Net and Olyset Duo) than with the control or pyriproxyfen-treated nets (Table 3). There was no difference in exit rates between Olyset Net and Olyset Duo whether washed or unwashed. There was some decrease in the proportion of mosquitoes exiting the huts with nets washed 20 times.

Personal protection (the number of mosquitoes prevented from blood feeding relative to the untreated control net) and killing effect (the number of mosquitoes killed relative to the untreated control net) were higher with the unwashed Olyset Duo net (72 and 51%, respectively) than with the unwashed Olyset Net (58 and 39%, respectively; $P < 0.05$) (Table 3). Although personal protection did decrease after 20 washes with both types of long-lasting insecticidal net, this decrease was smaller with Olyset Duo (from 72 to 50% decrease) than with Olyset Net (from 58 to 13% decrease). The percentage of blood-fed mosquitoes collected was significantly less with Olyset Duo than with Olyset Net both before and after washing (Fig. 5). Hence, Olyset Duo provided greater blocking of blood feeding (79 and 63% before and after washing, respectively) than did Olyset Net (66 and 34%) (Table 3).

Mosquito mortality was highest with the unwashed Olyset Duo net (40%). The unwashed pyriproxyfen-treated net and the Olyset Net induced 30 and 32% mortality, respectively (Fig. 6). With nets washed 20 times, mortality rates decreased significantly to 22% with the pyriproxyfen-treated net and 27% with the Olyset Net ($P < 0.05$). In contrast, mortality with the Olyset Duo net (39%) did not change significantly ($P = 0.47$).

Tables 4 and 5 show the effects of the nets on the reproduction of surviving blood-fed *A. gambiae* female mosquitoes. The proportion of females that oviposited was high with the untreated control net (55%) and unwashed permethrin-treated Olyset Net (55%) but was significantly reduced with the unwashed pyriproxyfen-treated net (12%; $P < 0.001$) and the unwashed Olyset Duo net (31%; $P < 0.05$) (Table 4). A similar trend was observed with respect to fecundity (number of eggs laid per female) and the number of larvae

Table 2. Offspring of surviving pyrethroid-resistant *A. gambiae* VKPer mosquitoes in tunnel tests.

Net type	No. of washes	Total eggs laid	Eggs per female observed (95% CI)*	% Reduction in fecundity	Total larvae hatched	Hatch rate (%)	Larvae per female observed (95% CI)*	% Reduction in offspring (95% CI)
Control	—	14,012	80 (75–85) ^a	—	9856	70	56 (49–61) ^a	—
Pyriproxyfen net	0	530	3 (1–4) ^b	97 (95–99)	131	25	1 (0–2) ^b	99 (96–100)
	10	12,400	71 (65–75) ^c	11 (3–19)	4125	33	24 (21–27) ^c	58 (53–63)
	20	8,206	44 (40–48) ^d	45 (40–50)	3765	46	20 (18–23) ^c	64 (60–68)
Olyset Net	10	2,828	101 (94–109) ^e	0 (0–18)	1740	62	62 (51–71) ^a	0 (0–9)
	20	924	58 (48–69) ^f	28 (16–40)	729	79	46 (34–56) ^a	19 (0–39)
Olyset Duo	10	625	30 (25–35) ^g	63 (56–69)	411	66	20 (14–25) ^c	65 (55–75)
	20	425	35 (19–49) ^{d,g}	56 (36–76)	324	47	27 (18–33) ^c	52 (36–68)

*Values down a column bearing the same superscript are not significantly different at the 5% level ($P > 0.05$). Kruskal-Wallis test.

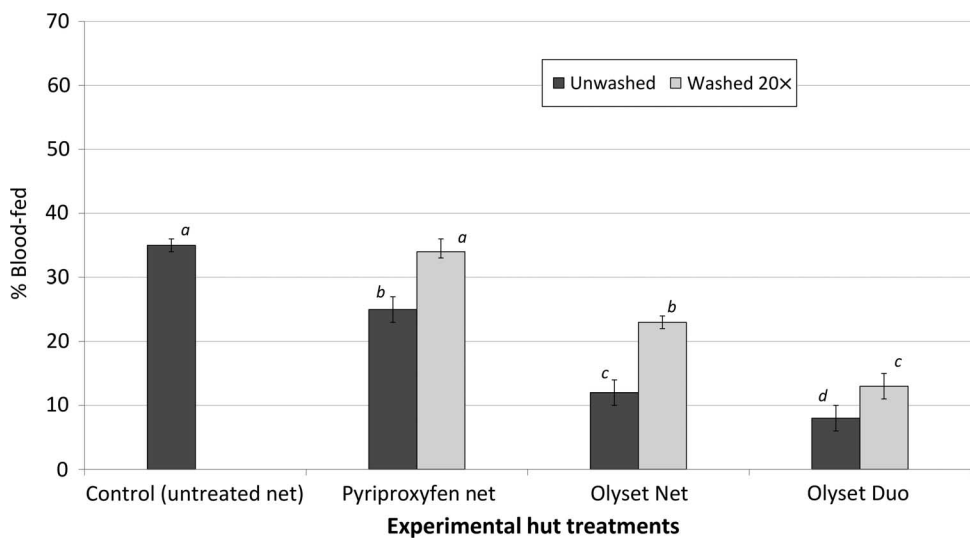


Fig. 5. Blood feeding by wild free-flying pyrethroid-resistant *A. gambiae* mosquitoes in experimental field huts. Consenting adult human volunteers slept in experimental huts in Cove, Benin, from 8:00 p.m. to 5:00 a.m. each night of the study to attract mosquitoes into the huts. Three replicate nets in each hut were tested, and these nets were rotated in the hut every 2 days for each week of the trial. Mosquitoes were collected in the morning and were scored for blood-feeding status. Each bar represents percentage of blood-fed mosquitoes (relative to the control untreated net) at each wash point (0 and 20 washes) for the different bed net types (pyriproxyfen-treated net, Olyset Net, and Olyset Duo). Bars bearing the same letter label are not significantly different at the 5% level ($P > 0.05$) (generalized linear mixed models). Error bars represent 95% CIs. Blood-feeding rates with Olyset Duo either unwashed or after 20 washes were much lower than with Olyset Net ($P < 0.05$).

produced per female (Table 5). This resulted in a significant reduction in the number of offspring relative to the untreated control net: 90% reduction with the unwashed pyriproxyfen-treated net and 71% with the unwashed Olyset Duo net. Relative to the unwashed long-lasting insecticidal nets, the proportion of females that oviposited, their average fecundity, and the number of viable offspring all increased after the pyriproxyfen-treated net and Olyset Duo net were washed 20 times. Hence, the proportional reduction in the average number of offspring produced relative to the control net went from 90 to 38% reduction with the pyriproxyfen-treated net after 20 washes and from 71 to 43% with the Olyset Duo net after 20 washes.

When the combined effect of adult mosquito mortality and suppression of reproduction was taken into consideration, the overall reduction in reproductive rate R_0 revealed differences between the different nets. The reduction in reproductive rate relative to untreated nets was greater with the pyriproxyfen-treated net (93% reduction) and Olyset Duo net (94% reduction) than with the Olyset Net (75% reduction) before washing. After 20 washes, this effect was reduced with the pyriproxyfen-treated net (36% reduction) and Olyset Net (48% reduction), but not with the Olyset Duo net (78% reduction).

Cylinder bioassays and tunnel tests with netting pieces hand-treated with pyriproxyfen and permethrin
We tested whether the increased mortality observed in the experimental hut trial with the Olyset Duo net treated with a mixture of insecticides relative to nets treated with permethrin alone or pyriproxyfen alone was due to an additive interaction between the two insecticides rather than to higher surface concentrations of permethrin

caused by accelerated release rates in the mixture-treated net. Netting pieces hand-treated with permethrin alone, pyriproxyfen alone, or a mixture were tested in the cylinder bioassay (3-min exposure) and the tunnel test (overnight exposure) for their activity against the susceptible laboratory *A. gambiae* Kisumu strain. The results are presented in Fig. 7 for mortality in the cylinder bioassay and Table 6 for mortality in the tunnel test. Mortality rates with the mixture-treated netting were not increased relative to the nets treated with a single active ingredient in either the cylinder bioassay or the tunnel test. Hence, with the concentrations tested, the data failed to show an additive or synergistic effect between both active ingredients upon mosquito mortality.

Table 3. Results for wild free-flying pyrethroid-resistant *A. gambiae* mosquitoes in experimental huts in Cove, Benin.

Net type	Control	Pyriproxyfen net		Olyset Net		Olyset Duo	
No. of washes	—	0	20	0	20	0	20
Total females caught	2874	3007	2897	3804	3782	3840	3293
Average catch per night	68	72	69	91	90	91	78
% Deterrence	—	0	0	0	0	0	0
Total exiting	1325	1367	1286	2493	2177	2458	1943
% Exiting (95% CI)*	46 (42–48) ^a	45 (42–47) ^a	44 (41–47) ^a	65 (62–68) ^b	58 (56–60) ^c	64 (62–68) ^b	59 (57–61) ^c
% Inside net (95% CI)	21	15	26	5	11	3	6
Total blood-fed mosquitoes	1004	720	1031	444	875	281	497
Inhibition of blood feeding (%)*	—	31 (28–34) ^a	0 (0–8) ^b	66 (61–71) ^c	34 (31–37) ^a	79 (75–83) ^d	63 (58–68) ^c
Personal protection (%)*	—	28 ^a	0 ^b	58 ^c	13 ^d	72 ^e	50 ^c
No. of dead after 24 hours	159	868	660	1228	1012	1536	1314
Killing effect (%)*	—	26 ^a	18 ^b	39 ^c	31 ^d	51 ^e	43 ^c

*Values along a row bearing the same superscript are not significantly different at the 5% level ($P > 0.05$). Generalized linear mixed models.

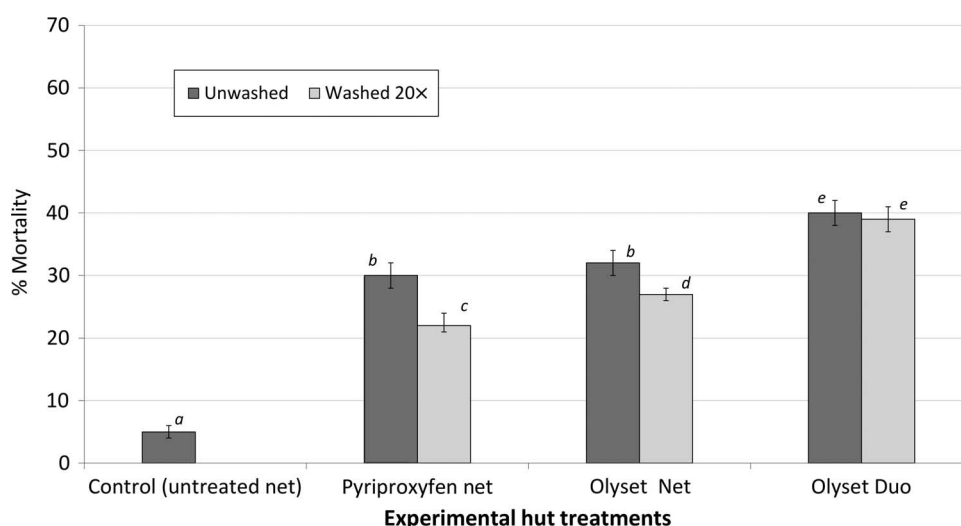


Fig. 6. Mortality of wild free-flying pyrethroid-resistant *A. gambiae* mosquitoes in experimental field huts. Consenting adult human volunteers slept in experimental huts in Cove, Benin, from 8:00 p.m. to 5:00 a.m. each night of the study to attract mosquitoes into the huts. Three replicate nets in each hut were tested, and these nets were rotated in the hut every 2 days for each week of the trial. Mosquitoes were collected in the morning, and mortality was recorded after a 24-hour holding period. Each bar represents percentage of mortality at each wash point (0 and 20 washes) for the different bed net types [control (untreated net), pyriproxyfen-treated net, Olyset Net, and Olyset Duo]. Bars bearing the same letter label are not significantly different at the 5% level ($P > 0.05$) (generalized linear mixed models). Error bars represent 95% CIs. Mortality rates with Olyset Duo either unwashed or after 20 washes were higher than with Olyset Net ($P < 0.05$).

Chemical analysis to determine changes in insecticide content after net washing

To assess changes in insecticide content after net washing, we analyzed samples of the pyriproxyfen-treated net and Olyset Duo net washed 0, 5, 10, 15, and 20 times using high-performance liquid chromatography (Table 7). The initial concentrations of insecticide in both net types were close to the target of 20 g/kg for permethrin and 10 g/kg for pyriproxyfen. There was better retention of permethrin than pyriproxyfen over

the wash cycles. By the 20th wash, the concentration of pyriproxyfen had decreased to 50% of the initial concentration in the Olyset Duo net and to 33% of the initial concentration in the pyriproxyfen-treated net, whereas the concentration of permethrin in the Olyset Duo net was 68% of the initial concentration. Nevertheless, a considerable amount of pyriproxyfen (50%) remained in the Olyset Duo net after 20 washes.

DISCUSSION

The Olyset Duo net incorporates both permethrin and pyriproxyfen to control pyrethroid-resistant mosquito populations and to prevent malaria transmission. Pyriproxyfen was added to the net to sterilize mosquitoes that can survive the permethrin component. By combining the adulticidal toxicity of permethrin and the sterilizing effect of pyriproxyfen on parental females, Olyset Duo, when used on a large scale, is expected to improve prevention of malaria transmission by inducing reductions

in vector populations across generations compared to standard nets treated only with pyrethroid insecticides. The results obtained with the unwashed nets in both phase 1 and 2 studies demonstrated increased mortality rates with Olyset Duo and significant reductions in the fertility of females surviving exposure to Olyset Duo or the pyriproxyfen-treated net compared to the untreated control net (10, 12). Mosquito nets are subjected to vigorous washing by home users; thus, as required by WHOPES, the effects of Olyset Duo must resist repeated

Table 4. Oviposition rate for surviving wild blood-fed pyrethroid-resistant *A. gambiae* mosquitoes in experimental hut studies.

Net type	No. of washes	No. of females observed (blood-fed alive at 24 hours)	No. of females laying eggs	% Blood-fed females laying eggs (95% CI)*	% Oviposition inhibition (95% CI)
Control	—	401	219	55 (50–60) ^a	—
Pyriproxyfen net	0	294	34	12 (8–16) ^b	78 (71–85)
	20	444	201	45 (40–50) ^c	18 (9–27)
Olyset Net	0	229	125	55 (49–61) ^a	0 (0–11)
	20	361	210	58 (53–63) ^a	0 (0–4)
Olyset Duo	0	120	37	31 (23–39) ^d	44 (30–58)
	20	227	106	47 (41–53) ^c	15 (4–25)

*Values down a column bearing the same superscript are not significantly different at the 5% level ($P > 0.05$). Generalized linear mixed models.

Table 5. Offspring of surviving wild blood-fed pyrethroid-resistant *A. gambiae* mosquitoes in experimental hut studies.

Net type	No. of washes	Total eggs laid	Eggs per female observed*	% Reduction in fecundity	Hatch rate (%)	Larvae per female observed*	% Reduction in offspring	Reproductive rate R_0	% Reduction in R_0 (95% CI)
Control	—	25,067	63 (61–65) ^a	—	33	21 (18–24) ^a	—	7.1	—
Pyriproxyfen net	0	2,833	10 (6–13) ^b	84 (78–90)	22	2 (0–4) ^b	90 (81–100)	0.5	93 (86–100)
	20	20,273	46 (39–51) ^c	27 (19–38)	30	13 (10–15) ^c	38 (29–52)	4.6	36 (33–39)
Olyset Net	0	13,994	61 (57–65) ^a	3 (0–10)	26	16 (12–21) ^c	24 (0–42)	1.8	75 (71–79)
	20	23,918	66 (58–74) ^a	0 (0–8)	26	17 (15–19) ^c	19 (10–29)	3.7	48 (44–53)
Olyset Duo	0	2,965	25 (19–30) ^d	60 (50–70)	23	6 (4–8) ^b	71 (62–80)	0.4	94 (88–100)
	20	12,440	55 (51–58) ^e	13 (7–19)	22	12 (8–15) ^c	43 (29–62)	1.6	78 (74–82)

*Values down a column bearing the same superscript are not significantly different at the 5% level ($P > 0.05$). Kruskal-Wallis test.

washings over an average 3-year life span for the net to induce a lasting impact on malaria transmission (13).

The WHOPES phase 1 and 2 wash resistance test results demonstrated that mortality and blood-feeding inhibition induced by Olyset Duo showed little or no decrease after 20 washes, indicating strong wash retention of permethrin in the Olyset Duo net. On the basis of the wash resistance results with permethrin alone, Olyset Duo may warrant recommendation by WHOPES as a pyrethroid long-lasting insecticidal net. However, to obtain this WHOPES recommendation, both active ingredients need to be sufficiently wash-resistant (13). Unlike with the permethrin component, the wash resistance studies showed a decline in the sterilizing effects of pyriproxyfen after washing in both laboratory and field studies. This decline in effect was, however, less evident in the tunnel test and hut trial than in the cone bioassay where the sterilizing effect with both types of pyriproxyfen-treated net appeared to be lost after only five washes. The difference in wash resistance of pyriproxyfen between the different test methods can be attributed to the shorter exposure time in the cone bioassay compared to the tunnel test and hut trial where mosquitoes were free to contact the net ad libitum. The 3-min exposure in the cone bioassay was insufficient to allow pick up of adequate amounts of pyriproxyfen from washed nets to cause the sterility that occurs from exposure to nets under household conditions. Recent phase 1 and 2 evaluations of a new chlorfenapyr-pyrethroid-treated net also showed poor performance of nonpyrethroid insecticides in the cone bioassay compared

to the tunnel test or hut trial (15, 16). These findings raise questions about the adequacy of current WHO guidelines for evaluating long-lasting insecticidal nets (13), particularly the duration of exposure in the cone bioassay, which remains central to phase 1 testing and predictions of activity of nonpyrethroid insecticides on mosquito nets. Identifying new active ingredients poses a major challenge to the development of new-generation mixture nets, and it is essential that the method of screening does not miss suitable compounds. The tunnel test is superior for this purpose because mosquitoes contact the netting when actively host-seeking at night in the same way they do under field or household conditions (15).

Unlike standard pyrethroid-treated nets and mixture nets that incorporate a synergistic agent as a complementary active ingredient (17–19), the Olyset Duo net has multiple effects on mosquitoes in addition to direct mortality and prevention of blood feeding. These effects include sterilization of mosquitoes, reduced offspring viability, and reduced adult longevity (10). Testing for some of the effects of Olyset Duo is not yet the subject of WHOPES guidelines or standard methods. It is anticipated that the methods and findings reported in the present study will contribute to new consensus and revision of WHOPES guidelines (14).

Despite some diminished capacity of Olyset Duo and the pyriproxyfen-treated net to sterilize mosquitoes after multiple washes, the continuing impact on reproductive rate R_0 (which combines the insecticide-induced reduction in adult life span and the sterilizing effect on the next generation)

showed that the impact of Olyset Duo remained high after washing, with R_0 reduced by 94% with the unwashed Olyset Duo net and by 78% with the Olyset Duo net washed 20 times. We predict that Olyset Duo would continue to suppress vector population densities relative to Olyset Net in field situations after multiple washes. This prediction requires field validation. As the first mixture long-lasting insecticidal net involving an insect growth regulator to be developed commercially, Olyset Duo constitutes a new paradigm in malaria vector control, which, according to the Vector Control Advisory Group, requires evaluation and validation in cluster-randomized malaria control trials (14). A community-randomized trial of Olyset Duo compared to Olyset Net is currently under way in Burkina Faso (20).

The chemical analysis of total content showed that a considerable amount of pyriproxyfen was retained within the net after 20 washes.

Previous laboratory bioassay studies using nets hand-treated with pyriproxyfen showed that surface concentrations as low as 10 mg/m² were enough to sterilize adult female mosquitoes after tarsal contact (10). This implies that the reservoir of the insect growth regulator may remain largely trapped in the fibers of Olyset Duo and pyriproxyfen-treated nets after repeated washing. The loss of bioactivity of pyriproxyfen after washing could therefore be attributed to the loss of bioavailability on the surface of the Olyset Duo net. The release rate of pyriproxyfen from within the fibers is being optimized within the current net specifications to improve diffusion rates to the surface after washing.

As with the preliminary experimental hut study in Benin (12), the pyriproxyfen-treated net induced some adult mosquito mortality in the experimental huts (~30%), suggesting that the high mortality with Olyset Duo could be due to a positive interaction between permethrin

and pyriproxyfen that increased toxicity. Hand treatment of nets by dipping with permethrin and pyriproxyfen formulations failed to show improved mortality with the combination of dosages tested. This indicates that the added mortality observed with Olyset Duo over Olyset Net is more likely due to the higher release rate and surface concentration of permethrin in Olyset Duo than to a synergistic interaction between the two compounds.

The experimental hut trial provided ample data on the sterilizing effects of the unwashed Olyset Duo net. However, it was not possible to demonstrate this effect in the phase 1 evaluation owing to the high mortality rates (98 to 100%) induced by the high permethrin concentration in the unwashed Olyset Duo net in the cone bioassay and tunnel test using the *A. gambiae* laboratory strains available. This shows one of the challenges encountered in laboratory evaluation of mixture bed nets involving

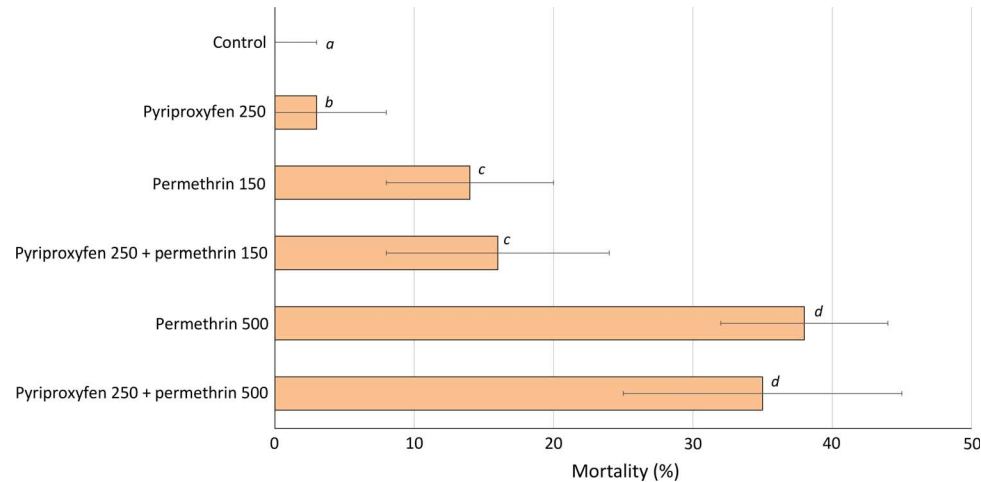


Fig. 7. Mortality of susceptible *A. gambiae* Kisumu mosquitoes exposed to net pieces treated with permethrin and/or pyriproxyfen. Fifty 3- to 5-day-old mosquitoes of the *A. gambiae* Kisumu strain were exposed (in batches of 10) for 3 min to net samples hand-treated with pyriproxyfen, permethrin, or a mixture of the two insecticides in WHO cylinders. Mortality was recorded after a 24-hour holding period. The doses of active ingredients applied (y axis) are expressed in mg/m². Each bar represents the percentage of mortality for each dose or combination of doses applied. Bars bearing the same letter label are not significantly different at the 5% level ($P > 0.05$) (generalized linear mixed models). Error bars represent 95% CIs. Mortality rates with nets hand-treated with a mixture of pyriproxyfen and permethrin did not increase compared to nets treated with either active ingredient alone ($P > 0.05$).

Table 6. Tunnel test results for susceptible *A. gambiae* Kisumu mosquitoes exposed to net pieces hand-treated with permethrin and/or pyriproxyfen.

Net treatment (mg/m ²)	No. of exposed	% Penetration*	% Blood-fed*	% Blood-feeding inhibition	% Mortality*
Control	98	100 ^a	97 ^a	—	3 ^a
95% CI		95–100	94–100		0–6
Pyriproxyfen (250)	101	99 ^a	98 ^a	0	8 ^b
95% CI		97–100	95–100		3–13
Permethrin (150)	105	40 ^b	30 ^b	69	46 ^c
95% CI		31–49	21–39		36–55
Permethrin (150) + pyriproxyfen (250)	104	43 ^b	41 ^b	58	35 ^c
95% CI		31–55	33–52		26–44

*Values down each column sharing the same superscript are not significantly different at the 5% level ($P > 0.05$). Generalized linear mixed models.

Table 7. Analysis of total active ingredients in Olyset Duo and pyriproxyfen-treated nets.

No. of wash cycles	Permethrin (g/kg)	% Retention of permethrin	Pyriproxyfen (g/kg)	% Retention of pyriproxyfen
Olyset Duo				
Unwashed	19 ± 0.2	—	10 ± 0.2	—
5	15 ± 0.3	79	7 ± 0.3	70
10	14 ± 0.4	74	6 ± 0.4	60
15	13 ± 1.8	68	6 ± 1.8	60
20	13 ± 0.2	68	5 ± 0.2	50
Pyriproxyfen net				
Unwashed	—	—	9 ± 0.2	—
5	—	—	5 ± 0.1	55
10	—	—	4 ± 0.1	44
15	—	—	4 ± 0.1	44
20	—	—	3 ± 0.1	33

pyrethroids. To help mitigate this problem, a net treated with pyriproxyfen alone was developed to similar technical specifications (pyriproxyfen concentration, release rate, fiber, and mesh size) as the Olyset Duo to serve as a suitable control.

In conclusion, the Olyset Duo net provided superior mortality and reduced blood feeding of pyrethroid-resistant mosquitoes compared to the standard pyrethroid-only Olyset Net in laboratory and experimental hut studies and also effectively sterilized surviving blood-fed mosquitoes. The mortality and blood-feeding inhibition effects observed with Olyset Duo persisted after 20 washes. The sterilizing effects gradually diminished during the wash resistance studies, but this was less evident in the tunnel test and hut trials than in the cone bioassay. The 3-min cone bioassay test was unable to predict pyriproxyfen efficacy under household conditions and was ineffective as a screening and evaluation tool for nets such as Olyset Duo treated with an insect growth regulator. Chemical analysis showed that a significant proportion of pyriproxyfen remained in the fibers of the net after washing. These findings demonstrate that the Olyset Duo net has the potential to control pyrethroid-resistant mosquito vector populations and warrants further testing in communities of high malaria transmission due to pyrethroid-resistant mosquitoes.

MATERIALS AND METHODS

Study design

The study was designed to assess whether Olyset Duo, a new long-lasting insecticidal net treated with permethrin and pyriproxyfen, would improve control of pyrethroid-resistant mosquito vectors compared to a standard net treated only with permethrin (Olyset Net) and whether this improvement would remain after multiple washes. In accordance with WHO guidelines (13), we performed phase 1 laboratory tests (cone bioassay and tunnel test) with laboratory-maintained

strains of *A. gambiae* mosquitoes. We also performed a small-scale phase 2 trial with human volunteers in experimental huts in a pyrethroid-resistant malaria-endemic area of southern Benin. Efficacy was measured in terms of the capacity of the different nets before and after washing to kill pyrethroid-resistant mosquitoes, to prevent blood feeding, and to suppress mosquito reproduction.

The study was approved by the Ethical Review Board of the London School of Hygiene and Tropical Medicine and by the Ministry of Health in Benin. Guinea pigs used in the study were kept in accordance with institutional guidelines for animal care. The volunteer experimental hut sleepers gave written informed consent before participating in the study. They were provided chemoprophylaxis before the study and were also checked regularly by a stand-by nurse for any signs of fever. Any sleepers testing positive for malaria were withdrawn from the study and treated accordingly.

Rationale and outcome measures

The following four types of mosquito net were compared in the phase 1 and 2 efficacy and wash resistance studies: (i) untreated control mosquito net; (ii) pyriproxyfen-treated long-lasting net (Sumitomo Chemical Co. Ltd.); (iii) Olyset Net (Sumitomo Chemical Co. Ltd.), a WHOPES-recommended standard permethrin-treated long-lasting insecticidal net; and (iv) Olyset Duo (Sumitomo Chemical Co. Ltd.), a new long-lasting insecticidal net treated with 1% (w/w) pyriproxyfen and 2% (w/w) permethrin.

All four types of mosquito net were made of white polyethylene woven material and were similar in physical appearance. Olyset Duo incorporates two active ingredients that induce differing effects on mosquitoes. Whereas permethrin acts primarily by killing mosquitoes and inhibiting mosquito biting, pyriproxyfen acts by reducing the capacity of the female mosquito to produce and oviposit fertile eggs and produce viable larval offspring. The effects of pyriproxyfen were investigated by detecting a reduction in reproductive capacity of female mosquitoes exposed to the treated nets. This was achieved by measuring the reduction in the size of the F_1 generation of female mosquitoes surviving exposure to the treated nets relative to unexposed control females. These reproductive effects included inhibition of oviposition and reduction in egg and larval output. After assessing the standard acute insecticidal effects on adult mosquitoes (blood-feeding inhibition, repellency and mortality, and personal protection) 24 hours after exposure in the phase 1 and 2 studies, surviving female mosquitoes from each treatment were chambered individually and allowed to oviposit. Mosquitoes were held in their own netted plastic cups containing about 50 ml of fresh water. The chambers were monitored daily for evidence of egg laying, 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 that contained eggs, and the numbers of larvae that hatched were recorded after another 4 to 6 days.

The following outcome measures were used to assess the reproductive effects of the different nets on surviving female mosquitoes:

1) % Reduction in oviposition rate: The reduction in the proportion of females ovipositing for a given treatment compared to the control. This was calculated as follows:

$$\frac{100(O_c - O_t)}{O_c}$$

where O_c is the proportion of surviving blood-fed females from the control that laid eggs, whereas O_t is the proportion of surviving blood-fed females from a given treatment that laid eggs.

2) % Reduction in fecundity: The reduction in the number of eggs per surviving blood-fed female for a given treatment relative to the control. This was calculated as follows:

$$\frac{100(E_c - E_t)}{E_c}$$

where E_c is the mean number of eggs per surviving blood-fed female observed in the control, whereas E_t is the mean number of eggs per surviving blood-fed female observed in a given treatment.

3) % Reduction in offspring: The percentage reduction in the number of larvae per surviving blood-fed female observed for a given treatment relative to the control. This was calculated as follows:

$$\frac{100(L_c - L_t)}{L_c}$$

where L_c is the mean number of larvae per surviving blood-fed female observed in the control, whereas L_t is the mean number of larvae per surviving blood-fed female observed in a given treatment.

4) % Reduction in reproductive rate R_0 : The percentage reduction in the overall number of offspring per female mosquito that entered the experimental hut. This was calculated as follows:

$$\frac{100(R_c - R_t)}{R_c}$$

where R_c (reproductive rate in control) is the mean number of larvae per female entering an untreated control hut, whereas R_t (reproductive rate in treatment) is the mean number of larvae per female entering a hut with a given treatment. The reproductive rate R_0 takes into account mortality of the adult, blood-feeding inhibition and reproductive effects. Because reproductive effects were assessed on a random selection of all live blood-fed females collected from the huts, the reproductive rate was adjusted to account for total surviving females that had successfully blood-fed. For each treatment, this adjustment was done by multiplying the total number of larvae produced by the ratio of the total surviving blood-fed females to the number of surviving blood-fed females that were observed for reproductive effects.

Phase 1 studies

Regeneration time and net-washing procedure. To assess the phase 1 biological efficacy of Olyset Duo after multiple washings, laboratory bioassays were performed on standard samples of unwashed and washed netting pieces from each type of mosquito net according to WHO procedures (13). Before washing and testing, preliminary bioassays were done to determine the “regeneration time,” which is the time taken for full insecticidal efficacy to be restored after washing. Insecticide regeneration occurs as a result of the diffusion of insecticide from the interior to the surface of the polyethylene fibers. Regeneration time is the minimum interval that should be applied between washes. Netting pieces were subjected to bioassay prewashing, then washed three times, and retested at daily intervals to determine the time taken to restore the killing and sterilizing properties of Olyset Duo to an effective and stable level. The tests revealed a 3-day regeneration time for Olyset Duo and pyriproxyfen net and 7-day regeneration time for Olyset Net. The net pieces of Olyset Duo, pyriproxyfen

net, and Olyset Net used for efficacy testing were washed at the respective regeneration time intervals.

The washing procedure followed WHO guidelines (13). Netting pieces were washed for 10 min with standardized soap solution (Savon de Marseille at 2 g per liter of deionized water) in shaker water baths set at 155 movements/min and 30°C. The samples were rinsed twice for 10 min in clean water under the same conditions as above and stored at 30°C and 75 to 85% relative humidity between washes and between tests.

WHO cone bioassays. For each type of long-lasting net and each wash point (0, 5, 10, 15, and 20 washes), 150 unfed 3- to 5-day-old insecticide-susceptible female *A. gambiae* Kisumu were tested for 3 min in replicates of 5 mosquitoes per cone. Knockdown was recorded after 1 hour and mortality after a 24-hour holding period. Mosquitoes that survived the cone bioassays were blood-fed overnight in cages using an anesthetized guinea pig, held in separate oviposition chambers for 13 to 15 days, and observed for reproductive effects (oviposition inhibition and reduction in fecundity and offspring), as described earlier.

Tunnel tests. About 200 adult female pyrethroid-resistant *A. gambiae* VKPer in four replicate tunnel tests were tested on each type of netting and each washing interval (0, 10, and 20).

The tunnel test consists of a glass chamber (25 cm high, 25 cm wide, and 60 cm long) divided into two sections by means of a netting frame slotted across the tunnel. In one section, an anesthetized guinea pig was housed unconstrained in a small cage, and in the other, 50 unfed female mosquitoes aged 5 to 8 days were released at dusk and left overnight. The net samples were holed with nine 1-cm-diameter holes to allow host-seeking mosquitoes to penetrate into the baited chamber; an untreated net sample served as the control. The tunnels were kept overnight in a dark room at 25° to 27°C and 75 to 85% relative humidity. The next morning, the numbers found alive or dead, fed or unfed, in each section were recorded. Live mosquitoes were provided with sugar solution, and delayed mortality was recorded after 24 hours. Blood-fed mosquitoes that remained alive after 24 hours were assessed for sterilizing effects of pyriproxyfen, as described earlier. The guinea pigs used in this study were kept in accordance with institutional guidelines for animal care.

Phase 2 studies: Experimental hut trial

Study site and experimental huts. The study was performed in the experimental hut station in Cove (7°14'N, 2°18'E), southern Benin. The station is situated in a rice-growing area, which provides extensive breeding sites for *A. gambiae* s.l. throughout the year. The rainy season extends from March to October. The study was performed for 7 weeks in seven experimental huts of West African design (13). The experimental huts are built on concrete plinths surrounded by water-filled moats to prevent entry of scavenging ants and are made of cement-plastered brick, with a corrugated iron roof and inner ceiling of palm thatch. Four baffled window slits on the walls allow host-seeking mosquitoes to enter and a veranda trap on the fourth wall captures the exiting mosquitoes. The local *A. gambiae* vector population is resistant to pyrethroids and DDT with a *L1014F kdr* allele frequency of 89%. Microarray studies also found CYP6P3, a P450 validated as an efficient metabolizer of pyrethroids (21), to be overexpressed in Cove (22).

Susceptibility tests. Mosquitoes 2 to 5 days old were exposed to 0.75% permethrin-treated papers for a range of times in four replicates of 25 mosquitoes. Comparison was made with the susceptible *A. gambiae* Kisumu laboratory strain. Deaths were scored 24 hours later. Log-time

mortality curves were generated using probit analysis, and estimates of the lethal time required to kill 50% of each strain (LT_{50}) and the resistance ratios relative to the susceptible laboratory colony were made (PoloPlus version 1.0).

Treatments and trial procedure. The following seven treatments were tested in the experimental huts: (i) untreated control mosquito net, (ii) pyriproxyfen-treated net unwashed, (iii) pyriproxyfen-treated net washed 20 times, (iv) Olyset Net unwashed, (v) Olyset Net washed 20 times, (vi) Olyset Duo unwashed, and (vii) Olyset Duo washed 20 times.

To simulate wear and tear, the bed nets were intentionally holed with six 16-cm² holes (two holes on each side and one on each end) according to WHOPES guidelines (13). Treatments were allocated to the experimental huts on a weekly basis using a randomized Latin square design to adjust for any variation in site attractiveness and minimize any carryover effect between treatments. Three nets of each type were prepared, and these were rotated every 2 days on each week (6 days) of the trial. Seven consenting adult volunteers slept overnight in the huts to attract mosquitoes into the huts; they were rotated between huts on successive nights to adjust for any variation in individual attractiveness to mosquitoes.

For each treatment, the insecticidal effects on adult female mosquitoes were assessed in terms of the following: (i) detergency: the percentage reduction in the number of mosquitoes caught in treated huts relative to the number caught in the control huts, (ii) treatment-induced exiting: expressed as the percentage of the mosquitoes collected from the veranda trap, (iii) blood-feeding rate: the proportion blood-fed, (iv) inhibition of blood feeding: the reduction in blood-feeding rate relative to the control, (v) personal protection: the proportional reduction in the number of mosquitoes blood-fed with a treatment relative to the untreated control, (vi) mortality: the percentage of dead mosquitoes, and (vii) potential mass killing effect: the number of mosquitoes killed by a treatment relative to the number entering the control hut.

Blood-fed mosquitoes that survived from the experimental huts after the 24-hour holding period were held in individual oviposition chambers for 13 to 15 days during which they were observed for reproductive effects (oviposition inhibition and reduction in fecundity and offspring), as described above.

Studies on interactions between permethrin and pyriproxyfen

The net pieces used for this study were treated with permethrin and pyriproxyfen alone and together by hand dipping in insecticide solutions. The following treatments were tested in the cylinder bioassays: (i) control untreated net, (ii) pyriproxyfen (250 mg/m²), (iii) permethrin (150 mg/m²), (iv) pyriproxyfen (250 mg/m²) + permethrin (150 mg/m²), (v) permethrin (500 mg/m²), and (vi) pyriproxyfen (250 mg/m²) + permethrin (500 mg/m²).

Mosquitoes were exposed for 3 min, and mortality was recorded after 24 hours. Dose combinations which showed mortality rates in cylinder bioassays that were low enough to allow demonstration of an interaction in the tunnels were then tested in the tunnel bioassays. A total of 100 *A. gambiae* Kisumu mosquitoes were exposed to each net sample in replicates of two tunnels.

Data analysis

Proportional outcomes (net penetration, blood feeding, exiting, mortality, and oviposition by surviving blood-fed females) related to each experimental hut treatment were assessed with binomial generalized linear mixed models with a logit link function, fitted using the

“lme4” package for R (version 2.15.0). 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 seven huts, between the seven 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 (overdispersion).

Differences in deterrence, personal protection, and mass killing effect between the treatments were analyzed with negative binomial regression based on numbers entering, blood feeding, and killed, respectively, with adjustment for the abovementioned covariates. The numbers of eggs laid and the numbers of larvae per surviving female were analyzed using the Kruskal-Wallis test. These analyses were performed using Stata version 11.1.

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Efficacy of the Olyset Duo net against insecticide-resistant mosquito vectors of malaria

Corine Ngufor, Raphael N'Guessan, Josias Fagbohoun, Damien Todjinou, Abibath Odjo, David Malone, Hanafy Ismail, Martin Akogbeto and Mark Rowland

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A net for all reasons

Long-lasting insecticide-treated mosquito nets are the most widely used intervention for preventing transmission of malaria by anopheline mosquitoes. Their effectiveness is threatened by the development of resistance to pyrethroid insecticides enabling the mosquito to survive contact with the net. Olyset Duo is a new type of bed net treated with pyrethroid and a new insecticide, pyriproxifen, that disrupts the maturation of eggs in the ovaries of blood-fed mosquitoes. Ngufor and colleagues evaluated the capacity of the Olyset Duo net to control pyrethroid-resistant mosquitoes in laboratory tests and under household conditions in West Africa. The Olyset Duo net killed more pyrethroid-resistant mosquitoes than did the standard pyrethroid net and also sterilized any mosquitoes that survived exposure to the net. By preventing mosquito reproduction, the new net has the potential to reduce mosquito populations and malaria transmission in areas of high pyrethroid resistance.

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