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Bio-efficacy of new long-lasting insecticide-treated bed nets against *Anopheles funestus* and *Anopheles gambiae* from central and northern Mozambique

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Abstract

Background: Long-lasting insecticide-treated nets (LLINs) are one of the main methods used for controlling malaria transmission in Mozambique. The proliferation of several types of LLINs and the re-emergence of insecticide resistance in the local vector populations poses challenges to the local malaria control programme on selecting suitable insecticide-based vector control products. Therefore, this study evaluated the insecticide susceptibility and bio-efficacy of selected new LLINs against wild populations of *Anopheles funestus* sensu lato and *A. gambiae s.l.* from Northern and Central Mozambique. The study also investigated whether the insecticide contents on the LINNs fabrics were within the WHOPES recommended target range.

Methods: The susceptibility of 2–5 day old wild female *A. funestus* and *A. gambiae* sensu stricto against the major classes of insecticides used for vector control, viz: deltamethrin (0.05 %), permethrin (0.75 %), propoxur (0.1 %), bendiocarb (0.1 %) and DDT (4 %), was determined using WHO cylinder susceptibility tests. WHO cone bioassays were conducted to determine the bio-efficacy of both pyrethroid–only LLINs (Olyset®, Permanet 2.0®, NetProtect® and Interceptor®) and, Permanet 3.0® a combination LLIN against *A. funestus s.s*, from Balama, Mocuba and Milange districts, respectively. The bio-efficacy of LLINs against the insectary-susceptible *A. arabiensis* (Durban strain) was assessed, as well. Untreated bed net swatches were used as negative controls. Chemical analyses, by high performance liquid chromatography, were undertaken to assess whether the insecticide contents on the LLINs fabrics fell within recommended target dose ranges. The frequency of *kdr* gene mutations was determined from a random sample of *A. gambiae s.s.* from both WHO susceptibility and cone bioassay experiments.

Results: *Anopheles funestus* from Balama district showed resistance to deltamethrin and possible resistance to permethrin, propoxur and bendiocarb, whilst *A. gambiae* from Mocuba district was susceptible to deltamethrin, bendiocarb and propoxur. There were no *kdr* mutants found in the sample of 256 *A. gambiae* tested. Overall, 186 LLIN swatches were tested. Mosquitoes exposed to Olyset® had the lowest knockdown (±standard error) and mortality rate (±standard error) in all studied sites regardless of vectors species tested. Permanet 3.0 showed the highest bio-efficacy independent of vector species tested and level of insecticide resistance detected. All types of LLINs effectively killed susceptible *A. arabiensis* Durban strain. The insecticide content of Olyset® and Permanet 2.0® was higher than the target dose but NetProtect® had a lower insecticide content than the target dose.

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Conclusion: The study shows evidence of considerable heterogeneity in both insecticide susceptibility and the level of bio-efficacy of commonly available types of LLINs against wild *A. funestus* and *A. gambiae* from Balama, Mocuba and Milange districts, located in north and centre of Mozambique. The findings suggest that vector control approaches combining different types of insecticides might help to tackle the apparent problem of pyrethroid resistance in the vector populations from these three sites. Results from bioassays on laboratory-susceptible *A. arabiensis* strongly suggest that LLINs can offer some protection against susceptible malaria vectors.

Keywords: LLINs, Bio-efficacy, *Anopheles funestus*, *A. gambiae*, Insecticide resistance, Insecticide content

Background

Long-lasting insecticide-treated bed nets (LLINs), often in association with indoor residual spraying (IRS), have for decades contributed to the reduction of malaria burden in sub-Saharan Africa [1, 2]. Notwithstanding recent reductions in morbidity and mortality, due to these interventions the disease remains a major problem of public health in Mozambique. The disease is responsible for nearly 45 % of the all cases observed among hospital outpatients and approximately 56 % of internments in paediatric wards [3]. Despite a decline the rate of mortality of malaria remains high, accounting for approximately 26 % of all hospital deaths [4]. LLINs continue to be the key measure for vector control in rural settings throughout the country and, since the introduction of mass distribution campaigns in 2000, it has been estimated that more than 7.6 million LLINs have been distributed, both by the Ministry of Health and partners [4]. Recently, a proliferation of several brands of LLINs in both rural and city markets has been observed. These largely derive from donations from public, private and civil organisations. Despite being beneficial to the population in needing of protection, an uncontrolled variety of nets might inadvertently, contribute to the development and spread of new foci of pyrethroid resistant strains of the local vector populations. Evaluation of LLINs against local vectors in laboratory and field studies should be performed before mass distribution of any LLIN. Moreover, studies have reported that the chemical contents of some brands of LLIN occasionally differ significantly from the recommended target doses [5]. These findings, emphasize the necessity for scrutiny and careful selection of insecticidal-based control measures since the exposure of local vectors to either sub-lethal or higher doses than that recommended for public health pesticides might potentially exacerbate the problem of insecticide resistance, as shown elsewhere [6, 7].

Anopheles funestus, *A. gambiae* sensu stricto (*s.s.*) and *A. arabiensis* are the most important malaria vectors found in Mozambique [8–11], whilst *A. merus* has been reported as playing secondary role on malaria transmission along the coastal regions [12].

High levels of phenotypic and metabolic resistance against the pyrethroids, deltamethrin, permethrin and alpha-cypermethrin and the carbamates propoxur and bendiocarb, have been reported in *A. funestus* from southern Mozambique [13]. However, the mosquito remained fully susceptible to DDT and malathion [14]. Resistance to lambda-cyhalothrin, permethrin and bendiocarb was reported among *A. funestus* from Zambézia Province, in the Central region [15], whereas low levels of pyrethroid and malathion resistance was detected in the provinces located in South (Maputo, Gaza and Inhambane) and Centre (Zambezia and Manica) of the country [16]. Published data on the status of insecticide susceptibility in the vector populations from Northern regions remain limited, notwithstanding, in 2006, Casimiro and colleagues [16] have reported full susceptibility to pyrethroids, carbamates and DDT in the population of *A. funestus* from Pemba city, Cabo Delgado Province; in *A. gambiae s.s.* and *A. arabiensis* from Namialo district and Nampula city, respectively, both at Nampula Province. This distribution of the patterns of malaria vector resistance against the major classes of insecticides, suggests that site-based evidence must be obtained to improve the sustainability of vector control programmes, as recommended in the WHO's Global Plans for Insecticide Resistance Management and Vector Control [17]. Therefore, laboratory study was conducted to evaluate the response of malaria vectors from Central and Northern Mozambique to selected types of WHOPES-recommended LLINs. The current status of vector susceptibility to selected insecticides from all major classes of insecticides, currently used for vector control, was also assessed, as well as, the concentration of insecticide on LLINs fabrics. The results are discussed with respect to current malaria control policies in Mozambique.

Methods

Description of study sites

The study was undertaken during the dry season, from June to August 2012, in Cabo Delgado (northern region) and Zambezia provinces (central region of Mozambique). In Cabo Delgado province larvae survey were undertaken in Balama district (13°20.914′S, 38°34.183′E), located in the southern part of the province, whilst in Zambezia larvae were collected in Mocuba (16°51.00′S, 36°59.00′E)

and Milange districts (16°5.810′S, 35°46.325′E) both located in the central and northeast part of Zambézia province, respectively. The three districts are among those having the highest malaria prevalence (≥40 %) in the country [18] with a low level of interventions [19].

Balama district is located at an altitude ranging from 200 to 570 m above the sea level. The climate is semi-arid with a rainy season from December to March. The mean annual precipitation ranges from 800 to 1200 mm, occasionally reaching a maximum of 1500 mm in those villages closest to the coast. The monthly air temperatures fluctuate from 20 to 25 °C. The Ruassa river is one of the most important sources of surface water in the district. The district hydrography has been dominated by underground rivers, which sometimes give rise to dispersed water bodies (locally known as Ndabo) due to either manmade excavations or through cracks that reach the surface.

Mocuba district is located at an altitude varying from 200 to 400 m above sea level. The wet season is from November to February, whilst the dry season ranges from March to October, between which some irregular rainfalls also occur. The mean annual rainfalls varies from 850 to 1300 mm and the mean monthly air temperature varies from 20 to 27 °C. Licungo and Lugela rivers are the most important sources of permanent water in the district.

Milange district is located at the northeast region of Zambézia province at an altitude varying from 200 to 1000 m above sea level. The district is boarded to the southeast by Mocuba district. The annual precipitation ranges from 800 to 1400 mm. The rainy season occurs between November and May and the mean monthly air temperature fluctuates from 24 to 26 °C.

In all three districts, during the wet season, the mean relative humidity varies from 60 to 80 %.

The people residing in the study sites are mainly subsistence farmers who grow crops such as rice, maize, beans, and manioc and cotton on the banks of small streams or rivers. Most houses are built of bamboo reinforced with mud and covered by either thatched roofs or corrugated zinc sheets. *Anopheles funestus* is the most common malaria vector in Balama and Milange district whilst *A. gambiae* sensu lato (*s.l.*) is the most common in Mocuba district. Other *Anopheles* and culicinae species, such as *A. tenebrosus*, *A. pharoensis*, *Mansonia* spp. and *Culex* spp. occur also.

Mosquito collection

Mosquito larvae were collected in both known and potential breeding sites located along the main rivers and water collections usually found in the three districts. Larvae were collected using pipettes, dippers and bowls, depending on whether the breeding site was small or large one [20].

In Balama district larvae were collected in four breeding sites; two situated in Kwekwe village (breeding site 1: 13°45.567′S; 38°24.767′E and breeding site 2: 13°57.139′S; 38°23.314′E) and the other two in Mavala (13°11.776′S; 38°18.345′E) and Impiri (13°19.861′S; 38°15.684′E) villages. In Mocuba district larvae were collected in Mocuba city (16°50.997′S; 36°59.000′E), whilst in Milange district collections were carried out in Majaua (16°16.919′S; 35°26.998′E) and Molumbo (15°47.301′S; 35°59.741′E) villages (Fig. 1).

Larvae collected in Balama district were brought to the insectary located in Pemba city, the capital of Cabo Delgado province, whilst those collected in Mocuba and Milange districts were brought to the insectary located in the Quelimane city, capital of Zambezia province. Larvae were transferred into bowls and held in the insectary at room temperature and humidity of 25.1 ± 2 °C and 80 ± 5.4 %, respectively, until eclosion to adult mosquitoes. Newly emerged adult females were sorted and identified morphologically according to available taxonomic keys [21]. *Anopheles funestus* and *A. gambiae s.l.* were kept in separate cages. Morphological identification was posteriorly confirmed by PCR analysis, for members of *A. funestus* group [22] and the *A. gambiae* complex [23].

Insecticide susceptibility tests

WHO susceptibility tests [24] were conducted to determine the susceptibility of collected vectors against permethrin (0.75 %), deltamethrin (0.05 %), bendiocarb (0.1 %), propoxur (0.1 %) and DDT (4 %). Only insecticides used to treat LLINs, as well as, those insecticides that have already been used or are currently being used for IRS. Twenty-five sugar-fed, 2–5 years old, females were transferred into testing cylinders containing papers impregnated with insecticide. The knockdown rate of mosquito exposed to the insecticides was recorded each 10 min, over 1 h exposure-period. At least four replicates were obtained for each type of insecticide tested, giving a minimum of 100 mosquitoes per insecticide. Concurrently, 50 mosquitoes (25 per cylinder) were exposed to papers impregnated with mineral oil to act as negative controls. Mosquitoes were later transferred into recovery cups and provided with cotton wool soaked in 10 % glucose solution and the final mortality was recorded 24 h later. If the mortality rate in the control cups was between 5 and 20 %, the final mortality rate was adjusted according to Abbott's formula. When the mortality rate in the controls was >20 %, the test was discarded. Vectors were considered as being susceptible to a given insecticide if mortality rate was ≥98 %, resistant if mortality <80 % or possibly resistant if mortality was between 80

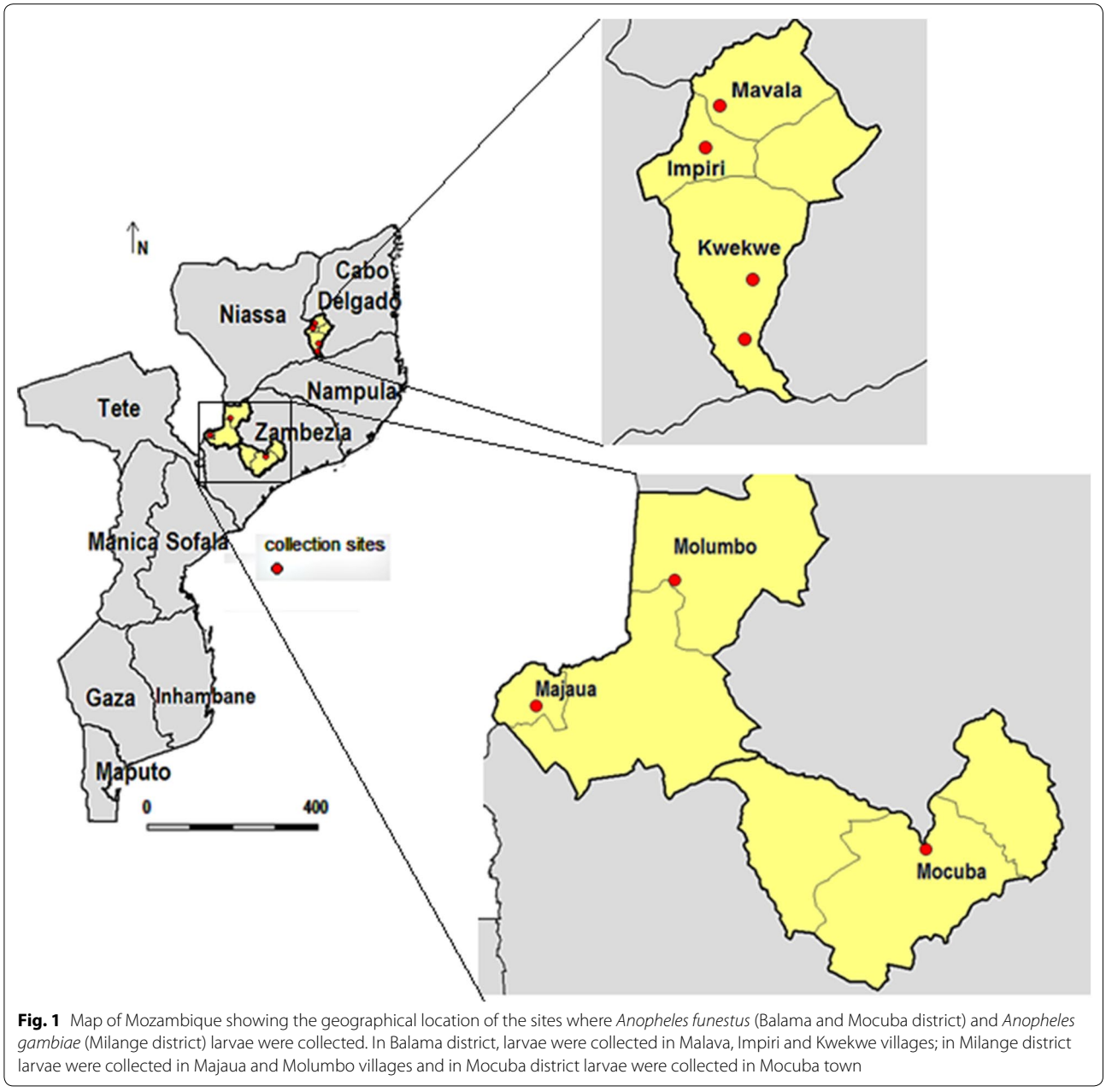

Fig. 1 Map of Mozambique showing the geographical location of the sites where *Anopheles funestus* (Balama and Mocuba district) and *Anopheles gambiae* (Milange district) larvae were collected. In Balama district, larvae were collected in Malava, Impiri and Kwekwe villages; in Milange district larvae were collected in Majaua and Molumbo villages and in Mocuba district larvae were collected in Mocuba town

and 98 %. Mosquitoes from this test were later stored in tubes continuing silica gel and, a random sample of 194 (94 *A. funestus* and 100 *A. gambiae s.l*) mosquitoes was taken for molecular identification of vector complexes members [23]. Those specimens identified as either *A. gambiae* or *A. arabiensis* were later screened for the presence of target-site resistance *kdr* (East and West) mutations by allele-specific polymerase chain reaction, as suggested by Martin-Torre et al. [25] and Ranson et al. [26]. These mutations have often been found associated with pyrethroid/DDT cross-resistance in populations of *A. gambiae s.s.*, *A. arabiensis* and other vectors [27] but not reported in members of the *A. funestus* group [28].

Extraction and preparation of LLINs sub-samples

The main goal was to determine the bio-efficacy and insecticide content of LLINs available in Mozambique. Five types of rectangular LLINs were investigated namely: pyrethroid-only Olyset® (polyethylene fabric incorporated with 20 g/kg of permethrin), Permanet 2.0® (polyester fabric coated with 55 mg/m^2 of deltamethrin), NetProtect® (polyethylene incorporated with

1.8 g/kg of deltamethrin), Interceptor® (polyester coated with 200 mg/m^2 of alpha-cypermethrin) and combination LLINs Permanet 3.0®. Permanet 3.0® is made mainly of polyethylene fabric incorporated with 2.1 g/kg ± 25 % of deltamethrin alone (on the upper sides) and 4 g/kg ± 25 % of deltamethrin combined with 25 g/kg of a synergist piperonil butoxide (PBO) on the roof and coated with 2.8 g/kg ± 25 % of deltamethrin on the lower sides, also called borders. The lower sides are reinforced with polyester fabric [29, 30]. The PBO acts by enhanced the penetration rate of the insecticide deltamethrin through the insect cuticle inhibiting, thereby, the insects defence mechanisms, particularly the effect of enzymes P450 monooxygenases [31]. Olyset® and Permanet 2.0® are usually distributed as part of either mass or antenatal distribution campaigns whilst NetProtect® and Interceptor® are available for purchase at some local markets. Therefore, Olyset® and Permanet 2.0® were obtained through public and private partners of the Mozambique Ministry of Health, currently supporting the National Malaria Control Programme (NMCP) whereas, NetProtect® and Interceptor® were obtained by convenience and availability from the local markets. The hygiene conditions in the particular place of selling, as well as, the storage conditions of the LLINs was carefully inspected before proceeding with the purchasing of the nets. The combination LLINs PermaNet 3.0® were kindly donated by Vestegaard Frandsen Ltd. All LLINs were carefully inspected to verify the physical integrity of the packet, manufacturing date and batch number.

Three samples of each LLIN were obtained. For each pyrethroid-only LLIN, (viz: Olyset®, Permanet 2.0®, NetProtect® and Interceptor®), three 30 × 30 cm swatches from each long side and from the roof of the net were taken, making a total of 9 (3 × 3 LLINs) swatches per type of LLINs, whilst for the combination LLIN (PermaNet 3.0®), two swatches from the long lower sides (borders), two from the long upper sides and one from the roof were taken, giving a total of 15 (5 × 3 LLINs) samples. Individual samples were wrapped in aluminium foil and placed inside plastic labelled zip lock bags to prevent possible cross-contamination between sub-samples.

WHO cone bioassay

WHO Cone bioassays were conducted with 2–5 day old sugar-fed females following standard WHO procedures [32]. Four cones, each containing five mosquitoes, were put in contact to 30 × 30 cm swatches taken from the sides and roof of pyrethroid-only LLINs (Olyset®, Permanet 2.0®, NetProtect® and Interceptor®) and combination LLIN (PermaNet 3.0®). Mosquitoes were exposed for 3 min after which were transferred into recovery paper cups and provided with cotton wool soaked in a solution of 10 % glucose. Each swatch was tested twice, giving a total of 40 mosquitoes tested per swatch, i.e., 20 mosquitoes per 2 replicates. Mosquito knockdown rate (KD) was recorded every 30 min during a 1-h post-exposure period (KD 60) and the final mortality rate (MT) was determined 24 h post-exposure. The mortality rate was corrected using Abbott's formula when mortality in the control was 5–20 %. Otherwise, if mortality rate in the control tube was >20 %, the bioassay round was discarded and a new test was conducted. A total of 360 mosquitoes (40 mosquitoes × 3 swatches × 3 LLINs samples) were used to test each type of pyrethroid-only LLIN whilst, 600 (40 mosquitoes × 5 swatches × 3 LLINs samples) were used to test Permanet 3.0®. A random sample of 477 (321 *A. funestus* and 156 *A. gambiae s.l.*) mosquitoes from this assay was used for molecular identification of vector complexes members and determination of *kdr* (West-East) resistance allele mutations, as above indicated [25]. Cone bioassays were also conducted against a susceptible colony of *A. arabiensis* (Durban strain) maintained at the entomology laboratory of the National Institute of Health (INS) in Maputo city. These tests were conducted at a room temperature and relative humidity of 25 ± 2 °C and 80 ± 5 %, respectively. The susceptibility status of the colony against the classes of insecticides commonly used for vector control has been assessed every 6 months. Sub-samples from an untreated bed-net were used concurrently as negative controls of the bioassays.

Chemical analysis for insecticide contents

Additional samples of netting from the sides of pyrethroid-only LLINs and Permanet 3.0® were collected for chemical analysis to determine if the insecticide content of the fabric was within the recommended target range. The insecticide content was determined through High Performance Liquid Chromatography (HPLC) using protocols developed by the Collaborative International Pesticides Analytical Council (CIPA) [33, 34]. Thus, deltamethrin was extracted in a mixture of iso-octane and 1,4-dioxane solution and the concentration was determined by normal-phase HPLC using dipropyl phthalate as internal standard and detection at 236 nm, whilst, alpha-cypermethrin was extracted with n-hexane and 1, 4-dioxane (95:5 v/v), shaken, sonicated and later filtered on a 0.45 mm Teflon membrane. Permethrin and piperonil-butoxide (PBO) were both extracted in the presence of hot xylene followed by drying, reconstitution and filtrations process before the final concentration was determined by HPLC. Insecticide concentration (IC) was calculated using the formula (An/As) × Cs × (Vn/ms), where An is the area of the insecticide peak in net sample, As is average area of the insecticide peak in the working standards (from a single point calibration prepared at

the target concentration), Cs is average concentration of the working standards (mg/ml), Vn is volume of sample solution (100 ml) and ms is mass of net sample [35].

Statistical data analysis

The significance of the differences between knockdown (KD 60) and mortality rates of mosquitoes exposed to different types of LLIN were analysed by Generalized Linear Mixed Models (GLIMM) using binomial error distribution and logit link function [36]. Initially, GLIMM tests were applied using lme4 v. 1.1–7 package [37], the type of LLIN was considered as fixed factor, whilst the sides and roof of it was considered as a random factor nested within each bed net type, so as to account for any possible non-constant variability of knockdown and mortality rates between the side of LLINs and any possible correlations between repeated measures taken from the same swatch. Subsequently, the fitted models for each study site and species tested were used to determine the significance of difference of KD 60 and mortality rate between the types of LLINs using the package multcomp v. 1.3–7 [38]. The Tukey HSD test was applied to assess the significance of the differences. The *p*-values estimated by the Tukey HSD test was adjusted to account for multiplicity and correlation between statistics using the Westfall truncated closed test procedure, implemented also with multcomp v. 1.3–7 [39]. Probit regression analysis was applied to mortality rates from the susceptibility tests to estimate the median exposure time necessary to kill 50 % (KDT_{50}) and 95 % (KDT_{95}) of the vector populations when exposed to each class of insecticides tested, using the package drc [38]. All statistical analysis were performed using R v. 3.1.2 [40].

Ethical considerations

The study received ethical approval by the National Committee of Bioethics of the Mozambique Ministry of Health, under the registration number 06/CNBS/12.

Results

Vector populations

1680 *A. funestus* from Balama and 1670 Mocuba districts and 1720 *A. gambiae s.l.* from Milange district were used to perform cone bioassays. 10 data points of Permanet 3.0 from Milange district were missing. Additionally, 500 *A. funestus* and 400 *A. gambiae* were used to undertake the susceptibility tests against Propoxur, Deltamethrin, Permethrin, Bendiocarb and DDT.

All 415 members of the *A. funestus* group analysed by PCR were *A. funestus s.s* and all 256 *A. gambiae s.l.* were *A. gambiae s.s*, S form. Therefore we presume that these were the only two vector species in the study.

Insecticide susceptibility

The knockdown rates of *A. funestus s.s.* and *A. gambiae s.s.* (henceforth *A. funestus* and *A. gambiae*) exposed to five selected insecticides are showed in Fig. 2a, b, respectively. The probability of an insect being knocked down during the first 30 min of exposure varied from 0 to 46 % (in *A. funestus*) and 0 to 50 % (in *A. gambiae*), suggesting that a high frequency of resistant strains in the two vector populations. These results were later corroborated by the estimates of the median time (in minutes) required to kill 50 % [KDT_{50} (±95 % CI)] and 95 % [KDT_{95} (±95 % CI)] of the vectors populations when exposed to the same insecticides (Tables 1, 2). There was no expressive difference between these estimates for either species. The smallest KDT_{50} estimate for *A. funestus* was observed when mosquitoes were exposed to propoxur [29.37 (27.17–31.58)], whilst the smallest KDT_{95} was observed against bendiocarb [58.84 (53.18–64.50)]. The shortest KDT_{50} and KDT_{95}, in *A. gambiae* was observed with deltamethrin [31.61 (29.81–33.41)], bendiocarb [33.28 (31.55–35.01)] and propuxor [34.9 (33.24–36.61)] whilst, the shortest KDT_{95} estimate was 62.29 (56.32–68.26) obtained against bendiocarb and propoxur, respectively.

In Balama district, the mortality rates of *A. funestus* recorded 24 h post-exposure, suggest that it might be resistance to virtually all four types of insecticides tested (Table 1). In Mocuba, on the other hand, results suggested that *A. gambiae* might be resistant to permethrin, propoxur and DDT and, susceptible to deltamethrin and bendiocarb (Table 2). However, molecular analysis failed to reveal the presence of kdr gene mutant alleles in random sample of 250 specimens of *A. gambiae* tested. Susceptibility tests were not performed on mosquitoes from Milange district due to the low number of mosquitoes collected.

Bio-efficacy of LLINs against wild-caught vector populations

135 LLINs swatches were obtained, 90 from pyrethroid-only LLINs and 45 from combination Permanet 3.0®. 84 swatches were tested against *A. funestus* from Balama (42/135) and Mocuba (42/135) districts and 51/135 against *A. gambiae* from Milange district, respectively the knockdown (KD 60) and mortality rates of the two species exposed to the five types of LLINs are depicted on Table 3 and Fig. 3. There was a significant difference in both knockdown ($F = 151.52$, $P < 0.0001$) and mortality rates ($F = 181.74$, $P < 0.0001$) of mosquitoes exposed to LLINs. In addition, there was also a significant correlation between the knockdown rate and mortality rate ($R^2 = 0.857$, $P < 0.0001$), when the data were stratified by species and study sites, indicating that previous exposure of mosquitoes to insecticides on bed nets explained

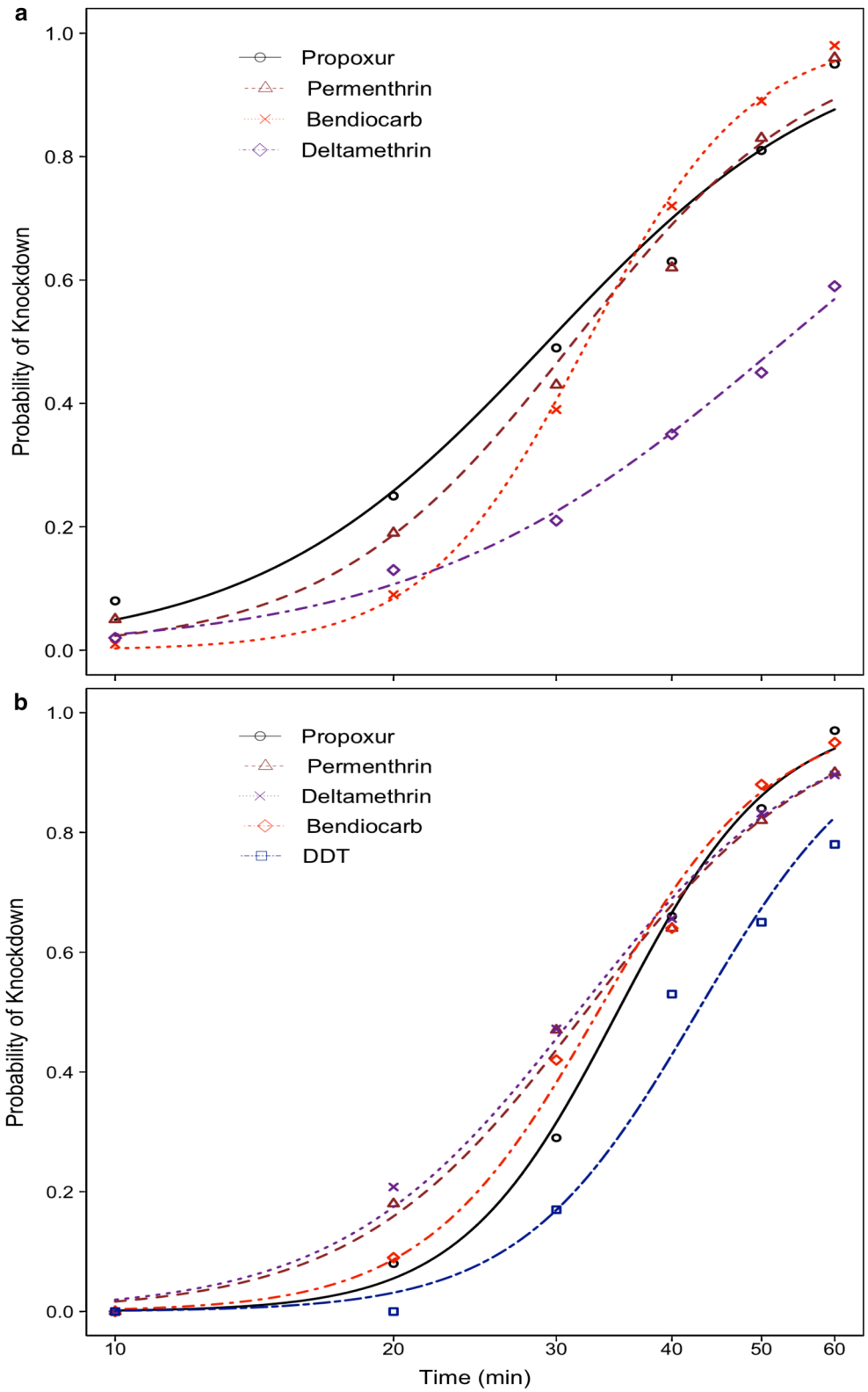

Fig. 2 Response curves showing the probability of knockdown of *Anopheles funestus* **a** from Balama distric and *Anopheles gambiae s.s* **b** from Mocuba district exposed to selected types of insecticides over 60 min exposure-time

Table 1 Mortality rate of *Anopheles funestus* from Balama district exposed to four types of insecticides and, the estimated median time (in minutes) required to kill 50 % (KDT_{50} ± 95 % CI) and 95 % (KDT_{95} ± 95 % CI) of the vector population when exposed to the same insecticides

Insecticide	Mosquito tested	KDT_{50} (± 95 % CI)	KDT_{95} (± 95 % CI)	Mortality rate
Deltamethrin (0.05 %)	100	52.81 (47.11–58.51)	203.07 (127.33–278.81)	85
Permethrin (0.75 %)	100	31.33 (29.28–33.38)	77.08 (65.88–88.29)	97
Bendiocarb (0.1 %)	100	32.42 (30.78–34.06)	58.84 (53.18–64.50)	92
Propoxur (0.1 %)	100	29.37 (27.17–31.58)	85.92 (70.97–100.88)	94

Table 2 Mortality rate of *Anopheles gambiae* from Mocuba district exposed to five types of insecticides and, the estimated median time (in minutes) required to kill 50 % (KDT_{50} ± 95 % CI) and 95 % (KDT_{95} ± 95 % CI) of the vector population when exposed to the same insecticides

Insecticide	Mosquito tested	KDT_{50} (95 % CI)	KDT_{95} (95 % CI)	Mortality rate
Deltamethrin (0.05 %)	125	31.61 (29.81–33.41)	75.24 (65.82–84.65)	99.2
Permethrin (0.75 %)	100	32.26 (30.25–34.27)	75.11 (64.80–85.42)	97
Bendiocarb (0.1 %)	100	33.28 (31.55–35.01)	62.29 (56.32–68.26)	99
Propoxur (0.1 %)	100	34.9 (33.24–36.61)	62.29 (56.33–68.26)	98
DDT (4 %)	100	42.6 (40.51–44.69)	81.59 (71.40–91.77)	97

Table 3 Knockdown (KD 60 ± standard error) and mortality (±standard error) rates of *A. funestus* (Balama and Mocuba district) and *A. gambiae* from Milange district tested against five brands of Long-lasting insecticide-treated bed nets (LLINs)

LLINs	Mosquito tested per site	Bio-efficacy indexes	Study districts		
			Balama (*A. funestus*)	Mocuba (*A. funestus*)	Milange (*A. gambiae*)
Olyset	360	KD 60 (±se)	*35.55 (±3.15)*	*49.14 (±2.47)*	*57.5 (±2.71)*
		Mortality rate (±se)	*20.9 (±2.34)*	*38.07 (±3.07)*	*40.77 (±2.82)*
Permanet 2.0	360	KD 60 (±se)	69.72 (±3.40)	78.61 (±2.18)	91.25 (±1.30)
		Mortality rate (±se)	60.48 (±3.64)	81.94 (±2.32)	89.65 (±1.65)
Permanet 3.0	600	KD 60 (±se)	93.33 (±1.12)	85.16 (±1.43)	99.64 (±0.36)
		Mortality rate (±se)	85.5 (±2.09)	90.16 (±1.27)	98.92 (±0.61)
NetProtect	360	KD 60 (±se)	*61.38 (±2.79)*	62.22 (±2.52)	83.88 (±1.61)
		Mortality rate (±se)	*23.95 (±2.34)*	63.61 (±2.95)	78.87 (±3.56)
Interceptor	360	KD 60 (±se)	–	–	80.83 (±1.87)
		Mortality rate (±se)	–	–	77.84 (±2.16)

(–) Not tested

Highlighted cell indicates where significant difference between knockdown and mortality rate was found at 5 % significance level

85.7 % of the total variation of mortality rates recorded 24 h post-exposure. Therefore, further statistical analyses were mainly focused on mortality rates as an indicator of bio-efficacy.

In general, the pyrethroid-only Olyset® (permethrin incorporated) showed the lowest bio-efficacy (mean ± standard error) against *A. funestus* from Balama (20.9 ± 2.34) and Mocuba (38.07 ± 3.07) and *A. gambiae* from Milange (40.77 ± 2.82) when compared to the same vectors exposed to other types of LLINs (Table 3). The highest bio-efficacy was observed with deltamethrin coated (Permanet 2.0®), deltamethrin incorporated (NetProtect®) and deltametrin incorporated/coated plus piperonyl-butoxide (PBO) incorporated Permanet 3.0® (Table 3). The LLIN Interceptor® (alpha-cypermetrin coated) was only tested against *A. gambiae* from Milange district. The mortality rate (±standard error) of *A. gambiae* exposed to Interceptor® was 77.84 ± 2.16. This was

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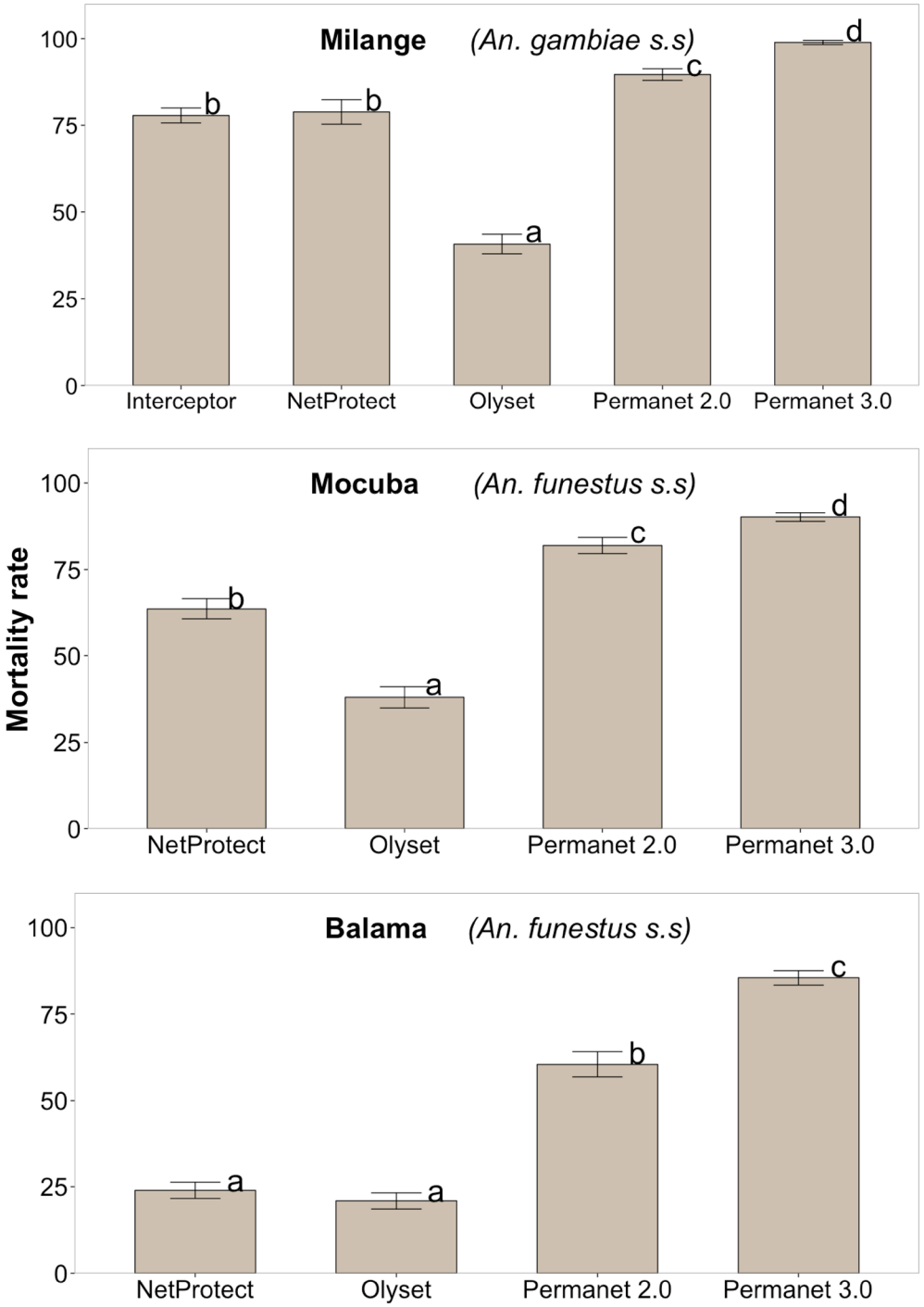

Fig. 3 Comparison of mortality rates of *Anopheles gambiae s.s* (Milange distric) and *Anopheles funestus* (Mocuba and Balama district) mosquito females exposed to different brands of LLINs. *Letters above each bar* display the significance of the difference of Mortality rates between pairs of bed nets, obtained by TukeyHSD at 5 % significance level. Mortality rates followed by the *same letter* are not statistically significant. The *letters* were sorted starting from lower (*a*) to higher (*d*) significant Mortality rate. P-values were adjusted using Westfall procedure (see Additional file 1 for further details)

similar to that obtained with Netprotect® (78.87 ± 3.56; $P = 0.839$). The mortality of *A. funestus* from Balama district exposed to Netprotect® (23.95 ± 2.34) and Olyset® (20.9 ± 2.34; $P = 0.129$) did not differ significantly (see Fig. 3; Additional file 1, for further details).

Results of mortality rates of mosquitoes exposed to Permanet 3.0® were stratified by site of the bed-net, namely, lower side (border), upper side and roof (Fig. 4). The mortality rate of *A. funestus* from Balama district exposed to roof sub-samples was significantly higher than of those exposed to samples from lower sides [Estimated difference ± standard error (se) = 18.54 ± 5.47; $P = 0.003$] and upper sides [Estimated difference ± se = 17.71 ± 5.46; $P = 0.003$]. A similar result was obtained with *A. funestus* from Mocuba district, i.e. estimated difference (±se) roof vs. lower side (11.88 ± 3.31; $P = 0.0013$); roof vs. upper side (10.63 ± 3.31; $P = 0.0017$). There was no significant difference of mortality rates of *A. gambiae* from Milange district, exposed to swatches from either sides of Permanet 3.0® (Fig. 4; Table 4; Additional file 2).

Bio-efficacy against colony susceptible vectors

A total of 51 swatches (36 from pyrethroid-only LLINs and 15 from combination Permanet 3.0®) were tested against the colony of susceptible *A. arabiensis* (Durban strain). The knockdown and mortality rates from this bioassay indicate that all type of LLINs performed well against this mosquito (Table 5). The mortality rate (±standard error) varied from 90.36 ± 1.34 % to 100 ± 0.00 % when mosquitoes were exposed to Olyset®, Permanet 2.0® and Permanet 3.0®, respectively. Comparisons between the mortality rates of wild-caught *A. funestus* and *A. gambiae* (see Table 4) and the *A. arabiensis* colony (Table 5) indicated that the mortality rate of the *A. arabiensis* exposed to both Olyset® and NetProtect® was two to four times higher than the rates obtained with *A. funestus* from Balama and Mocuba district and *A. gambiae* from Milange district (see Additional file 3). There was no expressive difference of the ratios between the mortality rates of susceptible and wild-caught mosquitoes exposed to Permanet 2.0®, Permanet 3.0® and Interceptor® (Additional file 3).

Insecticide contents on the LLINs

Fifty-one swatches were assessed for insecticide concentration, 36 from pyrethroid-only LLINs and 15 from combination Permanet 3.0®. The results indicated that, the insecticide concentration on the swatches from sides (1.0 g/kg) and roof (1.0 g/kg) of NetProtect® were below the target dose range (1.8 g/kg) whereas, the sides (23.2 g/kg) and roof (23.6 g/kg) of Olyset® and roof (73.2 mg/m^2) of Permanet 2.0® had insecticide content above those specified by manufacturers (Table 6).

Discussion

Vector susceptibility to insecticides

The results from WHO susceptibility tests indicate that the *A. funestus* population from Balama district, Cabo Delgado Province, has possibly became resistant to all the four types of insecticides tested two of which were pyrethroids and two carbamates viz: deltamethrin (0.05 %), permethrin (0.75 %), bendiocarb (0.1 %) and propoxur (0.1 %), respectively. Resistance against the two pyrethroids may be due the over expression of the enzymes P450 mono-oxigenases [41], whilst resistance to carbamates may be to an elevated production of acetylcholinesterase [27]. Prior to undertaking this study, there was no previous report about the susceptibility status of malaria vectors from Balama district. However, results from this study contrast with that obtained in 2006 by Casimiro et al. [16] in Pemba city, located at approximately 250 km from Balama district, which reported full susceptibility (100 % of mortality) of *A. funestus* to lambdacyalothrin (0.05 %), deltamethrin (0.05 %), propoxur (0.1 %), malathion (5 %) and DDT (4 %). The authors also detected an elevated expression of glutathione-S-transferase (GST) in the wild population of *A. funestus* compared to laboratory-resistant *Aedes aegypti* strains. As such, the resistance to DDT found in Balama district may probably be related to elevated expression of GST associated with resistance to DDT in several insect populations, including malaria vectors [27]. Recently, it has been demonstrated that a single mutation (GSTe2) in the sequence of the gene that encodes for GST in *A. funestus* from Benin, can confer resistance to both DDT and pyrethroids [42]. Previous studies from Southern Mozambique have also reported a high level of pyrethroid resistance in *A. funestus* [13] consistently associated with a high expression of cytochrome P450 mono-oxygenases [43, 44]. Unfortunately, in Mocuba city, the number of *A. funestus* collected was not enough to perform susceptibility tests, other than those used for the cone bioassay. Meanwhile, approximately 500 larvae of *A. gambiae* were collect. Adults derived from these larvae were used to perform the susceptibility tests (Fig. 2b; Table 2). Results indicate that *A. gambiae* from Mocuba city remains susceptible to bendiocarb (0.1 %), propoxur (0.1 %) and deltamethrin (0.05 %) but is possibly resistant to permethrin (0.75 %) and DDT (4 %). These findings contrast with those from Abilio and colleagues [15], who, in 2011, reported full susceptibility of *A. gambiae s.l.* to pyrethroids and DDT.

There were no *kdr* gene resistant mutants detected in a random sample of n = 256 *A. gambiae* tested, despite the susceptibility tests suggesting resistance to DDT and pyrethroids. The *kdr* resistance mechanism has been consistently associated with cross-resistance to pyrethroid

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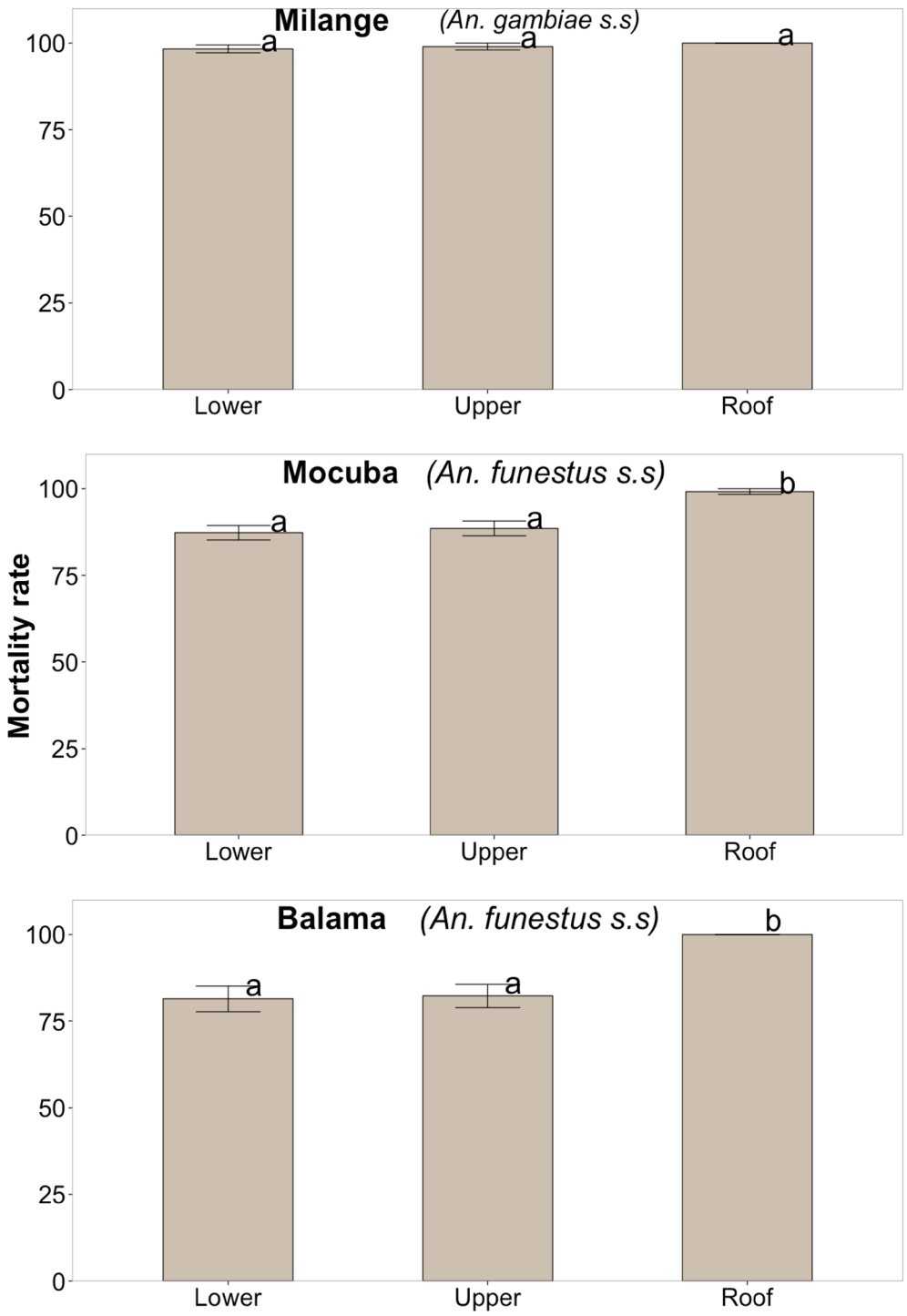

Fig. 4 Comparison of mortality rates of *Anopheles gambiae s.s* (Milange distric) and *Anopheles. funestus* (Mocuba and Balama district) mosquito females exposed to sides and roof of Permanet 3.0. *Letters above each bar* display the significance of the difference of Mortality rates between pairs of bed nets, obtained by TukeyHSD at 5 % significance level. Mortality rates followed by the *same letter* are not statistically significant. The *letters* were sorted starting from lower (*a*) to higher (*d*) significant Mortality rate. P-values were adjusted using Westfall procedure (see Additional file 2)

Table 4 Knockdown (±standard error) and mortality (±standard error) rates of *A. funestus* (Balama and Mocuba district) and *A. gambiae s.* from Milange district exposed to different sides of combination LLIN Permanet 3.0

Permanet 3.0 sides	Mosquito tested per site	Bio-efficacy indexes	Study sites		
			Balama (*A. funestus*)	Mocuba (*A. funestus*)	Milange (*A. gambiae*)
Lower side (border)	240	KD 60 (±se)	*90 (±2.06)*	84.58 (±1.91)	100 (±0.00)
		Mortality rate (±se)	*81.46 (±3.69)*	87.29 (±2.10)	98.33 (±1.15)
Upper side	240	KD 60 (±se)	*93.33 (±1.92)*	80 (±2.60)	99 (±1)
		Mortality rate (±se)	*82.29 (±3.35)*	88.54 (±2.13)	99 (±1)
Roof	120	KD 60 (±se)	100 (±0.00)	96.67 (±1.55)	100 (±0.00)
		Mortality rate (±se)	100 (±0.00)	99.17 (±0.83)	100 (±0.00)

Highlighted cell indicates where significant difference between knockdown and mortality rate of vector was found at 5 % significance level

Table 5 Knockdown (±standard error) and mortality rates (±standard error) of insectary-susceptible *A. arabiensis* (Durban strain) exposed to LLINs

Type of LLIN	Mosquito tested	KD 60 ± se	Mortality rate ± se
Olyset	360	68.33 ± 2.26	90.36 ± 1.35
Permanet 2.0	360	94.72 ± 1.12	100 ± 0.00
Permanet 3.0	600	98.17 ± 0.58	100 ± 0.00
NetProtect	360	83.89 ± 1.80	99.44 ± 0.39
Interceptor	360	80.56 ± 1.63	98.84 ± 0.57

and DDT in populations of *A. gambiae* and *A. arabiensis* [27]. The mechanism is yet to be identified in *A. funestus* [28]. Unfortunately, metabolic resistance assays were not carried out in this study. Therefore the insecticide resistance mechanism involved in conferring resistance among these insects is not known yet. Riveron et al. [42] have recently reported that a single amino acid change in the binding pocket of the glutathione-s-transferase epsilon 2 (GSTe2) gene confers a high level of DDT resistance and also cross-resistance to pyrethroids in *A. funestus*. The expression of GSTe2 mutation has also been widely documented in *A. gambiae* [45].

Bio-efficacy of pyrethroid-only LLINs

This study is the first to determine the response of wild-caught malaria vectors from Central (Mocuba and Milange districts) and Northern (Balama district) regions of Mozambique to commonly available types of LLINs. The results of cone bioassay indicated that the bio-efficacy of pyrethroid-only LLINs varied significantly depending on the vectors species tested (Fig. 3; Additional file 1). Overall, Olyset® and NetProtect® showed a dramatically lower bio-efficacy, regardless of vector species was tested (Table 3). Permanent 2.0® showed a higher bio-efficacy against both *A. funestus* from Balama and Mocuba city and against *A. gambiae* from Milange district, compared to either Olyset® or NetProtect®.

Table 6 Comparisons between measured and target dose of insecticide contents on swatches from sides and roof of LLINs

Net type	Active ingredient	Net section	Target mean dose	Target dose range	Measured mean dose	Measured dose within product target range?
Interceptor (IT)	Alpha-cypermethrin	Roof	200 mg/m^2	150.0–250.0	204.2 mg/m^2	Yes
	Alpha-cypermethrin	Sides	200 mg/m^2	150.0–250.0	204.2 mg/m^2	Yes
NetProtect (NP)	Deltamethrin	Roof	1.8 g/kg	1.35–2.25	1.0 g/kg	Under
	Deltamethrin	Sides	1.8 g/kg	1.35–2.25	1.0 g/kg	Under
Olyset (OL)	Permethrin	Roof	20 g/kg	17.0–23.0	23.2 g/kg	Over
	Permethrin	Sides	20 g/kg	17.0–23.0	23.6 g/kg	Over
Permanet 2.0 (PN2)	Deltamethrin	Roof	55 mg/m^2	41.25–68.75	73.2 mg/m^2	Over
	Deltamethrin	Sides	55 mg/m^2	41.25–68.75	65.8 mg/m^2	Yes
Permanet 3.0 (PN3)	Deltamethrin	Roof	4 g/kg	3.0–5.0	3.4 g/kg	Yes
	Deltamethrin	Lower side (border)	2.8 g/kg	2.1–3.5	3.0 g/kg	Yes
	Deltamethrin	Upper side	2.8 g/kg	2.1–3.5	3.1 g/kg	Yes
	PBO	Roof	25 g/kg	18.75–31.25	28.8 g/kg	Yes

However, in Balama district Permanet 2.0® had a lower bio-efficacy compared to that observed in Mocuba and Milange districts (Table 3). The lower performance of these two type of pyrethroid-only LLINs, particularly against *A. funestus* from Balama district, may be due to the existence of resistant individuals in the local vector population as demonstrated in the WHO susceptibility tests (Fig. 2a; Tables 1, 2). Olyset® and Permanet 2.0® have been the two main brands of LLINs usually distributed as part of mass and antenatal distribution campaigns in Mozambique. Thus, results from Balama district strongly suggest that Olyset® and Permanet 2.0® may not be effectively killing *A. funestus* in those regions where there are resistant population foci. Studies should be extended to other locations of Balama district in order to get the current picture on both phenotypic and metabolic insecticide resistance profile in the malaria vectors population and, thereby, be able to accurately predict the impact any control approach may have on the vector populations at district level. However, several studies have shown that LLINs still protect people against infectious mosquito bites despite insecticide resistance detected in the vector population, since the pyrethroids are also, to certain degree, repellent to mosquitoes [46] and, as long as the integrity of the fabric remains intact, the LLIN is also a physical barrier between sleepers and mosquitoes, [47]. In addition, more than 90 % of susceptible *A. arabiensis* were killed when exposed to LLINs in bioassays (Table 5; Additional file 3), suggesting that the LLINs can control susceptible mosquitoes. Interestingly, the mortality rate of *A. gambiae* from Milange exposed to both Interceptor® and NetProtect® was statistically similar ($P = 0.839$) (see Table 3; Fig. 3; Additional file 1); this suggest that the two types of LLINs might probably perform equally well in the field. Since they have been treated with different insecticide formulations then having both nets in use may reduce the selective pressures that favour the occurrence of resistant strains in the vector compared to the situation when a single type of insecticide or LLINs is used. Unfortunately, the bio-efficacy of Interceptor® against vectors from Balama and Mocuba was not assessed. However, the knockdown and mortality rate of *A. funestus* from Furvela village, in southern Mozambique, exposed to Interceptor® swatches, suggested that the vector population was resistant to the insecticide (JD Charlwood et al., *unpublished report*).

Bio-efficacy of combination Permanet 3.0®

Permanet 3.0® performed well against the two malaria vectors populations, irrespective of the level of resistance to pyrethroids. *Anopheles funestus* from Balama and Mocuba district exposed to swatches from the roof had the highest mortality compared to mosquitoes exposed to the upper and lower sides of the net whilst the mortality rates of *A. gambiae* from Milange district was independent of the location tested (Table 4; Fig. 4; Additional file 2). The higher mortality rates observed when mosquitoes were exposed to roofing swatches of Permanet 3.0® was probably due to the presence of the synergist PBO and the higher concentration of insecticide on the fabric of the roof of the net. In southern Mozambique, Brooke and colleagues [13] managed to revert the resistance of *A. funestus* against the lambda-cyalothrin after pre-exposing the insect to PBO. This prompted the authors to suspect that the mean metabolic resistance involved at the time (in 2001) was the over expression of enzyme mono-oxygenases; later reported in *A. funestus* from Belulane district [43] and recently in *A. funestus* from Chókwè villages [44]. The higher concentration of deltamethrin in the roofing fabrics compared to sides of Permanet 3.0® may have also caused higher mortality rate of mosquitoes exposed to it. However, increased insecticides concentration may be, per se, a counterproductive measure, since it can also contribute to rapid selection of resistant strains in the population, as discussed in [48]. Previous and recent field and laboratory works have reported better performance of combinations of "two-in-one" approaches, i.e. the combination of pyrethroid and non-pyrethroid insecticides applied to different parts of the bed nets [49]. However, recent reports have demonstrated that the better performance of Permanet 3.0® has been only achieved with unwashed bed nets [50, 51]. These studies have also noted that the biological activity of both deltamethrin and PBO tend to reduce significantly after a few washes, despite a high concentration of the two insecticidal compounds [52], suggesting that further investigation on insecticide retention by Permanet 3.0® fabrics must be done to improve the field performance of the net.

Insecticide concentration on bed nets

Chemical analysis of swatches from the sides and roof of the nets indicated that the insecticide content from the sides and roof of Olyset® and the roof of Permanet 2.0® was above the target dose. On the other hand, the insecticide concentration of NetProtect® was below that recommended dosage (Table 6). Intriguingly, Olyset® showed a low performed against both vectors species despite high level of insecticide found. This implies that different types of insecticide resistance mechanisms are involved. Laboratory and field evidence has shown that the insecticide concentration on the fabric of a LLIN decays over time, for instance after 6 months of intensive usage and washes, as recently reported in Permanet 3.0® [51] or due to bad storage. However, in the present study new nets were tested. The integrity of the packets and the expiration

date of each type of LLIN were carefully verified before the extraction of the sub-samples. Therefore, the low insecticide content observed in NetProtect® swatches was caused by unidentified factors. Similar studies have reported significant differences of insecticide contents between the sides and roof of Permanet 2.0® [5] and Permanet 3.0® [53]. These findings have obvious operational implications since the concurrent exposure of vectors to varying doses of the same insecticides might potentiate resistance in the vector [6].

All types of LLINs tested in this study performed remarkably very well against the colony of susceptible *A. arabiensis*, maintained at the insectary of the National Institute of Health (INS) in Maputo.

Conclusion

Considerable heterogeneity in both, insecticide susceptibility and the level of bio-efficacy of commonly available types of LLIN's was observed among pyrethroid resistant populations of wild-caught *A. funestus* and *A. gambiae* from northern and central Mozambique. The findings suggest that vector control approaches by combining different types of pyrethroid-based methods, particularly LLINs, might help to tackle the apparent problem of pyrethroid resistance in the malaria vectors such as these, as it would both increase the killing efficacy against the vectors and concurrently reduce the selective pressures favouring the occurrence of resistant strains. The on-going management of insecticide resistance in vector control programmes is, obviously, mandatory for an effective malaria control. Results from bioassays against susceptible *A. arabiensis* strongly suggested the LLINs tested will still kill susceptible mosquitoes and so can help reduce transmission. Similar studies should be extended throughout the country in order to fill the gaps in the current knowledge concerning the status of phenotypic and metabolic resistance of malaria vectors populations, as well as, to determine the extent to which vectors might respond to insecticide-based vector control approaches prior to their implementation.

Additional files

Additional file 1: Results of pair-wise comparisons, obtained by TukeyHSD test, of overall mortality rates of mosquitoes exposed to different types of LLINs.

Additional file 2: Results of pair-wise comparisons, obtained by TukeyHSD, of mortality rates of mosquitoes exposed to different sides of Permanet 3.0.

Additional file 3: Ratio between the mortality rate of insectary-susceptible *Anopheles arabiensis* (Durban strain) and the mortality rate of wild-caught *Anopheles funestus* (from Balama, Mocuba district) and *Anopheles gambiae s.s.* from Milange district. The figure shows that the LLINs can still remarkably killing higher number (mortality rate > 90 %) of susceptible mosquitoes.

Authors' contributions

APA conceived the study, helped write the protocol, co-ordinated and supervised the field work and drafted the manuscript; PM, helped coordinate and supervise the field work in Cabo Delgado Province; ND and FM, helped with the preparation of field work, co-ordination, logistics/material/reagents; PM helped coordinate and supervise field work in Zambézia Province and reviewed the manuscript; AK, helped design the study, write the protocol, coordinated and supervised the field work, analysed the data and wrote the final manuscript. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declared that they have no competing interests.

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WHO cone bio-assays of classical and new-generation long-lasting insecticidal nets call for innovative insecticides targeting the knock-down resistance mechanism in Benin

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Abstract

Background: To increase the effectiveness of insecticide-treated nets (ITN) in areas of high resistance, new long-lasting insecticidal nets (LLINs) called new-generation nets have been developed. These nets are treated with the piperonyl butoxide (PBO) synergist which inhibit the action of detoxification enzymes. The effectiveness of the new-generation nets has been proven in some studies, but their specific effect on mosquitoes carrying detoxification enzymes and those carrying both detoxification enzymes and the knock-down resistance gene in Benin is not well known. Thus, the objective of this study is to evaluate the efficacy of LLINs treated with PBO on multi-resistant *Anopheles gambiae* s.l.

Methods: The study occurred in seven cities in Benin, Abomey, Cotonou, Porto-Novo, Zangnanado, Parakou, Malanville and Tanguiéta, and included ten locations selected on a north–south transect. Mosquito larvae were collected from these sites, and adult females from these larvae were exposed to single-pyrethroid-treated nets (LifeNet, PermaNet 2.0, Olyset Net) and bi-treated nets (PermaNet 3.0 and Olyset Plus) based on their level of resistance and using WHO cone tests following WHO guidelines.

Results: The different LLINs showed 100% mortality of the susceptible laboratory strain Kisumu and the resistant strain Ace-1R Kisumu. However, with the resistant laboratory strain kdr-Kisumu, mortality was low (16–32%) for all LLINs except PermaNet 3.0 (82.9%). The mortality of local strains carrying only the *kdr* mechanism varied from 0 to 47% for the single-pyrethroid-treated LLINs and 9 to 86% for bi-treated LLINs. With local strains carrying several mechanisms of resistance (kdr + detoxification enzymes), the observed mortality with different LLINs was also low except for PermaNet 3.0, which induced significantly higher mortality, usually greater than 75% ($p < 0.001$), with multi-resistant strains. The inhibition of the mortalities induced by the LLINs (11–96%) on multi-resistant field populations was similar to the inhibition observed with the laboratory strain carrying only the knock-down resistance mechanism (kdr-Kisumu) ($p > 0.05$).

Conclusion: This study showed that the new-generation LLINs treated with pyrethroids and PBO showed better efficacy compared to conventional LLINs. Although the addition of PBO significantly increased the mortality of mosquitoes, the significant role of the *kdr* resistance gene in the low efficacy of LLINs calls for LLIN technology innovation that specifically targets this mechanism.

Keywords: LLINs, Bio-efficacy, Piperonyl butoxide, Resistant mosquitoes

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Background

Malaria is a major public health problem worldwide, and particularly so in Benin. It remains a permanent threat from its high morbidity (214 million) and mortality (438,000). Africa is the most endemic region affected (395,000 deaths per year) [1]. It affects one-fifth of the world population. However, this proportion has decreased significantly by 37% between 2000 and 2015 due to the effect of malaria prevention and treatment methods, including long-lasting insecticidal nets (LLINs), indoor residual spraying of residual insecticides (IRS), chemo-prevention for pregnant women and children, and therapeutic treatment with artemisinin-based combinations.

Among these prevention methods, LLINs have emerged in recent years as a privileged tool to prevent malaria. The insecticides selected by the World Health Organization (WHO) for LLIN treatment are pyrethroids, which have little toxicity to humans, are effective at low doses, are fast acting (knock-down effect) and, along with repellants, have an irritant effect [2]. The Abuja Conference, which brought together all the leaders of Africa and other UN representative states, donors and NGOs in April 2000, gave impetus to a political commitment to the fight against malaria with the use of insecticide treated nets (ITNs) [3]. Efforts are being made to increase accessibility for populations, especially pregnant women and children under five, who are vulnerable to malaria, a major cause of perinatal mortality, low birth weight and maternal anaemia [1].

Several research studies have been conducted and have shown the effectiveness of ITNs in the fight against malaria in Burkina Faso [4], Cameroon [5], Gambia [6–9], the Democratic Republic of Congo [10], Kenya [11], Ghana [12], Benin [13] and Côte d'Ivoire [14].

However, several studies have shown that *Anopheles gambiae* s.l. has developed strong resistance to pyrethroids and DDT in Benin, with a very high knock-down resistance frequency of approximately 80% in the urban areas of Cotonou and in rural areas [15–23].

Despite this resistance developed by *An. gambiae* s.l. to pyrethroids, LLINs remain effective in vector resistance areas [24] and provide protection through their mechanical barrier role [25]. However, Asidi et al. [26] showed a decrease in their effectiveness in areas of high resistance of Anopheles in southern Benin. Major developed resistance mechanisms are the targets of modification (*kdr* resistance and *ace-1R*) and metabolic resistance (overexpression of detoxification enzymes, oxidases, esterases, GST) [27]. The *kdr* mutation is associated with pyrethroid and DDT resistance, and *ace-1R* is associated with organophosphate and carbamate resistance (two classes of insecticides which are not used to treat LLINs) [15, 28].

To increase the effectiveness of ITNs in areas of high resistance, new nets treated with a so-called new-generation of chemicals has been developed. They are treated with a synergist called piperonyl butoxide (PBO). For some LLINs, the PBO is used on all sides of the net (Olyset Plus®). For others, only the upper part of the net is processed (PermaNet® 3.0). The principle of an ITN synergist is to inhibit the action of detoxification enzymes, which will result in increasing the effectiveness of the insecticide against resistant populations of mosquitoes.

Evidence of the efficacy of PermaNet 3.0 has been shown in some studies, particularly in Tanzania [29], but we do not know its specific action on mosquitoes carrying detoxification enzymes and on those carrying both detoxification and *kdr* mechanisms in West Africa, particularly in Benin. There have been limited data on the bio-efficacy of new-generation LLINs against multi-resistant mosquitoes in Africa in general and particularly in Benin. Thus, the objective of this study is to evaluate the efficacy of long-lasting insecticidal nets (LLINs) treated with PBO on multi-resistant *An. gambiae* s.l. populations in Benin. It aims to assess the bio-efficacy of LLINs in areas with a high frequency of molecular resistance genes (*kdr* and *ace-1R*) and over-expression of detoxification enzymes (oxidases, esterases, GST). The efficacy of the new-generation LLINs against pyrethroid-resistant Anopheles was also compared to that of conventional LLINs.

Methods

Study design

This study is transversal and compares variability of the efficacy of two different types of LLINs against *An. gambiae* s.l. carrying *kdr* resistance mutations and detoxification enzymes in Benin. The two types of LLINs included conventional LLINs only treated with pyrethroids (Olyset Net, LifeNet, and PermaNet 2.0) and a second type of new-generation LLIN treated with pyrethroids and piperonyl butoxide (PBO), which inhibits the action of enzymes, particularly oxidases.

The study was conducted in Benin, a West African country from June 2015 to March 2016. Among the 12 departments of Benin surveyed, seven were selected in this study (Atlantique, Littoral, Oueme, Zou, Borgou, Atacora and Alibori). Priority was given to areas where higher oxidase activity was observed compared to the susceptible strain *An. gambiae* Kisumu. They were represented by Abomey, Cotonou, Porto-Novo, Zangnanado, Parakou, Malanville and Tanguiéta districts. The assessment of oxidase activity was conducted on 50 *An. gambiae* s.l. collected from each district using haem-peroxidase assay as described by Brogdon et al. [30].

The larvae of these mosquito populations were collected in different ecological areas (vegetable, urban, rice and cotton areas). The study was also conducted on resistant laboratory strains (kdr-Kisumu and ace-1R-Kisumu).

Study sites

Malanville

Malanville district is bordered on the north by the Republic of Niger, on the south by Kandi and Segbana districts, on the west by Karimama district and on the east by the Republic of Nigeria. It has an area of 3016 km^2 and had a population of 144,843 inhabitants in 2013 (Fig. 1).

Tanguieta

It is bordered on the north by the Republic of Burkina Faso, on the south by Boukoumbe district, on the east by Kerou, Kouande and Tounkountouna districts and on the west by Materi and Cobly districts. It covers an area of 5456 km^2 and had a population of 77,987 inhabitants in 2013 (Fig. 1).

Abomey-Calavi

Abomey-Calavi is bounded on the north by Ze district, on the south by the Atlantic Ocean, on the east by Cotonou and So-Ava districts, and on the west Ouidah and Tori-Bossito districts. It has an area of 539 km^2 and had a population of 438,564 inhabitants in 2013 (Fig. 1).

Cotonou

Cotonou is bordered on the North by So-Ava district and Nokoue lake, on the south by the Atlantic Ocean, on the east by Seme-Podji and on the west by Abomey-Calavi district. It has an area of 79 km^2 and had a population of 947,917 inhabitants in 2013 (Fig. 1).

Porto-Novo

Porto-Novo is bounded on the north by Akpro-Missérete and Avrankou districts, on the south by Seme-Podji, on the west by Aguegues district and on the east by Adjarra district. It covers an area of 223,552 km^2 and had a population of 318,608 inhabitants in 2013 (Fig. 1).

Parakou

It is bordered on the north by N'Dali district and on the south, east and west by Tchaourou district; it has an area of 441 km^2 and had a population of 213,498 inhabitants in 2013 (Fig. 1).

Zangnanado

This town is bounded on the north by Dassa-Zoume district, on the south by Ouinhi and Zogbodomey districts, on the west by Cove, Zakpota and Djidja districts and on the east by Ketou and Adja-Ouere. It has an area of 540 km^2 and had a population of 52,387 inhabitants in 2013 (Fig. 1).

Larvae collection

Bio-efficacy tests were conducted at various selected sites. Such tests required mosquitoes of 2–5 days old, so the larvae were collected. These collections were conducted in the different localities mentioned above. *Anopheles gambiae* s.l. larvae and pupae were collected from different locations at each site and carried to the insectarium of the Entomological Research Center of Cotonou (CREC), where they were reared to adult stage at a relative humidity of 70–80% and a temperature of 25–30 °C. Female adults aged 2–5 days were used for bio-efficacy tests.

Highlighting resistance mechanisms

Before the bioassays, living and dead mosquito populations kept after susceptibility testing were analyzed by PCR to detect the genotypes of the *kdr* gene. The detection of *kdr* mutation L1014F was performed according to the method of Martinez-Torres et al. [31].

For the molecular characterization of insecticide resistance, two molecular markers were used for characterization of the resistance genes, *kdr* and *ace-1R*.

Similarly, for the biochemical characterization of resistance mechanisms, biochemical assays were performed to compare the activity levels of mixed function oxidases (MFO), non-specific esterases (NSE) and glutathione S-transferases (GST) according to the protocol described by Hemingway et al. [32] in susceptible Kisumu and field *An. gambiae* strains. The mosquitoes used for biochemical analysis had not been exposed to insecticides before the biochemical assessment. These enzyme activities were measured using a sample of 50 mosquitoes per site.

Mosquito nets

Five types of long-lasting insecticidal nets were evaluated in this study. The group of mono-treated LLINs included LifeNet (polypropylene LLIN with fiber coated with 340 mg/m^2 ± 25% deltamethrin), Olyset Net (polyethylene LLIN with permethrin incorporated into the fibers at 20 ± 3 g/kg), and PermaNet 2.0 (polyester LLIN with fiber coated with deltamethrin at 55 mg/m^2 ± 25%). The group of new-generation LLINs included: Olyset Plus (same characteristics as Olyset Net but with PBO incorporated throughout the LLIN) and PermaNet 3.0 (polyethylene roof with deltamethrin at 2.8 g/kg ± 25% and PBO at 4.0 g/kg ± 25% incorporated into the fibers, and polyester lateral sides with the fibers coated with deltamethrin at 2.8 g/kg ± 25%). All these nets were obtained from local markets. All nets included in the study are rectangular and were selected by type.

Cone test

The cone test is used to assess the effectiveness of an insecticide and its persistence on the net. It was conducted following the WHO protocol. This test aims to compare the behaviour of mosquitoes while in contact with treated mosquito nets without PBO or with PBO.

Cone tests were performed on five types of nets (Olyset Plus, Olyset Net, LifeNet, PermaNet 2.0 and PermaNet 3.0). These tests were carried out using fragments of LLINs (30 cm × 30 cm) cut from five (05) positions on each net. Two standard cones were fixed with a plastic sheet on each of the five (05) screen fragments. For PermaNet 3.0 LLIN, an additional two cones were added on the PBO-containing roof. Five unfed *An. gambiae* females aged 2–5 days (Kisumu or wild type) were introduced into each cone placed on the LLIN for 3 min. After exposure, the mosquitoes were removed from the cones using a mouth aspirator and then transferred into paper cups and provided 10% sugar solution. Mosquito knock-down was recorded every 5 min for 60 min. A negative control (untreated net) was included in each series of cone tests. After 24 h of observation, mortality post exposure was recorded. No correction of mortality with Abbott's formula was used as mortality in the control was <5%. All these operations were carried out at a temperature of 25 ± 2 °C and a humidity of 70 ± 10%.

Data analysis

According to the WHO, the bio-effectiveness threshold is 95% knock-down and 80% mortality for laboratory mosquitoes; but for resistant field mosquito populations, we used a threshold of 70% knock-down and 50% mortality. Therefore, all nets showing less than 95% knockdown for laboratory mosquitoes and 70% for field mosquitoes after 60 min, or less than 50% mortality for laboratory mosquitoes and 50% for field mosquitoes after 24 h of observation, were considered ineffective. These knock-down thresholds were chosen taking into account the *kdr* resistance level observed in the country in general (>50%).

The inhibition of mortality induced by resistance mechanisms was estimated using the following equation:

$$\text{Inhibition} = 1 - (p1 / p2) \times 100$$

where p1 = proportion of resistant mosquitoes dead and p2 = proportion of susceptible Kisumu mosquitoes dead.

To determine if there was any significance difference between the outcome variables (knock-down, mortality and inhibition), Poisson regression (for numeric data) and logistic regression (for proportional data) were used. The 50 and 95% knock-down times and their confidence intervals were obtained after log-probit regression using the method described by Finney [33].

Results

Characteristics of the studied mosquito populations

The majority of female mosquitoes were collected and identified morphologically as *An. gambiae* s.l. The biochemical and molecular analyses indicated that among ten sites, five showed significantly higher oxidase activity than the susceptible strain Kisumu (Table 1). Esterases were significantly expressed in the Tanguieta mosquito population (Table 1). Over-expression of glutathione-S-transferase was observed at four sites (Table 1). However, the allelic frequency of the *kdr* mutation was high at almost all sites and ranged from 0.03 to 0.93.

Knock-down (KD) and mortality of laboratory strains

Figure 2 shows the proportion of laboratory mosquitoes (ace-1R-Kisumu, kdr-Kisumu, and susceptible Kisumu) knocked down after 60 min for each LLIN. The Olyset Plus and PermaNet 3.0 LLINs induced 100% knock-down of *An. gambiae* Kisumu. The knock-down effect was 96.15% for Olyset, 90.2% for LifeNet and 93.22% for PermaNet 2.0.

With the ace-1R-Kisumu strain, which carries the acetylcholinesterase-1 resistance gene, there was a knock-down effect greater than 95% for all nets, with 98.11% for LifeNet, 100% for Olyset, 98.18% for Olyset Plus, 97.96% for PermaNet 2.0, and 98.78% for PermaNet 3.0 (Fig. 2).

For the kdr-Kisumu strain (carrying the resistance knock-down), the knock-down effects observed were 89.29% for LifeNet, 63.64 for Olyset Net, 71.43% for Olyset Plus, 45.78 for PermaNet 2.0 and 71.05% for PermaNet 3.0 (Fig. 2).

Kisumu and ace-1R-Kisumu (Fig. 3). With the kdr-Kisumu strain, mortality was 16% for Olyset Net, 26% for PermaNet 2.0, 28% for LifeNet, and 32.1% for Olyset Plus but was more than 82.9% for PermaNet 3.0. Therefore, based on the bio-efficacy threshold set by WHO (80%), PermaNet 3.0 was effective on all laboratory strains, and Olyset Plus was only effective on the susceptible and ace-R1-Kisumu strains (Fig. 3).

Inhibition of mortality conferred by the *kdr* resistance gene

Comparing the mortality observed with the susceptible Kisumu strain with that of the resistant kdr-Kisumu strain, the inhibition of mortality induced by the *kdr* gene regarding the effectiveness of LLINs was 84% for Olyset Net, 74% for PermaNet 2.0, 72% for LifeNet, 68% for Olyset Plus and 17% for PermaNet 3.0.

Knock-down (Kd) effect and mortality induced by mosquito nets on local *An. gambiae* s.l.

Approximately 2819 local *An. gambiae* s.l. mosquitoes and 889 *An. gambiae* Kisumu laboratory strain mosquitoes were tested on different types of LLINs. Tables 2 and

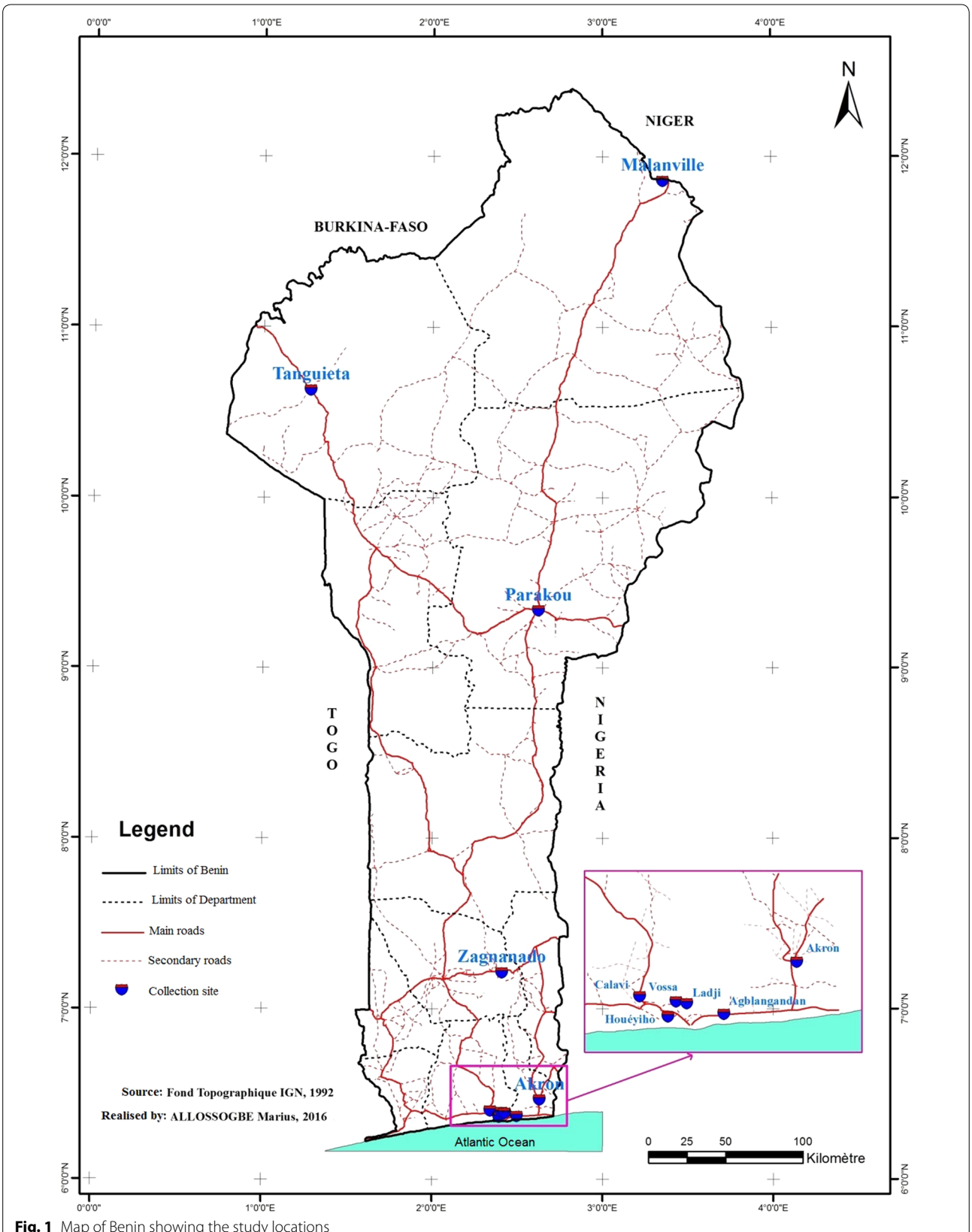

Fig. 1 Map of Benin showing the study locations

Table 1 Biochemical and molecular characteristics of the *Anopheles gambiae* s.l. populations tested

Strains of *An. gambiae* s.l.	Average oxidase activity (min/mg protein)	Average α esterase activity (min/mg protein)	Average β esterase activity (min/mg protein)	Average glutathione-S-transferase activity (min/mg protein)	*kdr* frequency
Kisumu	0.1015[a]	0.07409[a]	0.07655[a]	0.3846[a]	0[a]
Agblangandan	0.07966[a]	0.07883[a]	0.06117[a]	0.7319[b]	0.03[a]
Abomey-Calavi	0.08454[a]	0.07149[a]	0.05929[a]	0.4295[a]	*0.93*[b]
Akron	0.1604[b]	0.08589[a]	0.07897[a]	2.221[b]	*0.74*[b]
Houeyiho	0.17.39[b]	0.07694[a]	0.08774[a]	0.4042[a]	*0.9*[b]
Vossa	0.07566[a]	0.06897[a]	0.06389[a]	0.7078[a]	*0.84*[b]
Ladji	0.1737[b]	0.07146[a]	0.0774[a]	1.194[b]	*0.92*[b]
Bame	0.1106[a]	0.0588[a]	0.06223[a]	0.2901[a]	0.78[b]
Malanville	0.06549[a]	0.04949[a]	0.04871[a]	0.1723[a]	*0.90*[b]
Parakou	0.1536[b]	0.08124[a]	0.08871[a]	0.4698[a]	*0.74*[b]
Tanguieta	0.2267[b]	0.1585[b]	0.1442[b]	1.182[b]	*0.85*[b]

[a, b] Values with the same superscript do not differ significantly at α = 0.05

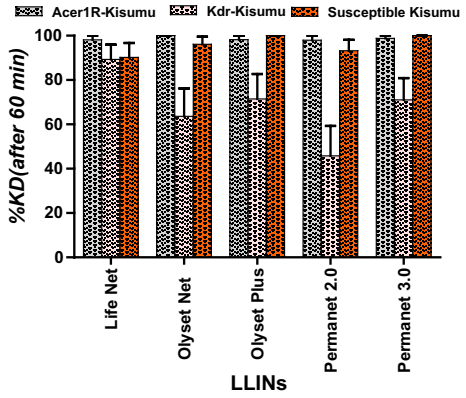

Fig. 2 Mosquito knock-down at 60 min post-exposure to LLINs

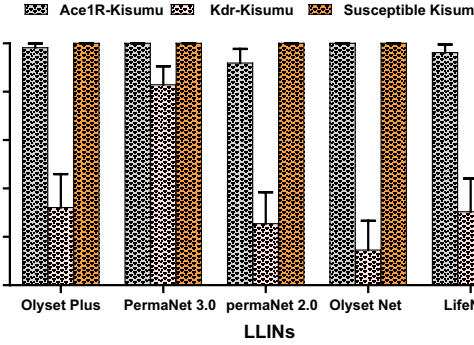

Fig. 3 Mosquito mortalities 24 h post-exposure to LLINs

3 show the percentage of local strain mosquitoes knocked down after 60 min for LifeNet, Olyset Net, Olyset Plus, PermaNet 2.0, and PermaNet 3.0.

Knock-down (KD) and mortality induced by the LLINs on mono-resistance mosquito strains

Only PermaNet 3.0, Olyset Plus and LifeNet LLINs showed a knock-down effect greater than 50% at Agblangandan, Vossa, Zangnanado and Malanville (areas of low resistance) (Table 2). These knock-down values varied between 51 and 95%. At Abomey, only PermaNet 3.0 and Olyset Plus LLINs showed a knock-down effect greater than 50%.

PermaNet 3.0 was the only LLIN that showed significantly higher mortality of greater than 50% in all localities where mosquitoes carried only the *kdr* gene. The average mortality for other types of LLINs tested in these areas varied from 5 to 47% (Table 2). These mortality rates varied from 0 to 14% for Olyset, 7 to 27% for LifeNet, from 9 to 22% for Olyset Plus, from 24 to 47% for PermaNet 2.0 and from 40 to 86% for PermaNet 3.0.

Inhibition of mortality in mono-resistant *An. gambiae* s.l. strains

The observed inhibition of mortality induced by *kdr* resistance of local mosquito strains on LLIN effectiveness was 100–86% for Olyset, 92–73% for LifeNet, 53–76% for PermaNet 2.0, 78–91% for Olyset Plus and 14–60% for PermaNet 3.0. These inhibition rates are similar to those observed with the kdr-Kisumu strain ($p > 0.05$).

Knock-down (KD) and mortality induced by the LLINs on multi-resistant mosquito strains (carrying *kdr* and biochemical resistance mutations)

In areas with multi-resistance, the knock-down effects observed were also low (Table 3).

At Akron, the percentage of mosquitoes knocked down after 60 min was 31.48% [19.52–45.55] and 74.55% [60.99–85.33] for Olyset Net and Olyset Plus,

Table 2 Distribution of the knock-down rate observed in localities where there was only one resistance mechanism (*kdr*)

Strains	LLINs	N mosquito tested	KD after 60 min	95% CI	Mortality after 24 h (%)
Malanville	LifeNet	55	72.27	[59.03–83.86]	27.27
	Olyset Net	53	30.19	[18.34–44.34]	05.56
	Olyset Plus	51	54.9	[40.34–68.87]	21.56
	PermaNet 2.0	59	28.81	[17.76–42.08]	47.46
	PermaNet 3.0	84	95.24	[88.25–98.69]	61.90
Abomey-Calavi	LifeNet	53	9.43	[3.13–20.66]	7.54
	Olyset Net	54	11.11	[4.18–22.63]	5.56
	Olyset Plus	55	29.09	[17.62–49.90]	20
	PermaNet 2.0	52	70.49	[57.43–81.84]	26.92
	PermaNet 3.0	72	81.94	[71.1–90.02]	86.11
Zagnanado (Bamè)	LifeNet	58	68.97	[55.45–80.46]	10.34
	Olyset Net	54	23.08	[12.53–36.84]	00
	Olyset Plus	55	33.96	[21.51–46.27]	09.43
	PermaNet 2.0	53	52.83	[38.63–66.7]	03.77
	PermaNet 3.0	75	63.93	[57.61–79.47]	62.67
Vossa	LifeNet	54	62.96	[48.74–75.71]	20.37
	Olyset Net	57	21.05	[11.37–33.89]	14.03
	Olyset Plus	53	41.51	[28.13–55.87]	15.05
	PermaNet 2.0	51	52.94	[38.45–67.07]	23.52
	PermaNet 3.0	73	79.45	[68.38–88.02]	39.75

N number, *KD* knock-down, *min* minutes, *CI* confidence interval, *h* hours

respectively; 70.49% [57.43–81.84] and 81.71% [71.63–89.38] for PermaNet 2.0 and PermaNet 3.0, respectively, and 30.77% [18.71–45.1] for LifeNet. At Houéyiho, the knock-down effect was 23.08% [12.53–36.84] and 49.15% [35.89–62.5] for Olyset Net and Olyset Plus, respectively; 46.3% [32.62–60.39] and 73.5% [61.46–83.97] for PermaNet 2.0 and PermaNet 3.0, respectively, and 61.11% [46.87–74.08] for LifeNet. It was generally observed that knock-down was significantly higher with Olyset Plus than with Olyset on multi-resistant Akron and Houéyiho strains ($p < 0.05$). The same observation was made with PermaNet 3.0, whose knock-down was significantly higher than that observed with PermaNet 2.0.

The same observations were made at Ladji, Parakou and Tanguiéta, where the KD induced by Olyset Plus was higher than that of Olyset. Similarly, PermaNet 3.0 (98%) was more effective than PermaNet 2.0 (39%) (Table 3). However, at Tanguieta, only three LLINs were tested. The three types of mosquitoes tested showed a KD effect ≥75%. Overall, in areas where there was high activity of oxidase enzymes associated with the *kdr* gene, only three LLINs (LifeNet, Olyset Plus, and PermaNet 3.0) showed a KD effect that was generally high. However, the mortality observed in these populations was generally low (Table 3). Only the PermaNet 3.0 LLIN induced significantly higher mortality ($p < 0.001$) that was generally greater than 75% (Table 3).

Inhibition of mortality in multi-resistant strains

The inhibition of the mortality induced by LLINs observed with strains carrying several resistance mechanisms (compared to the susceptible strain Kisumu) ranged from 60 to 96% for Olyset, 53 to 90.2% for LifeNet, 45 to 86% for PermaNet 2.0, 59 to 76% for Olyset Plus and 11 to 55% for Permanet 3.0. These inhibition rates are similar to those observed with the kdr-Kisumu strains ($p > 0.05$).

Knock-down time of LLINs on local *An. gambiae* s.l. strains

The average time estimated for knock-down of 50% of resistant local *An. gambiae* s.l. populations was significantly shorter with PermaNet 3.0 (12 min) ($p < 0.001$), followed by Olyset Plus and LifeNet (33 min). However, the time required for 95% of mosquitoes to be knocked down was high for all LLINs. Generally, there was a slower effect with LLINs treated with permethrin (Table 4).

Discussion

This study is one of the first conducted in Benin to compare the response of local malaria vectors in Benin to several LLINs recommended by the WHO. It helps to observe the variation in mortality of vectors submitted to different types of LLINs. This mortality was generally low, especially with LLINs only treated with pyrethroids.

Table 3 Distribution of the knock-down rate observed in localities where there were several resistance mechanisms (*kdr* + metabolic resistance)

Strains	LLINs	N mosquito tested	KD after 60 min	95% CI	Mortality (%)
Agblangandan	LifeNet	53	50.94	[36.83–64.96]	15.09
	Olyset Net	54	20.75	[10.84–34.11]	07.4
	Olyset Plus	55	50.91	[37.07–64.65]	34.72
	PermaNet 2.0	47	36.17	[22.67–51.58]	17.02
	PermaNet 3.0	66	60.61	[47.80–72.42]	65.15
Ladji	LifeNet	57	85.96	[74.2–93.74]	47.36
	Olyset Net	57	50.88	[37.28–64.37]	40.35
	Olyset Plus	56	42.86	[29.71–56.78]	41.07
	PermaNet 2.0	50	66	[51.23–78.79]	14
	PermaNet 3.0	69	88.41	[78.42–94.86]	44.93
Akron	LifeNet	52	30.77	[18.71–45.1]	15.38
	Olyset Net	54	31.48	[19.52–45.55]	5.56
	Olyset Plus	55	74.55	[60.99–85.33]	25.45
	PermaNet 2.0	61	70.49	[57.43–81.84]	54.09
	PermaNet 3.0	82	81.71	[71.63–89.38]	89.02
Parakou	LifeNet	51	43.14	[29.34–57.75]	09.80
	Olyset Net	52	26.92	[15.56–4 1.02]	07.69
	Olyset Plus	50	66	[51.23–78.79]	28
	Permanet 2.0	56	39.29	[26.49–53.25]	37.50
	Permanet 3.0	88	98.86	[93.83–99.97]	82.95
Houeyiho	LifeNet	54	61.11	[46.87–74.08]	14.81
	Olyset Net	52	23.08	[12.53–36.84]	3.84
	Olyset Plus	59	49.15	[35.89–62.5]	23.72
	Permanet 2.0	54	46.3	[32.62–60.39]	22.22
	Permanet 3.0	65	73.85	[61.46–83.97]	61.54
Tanguieta	LifeNet	–	–	–	–
	Olyset Net	–	–	–	–
	Olyset Plus	51	74.51	[60.36–85.67]	56.86
	PermaNet 2.0	62	75.81	[63.25–85.78]	32.26
	PermaNet 3.0	86	100	[88.78–100]	78.82

N number, *KD* knock-down, *min* minutes, *CI* confidence interval, *h* hours

Table 4 Probable time for 50 and 95% knock-down of *Anopheles gambiae* s.l. per LLIN

LLINs	50% KDT (min)	95% CI	95% KDT (min)	95% CI
LifeNet	33.12	[32.5–33.91]	425.13	[385.6–468.69]
Olyset Net	98.74	[90.4–107.85]	10,257.58	[7090.39–14,839.5]
Olyset Plus	33.44	[32.56–34.34]	674.68	[595.91–763.86]
PermaNet 2.0	42.3	[41.26–43.37]	468.28	[424.57–516.49]
PermaNet3.0	12.61	[12.30–12.93]	137.99	[131.6–144.69]

%KDT knock down time, *IC 95%* confidence interval at 95%, *min* minutes, *CI* confidence interval

Cone tests showed that LLINs treated with piperonyl butoxide and pyrethroids (especially PermaNet 3.0) have optimum efficacy on all strains of *An. gambiae* s.l. (mono and multi-resistant).

Several studies have shown a decrease in the bio-efficacy of LLINs against local pyrethroid-resistant vectors [34, 35]. The effectiveness of LLINs treated only with deltamethrin (PermaNet 2.0 and LifeNet) was found to be significantly lower compared to that of nets treated with deltamethrin and PBO. The same observation was made with the LLINs treated with permethrin only (Olyset Net) and those treated with permethrin and PBO. However, the effectiveness of LLINs treated with permethrin was generally lower than that of LLINs treated with deltamethrin, with lower mortality and a very slow knock-down

time (KDT 50 and 95%) compared to other LLINs. In a recent study conducted in Benin [36], Olyset Plus, treated with permethrin + PBO, demonstrated a higher efficacy than Olyset Net against wild multi-resistant *An. gambiae* s.l. in experimental huts, as observed in WHO cone tests used in the present study. In south-western Ethiopia [35] and in Uganda [34], a reduced efficacy of mono-treated LLINs was also observed against wild resistant *An. gambiae* s.l. in comparison with Permanet 3.0 treated with deltamethrin + PBO. The results are similar to those observed in this study. However, these studies did not include Olyset Plus, the second type of new-generation LLINs treated with permethrin + PBO.

The reduced efficacy of LLINs treated with permethrin would be related to the strong resistance of the local vectors to permethrin due to the resistance selection pressures generated by the use of the same class of insecticide for malaria vector control in public health and for pest control in agriculture [16, 17, 23, 37, 38].

The comparison of LLIN bio-efficacy performed in this study provides the necessary information for the selection of appropriate LLINs for mass distribution. The optimal and constant efficacy of PermaNet 3.0 LLINs on all vector populations shows that this combination of deltamethrin and PBO on LLINs is a most successful strategy against pyrethroid resistance in Benin. Variations in the mortality of vectors also showed that certain types of LLINs are more appropriate than others for distribution in specific regions. This is related to the fact that the effectiveness of an LLIN depends on the characteristics of the mosquito population tested and the chemical structure of the molecule (insecticide) used.

The mosquito populations assessed in the present study were characterized by a high frequency of the *kdr* gene. This high frequency was probably due to the massive use of pyrethroids in agriculture and public health. In some areas, such as Tanguieta, Parakou, Houeyiho, Akron, and Ladji, farmers and gardeners use huge amounts of insecticides to reduce pests in their crops, which explains the presence and strong expression of several resistance mechanisms in the mosquito populations [39, 40]. Overproduction of resistance enzymes in these areas would be linked to pressure on mosquito larvae from insecticides used by farmers to protect vegetable crops [41–43]. This expression of the *kdr* resistance gene induced a 17–84% reduction in LLIN efficacy against laboratory strains. These frequencies are similar to those observed in natural populations of *An. gambiae* s.l. This observation shows that the *kdr* gene is the main mechanism involved in the reduction of the effectiveness of LLINs. Although detoxification enzymes contribute to resistance, their impact is successfully inhibited by the presence of PBO on new-generation LLINs and the remaining part is more likely related to the presence of *kdr* gene in the mosquito populations. This also suggests that the search for new molecules or combinations of molecules that target the *kdr* resistance mechanism should be promoted.

The WHO recommends preventive measures against vector resistance to insecticides [44]. The results of this study therefore constitute important evidence that can guide decision making in the selection and distribution of high efficacy LLINs in specific regions of Benin. The use of LLINs that showed high bio-efficacy against the local vector populations should be encouraged to contribute substantively to reducing the transmission of malaria in Benin.

This study also suggests the need to develop a routine for monitoring the bio-efficacy of LLINs against local malaria vectors for the replacement of ineffective LLINs. However, community studies would be needed to evaluate the epidemiological impact of these LLINs to confirm whether or not the low efficacy observed is followed by a loss of the epidemiological impact of these nets.

Although the important results of this study, it had certain limitations. Strong evaluation would have been possible if tunnel tests were conducted on LLINs that did not meet the criteria of 80% mortality with resistant mosquito strains. In addition, a chemical analysis of the LLINs prior to the start of the study would also have improved the quality of the results. However, all the LLINs demonstrated a good performance with susceptible laboratory stain Kisumu (mortality > 80%), as recommended by WHO [45], and the focus of this study was to demonstrate the important role of resistance mechanisms on LLINs efficacy.

Conclusion

This study showed variable effectiveness of LLINs on *An. gambiae* s.l. populations from different localities surveyed from north to south in Benin. The new-generation LLINs with pyrethroids and PBO (PermaNet 3.0 and Olyset Plus) showed higher efficacy than conventional LLINs (PermaNet 2.0, LifeNet and Olyset net). However, the strong resistance of local vectors to permethrin suggests that the combination of deltamethrin + PBO is the most appropriate strategy against local vectors in Benin. Although the addition of PBO (targeting many biochemical mechanisms of resistance) significantly increased the mortality of mosquitoes, the significantly high role of the *kdr* resistance gene in the low efficacy of LLINs calls for LLIN technology innovation that specifically targets this mechanism.

Authors' contributions

MA, VG and MCA designed the study, supervised laboratory work, analyzed data and wrote the manuscript. BY, RA, FA and BA conducted field collections, laboratory tests and contributed in the writing of the manuscript. AH and GGP helped in the study design and revising the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
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Bioefficacy of long-lasting insecticidal nets against pyrethroid-resistant populations of *Anopheles gambiae s.s.* from different malaria transmission zones in Uganda

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Abstract

Background: There are major concerns over sustaining the efficacy of current malaria vector control interventions given the rapid spread of resistance, particularly to pyrethroids. This study assessed the bioefficacy of five WHO-recommended long-lasting insecticidal nets (LLINs) against pyrethroid-resistant *Anopheles gambiae* field populations from Uganda.

Methods: Adult *An. gambiae* from Lira, Tororo, Wakiso and Kanungu districts were exposed to permethrin (0.75%) or deltamethrin (0.05%) in standard WHO susceptibility tests. Cone bioassays were used to measure the bioefficacy of four mono-treated LLINs (Olyset®, Interceptor®, Netprotect® and PermaNet® 2.0) and one combination LLIN (PermaNet® 3.0) against the four mosquito populations. Wireball assays were similarly conducted to determine knockdown rates. Species composition and *kdr* mutation frequency were determined for a sample of mosquitoes from each population. Chemical assays confirmed that test nets fell within target dose ranges.

Results: *Anopheles gambiae s.s.* predominated at all four sites (86 - 99% of *Anopheles spp.*) with moderate *kdr* L1014S allelic frequency (0.34 – 0.37). Confirmed or possible resistance to both permethrin and deltamethrin was identified for all four test populations. Reduced susceptibility to standard LLINs was observed for all four populations, with mortality rates as low as 45.8% even though the nets were unused. The combination LLIN PermaNet®3.0 showed the highest overall bioefficacy against all four *An. gambiae s.l.* populations (98.5 - 100% mortality). Wireball assays provided a more sensitive indicator of comparative bioefficacy, and PermaNet 3.0 was again associated with the highest bioefficacy against all four populations (76.5 – 91.7% mortality after 30 mins).

Conclusions: The bioefficacy of mono-treated LLINs against pyrethroid-resistant field populations of *An. gambiae* varied by LLIN type and mosquito population, indicating that certain LLINs may be more suitable than others at particular sites. In contrast, the combination LLIN PermaNet 3.0 performed optimally against the four *An. gambiae* populations tested. The observed reduced susceptibility of malaria vectors to mono-treated LLINs is of particular concern, especially considering all nets were unused. With ongoing scale-up of insecticidal tools in the advent of increasing resistance, it is essential that those interventions with proven enhanced efficacy are given preference particularly in areas with high resistance.

Keywords: Long-lasting insecticidal nets (LLIN), Pyrethroid-resistant *An.gambiae* s.s, Uganda

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Background

Malaria remains a major public health problem, causing an estimated 225 million disease cases and 781,000 deaths per year, especially among children aged less than five years [1]. The disease is transmitted by anopheline mosquitoes and vector control is one of the most important means of malaria prevention. There is evidence that the use of insecticide-treated nets (ITNs) on a large scale decreases malaria related morbidity and mortality [2,3] and for this reason, the use of ITNs has been considered an important tool in the Roll Back Malaria (RBM) strategy.

Unlike conventional ITNs which lose effective insecticide after one or two washes and maintain bioefficacy for a maximum of 6–12 months, long-lasting insecticidal nets (LLINs) in which insecticide is either incorporated into the fibre during extrusion or coated on the fibre following extrusion, retain effectiveness against susceptible *Anopheles* spp. vectors for up to 20 standard WHO laboratory washes and 3 years of recommended usage under field conditions [4]. All LLINs are currently treated with pyrethroids due to their relative safety for humans at low dosage, repellent properties, rapid knockdown rates and killing effects [5]. However, pyrethroid resistance in mosquito vectors as reported in many African countries [6] could limit the efficacy of LLINs as shown by findings of decreased efficacy of LLINs in Benin, Mali and Zanzibar [7-9].

Insecticide resistance is mediated either by mutations in the target site of the insecticide or its active metabolites (target site resistance), through enzymatic modification of insecticides to produce non-toxic metabolites (metabolic detoxification), via behaviour resistance or through reduced penetration of the insecticide into the vector species [10]. Several factors can select for resistance in mosquito vector species, such as overuse of insecticide, whether in ITNs, indoor residual spraying (IRS) or through agricultural applications which account for huge insecticide inputs of almost all available classes of insecticides [11,12]. In Uganda, there is widespread insecticide resistance in the main malaria vector species, *An. gambiae s.s, An. arabiensis* and *An. funestus* [13-18]. This resistance is due to both target site (*kdr*) and metabolic mechanisms and there is cross-resistance between DDT and pyrethroids. There are currently no reports of organophosphate resistance but resistance to carbamates including propoxur has been reported [13-18].

The current strategy of the National Malaria Control Programme (NMCP) in Uganda is based on effective case management and vector control using LLINs and IRS. Insecticide resistance monitoring is therefore essential to guide implementation of more effective and sustainable vector control. There have been limited data on comparative efficacy of World Health Organization (WHO)-recommended LLINs against field-derived populations of *Anopheles* spp. from different transmission zones within single countries. Rather, efficacy has largely been measured in specific areas via experimental huts with only one or two nets assessed in relation to controls. In Uganda, one study showed progressive reductions over a 10 year period in susceptibility of *An. funestus* from the western region to nets treated with three different insecticides. However, non-standard bioassay techniques were used and mosquitoes from five parishes were pooled for assessments [15]. In the absence of experimental huts and in order to assess susceptibility to LLINs of multiple local malaria vector species, the Uganda NMCP initiated the present study using WHO-recommended LLINs against local *Anopheles gambiae* populations. Outcomes are expected to be applied in evidence-based decision making on the most appropriate LLINs for application in malaria prevention and control in specific regions of the country.

Methods

Mosquito collections

Collections were conducted in April and October 2011 in the districts of Lira, Tororo, Wakiso and Kanungu located in Northern, Eastern, Central and Western regions of Uganda, respectively (Figure 1). In these four districts,

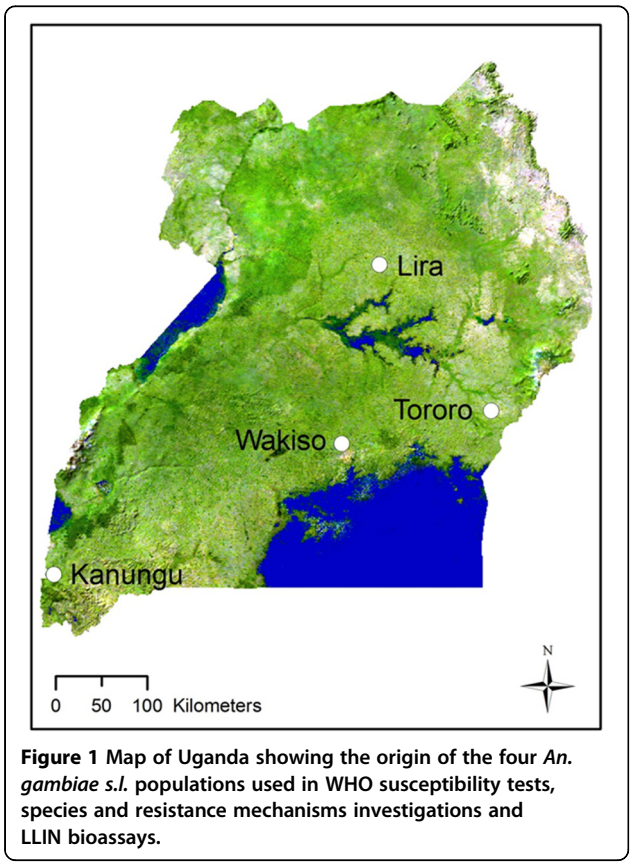

Figure 1 Map of Uganda showing the origin of the four *An. gambiae s.l.* populations used in WHO susceptibility tests, species and resistance mechanisms investigations and LLIN bioassays.

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malaria transmission levels range from very high (Lira, Tororo) to medium-high (Wakiso) to low (Kanungu) (Additional file 1). Previous studies identified the presence of *kdr* mutations in *An. gambiae s.l.* from Tororo district in the eastern part of the country, Apac district in northern Uganda near the current study district of Lira, in the central part of the country and in Kyenjojo and Kanungu in the western part of the country, with *kdr* frequencies ranging from 25% to 30% in these districts [14,16-19]. Metabolic resistance mechanisms have also been implicated in populations from Tororo with a significant increase in esterase activity detected [17].

Female *Anopheles* spp. adult mosquitoes were collected via standard resting catches from houses and larvae were collected from breeding sites within the study districts, and all were transported to the Vector Control Division (VCD) insectary in Kampala. Blood-fed and gravid females were allowed to oviposit, eggs were hatched and larvae were pooled for each collection district. Field-collected larvae were also pooled by collection district. All preimaginal stages were reared to adults under conditions of ambient temperature and humidity with 12:12 hours of light: dark cycle. Unfed adult females at 2 – 5 days post-emergence were identified morphologically as previously described [20] at the Centre for Research on Infectious Diseases Laboratory, College of Health Science Makerere University, Kampala. Only *An. gambiae s.l.* mosquitoes were used in WHO susceptibility tests and cone bioassays.

Insecticide susceptibility and molecular testing

Standard WHO susceptibility tests were conducted to determine mortality rates (MT) following one hour of exposure to papers treated with either permethrin (0.75%) or deltamethrin (0.05%). Concurrent negative controls were run using untreated papers. Test populations were classified as susceptible (≥98% MT), possibly resistant (80-97%) or confirmed resistant (<80% MT) [21]. Mosquitoes used in controls were stored in contact with silica gel desiccant. A random sample of 100 mosquitoes from each site was used in species identification by restriction fragment length polymerase chain reaction [22] and determination of *kdr* mutation frequency by allele-specific polymerase chain reaction [23,24]. All PCR runs for *kdr* analyses included controls of wild type homozygote, heterozygote and *kdr* homozygote mosquitoes for both L1014S and L1014F.

LLIN samples

All LLINs as well as the untreated control nets were obtained from the local market. All had an unknown storage history but were within the specified product shelf-life. LLINs included in the study were: Olyset® Net (polyethylene with permethrin incorporated at 20 g/kg ± 3 g/kg), Interceptor® (polyester coated with alphacypermethrin at 200 mg/m^2 ± 25%), NetProtect® (polyethylene with deltamethrin incorporated at 1.8 g/kg ± 25%), PermaNet® 2.0 (polyester coated with deltamethrin at 55 mg/m^2 ± 25%) and PermaNet® 3.0 (polyethylene roof with deltamethrin incorporated at 2.8 g/kg ± 25% and piperonyl butoxide (PBO) incorporated at 4.0 g/kg ± 25% in the roof and sides coated with deltamethrin at 2.8 g/kg ± 25%). PBO is a synergist that increases the rate of penetration of insecticide into the insect [25] and inhibits the metabolic enzymes the mosquito uses to sequester or break-down the insecticide [26].

All LLINs were rectangular with sub-samples of 30 × 30 cm taken from the roof section (2 per net) and side sections (1 each from the upper and lower part of the two long sides of each net) to give a total of 6 sub-samples for each net for use in bioassays. Four nets of each type were used for a total of 24 sub-samples of each net type assessed via cone bioassays. Identical sub-sampling was performed in adjacent areas for reference samples used in chemical assays. All samples were rolled up and placed individually in a labelled clean aluminium foil prior to assays.

LLIN chemical analyses

Assays were conducted via high performance liquid chromatography (HPLC) to confirm whether chemical concentrations were within product specifications for each individual LLIN. Analyses were conducted at an ISO IEC 17025-accredited laboratory. Deltamethrin was assessed by normal-phase HPLC according to CIP333/LN. Alpha-cypermethrin was extracted with n-hexane and 1, 4-dioxane (95:5 v/v) with the mixture then shaken and sonicated and filtered on a 0.45 mm teflon membrane, whereas for permethrin and PBO, hot xylene extraction was followed by drying, reconstitution and filtration after which both were assessed via HPLC.

LLIN bioassays

Standard WHO cone bioassays [4] were used to determine bioefficacy of LLINs against field-derived populations as well as against a susceptible laboratory-reared *Anopheles gambiae s.s.* strain (Kisumu). The Kisumu colony was established at the Vector Control Division (VCD) of the Ministry of Health in Kampala in 2011, with full susceptibility (100% mortality) to permethrin (0.75%) and deltamethrin (0.05%) confirmed via standard WHO susceptibility tests prior to assays. At the VCD insectary, five non-blood fed 2-to 5-day old *Anopheles* females were exposed to each sub-sample for 3 minutes, removed and kept in holding containers with access to sugar solution. Knockdown (KD) was recorded at 60 minutes post-exposure and mortality (MT) was recorded after 24 hours. Two cone tests were conducted per sub-

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sample and per mosquito population including for the laboratory susceptible population such that 240 mosquito of each of the five populations were tested for each net type. Mosquitoes exposed to untreated nets were used as controls with all concurrent results discarded if MT was ≥20% and Abbott's adjustment applied if MT was >5% for the controls.

Wireball assays were used to measure knockdown following 30 and 60 mins of continuous exposure to an LLIN in a wireball. This approach was included as it is of use where mortality rates may be lower and hence longer exposure times are required, or where high repellency of the insecticide may compel mosquitoes to rest on the cone interior rather than on the LLIN. Net subsamples were wrapped around a wire frame of three intersecting circles of 15 cm in diameter with the netting secured around the frame in such a way that a "sleeve" was left through which 11 mosquitoes were introduced. Numbers of mosquitoes knocked down after 30 mins (KD_{30}) and 60 mins (KD_{60}) were recorded. Mosquitoes were then transferred to holding cups for 24-hour post-exposure readings. For each individual sub-sample, four wire-ball tests were conducted such that 44 mosquitoes were tested per sub-sample. With 3 sub-samples of each individual net and 3 nets of each type, a total of 396 mosquitoes were tested for each net type. Controls were run concurrently with interpretation as for cone bioassays.

Data analyses

For cone bioassays, KD and MT were compared for individual samples via regression analyses. Data, aggregated for mosquito population, net type and net section, were assessed via ANOVA with Duncan's multiple comparison procedure. Data were then combined for net sections and assays were repeated. Wireball assay data for KD_{30} and KD_{60} were similarly analyzed.

Results

Population characterisation and insecticide susceptibility

For the population analyses, the majority of collected females were morphologically identified as *An. gambiae s.l.* (391/400) with a small proportion identified as *An. funestus s.l.* (9/400). Molecular analyses indicated that at all four sites, *An. gambiae s.s.* predominated (Table 1). The *kdr* mutation L1014S was detected in 257 of the 363 *An. gambiae s.s.* successfully tested, with overall 29.2% homozygous wild type (SS), 70.5% heterozygous (RS) and one single homozygous resistant (RR) mosquito detected from Kanungu. The *kdr* allelic frequency was moderate at all sites, and varied from 0.34 at Lira to 0.37 at Wakiso. Genotype frequencies for all populations did not adhere to Hardy-Weinberg expectations. All *An. arabiensis* tested (14) were wild type. No L1014F mutations were observed in any species.

Only *An. gambiae s.l.* were used in further assays. WHO susceptibility tests confirmed resistance to both permethrin and deltamethrin for the populations from Lira and Tororo (Table 2). There was confirmed resistance to permethrin and possible resistance to deltamethrin for the population from Kanungu, and possible resistance to both pyrethroids for the population from Wakiso. At all the four sites, higher resistance to permethrin was identified than to deltamethrin at the standard tested dosages. The Kisumu laboratory strain of *An. gambiae s.s.* was 100% susceptible to both pyrethroids.

LLINs and bioassays

All LLIN sub-samples had optimal bioefficacy (100% KD and 100% MT), against the susceptible *An. gambiae s.s.* (Kisumu), strain in cone bioassays, with the exception of Interceptor (78.8% and 80.0% KD for upper and lower sides respectively, and 97.5% MT for upper side) and Olyset (92.5% KD for upper side). For wireball assays with the susceptible strain, KD_{30} ranged between 84.8 and 93.3% with 100% MT at 24 hours post-exposure for all LLINs. Chemical analyses confirmed that all LLINs exceeded the specified lower cut-off level for insecticide (or synergist) concentration though there were two instances where LLIN sub-samples slightly exceeded the upper limits i.e., roof of PermaNet 2.0 and sides of Interceptor (Table 3).

An overall association was identified between KD and MT for cone bioassays on individual sub-samples ($n = 720$; $R^2 = 0.8903$; $P < 0.0001$), while associations on aggregated data showed correlation between KD and MT for Interceptor against all four mosquito populations, Olyset for 3 populations, and for the remaining LLINs two populations only ($P < 0.05$ for all specified).

Table 1 Species composition of *Anopheles spp.* and *kdr* mutation frequency in *An. gambiae s.s.* from the four study sites

Study site	Species				*An. gambiae s.s. kdr* mutation	
	Identified (no.)	*An. funestus* (%)	*An. arabiensis* (%)	*An. gambiae s.s.* (%)	Genotyped (no.)	L1014S frequency (%)
Kanungu	98	2.0	0.0	98.0	94	36.7
Lira	100	0.0	1.0	99.0	97	33.5
Tororo	99	1.0	13.1	85.9	79	35.4
Wakiso	100	6.0	0.0	94.0	93	36.6

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Table 2 Susceptibility to permethrin and deltamethrin of *An. gambiae* adult female mosquitoes collected from four sites in Uganda and the laboratory *An. gambiae s.s.* (Kisumu) strain determined via standard WHO susceptibility tests

	Permethrin (0.75%)			Deltamethrin (0.05%)		
Mosquito population	Number exposed	24 h mortality (%)	Susceptibility status	Number exposed	24 h mortality (%)	Susceptibility status^
Kanungu	100	68	Confirmed resistant	100	97	Possibly resistant
Lira	100	60	Confirmed resistant	100	71	Confirmed resistant
Tororo	100	53	Confirmed resistant	100	66	Confirmed resistant
Wakiso	100	90	Possibly resistant	100	94	Possibly resistant
Kisumu	100	100	Susceptible	100	100	Susceptible

^ Susceptible (≥98%), possibly resistant (80–97), confirmed resistant (<80%).

Comparisons of the bioefficacy of net sections (roof, upper sides, lower sides) indicated no difference among net types with the exception of Olyset against both the Kanungu and Tororo strains for both KD and MT ($P < 0.05$). Thus, there was no significant difference observed in the deltamethrin plus PBO roof and the deltamethrin-only sides of PermaNet 3.0, presumably because bioefficacy of the three sections was high against all four populations (≥87.9% KD and ≥ 97.5% MT). As such, data were aggregated by net type for subsequent analyses with data presented for MT.

Reduced susceptibility to LLINs was observed for all four field populations of *An. gambiae*. Bioefficacy varied between LLINs in cone bioassays with each of the four populations for both KD ($P < 0.001$ for all) and MT ($P < 0.001$ for all). Mean MT differed by 46.7% (range: 53.3-100%) for the Kanungu population, 54.2% (range: 45.8-100%) for the Lira population, 35.0% (range: 63.5-98.5%) for the Tororo population and 40.0% (range: 60.0-100) for the Wakiso population (Figure 2). PermaNet 3.0 exhibited the highest bioefficacy against all the four populations (98.5 – 100%). When data were analyzed via multiple comparison methods, PermaNet 3.0 performed significantly better than the mono-treated LLINs at Lira and Wakiso, and equal best with NetProtect at Kanungu and Olyset at Tororo. Each of the mono-treated LLINs also varied in bioefficacy for the four different populations for both KD ($P < 0.001$) and MT ($P < 0.001$). For PermaNet 3.0, there was no identifiable difference in bioefficacy against the four populations for either KD ($P = 0.1011$) or MT ($P = 0.0890$), presumably because bioefficacy was high against all populations. Conversely, there was also no significant difference in bioefficacy of the untreated net between populations since KD and MT were minimal for all.

Wireball assays also indicated differences in LLIN bioefficacy between net types for each of the four field populations, with PermaNet 3.0 resulting in the highest KD_{30} against all populations (76.5 – 91.7%) (Table 4). Bioefficacy also varied against the susceptible *An. gambiae s.s.* (Kisumu) strain ($P < 0.001$) and was highest for PermaNet 3.0 followed by PermaNet 2.0 and then the other LLINs, indicating that KD_{30} from wireball assays may be a more sensitive indicator of bioefficacy than KD and MT from cone bioassays. Bioefficacy of specific net types also varied against the different populations for the mono-treated LLINs ($P < 0.001$) and in contrast to the cone bioassay data, also varied across populations for PermaNet 3.0 ($P = 0.0063$) with the lowest KD_{30} (76.5%) observed against the Kanungu population. There was a significant overall association between KD_{30} and KD_{60} ($n = 348$; $R^2 = 0.8844$;

Table 3 Target concentration and range and mean insecticidal or synergist concentration measured via high performance liquid chromatography for roof and side sub-samples of five different LLIN types used in bioefficacy evaluations

		Target concentration			Mean measured concentration	
Net type	Chemical	Units	Mean	Range	Roof	Sides
PermaNet 3.0	Deltamethrin	g/kg	2.8 (sides)	2.1 - 3.5	-	3.1
		g/kg	4 (roof)	3.0 - 5.0	3.9	-
	Piperonyl butoxide	g/kg	25 (roof)	18.8 - 31.3	18.7	-
PermaNet 2.0	Deltamethrin	mg/m^2	55	41.3 - 68.8	69.4	65.6
NetProtect	Deltamethrin	g/kg	1.8	1.4 - 2.3	1.6	1.6
Interceptor	Alpha-cypermethrin	mg/m^2	200	150.0 - 250.0	171.0	251.0
Olyset	Permethrin	g/kg	20	17.0 - 23.0	21.0	21.6

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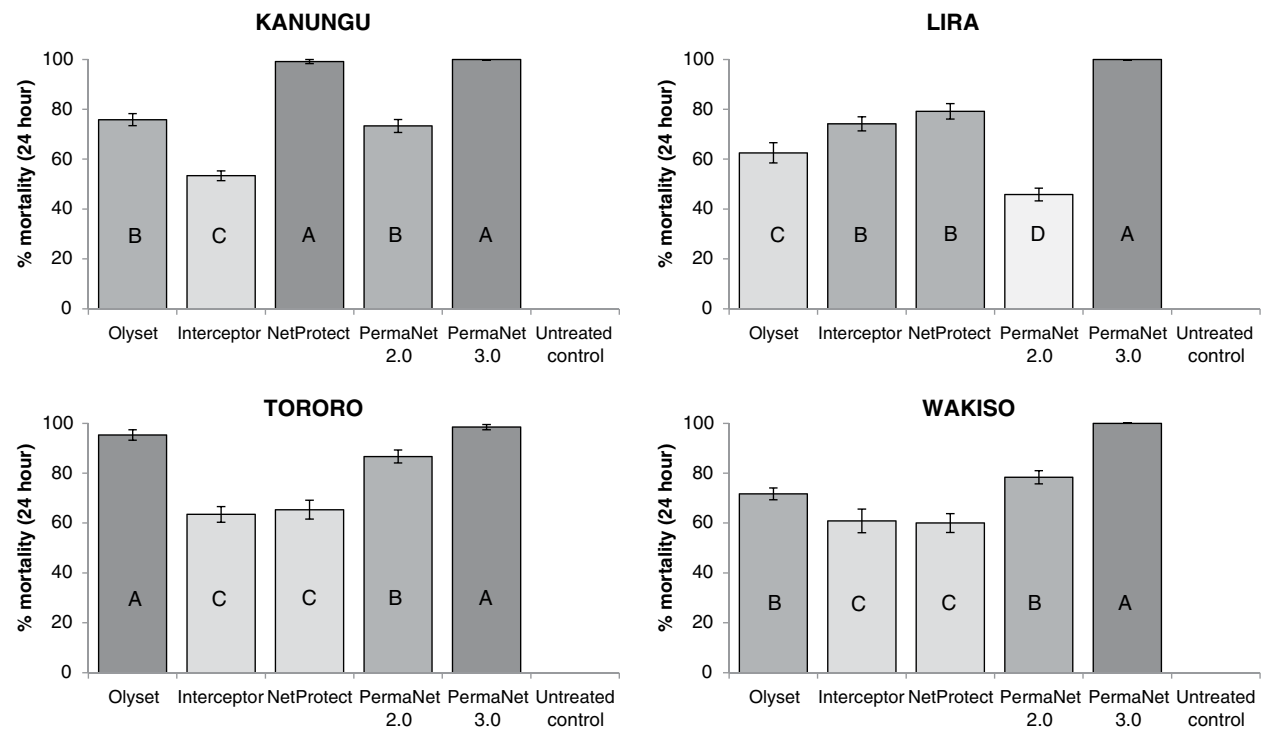

Figure 2 Bioefficacy (mean % 24-hour mortality) in cone bioassays of WHO-recommended LLINs against field-derived *An. gambiae* populations from four sites in Uganda. Vertical lines indicate standard error (n = 24). The same letter and shading within graphs indicates no significant difference (via Duncan's multiple comparison procedure at P < 0.05).

P <0.0001), and bioefficacy of the net sections differed only for PermaNet 3.0 against the Kanungu population (P = 0.0160) and Olyset against the Lira population (P = 0.0240).

There was some concurrence between cone bioassays and wireball assays, especially for PermaNet 3.0, which exhibited high bioefficacy in both assay types (Figure 3). Considering both assay types, PermaNet 3.0 performed best or equal best against all four populations. NetProtect also performed well in cone bioassays against the Kanungu population and in wireball assays against the Lira population. Olyset performed well in cone bioassays against the Tororo population and three mono-treated LLINs also performed well in wireball assays against this population, indicating that the Tororo population was overall the most susceptible to LLINs. In general, cone bioassays indicated that LLINs had the lowest efficacy against the Lira and Wakiso populations (72.3 and 74.2% MT, respectively), but wireball assays indicated the lowest efficacy against the Kanungu population

Table 4 Bioefficacy in wireball assays (mean % 30-minute knockdown) of WHO-recommended LLINs against field-derived *An. gambiae* populations from four sites in Uganda and a susceptible laboratory *An. gambiae s.s.* strain (Kisumu)

Mosquito population	Net type						P-value
	Olyset	Interceptor	NetProtect	PermaNet 2.0	PermaNet 3.0	Untreated control	
Kanungu	42.4^{C}	45.5^{C}	47.7^{C}	63.6^{B}	76.5^{A}	0.0^{D}	<0.0001
Lira	67.4^{C}	$76.5^{B,C}$	$83.3^{A,B}$	70.5^{C}	86.4^{A}	0.0^{D}	<0.0001
Tororo	$76.5^{A,B}$	$76.5^{A,B}$	83.3^{A}	70.5^{B}	86.4^{A}	nt	0.0241
Wakiso	75.8^{B}	41.7^{D}	65.9^{C}	81.8^{B}	91.7^{A}	0.0^{E}	<0.0001
Kisumu	84.9^{B}	86.4^{B}	84.9^{B}	$90.2^{A,B}$	93.3^{A}	0.0^{C}	<0.0001

Same letter in rows indicates no significant difference (via Duncan's multiple comparison procedure at P < 0.05).
nt: not tested due to limited numbers of mosquitoes available.

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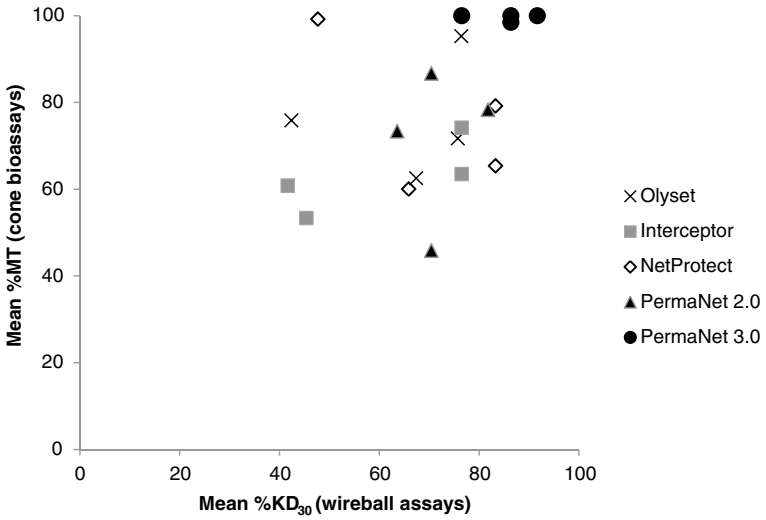

Figure 3 Comparative bioefficacy of different LLIN types as determined via wireball versus cone bioassays for four populations of *An. gambiae* from Uganda.

(55.2% KD_{30}). Bioefficacy was highest against the Tororo population for both bioassays.

Discussion

This is the first study to compare the response of field populations of malaria vectors from multiple sites in Uganda to WHOPES-recommended LLINs. Wide variations were observed in susceptibility to the different net types even within specific mosquito populations, with reduced susceptibility to pyrethroid-only LLINs observed for all four populations. Cone bioassays indicated that for two populations, a single mono-treated LLIN performed well (i.e., NetProtect at Kanungu and Olyset at Tororo), while optimal efficacy of the combination LLIN PermaNet 3.0 was observed for all four populations. Bioefficacy of LLINs differed by almost 50% against some populations (e.g. Kanungu had 53.3% MT with Interceptor and 100% MT with PermaNet 3.0 in cone bioassays).

Yewhalaw and colleagues [27] similarly observed reduction in the bioefficacy of standard LLINs against four pyrethroid-resistant *An. arabiensis* populations from the Jimma region of Ethiopia. In contrast to results herein, the PBO plus deltamethrin roof had higher bioefficacy than the pyrethroid-only sides of PermaNet 3.0. While the Ethiopia data provide evidence to indicate that PBO was effectively restoring susceptibility of the *An. arabiensis* populations to deltamethrin, this could not be demonstrated in the current investigation since bioefficacy of the deltamethrin-only sides of PermaNet 3.0 was optimal against the Uganda *An. gambiae* populations. Studies comparing the bioefficacy of PermaNet 3.0 versus deltamethrin- or permethrin-only LLINs in experimental huts have been conducted in numerous countries with results indicating that comparative bioefficacy will largely depend on the levels and types of resistance mechanisms present in the local vector species [28-32]. Data from some of these studies were applied in a malaria transmission model to compare PermaNet 3.0 to the deltamethrin-only PermaNet 2.0 under conditions of high net coverage (80%), with outputs indicating that PermaNet 3.0 (new and washed 20 times) had consistently higher impact on entomological inoculations rates across four sites with pyrethroid resistant *Anopheles spp.* [33].

Observed variation in susceptibility of *Anopheles* populations to pyrethroid-only LLINs indicates that particular LLINs may be more suitable for deployment in specific regions. This is due to anticipated differences in bioefficacy depending on characteristics of individual mosquito populations. This is currently seldom a consideration in selection of LLINs for wide-scale deployment, which is usually guided by availability, price and other factors such as user acceptability of the polymer type (e.g. polyester versus polyethylene). Reliance on phenotypic susceptibility status to select nets by active ingredient is also not appropriate since results from WHO susceptibility tests cannot be extrapolated to expected results from LLIN bioassays. In this study, susceptibility to the permethrin LLINs was highest for the population found to be least susceptible to permethrin (Tororo). Comparative bioefficacy evaluations using local vector populations such as presented in this study provide valuable data to inform selection of appropriate interventions. The consistently optimal bioefficacy of PermaNet 3.0 indicates that this combination LLIN represents a viable option for areas with pyrethroid-resistant *Anopheles spp*.

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The current study provides compelling further evidence of increasing pyrethroid resistance in Uganda, which is consistent with observations from other studies [13-19]. The *kdr* mutation (L1014S) was detected at a moderate frequency (34–37%) in *An. gambiae s.s.* across all four sites. Although metabolic resistance assays were not conducted, it is likely that these *kdr* mutations may in part be contributing to the observed reductions in efficacy of the standard LLINs. In another study in selected areas in Uganda with resistant vector populations, *kdr* frequency was found to be notably higher in *P. falciparum*-infected mosquitoes, which contributed to 70% of the malaria transmission during the dry season [18]. Although fitness cost was not assessed, this potential for higher infectivity may have enormous implications for malaria transmission and might jeopardize current resistance management strategies. It also indicates that such resistance may be affecting the bioefficacy of insecticide-based vector control interventions, such as LLINs. This requires confirmation, using standard WHO approaches such as Phase II experimental hut trials or robust longitudinal and multi-site village trials since the observations in this study were based only on cone and wireball bioassays conducted under laboratory conditions. However, the low KD and MT rates observed give some indication that there may be reductions in the ability of mono-treated LLINs to kill mosquitoes under field conditions [34], and that their continued use may have limited impact on malaria prevention and control in Uganda. Reductions in efficacy of insecticidal interventions ,due to resistance, has been noted elsewhere, such as in South Africa, Benin, Mali and Equatorial Guinea [7,8,35,36]. Accordingly, further investigations in Uganda are warranted.

The reduced susceptibility to permethrin and deltamethrin observed for the four field populations of *An. gambiae s.l.* was similarly noted in assessments conducted between 2004 to 2006 and in 2009 and 2011 in Central and Eastern Uganda, with *kdr* identified as the main resistance mechanism and metabolic resistance also implicated for Tororo district [18]. In the current study, resistance was higher in Tororo, Kanungu and Lira than in Wakiso districts perhaps due to the historical widespread use of insecticides such as organochlorines and pyrethroids in the cotton growing districts of Tororo and Lira and in the tea cultivation fields of Kanungu. Resistance may also have arisen from selective pressure exerted due to the rapid scale-up of malaria interventions, such as LLINs and IRS. The fact that the *kdr* mutational assortments in the four tested populations did not meet Hardy-Weinberg expectations is a further indication that the populations are likely currently undergoing selective pressure. Interestingly, the frequency of L1014S in the *An. gambiae s.s.* from Tororo in this study (35%), was significantly lower than that reported in 2008 (86%), but was more similar to earlier reports from 2006 (47%) and 2002 (29%) [16,18]. While rapid geographical spread of insecticide resistance alleles has been noted from ongoing longitudinal studies [37], evidence of such rapid reversion to wild type is limited and thus this warrants further investigation. The presence of multiple resistance mechanisms in malaria vector species in Tororo may have severe implications for control efforts and further testing for metabolic resistance mechanisms in Uganda should be prioritized.

The WHO recommends that action against insecticide resistance should be immediate and pre-emptive, not responsive [37]. Data as presented here provides evidence for guiding decisions on the selection of LLINs with the highest efficacy for use in specific regions of Uganda. Evidence-based decision making was successfully applied by the Uganda National Malaria Control Programme in 2009 when there was a switch from the use of pyrethroids to carbamates for IRS, following results from resistance studies indicating reduced susceptibility to pyrethoids in major malaria vectors (Additional files 2 and 3) in Northern Uganda. More comprehensive studies will be needed to ascertain the bioefficacy of LLINs in Uganda, although the WHO cautions against awaiting indisputable proof of control failures before taking action against insecticide resistance [37]. With the new global initiative of the Roll Back Malaria (RBM) partnership to scale up for impact (SUFI), PermaNet 3.0 may be the most appropriate LLINs to use for malaria prevention particularly in the Northern and Central regions of Uganda where pyrethroid-resistance is already high and there is proof of increased bioefficacy relative to standard LLINs. Despite concerted efforts by the Ministry of Health to control malaria in Lira and neighbouring districts in the Northern region, malaria has remained a challenge. A survey conducted in the adjacent district of Apac in 2001–2002 found perennial holoendemic malaria with parasite prevalence rates of 70-90% in children less than 10 years of age [38]. In the subsequent 2009 survey conducted in Apac district, age sero-prevalence curves gave no indication of recent changes in malaria transmission intensity in the area [39]. This calls for urgent scale up of malaria prevention interventions with proven bioefficacy to rapidly achieve high coverage and resulting individual and community protection from malaria.

Current WHO guidelines recommend combining ITNs and IRS in various transmission settings, especially in areas with holoendemic and epidemic malaria [40]. LLINs and IRS could be used together in the same households in Northern and Eastern regions to suppress malaria transmission. However, if LLINs are to be combined with IRS for malaria prevention and control, the

selection of appropriate LLIN types and IRS chemicals should be done with caution to avoid further exacerbating existing resistance. Products with the highest proven bioefficacy against local vector populations should be selected and IRS chemicals should differ from pyrethroids in their mode of action. In the absence of novel classes of insecticides, organophosphate- or carbamate-based IRS could be used where both LLIN and IRS are applied to form part of an insecticide resistance management strategy [41]. Encouragingly, recent insecticide susceptibility evaluations in Uganda found high susceptibility to carbamates and organophosphates in malaria vector populations (Additional file 2). A parasitemia survey in children conducted in late 2010 in three contiguous districts of Northern Uganda found that parasitemia levels were lower in two districts that had been sprayed with carbamates (37.0% and 16.7% positive smears) compared to a non-sprayed district (49.8% positive smears) [42]. There is a need for routine resistance surveillance and ongoing LLIN and IRS bioefficacy assessments against local vector populations so that products with significantly reduced efficacy relative to other available options can be replaced accordingly.

Conclusions

Pyrethroid resistance in malaria vectors in Uganda is high and is likely to limit the impact of LLINs. Evaluation of the efficacy of various LLINs against *An. gambiae* populations from different malaria transmission zones has provided valuable information on wide variations depending on the population and LLIN being tested. Such information can be used to make rational decisions for selecting LLINs with the highest anticipated bioefficacy without waiting for indisputable proof of control failures from more comprehensive studies. Monitoring the efficacy of LLINs should be undertaken regularly in order to guide policy on selection and distribution of LLINs.

Additional files

Additional file 1: Map to show malaria endemicity by district and entomological inoculation rates for specific sites in Uganda.

Additional file 2: Susceptibility to selected insecticides of adult *An. gambiae s.l.* from various districts in Uganda between August and October 2009.

Additional file 3: Susceptibility to selected insecticides of adult *An. gambiae s.l.* from various districts in Uganda between October and November 2011.

Competing interests

The authors declare no competing interests.

Authors' contributions

MO, JK, AB, SA and JR conceived the study and designed the experiments. FK supervised the genotyping for species identification and *kdr* determinations. MO, JK AB, RN and FK analysed the data, drafted and wrote the manuscript. All authors have read and approved the final manuscript.

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RESEARCH Open Access

Bio-efficacy of selected long-lasting insecticidal nets against pyrethroid resistant *Anopheles arabiensis* from South-Western Ethiopia

Delenasaw Yewhalaw[1*], Abebe Asale[1], Kora Tushune[2], Yehenew Getachew[3], Luc Duchateau[4] and Niko Speybroeck[5]

Abstract

Background: The emergence and spread of insecticide resistance in the major African malaria vectors *Anopheles gambiae s.s.* and *Anopheles arabiensis* may compromise control initiatives based on insecticide-treated nets (ITNs) or indoor residual spraying (IRS), and thus threaten the global malaria elimination strategy.

Methods: We investigated pyrethroid resistance in four populations of *An. arabiensis* from south-western Ethiopia and then assessed the bio-efficacy of six World Health Organization recommended long lasting insecticidal nets (LLINs) using these populations.

Results: For all four populations of *An. arabiensis*, bottle bioassays indicated low to moderate susceptibility to deltamethrin (mortality at 30 minutes ranged between 43 and 80%) and permethrin (mortality ranged between 16 and 76%). Pre-exposure to the synergist piperonylbutoxide (PBO) significantly increased the susceptibility of all four populations to both deltamethrin (mortality increased between 15.3 and 56.8%) and permethrin (mortality increased between 11.6 and 58.1%), indicating the possible involvement of metabolic resistance in addition to the previously identified *kdr* mutations. There was reduced susceptibility of all four *An. arabiensis* populations to the five standard LLINs tested (maximum mortality 81.1%; minimum mortality 13.9%). Bio-efficacy against the four populations varied by net type, with the largest margin of difference observed with the Jimma population (67.2% difference). Moreover, there were differences in the bio-efficacy of each individual standard LLIN against the four mosquito populations; for example there was a difference of 40% in mortality of Yorkool against two populations. Results from standard LLINs indicated reduced susceptibility to new, unused nets that was likely due to observed pyrethroid resistance. The roof of the combination LLIN performed optimally (100% mortality) against all the four populations of *An. arabiensis*, indicating that observed reductions in susceptibility could be ameliorated with the combination of PBO with deltamethrin, as used in PermaNet® 3.0.

Conclusion: Our results suggest that bio-efficacy evaluations using local mosquito populations should be conducted where possible to make evidence-based decisions on the most suitable control products, and that those combining multiple chemicals such as PBO and deltamethrin should be considered for maintaining a high level of efficacy in vector control programmes.

Keywords: Bio-efficacy, Long-lasting insecticidal nets, Insecticide resistance, *Anopheles arabiensis*, Ethiopia

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Background

Insecticide-treated nets (ITNs) lead to a reduction of human-vector contact by providing a physical barrier and through insecticidal and/or repellent effects. Wide-scale deployment of ITNs protects users as well as non-users through personal and community level protection gained with high coverage rates [1,2]. In this way, ITNs have been shown to reduce the burden of malaria in pregnant women and young children [3] and reduce the incidence of uncomplicated malarial episodes by around 40% in areas of both stable and unstable malaria relative to untreated nets [4]. Long-lasting insecticidal nets (LLINs) pre-treated with insecticides designed to last the life span of the mosquito net were developed to avoid the need for retreatment every 6 months [5]. To be classified as an LLIN, nets must retain their effective biological activity without re-treatment for at least 20 WHO standard washes under laboratory conditions and three years of recommended use under field conditions [6]. Two techniques have been developed to maintain biological activity: incorporating the insecticide into the textile polymer through extrusion (as with polyethylene and polypropylene), and mixing the insecticide with a wash-resistant resin that is bound around the fibers of the polymer (polyester). Pyrethroids are the only class of insecticide currently recommended to treat mosquito nets. Twelve net types are currently recommended by the WHO Pesticide Evaluation Scheme (WHOPES), and use permethrin, deltamethrin or alpha-cypermethrin, with one combination net using deltamethrin combined with the synergist piperonylbutoxide (PBO) in the roof of the product. However, there are increasing reports of malaria vectors that have developed resistance to the pyrethroids commonly used in LLINs and pyrethroid resistance is now firmly established throughout Africa [7-9]. This resistance to pyrethroids may compromise malaria control as LLINs may lose efficacy, although at present there are no studies linking insecticide resistance to LLIN control failure.

In Ethiopia, ITN use started in 1997 and scaling up commenced in 2005 with the aim of obtaining a high coverage towards effective malaria control. The National Malaria Control Programme (NMCM) distributed 36 million LLINs between 2005 and 2010, targeting 52 million people at risk [10]. Indoor residual spraying has also been conducted using deltamethrin, malathion and bendiocarb.

An. arabiensis Patton is the primary malaria vector species in the south-west of the country, and is the only vector species of the *An. gambiae* complex present in the study area. Previous studies within the area indicated that populations of *An. arabiensis* were resistant to DDT, permethrin, deltamethrin, malathion [11,12] and lambdacyhalothrin (D. Yewhalaw *et al.*, unpublished). The West African *kdr* mutation (L1014F) was the underlying resistance mechanism observed in these mosquito populations with an allelic frequency of over 98% [11,12]. However, the relationship between *kdr* frequency and phenotypic resistance remains poorly defined; for instance, rapid increases in *kdr* frequency in *An. gambiae s.s.* from western Kenya were not linked to concurrent increases in phenotypic resistance [13]. Moreover, despite *kdr* reaching fixation, LLINs appeared to remain effective. Thus, observed resistance in *An. arabiensis* in the study area may not be solely attributable to target-site resistance, though investigations of other mechanisms have been lacking due to limited capacity to conduct biochemical assays on fresh field-collected specimens, which is required for detection of upregulated esterases, oxidases or GSTs. Furthermore, little is known about the implications of any observed resistance on the anticipated bio-efficacy of insecticidal interventions such as LLINs.

Therefore, this study was conducted to: 1) monitor insecticide resistance and assess the presence of resistance mechanisms other than *kdr* in these mosquito populations and 2) determine the bio-efficacy of six WHOPES-recommended LLINs against pyrethroid resistant populations of *An. arabiensis* from south-western Ethiopia.

Methods

Study area and period

Mosquitoes were collected from villages located in Jimma, TiroAfeta, OmoNada and Kerssa districts (*weredas*) in south-western Ethiopia, from November 2011 to January 2012. TiroAfeta, Omo Nada and Kerssa districts are located approximately 255 to 297 km southwest of the capital Addis Ababa, whereas Jimma is located 335 km southwest of the capital. The study area lies between latitudes 7°42'50"N and 07°53'50"N and between longitudes 037°11'22"E and 037°20'36"E, at an altitude of 1,672–1,864 m above sea level. The area has a sub-humid, warm to hot climate, receives between 1,300 and 1,800 mm of rain annually and has a mean annual temperature of 19°C. The rainfall pattern of the area is similar to other parts of Ethiopia, with the long rainy season starting in June and extending up to September while the short rainy season begins in March and extends to April/May. The main socio-economic activities of the local communities in the 3 districts (TiroAfeta, Omo Nada and Kerssa) are mixed farming involving the cultivation of staple crops (maize, teff and sorghum), and cattle and small stock-raising.

Previous assessments showed that *An. arabiensis* was the predominant species present in the area, and populations from all four sites exhibited high resistance to DDT (0–2.7% mortality) in WHO susceptibility tests [11]. Resistance to pyrethroids was also noted for all populations, with mortalities of 10.0, 4.5, 37.3 and 42.7% after exposure to permethrin and 55.5, 56.9, 53.6 and 78.6% after exposure to deltamethrin for *An. arabiensis* populations from Jimma, Omo Nada, Kerssa and TiroAfeta, respectively. Resistance to malathion (60.0–81.8% mortality) but susceptibility to

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propoxur (99.1–100% mortality) was also noted. Very high (95–100%) allelic frequencies of *kdr*-L1014F mutation were found in all four populations but the ace-1^R mutation was not detected [11].

Mosquito collections

Adult female mosquitoes were collected from inside houses and cow sheds by two teams of two people from 5:00 h to 7:30 h using a torch and aspirator in each of the study districts. Adults were transported to the Vector Biology Laboratory, Asendabo for direct use in CDC bottle assays.

Mosquito larvae were collected from different breeding habitats in the four districts, transported to the Vector Biology Laboratory, Asendabo and were reared to adult stage feeding on dog biscuits and baker's yeast for use in WHO cone bioassays. All adult mosquitoes were identified morphologically using standard taxonomic keys [14].

CDC bottle assays

CDC bottle assays were carried out on populations of *An. arabiensis* from the four study districts in order to monitor susceptibility to permethrin and deltamethrin. The bottle assay was conducted following standard procedures [15,16]. Reagent bottles (Wheaton bottles, 250 ml) were coated with 1 ml of either permethrin (21.5 μg/bottle) or deltamethrin (12.5 μg/bottle), which were diluted with factory-grade acetone. Assays with both insecticides were also run following a pre-exposure step in which mosquitoes were exposed to the synergist piperonylbutoxide (PBO, 400 μg/bottle) for one hour before undergoing the standard bottle assays. Each bottle was rolled and inverted in such a way that all interior surfaces were exposed to the solution as the acetone was allowed to evaporate. The bottles and caps were inverted on paper over night in a dark cabinet. Approximately 10–15 field collected adult mosquitoes were introduced into each bottle by mechanical aspiration at time = 0 and mortality was recorded at 15 minutes intervals up to 120 minutes. Mortality was recorded for mosquitoes that could not rest the right way up or fly when the test bottles were slowly rotated. After 120 minutes, mosquitoes were transferred to recovery cups and observed 24 hour later. Mortality after 30 minutes (the resistance threshold for deltamethrin and permethrin in our test conditions) and 24 hour recovery were recorded. Each test had 4 replicates with approximately equal numbers of mosquitoes that were introduced into control bottles coated with acetone only; assays were run simultaneously. For the pre-exposure step, an equal number of mosquitoes were concurrently exposed in a bottle coated with acetone only.

LLIN sample preparation and chemical assays

Three rectangular nets of 6 net types plus untreated nets to be used as a negative control were purchased from the local market in Uganda due to availability. The production date and batch number of all nets were recorded. For standard LLINs (Olyset®, Netprotect®, Interceptor®, Yorkool® and PermaNet® 2.0), three sub-samples per net were taken and prepared for cone tests by cutting 30 cm x 30 cm pieces: one from the roof and two others with one from each long side of the net. For the combination net PermaNet® 3.0, five sub-samples were prepared for cone tests: one piece from the roof, two samples from the upper half of each long side, and two samples from the lower half of each long side of the net. This was done to verify if there were any differences in bio-efficacy between the lower border region of the sides of the net and the upper region of the sides of the net. Three or five sub-samples were similarly taken adjacent to cone test sub-samples to be used as reference samples in chemical assays. Each sub-sample was rolled up in new aluminium foil, labelled (by net type, net number and sample area) and kept individually in a refrigerator prior to assays. Reference samples were tested for chemical content at an ISO IEC 17025-accredited laboratory to confirm that all nets were within product target doses. For deltamethrin, normal-phase high performance liquid chromatography (HPLC) was conducted as per standard protocols (CIP 333/LN (M)). For alpha-cypermethrin, extraction was conducted with n-hexane and 1,4-dioxane (95:5 v/v) with the mixture then shaken and sonicated and filtered on a 0.45 mm teflon membrane, whereas for permethrin hot xylene extraction was followed by drying, reconstitution and filtration, with both then assessed via HPLC. The precision as measured by the Relative Standard Deviation was 0.79% and 1.79%, respectively and the recovery was 101 and 102%, respectively.

WHO cone bioassays

For each individual sub-sample prepared for cone tests from both standard LLINs and the combination LLIN, four cone tests were conducted at a time following standard WHO procedure [6] using mosquitoes from each collection district. Five non-blood fed two to three day old adult female *An. arabiensis* were introduced into each cone and exposed to each bed net sample for 3 minutes before being transferred to paper cups and held with access to 10% sugar solution. Knockdown (KD) was recorded at 1, 3, 5, 10, 15, 30, 45 and 60 minutes and mortality (MT) was recorded 24 hours post-exposure. A total of 180 mosquitoes were tested for each net type (20 mosquitoes x 3 sub-samples x 3 nets) for standard LLINs while 300 mosquitoes were tested for the combination net (20 mosquitoes x 5 sub-samples x 3 nets) for each of the four mosquito populations. Replicates of cone assays with sub-samples taken from untreated nets were also conducted concurrently as a negative control. Mortality was corrected using Abbott's formula when mortality in the control exceeded 5% [17]. Bioassays were carried out at 27 ± 2°C and 80 ± 4% relative humidity.

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Data analysis

Data were analysed using SAS software package. Association between % knockdown and % mortality by site, type of net and net section were assessed vialine arregression. Differences in mean % mortality for the sections of specific net types were assessed via Student's *t*-test for standard LLINs and via ANOVA for the combination net. Variations in mean % mortality between the 5 net types, and for each net type between the 4 mosquito populations, were assessed via ANOVA with Duncan's method applied to identify groupings. The alpha value was set at 0.05 with $P < 0.05$ considered significant in the analysis.

Results

Bottle bioassays

Results of the susceptibility status of populations of *An. arabiensis* from the 4 collection sites as determined in CDC bottle bioassays are presented in Figure 1. At the 30 minute diagnostic period, all four populations showed low to moderate susceptibility to deltamethrin (mortality ranged between 43% and 80%) and permethrin (mortality ranged between 16% and 76%). Susceptibility to deltamethrin was highest for the Jimma and Omo Nada populations (79.7 and 76.5% mortality, respectively), though susceptibility to permethrin was highest for the Omo Nada population only (75.9% mortality) with mortality % ≤60% for all other situations. The synergist PBO reduced the expression of deltamethrin and permethrin resistance in the four populations of *An. arabiensis*. Following pre-exposure for 1 hour to PBO, the susceptibility of mosquito populations increased at all four sites to both deltamethrin (mortality increased from 18.0 to 56.8%, to range from 91.8 to 100%) and permethrin (mortality increased from 11.6 to 58.1% to range from 73.9 to 100%). The increase in mortality following exposure to PBO was greatest at Jimma and TiroAfeta for deltamethrin and at Kerssa and TiroAfeta for permethrin, however for the Jimma population there was not such a marked increase in susceptibility to permethrin following pre-exposure to PBO with mortality remaining relatively low (73.9%).

Cone bioassays

Overall, there was a significant relationship between % knockdown and % mortality ($R^2 = 0.53$, $n = 959$, $p < 0.001$), noting that one data point (single sample of PermaNet® 2.0 side) was missing from the bio-efficacy data set. When data were stratified by site and net type, there was a significant association between mean % knockdown and % mortality for PermaNet® 3.0, Interceptor® and Olyset® against all mosquito populations ($p < 0.05$) (Figure 2). For PermaNet® 2.0, Netprotect® and Yorkool®, there was an association between mean % knockdown and % mortality for two populations only, although there was no consistency in populations where an association was found. Based on observed associations, further assessments of bio-efficacy focused on mortality data.

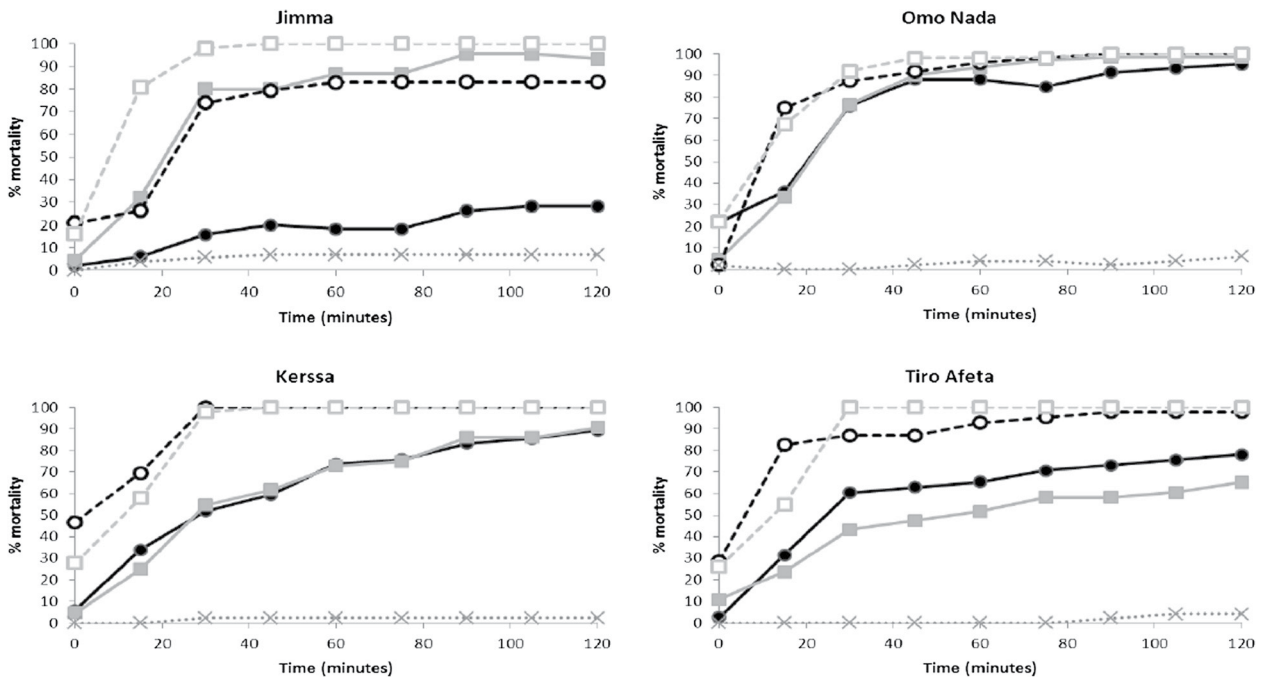

Figure 1 Susceptibility of populations of *An. arabiensis* adult female mosquitoes collected from four sites in Ethiopia to permethrin alone (black filled circle), permethrin following 60 mins pre-exposure to PBO (black unfilled circle), deltamethrin alone (grey filled square), and deltamethrin following 60 mins pre-exposure to PBO (grey unfilled square) in bottle bioassays. Average of all controls is also indicated (x).

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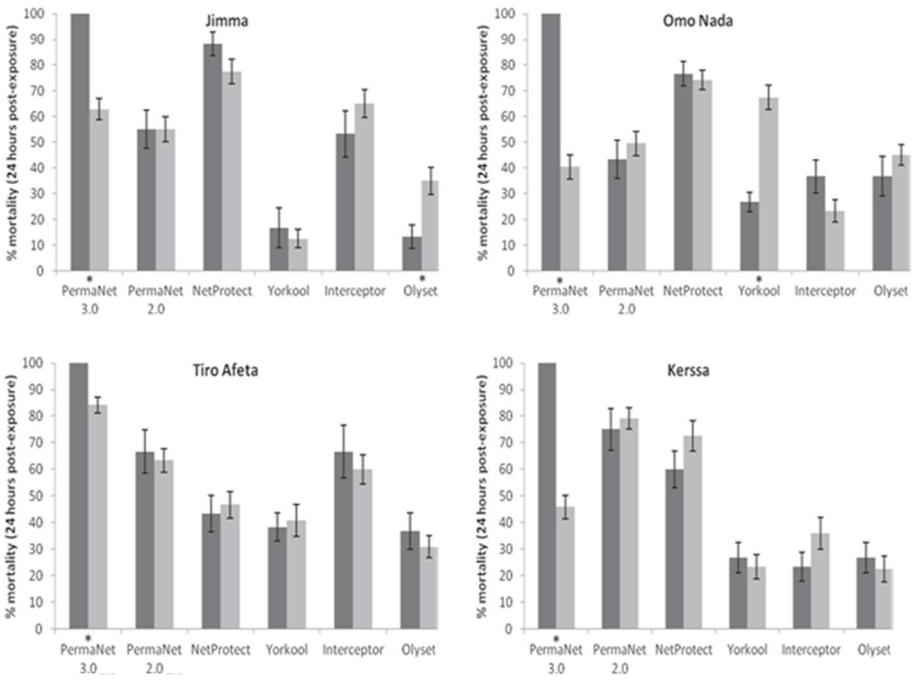

Figure 2 Bio-efficacy of roof (black rectangles) and side (grey rectangles) samples of six long-lasting insecticidal net types against *An. arabiensis* adult female mosquitoes collected from four sites in Ethiopia following 3-minutes exposure in standard WHO cone bioassays. Bars show mean percentage mortality ± standard error, asterisks indicate significant difference detected between roof and sides ($P < 0.05$).

When mean % mortality was compared between the different net sections for each net type for each study site, there were significant differences observed in the bio-efficacy of net sections for PermaNet® 3.0 against all four populations ($p < 0.05$ for all), for Olyset® against the Jimma population ($p = 0.012$) and for Yorkool® against the Omo Nada population ($p < 0.05$). However, for PermaNet® 3.0 there was a clear grouping of lower and upper side data (mortality of 59.2 and 66.7%, respectively), with roof data significantly higher (100%). Based on observed associations, data for sections of the specific net types were grouped together except for PermaNet®3.0 for which the roof and side panels were assessed separately.

Although there was an association between mean % knockdown and % mortality for 16 of the 40 other net types–net section–collection site groupings, there was no observable pattern. There was a particularly large disparity in the mean % knockdown and mortality data for Yorkool® roof sections against the Omo Nada *An. arabiensis* population.

Table 1 shows the bio-efficacy of the six LLINs tested against the four *An. arabiensis* populations. Bio-efficacy against each population varied significantly between net types: Jimma ($F = 39.24$, $n = 240$, $p < 0.001$); Omo Nada ($F = 21.24$, $n = 239$, $p < 0.001$), Kerssa ($F = 34.21$, $n = 240$, $p < 0.001$); TiroAfeta ($F = 28.73$, $n = 240$, $p < 0.001$). The greatest variation in bio-efficacy was observed for the Jimma population (PermaNet® 3.0 roof: 100%, Yorkool®: 13,9%), with the least variation observed against the TiroAfeta population (PermaNet® 3.0 roof: 100%, Yorkool®: 40.0%).

The bio-efficacy of the roof section of PermaNet® 3.0 was consistently high against all mosquito populations (all 100%). Apart from this, the bio-efficacy of each specific net type varied significantly between mosquito populations: PermaNet® 3.0 sides ($F = 22.78$, $n = 192$, $p < 0.001$); PermaNet® 2.0 ($F = 11.11$, $n = 143$, $p < 0.001$); Netprotect® ($F = 16.83$, $n = 144$, $p < 0.001$); Yorkool® ($F = 18.70$, $n = 144$, $p < 0.001$); Interceptor® ($F = 17.37$, $n = 144$, $p < 0.001$); Olyset® ($F = 4.34$, $n = 144$, $p < 0.0058$). This indicates that with the exception of the combination roof of PermaNet® 3.0, the standard LLINs performed differently against the different *An. arabiensis* populations.

Target insecticide and/or synergist concentrations for all LLINs fell within manufacturer specifications (Table 2).

Discussion

Bottle bioassays revealed that populations of *An. arabiensis* from all four localities in south-western Ethiopia had low to moderate susceptibility to both permethrin and deltamethrin for the diagnostic dose and time used. Although no historical data for the same populations or reference data from a susceptible *An. arabiensis* strain were available, previous WHO susceptibility tests also indicated reduced susceptibility of mosquito populations from the same study area to these insecticides [11,12]. Moreover, the susceptibility of mosquito populations to both permethrin and deltamethrin increased

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Table 1 Bio-efficacy (in mean percentage mortality) of samples of six long-lasting insecticidal net types against *An. arabiensis* adult female mosquitoes collected from four sites in Ethiopia following 3-minutes exposure in standard WHO cone bioassays

	Net type/section							
Collection site	PermaNet® 3.0		PermaNet® 2.0	NetProtect®	Yorkool®	Interceptor®	Olyset®	F statistic; P-value
	Roof	Side						
Jimma	100a	62.92c	55.00c	81.11b	13.89e	61.11c	27.78d	39.24; <0.0001*
Omo Nada	100a	40.42c,d	47.43c	75.00b	53.89c	27.78d	42.22c	21.24; <0.0001*
Kerssa	100a	45.83c	77.78b	68.33b	24.44d	31.67d	23.89d	34.21; <0.0001*
TiroAfeta	100a	84.17b	64.44c	45.56d	40.00d	62.22c	32.78d	28.73; <0.0001*

*Differences in mean % MT between net types at a specific collection site were significant ($p < 0.05$; ANOVA and Duncan's test); Means within a row followed by the same letter (s) are not significantly different from each other ($p \geq 0.05$).

significantly when synergized by PBO, suggesting the presence of metabolic-based resistance mechanisms. Since PBO inhibits two major metabolic systems (P450s and non-specific esterases) that are otherwise responsible for degrading or sequestering the insecticide [18] and also enhances cuticular penetration thereby increasing the rate of uptake into the mosquito [19], it is difficult to know which mechanisms are operating without conducting a battery of other tests such as esterase-only synergist biochemical assays or genetic analyses. This was beyond the scope of this initial evaluation but further investigations of resistance mechanisms are clearly warranted to better define and quantify resistance mechanisms present in the test populations and verify the preliminary evidence of metabolic-based mechanisms as indicated by bottle bioassays.

Low knockdown and mortality of the four *An. arabiensis* populations following exposure to standard LLINs may be explained by either limited bioavailability of active ingredient on the LLIN surface or by physiological resistance of mosquitoes to the insecticide. Chemical assays indicated that pyrethroid content was satisfactory for all LLIN types, and as nets were new and had not been washed it was assumed that surface chemical content was satisfactory. It was most likely that reductions in efficacy were due to previously-identified *kdr* mutations and/or suspected metabolic resistance mechanisms. This was supported by the observed bio-efficacy of the roof of PermaNet 3.0, since the deltamethrin and PBO combination clearly restored optimal bio-efficacy against all four populations. While loss in efficacy of pyrethroid ITNs has been associated with high *kdr* mutation frequency in *An. gambiaes.s.* in Burkina Faso [20], in Western Kenya a high *kdr* frequency was not associated with a reduction in ITN efficacy [13]. General consensus among experts is that metabolic resistance is considered more of a threat than *kdr*, with major loss of efficacy of permethrin-treated nets in experimental huts associated with oxidase-based metabolic resistance in *An. gambiae* in Cameroon [21] and *An. arabiensis* in Cameroon [20]. Co-occurrence of *kdr* and P450- based resistance has been reported in mosquito populations from several countries [22,23], leading to extremely high levels of pyrethroid resistance [24,25] and extreme reduction in LLIN efficacy against *An. gambiae* in Akron, Benin [20]. The likely co-existence of multiple resistance mechanisms in *An. arabiensis* from the

Table 2 Mean (± standard error) insecticidal or synergist concentration and % as proportion of target concentration for roof and side samples from six different LLINs types as determined via high performance liquid chromatography

		Target dose		Roof	Side
Net type	Chemical	Mean	Range	Mean	Mean
PermaNet® 3.0	Deltamethrin	2.8 g/kg (sides)	2.1–3.5	n/a	2.4 ± 0.1
		4 g/kg (roof)	3.0–5.0	3.8 ± 0.1	n/a
	Piperonylbutoxide	25 g/kg (roof)	18.75–31.25	24.3 ± 1.0	n/a
PermaNet®2.0	Deltamethrin	55 mg/m2	41.25–68.75	60.8 ± 1.0	62.5 ± 4.1
NetProtect®	Deltamethrin	1.8 g/kg	1.35–2.25	1.9 ± 0.0	1.9 ± 0.0
Yorkool®	Deltamethrin	55 mg/m2	41.25–68.75	56.2 ± 8.3	59.9 ± 9.4
Inteceptor®	Alpha-cypermethrin	200 mg/m2	150.0–250.0	223.6 ± 20.8	196.0 ± 33.7
Olyset®	Permethrin	20 g/kg	17.0–23.0	22.4 ± 0.1	22.2 ± 0.1

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four areas in Ethiopia and the observed significant reductions in their susceptibility to LLINs in cone bioassays raises major concerns for the performance of pyrethroid interventions in Ethiopia.

In Ethiopia, DDT has been extensively used in indoor residual spraying (IRS) in alternation with malathion for over five decades. ITN use started in 1997 with significant scale up since 2005 (mainly LLINs) with the aim of obtaining a high coverage towards upgraded malaria control. In addition, pyrethroids (deltamethrin) were used in indoor residual spraying in 2009 [26]. The prolonged use of DDT and malathion, the high coverage of LLINs and the recent use of pyrethroids for indoor residual spraying are likely to have enhanced the selection pressure for insecticide resistance in the *An. arabiensis* populations in Ethiopia. The increasing trend in use of pyrethroid for indoor residual spraying may not be consistent with the need to preserve the effectiveness of LLINs [26]. Trape *et al.* [27] also reported that LLINs may result in mosquito resistance to insecticides and that the increase in pyrethroid resistance of *An. gambiae* likely caused the rebound of malaria morbidity in Senegal. In 2011, Ethiopia switched from pyrethroids (deltamethrin) to carbamates (bendiocarb) for IRS because of resistance reported to other classes of insecticides [28]. The carbamate class is the only class of insecticides to which these mosquito populations are susceptible in Ethiopia. Unfortunately, evidence of resistance to carbamates (bendiocarb) has also emerged in Afro-tropical malaria vectors from elsewhere [29-33].

If resistance and control failure is shown to both pyrethroids and DDT, programs will need to consider carbamates and organophosphates [34]. High levels of control have been achieved with certain carbamates and this insecticide class has been evaluated for potential use on ITNs [35]. However, safety remains a concern with carbamates, and formulations with low toxicity or methods of delivery that limit human contact may be potential options alone or in combination with pyrethroid-treated nets [36]. Combining two classes of insecticides on nets may also present a method for managing resistance, by exposing mosquitoes to two insecticides with different modes of action [37,38]. However, since there are currently no non-pyrethroid LLINs available combining these insecticides with a synergist such as PBO offers a viable and readily-available alternative to standard LLINs for areas with pyrethroid-resistant *Anopheles* populations.

While cone bioassays on new nets are by no means a definitive indication of anticipated net performance under field conditions, these assays can provide valuable comparative information across numerous sites, where experimental huts are not available. Non-uniformity of nets such as PermaNet® 3.0 complicate evaluations where net sections are assessed separately; since anophelines most frequently make contact with the roof of bed nets (37, P. McCall personal communication), emphasis would be well placed on outcomes from roof sections. Further studies are warranted to investigate the impact of observed resistance on LLIN bio-efficacy, and also to better define the relationship between results from cone bioassays, experimental hut trials and real-life use. In Mali, *An. gambiaes.l.* populations from two sites showed no apparent differences in susceptibility to alpha-cypermethrin nets when tested in laboratory cone bioassays yet one population showed reduced susceptibility to the same nets in experimental hut trials [39].

This study was the first attempt to establish the comparative bio-efficacy data of six types of WHO-recommended LLINs against pyrethroid resistant populations of *An. arabiensis* from Ethiopia. Although comparisons to a susceptible strain were not incorporated due to logistical limitations, the low bio-efficacy of new LLINs against these populations suggests that the standard LLINs tested would have sub-optimal efficacy under field conditions. We also report for the first time the likely existence of metabolic resistance in addition to *kdr* mutations in Ethiopia. The underlying mechanisms involved in metabolic resistance should be further assessed using esterase and glutathione-S-transferase synergists as well as at the genetic level using the microarray technique. LLINs should be assessed at additional sites across the country to compare bio-efficacy against populations with different resistance levels or mechanisms, and attempts need to be made to relate results to observed phenotypic resistance and observed or reported LLIN failure.

Conclusion

Relatively low knockdown and mortality rates were observed for four pyrethroid resistant populations of *An. arabiensis* from south-western Ethiopia following exposure to new, unused WHO-recommended standard LLINs. Conversely, optimal bio-efficacy was observed for the deltamethrin + PBO roof of PermaNet® 3.0 against all four populations. Although the approach used cone bioassays with new nets only, it provided compelling information suggesting that pyrethroid resistance may be a cause for concern for sustained efficacy of pyrethroid-based interventions in Ethiopia. It also indicates the utility of conducting comparative bio-efficacy studies using local mosquito populations, and underscores the urgent need to establish an insecticide resistance management (IRM) strategy for Ethiopia.

Competing interests

Authors declare that they have no competing interests.

Authors' contribution

DY conceived and designed the study, was involved in field supervision and drafted the manuscript; AA & DY were involved in WHO cone bioassays and CDC bottle assays; KT, YG, LD & NS reviewed the manuscript. All authors read and approved the final version of the manuscript.

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Experimental hut studies

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Research Article

Efficacy of a combination long lasting insecticidal net (PermaNet® 3.0) against pyrethroid resistant *Anopheles gambiae s.s* and *Culex quinquefasciatus* : an experimental hut trial in Nigeria

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Summary

PermaNet® 3.0: a mosaic combination long-lasting insecticidal net (LLIN) combining deltamethrin coated polyester side panels and a deltamethrin with PBO incorporated polyethelene roof was designed to give increased efficacy against pyrethroid-resistant malaria vectors. Evidence supporting the efficacy of this product is mainly limited to comparison with PermaNet® 2.0: a product relying on deltamethrin alone. Here we report on performance of PermaNet® 3.0 on free flying *Anopheles gambiae* and *Culex quinquefasciatus* in comparison to permethrin-impregnated Olyset® nets in an area with multiple pyrethroid resistance mechanisms. Prior to the field test, net samples were washed using standard WHOPES washing protocols and bio-efficacy conducted with the reference susceptible Kisumu strain of *A. gambiae*. Additional bioassay tests were conducted on the net samples with a resistant strains of *A. gambiae s.s* and *C. quinquefasciatus* prior to and after the field trial. Field efficacy was expressed in terms of deterrence in hut entry, blood-feeding inhibition, induced exophily and mortality. Laboratory cone bioassays prior to and after field test showed high mortality of *A. gambiae s.s.* (>70%)in PermaNet® 3.0 which was consistent with the field data. Increased bio-efficacy likely due to the synergist effect of PBO and deltamethrin was obvious from *in situ* bioassays, with significant mortality of resistant *A. gambiae s.s.* recorded following exposure to the roof panel. Experimenal field data showed that PermaNet® 3.0 induced a level of deterrence and exophily against *A. gambiae* and *C. quinquefasciatus* similar to that of the Olyset® net, but the feeding rate of *A. gambiae s.s.* in PermaNet® 3.0 was highly minimized compared to the Olyset® net. In spite the presence of both *kdr* and MFO resistance mechanisms, the proportion of *A. gambiae s.s* and *C. quinquefasciatus* killed by PermaNet® 3.0 was significantly higher than the Olyset® net ($P < 0.01$) confirming the increased bioefficacy of PermaNet® 3.0.PermaNet® 3.0 outperformed the Olyset® net and could provided additional protection in terms of reduction in blood feeding and increase in mosquito mortality: a plausible tool against pyrethroid resistant mosquitoes.

Key words: long-lasting nets, PermaNet®, P.B.O, *Anopheles gambiae , Culex quinquefasciates, kdr,*

Introduction

The development of long-lasting insecticidal net technology has been described as a breakthrough in malaria prevention[1]. However, there is increasing evidence from West[2,3,4] Central[5,6] and East Africa[7,8] on the

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reduced efficacy of insecticide treated nets (ITNs) and long lasting insecticidal nets (LLINs) in areas with high levels of mosquito resistance to pyrethroid. Although alternative insecticides on nets samples have been tested against pyrethroid resistant *Anopheles*[9,10], none is as effective as pyrethroid in terms of the excito-repellency properties and fast knock down action of pyrethroid which are major features that provide protection against mosquito bites.

Two major mechanisms play an important role in *Anopheles* resistance to insecticides: target site insensitivity and metabolic enzyme-based resistance[11,12]. Target site insensitivity to pyrethroid is associated with a single point mutation commonly referred to as knock down resistance (*kdr*) leading to modification of the voltage-gated sodium channel gene making it less susceptible to the binding of pyrethroid[13] . Metabolic-based resistance mechanism is principally associated with mixed function oxidases (MFO) [14] . Evidence of both resistance mechanisms had been reported in the malaria mosquito: *Anopheles gambiae s.s* from Nigeria[15,16]. Insecticide resistance management strategies include, rotational use of insecticide, insecticide mixtures and insecticide synergism. Insecticide synergism in particular is used to enhance the potency of commercial aerosols[17].

With the threat of insecticide resistance, newer methods of preserving the efficacy of available public health insecticides need to be put in place. PermaNet® 3.0, a new LLIN, was designed to give increased efficacy against pyrethroid-resistant malaria vectors. This mosaic LLIN combines deltamethrin coated polyester side panels and deltamethrin with the synergist piperonyl butoxide (PBO) incorporated in the polyethylene roof. PBO is an inhibitor of MFO with potential to reduce activity of enzymes associated with this resistance mechanism. The principle behind the development of PermaNet® 3.0 is based on the notion that a combination of a pyrethroid and PBO will also enhance the rate of insecticide penetration in the insect and increase the efficacy of the net. The World Health Organization Pesticide Evaluation Scheme (WHOPES) is yet to set criteria for evaluating products that have an effect on insecticide resistant vectors. A practical approach is to compare the efficacy of PermaNet®3.0 with LLINs that have received full WHOPES recommendation. At present, only PermaNet® 2.0 and Olyset® nets have met these criteria[18-20] with Yorkool® nets given a full recommendation but based only on equivalence with PermaNet® 2.0. Aside from a parallel study in Benin[21], previous field evaluations of PermaNet® 3.0 were made in comparison with PermaNet® 2.0, a product from the same manufacturer but relying on deltamethrin alone[22,23]. Following a review of the available evidence on the efficacy of PermaNet® 3.0[24], an interim recommendation was granted by WHOPES based on the need for additional proof of evidence as a requirement for developing full recommendations on the use of the product.

The current study was undertaken to compare the performance of PermaNet® 3.0 with permethrin-incorporated Olyset® nets in experimental huts in north-central Nigeria.

Materials and method

Study site and experimental huts: The study was conducted in experimental huts situated at the Nigerian Institute of Medical Research field station at New Bussa (9° 53'N; 4° 31' E). The malaria mosquito *Anopheles gambiae s.s.* in the area exhibits a high level of pyrethroid resistance associated with both knock down resistance (*kdr*) and metabolic-based resistance with mixed function oxidases (MFO) (Awolola unpublished). Six experimental huts were built on a concrete floor following the pattern of huts used in West Africa[25]. The styles of the huts simulates domestic habitations and were purposely built with

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the front side facing perennial mosquito breeding sites.

Insecticide susceptibility test: Prior to the commencement of the experimental hut evaluation, *Anopheles* and *Culex* larvae were collected around the field site and reared to adulthood. Insecticide susceptibility test was conducted with permethrin (0.75%) and deltamethrin (0.05%) on 2-3 day old non-blood fed female mosquito using WHO test kits[26]. Mosquitoes tested were identified morphologically and specimens belonging to the *A. gambiae s.l.* further analysed by PCR[27]. A population *of Anopheles gambiae* that survived the insecticide exposure was divided into two: a subset was analysed for the presence of the *kdr* mutation[13]. The second subset was induced to lay eggs in the laboratory insectaries, and the F1 progeny used for synergist and biochemical analysis using the protocol described in our previous study[16] with reference to the Kisumu susceptible strain of *A. gambiae* .

Bioassay on net samples: Before field test, net samples: PermaNet® 3.0, Olyset® and conventionally treated polyester net (CTN) samples were washed according to the WHOPES phase 1 protocol[25]. Because of the limited information on insecticide regeneration on Olyset®, incubation of the Olyset® net samples was adapted from a previous phase 1 study in our laboratoryand bio-efficacy evaluations conducted with the Kisumu susceptible reference strain of *A. gambiae s.s.* Separate bioassay tests were conducted with resistant strains of *A. gambiae s.s* and *C. quinquefasciatus* prior to and after the field test.

Experimental hut evaluation

The experimental set up was made of six treatment arms:

1. Untreated net
2. PermaNet® 3.0 unwashed
3. Olyset® unwashed
4. PermaNet® 3.0 washed 20 times
5. Olyset® washed 20 times
6. Polyester net conventionally treated (by deltamethrin at 25 mg/m²) and washed until just before exhaustion (<80% mortality in cone bioassay or <95% knockdown after 1h).

Each treatment arm consisted of 7 whole nets, 6 of which were used in the experimental huts. The seventh net of each arm was used as a reference sample and preserved for chemical analysis. Nets were purposely holed with 6 holes of 4 x 4 cm cut in each net. The conventionally treated net (CTN) washed until just before exhaustion (CTN) was used as the positive control. The 12 weeks Latin Square design was adapted from WHO guidelines for phase 2 field trials[25]. Each treatment arm was rotated each week among the huts, with rotation of adult male volunteers who slept under the nets each night. Field efficacy was expressed in terms of mosquito deterrence in hut entry, blood-feeding inhibition, induced exophily and mortality.

Chemical analysis: The 7th net of each treatment arm was not tested in the huts but instead stored at ambient temperature and processed for chemical assays together with samples used in the field. Chemical assays were carried out according to CIPAC method at an independent laboratory (TUV SUD PSB Pte Ltd, Singapore: test report reference no. 719186073-CHM10/02-CSY &719186073-CHM10-JS-CR01).

Statistical analysis

Because of the variation in the number of mosquitoes collected over different nights, the number of mosquitoes entering the huts was analysed using non-parametric Kruskall-wallis tests. The proportion of mosquitoes that exited into the traps, the proportion killed within the hut and the proportion that was blood fed in each experimental arm were analysed using logistic regression (STATA 6 Software).

Results

Mosquito resistance status

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The *Anopheles* population for the resistance test consisted of 61.6% *A. gambiae s.s* and 38.4% *A. arabiensis*. *Anopheles gambiaes.s* was resistant to permethrin and deltamethrin with an average mortality rate of 75.7 and 79.5% for permethrin and deltamethrin respectively. *Anopheles arabiensis* was susceptible to both insecticides (Table 1). The mortality rate for *Culex quinquefasciatus* was 61.3 and 74.1% in permethrin and deltamethrin respectively (Table 1).

Table 1: Susceptibility status of *Anopheles gambiae s.s, A. arabiensis, Culex quinquefasciatus* and synergist test comparing pyperonyl butoxide synergized and unsynergized resistant population of *A. gambiae s.s* collected at the study site

Mosquito species	No. exposed (24 h % mortality)[a]			
	Permethrin (0.75%)	4% PBO + 0.75% permethrin	Deltamethrin (0.05%	4% PBO + 0.05% deltamethrin
Anopheles gambiae s.s.	140 (75.7)	120 (88.8)	132(79.5)	120 (94.5)
Anopheles arabiensis	102 (100)	ND	118(100)	ND
Culex quinquefasciatus	320 (61.3)	ND	310 (74.1)	ND

PBO: Piperonyl Butoxide. ND: not determined
[a] Figures in parentheses denote % mortality of the mosquitoes exposed

The *kdr* frequency was 45% in the resistant *A. gambiae* population. The difference in mortality 24 h post-exposure between synergized and unsynergized field population of *A. gambiae* exposed to permethrin was highly significant ($P < 0.001$). Synergized and unsynergized samples exposed to deltamethrin gave similar results (Table 1). Biochemical analysis revealed a significantly increased level of monooxygenase in the resistant *A. gambiae*population compared with the reference susceptible Kisumu strain(P= 0.027).

Laboratory bio-efficacy of net samples: The unwashed PermaNet® 3.0 and the Olyset® net samples produced 100% mortality against the susceptible Kisumu reference strain of *A. gambiaes.s* and remained effective after 20 washes. The CTN samples declined with successive washes and after 5 washes efficacy had decreased from 100% at baseline to 80%. Five washes was therefore selected as the number of washes required before CTN exhaustion. Bioassay tests of washed and unwashed net samples with the resistant mosquitoes population prior to the field test revealed high mortality of *A. gambiaes.s* (70-89%) and *C.quinquefasciatus* (60-78%) only in PermaNet® 3.0 (Figure 1).
There was a significant increase in mortality of *A. gambiaes.s* (85- 96%) and *C. quinquefasciatus*(74-89 %)in the roof panel of PermaNet®3.0 compared to the side panels. This however declined with a margin of 7% with *C. quinquefasciatus*after 20 washes.

Efficacy of treatments in experimental huts

Vector population: The 432 hut-night collections (72 nights x 6 huts) produced 2306 female *Anophelesgambiaes.l*(62.2 % *A. gambiae*s.s.; 37.8% *A. arabiensis*) and 4873*C.quinquefasciatus*. The overall mean number of female mosquitoes collected per

hut per day was 5.3% *A.gambiaes.l* and 11.3 *C.quinquefasciatus.* The final results for the field efficacy (Table 1 and 2) is presented for *A. gambiaes.s* and *C. quinquefasciatus* were resistant to deltamethrin and permethrin.

After 12 weeks use in the experimental hut, both PermaNet® 3.0 and the Olyset® net still showed high efficacy (>98%) against the susceptible Kisumu strain of *A. gambiaes.s*. There was however, a significant decline in the bio-efficacy of the Olyset® relative to PermaNet® 3.0 when tested on the resistant strain of *A. gambiae*and *C. quinquefasciatus.*

Deterrence in hut entry: The number of mosquitoes in the huts with the untreated net was higher than with any of the other treatment arms. The treatment arms with insecticide deterred more *Anopheles* than *Culex.* There was >13% reduction in huts entry of *Anopheles* in each of the treatments when compared with the huts with the untreated net. Although there was an increase in the deterrence of *A. gambiae* in huts with PermaNet® 3.0 compared to Olyset®, the difference was not statistically significant. The percentage deterrency of *C.quinquefasciatus*was also similar for both PermaNet® 3.0 and Olyset® (Table 3).

Figure 1: Bio-efficacy of PermaNet® 3.0, Olyset® and CTN against resistant field population of *Anopheles gambiae s.s* and *Culex quinquefasciatus* prior to field use.

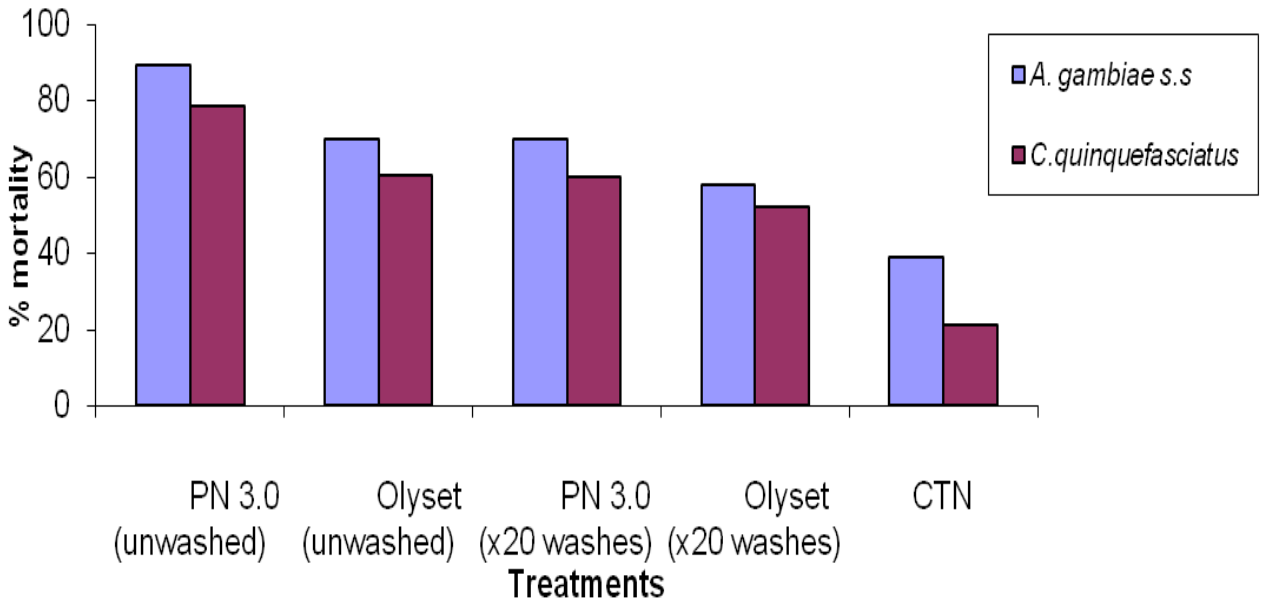

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Table 2: Summary of experimental hut trial for *Anopheles gambiae s.s.*

Outcome Measures	Control untreated net	PermaNet 3.0 (unwashed)	Olyset (unwashed)	PermaNet 3.0 (x20 washes)	Olyset (x20 washes)	CTN (x5 washes)
ENTRY RATE						
Total females Caught	470	344	368	356	376	392
Females caught Per night	6.5[a]	4.8[b]	5.1[ab]	4.9[a]	5.2[ab]	5.4[ab]
% deterrence (Tc-Tt)/Tc x 100	-	26.8	21.7	24.2	20.0	16.5
EXIT RATE						
Total females in traps*	110	142	162	139	161	132
% exophily (95% CI)	23.4[a] (20.2-25.8)	41.3[b] (39.6-44.4)	44.0[b] (43.1-50.4)	39.0[b] (37.8-43.9)	42.8[b] (40.9-45.1)	33.7[ab] (30.8-37.9)
BLOOD FEEDING RATE						
Total females blood fed	270	10	47	16	68	108
% blood fed (95% CI)	57.5[a] (54.8-59.1)	2.9[b]	12.8[c] (10.2-13.8)	4.5[b]	18.1[c] (16.5-21.2)	27.5[d] (24.5-30.1)
% blood feeding inhibition (%BFc-%BFt)/BFcx 100	-	94.9	77.7	92.1	68.5	52.2
% personal protection (BFc-BFt)/ (BFc x 100)	-	96.2[a]	82.6[b]	94.1[a]	74.8[b]	60.0[c]
MORTALITY RATE						
Total females dead	20	252	144	246	104	70
% overall mortality	4.3[a]	73.3[b]	39.1[c]	69.1[b]	27.6[d]	17.9[e]
(95% CI)	-	(70.6-75.9)	(37.1-41.8)	(64.6-72.9)	(24.1-29.2)	(15.8-19.9)
Overall insecticidal effect (Dt-DC/Tc x 100)	-	49.4[a]	26.4[b]	48.1[a]	17.9[c]	10.6[d]

* Number on the same row with similar superscript do not differ significantly (P >0.05).

Tc = Total number of female *Anopheles gambiae s.s* collected in the control arm (non-treated net)

Tt = Total number of female *Anopheles gambiae s.s* collected in each treated arm

BFc = Total number of blood fed female *Anopheles gambiae s.s* collected in the control arm

BFt = Total number of blood fed female *Anopheles gambiae s.s* collected in the treated arm

Dc = Total number of dead female *Anopheles gambiae s.s* in the control arm

Dt = Total number of dead female *Anopheles gambiae s.s* in the treated arm

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Table 3 Summary of experimental hut trial for *Culex quinquefasciatus*

Outcome Measures	Control untreated net	PermaNet 3.0 (unwashed)	Olyset (unwashed)	PermaNet 3.0 (x20 washes)	Olyset (x20 washes)	CTN (x5 washes)
ENTRY RATE						
Total females Caught	990	748	780	762	788	805
Females caught Per night	13.8[a]	10.4[b]	10.8[b]	10.6[b]	10.9[b]	11.2[b]
% deterrence (Tc-Tt)/Tc x 100	-	24.4	21.2	23.0	20.4	18.9
EXIT RATE						
Total females in traps	182	272	255	242	234	194
% exophily	18.3[a]	36.4[b]	32.6[b]	31.8[b]	29.7[b]	24.1[c]
(95% CI)	(16.9-20.1)	(32.5-39.2)	(29.2-35.6)	(28.6-34.9)	(25.8-33.4)	(21.2-28.8)
BLOOD FEEDING RATE						
Total females blood fed	620	72	197	88	251	307
% blood fed	62.7[a]	9.6[b] 25.2[c]	11.5[b]	31.9[cd]	38.1[d]	
(95% CI)	(57.9-65.1)	(6.8-11.2)	(20.4-27.9)	(8.2-13.5)	(28.6-35.6)	(33.1-42.4)
% blood feeding inhibition (%BFc-%BFt)/BFcx 100)	-	84.6	59.8	81.6	49.2	39.2
% personal protection	-	88.3[a]	68.2[b]	85.8[a]	59.5[bc]	51.9[c]
MORTALITY RATE						
Total females dead	28	487	164	315	113	118
% overall mortality	2.8[a]	65.1[b]	21.0[c]	41.3[d]	14.3[e]	14.6[e]
(95% CI)	-	(60.2-69.6)	(16.2-25.8)	(37.8-46.2)	(10.9-16.3)	(11.2-16.9)
Overall insecticidal effect (Dt-DC/Tc x 100)	-	46.4[a]	13.7[b]	28.9[c]	8.9[d]	9.0[d]

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Induced exophily: All treatment arms increased the proportion of *A. gambiae* and *C. quinquefasciatus* in the veranda and exit traps compared to the untreated control. Both PermaNet® 3.0 and Olyset® nets induced greater exophily against resistant *A. gambiaes.s*than *C. quinquefasciatus* . The extent of induced exophily in PermaNet® 3.0 ranged from 32 to 41 % for *A. gambiae* and 23 to 25% for *C. quinquefasciatus*. There was no significant increase in the exit rate of *A. gambiae*and *C. quinquefasciatus* in huts with PermaNet® 3.0 and Olyset®.

Blood feeding: Both *A. gambiae* and *C. quinquefasciatus* showed a high rate of blood feeding (>57%) in the hut with the untreated net (Figure 2) but *C. quinquefasciatus* showed a significantly higher feeding rate than *A. gambiae* in all treatment arms with insecticide, resulting in lower blood feeding inhibition of the former (Table 3). There was >92 % blood feeding inhibition of *Anopheles* in huts with PermaNet® 3.0 (Table 2). In contrast to the Olyset® net, the blood feeding inhibition of *A.gambiae*and *C. quinquefasciatus*in huts with unwashed PermaNet® 3.0 did not differ from the huts with the washed nets (Table 2 and 3).

Mortality: PermaNet® 3.0 induced significantly greater mortality against resistant A. gambiaes s. than C.

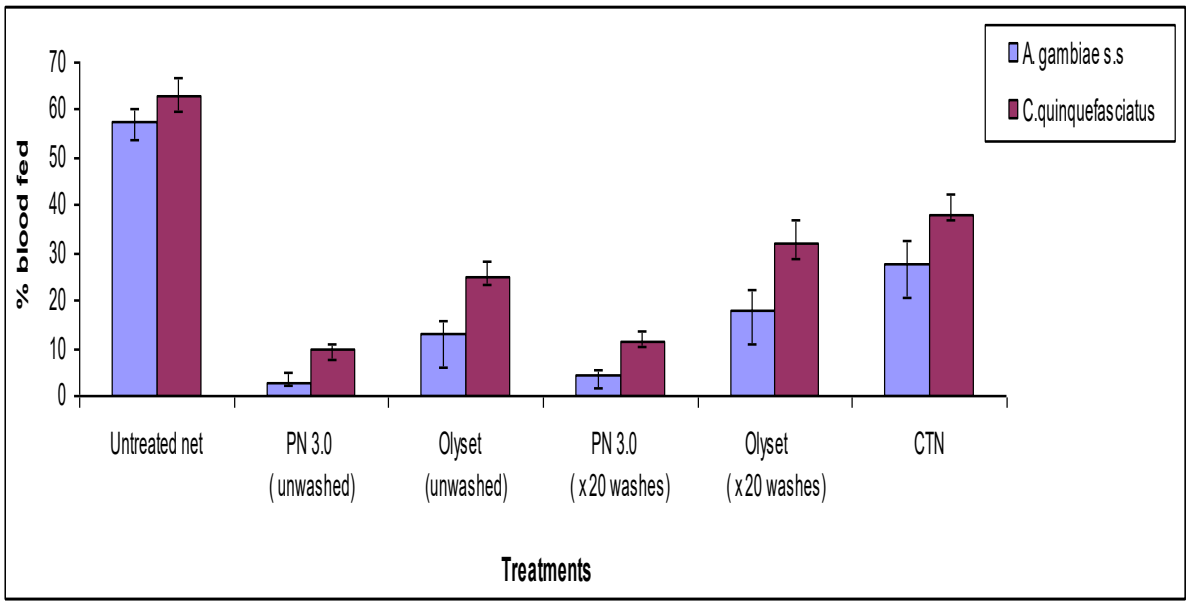

Figure 2: Proportion of blood fed Anopheles gambiae s.s. and Culexquinquefasciatus in experimental huts with PermaNet® 3.0,Olyset® net and CTN.

quinquefasciatus(Figure 3).The unwashed PermaNet® 3.0 produced the highest mortality rates:73.5 and 65.1% for *A. gambiae*and *C. quinquefasciatus* respectively. PermaNet® 3.0 had significantly higher mortality for both *A. gambiae*and *C. quinquefasciatus* when compared to the Olyset® nets (P<0.01). Washing PermaNet® 3.0 up to 20 times did not significantly reduced the mortality of *A. gambiae*but mortality declined with *C. quinquefasciatus*after 20 washes (P<0.01). Washed Olyset® significantly reduced the mortality of both *A. gambiae s.s.* and *C. quinquefasciatus*.

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Figure 3: Mortality rate of *Anophelesgambiaes.s.* and *Culexquinquefasciatus* in experimental huts with PermaNet® 3.0,Olyset® net and CTN

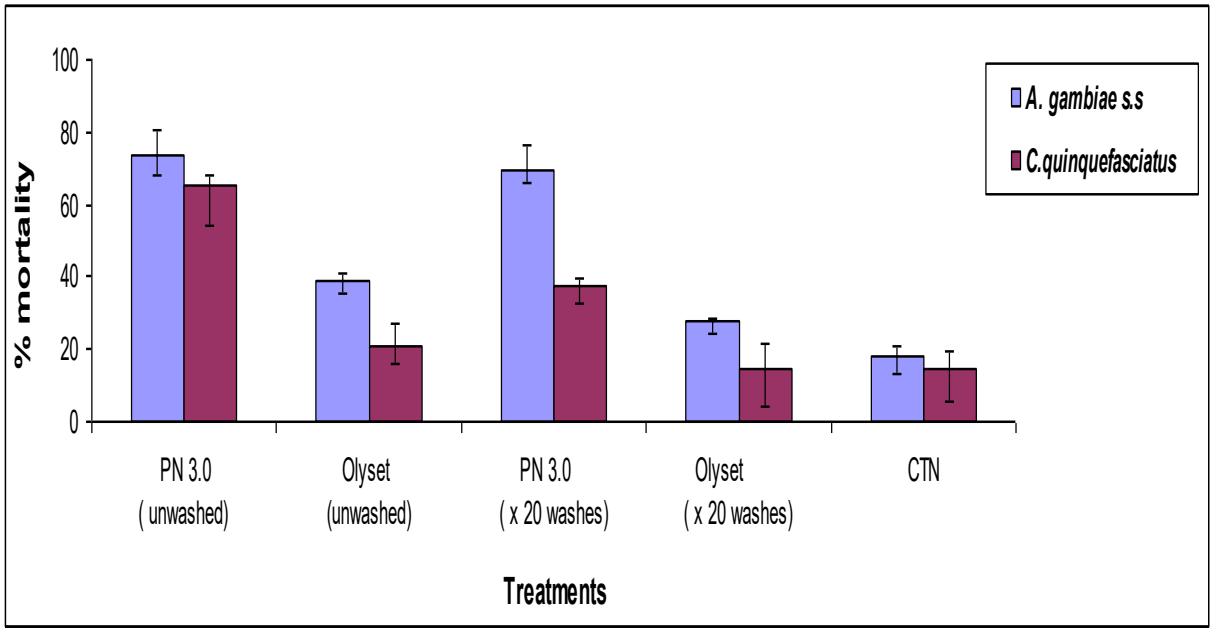

Figure 4: (A) Chemical analysis of deltamethrin in side panel of PermaNet® 3.0 and (B) permethrin in Olyset® nets used in the experimental hut tria

Fig 4A

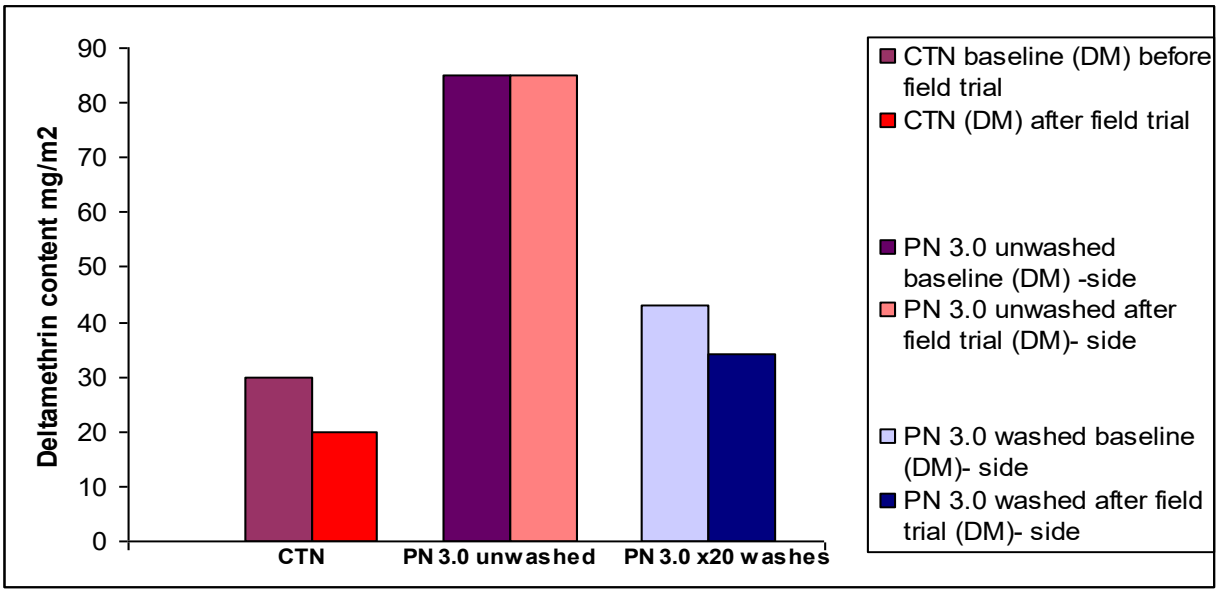

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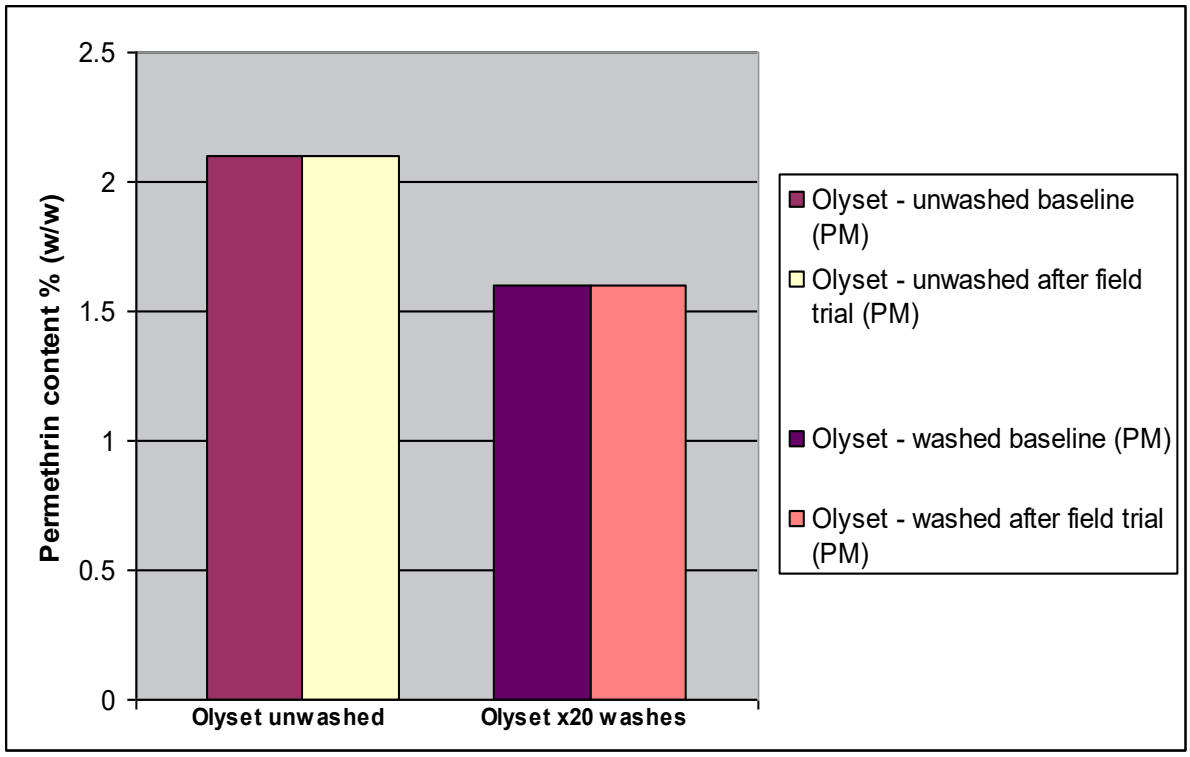
Fig 4B



Chemical analysis of net samples: Figure 4 shows the results of chemical analysis of residual deltamethrin content on the side panel of PermaNet® 3.0. The mean deltamethrin content of the unwashed side panel prior to and after field test was 85 mg/m^2 and fell to <43 mg/m^{2}after 20 washes but was still significantly higher than the CTN. The roof panel washed 20 times also lost 25% of the original (unwashed) baseline deltamethrin content and remained at this level post field trial. PBO content of the roof panel also declined from 25g/kg for unwashed net samples at baseline to 15g/kg after 20 washes and remained the same post field trial. The CTN samples had <65% of its original deltamethrin content after five washes. There was less decline in permethrin content of the Olyset® nets after 20 washes. The Olyset® nets still retained >75% of initial insecticide content and remained so at the end of the field trial (Figure 4).

Discussion

Proof of evidence of innovative resistance managementtools is needed from different resistance spectrum prior to large scale field trials. In this study, both *kdr* and MFO-based resistance mechanisms were found in *Anopgeles gambiae s.s.* The synergist data and the increased level of monooxygenase in the resistant *Anopheles* population suggest the involvement of monooxygenase inpyrethroid metabolism. Laboratory cone bioassays using resistant *A. gambiae s.s.* prior to and after experimental hut evaluations showed high mortalityin PermaNet® 3.0, which was consistent with the field data.Increased bio-efficacy likely due to the synergistic effect of PBO and deltamethrin was obvious from *in situ* bioassays, with significant mortality of resistant *A. gambiae s.s.* recorded following exposure to the roof panel. This property

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however declined in bioassays with *C. quinquefasciatus* after 20 washes.

There is currently limited information on insecticide regeneration of Olyset® that has made comparison of PermaNet® 3.0 with the Olyset® net difficult to conduct. While self regeneration of Olyset® has been reported to take up to 2 weeks at room temperature[27], Olyset® net held at 30°C elsewhere did not regenerate after the same period of time[27].The incubation protocol adapted for the Olyset® in the present study allows for full insecticide regeneration between each round of net washing as evident in laboratory bioassay of Olyset® net samples against the reference susceptible stain of *A. gambiaes.s.*Therefore, the decline in bio-efficacy observed after 20 repeated washes of the Olyset® net samples was likely not due to insufficient insecticide within the fibres of the net but probably to problems associated with bioavailability of active ingredient on the surface of the net.

Analysis of the twelve weeks experimental hut data showed that PermaNet® 3.0 induced a level of deterrence against *A. gambiae and C. quinquefasciatus*similar to that of the Olyset® net. Both nets still deterred hut entry after 20 successive washes. The similarity in deterrency of both nets could explain the negligible difference in the overall number of mosquito collected in the different treatment arms. The excito-repellency property of both net types was also similar, but the feeding rate of *A. gambiaes.s*. in huts with PermaNet® 3.0 was highly minimized and the protective effect was not lost after 20 washes compared with the Olyset® net. The overall proportion of *A. gambiaes.s*. that successfully blood fed in the Olyset® net was triple that for PermaNet® 3.0. Significantly more *A. gambiae s.s.* was recorded blood fed with the CTNs washed to just before exhaustion. This result suggests that vectorcontrol with the Olyset® net or CTNs in the study site will be undermined by pyrethroid resistance as previously reported in neighbouring Benin Republic with reduced efficacy of ITNs and IRS due to high level of *kdr* and metabolic resistance[3] . Mortality of *A. gambiae* and *C. quinquefasciatus* in the untreated net was negligible compared to the insecticide treatment arms thereby making a correct deduction of the overall insecticide effect on mosquitoes more reliable. Despite the presence of both *kdr* and MFO resistance mechanisms, the proportion of *C. quinquefasciatus* and *A. gambiae s.s.* killed by PermaNet® 3.0 was high, even with the washed samples containing <50% of the baseline deltamethrin content, indicating the higher efficacy of PermaNet® 3.0 compared with the Olyset® net. Although there was a slight decline in mortality after twenty repeated washes, mortality data were also consistent with the laboratory bioassay. Previous studies in West Africa[23] comparing PermaNet® 3.0 and PermaNet® 2.0 have shown a higher mosquito mortality in PermaNet® 3.0; linked to the high deltamethrin content relative to PermaNet® 2.0. The higher efficacy of PermaNet® 3.0 relative to the Olyset® in this study could be due to the higher rate of resistance against permethrin in this area. The fact that PermaNet® 3.0 outperformed the CTN by far, showed that PermaNet® 3.0 fulfilled the WHOPES criteria for an LLIN. It also suggests that despite the high level of deltamethrin resistance, PermaNet® 3.0 is performing better, which could be attributed to the higher deltamethrin content as previously noted[23] or the presence of PBO in the roof, or a combination of these two features.

Taken together, our data revealed that PermaNet® 3.0 showed an increase efficacy over the CTNs and fulfilled the WHOPES criteria for LLINs and outperformed the Olyset® against resistant *A. gambiae s.s* and *C. quinquefasciatus.* With the increasing spread of pyrethroid resistance in African malaria mosquitoes, care must be taken to evaluate and monitor tools to find out if they have an effect on resistance. In the absence of such a tool, it is unlikely that LLINs will continue to provide the much needed

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protection in areas with insecticide resistance.

Conclusion

This study demonstrated under experimental field conditions that PermaNet® 3.0outperformed theOlyset® net and could provide additional protection in terms of reduction in blood feeding and increase in mosquito mortality: a plausible tool against pyrethroid resistant mosquitoes.

Ethics and conflict of interest: The study was approved by the Institutional Review Board and the Research Ethics Committee of the Nigerian Institute of Medical Research and funded by Vestergaard Frandsen, Switzerland. The authors followed the WHOPES guideline for phase II evaluation of LLINs and have no commercial interest with the net manufacturers.

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Field efficacy of a new mosaic long-lasting mosquito net (PermaNet® 3.0) against pyrethroid-resistant malaria vectors: a multi centre study in Western and Central Africa

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Abstract

Background: Due to the spread of pyrethroid-resistance in malaria vectors in Africa, new strategies and tools are urgently needed to better control malaria transmission. The aim of this study was to evaluate the performances of a new mosaic long-lasting insecticidal net (LLIN), i.e. PermaNet® 3.0, against wild pyrethroid-resistant *Anopheles gambiae s.l.* in West and Central Africa.

Methods: A multi centre experimental hut trial was conducted in Malanville (Benin), Vallée du Kou (Burkina Faso) and Pitoa (Cameroon) to investigate the exophily, blood feeding inhibition and mortality induced by PermaNet® 3.0 (i.e. a mosaic net containing piperonyl butoxide and deltamethrin on the roof) comparatively to the WHO recommended PermaNet® 2.0 (unwashed and washed 20-times) and a conventionally deltamethrin-treated net (CTN).

Results: The personal protection and insecticidal activity of PermaNet 3.0 and PermaNet® 2.0 were excellent (>80%) in the "pyrethroid-tolerant" area of Malanville. In the pyrethroid-resistance areas of Pitoa (metabolic resistance) and Vallée du Kou (presence of the L1014F *kdr* mutation), PermaNet® 3.0 showed equal or better performances than PermaNet® 2.0. It should be noted however that the deltamethrin content on PermaNet® 3.0 was up to twice higher than that of PermaNet® 2.0. Significant reduction of efficacy of both LLIN was noted after 20 washes although PermaNet® 3.0 still fulfilled the WHO requirement for LLIN.

Conclusion: The use of combination nets for malaria control offers promising prospects. However, further investigations are needed to demonstrate the benefits of using PermaNet® 3.0 for the control of pyrethroid resistant mosquito populations in Africa.

Background

Malaria remains a major public health problem. Last global estimates of the malaria disease burden in 2006 indicate that at least 250 million clinical cases occurred each year, with around 1 million deaths of which 90% occurred in sub-Saharan Africa [1,2]. Recommendations of the World Health Organization (WHO-Roll Back Malaria programme) to combat malaria include artemisinin-based combination therapy (ACT) and long-lasting insecticidal nets (LLIN), supported by indoor residual spraying of insecticide (IRS) and intermittent preventive treatment in pregnancy (IPT) [2]. Recent deployment of such strategies has showed important reduction in malaria-associated morbidity and mortality in settings with moderate to high transmission levels in sub-Saharan Africa [3-5]. Eight LLINs are now recommended by WHOPES for malaria control [6]. All of them contain pyrethroids because of their fast and high insecticidal properties on mosquitoes as well as their low mammalian toxicity [7].

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Unfortunately, pyrethroid resistance is now widespread in malaria vectors including Western [8], Central [9], Eastern [10], and Southern Africa [11,12]. Resistance mechanisms are divided into two groups: metabolic (i.e. alterations in the levels or activities of detoxification proteins) and target site (i.e. non-silent point mutations within structural receptor genes) [13]. Mutations (L1014F or L1014S) on the gene encoding for the sodium channel, known as knockdown resistance (*kdr*), cause resistance to DDT and/or pyrethroid insecticides [14,15]. Over-expression of enzymes related to insecticide resistance involves the cytochrome P450-dependent monooxygenases (P450), the carboxylesterases (COE), and the glutathione-S transferases (GST) [16]. Among these three families, P450s can play a primary role in pyrethroid detoxification and resistance in malaria vectors as recently shown in Benin [17], Cameroon [18], Ghana [19] and South Africa [20].

There are more and more evidences in the recent literature to support that pyrethroid resistance may seriously impact on malaria vector control [21]. An experimental hut study carried out in southern Benin in 2004 (Ladji) showed a rather low insecticidal effect of permethrin-treated nets, at WHO recommended dosages against *Anopheles gambiae* [22]. A recent study carried out in the same locality with lambda-cyalothrin used for ITNs and IRS showed a major loss of efficacy associated with *kdr* resistance [23]. Reduced efficacy of permethrin-impregnated bed nets against *An. gambiae* strain sharing oxidase-based pyrethroid tolerance was also reported in Cameroon [24] and Kenya [25,26]. Moreover, an increasing number of countries (such as Benin, Ghana and Nigeria) reported the co-occurrence of the L1014F *kdr* mutation and increased levels of P450s within the same Anopheline populations [17,19]. As demonstrated in *Culex quinquefasciatus*, multiplicative interaction (epistasis) between these two types of resistance can lead to extremely high level of resistance to pyrethroids [27,28]. Thus, the challenge is not only to manage and control pyrethroid-resistant mosquitoes, but also to deal with the development of "multiple resistance" that may confer resistance to all insecticide classes used in public health (i.e. DDT, carbamates, etc.). Innovative tools are then urgently needed to ensure more effective control of resistant malaria vectors and to help developing countries to achieve the malaria-related Millennium Development Goals i.e. 75% reduction of malaria burden until 2015 [2].

Among the new tools available in public health, PermaNet® 3.0, has been designed to improving efficacy against pyrethroid-resistant mosquito populations [29]. PermaNet® 3.0 is a mosaic net combining deltamethrin-coated-polyester side panels and a deltamethrin plus piperonyl butoxide (PBO) incorporated-polyethylene roof. PBO has been incorporated to the net as it showed to enhance the effects of deltamethrin against insects by inhibiting metabolic defence systems, mainly P450s [30].

In this paper, a multi centre study was carried out in western and central Africa to evaluate the performances of this new LLIN technology (PermaNet® 3.0) in comparison with the classical PermaNet® 2.0 recommended by WHO. Experimental hut trials were conducted in Malanville (Benin), Pitoa (Cameroon) and Vallée du Kou (Burkina Faso), where *An. gambiae* populations showed different levels and types of pyrethroid resistance (i.e. metabolic *versus* target site modification). Standard WHO procedures in phase II were followed to investigate the efficacy of unwashed and 20 times washed LLINs in terms of induced exophily, blood-feeding inhibition and mortality.

Methods

Study areas

This study was conducted in three experimental stations belonging to the Anopheles Biology & Control (ABC) network (Figure 1). Two stations are located in western Africa whereas the third one is located in Central Africa. Each site presents different pattern of pyrethroid-resistance among *An. gambiae* s.l. populations.

Malanville (11°87N; 03°38E) is in northern Benin, 800 km from Cotonou, in an irrigated rice-growing valley. The climate is tropical soudanian, characterized by a dry season from December to June and a rainy season from July to November. *Anopheles gambiae s.s.* M cytotype is the main malaria vector in this area and presents very low levels of pyrethroid resistance [31].

Pitoa (9°21N; 13°31E) is a small village, with around 5,000 inhabitants, located at 15 km from Garoua, in an area of extensive cotton cultivation in Northern Cameroon (around 35 000 ha cultivated). *Anopheles gambiae s.l.* and *Anopheles funestus s.l.* are the main malaria vectors in this area. *Anopheles arabiensis* is predominant and showed moderate level of resistance to permethrin, deltamethrin and DDT [32] due to increased oxidase and esterase activity [18,33].

Vallée du Kou is a large rice-growing area (1,200 ha), located at 30 km Northern Bobo-Dioulasso and comprised seven villages, surrounded by wooded savannah. VK7 (11°24N; 4°24E) is a village located on the outskirts of rice fields. Both M & S molecular forms of *An. gambiae* co-exist in sympatry but the M form is mostly present during the dry season [34]. *Anopheles gambiae* showed high level of resistance to pyrethroids due to the presence of the *Kdr* mutation occurring at high allelic frequency among the molecular M and S forms [35].

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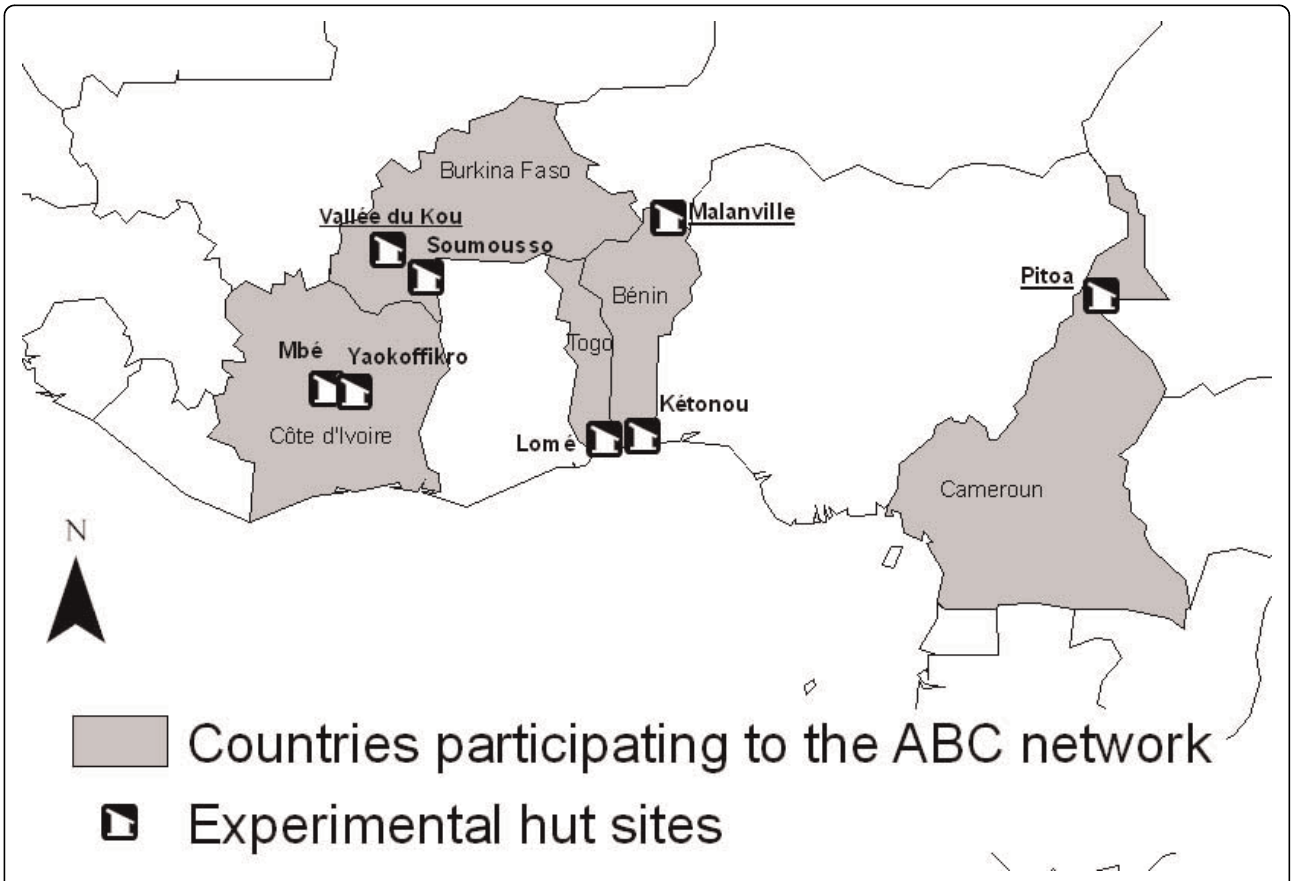

Figure 1 Experimental hut stations belonging to the Anopheles Biology & Control (ABC) network. Huts from Malanville (Northern Benin), Pitoa (Northern Cameroon), and Bobo-Dioulasso (Southern Burkina Faso) were used in this study.

Determination of the pyrethroid resistance status of *An. gambiae s.l*

In each site (Malanville, Pitoa and Vallée du Kou) and just prior to the trials, resistance of *An. gambiae s.l.* to pyrethroids was checked using WHO cylinder test [36]. Four batches of 25 unfed females, aged 2-5 days, were exposed to deltamethrin-impregnated papers for 1 h (0.05%) and held to observe mortality after 24 h, then stored at 4°C for further molecular studies. A sub-sample of 30 mosquitoes per locality was identified to sibling species and for the relative frequency of the molecular M & S forms using standard PCR methods [37,38]. The method of Martinez-Torrez *et al* [14] was used for the molecular detection of the L1014F *kdr* mutation.

Study design and experimental huts

Experimental huts are specially designed to test vector control products against freely entering mosquitoes under natural but controlled conditions [36]. The 3.5 × 2 × 2 m huts were built with local materials and designed with four entry baffles that enabled mosquitoes to fly into the hut but then hindered their escape from the hut. A veranda trap made of polyethylene sheeting and mesh screening (2 m long × 1.5 m wide × 1.5 m high) projected from the back wall of each hut. Movement of mosquitoes between the huts and the verandas was unimpeded during the night. Each hut rested on a concrete base surrounded by a water-filled moat to prevent entry of ants that would otherwise eat mosquitoes knocked down on the floor of the hut.

In each country, the treatments were randomly allocated to six experimental huts, which did not differ between them for their mosquito attractiveness in absence of treatment. Adult volunteers have been recruited among the inhabitants of the villages where experimental huts were implemented. They have been informed on the objective of this study and signed (or through a literate witness if illiterate) an informed consent. They entered the hut at dusk (7:00pm) and remained inside until dawn (5:30 am) of the next morning. Early in the morning, dead mosquitoes were collected from the floor of the hut as well as from the exit traps and inside the nets; resting mosquitoes were collected using aspirators from inside the net and from the walls and roof of the hut and exit traps. Mosquitoes were scored by location as dead or alive and as fed or

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unfed. Live mosquitoes were placed in small cups and provided with access to sugar solution for 24 hours to assess the delayed mortality. To minimize bias related to mosquito attractiveness of each volunteer and spatial variation in mosquito densities, the volunteers and bed nets were rotated between huts each day according to a Latin square design [36].

Efficacy of each treated arms was expressed in terms of deterency, induced exophily, blood-feeding inhibition, and mortality. This multi-centre trial included the determination of the efficacy of unwashed and 20 times washed PermaNet® 3.0 comparatively to the WHO recommended PermaNet® 2.0 and a conventional deltamethrin-treated net washed to just before exhaustion (as defined by WHOPES guidelines [33]). Their impact on the behaviour of wild pyrethroid resistant *An. gambiae s.l.* and *An. arabiensis* mosquitoes was also evaluated.

Mosquito net treatments

In each country, six treated arms were randomly allocated to huts:

1. Untreated net (same fabric - polyester on the side with a strengthened 70 cm lower border/polyethylene on top)
2. PermaNet® 3.0 unwashed
3. PermaNet® 2.0. unwashed
4. PermaNet® 3.0 washed 20 times
5. PermaNet® 2.0 washed 20 times
6. Polyester net conventionally treated with deltamethrin at 25 mg a.i./m^2 and washed to just before exhaustion i.e. 95% Knock down after 1 h of contact and/or 80% mortality after 24 h [36].

The LLINs (PermaNet® 2.0 and PermaNet® 3.0) were provided by Vestergaard Frandsen SA, (Switzerland). PermaNet® 2.0 is a deltamethrin-coated LN, made of knitted multi-filament polyester fibres and is treated with deltamethrin at a target concentration of 55 mg/m^2 (= 1.8 g/kg for a 75-denier net used in Malanville and Pitoa and = 1.4 g/kg for a 100-denier net in Vallée du kou). PermaNet® 2.0 received WHOPES full recommendation for LLIN in 2009. PermaNet® 3.0 product is a combination of different long-lasting technologies. The roof of PermaNet® 3.0 utilizes deltamethrin and PBO incorporated into monofilament polyethylene yarn of 100 denier (warp-knitted fabric, with weight of 40 ± 15% g/m^2) at the target dosage of 4.0 g AI/kg and 25 g AI/kg of netting material, respectively. The side panels of PermaNet® 3.0 are made of multi-filament polyester fibres, treated with deltamethrin in a resin coating (75 denier, warp-knitted fabric, atlas construction). The side netting has two parts: a strengthened lower part, so-called border (70 cm) by using 75 denier yarn (weight 40 ± 10% g/m^2) and a side panel made of 75 denier (weight of 30 ± 10% g/m^2). The target dose of deltamethrin in the side panels is 2.8 g AI/kg of netting material, i.e. 115 mg AI/m^2 of the border and 85 mg AI/m^2 of the remaining of the side panels.

The polyester net was conventionally treated with deltamethrin at 25 mg AI/m^2 and washed to just before the point of exhaustion (i.e. <80% mortality or <95% knock down). This treatment was used as a positive control. Each net was deliberately holed with six holes (4 cm × 4 cm) to simulate a torn net [36].

Residual activity and wash resistance of the net treatments

The bio-efficacy of each treatment was determined before washing and after field testing by exposing 2 to 5 days old unfed females of the susceptible *An. gambiae* Kisumu strain in WHO cone bioassays [36]. This test consists to expose female mosquitoes to each part of the nets for 3 min and to measure the knock down time after 60 minutes and the mortality after 24 H. A mean of 50 mosquitoes was tested per net and results pooled for analysis. Sugar solution was provided during the 24-h holding period, and the temperature was kept at around 25°C. The standardized WHO protocol was used for washing the nets [36].

Chemical analysis

Determination of deltamethrin and PBO content on nets, before washing and after the field testing was investigated using a new method developed by the WHO Collaborating Centre for the Quality Control of Pesticides (Walloon Agricultural Research Centre, Gembloux, Belgium)[39]. In each country, five pieces of netting (about 30 cm × 30 cm) were cut from the roof and side panel and stored in aluminium foil for subsequent chemical analysis. The side panels and roof were tested separately for the PermaNet® 3.0. The chromatographic determination of deltamethrin, deltamethrin R-isomer and piperonyl butoxide was performed by gas chromatography with flame ionization detection (GC-FID) after extraction by refluxing with xylene. Before the analysis of samples, the analytical method was successfully validated for its specificity, linearity of detector response, accuracy, repeatability and reproducibility.

Statistical analysis

Data from *in situ* bioassays were compared between each net using a Chi square test at 95% confidence interval, using the Minitab software version 12.2. In each study site, the number of mosquitoes of each species entering the huts was compared by species and analysed using the non parametric Kruskal-Wallis test. The proportion of mosquitoes that exited early (induced exophily), the proportion that was killed within the hut (mortality) and the proportion that successfully blood fed (blood feeding rate) were compared and analysed

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using the logistic regression (Addinsoft, 2009, XLSTAT 2006). The percentage personal protection (PP) was calculated as (BFC-BFT)/(BFC) * 100, where BFC is the total number of blood fed females in the control hut and BFT the total number of blood-fed female mosquitoes in the treated hut. The overall killing effect (KE) of a treatment was calculated as (DT-DC)/(TC) * 100, where DT is the total number of dead mosquitoes in the treated hut, DC the total number of dead mosquitoes in the control hut and TC is the total number of mosquitoes collected in the control hut [36].

Results

Vector population and pyrethroid resistance

Table 1 summarizes the sibling species, molecular forms and pyrethroid resistance status of *An. gambiae s.l.* collected in the three experimental hut stations. *Anopheles gambiae s.s.* was predominant in Malanville (95%) and Vallée du Kou (100%), whereas *An. arabiensis* was predominant (95%) in Pitoa. The composition of *An. gambiae s.s.* was 100% M form for the Malanville sample and 80%/20% S/M molecular forms for the Kou Valley sample. Different levels of deltamethrin resistance were reported in the three study sites; the most "susceptible" population was found in malanville (i.e. 85% mortality to deltamethrin), the most resistant in Vallée du Kou (23% mortality) and the population of Pitoa being intermediate (70% mortality). The *kdr* mutation was present at very high frequency (>80%) in both molecular M & S forms in Vallée du Kou whereas it was only 16% in the M form in Malanville. In Pitoa, the *kdr* mutation was almost absent (<5%) and deltamethrin resistance in *An. arabiensis* was associated with elevated esterase and oxidase activities as described previously [18,33].

Insecticide residual activity

With conventionally deltamethrin-treated nets (CTN), KD and mortality decreased below the WHO threshold (95% and 80% respectively) after four washes (respectively 73% and 71%). Hence, three washes were considered as the number of washes required before exhaustion. Residual activity of PermaNet® 2.0 and PermaNet® 3.0 as measured by WHO cone bioassay tests showed no significant decrease in efficacy after washing and/or field testing (Table 2).

Efficacy of treatments in experimental huts

The experimental hut trials were conducted from September till November 2007 in the Vallée du Kou and from July till September 2008 in Malanville and Pitoa. Thirty-six night collections (one Latin square) were required in Vallée du Kou and Pitoa to collect sufficient number of Anopheline mosquitoes for statistical analysis, whereas 72 nights collection (two Latin squares) were required in Malanville to obtain a correct density. In overall, 1,594 *An. gambiae s.l.* mosquitoes were collected in the control (untreated) huts among which 908 (equivalent 19 Anopheles bites per man per night), 401 (eq. 5.8 bites per man per nigh) and 285 (eq.1.5 bites per man per man) were found in Vallée du Kou, Pitoa and Malanville, respectively.

Deterency

A significant reduction in entry rates (deterrency) was noted with the unwashed PermaNet® 2.0 and 3.0 in Vallée du Kou and Pitoa compared to the untreated (control) arm whereas no significant reduction was noted in Malanville regardless the treatments ($P < 0.05$, see Additional file 1).

Induced exophily

In the control huts, the exophily in Pitoa did not differ significantly from the two others study sites, but the exophily rate in Malanville was significantly higher than in Vallée du Kou ($p = 0.018$) (see Additional file 1). The exophily induced by each treated hut is illustrated in Figure 2 and summarized in Additional file 2. The proportion of mosquitoes found in the veranda trap with

Table 1 Species, molecular forms and pyrethroid resistance status of *An. gambiae s.l* in the three experimental hut stations.

Country	Species		Molecular form *		Kdr frequency		Mortality % **	Resistance status ***
	An. gambiae s.s.	*An. arabiensis*						
Malanville (Benin)	95%	5%	100% M		16%		85%	**Resistance suspected** (kdr mutation, oxidase)
Pitoa (Cameroon)	5%	95%	100% S		<5%		70%	**Resistance** (oxidase +Esterase)
Vallée du kou (Burkina Faso)	100%	0%	15% M	85% S	>80 % M	>80 % S	23%	**Resistance** (kdr mutation)

An. gambiae s.s. **deltaméthrine 0.05%, *** from [30 and][17]

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Table 2 Residual activity (as determined by WHO cone bioassays on susceptible Kisumu strain) of unwashed and washed Permanet 2.0 and Permanet 3.0 in comparison with Conventionally Treated nets (CTN) washed to just before exhaustion in the three experimental hut stations.

Country	Conditions	Untreated net	Permanet 2.0 unwashed	Permanet 2.0 20× washed	Permanet 3.0 unwashed	Permanet 3.0 20× washed	CTN*
	Before washing	0.0[a] (52)	100[b] (52)	100[b] (53)	100[b] (54)	100[b] (53)	100[b] (53)
Malanville (Benin)	After washing or field testing	0.0[a] (56)	100[b] (63)	97[b] (62)	100[b] (61)	100[b] (59)	89[c] (64)
	Before washing	0.0[a] (52)	100[b] (55)	100[b] (55)	100[b] (50)	100[b] (56)	100[b] (54)
Pitoa (Cameroon)	After washing or field testing	3.6[a] (56)	100[b] (63)	98[b] (61)	100[b] (65)	100[b] (60)	81[c] (63)
	Before washing	0.0[a] (56)	100[b] (55)	100[b] (57)	100[b] (59)	100[b] (59)	100[b] (58)
Vallée du Kou (Burkina Faso)	After washing or field testing	0.0[a] (62)	100[b] (63)	98[b] (59)	100[b] (55)	100[b] (54)	95[b] (57)

* Three washes were required to reach the point to just before exhaustion.

Two Values in the same raw sharing same letter do not significantly differ (P > 0.05).

Values in bold represent the number of tested mosquitoes per treatment.

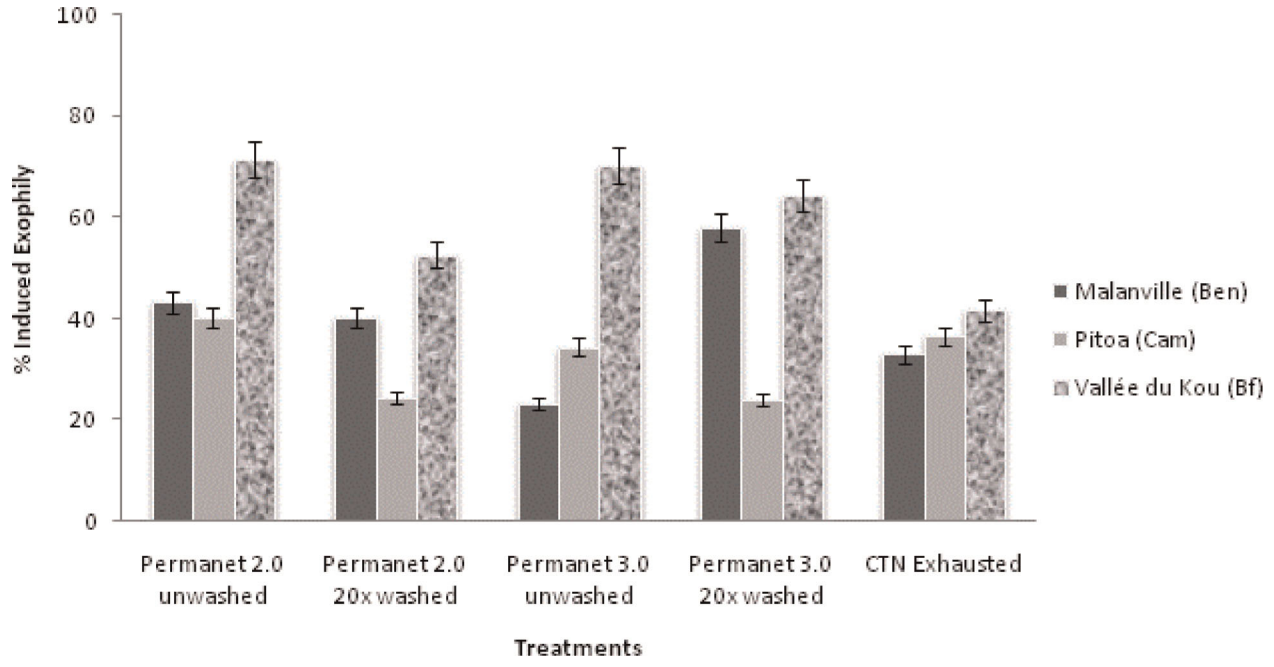

Figure 2 Comparison of Induced Exophily obtained for unwashed and washed PermaNet® 2.0, PermaNet® 3.0 and CTN against free flying *An.gambiae s.l.* in experimental huts.

PermaNet® 2.0 and 3.0 (washed or unwashed) was greater in Vallée du Kou (from 67 to 80%) than in Pitoa and Malanville (from 51 to 67%). Both LLINs (washed or unwashed) induced significantly more exophily than the untreated nets, regardless of the ecological settings. PermaNet® 3.0 (washed or unwashed) did not induce significantly higher exophily than PermaNet® 2.0 (washed or unwashed), except in Vallée du Kou where the proportion of mosquitoes found in the veranda trap was higher with PermaNet® 3.0 washed 20 times (75%) than PermaNet® 2.0 washed 20 times (67%) (P < 0.05).

BFI and personal protection

The proportion of mosquitoes that succeeded to take a blood meal with untreated holed nets was significantly

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lower from the area of Malanville (38%), to Pitoa (52% of blood fed females caught; p = 0.0205) and Vallée du Kou (75% of blood fed females caught; p = 0.0002) (see Additional file 2).

The blood feeding inhibition (BFI) rates induced by each treated hut are illustrated in the Figure 3 and summarized in Additional file 2. The proportion of mosquitoes that succeeded to take a blood meal in the treated huts differed according to the study site; the BFI ranged from 65 to 98% in Malanville, from 45 to 71% in Pitoa and from 34 to 72% in Vallée du Kou. In Malanville, the BFI of PermaNet® 3.0 washed 20 times (65%) was significantly lower than for Permanet® 3.0 unwashed (98%) and PermaNet® 2.0 unwashed or washed 20 times (respectively 90% and 84%). In Pitoa, PermaNet® 2.0 induced a higher BFI, although this was only significant for unwashed nets. In contrast, BFI was higher with PermaNet® 3.0 over PermaNet® 2.0 in Vallée du Kou for both unwashed and washed bed nets (P < 0.05).

The personal protection of PermaNet® 2.0 and 3.0 was good when the LLIN was unwashed (from 80% in Pitoa to 99% in Malanville) but much lower when the nets were washed 20 times, especially in the pyrethroid resistance area of Vallée du Kou (44% and 62% for PermaNet® 2.0 and PermaNet® 3.0 respectively). In this resistance area of Vallée du Kou, PermaNet® 3.0 conferred a significantly better protection than the PermaNet® 2.0 (p = 0.0006 for unwashed LLINs; p = 0.0024 for washed LLINs).

Insecticidal activity

Mortality of mosquitoes in the control huts was low (around 5%) in Malanville and Vallée du Kou where *An. gambiae s.s.* was predominant. In contrast, higher mortality (13%) was recorded in Pitoa where *An. arabiensis* was found in higher proportion.

The mortality induced by each treated arm is illustrated in Figure 4 and summarized in Additional file 3. As for the blood feeding behaviour, the proportion of *An. gambiae* killed by the treated nets greatly differed according to the entomological setting. The corrected mortality was high in Malanville (from 61% for CTN to 96% for PermaNet® 3.0) and, in a lesser extent, Pitoa (from 41% for CTN to 93% for PermaNet® 3.0). In contrast, the mortality in Vallée du Kou ranged from 28% for CTN to 78% for PermaNet® 3.0. Regarding the LLINs only, mortality (~69%) was similar between PermaNet® 3.0 and PermaNet® 2.0 washed 20 times in Malanville whereas in Pitoa and Vallée du Kou PermaNet® 3.0 (washed or unwashed) induced significantly more mortality than PermaNet® 2.0 (p < 0.05). In all settings, washing the nets 20 times significantly reduced the number of mosquitoes being killed by the LLINs (p < 0.05).

The overall insecticidal effects of unwashed PermaNet® 2.0 and PermaNet® 3.0 were high in Malanville and Pitoa (from 80 to 96%) comparatively to Vallée du Kou (from 41 to 77%). This trend was stronger with washed LLINs especially in Vallée du Kou where the insecticidal

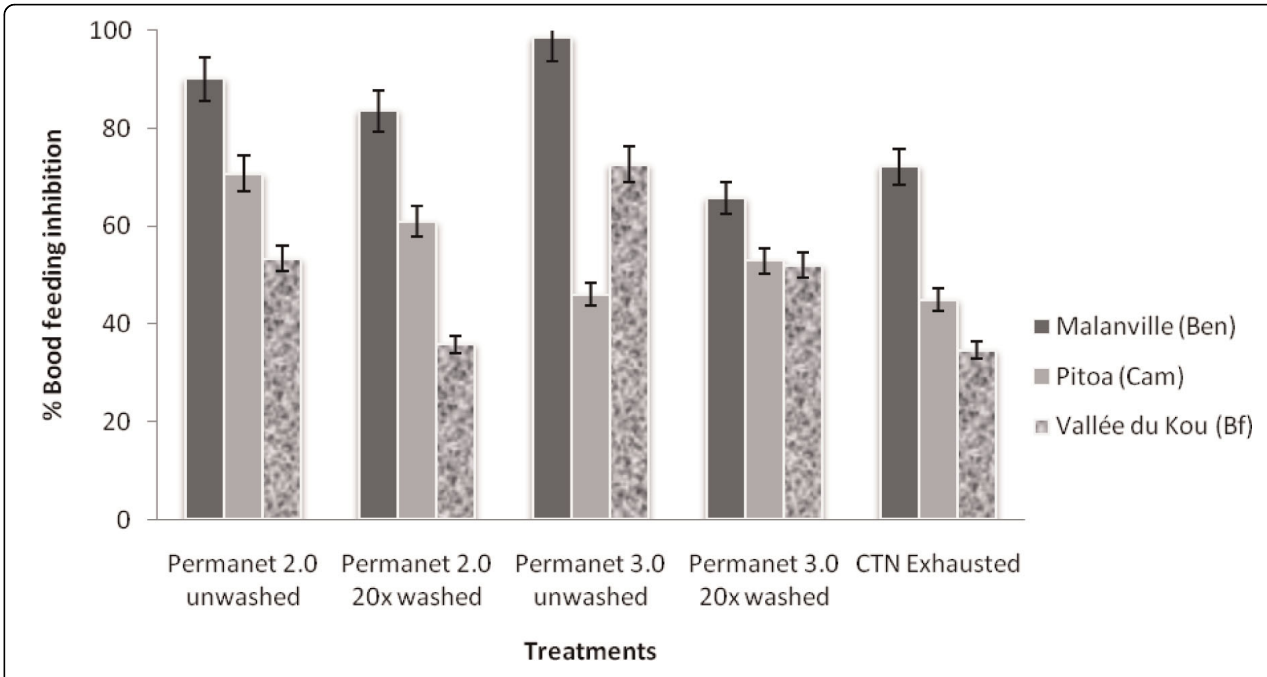

Figure 3 Comparison of Blood Feeding Inhibition (BFI) rates obtained for unwashed and washed PermaNet® 2.0, PermaNet® 3.0 and CTN against free flying *An.gambiae s.l.* in experimental huts.

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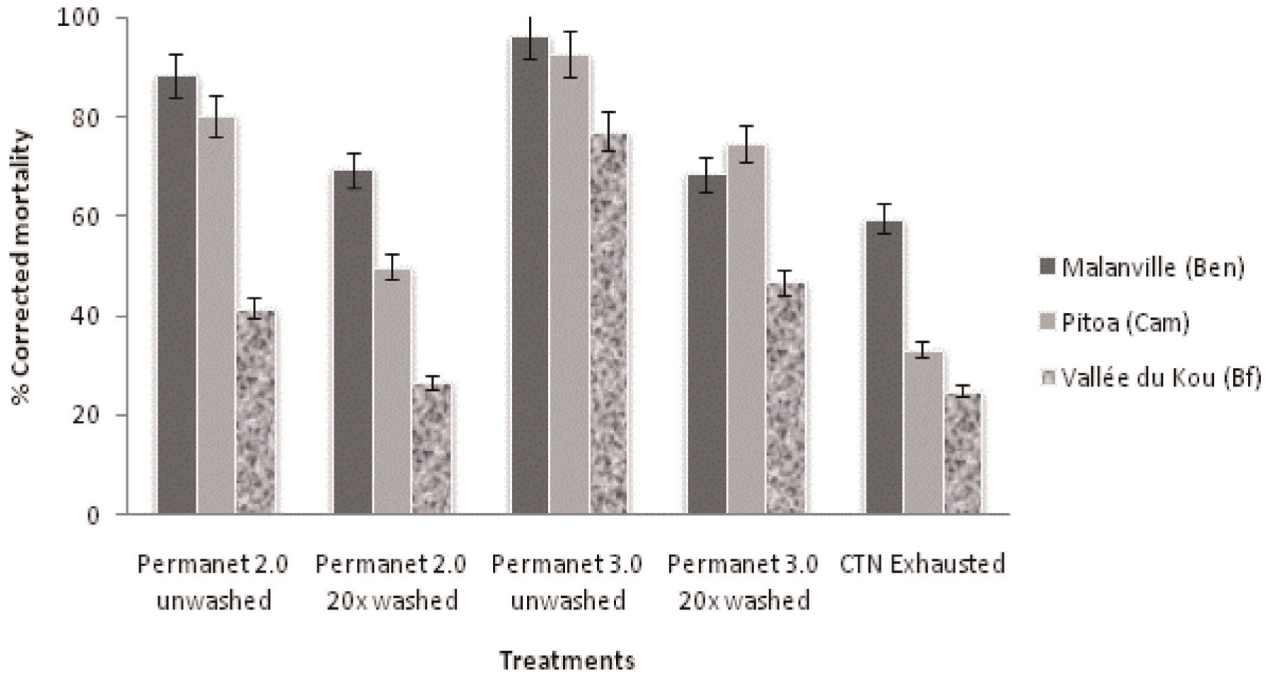

Figure 4 Comparison of corrected mortality rates obtained for unwashed and washed PermaNet® 2.0, PermaNet® 3.0 and CTN against free flying *An.gambiae s.l.* in experimental huts.

activity of PermaNet® 3.0 and PermaNet® 2.0 decreased to 46% and 26%, respectively (see Additional file 3). The side effect questionnaires collected during the field trials did not reveal any adverse effects (symptoms or troubles) related to the use of any treated arms.

Deltamethrin and PBO content on mosquito nets

In the three study sites, neither deltamethrin nor PBO was detected (limit of quantification = 0.01 g/kg for deltamethrin and 0.1 g/kg for PBO) in the untreated nets, hence confirming that no contamination occurred during the rotations of the nets (Table 3). The active ingredient content for unwashed PermaNet® 3.0 (deltamethrin and PBO) and PermaNet® 2.0 (deltamethrin) complied with the target doses (± 25%), except for one PermaNet® 2.0 (Pitoa) for which the average deltamethrin content on the side panels (2.53 g AI/kg) was just above the upper limit (2.25 g AI/kg) of the target dose. No major loss of deltamethrin and/or PBO content (from nil to 17%) was reported for unwashed PermaNet® 2.0 (deltamethrin) and PermaNet® 3.0 (deltamethrin and PBO) after the field testing. However, the loss of deltamethrin after 20 washes was relatively important for both PermaNet® 2.0 (from 59 to 85%) and PermaNet® 3.0 in the side panels (from 71 to 87%). However, the deltamethrin and PBO content in the roof panel remained high (>60% retention) in PermaNet® 3.0 after 20 washes. The deltamethrin content for unwashed CTN (0.8 g AI/kg) complied with the initial target doses (± 25%). After 3 washes the loss of active ingredient ranged from 85 to 91%.

Discussion

A multi-centre experimental hut study was carried out to assess the efficacy of PermaNet® 3.0 against wild pyrethroid-tolerant *An. gambiae s.s.* (Malanville), *kdr*-resistant *An. gambiae s.s.* (Vallée du Kou) and pyrethroid-resistant *An. arabiensis s.l.* showing metabolic resistance (Pitoa). The ABC network offers ideal conditions to address this objective based on the existence of eight experimental hut stations in different ecological and entomological settings (Figure 1). In the present study, three stations where *An. gambiae s.l.* showed different level and type of resistance to pyrethroids were selected to assess whether PermaNet® 3.0 may represent a more potent technology than PermaNet® 2.0 against pyrethroid resistant mosquito populations.

Differences in behavioural responses between wild Anopheline populations

The results from the control huts showed that the behavioural preferences of Anopheline populations (in terms of endophily/exphily) significantly differ between the three sites as expected from literature on trophic behaviour of malaria vectors [40-42]. It confirms that the behaviour of Anopheline populations depend on several

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Table 3 Determination of deltamethrin and PBO content on mosquito nets in the three experimental hut trials.

Country	Treatment	Target dose*	Deltamethrin/PBO content (g/kg)		Loss of active ingredient (%)
		g/kg (IC95)	Before washing and testing	After testing	g/kg (IC95)
Malanville (Benin)	Untreated net	0	<0.01	<0.01	
	Permanet 2.0	1.8 [1.35-2.25]	2.09	1.74	17%
	Permanet 2.0 20×	1.8 [1.35-2.25]	**2.41**	0.99	59%
	Permanet 3.0	2.8 [2.1-3.5]	2.61	2.32	11%
	Permanet 3.0 Roof	4 [3-5]	3.69	3.00	19%
	Permanet 3.0 20×	2.8 [2.1-3.5]	2.58	0.53	79%
	Permanet 3.0 roof 20×	4 [3-5]	3.69	3.16	14%
	CTN exhausted	0.8 [0.6-1.0]	0.59	0.09	85%
	PBO Permanet 3.0	25 [18.75-31.25]	20.8	23.1	+11%
	PBO Permanet 3.0 20×	26 [18.75-31.25]	20.7	12.4	40%
Pitoa (Cameroon)	Untreated net	0	<0.01	<0.01	
	Permanet 2.0	1.8 [1.35-2.25]	**2.53**	2.51	1%
	Permanet 2.0 20×	1.8 [1.35-2.25]	**2.65**	0.41	85%
	Permanet 3.0	2.8 [2.1-3.5]	2.44	2.50	-3%
	Permanet 3.0 Roof	4 [3-5]	3.21	3.22	0%
	Permanet 3.0 20×	2.8 [2.1-3.5]	2.48	0.33	87%
	Permanet 3.0 roof 20×	4 [3-5]	3.38	2.57	24%
	CTN exhausted	0.8 [0.6-1.0]	0.88	0.08	91%
	PBO Permanet 3.0	25 [18.75-31.25]	26.0	29.8	+15%
	PBO Permanet 3.0 20×	26 [18.75-31.25]	28.0	17.7	37%
Vallée du kou (Burkina Faso)	Untreated net	0	<0.01	<0.01	
	Permanet 2.0	1.4 [1.05-1.75]	1.31	1.32	-1%
	Permanet 2.0 20×	1.4 [1.05-1.75]	1.30	0.27	79%
	Permanet 3.0	2.8 [2.1-3.5]	2.89	2.94	+2%
	Permanet 3.0 Roof	4 [3-5]	4.33	4.07	6%
	Permanet 3.0 20×	2.8 [2.1-3.5]	3.10	0.90	71%
	Permanet 3.0 roof 20×	4 [3-5]	4.18	3.36	20%
	CTN exhausted	0.8 [0.6-1.0]	0.72	0.11	85%
	PBO Permanet 3.0	25 [18.75-31.25]	23.1	20.4	12%
	PBO Permanet 3.0 20×	26 [18.75-31.25]	22.9	15.1	34%

* Target dose for Permanet 2.0 was 1.8 g/kg ± 25% for 75 denier's nets in Benin and Cameroon and 1.4 g/kg ± 25% for 100 deniers net in Burkina Faso

factors including the species, the molecular forms, the resistance mechanisms and other environmental variables [40-42]. Interestingly, there is a difference in mortality rate in the control huts between *An. arabiensis* collected in Pitoa (12%) and the two others *An. gambiae* populations from Malanville and Vallée du Kou (<5%). Unfortunately, this study did not allow to decipher on the causes of this difference of mortality (behavioural preference, environmental conditions, etc.) but other authors have already reported similar mortality rates of *An. arabiensis* (10%) in experimental huts [43]. Nevertheless these differences shed light on the need for further investigations on behavioural preferences of wild populations of *An. gambiae s.s.* and *An. arabiensis*.

Comparison between PermaNet® 2.0 and 3.0

The chemical analysis confirmed that in overall, unwashed nets were impregnated with the appropriate target dose of deltamethrin and PBO. Although efficacy of 20 times washed PermaNet® 3.0 and PermaNet® 2.0 was good, a rather high loss of insecticide was noted in the side panels (Table 3). Nevertheless, the deltamethrin and PBO retention in the roof was around 2.5 times higher than that of deltamethrin in the side panels, showing that the retention is better with incorporated polyethylene than with coated polyester [39].

This study first demonstrated a better or equal impact of PermaNet® 3.0 washed 20 times on mortality and blood feeding inhibition of major malaria vectors compared with

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that of the conventionally treated polyester nets (25 mg/m^2 AI) washed until just before exhaustion. This confirms that the PermaNet® 3.0 fulfils the WHOPES efficacy criteria of Phase II studies for LLIN.

Regarding the two LLINs, unwashed Permanet 3.0 induced significantly higher BFI and mortality than Permanet 2.0 in Vallée du Kou and Malanville. In the locality of Pitoa, the BFI was however higher with Permanet 2.0 than Permanet 3.0 but the mortality was still higher with Permanet 3.0. After 20 washes, the PermaNet® 3.0 also induced higher insecticidal effect than PermaNet® 2.0 in the pyrethroid resistance areas of Pitoa and Vallée du Kou, but performed equally in the area of Malanville.

One should note that in areas with high resistance levels (Vallée du Kou) 50% of resistant mosquitoes survived after exposure to PermaNet® 3.0 relative to 75% survival after exposure to PermaNet® 2.0. It remains to be seen if the gain of efficacy of PermaNet® 3.0 over PermaNet® 2.0 is enough to control highly pyrethroid-resistant malaria vector populations. Here, it is difficult to conclude on the benefit of using PBO on the roof because the deltamethrin content on PermaNet® 3.0 was approximately twice higher than that of PermaNet® 2.0. So the better efficacy on resistant mosquitoes could be impeded either to the higher deltamethrin concentration or to the PBO itself or both. Other field studies did not show an increase of efficacy on resistant *Culex* and pyrethroid susceptible *An. gambiae s.s.* [44] as well as deltamethrin-resistant *Anopheles epiroticus* [45].

The threat of insecticide resistance mechanisms

This multi-centre study provided also more evidence that pyrethroid resistance can seriously reduce the efficacy of pyrethroid -treated materials in malaria vectors [21,22]. Results obtained in Vallée du Kou showed a strong reduction of ITNs efficacy where the *kdr* mutation frequency was high (e.g. personal protection of CTN washed to just before exhaustion ranged from 88% in Malanville to 24% in Vallée du Kou and the insecticidal effect ranged from 60% in Malanville to 25% in Vallée du Kou). The same trend was observed with PermaNet® 2.0, confirming that the *Kdr* mutation is an important predictor of pyrethroid resistance phenotype in malaria vectors as previously described [23,46]. Lower insecticidal activity and personal protection were already demonstrated in West Africa with pyrethroid resistant mosquito populations using either Olyset® net or PermaNet® [47] and also insecticide treated plastic sheetings [48]. Unfortunately, in most malaria endemic countries, *An. gambiae* populations are sharing very high frequency of *Kdr* mutation [8,49-51] alone or in combination with metabolic resistance [16,18]. In Pitoa, where *An. arabiensis* show higher metabolism through elevated oxidase and esterase activity [33], CTN efficacy was intermediate (PP and IE were 63.6% and 33.2%, respectively), suggesting that metabolic resistance could also reduce ITN efficacy [24]. This finding supports the global warning about the spread of the pyrethroid resistance although there is no evidence yet for a malaria control failure using LLIN at an operational scale [52].

Conclusion

To summarize, the present study showed that the new long-lasting bed nets PermaNet® 3.0 caused better efficacy against both *Kdr* and metabolic resistant malaria vectors than PermaNet® 2.0. Nevertheless in areas of strong resistance like the Vallée du Kou, a large number of exposed mosquitoes survived after exposure to both LLINs. Then as a short term prospect, it seems essential to evaluate this tool in others areas of strong resistance like southern Benin, southern Nigeria and Côte d'Ivoire. It is also crucial to strengthen the collaboration between companies and Research Institutions to find alternative tools for malaria vector control (e.g. using mixtures of unrelated compounds for LLINs [53-57] and/or the use of insecticide-treated plastic sheeting and LLINs [58]), because the race towards an insecticide with a new mode of action will be long and expensive.

Additional file 1: Comparison of exophily obtained for free flying wild *Anopheles gambiae* in experimental huts of all countries. Raw data from the experimental hut trials.

Additional file 2: Comparison of blood feeding rates obtained for free flying wild *Anopheles gambiae* in experimental huts of all countries. Raw data from the experimental hut trials.

Additional file 3: Comparison of mortality rates obtained for free flying wild *Anopheles gambiae* in experimental huts of all countries. Raw data from the experimental hut trials.

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Authors' contributions

VC designs the study and drafted the manuscript. JC, RDD, JE, PN carried out bioassays and conducted the experimental hut trials. OP conducted the chemical analysis on nets. MA and JMH helped design the study and critically revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors received financial support from Vestergard Frandsen Company to carry out the experimental huts trials in Burkina Faso and Cameroon. However, the authors have strictly followed the WHOPES procedures for testing and evaluation of the efficacy of Permanet 3.0 against malaria vectors. The Research teams involve in this study (i.e. IRD, CREC, OCEAC, CM and CRA-W) have no competing and commercial interests with the manufacturer.

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RESEARCH ARTICLE

Efficacy of two PBO long lasting insecticidal nets against natural populations of *Anopheles gambiae s.l.* in experimental huts, Kolokopé, Togo

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Abstract

LLINs containing an insecticide plus the synergist, piperonyl butoxide (PBO) have been designed for increased efficacy against pyrethroid-resistant malaria vectors. In this study, two LLINs with PBO, PermaNet® 3.0 and Olyset® Plus, and a pyrethroid-only LLIN, Yorkool®, were evaluated in experimental huts against a free-flying, wild population of *Anopheles gambiae s.l.* in Kolokopé, a cotton cultivated area of Togo. WHO susceptibility tube tests and subsequent molecular assays determine the *An. gambiae s.l.* populations to be resistant to pyrethroids and DDT with both target site *kdr* and metabolic resistance mechanisms involved in the resistance observed. *Anopheles gambiae s.s.* and *An. coluzzi* were present in sympatry though the *kdr* (L1014F) mutation was observed at a higher frequency in *An. gambiae s.s.* The experimental hut results showed that both PermaNet® 3.0 and Olyset® Plus nets induced similar levels of deterrence, exophily, and reduced blood feeding rate against wild *An. gambiae s.l.* in contrast to the pyrethroid only LLIN, Yorkool®. The proportion of wild *An. gambiae s.l.* killed by unwashed PermaNet® 3.0 was significantly higher than unwashed Olyset® Plus (corrected mortality 80.5% compared to 66.6%). Similar blood feeding inhibition rates were observed for unwashed PermaNet® 3.0 and Olyset® Plus; however, PermaNet® 3.0 washed 20 times demonstrated significantly higher blood feeding inhibition rate than Olyset® Plus washed 20 times (91.1% compared with 85.6% respectively). Yorkool® performed the worst for all the parameters evaluated. In an area of pyrethroid resistance of *An. gambiae s.l* involving *kdr* target site and metabolic resistance mechanisms, LLINs with PBO can provide additional protection in terms of reduction in blood feeding and increase in mosquito mortality compared to a pyrethroid-only net, and should be considered in malaria vector control strategies.

Introduction

Long lasting insecticidal nets (LLINs) continue to be one of the primary interventions against malaria vectors. Currently, a LLIN is expected to retain its biological activity for at least 20 standard washes under laboratory conditions and three years of recommended use under field conditions, as defined in WHO guidelines [1]. WHO Pesticide Evaluation Scheme (WHOPES) Phase II experimental hut studies are conducted on nets that pass Phase I laboratory investigations to determine comparative efficacy against free-flying, wild mosquito populations.

In Togo, insecticide susceptibility status of *Anopheles* populations was effectively reported for the first time in 2005 [2]. Pyrethroid resistance with the presence of *kdr* (L1014F) mutation was recently detected in malaria vectors in the south of the country [3]. It was also known that carboxylesterases (COEs), glutathione-S-transferases (GSTs) and cytochrome P450-dependent monoxygenases (P450s) are the three main groups of enzymes involved in the metabolic resistance to pyrethroids used for malaria vector control [4].

LLINs containing an insecticide plus the synergist piperonyl butoxide (PBO) have been designed for increased efficacy against pyrethroid-resistant malaria vectors. PBO is an inhibitor of mixed function oxidases (MFO) implicated in pyrethroid resistance, and also increases the rate of insecticide uptake through the mosquito cuticle [5]. In 2014, at the time of the evaluation, two LLINs with PBO were available: PermaNet® 3.0[6] and Olyset® Plus [7] PermaNet® 3.0 is a LLIN with PBO and deltamethrin incorporated on polyester side panels and a mixture of deltamethrin and PBO incorporated in the polyethylene top panel. Olyset® Plus LLIN is made of polyethylene netting incorporating permethrin and PBO. A third LLIN with PBO, Veeralin® LN received WHOPES interim recommendation in 2016 [8]. In contrast to LLINs with PBO, the pyrethroid-only LLIN included in this study, Yorkool® is a multifilament polyester net coated with deltamethrin.

In this experimental hut trials, the primary objective was to determine the comparative efficacy between LLINs with PBO and a pyrethroid-only net in an area of pyrethroid resistance with the involvement of metabolic resistance mechanisms. As per standard outcomes measures for experimental hut trials, efficacy was measured in terms of blood-feeding inhibition, deterrence, induced exophily and mortality. Characterisation of the wild mosquito population including species composition, susceptibility to the pyrethroid active ingredient in the LLINs (deltamethrin and permethrin), frequency of the target site mutation (*kdr* L1014F) and upregulated metabolic enzymes were also determined. The trial was conducted from June to December 2013 in a West African experimental hut design at Kolokopé, Togo.

Methods

Study site

This evaluation was conducted in experimental huts located at Kolokopé, Togo (07˚47'59'N, 01˚18'00E) from June to December 2013. The village is situated in the plateau region of the country and at 200 km from Lomé. The area is a cotton cultivation site covering approximately 236 hectares and produces an estimated 1000 tons of cotton per year. To protect farms, pyrethroid based insecticides are commonly used to spray fields [9]. The region is characterised by a long rainy season from March to October and a dry season from November to February. The annual rainfall is estimated to 1300-1500mm per year.

WHO susceptibility Test

To characterise the wild *An. gambiae s.l.* mosquito population in Kolokopé, WHO susceptibility tests were conducted according to WHO standard protocols [10]. Mosquitoes were assayed

using WHO discriminating dosages of nine insecticides belonging to four chemical classes: (1) pyrethroids (0.05% deltamethrin, 0.75% permethrin and 0.05% lambdacyhalothrin), (2) organochlorine (4% DDT), (3) organophosphates (1% fenitrothion; 5% malathion and 0.4% chlorpyrifos methyl), and (4) carbamates (0.1% propoxur and 0.1% bendiocarb). In addition, synergist assays with 5% PBO impregnated papers were conducted to determine the presence of metabolic mechanisms such as P450 enzymes. *Anopheles gambiae s.l.* mosquito larvae were collected in the surroundings of the village and reared to adults at the field site laboratory. Twenty to twenty-five non-blood fed female *An. gambiae s.l.*, aged 3–5 days were exposed for one hour to the different insecticides and two hours specifically for fenitrothion. For the synergist assay, mosquitoes were pre-exposed to PBO for one hour before exposure to the insecticide for an additional hour. The number of mosquitoes knocked down was recorded at 60 minutes and mortality recorded after 24 hours [10]. Tests with silicone and olive oil impregnated papers were run in parallel and served as controls. Following the susceptibility tests, all mosquitoes (including controls) were kept at −20˚C for further identification of *An. gambiae* species complex and characterization of the *kdr* mutation.

Species identification and kdr L1014F detection

Anopheles specimens were randomly selected from the susceptibility testing and analyzed using SINE-PCR for species identification [11]. The detection of *kdr* L1014F was conducted following the methods of Martinez-Torres *et al.*[12] with additional confirmation using real time-PCR following the protocol of Bass *et al.* [13].

Experimental hut design

The experimental huts are made of concrete bricks with a corrugated iron roof, a ceiling of thick polyethylene sheeting, and a concrete base surrounded by a water-filled channel to prevent entry of ants [14]. Mosquito access is via four window slits constructed from pieces of metal, fixed at an angle to create a funnel with a 1 cm wide gap. Mosquitoes fly upward to enter through the gap and downwards to exit; this precludes or greatly limits exit though the aperture enables the majority of entering mosquitoes to be accounted for. A single verandah trap made of polyethylene sheeting and screening mesh measuring 2 m long, 1.5 m wide and 1.5 m high, projects from the back wall of each hut. Movement of mosquitoes between hut and verandah is unimpeded during the night.

Treatment arms. Washed and unwashed LLINs were evaluated using experimental huts for their effects on free-flying, wild mosquitoes and for their ability to deter entry, repel or drive mosquitoes out of houses (i.e. induced exophily), induce mortality, and inhibit blood-feeding. Yorkool® LLIN was used as a positive control and untreated polyester net was used as a negative control.

The following treatment arms were tested using seven nets per arm for the study:

1. Untreated net
2. PermaNet® 3.0 unwashed
3. PermaNet® 3.0 washed 20 times
4. Olyset® Plus unwashed
5. Olyset® Plus washed 20 times
6. Yorkool® unwashed
7. Yorkool® washed 20 times

Washing of the nets. The nets were washed according to standard WHO Phase II washing procedure. Nets were washed in aluminium bowls containing 10 litres of clean water and containing 2g/litre of soap ("Savon de Marseille") using manual agitation. Nets were rinsed twice and dried horizontally in the shade then stored at ambient temperature between daily washes. One day regeneration time was considered between each washing of all the nets following previous study results of the same treatment arms [6, 15].

Before testing in the experimental huts, the nets (including control) were deliberately holed. Six holes were made in each net: two holes in each of the long sides and one hole at each short side. Each hole measured 4cm x 4cm.

Each week, the treatment arms were rotated among the huts according to a Latin square scheme. Seven nets were used per treatment arm and each of the seven nets was tested one night during the week. At the end of the week, the huts were carefully cleaned and aired to remove potential contamination. The treatment was then rotated to a different hut.

Study design. Adult volunteers slept under each individual net per night. They were recruited among the inhabitants of the villages close to the site. Nets were evaluated from 23 June to 22 August 2013 for the first Latin square and from 20 October to 19 December 2013 for a second Latin square, corresponding to 98-night collections per hut to obtain sufficient number of mosquitoes for adequate statistical analysis.

Sleepers were rotated randomly among huts each night of the study. They entered a hut at dusk and remain inside until dawn. In the morning, dead and alive mosquitoes were collected from the floor of the hut as well as from the veranda traps and inside the nets; resting mosquitoes were collected using aspirators from inside the net, from the walls and roof of the hut, and veranda traps. Mosquitoes were scored by location as dead or alive and as fed or unfed. Alive mosquitoes were placed in disposable cups and provided with access to 10% sugar solution for 24 hours to assess delayed mortality.

The primary outcomes measured in experimental huts were:

- deterrence (reduction in hut entry relative to the control hut fitted with untreated nets);
- induced exophily (the proportion of mosquitoes that exited early and were found in exit traps);
- blood-feeding inhibition (the reduction in blood feeding compared with that in the control hut);
- Immediate and delayed mortality (the proportion of mosquitoes that were killed).

Outcome measures were calculated as per standard procedures. The primary analysis was a test of the non-inferiority of the candidate LLINs with PBO (PermaNet® 3.0 and Olyset® Plus) washed 20 times relative to the standard LLIN (Yorkool®) washed 20 times. According to WHOPES, a candidate LLIN is considered to meet the Phase II efficacy criteria if, after 20 washes, it performs as well as or better than the reference LN when washed 20 times in terms of blood feeding inhibition and mortality.

The percentage personal protection was calculated as follows [16]:

$$\%\ personal\ protection\ = 100 \times \frac{BFC - BFT}{BFC}$$

BFC = total number of blood fed females in the control hut
BFT = total number of blood-fed female mosquitoes in the treated hut

The insecticidal effect or overall killing effect of a treatment was calculated using the following formula [16]:

$$\textit{Overall insecticidal effect}\ (\%)\ =\ 100 \times \frac{\mathrm{DT} - \mathrm{DC}}{\mathrm{TC}}$$

DT = total number of dead mosquitoes in the treated hut
DC = total number of dead mosquitoes in the control hut
TC = total number of mosquitoes collected in the control hut

WHO cone bioassays. Cone bioassays were conducted according to the WHO procedures [10] on one net of each treatment arm before the first wash on the 27 May 2013, for a 2nd time when all washings were completed on 17 June 2013, and for a 3rd time at the end of the field experiment on the nets used in huts. For each net, 5 cones each were placed on the 5 sections of the net (roof and 4 sides). Ten females of *An. gambiae* s.s. Kisumu, the susceptible reference strain were introduced per cone and exposed for 3 min to the net giving an average of 50 mosquitoes. Knockdown was recorded 60 minutes after exposure and mortality was checked 24 hours after exposure. Bioassays were also conducted against wild *An. gambiae s.l.* from Kolokopé. Mosquitoes were collected at larval stage from the site, brought to the insectary, and reared until adults. WHO cone bioassays were conducted on non-blood fed adults 3 to 4 days post emergence.

Chemical content analysis of nets. Chemical analysis was conducted on LLIN samples pre-washing, post-washing, and post hut trial. Each net sample (10cm x 10cm) was homogenized and an analytical portion of 300mg was taken for determination of permethrin, deltamethrin, and/or PBO. Following CIPAC (Collaborative International Pesticide Analytical Council) methods deltamethrin, deltamethrin R-isomer, and PBO were extracted by heating under reflux for 60 min with xylene and were determined by gas chromatography with flame ionization detection (GC-FID) using the internal standard calibration. Permethrin and PBO was extracted in a water bath with heptane for 45 minutes and similarly determines by GC-FID.

Statistical analysis

The analysis of each mosquito species that entered the huts was compared among the different treatment arms by a non-parametric Kruskal-Wallis test. The proportion of mosquitoes that exited early, the proportion that were killed within the hut and the proportion that successfully blood fed was compared by species and then analyzed using a logistic regression or generalized linear mixed models, which provide a framework for regression modeling of non-normal outcome data using XLSTAT software (version 2011).

Ethics statement

In addition to approval from the traditional head of district, ethics approval was obtained from national ethics committee and the Ministry of Health of Togo. Sleeper volunteers were informed of the objective of this study and informed consent was obtained from each volunteer. A medical doctor was on hand during the trial to respond to any side effects of the treated nets or to treat any cases of fever. Any confirmed case of *P. falciparum* parasitaemia was treated with Coartem (artemether 20mg/lumefantrine 120 mg). Perceived adverse or beneficial side effects of the washed and unwashed nets were also noted by the seven volunteers during the experiment.

Results

WHO susceptibility tests

The results of the susceptibility testing in Table 1 showed that *An. gambiae s.l.* population of Kolokopé is resistant to both pyrethroids and DDT, but susceptible to organophosphates and carbamates. Among the pyrethroids, particularly low mortality (1.2%) was recorded for lambdacyhalothrin. Resistance to deltamethrin and permethrin were 14.8% and 7.5% mortalitiy respectively.

Alongside WHO susceptibility tests, synergist assays conducted with pre-exposure to PBO enhanced the mortality of permethrin from 7.5% to 92.8% and deltamethrin from 14.8% to 100%.

Species identification and determination of resistant mechanisms

The results of the species identification and the *kdr* genotype are shown in the Table 2. Out of the 270 *An. gambiae s.l.* analyzed, 133 (49.3%) were *An. coluzzii* and 137 (50.7%) were *An. gambiae s.s.* The frequency of the *kdr* mutation (L1014F) was 0.62 within the population of *An. coluzzii* and 0.96 for *An. gambiae s.s.*

WHO cone bioassays

Full bioefficacy (meeting WHO cut-offs of >80% mortality or >95% knockdown) was observed in all unwashed and washed nets against susceptible *An. gambiae* Kisumu (Table 3). Bioassays against the wild resistant populations of *An. gambiae s.l.* from Kolokopé demonstrated that PermaNet® 3.0 unwashed (roof portions containing deltamethrin and PBO) retained full bioefficacy before and after the hut trial (Table 4). PermaNet® 3.0 washed 20 times and Olyset® Plus (unwashed and washed) reported low bioefficacy against wild resistant populations. Yorkool® a deltamethrin-only net delivered nearly no mortality against wild resistant *An. gambiae s.l* populations irrespective to the wash status.

Experimental hut trial

In total, 4,716 mosquitoes were collected in the experimental huts during the evaluation: 2,591 (54.9%) were *An. gambiae s.l.*, 1,037 (22.0%) were *Culex* species, and 1,088 (23.1%) were other species predominantly *Mansonia*. The trial results are outlined in Table 5 for *An. gambiae s.l.*

An. gambiae s.l.

Control hut. During the 98-night collections, 835 culicidae were collected in the control hut. Among them 465 *An. gambiae s.l.* were recorded. A mean number of 5 *Anopheles* females were caught per night and 84.1% of them were blood fed. This corresponded to an average of 3.9 bites per man per night in the control. Natural exophily (13.1%) and natural mortality remained low throughout the experiment (1.9%).

Treated huts. A significant reduction in entry rates (deterrence) was noted with both unwashed PermaNet® 3.0 and Olyset® Plus yielding a deterrence of 33.3% and 34.6%, respectively compared with the negative control ($p<0.0001$). The same trend was observed for washed PermaNet® 3.0 and Olyset® Plus with a deterrence of 25.2% and 23.4%, respectively ($p<0.0001$). Both unwashed and washed LLINs with PBO deterred more *An. gambiae s.l.* compared with unwashed and washed pyrethroid-only Yorkool® LLIN. Notably, the deterrence of unwashed and washed Yorkool® did not differ significantly compared to the untreated net ($p = 0.181$ and $p = 0.143$, respectively).

Table 1. Knockdown (60 min) and mortality (24 hours) of wild adult *An. gambiae s.l.* mosquitoes of Kolokopé using WHO tube test.

Insecticides	N	KD60min (%)	Mortality 24hrs (%)	Resistance status
DDT 4%	96	0	1.0	Resistant
Deltamethrin 0.05%	74	18.9	14.8	Resistant
PBO 5% + Deltamethrin 0.05%	87	100	100	Susceptible
Permethrin 0.75%	80	2.5	7.5	Resistant
PBO 5% + Permethrin 0.75%	83	85.5	92.8	Suspected resistant
Lambdacyhalothrin 0.05%	85	3.5	1.2	Resistant
Propoxur 0.1%	87	100	97.7	Suspected resistant
Bendiocarb 0.1%	85	100	98.8	Susceptible
Malathion 5%	95	100	100	Susceptible
Fenitrothion 1.0%	94	100	100	Susceptible
Chlorpyriphos methyl 0.4%	86	100	100	Susceptible

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High exophily rates were induced by PermaNet® 3.0 and Olyset® Plus. In contrast, exophily rates were low for the Yorkool® washed and unwashed arms (28.5% and 22.9% respectively).

A decrease of the number of blood fed mosquitoes was observed with all six treatments and especially with unwashed PermaNet® 3.0 and Olyset® Plus, recording 93.9% and 96.1% blood feeding inhibition, respectively. However, the bloodfeeding inhibition of both unwashed LLINs with PBO were not significantly different ($p = 0.263$), the blood feeding inhibition of PermaNet® 3.0 washed 20 times was significantly higher (91.1%) than Olyset® Plus washed 20 times (85.6%) ($p = 0.043$).

A significantly higher corrected mortality of 80.5% was observed with PermaNet® 3.0 unwashed arm compared to other treatments ($p<0.05$). The corrected mortality of Olyset® Plus unwashed was 66.6%. As with other parameters washed Yorkool® LLIN performed the worst with a corrected mortality of 40.7%.

Personal protection and insecticidal effect. The personal protection rates measured for PermaNet® 3.0 unwashed and washed were 95.9% and 93.4% respectively; Olyset® Plus unwashed and washed, 97.4% and 89.0% respectively and Yorkool® unwashed and washed, 80.3 and 65/0% respectively.

The insecticidal effect showed a similar trend: PermaNet® 3.0 unwashed and washed, 55.3% and 46.7% respectively; Olyset® Plus unwashed and washed, 44.3% and 42.4% respectively and Yorkool® unwashed and washed, 46.2% and 37.2% respectively.

Other species. Similar outcomes like those of *An. gambiae s.l.* were noted for all the other species including *Culex* and *Mansonia* species for induced exophily, blood feeding inhibition and mortality parameters. Data on *Culex* species are available from S1 Table.

Table 2. Characterization of the *An. gambiae s.l.* mosquito populations from Kolokopé.

Species	N (%)	*kdr* mutation (L1014F)	N	Frequency (per species)
An. coluzzi	133 (49.3%)	RR	83	0.62
		RS	1	
		SS	49	
An. gambiae s.s.	137 (50.7%)	RR	131	0.96
		RS	1	
		SS	5	

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Table 3. WHO cone bioassay against susceptible *An. gambiae s.s.* Kisumu of nets before and after washing, and after the hut trial.

Treatment arm	Unwashed		After 20 washes		After hut trial	
	KD60 (%)	Mortality (%)	KD60 (%)	Mortality (%)	KD60 (%)	Mortality (%)
1. Control	0.00[a]	0.00[a]	0.0[a]	0.0[a]	0.00[a]	0.00[a]
2. PermaNet® 3.0 0X	100[b]	100[b]	n/a	n/a	100[b]	100[b]
3. PermaNet® 3.0 20X	100[b]	100[b]	100[b]	100[b]	100[b]	100[b]
4. Olyset® Plus 0X	100[b]	100[b]	n/a	n/a	100[b]	100[b]
5. Olyset® Plus 20X	100[b]	100[b]	100.0[b]	96.3[b]	100[b]	98.03[b]
6. Yorkool® 0X	100[b]	100[b]	n/a	n/a	100[b]	100[b]
7. Yorkool® 20X	100[b]	100[b]	100.0[b]	94.1[b]	100[b]	96[b]

Values in the same column sharing the same letter superscript do not differ significantly ($P > 0.05$)

n/a = not applicable

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Chemical analysis

The mean concentration of deltamethrin, permethin, and PBO against the target concentration indicated by the manufacturer is outlined in Table 6. At the start of the trial, prior to any washing, all LLINs reported active ingredient concentrations within the target range provided by the manufacturer. Substantial active ingredient loss was noted on the side portions (deltamethrin only) of PermaNet® 3.0. A similar loss of deltamethrin was also observed with Yorkool® net. Also, the loss of PBO active ingredient after 20 washes was 44.0% and 56.6% lower than the initial concertation for Olyset® Plus and PermaNet® 3.0, respectively.

Discussion

*An. gambiae s.l*is the main malaria vector in Kolokopé. PCR testing for species identification found both *An. coluzzii* and *An. gambiae s.s* living in sympatry at similar proportions. WHO susceptibility testing conducted from larval collections determined resistance to pyrethroid insecticides and DDT. Higher *kdr* frequency was found in *An. gambiae s.s.* (96%) compared to *An. coluzzi* (62%). This data is in line with previous works done in Burkina Faso demonstrating a similar predominance of the *kdr* allele frequency of *An. gambiae s.s.* [17–19].

Synergist assays conducted using pre-exposure to PBO restored the susceptibility against permethrin and deltamethrin, thus indicating resistance mediated by elevated P450 mono-

Table 4. WHO cone bioassay against wild resistant *An. gambiae s.l.* Kolokopé of all treatment arms before and after the hut trial.

Treatment arm	Before hut trial		After hut trial	
	KD60 (%)	Mortality (%)	KD60 (%)	Mortality (%)
1. Control	0.0[a]	0.0[a]	0.0[a]	0.0[a]
2. PermaNet® 3.0 0X –side	100.0[b]	17.9[b]	22.5[b]	5.0[a]
2. PermaNet® 3.0 0X –roof	100.0[b]	100.0[c]	90.0[c]	100.0[b]
3. PermaNet® 3.0 20X –side	2.6[a]	5.1[a,b]	16.2[b]	8.1[a]
3. PermaNet® 3.0 20X –roof	80.0[b,c]	60.0[b]	10.0[a,b]	20.0[c]
4. Olyset® Plus 0X	50.0[c]	15.2[b]	16.3[b]	6.1[a]
5. Olyset® Plus 20X	0.0[a]	2.0[a]	2.0[a]	2.0[a]
6. Yorkool® 0X	0.0[a]	0.0[a]	0.0[a]	0.0[a]
7. Yorkool® 20X	0.0[a]	0.0[a]	4.0[a]	2.0[a]

Values in the same column sharing the same letter superscript do not differ significantly ($P > 0.05$)

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Table 5. Summary of trial results obtained for free flying *An. gambiae s.l.* in experimental huts (98 nights) in Kolokopé, Togo.

	Control	PermaNet® 3.0–0 wash	PermaNet® 3.0–20 washes	Olyset® Plus 0 wash	Olyset® Plus 20 washes	Yorkool® 0x	Yorkool® 20x
Total females caught	**465[a]**	**310[b]**	**348[b]**	**304[b]**	**356[b]**	**389[a]**	**419[a]**
Females caught per night	4.75	3.16	3.55	3.10	3.63	3.97	4.27
Deterrence (%)	-	33.33	25.16	34.62	23.44	16.34	9.89
Total females inside verandah	**61[a]**	**174[d]**	**159[b]**	**178[d]**	**141[b]**	**111[c]**	**96[c]**
Exophily (%)	13.12	56.13	45.69	58.55	39.61	28.53	22.91
95% confidence interval	10.05–16.19	50.60–61.65	40.46–50.92	53.01–64.09	34.53–44.69	24.05–33.02	18.89–26.94
Induced exophily (%)	-	49.50	37.49	52.29	30.49	17.74	11.27
Total females blood fed	**391[a]**	**16[c,e]**	**26[c]**	**10[e]**	**43[b]**	**77[f]**	**137[d]**
Blood fed (%)	84.09	5.16	7.47	3.29	12.08	19.79	32.70
95% confidence interval	80.76–87.41	2.70–7.62	4.71–10.23	1.28–5.29	8.69–15.46	15.83–23.75	28.21–37.19
Blood feeding inhibition (%)	-	93.86	91.11	96.09	85.64	76.46	61.11
Overall mortality	**9[a]**	**257[e]**	**226[b,d]**	**215[d]**	**206[b]**	**224[b]**	**182[c]**
Overall mortality (%)	1.94	82.90	64.94	70.72	57.87	57.58	43.44
95% confidence interval	0.68–3.19	78.71–87.09	59.93–69.96	65.61–75.84	52.74–62.99	52.67–62.49	38.69–48.18
Corrected mortality (%)	-	80.52	61.53	66.56	55.05	55.24	40.67

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

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oxygenase mechanisms. Similar trends were observed in Côte d'Ivoire and Benin where synergist assays using PBO also indicated the involvement of P450*s* [20, 21]. Similarly, previous studies have reported *kdr* L1014F mutation [3] and other resistance mechanisms [22] in Togo. The DDT and pyrethroid resistance observed at the experimental hut site in Kolokopé is likely conferred by both *kdr* L1014F and metabolic mechanisms. However, microplate enzyme activity experiments should be conducted to further explore the level and role metabolic mechanisms play in insecticide resistance in Togo. The confirmation of elevated P450 based mechanisms as indicated by the synergist assays also demonstrates the increased efficacy expected from LLINs with PBO (PermaNet® 3.0 and Olyset® Plus) against natural resistant *An. gambiae s.l.* populations.

WHO cone bioassays conducted against susceptible *An. gambiae* s.s. Kisumu indicated that all LLINs met WHO cut-offs of greater than 80% mortality or 95% knockdown before and after the hut trial. Against resistant wild *An. gambiae s.l.* only PermaNet® 3.0 (roof) unwashed and washed 20 times were able to exceed 80% mortality performance cut-off. Olyset® Plus

Table 6. Active ingredient and synergist contents of each net sample before and after the experimental hut trial.

Net type	Chemical	Target concentration		Mean concentration			Loss of active ingredient (%)
		Mean	Range	0 wash	20 washes	20 washes after hut trial	
PermaNet® 3.0	Deltamethrin (roof)	4g/kg	3.0–5.0	3.53	2.98	2.90	**17.8**
	PBO (roof)	25g/kg	19.75–31.25	23.4	15.3	13.1	**44**
	Deltamethrin (side)	2.8g/kg	2.1–3.5	2.57	0.83	0.96	**62.6**
Olyset® Plus	Permethrin	20g/kg	17.0–23.0	19.8	14.0	15.1	**23.7**
	PBO	10g/kg	7.0–13	7.67	2.5	3.33	**56.6**
Yorkool®	Deltamethrin	1.8g/kg	1.35–2.25	1.79	0.59	0.67	**62.6**

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delivered overall low to minimal bioefficacy against wild pyrethroid resistant *Anopheles* populations. Washed and unwashed Yorkool® nets were found to have no bioefficacy against the resistant wild *An. gambiae s.l.* population, even though full bioefficacy was noted against susceptible *An. gambiae s.s.* This suggests that pyrethroid-only Yorkool® nets would not be effective in this area and would perform like an untreated net.

PermaNet® 3.0 and Olyset® Plus exhibited high mortality and high blood feeding inhibition in free flying population of *An. gambiae s.l.* A similar trend was observed in deterrence and exophily rates of PermaNet® 3.0 and Olyset® Plus compared to the pyrethroid-only Yorkool®. These findings are in accordance with studies of Corbel *et al.* [15], Tungu *et al.* [23] and Koudou *et al.* [24] that demonstrated that PermaNet® 3.0 fulfils the WHOPES efficacy criteria of Phase II studies. An experimental hut study comparing Olyset® Plus and Olyset® net also reported similar evidence for the advantage of incorporating PBO with pyrethroid insecticides in a LLIN for increased efficacy [25].

Yorkool® net performed markedly worse than the other LLINs tested on all parameters measured. In an area with pyrethroid resistant malaria vectors, PermaNet® 3.0 and Olyset® Plus (LLINs with PBO) can provide additional protection in terms of reduction in blood feeding and increase in mosquito mortality, compared to a pyrethroid-only net.

The different amount of active ingredient noted for all three LLINs after analysis are similar to some previous studies [15, 25] and as reported in WHOPES reports [6, 7]. As observed in the WHOPES report, PermaNet® 3.0 deltamethrin was lost more substantially from the sides than the roof of the net. It is noted that a study published after this evaluation reported a two day regeneration time for Olyset® Plus [25]. While a one day washing interval was used for this study, the chemical content analysis for both permethrin and PBO were in line with previous reports [25]. Similarly, the WHOPES report for Yorkool® reported 21.6% retention following 20 washes [26]; this study reported a 62.6% loss of deltamethrin, which corresponds to a retention rate of 37.4%. The percentage of loss of active ingredient is more considerable for deltamethrin treated nets than permethrin.

Apart from demonstrating the efficacy of PermaNet® 3.0 and Olyset® Plus, this is one of the first experimental hut studies on two available WHOPES approved LLINs with PBO that emphasises the potential benefit of LLINs with PBO to better control resistant malaria vectors compared to a pyrethroid-only LLIN. In an area with pyrethroid resistant malaria vectors with both *kdr* target site and P450-based metabolic mechanisms, PermaNet® 3.0 and Olyset® Plus (LLINs with PBO) can provide additional protection in terms of reduction in blood feeding and increase in mosquito mortality, compared to a pyrethroid-only net.

Conclusion

To conclude, the present study showed efficacy of LLINs with PBO in experimental huts. PermaNet® 3.0 and Olyset® Plus showed significantly better performance against pyrethroid resistant populations of *An. gambiae s.l.* than the pyrethroid-only Yorkool® LN. These results are encouraging and LLINs with PBO should be taken into consideration in malaria vector control strategies.

Supporting information

S1 Table. Summary of results obtained for free flying all other species in experimental huts (98 nights) in Kolokopé, Togo.
(DOCX)

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Do bednets including piperonyl butoxide offer additional protection against populations of *Anopheles gambiae s.l.* that are highly resistant to pyrethroids? An experimental hut evaluation in Burkina Faso

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Abstract. Malaria control is dependent on the use of longlasting insecticidal nets (LLINs) containing pyrethroids. A new generation of LLINs containing both pyrethroids and the synergist piperonyl butoxide (PBO) has been developed in response to increasing pyrethroid resistance in African malaria vectors, but questions remain about the performance of these nets in areas where levels of pyrethroid resistance are very high. This study was conducted in two settings in southwest Burkina Faso, Vallée du Kou 5 and Tengrela, where *Anopheles gambiae s.l.* (Diptera: Culicidae) mortality rates in World Health Organization (WHO) discriminating dose assays were < 14% for permethrin and < 33% for deltamethrin. When mosquitoes were pre-exposed to PBO in WHO tube assays, mortality rates increased substantially but full susceptibility was not restored. Molecular characterization revealed high levels of *kdr* alleles and elevated levels of P450s previously implicated in pyrethroid resistance. In cone bioassays and experimental huts, PBO LLINs outperformed the pyrethroid-only equivalents from the same manufacturers. Blood feeding rates were 1.6–2.2-fold lower and mortality rates were 1.69–1.78-fold greater in huts with PBO LLINs vs. non-PBO LLINs. This study indicates that PBO LLINs provide greater personal and community-level protection than standard LLINs against highly pyrethroid-resistant mosquito populations.

Key words. insecticide resistance, insecticide resistance management, longlasting insecticidal nets, PBO.

Introduction

Use of the longlasting insecticidal net (LLIN) is pivotal in the fight against malaria in Africa. A massive scaling up of the distribution of this commodity has occurred over the past 15 years, with 178 million LLINs delivered for use in sub-Saharan Africa (SSA) in 2015 alone [World Health Organization (WHO), 2016]. Although reliable estimates of LLIN usage are very hard to obtain, the WHO estimates that 53% of the population at risk in SSA slept under an LLIN in 2015 (WHO, 2016). The results have been dramatic: an estimated 450 million clinical cases of malaria were averted in the last 15 years by the use of LLINs (Bhatt *et al.*, 2015).

All LLINs in current use contain pyrethroid insecticides, but there is growing recognition that increases in the prevalence and intensity of pyrethroid resistance, driven at least in part by the scale-up in the use of LLINs, could jeopardize recent gains in malaria control (WHO, 2012). Direct evidence that pyrethroid

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resistance is reducing either the personal or community-level protection provided by LLINs is challenging to obtain (Kleinschmidt *et al*., 2015; Ranson & Lissenden, 2016), but models of malaria transmission predict that even relatively low levels of resistance can substantially reduce the public health benefits of LLINs (Churcher *et al*., 2016). In countries that rely on the LLIN as the primary malaria prevention tool, the only currently available alternatives to conventional pyrethroid-only LLINs are nets in which the synergist piperonyl butoxide (PBO) has been included in the fibres making up all, or part, of the net. Piperonyl butoxide inhibits cytochrome P450s, which comprise one of the most important enzyme families involved in pyrethroid resistance, and exposure to PBO has been shown to reduce resistance and sometimes to restore susceptibility to pyrethroids in malaria vectors (Jones *et al*., 2013; Edi *et al*., 2014).

Four brands of LLIN containing PBO have received interim approval from the WHO as conventional LLINs. These are the PermaNet® 3.0 (deltamethrin + PBO) (Vestergaard Frandsen Holding SA, Lausanne, Switzerland), the Olyset® Plus (permethrin + PBO) (Sumitomo Chemical Asia Pte Ltd, Health and Crop Sciences Sector, Tokyo, Japan), the Veeralin® (alpha cypermethrin + PBO) (VKA Polymers Pte Ltd, Karur, India) and the DawaPlus® (deltamethrin + PBO) (Tana Netting Co. Ltd, Dubai, U.A.E.). The benefit of the addition of PBO is only expected to manifest in areas in which mosquito populations are resistant to pyrethroids and experimental hut trials of PBO LLINs in areas of resistance have supported this prediction. Increased mosquito mortality was observed in experimental huts containing PBO LLINs compared with conventional LLINs in Ivory Coast, Benin and Burkina Faso (Corbel *et al*., 2010; Ngousso *et al*., 2010; Koudou *et al*., 2011; Pennetier *et al*., 2013) and reductions in blood feeding rates were also reported in trials in the latter two countries. All of these sites reported high levels of pyrethroid resistance.

There has been a dramatic escalation in the strength of pyrethroid resistance in southwestern Burkina Faso since earlier trials of PBO LLINs in 2007. World Health Organization cone bioassays performed in 2012 revealed that none of the conventional LLINs were effective in killing local vector populations and that the performance of the PBO LLIN PermaNet® 3.0 was also compromised in these assays (Toe *et al*., 2014). Although the numbers of malaria deaths in Burkina Faso have fallen over the past 10 years, numbers of malaria cases have risen year on year despite countrywide LLIN distribution campaigns [National Malaria Control Programme (NMCP), personal communication; K.H.Toe, 2017]. In order to advise the NMCP on whether a switch to PBO LLINs may be warranted to target these highly resistant populations, an experimental hut trial was undertaken in two rice-growing areas in the southwestern region of Burkina Faso.

Materials and methods

Study sites

The experimental hut studies were carried out at two field stations in southwest Burkina Faso: the first is located in the Vallée du Kou 5 (VK5) near Bobo-Dioulasso (11°39' N, 04°41' W) and belongs to the Institut de Recherche en Science de la Santé (IRSS)/Centre MURAZ, and the second is located at Tengrela (10°40' N, 04°50' W) near Banfora and is maintained by the Centre National de Recherche et de Formation sur le Paludisme (CNRFP). These two sites are separated by approximately 120 km. Previous surveys revealed *Anopheles coluzzii* (formerly *Anopheles gambiae s.s*. M molecular form) to be the predominant *Anopheles* species at both sites. High levels of resistance to both DDT and pyrethroids have been reported previously at both sites (Ngufor *et al*., 2014; Toe *et al*., 2015).

Characterization of mosquito populations

Anopheles gambiae s.l. larvae were collected in Tengrela and VK5 and reared to adults in local insectaries (mean relative humidity: 75 ± 10%; mean temperature: 27 ± 2 °C). To assess susceptibility to pyrethroids, batches of approximately 25 non-blood-fed *An. gambiae s.l.* females aged 3–5 days were exposed to papers treated with 0.75% permethrin or 0.05% deltamethrin. The papers and susceptibility test kits were purchased from the Universiti Sains Malaysia (Penang, Malaysia). In parallel bioassays, mosquitoes were exposed to papers treated with 4% PBO [prepared by the Liverpool School of Tropical Medicine (LSTM)] for 1 h before they were transferred to tubes containing either insecticide-treated papers or insecticide-free papers and exposed for a further 1 h. Knock-down was recorded at the end of exposure and mortality was recorded 24 h later.

The bio-efficacy of the LLINs was tested using non-blood-fed mosquitoes aged 3–5 days from local larval collections, and from the Kisumu susceptible laboratory strain, using the WHO cone bioassay procedure (WHO, 2013). For each LLIN type, two unwashed nets were tested under insectary conditions. Ten mosquitoes per cone were exposed for 3 min to 30 cm × 30 cm net pieces sampled from the top, the short and the long sides of the net. During exposure, the set-up was kept at an angle of 45 degrees as recommended (Owusu & Müller, 2016). Knock-down and mortality were recorded at 60 min and 24 h after exposure, respectively. Conventional LLINs were compared with PBO LLINs using Fisher's exact test (2 × 2 contingency table, significance level of 0.05) (http://wwwgraphpadcom/quickcalcs/contingency2) (Roberts & Andre, 1994).

Molecular analysis

DNA was extracted from mosquito legs by heating at 90 °C for 30 min. Species were identified using the SINE200 protocol (Santolamazza *et al*., 2008) and then screened for the voltage gated sodium channel (VGSC) 1014F and 1575Y alleles using Taqman assays (Bass *et al*., 2007; Jones *et al*., 2012).

Total RNA was extracted from six pools of 10 5-day-old non-blood-fed female *An. coluzzii* from larval collections from Tengrela and VK5 using the RNAqueous®-4PCR Kit for isolation of DNA-free RNA (Ambion, Inc., Austin, TX, U.S.A.) according to the manufacturer's procedures. The RNA was eluted in 50 µL of elution solution and treated with DNase. The quality and quantity of all the RNA used were assessed

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using a NanoDrop ND1000 (Thermo Fisher Scientific UK Ltd, Renfrew, U.K.).

The expression profiles of five P450 genes (*CYP6M2*, *CYP6Z2*, *CYP6Z3*, *CYP6P3*, *CYP6P4*), previously found to be over-expressed in pyrethroid-resistant field populations from Burkina Faso (Toe *et al*., 2015) and/or known to metabolize pyrethroids (Müller *et al*., 2008; Mitchell *et al*., 2012) were quantified using reverse-transcription quantitative polymerase chain reaction (RT-qPCR). The qPCR analysis was conducted at the LSTM and used the following mosquito populations: Ngousso, an insecticide-susceptible strain originating from Ngousso in Cameroon in 2006 and maintained in the insectary at LSTM; Tengrela specimens reared from larval collections in October–November 2014, and VK5 specimens reared from larval collections in October–November 2014. Approximately 600 ng of RNA was reverse-transcribed to first-strand cDNA using SuperScript™ III reverse transcriptase (Invitrogen, Inc., Carlsbad, CA, U.S.A.) according to the manufacturer's procedures. Samples were then purified using the Qiagen Easy Purification Kit (Qiagen Benelux BV, Venlo, the Netherlands) before proceeding to qPCR. Each of the six pool replicates were run in triplicate using 2X SYBR Brilliant III (Agilent Technologies, Inc., Palo Alto, CA, U.S.A.), forward and reverse primers (300 nM) [sequence available in Toe *et al.* (2015)] on the Mx3005P qPCR system (Agilent Technologies, Inc., Palo Alto, California) with the following cycling protocol: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 10 s. The qPCR data were analysed using the delta Ct values method, taking into account the PCR efficiency (Pfaffl, 2001). The candidate Ct values were normalized against three housekeeping genes, encoding ribosomal protein L40 (ubiquitin) (*AGAP007927*), an elongation factor (*AGAP005128*) and the S7 ribosomal protein (*AGAP010592*). The normalized Ct values of each gene were then compared with the normalized Ct values of the susceptible Ngousso strain.

Experimental hut trials

Each station consisted of six experimental huts built according to the West African style (WHO, 2013). The study had six arms that used, respectively, five different LLINs and one net with no insecticide treatment as a negative control (Table 1). The nets were obtained from the manufacturers and were unpacked and kept in the shade for 24 h, but not washed prior to testing. Two of the nets, the OlysetPlus and PermaNet 3.0, contain PBO, whereas the other three LLINs contain only pyrethroids. The LLINs were holed according to WHO standard procedures (WHO, 2013). A total of six holes (4 cm × 4 cm) per net were cut, two on each of the long sides and one on each of the short sides.

Study participants (male sleepers) spent 6 nights per week under a net in an experimental hut from 20.00 hours to 05.00 hours, followed by 1 day of break. The sleepers were rotated through the six huts so that each sleeper spent 1 night per week under each net type. To complete a full Latin square rotation with all combinations of sleeper, net type and hut, the study ran over 36 days from 8 September to 22 October 2014.

Each morning at 05.00 hours, mosquitoes were collected manually by the sleepers, with supervision, from under the net, inside the hut and on the exit veranda. The collected specimens were morphologically identified to genus and, where possible, to species level (Gillies & Coetzee, 1987), grouped according to their gonotrophic stage (blood-fed, unfed or gravid), and scored as dead or alive. Live mosquitoes were transferred to paper cups, provided with 10% sugar water and kept in the insectary described above for 24 h, after which delayed mortality was recorded. All specimens were stored on silica gel for further molecular analysis.

Data analysis was performed in the open-source statistical software R Version 3.3.2 (R Development Core Team, 2011) using the libraries 'lme4' (Bates *et al*., 2012) and 'glmADMB' (Skaug *et al*., 2012) for generalized linear mixed models (GLMMs). Plots were then generated with the package 'ggplot2' (Wickham, 2009).

In the statistical analysis of hut trial data, in order to increase the number of replicates, give more power and increase confidence in the analysis, data collected from both sites (Tengrela and VK5) were pooled and the following four outcomes for *An. coluzzii* were compared between the LLINs and the untreated control net, as well as between the PBO and non-PBO nets from the same manufacturer: (a) deterrence (i.e. the reduction in hut entry relative to the control or non-PBO net); (b) induced exophily (i.e. the ratio of the odds of a mosquito being found in the veranda trap compared with the hut); (c) blood feeding inhibition (i.e. the ratio of the odds of blood

Table 1. Treatment arms and descriptions of longlasting insecticide-treated nets.

Treatment arm	Description	Manufacturer
Untreated net	Net manufactured manually using netting material from market	Local market
Olyset® Net	8.6×10^{-4} kg/m^2 of permethrin incorporated into polyethylene	Sumitomo Chemical
Olyset® Plus	8.6×10^{-4} kg/m^2 of permethrin and 4.3×10^{-4} kg/m^2 of PBO incorporated into polyethylene	Sumitomo Chemical
PermaNet® 2.0	5.5×10^{-5} kg/m^2 of deltamethrin coated on polyester	Vestergaard Frandsen
PermaNet® 3.0	Combination of 2.8 g/kg of deltamethrin coated on polyester with strengthened border (side panels) and deltamethrin (4.0 g/kg) and PBO (25 g/kg)	Vestergaard Frandsen
Dawa® Plus 2.0	8.0×10^{-5} kg/m^2 of deltamethrin coated on polyester	TANA Netting

PermaNet is a registered trademark of Vestergaard Frandsen Holding SA. Olyset is a registered trademark of Sumitomo Chemical Co. Ltd. DawaPlus is a registered trademark of Tana Netting Co. Ltd.
PBO, piperonyl butoxide.

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fed vs. unfed mosquitoes), and (d) induced mortality [i.e. the ratio of the odds of dead vs. alive mosquitoes] (The original dataset for *An. gambiae s.l.* is supplied in Table S2.) Immediate mortality and mortality at 24 h post-collection were combined for the analysis. Deterrence was analysed as the ratio in total numbers between the treatment arms (or PBO net) vs. the control arm (or non-PBO net). The numbers of mosquitoes in the room and the veranda were combined and analysed using a GLMM with a negative binomial distribution and a log link function using the R function 'glmmadmb()' in the 'glmmADMB' package. In the model, the net type was the fixed effect term and random intercepts were introduced for the sleeper and the hut, and a random slope for the day depending on the location. For proportional outcomes of induced exophily, blood feeding inhibition and induced mortality, the negative binomial model was replaced by a GLMM with a binomial distribution and logit link function using the R function 'glmer()' in the 'lme4' package. In the models, in addition to the terms listed above, a random intercept was introduced for each observation to account for unexplained overdispersion. For statistical testing, the level of significance was set at $\alpha = 0.05$.

In addition to the ratios described above, averages (i.e. the modes) and 95% confidence intervals (CIs) of the crude values underlying the outcomes were computed by the same models as above but using the individual nets as the intercept. These corresponding crude values were: (a) entry rate (i.e. the number of mosquitoes entering a hut); (b) exit rate (i.e. the number of mosquitoes collected from the veranda trap; (c) blood feeding rate (i.e. the proportion of blood-fed mosquitoes), and (d) mortality rate (i.e. the proportion of dead mosquitoes at 24 h post-collection).

The study participants were recruited from the local communities and gave informed consent. Ethical approval was obtained from the Ethical Committee for Health Research of the Ministry of Health and Ministry of Research in Burkina Faso (Deliberation No. 2013-07-057, 11 July 2013). Malaria chemotherapy was not offered to study participants in line with Ministry of Health recommendations. However, medical supervision was provided throughout the study and any malaria case was treated according to national requirements.

Results

Pyrethroid resistance in Tengrela and VK5 and associated mechanisms

In VK5, very low levels of mortality were observed after exposure to the discriminating dose of deltamethrin and permethrin, but pre-exposure to PBO significantly increased mortality rates from 2.5% ($n = 163$) to 45% ($n = 158$) and from 5% ($n = 153$) to 26% ($n = 156$) for deltamethrin and permethrin, respectively (Fisher's exact test, $P < 0.0001$) (Fig. 1). In Tengrela, mortality rates of 34% ($n = 85$) and 14% ($n = 101$) were recorded for deltamethrin and permethrin, respectively. When PBO was used, mortality rates increased significantly to 63% ($n = 84$) and 42% ($n = 104$), respectively (Fisher's exact test, $P < 0.0001$) (Fig.1). Although there was evidence of synergism, pre-exposure to PBO did not fully restore susceptibility in either site to either pyrethroid.

Anopheles coluzzii was the only species of the *An. gambiae* complex identified by PCR of a subset of 80 specimens from Tengrela and VK5. High frequencies of the 1014F allele of the VGSC were recorded in Tengrela (0.819, 95% CI 0.750–0.875) and VK5 (0.885, 95% CI 0.824–0.930) with the 1575Y allele present at lower frequencies of 0.169 (95% CI 0.114–0.236) and 0.221 (95% CI 0.158–0.295), respectively. Samples were not genotyped for the 1014S allele as previous extensive surveys have not detected this allele in *An. coluzzii* from these sites (Toe *et al.*, 2015). There was no statistically significant difference in the frequency of either the 1014F or 1575Y allele between the two sites (Table 2).

Several cytochrome P450 genes previously associated with pyrethroid resistance showed elevated expression levels in the Tengrela and the VK5 *An. coluzzii* populations compared with the susceptible laboratory Ngousso strain (Fig. 2). *CYP6P3*, *CYP6M2*, *CYP6Z3*, *CYP6P4* and *CYP6Z2* were found to be

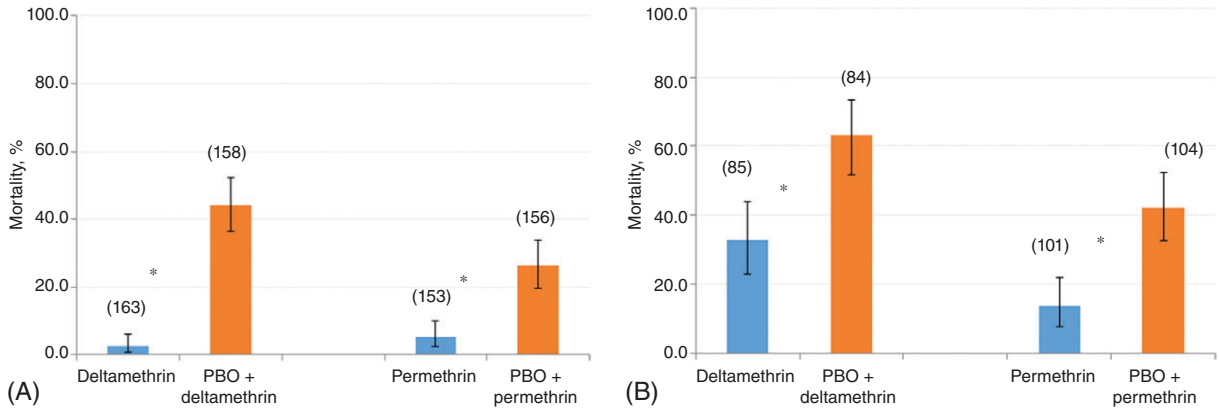

Fig. 1. Mortality rates (with binomial confidence intervals) after exposure to deltamethrin and permethrin in World Health Organization discriminating dose assays with or without pre-exposure to piperonyl butoxide (PBO) in (A) Vallée du Kou 5 and (B) Tengrela (October 2014). *Significant differences in mortality: $P < 0.0001$. Numbers in brackets are the total numbers of mosquitoes tested. [Colour figure can be viewed at wileyonlinelibrary.com].

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Table 2. Frequencies of 1014F and 1575Y *kdr* alleles in *Anopheles coluzzii*, in Tengrela and Vallée du Kou 5 (VK5).

	1014F mutation					
	Total *n*	LL	LF	FF	f (1014F)	*P*-value
Tengrela	80	2	25	53	0.819	0.26
VK5	78	0	18	60	0.885	
	1575Y mutation					
	Total *n*	NN	NY	YY	f (1575Y)	*P*-value
Tengrela	80	57	19	4	0.169	0.39
VK5	77	49	22	6	0.221	

Chi-squared test (http://vassarstats.net/) for the comparison of the frequency of the 1014F and 1575Y mutations in *Anopheles coluzzii* populations from Tengrela and VK5 (October 2014). No statistical difference was observed in the frequencies of the *kdr* alleles in the two sites.

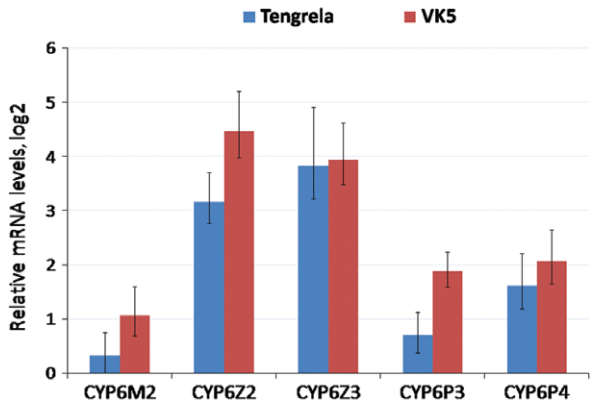

Fig. 2. Expression levels of candidate genes previously associated with pyrethroid resistance in Tengrela and Vallée du Kou 5. The analysis was performed using the $2^{-\Delta\Delta}$Ct method with data normalized against three control genes and presented as a ratio of expression levels in the Ngousso susceptible laboratory strain. Relative gene expressions were transformed to log scale before plotting to minimize large differences in gene expression. [Colour figure can be viewed at wileyonlinelibrary.com].

overexpressed in both field strains compared with the susceptible laboratory colony (fold change: > 2) with the most highly overexpressed genes in both populations being *CYP6Z2* and *CYP6Z3* (Fig. 2).

Efficacy of LLINs under laboratory conditions

All LLINs tested showed good bio-efficacy in cone bioassays against the susceptible laboratory Kisumu strain with 60 min knock-down and 24 h mortality rates of all LLINs above the 98% and 80% WHO thresholds (WHO, 2005) (Table S1). By contrast, in tests using the field-caught mosquitoes, the mortality threshold was met only by the top panels of the PermaNet 3.0 (Fig. 3). Knock-down rates exceeded the 98% threshold for the PermaNet 3.0 in VK5 only (Table S1).

Experimental hut results

Entry rate and deterrence. In total, 12 915 specimens from four different mosquito genera were collected inside the huts (sleeping rooms and veranda traps) over the 6-week trial (Tengrela, $n = 5808$; VK5, $n = 7107$). Most specimens collected from the huts belonged to the *An. gambiae s.l.* species complex, accounting for 75.4% ($n = 4379$) in Tengrela and 98.8% ($n = 7020$) in VK5. The second most frequently collected *Anopheles* species was *An. pharoensis*, of which 49 and 19 specimens were collected in Tengrela and VK5, respectively. Other *Anopheles* mosquito species, including *An. funestus* ($n = 3$), *An. nili* ($n = 2$) and *An. coustani* ($n = 3$), were collected in Tengrela. Additional mosquito taxa were also collected, including *Mansonia* sp. ($n = 1330$ in Tengrela and $n = 45$ in VK5), *Culex* sp. ($n = 41$ in Tengrela and $n = 23$ in VK5) and *Aedes* sp. ($n = 1$ in Tengrela) (all: Diptera: Culicidae). Because of the low numbers of other genera, only data for *An. gambiae s.l.* were included in the analysis.

Within the total of 11 399 *An. gambiae s.l.* caught at both field sites, there was considerable variation between weeks and treatments in the numbers of mosquitoes entering the huts in both study locations (Fig. 4). The average number of mosquitoes caught per night/per hut was between 13 (PermaNet 3.0) and 20.7 (Olyset Net) mosquitoes (Table S3) and induced deterrence was found only for the Olyset Net, albeit at a low ratio of 1.31 (95% CI 1.02–1.66; $P < 0.05$) (Fig. 4).

Exit rates and induced exophily

Exit rates refer here to the proportion of mosquitoes present in the veranda trap. As with entrance rates, exit rates varied throughout the study (Fig. 4). On average, the induced exophily rates were between 26.4% (control net) and 48.5% (Olyset Net) (Table S4). The odds of finding a mosquito in the veranda trap were significantly increased in the huts with treated nets, with the exception of the DawaPlus, although even for the DawaPlus a tendency to induce exophily was observed (Fig. 4, Table S4).

Blood feeding rates and inhibition. Average blood feeding rates were between 17.0% (PermaNet 3.0) and 56.4% (control net) (Table S4). With the exception of the DawaPlus, all treated nets reduced blood feeding (Fig. 4, Table S4), and the effect was most prominent with the PBO nets PermaNet 3.0 and Olyset Plus (Fig. 4, Table S4), for which the odds ratios (ORs) of blood feeding relative to control nets were 0.19 (95% CI 01.3–0.26) and 0.16 (95% CI 0.11–0.23), respectively.

Mortality rates and induced mortality. Average mortality rates ranged from 9.5% in the control arm to 46.1% in the PermaNet 3.0 arm (Table S4). Mortality was statistically higher in all treated net conditions than in the control huts. As with blood feeding inhibition, the PBO nets showed the largest effects in increasing mortality (Fig. 5, Table S4); the ORs for mortality relative to control nets were 5.56 (95% CI 3.92–7.89) and 8.14

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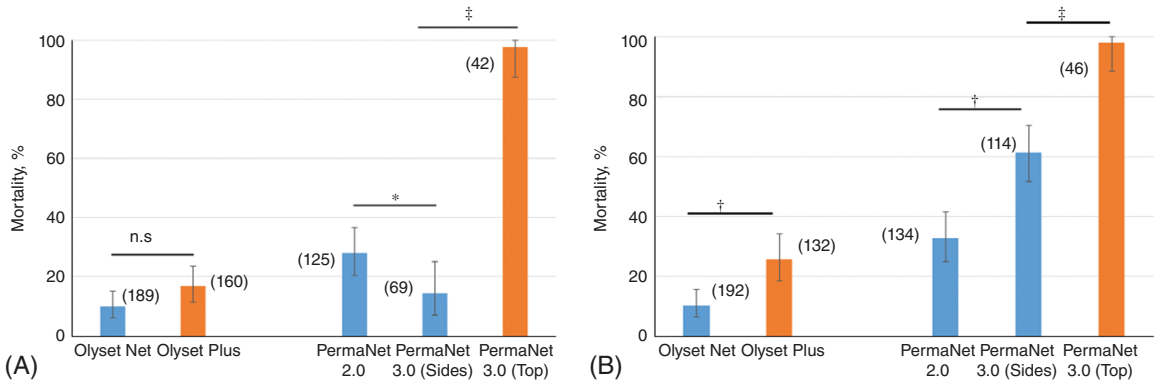

Fig. 3. Mean mortality rates with binomial confidence intervals for mosquitoes collected from (A) Tengrela and (B) Vallée du Kou 5 after exposure to longlasting insecticidal nets (LLINs) for 3 min. Significant differences in mortality between pyrethroid-only and pyrethroid plus piperonyl butoxide-treated LLINs are indicated by * ($P < 0.001$), † ($P < 0.0001$), n.s. (non-significant), ‡ ($P < 0.0001$) for the comparison between the sides and top of the PermaNet 3.0. Numbers in brackets are the total numbers of mosquitoes tested. [Colour figure can be viewed at wileyonlinelibrary.com].

(95% CI 5.64–11.75) for the Olyset Plus and PermaNet 3.0, respectively, compared with 2.65 (95% CI 1.87–3.75) and 3.33 (95% CI 2.35–4.74) for the Olyset Net and PermaNet 2.0, respectively.

Impact of PBO on LLIN efficacy in pyrethroid-resistant mosquitoes. The performance of nets containing PBO as compared with pyrethroid-only nets from the same manufacturer is shown in Fig. 5. The PermaNet 3.0 deterred more mosquitoes than the PermaNet 2.0 (OR = 0.67, $P < 0.01$) (Fig. 5, Table S5), but there was no significant difference between the Olyset Plus and Olyset Net ($P = 0.412$). The Olyset Net induced more exophily than the Olyset Plus (OR = 0.76, $P < 0.05$), but there was no significant difference in exophily between the PermaNet 2.0 and 3.0 ($P = 0.727$) (Table S5). The PBO nets from both manufacturers considerably reduced blood feeding compared with non-PBO nets with ORs of 0.34 ($P < 0.001$) and 0.55 ($P < 0.01$) for the PermaNet 3.0 and Olyset Plus, respectively (Fig. 5, Table S4). In addition, the PBO nets killed significantly more *An. gambiae s.l.* than the non-PBO nets. The ORs for mortality were 2.45 ($P < 0.001$) for the PermaNet 3.0 and 2.1 ($P < 0.001$) for the Olyset Plus (Fig. 5, Table S5).

Discussion

Pyrethroid resistance in southwest Burkina Faso

Very low mortality rates were obtained for both permethrin and deltamethrin in the two study sites following an hour of exposure to WHO diagnostic doses. Southwestern Burkina Faso is known as a hotspot of pyrethroid resistance (Dabiré *et al.*, 2012; Namountougou *et al.*, 2012). Rapid changes in the prevalence of pyrethroid resistance have been observed since the first national LLIN distribution programme in 2010: in 2009, mosquito mortality following deltamethrin exposure was 25% (Dabiré *et al.*, 2012), in VK has since fallen to just 2.5%. The frequency of the 1014F *kdr* mutation also increased from 0.28 in 2006 (Dabiré *et al.*, 2009) to 0.88 in the current study. Similar increases in the prevalence of pyrethroid resistance have been witnessed in Tengrela. Mortality rates of 93% and 46% for deltamethrin and permethrin, respectively, were recorded in 2011 (Namountougou *et al.*, 2012; K. H. Toe, unpublished data 2011), but these mortality rates had reduced to 33% and 13% in 2014, the year of the current study (Toe *et al.*, 2015). In the present study, pyrethroid mortality was significantly increased by pre-exposure to PBO. This, together with the qPCR data showing elevated expression of multiple P450s in both field populations compared with a susceptible laboratory strain, indicate that oxidases are an important resistance mechanism in *An. coluzzii* in southwestern Burkina Faso. It is noted that resistance was not fully restored by PBO pre-exposure, indicating that additional resistance mechanisms, such as target site resistance and possibly cuticular modifications (Toe *et al.*, 2015), may contribute to the pyrethroid resistance phenotype in these populations.

Bio-efficacy of LLINs in cone bioassays

The low levels of mortality observed in cone bioassays in this study are similar to those reported previously in VK7, a neighbouring village to VK5, in the Vallée du Kou rice-growing region. However, whereas the current study found that exposure to the tops of the PermaNet 3.0 nets resulted in mortality levels exceeding the WHO threshold of 80%, equivalent bioassays conducted in the VK7 population in 2012 showed mortality of only 43%. Cone bioassays are not a reliable method of comparing the performance of LLINs containing different pyrethroids because of the inherent differences in excito-repellency within this class, which can affect exposure time (Siegert *et al.*, 2009). In particular, cone tests may underestimate the performance of permethrin LLINs; this is indicated by comparison of the cone bioassay results in Fig. 3, in which the Olyset Net was found to induce considerably lower mortality than the PermaNet 2.0, although experimental hut results showed similar levels of mortality in huts with these two net types (Table S3).

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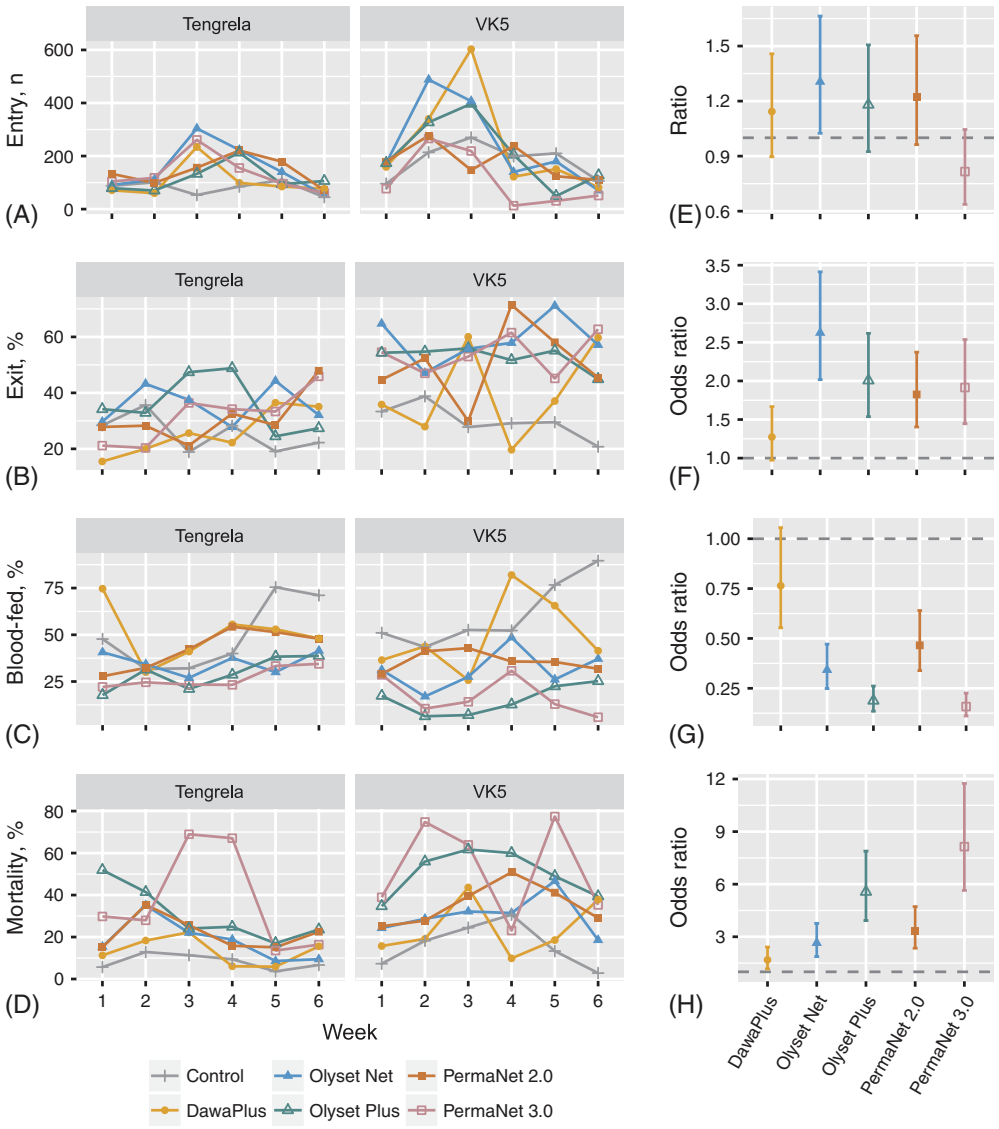

Fig. 4. Primary outcomes measured in the experimental hut trials in Tengrela and Vallée du Kou 5 (VK5). (A–D) Crude data for the measured outcomes. (A) Entry, number of mosquitoes per night entering a hut. (B) Exit, proportion of mosquitoes collected from the veranda trap. (C) Blood feeding, proportion of mosquitoes found blood-fed. (D) Mortality, proportion of mosquitoes dead at 24 h post-collection. (E–H) These outcomes measured relative to the control (i.e. untreated mosquito net) for both sites combined. (E) Deterrence, the ratio of mosquitoes entering a hut relative to the control hut. (F) Exophily, the odds ratio (OR) for a mosquito being found in the veranda trap. (G) Blood feeding inhibition, the OR for a mosquito being blood-fed. (H) Mortality, the OR for a mosquito being dead in the morning (immediate mortality) or after being caught alive and held for 24 h (delayed mortality). Symbols show the average and whiskers show the limits of the 95% confidence intervals around the average. The dashed line shows the ratio or OR of 1, indicating no association of the outcome with the treatment. The data used to produce the plot together with the *P*-values are provided in Table S3. [Colour figure can be viewed at wileyonlinelibrary.com].

Nevertheless, the results of these cone bioassays provide further evidence that resistance can be at least partially ameliorated by exposure to PBO.

Performance of PBO LLINs in experimental hut studies

The enhanced performance of PBO-containing LLINs over conventional LLINs was further supported by the experimental hut results. Both blood feeding inhibition and mortality rates were significantly higher in huts containing PBO LLINs than in those containing conventional LLINs from the same manufacturers. An improved performance of the PermaNet 3.0, which contains both higher concentrations of deltamethrin compared with the PermaNet 2.0, plus PBO on the roof of the net, has also been reported in pyrethroid-resistant populations of malaria vectors in Ivory Coast (Koudou *et al.*, 2011), Benin (N'Guessan *et al.*, 2010) and Burkina Faso (Corbel *et al.*, 2010).

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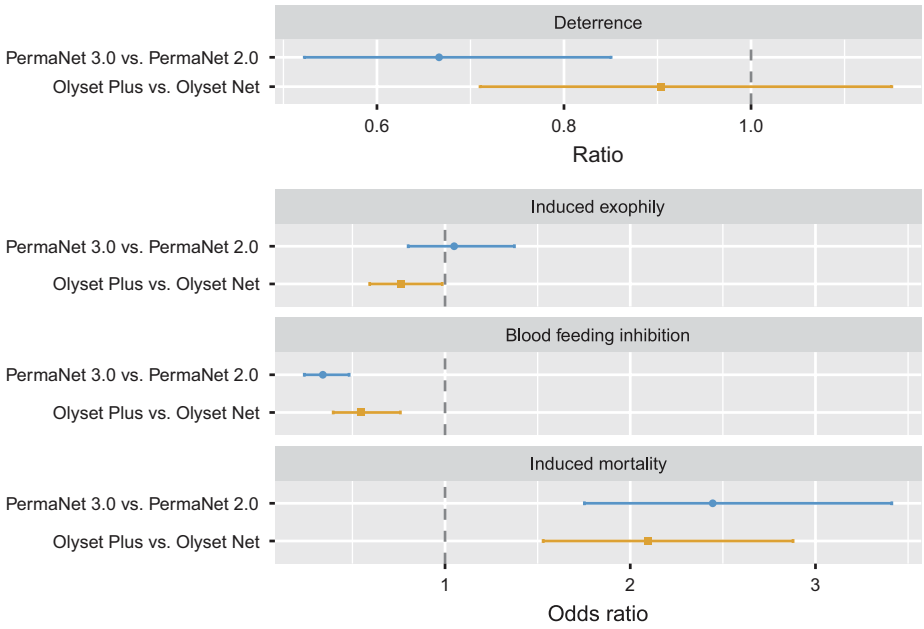

Fig. 5. Outcome measures comparing piperonyl butoxide (PBO)-treated and non-PBO-treated nets from the same manufacturers. Deterrence, the ratio of mosquitoes entering a hut relative to the control hut. Exophily, the odds ratio (OR) for a mosquito being found in the veranda trap. Blood feeding inhibition, the OR for a mosquito being blood-fed. Mortality, the OR for a mosquito being dead in the morning (immediate mortality) or after being caught alive and held for 24 h (delayed mortality). [Colour figure can be viewed at wileyonlinelibrary.com].

The magnitude of this effect differs among studies, with previous studies reporting increases in mortality rates of 1.3–1.8-fold, whereas the current study reports an OR of 2.45, which corresponds to a 1.78-fold increase. Only one experimental hut study comparing the Olyset Plus with the Olyset Net has been published to date (Pennetier *et al*., 2013). This study, conducted in Benin in 2013, reported mortality rates 1.9-fold higher in the PBO arm than in the conventional LLIN arm (Pennetier *et al*., 2013), which is in line with the findings of the current study, which found a 1.89-fold (OR 2.1) elevation in mortality rates in the Olyset Plus arm.

In addition to increased mortality rates, the current study, plus two of the previous experimental hut studies, reported significantly higher rates of blood feeding inhibition for PBO vs. conventional LLINs (Corbel *et al*., 2010; N'Guessan *et al*., 2010), indicating that PBO LLINs afford an enhanced level of personal protection in areas where vectors are resistant to pyrethroids. It should be noted that the current study was performed using unwashed nets only. Previous studies have found that the efficacy of PBO LLINS against resistant mosquitoes decreases substantially after the nets have been washed 20 times according to WHO protocols (Corbel *et al*., 2010; Tungu *et al*., 2010; Koudou *et al*., 2011). Thus further studies on the durability of the bio-efficacy of PBO LLINS under field conditions are urgently needed.

Conclusions

The results of these experimental hut studies with entomological endpoints suggest that substituting conventional LLINs with PBO LLINs in areas where there is a high prevalence of pyrethroid resistance in local vectors may be an effective strategy to maintain the efficacy of malaria vector control. The public health benefit of this would depend on a wide range of factors, including the level of malaria endemicity, LLIN coverage rates and the predominant mosquito vectors present, but a recent modelling exercise predicts that in some settings, a switch to PBO LLINs could avert up to 0.5 clinical cases per person per year (Churcher *et al*., 2016). These predictions from models are now being evaluated in large-scale field trials. A recent study in Tanzania reported a 33% protective efficacy of PBO LLINs over conventional LLINs after 2 years of use (Protopopoff *et al*., 2018). A larger trial, involving the distribution of over 10.7 million nets in Uganda, is evaluating whether PBO nets reduce malaria prevalence under programmatic conditions (https://www.againstmalaria.com, https://www.pmi.gov).

In light of the results from experimental hut studies, including the current study, and after reviewing data from the first clinical trial of a PBO LLIN, the WHO recently made a policy recommendation that national malaria control programmes should consider deployment of PBO LLINs in areas with pyrethroid-resistant vectors (WHO, 2017). If deployed at scale, PBO LLINs may play an important role in reducing the immediate threat of pyrethroid resistance to malaria control.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article. DOI: 10.1111/mve.12316

Table S1. Knock-down at 60 min and 24 h mortality rate with 95% binomial confidence intervals of the longlasting insecticidal nets against Kisumu, Tengrela and Vallée du Kou 5 colonies. Data for the PermaNet 3.0 refer to combined results from the top and sides of the net.

Table S2. Original dataset with data for *Anopheles gambiae s.l.*

Table S3. Outcomes measured in the experimental hut trial when pooling data from both study sites.

Table S4. Comparisons of longlasting insecticidal nets vs. the untreated control net for each brand. Data from both sites were combined for this analysis.

Table S5. Comparison of piperonyl butoxide (PBO)-treated longlasting insecticidal nets (LLINs) with the respective non-PBO-treated LLIN.

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The authors declare no conflicts of interest.

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Research Article

Village-Scale Evaluation of PermaNet 3.0: an Enhanced Efficacy Combination Long-Lasting Insecticidal Net Against Resistant Populations of *Anopheles gambiae s.s.*

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Abstract *Background.* PermaNet® 3.0 (PN 3.0) is a combination long-lasting insecticidal net (LLIN) designed to have increased efficacy against pyrethroid-resistant malaria vectors. Field testing of this new tool under normal use has been limited. Here we report on a small-scale village trial carried out at two localities where malaria vectors were resistant to pyrethroid insecticides. *Methods.* Nets were distributed to cover all sleeping spaces and evaluated for insecticidal activity. Households were visited to assess net usage and reported side effects. Entomological data were collected on a monthly basis for 12 months. *Results.* Bioassays repeated on domestically used PN 3.0 over 12 months showed persistent bioefficacy although bioefficacy of Olyset decreased over this period (< 80% mortality). The overall results demonstrated that PN 3.0 was well accepted by nets users and resulted in 8–11% and 34–37% reductions in blood feeding relative to the Olyset and the untreated control respectively. *Anopheles gambiae s.s.* mortality was also greater for PN 3.0 (> 65% mortality) compared to the Olyset nets (< 45%). *Conclusion.* This study provides persuasive evidence on the increased efficacy of PN 3.0 against malaria vectors with *kdr* only and *kdr* plus metabolic-based pyrethroid resistance mechanisms under realistic LLIN use scenarios.

Keywords PermaNet 3.0; village trial; efficacy; resistance; *Anopheles gambiae*

1 Background

The use of insecticide-treated nets (ITNs) is a key strategy for protection against malaria infection [6,22]. The bio-efficacy of conventionally-treated nets is known to diminish due to repeated washing and handling, necessitating re-treatment at six to twelve month intervals in order to retain bio-efficacy. The development and promotion of long-lasting insecticidal nets (LLINs) has circumvented the problems associated with re-treatment of nets [16]. Long-lasting nets are manufactured with the aim that the net is more resistant to washing than conventionally treated nets, with minimum criteria of withstanding 20 standard washes under laboratory conditions and 3 years of recommended usage under field conditions. The production of LLINs employs two main technologies. The first involves incorporation of the insecticide into the mixture prior to extrusion of the fibre, such as for Olyset Net® which incorporates permethrin into polyethylene [34]. A second strategy is by coating a resin containing insecticide onto the pre-extruded fibre, such as employed in the development of PermaNet® which uses deltamethrin mixed in a resin and bound around polyester fibres [35].

Pyrethroids are currently the only class of insecticides recommended for use in LLINs. Resistance to pyrethroids has become widespread and is a threat to the success of malaria control programs [12,13,24,31]. Pyrethroid resistance in African malaria vectors is normally associated with two major mechanisms: target site insensitivity and metabolic-based resistance [18,27]. Target site insensitivity to pyrethroid is due to a single point mutation commonly referred to as knock down resistance (*kdr*) leading to modification of the voltage-gated sodium channel making it less susceptible to the binding of pyrethroids [27]. Metabolic-based resistance mechanisms are principally associated with three enzymes families: the cytochrome P450 monooxygenases, carboxylesterases and glutathione-S-transferases [18,27].

Synergists have been used commercially for over 50 years and have contributed significantly to improve the efficacy of insecticides [8,9,19]. This can be attributed to their enzyme-inhibiting action, restoring the susceptibility of insects to the chemical which would otherwise require higher levels of the toxicant for their control [11]. Synergists are also useful for laboratory investigation of resistance mechanisms through their ability to inhibit specific metabolic pathways [11]. PermaNet® 3.0 (PN 3.0)

is a mosaic LLIN which combines deltamethrin-coated polyester side panels and deltamethrin with the synergist piperonyl butoxide (PBO) incorporated in the polyethylene roof [35]. PBO is an inhibitor of mixed function oxidases with potential to reduce activity of enzymes associated with resistance [11] as enhancing penetration of deltamethrin across the insect cuticle [1]. Data from experimental hut trials in West and East Africa have shown the potential of PN 3.0 in controlling resistant malaria mosquitoes when compared to standard LLINs (PermaNet 2.0 and Olyset) that received full WHOPES recommendation [7,21,23,30,32], but there is a paucity of field testing under normal use conditions. The present study was conducted in areas where the malaria vector *Anopheles gambiae sensu stricto* is resistant to pyrethroids. Product acceptance, perceived side effects and user perception of effectiveness were also investigated.

2 Methods

2.1 Study sites

Three villages at Ikorodu (Igbokuta, Agundun, and Lantoro) in south-western Nigeria and three others at Kainji (Monai, Dongogari, and Sabogari) in north-central Nigeria were selected for the study based on available pyrethroids resistance data [2,3,10,25]. The study area at Ikorodu is at the outskirts of Lagos. The three villages have a combined population of 500 people and similar sleeping pattern with an average of three persons per room. The area is usually flooded during the rainy season and provides mosquito breeding sites year round. Previous studies have shown that the main malaria vector in this area, *Anopheles gambiae sensu stricto*, is resistant to pyrethroids by the *kdr*-based resistance mechanism [25]. The study area at Kainji, with a population of 950 people, is located around the Kainji Dam. The three villages have similar housing structures (mainly traditional houses built with mud and a thatched roof) and sleeping pattern with an average of four persons per room. *Anopheles gambiae sensu stricto* and *An. arabiensis* are the predominant malaria vectors at the site. *Anopheles gambiae* is resistant to pyrethroids by the *kdr* and metabolic P450-based resistance mechanisms [2,25].

2.2 Insecticide susceptibility test and synergist study

Insecticide susceptibility tests were conducted on mosquitoes collected from the 6 villages in April 2010. Two to three day old adult *An. gambiae s.l.* reared from larval collection in each village were identified morphologically [14,15] and were exposed to permethrin (0.75%) and deltamethrin (0.05%). The 1 h insecticide exposure followed the standard WHO protocol and test kits [33]. For each village, the population of *An. gambiae s.l.* that survived the insecticide exposure was divided into two: (1) the first subset was analyzed together with dead mosquito to species level using PCR [28] and also for the presence of the *kdr* mutation using allele-specific PCR diagnostic tests designed for the West and East African *kdr* mutation [13,26]; (2) the second subset was induced to lay eggs in the insectary and F1 progeny were used for synergist and biochemical analyses as previously described [5]. In brief, PBO was tested for synergistic activity with permethrin or deltamethrin; mortality was compared between mosquitoes exposed and unexposed to PBO to determine the role of metabolic degradation as a mechanism for pyrethroid resistance. To investigate the relative role of specific metabolic pathways inhibited by this synergist, enzyme assays were carried out on live mosquitoes to measure esterase, glutathione S-transferase (GST) and cytochrome P450 monooxygenase activity [4,5]. All mosquitoes tested were identified to species level by PCR [28].

2.3 Mosquito nets

PermaNet® 3.0 nets were provided by Vestergaard Frandsen, Switzerland. Olyset® nets (Sumitomo, Japan) were procured from a local market in Kampala, Uganda with a production date of October 2009. Untreated polyester nets were procured from a local market in Lagos, Nigeria. Before the commencement of the study, village group meetings were held and volunteers were educated on the objectives of the study. Householders were provided with basic information on correct net usage. A survey of sleeping patterns was then carried out and used to estimate the total number of existing nets for each village. Existing nets were collected except in the control village where they were retained. Study nets were given a unique code by sewing a label onto them. A "net master list" was then developed for each village for follow-up. Net distributions were conducted on 1st May 2010. At Kainji, the village of Monai was randomly assigned to PN 3.0 and 125 nets were distributed to cover all sleeping spaces. 50 Olyset nets were distributed at Dongogari and 50 untreated polyester nets in the control village (Sabogari). At Ikorodu, the village of Igbokuta was randomly assigned to PN 3.0 with 50 nets; 50 Olyset nets were distributed at Agundun and 50 untreated polyester nets in the control village (Lantoro). In each case, nets were distributed to cover all sleeping spaces. The nets were washed on April 28th 2010 prior to the initial distribution and every three months following distribution, nets were collected and washed (July 2010, October 2010, January 2011, and April 2010). Net washing was carried out at a central location using the standard WHOPES washing guideline 33. Nets were then dried in the shade and returned to the same households.

2.4 Bioassays on nets

Before each washing round, the same 10 randomly-selected nets from each village were used in bioassay. Bio-efficacy was assessed first using the reference Kisumu susceptible laboratory strain of *An. gambiae s.s.* in a standard WHO

conical exposure chamber [36]. Additional bioassays were then carried out with a laboratory resistant strain of *An. gambiae s.s.* from Nigeria named "AGN." This strain was colonised in 2005 from larvae collected from "Ipokia" near Lagos in South Western Nigeria and exhibited resistance to deltamethrin (72% mortality) and permethrin (58% mortality) in WHO susceptibility tests [2]. For all net types, four side panels and the roof panel of each net were tested [36]. One cone test was conducted per side panel, with five (2–3 day old non-bloodfed) female mosquitoes used per cone for a total of 25 mosquitoes of each strain tested on each net. In all, 500 mosquitoes (250 *An. gambiae* Kisumu strain and 250 AGN strain) were used per village in each bioassay round. Mosquitoes concurrently exposed to an untreated net were used as the control.

2.5 Monthly entomological evaluation

Adult mosquitoes were collected in a total of 10 randomly-selected houses (one room per house) in each village once prior to net distribution, a month following distributions and thereafter once per month for 12 months. The same houses were used for the duration of the study. Mosquito densities were measured in the trial and control villages by the following methods:

2.5.1 Floor sheet collection

White floor sheets were placed in the 10 randomly selected rooms per village each evening preceding collections. In the morning, the floor sheets were carefully removed and all dead or moribund mosquitoes were collected and counted [29].

2.5.2 Indoor resting collection

A 10 minute search using a flash light was conducted in the same room used for the floor sheet collection and all mosquitoes found were collected with a suction tube.

2.5.3 Window exit trap collection

A square exit trap (50×50 cm) with a conical aperture [29] was mounted on a window of each selected room at 18.00 h the day preceding the evaluation. The next morning, all mosquitoes in the exit trap were collected.

All collected *Anopheles* spp. were numbered by house and their status (i.e., dead/alive, blood fed/unfed) was recorded. Live mosquitoes from indoor resting catches and exit trap collections were transferred to paper cups, provided sucrose solution (10%), and were kept for 24 h in the laboratory to measure delayed mortality. Samples were identified using morphological keys [14, 15]. Those belonging to the *An. gambiae* complex were further analyzed for species using PCR [28].

2.6 Net tracking and household questionnaires

Two methods were used to collect data. Initially, house-to-house surveys for net usage and physical status of nets were conducted monthly. Using the net master list, all self-identified heads of households were interviewed. The questionnaires were used to determine people's perception of the benefits and/or side effects during use of nets. Where nets were no longer available, interviews were conducted once to determine reasons for halted usage. Focus group discussion were conducted after the 12th month to obtain descriptive information on volunteers' perception on the use of LLINs. Two focus group discussion guided by a member of the research team were held in each village, with one each with the households heads and individuals sleeping under the nets.

2.7 Data analysis

Data collected were analyzed using the STATA statistical package (STATA Corp LP, USA, version 9.1). Results from the insecticide susceptibility tests were analyzed according to the recommendations of WHO [33]. Four parameters were compared amongst PN 3.0, Olyset nets and the untreated nets: (i) percentage of house entering, (ii) mosquito densities over the period, (iii) blood feeding rate and (iv) mortality rate. For each entomological parameter, comparisons amongst treatment groups were made by ANOVA and a chi square tests with the significance level set to p-value < 0.05.

3 Results

3.1 Insecticide resistance and synergist analysis

Species composition varied by field site, with mosquitoes tested identified as a mix of 65% *Anopheles gambiae s.s.* and 35% *An. arabiensis* (Kainji) or as pure collection of *An. gambiae s.s.* (Ikorodu). Insecticide susceptibility tests carried out on wild-caught *An. gambiae s.l.* from the three villages in Kainji showed that *An. gambiae s.s.* exhibited possible or confirmed resistance to permethrin (62–75% mortality) and deltamethrin (77–81% mortality) (Table 1). *Anopheles gambiae s.s.* from the three villages at Ikorodu showed possible or confirmed resistance to permethrin (69–82% mortality) and confirmed resistance to deltamethrin (75–79% mortality) (Table 1).

The *kdr* assays detected the West African *kdr* mutation (*kdr-w*) while the East African (*kdr-e*) was not found in any specimens tested. The overall *kdr* frequency was 26–40% at Kainji without significant variation ($p > 0.05$) amongst the three villages (Table 1). In contrast, the *kdr* frequency at Ikorodu was 61–78% and was similar for the three villages ($p > 0.05$). Progeny of surviving mosquitoes from Kainji exposed to PBO followed by permethrin or deltamethrin exposure showed a significant increase in mortality (87–94%) compared to those exposed to permethrin ($p = 0.026$) or deltamethrin ($p = 0.023$) only (Table 2), indicating the likely presence of monooxygenase-mediated metabolic resistance. However, surviving mosquitoes from the three villages at

Table 1: Final 24 h mortality of *Anopheles gambiae s.s.* following exposure to permethrin and deltamethrin for 1 h, and the corresponding knock down resistance (*kdr*) allelic frequencies in populations from the study sites at Kainji and Ikorodu in Nigeria.

Study area/villages	No. exposed (24 hrs % mortality) 0.75% Permethrin	Genotype and frequency of the *kdr* alleles (%)				No. exposed (24 hrs % mortality) 0.05% Deltamethrin	Genotype and frequency of the *kdr* alleles (%)			
Kainji		RR	RS	SS	F(R)		RR	RS	SS	F(R)
Monai	156 (62.2)	28.8	3.8	67.4	32.6	130 (76.9)	19.2	12.9	67.9	26.1
Dongogari	130 (68.5)	21.5	7.7	70.8	29.2	118 (80.5)	17.8	16.9	65.3	34.7
Sabogari	104 (75.0)	17.3	23.1	59.6	40.4	101 (77.2)	22.8	15.8	61.4	38.6
Ikorodu										
Igbokuta	130 (73.8)	35.4	26.9	37.7	62.3	140 (75.0)	24.3	36.4	39.3	60.7
Agundun	150 (69.3)	35.3	42.7	22.0	78.0	140 (79.3)	20.0	44.3	35.7	64.3
Lantoro	125 (82.4)	17.6	47.2	35.2	64.8	120 (79.2)	20.0	42.5	37.5	62.5

F(R): frequency of the *kdr* alleles.

Table 2: Bioassay results comparing 24 h mortality of pyrethroid-resistant populations of *Anopheles gambiae s.s.* from six villages in Nigeria following exposure to permethrin and deltamethrin in the presence and absence of pre-exposure to piperonyl butoxide.

	No. exposed (24 h % mortality)[a]					
	0.75% Permethrin	4% PBO + 0.75% permethrin	*p*-value	0.05% Deltamethrin	4% PBO + 0.05% deltamethrin	*p*-value
Kainji						
Monai	108 (65.7)	115 (94.8)	0.026	122 (76.2)	120 (87.5)	0.023
Dongogari	120 (70.0)	108 (91.7)		114 (78.1)	114 (89.5)	
Sabogari	110 (71.8)	112 (88.4)		116 (75.0)	118 (92.4)	
Ikorodu						
Igbokuta	112 (72.3)	116 (76.7)	0.062	120 (77.5)	115 (79.2)	0.072
Agundun	118 (65.2)	112 (70.5)		116 (81.9)	118 (83.1)	
Lantoro	110 (79.1)	115 (81.7)		118 (82.2)	112 (83.0)	

PBO: piperonyl butoxide.
[a]Figures in parentheses denote % mortality of the mosquitoes exposed.

Ikorodu exposed to permethrin or deltamethrin after PBO exposure did not show a significant increase in mortality when compared to those exposed to permethrin and deltamethrin only ($p > 0.05$ for both insecticide) (Table 2). Biochemical analysis revealed a significant increased level ($p = 0.022$) of monooxygenase in the resistant mosquito population from Kainji compared to either the Kisumu or Ikorodu strain (Figure 1), further suggesting monooxygenase involvement in pyrethroid metabolism in the Kainji population. The difference in the mean GST or esterase activity between the Kainji and Kisumu or Ikorodu strains was not significant ($p > 0.05$ for both GST and Esterase).

3.2 Bioassays

Bioassays conducted on PN 3.0 at baseline (April 2010) and during quarterly evaluations showed that all PN 3.0 produced 100% knockdown and 100% mortality against the reference Kisumu susceptible strain and also the resistant strain of *Anopheles gambiae s.s.* The Olyset nets also produced 100% knockdown and 100% mortality against the Kisumu susceptible strain during the same period, but the mean knock down rate against the resistant strain of *An. gambiae s.s.* during the period of the study at both Kainji and Ikorodu was < 90 (Figure 2). Similarly, mortality in the Olyset net against the resistant strain of *An. gambiae s.s.* showed greater than 90% mortality only for the first quarter, declining to78% and 72% mortality at the end of the study in Kainji and Ikorodu, respectively (Figure 3).

3.3 Mosquito room entry rate

Entry rates of mosquitoes per room were calculated by pooling all mosquitoes collected using floor sheets, hand catches, and window exit traps in the ten randomly selected rooms for each village (Table 3). Before net distributions, there was no significant difference in entry rates for the three villages at either Kainji and Ikorodu ($p > 0.05$ at both). The impact of the introduction of PN 3.0 and Olyset nets on the entry rate was noticeable with a significant decrease in entry rates observed for villages with LLINs while an increase was observed for those with untreated nets at both Kainji

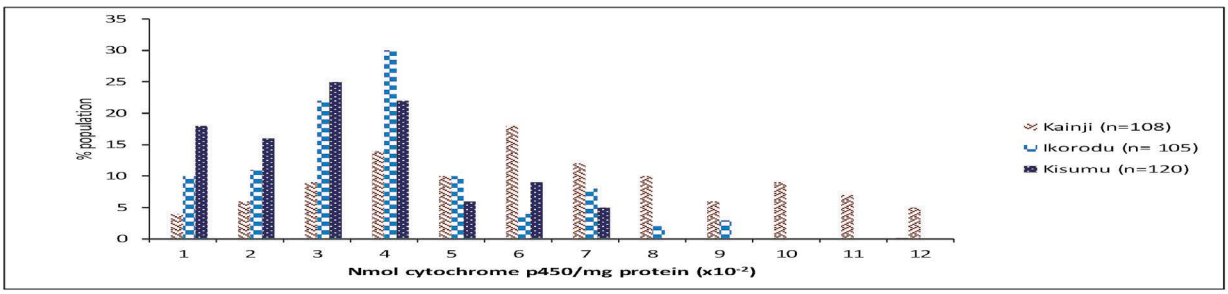

Figure 1: Frequency distribution of monooxygenase level detected in pyrethroid-resistant *An. gambiae s.s.* populations from Kainji and Ikorodu and in the susceptible *An. gambiae s.s.* Kisumu strain via biochemical assays.

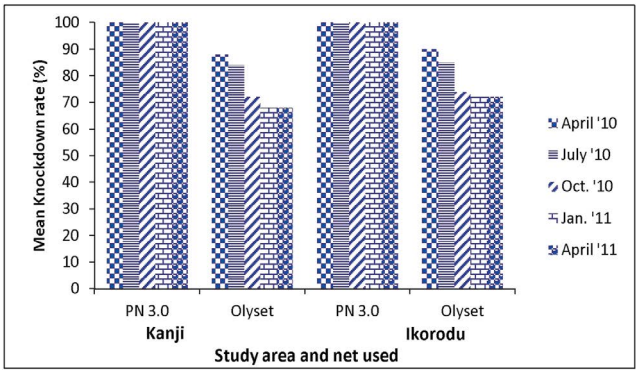

Figure 2: Mean knock down rates (KD) of pyrethroid-resistant laboratory strain of *Anopheles gambiae s.s.* (AGN) based on 3-minutes exposure to PermaNet 3.0 and Olyset nets in WHO cone bioassays prior to (April 2010) and following field usage for 3, 6, 9 and 12-months.

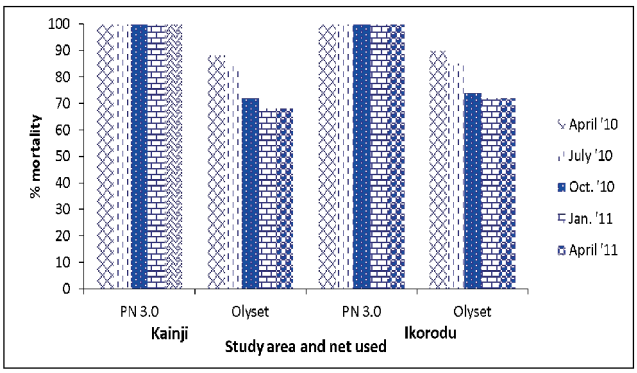

Figure 3: Bio-efficacy of PermaNet 3.0 and Olyset nets prior to and following field usage for 3, 6, 9 and 12-months based on % mortality in 3-minutes exposure in WHO cone bioassays using a pyrethroid-resistant laboratory strain of *Anopheles gambiae s.s.* (AGN).

and Ikorodu. There was no difference in mean monthly entry rates of *An. gambiae s.l.* in villages with PN 3.0 compared to Olyset at either Kainji and Ikorodu ($p > 0.05$ at both).

3.4 Impact of intervention on *Anopheles* densities

Before intervention in April 2010, there was no significant difference in the room density for the three villages at either Kainji and Ikorodu ($p > 0.05$ at both) showing that all three villages at each location were similar in relation to *Anopheles* productivity (Figures 4 and 5). However, following LLIN distribution in May 2010, there was a sharp decline ($> 50\%$) in the density *An. gambiae* in the PN 3.0 village in Kainji compared to the untreated net, and this remained significant for 12 months ($p = 0.006$). The impact of the introduction of the PN 3.0 was also noticeable compared to the untreated net at Ikorodu (Figure 5). A similar trend was observed with the introduction of the Olyset net at Kainji (Figure 4) and Ikorodu (Figure 5) when compared to the villages with the untreated nets. However, there was no significant difference in the density of *An. gambiae s.l.* in PN 3.0 and the village with the Olyset net at Ikorodu ($p=0.17$) or Kainji ($p=0.56$).

3.5 Mosquito mortality

Total mosquito mortality in each village was recorded as a sum of the immediate and delayed mortality divided by the total number of mosquitoes collected. Similarly low mortalities were observed for mosquitoes collected at the three villages in Kainji ($< 1\%$) and Ikorodu ($< 2\%$) prior to net distribution. Following net distribution, virtually all *An. arabiensis* collected in either PN 3.0 or Olyset net villages at Kainji were found dead (98.6% mortality). Overall, mortality of *An. gambiae s.s.* varied between villages at both Kainji and Ikorodu (Figure 6). In villages with PN 3.0, mortality was $> 65\%$, the overall mortality in villages using Olyset nets was $< 45\%$ while in the villages with untreated nets mortality was $< 3\%$.

3.6 Mosquito feeding success

Prior to net distribution, there was no significant difference in the proportion of *An. gambiae s.l.* that had bloodfed at the three villages at either Kainji (32–43%) or Ikorodu (37–46%) ($p > 0.05$ for both). Following net distribution, the proportion of blood-fed *An. gambiae s.s.* varied significantly

Table 3: Number of *Anopheles* caught monthly (entering rate) by indoor resting catch (by hand), window exit trap and floor sheet collection in 10 randomly selected rooms before and after distribution of PN 3.0, Olyset or untreated nets at three villages each in Kainji and Ikorodu in Nigeria from April 2010 to April 2011.

Location	Treatment	Total, before net distribution ($n = 1$)				Monthly mean (±SD), after net distribution ($n = 12$)			
		Indoor resting catch	Exit trap	Floor sheet collection	Total*	Indoor resting catch	Exit trap	Floor sheet collection	Total*
Kainji									
Monai	PermaNet 3.0	18	10	0	28	3.2 (±1.11)	1.2 (±0.79)	10.8 (±0.18)	15.3
Dongogari	Olyset	14	11	0	25	5.1 (±1.06)	6.5 (±0.02)	11.7 (±1.69)	23.3
Sabogari	Untreated net (control)	16	11	0	27	28.9 (±5.95)	8.3 (±0.12)	1.1 (±0.51)	38.3
Ikorodu									
Igbokuta	PermaNet 3.0	23	22	1	46	1.6 (±0.95)	8.2 (±0.11)	18.7 (±3.67)	28.5
Agundun	Olyset	20	23	0	43	7.6 (±2.06)	11.4 (±0.16)	12.2 (±2.01)	31.2
Lantoro	Untreated net (control)	20	25	0	45	30.1 (±4.83)	15.9 (±0.32)	1.2 (±0.69)	47.2

Mosquito collections were made in 10 rooms once per month in villages with PN 3.0, Olyset and untreated nets before and after nets distribution.
*Total = Indoor resting catch + exit trap + floor sheet collection.

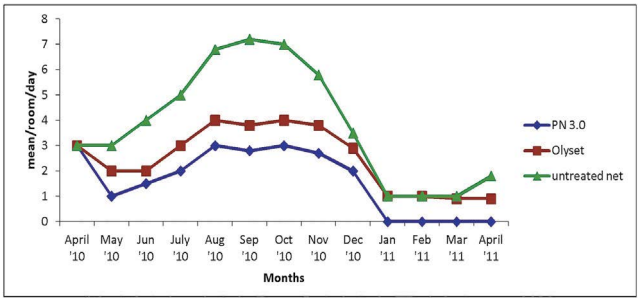

Figure 4: Mean number of *Anopheles gambiae s.s.* per room (pooled from monthly indoor resting, exit trap and floor sheet collections) at 10 houses with PN 3.0, Olyset and untreated nets at Kainji during the pre-intervention (April 2010) and intervention period (May 2010–April 2011).

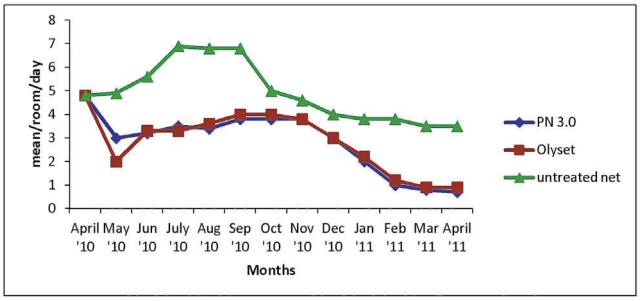

Figure 5: Mean number of *Anopheles gambiae s.s.* per room (pooled from monthly indoor resting, exit trap and floor sheet collections) at 10 houses with PN 3.0, Olyset and untreated nets at Ikorodu during the pre-intervention (April 2010) and intervention period (May2010–April 2011).

between villages with PN 3.0, Olyset nets or untreated nets at both Kainji ($p = 0.021$) and Ikorodu ($p = 0.032$) (Figure 7). At Kainji, there were no blood fed *An. arabiensis*; all bloodfed mosquitoes were identified as *An. gambiae s.s.* by PCR. The overall proportion of bloodfed females was < 3.0% for villages with PN 3.0, three times higher (10–13%) in villages with the Olyset nets, and twelve times higher in villages with untreated nets (37–39%). Overall, the use of PN 3.0 resulted in 8–11% and 34–37% reductions in blood feeding relative to the Olyset nets and the untreated controls, respectively.

3.7 Net usage and households perceived effectiveness

Data were analysed separately for each village and pooled when no significant difference was found between villages with the same net at Kainji and Ikorodu. At the commencement of the study, all households in the six villages indicated their willingness to participate and gave consent. However, two months after the study began, 81% of the 100 people with the untreated nets (control villages) said it provided no protection against mosquitoes bites and only 40% of them had the nets by the end of the study. Almost all LLINs were still in use at the end of the study (99% for both PN 3.0 villages and 99% for both Olyset villages). Although a slightly higher proportion of people sleeping under PN 3.0 reported a reduction in the number of mosquito bites (95%) compared to the Olyset nets (92%), the difference was not statistically significant ($p > 0.05$). Sneezing was the main side effect reported by 18.5% of the 173 people that slept under PN 3.0. The proportion of people that reported sneezing for PN 3.0 was significantly lower than for Olyset net ($p = 0.040$). In addition, dizziness (18%) and skin irritation (12%) were also reported as main side effect among the 99 people that slept under Olyset

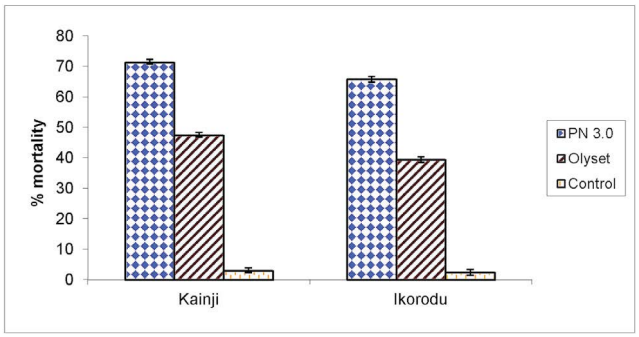

Figure 6: Mean mortality rate (%) based on immediate and delayed mortality of wild-caught female *Anopheles gambiae s.s.* (pooled from monthly indoor resting, exit trap and floor sheet collections) from 10 houses each in villages with PermaNet 3.0, Olyset nets or untreated nets at Kianji and Ikorodu from May 2010 to April 2011.

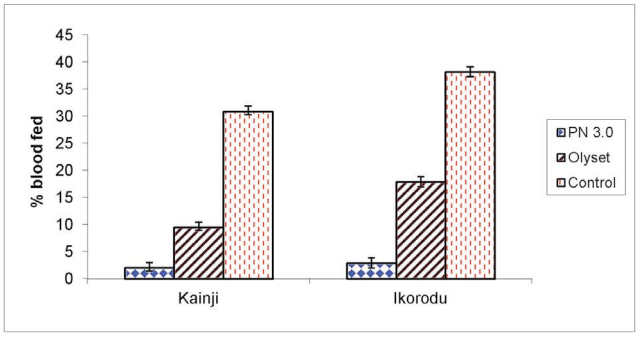

Figure 7: Mean proportion bloodfeeding (%) of wild-caught female *Anopheles gambiae s.s.* (pooled from monthly indoor resting, exit trap and floor sheet collections) from 10 houses each in villages with PermaNet 3.0, Olyset nets or untreated nets at Kainji and Ikorodu from May 2010 to April 2011.

(Table 4). Approximately 25% also complained about the smell of the Olyset nets. A significantly higher proportion of people using PN 3.0 (89.6%) versus Olyset (69.7%) indicated that the intervention was beneficial ($p = 0.043$). The descriptive data from the focus group discussion (data not shown) indicated this was because it also reduced the number of mosquitoes, bed bugs and cockroaches during the study. Thus, they indicated a preference for PN 3.0 over nets previously distributed by the Local Authority.

4 Discussion

This study evaluated the new LLIN, PermaNet 3.0, which consists of a combination of deltamethrin and the synergist PBO to improve bioefficacy against pyrethroid-resistant malaria vectors. A number of experimental hut studies in Africa have evaluated PN 3.0 in comparison to PN 2.0 or Olyset nets with variable reports on the efficacy

Table 4: Net users' perceptions of side effects and benefits of PermaNet 3.0 and Olyset nets.

	Proportion (%) of net owners[†]	
	PN 3.0 $n = 173$[‡]	Olyset $n = 99$[‡]
Unpleasant smell	3 (1.7)	25 (25.2)
Dizziness	2 (1.1)	18 (18.2)
Running nose	5 (2.9)	8 (8.1)
Fever	2 (1.1)	2 (2.0)
Headache	3 (1.7)	1 (1.0)
Sore eyes	0	5 (5.0)
Skin irritation	8 (4.6)	12 (12.1)
Coughing	0	0
Vomiting	0	0
Sneezing	32 (18.5)	28 (28.3)
Sleeplessness	3 (1.7)	1 (1.0)
Was the net beneficial?	155 (89.6)	69 (69.7)
Did the use of the net reduced mosquito bites	164 (94.8)	91 (91.9)
Would you continue sleeping under the net?	167 (96.5)	70 (70.7)

[†]Data were analysed separately for each village and pooled when no significant difference was found between villages with the same type of net.
[‡]Two PN 3.0 and one Olyset net user did not have the nets after 6 months and were excluded from the final analysis.

of PN 3.0 against pyrethroid resistant *Anopheles* and *Culex* species depending on the main vectors and levels and types of resistance mechanisms [7,21,23,30]. Based on modelling of PN 3.0 data from the experimental hut studies in Vietnam, Cameroon, Burkina Faso, and Benin, observed increases in bioefficacy against *Anopheles* vectors (relative to a deltamethrin-only LLIN) were associated with marked decreases in the simulated intensity of malaria transmission [20]. The results of the present study are based on comparative data collected from six different villages using PN 3.0, Olyset nets and untreated nets over a one-year period in areas where the main malaria vector *An. gambiae s.s.* is resistant to permethrin and deltamethrin. The resistance status of the malaria vector to permethrin and deltamethrin as ascertained by WHO susceptibility test remained unchanged and showed comparable results with previous reports from the same area [2,25]. Molecular, synergist, and biochemical analysis provided supporting evidence of *kdr* and metabolic-based resistance in the villages at Kainji. This presents further evidence of multiple pyrethroid resistance mechanisms in *An. gambiae s.s.* reported in our earlier study in Nigeria [2]. Similar findings have been reported in neighboring countries [10,17].

The bioassay data on nets showed that field-used and washed PN 3.0 maintained 100% mortality against a resistant laboratory strain of *An. gambiae s.s.* during the 12 months of the study. In contrast, the Olyset nets showed reduced efficacy over the same period. This is

consistent with results from an earlier experimental hut study comparing PN 3.0 and Olyset nets in Nigeria, in which bioefficacy against resistant mosquitoes was maintained following 20 standard washes for PN 3.0 but not for Olyset [Awolola unpublished].

The results of the monthly mosquito collections showed that although there was a reduction in the entry rate and density of *An. gambiae* following LLIN distribution, there was no difference in these parameters between PN 3.0 and Olyset villages at either Kainji or Ikorodu. However, PN 3.0 caused more than 65% mortality in all *Anopheles gambiae s.s.* entering the houses and provided better protection compared to the Olyset net. This indicated enhanced comparative efficacy of PN 3.0 in areas with *kdr* resistance and kdr plus metabolic resistance in *An. gambiae s.s.* As evident in the synergist analysis of the resistant mosquito populations from Ikorodu, it could be argued that if the rationale behind combining PBO with a pyrethroid is to increase the efficacy of deltamethrin through the synergist's action as a metabolic enzyme inhibitor, then the efficacy of the product in term of mosquito mortality should be less pronounced in an area such as Ikorodu where metabolic resistance was absent. A possible explanation for the improved efficacy in the area with only *kdr* resistance may be connected to the higher deltamethrin content in PN 3.0 in relation to similar nets by the same manufacturer, although this cannot be ascertained as no side-by-side comparison was conducted. Even so, the observed variation in mosquito mortality and feeding success rate between villages with PN 3.0 and Olyset suggests that PN 3.0 may be useful in areas of pyrethroid-resistance.

PN 3.0 was also well accepted by the users. Aside from sneezing, none of the people that used the nets complained of major side effect as a result of sleeping under the nets. Most preferred the nets to those previously distributed in the villages. Among the advantages given were that the use of PN 3.0 reduced mosquito bites in the rooms and that the intervention was beneficial as it killed more bed bugs, cockroaches and spiders compared to nets previously distributed. Further studies should explore this potential advantage, as it may increase user acceptability.

5 Conclusion

We demonstrated that the use of PN 3.0 resulted in substantial reductions in blood feeding rates, and increased the mortality of wild populations of pyrethroid-resistant *An. gambiae s.s.* in two areas of Nigeria. It is recommended that this tool be considered for strategic implementation particularly in areas where pyrethroid resistance has been identified or LLINs have shown reduced efficacy.

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Impact of PermaNet 3.0 on entomological indices in an area of pyrethroid resistant *Anopheles gambiae* in south-western Nigeria

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Abstract

Background: PermaNet® 3.0 is an insecticide synergist-combination long-lasting insecticidal net designed to have increased efficacy against malaria vectors with metabolic resistance, even when combined with *kdr*. The current study reports on the impact of this improved tool on entomological indices in an area with pyrethroid-resistant malaria vectors in Nigeria.

Methods: Baseline entomological indices across eight villages in Remo North LGA of Ogun State provided the basis for selection of three villages (Ilara, Irolu and Ijesa) for comparing the efficacy of PermaNet® 3.0 (PN3.0), PermaNet® 2.0 (PN2.0) and untreated polyester nets as a control (UTC). In each case, nets were distributed to cover all sleeping spaces and were evaluated for insecticidal activity on a 3-monthly basis. Collection of mosquitoes was conducted monthly via window traps and indoor resting catches. The arithmetic means of mosquito catches per house, entomological inoculation rates before and during the intervention were compared as well as three other outcome parameters: the mean mosquito blood feeding rate, mean mortality and mean parity rates.

Results: *Anopheles gambiae s.l.* was the main malaria vector in the three villages, accounting for >98% of the *Anopheles* population and found in appreciable numbers for 6–7 months. Deltamethrin, permethrin and lambdacyhalothrin resistance were confirmed at Ilara, Irolu and Ijesa. The *kdr* mutation was the sole resistance mechanism at Ilara, whereas *kdr* plus P450-based metabolic mechanisms were detected at Irolu and Ijesa. Bioassays repeated on domestically used PN 2.0 and PN 3.0 showed persistent optimal (100%) bio-efficacy for both net types after the 3rd, 6th, 9th and 12th month following net distribution. The use of PN 3.0 significantly reduced mosquito densities with a 'mass killing' effect inside houses. Households with PN 3.0 also showed reduced blood feeding as well as lower mosquito parity and sporozoite rates compared to the PN 2.0 and the UTC villages. A significant reduction in the entomological inoculation rate was detected in both the PN 2.0 village (75%) and PN 3.0 village (97%) post LLIN-distribution and not in the UTC village.

Conclusion: The study confirms the efficacy of PN 3.0 in reducing malaria transmission compared to pyrethroid-only LLINs in the presence of malaria vectors with P450-based metabolic- resistance mechanisms.

Keywords: PermaNet 3.0, Pyrethroid resistance, *Anopheles gambiae*

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Background

The use of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) remains the mainstay for malaria prevention. However, the development of resistance by *Anopheles* mosquitoes to all classes of WHO-recommended adult insecticides, particularly pyrethroids, is a serious concern and threat to malaria control [1,2]. Of the four classes of insecticides (pyrethroid, organochlorine, organophosphate and carbamate) currently recommended for malaria vector control, only pyrethroid is currently approved for LLINs because of its safety, residuality and cost effectiveness. A key issue is to maintain the effectiveness of these vector control tools in an era of growing resistance.

In Nigeria, the first case of pyrethroid resistance in malaria vectors was reported in 2002 [3,4]. Evidence of resistance has since then increased, and is now reported in 16 States affecting the two most important malaria vectors: *Anopheles gambiae s.s.* and *Anopheles arabiensis* [5-7]. While other causal factors of resistance have been identified, such as agricultural usage of insecticides, the significant increase in insecticide-based malaria vector control in the last 10 years has likely exerted significant insecticide selection pressure on *Anopheles* populations in the country. Two main mechanisms of resistance (target-site *kdr* mutations and metabolic alterations) have been identified in different areas [8] but resistance data are still limited. The reality and impact of resistance at the program level is unfolding and it is believed that the loss of pyrethroid effectiveness will lead to increases in preventable deaths particularly in the most vulnerable groups. Consequently, the World Health Organization recommends immediate and pre-emptive action to delay resistance [9]. This requires tools with high efficacy. Current WHO-recommended strategies for insecticide resistance management include: (i) rotational use of insecticides with different modes of action, (ii) combination of interventions, (iii) mosaic spraying, and (iv) application of mixtures of insecticides [9]. Unfortunately, these strategies are most appropriate for IRS. For LLINs, tools with improved efficacy against resistant mosquitoes are limited because pyrethroid is the only insecticide class currently used on LLINs.

Two next generation LLINs have been developed to provide additional efficacy against pyrethroid-resistant mosquitoes through a combination of a pyrethroid with the synergist piperonyl butoxide (PBO), known to affect resistant mosquitoes by inhibiting metabolic enzymes responsible for breaking down pyrethroid molecules. The first combination LLIN was PermaNet® 3.0, which received a WHO interim recommendation as an LLIN in 2008 [10]. This LLIN combines deltamethrin coated polyester side panels and deltamethrin with PBO incorporated in the polyethylene roof. More recently, OlysetPlus® received a WHO interim recommendation in 2012 [11]. OlysetPlus® combines permethrin with PBO incorporated in the polyethylene roof and sides.

In line with the policy of the Nigerian National Malaria Control Program prior to the introduction of an improved vector control tool, a village-wide impact study of PermaNet® 3.0 against pyrethroid resistant malaria vectors was conducted in relation to a pyrethroid only LLIN (Olyset nets) in 2010 [12]. Data from this study have shown the potential of PN 3.0 in controlling resistant malaria vectors when compared to a pyrethroid only LLIN (Olyset nets). The present study was designed to compare the efficacy of PermaNet® 3.0 to another standard pyrethroid only LLIN (PermaNet® 2.0) commonly used in Nigeria. Product acceptance and user perception of efficacy were also investigated.

Methods

Study area

The study was carried out in Remo North Local Government Area of Ogun State, South Western Nigeria. The climate of the area is characteristic of the forest zone with two distinct seasons. The rainy season from April to October and dry season from November to March. The mean annual rainfall is 2000 mm with a mean relative humidity of 78% [13]. The mean temperature is 24°C during the wet and 30°C during the dry season. The area consists of fifteen agrarian communities of approximately 5000 people. Around these communities are small cocoa and palm tree plantations in addition to small vegetable gardens. Herds of cattle and goat kept by nomadic Fulani herdsmen are common in the area. Housing structures consist of both traditional houses (20–35%: mud wall with thatched roof) and modern houses (60–65%: brick houses with corrugated iron roof). The inhabitants are mainly of the Yoruba ethnic group with similar culture and traditions. Malaria is endemic with perennial transmission associated with *Anopheles gambiae s.s* [14]. As a result of baseline insecticide resistance data collected in the area, three villages between 3–5 km apart: Ilara (06° 55.186' N; 003° 48.200' E), Irolu (06° 54.423' N; 003° 44.737'E) and Ijesa (06° 54.659' N; 003° 46.160' E) were selected for comparing the efficacy of PN 3.0 with PN 2.0 and the UTC. The villages are similar in term of size, housing structure and population. However, the most important criteria for their selection is the presence of insecticide resistance.

Baseline data

Insecticide susceptibility test and synergist study

Insecticide susceptibility tests were conducted on mosquitoes collected from the 8 villages in March 2012. Two to three day old adult *An. gambiae s.l.* reared from larval collection in each village were identified morphologically

[15,16] and were exposed to permethrin (0.75%), deltamethrin (0.05%), lambdacyhalothrin (0.05%) and DDT (4%) for 1 h, following the standard WHO protocol [17]. For each village, 100–140 female *Anopheles* (5–7 replicates of 20 mosquitoes) were used per test paper. Three villages (Irolu, Ijesa and Ilara) had the highest rate of insecticide resistance. The population *of An. gambiae s.l.* that survived the insecticide exposure in these three villages was divided into two: (1) the first subset was analyzed together with dead mosquitoes to species level using PCR [18] and also for the presence of the *kdr* mutation using allele-specific PCR diagnostic tests designed for the West African *kdr* mutation [19]; (2) the second subset was induced to lay eggs in the insectary and F1 progeny were used for synergist and biochemical analyses as previously described [20]. In brief, PBO was tested for synergistic activity with permethrin or deltamethrin; mortality was compared between mosquitoes exposed and unexposed to PBO to determine the role of metabolic degradation as a mechanism for pyrethroid resistance. To investigate the relative role of specific metabolic pathways inhibited by this synergist, enzyme assays were also carried out on live mosquitoes to measure esterase, glutathione S-transferase (GST) and cytochrome P450 monooxygenase activity [20-22]. All mosquitoes tested were identified to species level by PCR [18].

Adult mosquito collection

Adult mosquitoes were sampled once prior to net distribution in 35 houses in each of Irolu, Ijesa and Ilara using exit trap and indoor resting collections. The baseline data enabled the determination of vector species, indoor resting densities, blood feeding rates, mortality and determination of sporozoite rates prior to net distribution.

Mosquito nets and treatment arms

PermaNet 2.0 and PermaNet® 3.0 were provided by Vestergaard Frandsen, Switzerland with a production date of October 2010. Untreated polyester nets were procured from a local market in Lagos, Nigeria. Each village was randomly assigned to a treatment arm: PermaNet® 3.0 to Irolu, PermaNet® 2.0 to Ijesa and untreated nets to Ilara. Following house enumeration and completion of households records, 137 PN 3.0 were distributed at Irolu to cover all sleeping spaces, 147 PN 2.0 were distributed at Ijesa resulting in 100% coverage of all sleeping spaces and 150 untreated polyester net were provided at Ilara, also covering all sleeping places. The nets were distributed on the same day (15th March 2012) in the three villages. Nets were given a unique code and a "net master list" developed for each village for follow-up. Householders were provided with basic information on correct net usage. Prior to the distribution, existing nets at Irolu and Ijesa were collected and replaced with test nets. Existing nets in the control village were left with net owners. Before the commencement of the study, village group meetings were held and people were educated on the objectives of the study. Householders were provided with basic information on correct net usage.

Net selection for *in situ* bio-assay cone test

WHO guidelines for phase 3 trials [23] recommend that at least 30 nets per experimental arm are tested in bioassays. Therefore, 35 households were selected randomly from each treatment arm to account for potential drop-outs later in the study. From each of these households, a room where one man slept under the net (one room housing a single man) was selected. The same nets were tested at baseline (March 2012) and were then evaluated during each quarterly bioassay test (June 2012, September 2012, December 2012 and March 2013).

Bio-efficacy was assessed using the reference Kisumu susceptible laboratory strain of *An. gambiae s.s.* in a standard WHO cone test [23]. For all net types, four side panels and the roof panel of each net was tested. One cone test was conducted per side panel, with five 2–3 day old non-blood-fed female mosquitoes used per cone for a total of 25 mosquitoes tested on each net.

Entomological assessment

Mosquito collection and identification

Adult mosquitoes were sampled from 35 houses with nets previously selected for quarterly cone bioassay. One room housing a single man was used; collections were made once prior to net distribution in March 2012, and thereafter once per month for 12 months (April 2012 to March 2013). The same houses were used for the duration of the study. After net distribution, mosquitoes were collected on the 15thday of each month by a team of entomologists per village. The three teams were randomly rotated and allocated to a village each month. Mosquito densities were measured by the following methods:

(i) Window exit trap collection: 35 window traps were used in the selected houses in each village. Traps were in place by 18.00 hrs and mosquitoes were collected from it the following morning (06.00 hr). Locally sourced field workers including householders in whose dwellings the traps were placed were trained to support the entomology technicians for mosquito collection. They were instructed and shown how to block the exit trap by 06.00 hrs and collect live and dead mosquitoes from the window traps. Mosquitoes were placed into pre-labelled tubes with the number, name of the site and name of the householder marked. Alive and dead mosquitoes were placed in different tubes for further analysis.

(ii) Indoor resting collection: Sampling took place in rooms without window traps, and the same houses were used for each of the monthly samples with the houses

being sampled in the same order each month. 35 sleeping rooms with LLINs selected for periodic cone bioassay were included in indoor resting catches. Resting catches were carried out using a standard methodology (a 10 minute search) between 06.00-08.00 hrs using a flash light [24]. The number of mosquitoes collected in each house and their physiological status (unfed, blood fed, gravid) were recorded and *Anopheles* mosquitoes were identified using morphological keys. All *An. gambiae s.l.* were preserved individually on desiccated silica gel for PCR identification and *kdr* status. Host blood feeding preference was assessed by ELISA tests in the laboratory [25].

Parity rate and determination of source of blood meal and Plasmodium infection in mosquitoes by ELISA

Live mosquitoes collected were dissected to determine the parity rate, including all *An. gambiae s.s.* collected at baseline and each month during the 12 months evaluation in the LLIN villages together, with 3590 representing 50% of the total collected in the UTC village post-intervention. The blood meal analysis included all blood fed mosquitoes collected at baseline and in the LLIN villages during 12 months following net distribution together with 2000 (about 50%) blood fed mosquitoes collected from the UTC village over the same period. To estimate the *Plasmodium* infection rate in the mosquito populations, the head and thorax of all female *Anopheles* mosquitoes collected were cut and processed using an ELISA assay [26].

Net tracking and household questionnaires

Two methods were used to collect data. Initially, house-to-house surveys for net usage and physical status of nets (identification, counting and measurement of size of holes in the nets) were conducted monthly. Using the net master list, all self-identified heads of households were interviewed. The questionnaires were used to determine people's perception of the benefits and/or side effects during use of nets. Where nets were no longer available, interviews were conducted once to determine reasons for halted usage. Focus group discussions were conducted after 12th months with the household heads and individuals sleeping under the nets to obtain descriptive information on the households' perception on the use of LLINs.

Determination of chemical content of nets

Five PN 2.0 and five PN 3.0 were randomly collected from net owners and replaced with new nets after the 6th and 12th month of field use. 25 × 25 cm samples were cut from each of the four side panels and the roof panel of each net and were processed for chemical assays according to CIPAC method at an ISO-certified laboratory in Vietnam. A second set of samples (25 × 25 cm) from the same nets were stored at 4°C for reference purposes.

Data analysis

Data collected were analyzed using the STATA statistical package (STATA Corp LP, USA, version 9.1). Treatment arms and net allocation per village was blinded to the statistician to avoid potential bias. There was a positive skew in distribution of the data with a number of zero counts. A logarithmic transformation was therefore used for an approximation to a normal distribution. Counts of mosquitoes from each village were log transformed [ln (n + 1)] to normalize the data with the geometric mean modified to Williams mean to accommodate zero values [27]. The modified geometric means of mosquito catches per village before and during the intervention were compared as well as three other outcome parameters: the geometric means of mosquito blood feeding, mortality and parity rates amongst PN3.0, PN 2.0 villages and the village with untreated nets. For each entomological parameter comparisons amongst treatment groups were made by chi square tests with the significance level set to p-value <0.05.

Biting rates per room per day were calculated by dividing the total number of blood-fed mosquitoes caught by the number of persons sleeping in the room the night preceding the collection [28]. Entomological inoculation rates were calculated as the product of the sporozoites and man biting rates [28,29].

Survey questionnaires were summarized on excel spread sheets and analysed using an excel database. Comparisons of proportions between categorical variables were performed using a chi square test.

Results

Mosquito species and abundance

A total of 13, 030 anophelines were collected during the study, of which 12, 788 (98.1%) were *Anopheles gambiae s,l.*, the remainder being *Anopheles nili,* or *An. funestus* with no significant difference in proportion of these species found in the exit trap and room collections in any of the treatment arms. The 12, 788 *An. gambiae s.l.* correspond to 2,015 at baseline and 10,773 during the 12 months following net distribution (Table 1). PCR analysis of the *An. gambiae* s.l. showed that all samples from Ilara were *Anopheles gambiae s.s.* A predominance of *An. gambiae s.s.* was also recorded at Irolu (95% *An. gambiae s.s*, 4.5% *An. arabiensis*) and Ijesa (98.1% *An. gambiae s.s*, and 1.6% *An. arabiensis*). The percentage of *An. gambiae s.s.* during the 12 months post intervention in the three villages was similar to baseline (100% at Ilara, 96% at Irolu and 99% at Ijesa). PCR analysis for the molecular form of *Anopheles gambiae s.s.* identified the collections either as a mix of approximately 80% of

Table 1 Numbers of *Anopheles gambiae s.l.* collected in each village with the average room

Site	Baseline		After	
	No collected*	Average room density	No collected**	Average density
Ilara (UTC)	568	16.2	7182	17.1
Irolu (PN 3.0)	702	20.1	573	1.4
Ijesa (PN 2.0)	745	21.3	3018	7.2
Total	2,015		10,773	

Density prior to net distribution and during the following 12 months.
*Number of *Anopheles gambiae s.l.* collected in 35 rooms once prior to net distribution in each village.
**Total number of *Anopheles gambiae s.l.* collected in 35 rooms once per month following nets distribution from April 2012 to March 2013.

the S and 19% of the M form at Ijesa (PN 2.0 village) or as pure collections of the S form at Ilara (UTC village) and Irolu (PN 3.0 village) respectively. This proportion did not change during the 12 months following net distribution.

Phenotypic resistance

Using WHO criteria [17], permethrin, deltamethrin, lambdacyhalothrin and DDT resistance were found in the three villages (Ilara, Irolu and Ijesa) during the baseline survey. In addition, DDT and permethrin resistance was found in four other villages in the study area. The 24 h post exposure mortality at baseline for deltamethrin in the three villages was < 64% (Table 2). Twelve months after the intervention, the resistance status of the *Anopheles* populations in the three villages was similar to the pre-intervention level, with the highest resistance still occurring at Irolu (PN 3.0 village; mean 24 h post exposure mortality for all four insecticides of < 63%).

Resistance mechanisms

kdr mutations: Kdr alleles were detected at a high level in the villages where resistance was confirmed and at a low level where the mosquito population was susceptible to at least one of the four insecticides tested. The *kdr* frequencies in the three villages ranged between 55–78% at baseline and 52-72% after the intervention. The highest values, 78% at baseline and 72% following intervention were recorded at Ilara (UTC village).

Metabolic alterations

Figure 1 shows biochemical analyses indicating that *An. gambiae s.s* from Irolu (PN 3.0 village) and Ijesa (PN 2.0 village) had an increased level (>2 fold) of P450 activity compared with the standard Kisumu strain (Irolu, p = 0.049; Ijesa p = 0.047). The mean P450 activity of *An. gambiae s.s.* from Ilara was similar to that of the Kisumu strain (p = 0.891). There was no significant difference between baseline and post intervention P450 activity for the three villages (P > 0.05). Esterase and GST activities were low in all mosquitoes tested at pre- and post-intervention. The mean esterase activity for mosquitoes from the three villages were similar to that of the Kisumu reference strain (Irolu, p = 0.660, Ijesa, p = 0.723; Ilara, p = 0.755). The mean GST activity for each of the three villages was also similar to that of the reference Kisumu strain indicating that there was no esterase or GST resistance in the mosquitoes from the three villages.

Bioefficacy of PermaNet 3.0 and PermaNet 2.0

Baseline bioassay conducted on the net samples prior to net distribution showed high efficacy of PN 3.0 and PN 2.0 with 100% mortality against the susceptible Kisumu reference strain of *An. gambiae* s.s. The efficacy remained the same (100%) for both net types after the 3rd, 6th, 9th and 12th month following net distribution (Figure 2).

Table 2 Summary of main entomological findings for each village at baseline and monthly mean during the 12-months post-intervention period

Site		Villages					
		Ilara		Irolu		Ijesa	
Intervention		Baseline	UTC	Baseline	PermaNet 3.0	Baseline	PermaNet 2.0
WHO susceptibility test*	% Mortality	72.5	76.0	62.5	64.0	66.7	70.0
	N	120	100	120	100	120	100
Density (mean per house)		16.2	17.1	20.1	1.4	21.3	7.2
Mean mortality (%)		0.65	0.9	1.0	55.1	1.7	24.2
Mean blood feeding rate (%)		52.1	57.3	47.3	3.9	48.1	19.9
Overall mean parity rate (%)		48.7	45.9	48.1	10.7	40.9	22.8
Overall mean sporozoites rate (%)		1.76	2.09	2.14	0.87	3.08	2.81
EIR		28.5	26.9	43.0	1.1	65.6	20.2
Resistance mechanisms identified		*kdr*		*kdr* + metabolic (p450)		*kdr* + metabolic (p450)	

*with deltamethrin (0.05%).
UTC: untreated control.

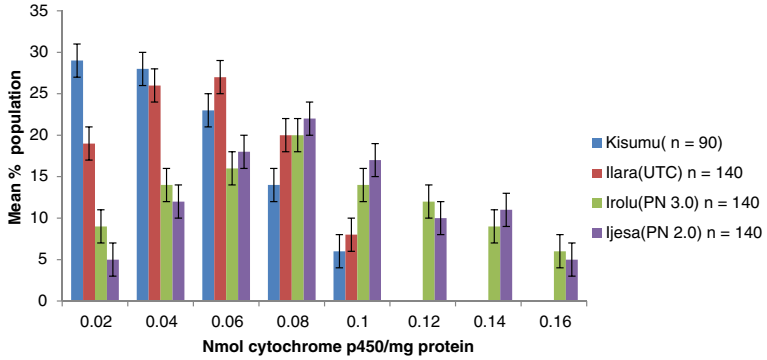

Figure 1 Mean level of P450 monooxygenase activity in pyrethroid resistant *Anopheles gambiae s.s.* from Ilara, Irolu and Ijesa in relation to the standard reference susceptible Kisumu strain of *Anopheles gambiae s.s.*

Chemical content of nets of LLINs

The amount of deltamethrin in the LLINs was within the original target dose at 6 months for both the roof and sides of PN3.0 and PN2.0. However, by 12 months the amount of deltamethrin had reduced for PN3.0 and PN2.0 sides, but remained high in the PN3.0 roof. Although the amount of PBO had decreased below the target dose after 6 months of use, there was no further reduction between 6 and 12 months post-distribution (Table 3).

Impact of PN 3.0 and PN 2.0 on malaria transmission indices

Vector densities

The average number of *Anopheles* found per room, as assessed by exit trap and indoor resting catches at the start of the study in March 2012, was similar in the three villages with a mean of 16.2 at Ilara (UTC village), 20.1 at Irolu (PN 3.0 village) and 21.3 at Ijesa (PN 2.0 village) (Table 1). The numbers in Ilara were elevated at the start of the rainy season in May (Figure 3) and remained so until October before declining to a lower level in February. Here, the malaria vector occurred in large numbers for 6–7 months (May–November) mainly during the wet season with a Williams mean density of 17.1 for the 12 months post-intervention period. On average, a lower density of mosquitoes was detected starting from November to February. This pattern of seasonal abundance was also shown at Ijesa (Figure 3) in spite of the decline in *Anopheles* density following PN 2.0 distribution, but could not be established at the PN 3.0 village because of the significant reduction in mosquito density immediately following net distribution and throughout the following 12 months (Figure 3).

Vector mortality, blood feeding and parity rates

The baseline data prior to net distribution showed similar rates of mosquito mortality (0.65–1.5%), blood feeding (47–52%) and parity (41–48%) in the three villages (Table 2).

The use of PN 3.0 at Irolu resulted in high mosquito mortality (Figure 4) with a Williams mean of 50.9% (CI:47.8–58.5) compared to Ijesa (PN 2.0) (mean mortality of 22.7% (CI: 19.8–25.4) and Ilara (UTC control village) (<1% mosquito mortality) (Figure 4). PN 3.0 resulted in a lower blood feeding rate with a mean of 7.3% (CI: 2.8–8.1) compared to Ijesa (PN 2.0) with a mean of

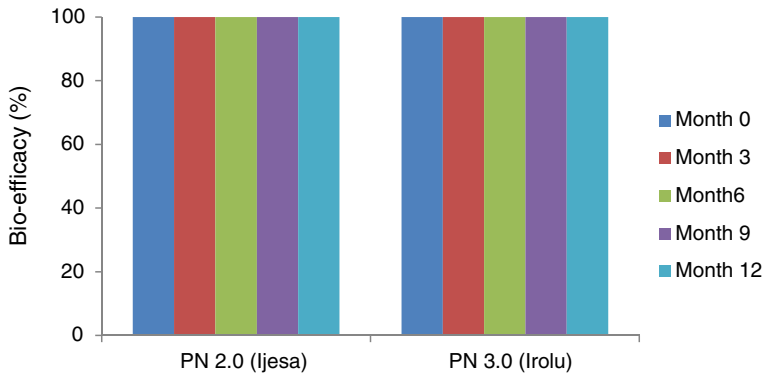

Figure 2 Bio-efficacy of PermaNet 2.0 and PermaNet 3.0 following 0, 3, 6, 9 and 12 months of field usage, as measured against a susceptible strain of *Anopheles gambiae s.s* (Kisumu) in WHO cone bioassays.

Table 3 Chemical content of PermaNet 3.0 and PermaNet 2.0 LLINs after 6 and 12 months of use in Irolu and Ijesa, respectively

Net type	Net section	Chemical	Units	Initial target dose before use		After 6 months in use	After 12 months in use
				Mean	Range	Mean ± SD	Mean ± SD
PN 3.0	Sides	Deltamethrin	g/kg	2.8	2.1 - 3.5	2.39 ± 0.28	2.67 ± 0.81
	Roof	Deltamethrin	g/kg	4.0	3.0 - 5.0	3.71 ± 0.26	3.63 ± 0.20
		PBO*	g/kg	25.0	18.8 - 31.3	12.0 ± 2.28	12.8 ± 4.34
PN 2.0	Sides	Deltamethrin	g/kg	1.8	1.4 - 2.3	1.60 ± 0.6	1.26 ± 0.42
	Roof					1.78 ± 0.49	1.42 ± 0.47

*Piperonyl butoxide.

22.2% (CI: 18.4–26.5) and Ilara (UTC) with a mean of 56.9% (CI: 51.2–62.8). The use of PN 3.0 at Irolu also reduced mosquito parity rates (Figure 4) with a mean of 13.6% (CI: 7.6–15.2) compared to a mean of 24.2% in the PN 2.0 village (CI: 19.6–26.8) and 46.1% (CI: 41.1–52.5) in the UTC village. The relatively low parity rate at Irolu is an indication of the high efficacy of PN 3.0 resulting in high mortality of *Anopheles* that had completed a gonotrophic cycle compared to Ijesa (PN 2.0) and the UTC village.

Source of mosquito blood meal and vector sporozoite rates

At baseline, 80-85% of mosquito blood meals from the three villages were from humans and the remainder were from cattle or other hosts (Table 2). This remained the same (81.1%) in the UTC village during the post intervention period. In contrast, following LLIN distribution, there was a significant reduction in the number of human blood meals in mosquitoes from the PN 3.0 village (P = 0.042) with a corresponding increase in cattle blood meals (mean 70.4%). There was also a reduction in human blood meals in mosquitoes from the PN 2.0 village when compared to baseline but this difference was not significant.

Results from the sporozoite ELISAs for the three villages are shown in Table 4. At baseline, *Plasmodium falciparum* sporozoite rates in the mosquito population were 1.8% at Ilara (UTC village), 2.1% at Irolu (PN 3.0 village) and 3.1% at Ijesa (PN 2.0 village). The use of PN 3.0 at Irolu resulted in a significant reduction in the sporozoite rate (declined to 0.9%) (P = 0.022). The sporozoite rate in the PN 2.0 and the UTC villages remained statistically similar post-intervention as at baseline (Table 4). The estimated monthly entomological inoculation rate (EIR) before bed net distribution was 28.5 at Ilara, 43.0 at Irolu and 65.6 at Ijesa. The use of LLINs at Ijesa (PN 2.0) and Irolu (PN 3.0) reduced the risk of malaria transmission by close to 75 and 97% respectively compared to the UTC village (Table 2).

Net use and performance

Data from the baseline survey showed that 52-58% of respondents from the three villages attested to the use of aerosols as the main practice for controlling mosquito bites. The use of LLINs was not a common practice in the three villages.

The post intervention follow up showed that three months after the commencement of the study, about 75% of the 150 households in the UTC village had removed the untreated nets from their beds. The reasons given by all respondents were that the untreated nets provided no protection against mosquito bites and none of them had the nets by the 12th month following distribution. In contrast, all of the 137 households with PN 3.0 and 147 with PN 2.0 still had the net mounted in their room 12 months after net distribution. However, when individuals were

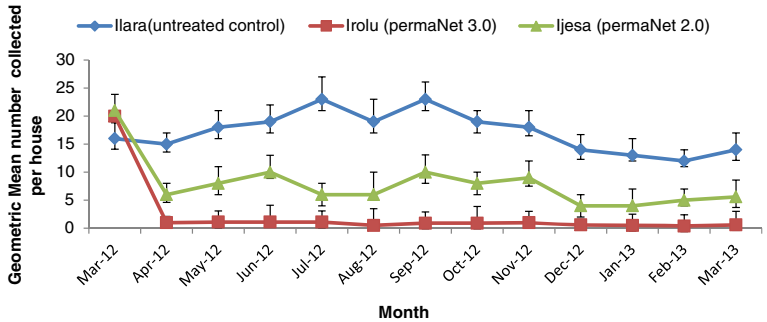

Figure 3 Geometric mean densities of *An. gambiae s.s.* collected per house at baseline (March 2012) and then monthly for 12-months post intervention at Ilara, Irolu and Ijesa.

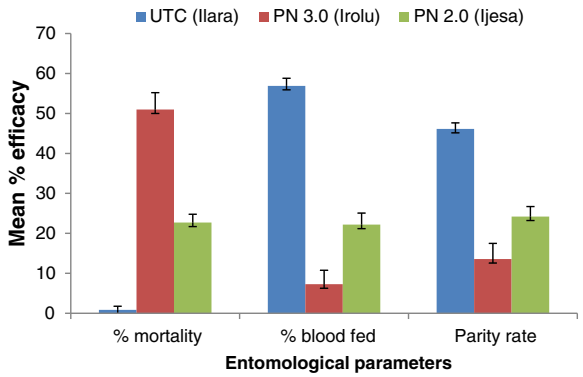

Figure 4 Geometric mean of percentage mortality, blood feeding and parity rate for *An. gambiae s.s.* collected indoor during 12-months post intervention at Ilara, Irolu and Ijesa.

asked whether they were still sleeping regularly under the LLINs, 98 and 84% of households in PN 3.0 and PN 2.0 respectively still used the nets with a significant difference in net usage by village (Fisher's exact test, p = 0.032).

Physical examination of nets after 12 months of field use showed that most of the PN 3.0 (98.5%) and PN 2.0 (93.8%) were in good condition, having no holes. Only two PN 3.0 had 3–5 holes (mean diameter = 2.5 cm) while 10 PN 2.0 had 3–8 holes (mean diameter = 2.8 cm).

Skin irritation was the main side effect reported by 19.7% and 16.3% of households using PN 3.0 and PN 2.0 respectively. A similar proportion of people (about 15%) from both LLIN villages also reported sneezing. Overall, a significantly higher proportion of people using PN 3.0 (92.7%) versus PN 2.0 village (74.1%) indicated that the intervention was beneficial (p = 0.036). The descriptive data from the focus group discussion indicated this was because PN 3.0 was perceived to reduce the number of mosquitoes, bed bugs and cockroaches during the study compared to nets previously distributed in the area.

Discussion

This study compared the efficacy of two LLINs at two individual villages with untreated nets at another village. An obvious limitation of this study is the lack of replication, as only one village per net-type was used, however, the similarity in baseline entomological indices, mosquito control practices and demographic characteristics of the villages in the study area in part explains the reason for employing this study design.

Anopheles gambiae s.s., the major malaria vector in the area, occurred in large numbers for 6–7 months, mainly during the wet season as earlier reported [14]. On average, fewer mosquitoes were found from November until February in the UTC control village. This seasonal abundance pattern would be expected to be similar for other villages in the area where transmission of *Plasmodium falciparum* continues to occur mainly in the wet season, although more control villages would be required to verify this. Seasonality in vector densities was clearly evident in the UTC village, partially evident in the PN 2.0 village but not evident in the PN 3.0 village, largely due to the consistently low vector densities post-intervention in the PN 3.0 village.

Pyrethroid and DDT resistance were found in the three villages during the baseline and post intervention surveys with a similar level of phenotypic resistance in both pre- and post-intervention periods. The *kdr* mutation was the sole resistance mechanism detected at Ilara, with *kdr* + metabolic p450-based resistance mechanisms detected at Irolu and Ijesa. The presence of both *kdr* + metabolic p450-based resistance mechanisms in the mosquito population from this study area alludes to an earlier notion of the presence of multiple pyrethroid resistance mechanisms in the malaria vector *An. gambiae s.s* in Nigeria [8]. However, in spite of the presence of these resistance mechanisms, the use of PN 3.0 at Irolu significantly reduced not only the mosquito density per house, but also the blood feeding and parity rates compared to the PN 2.0 and UTC control villages. This decrease was consistent during the twelve months following PN 3.0 distribution. Aside from the 'mass killing' effect of *Anopheles* caused by PN 3.0, the low parity rate in the PN 3.0 compared to the PN 2.0 village is an indication of the reduction in the parous population and the resultant

Table 4 Blood feeding preference and sporozoite rates for *An. gambiae s.s.*collected from the three study villages, at baseline and monthly mean during the 12-months post-intervention period

Mosquito population	Treatment	Time of testing	Blood positivity rate (%)					Sporozoite positivity rate		
			No. tested	Human only	Cattle only	Other	p value	No. tested	Positive (%)	p value
Ilara	UTC	Baseline	295	82.7	13.4	3.4	0.558	568	1.76	0.709
		Post-intervention	1500	81.1	13.0	10.5		4345	2.09	
Irolu	PN 3.0	Baseline	350	80.9	17.1	2.0	0.042	702	2.14	0.022
		Post-intervention	27	29.6	70.4	0		573	0.87	
Ijesa	PN 2.0	Baseline	360	85.0	13.9	1.1	0.091	745	3.08	0.832
		Post-intervention	548	60.0	36.0	4.0		2415	2.81	

p value < 0.05 indicates a significant difference between baseline and post-intervention monthly mean.

reduction in risk of malaria transmission, as reflected in the appreciable reduction in the post intervention entomological inoculation rates. This indicates that PN 3.0 may have resulted in a reduced mosquito life span and survival rate. The results also showed a shift in host preference after PN 3.0 distribution with a significant number of mosquitoes feeding on cattle in contrast to humans during the baseline period. This is a surprising finding, given the strong human feeding preference of *An. gambiae s.s.*, and could be a consequence of the lower sample size as there were far fewer mosquitoes to test during the post-intervention period. It could be that the use of PN 3.0 induced changes in the endophilic tendencies in *An. gambiae* populations, such that a higher level of excito-repellency occurred that may induce outdoor biting behaviour. This effect coupled with the high mosquito mortality due to the use of PN 3.0, may result in outdoor locations becoming an important venue for host-seeking *An. gambiae s.s* during the use of PN 3.0.

Analysis of chemical content of nets of LLINs showed a marked loss of PBO content from PermaNet® 3.0 at 6 months post-distribution. However, there was no change in PBO content evident between 6 and 12 months post-distribution. The rapid initial loss may be due to an accumulation of PBO on the surface of new nets, which is rapidly depleted through washing, handling and evaporation at the onset of usage. It may also indicate stabilization of the PBO migration rate throughout the polymer during early usage leading to minimal loss over the subsequent 6 months period. Related studies with permethrin-PBO combination LLIN (Olyset® Plus) in Benin and Cameroon [29] showed that after just three washes there was a loss in killing effect against resistant strains of *An. gambiae* from Benin (92% before and 56% after washing) and Cameroon (98% before and 69% after washing), also indicating rapid loss of PBO in permethrin-PBO combination [30]. Regardless of the initial depletion of PBO from PermaNet® 3.0, this combination LLIN exhibited enhanced efficacy when compared to the deltamethrin-only PermaNet® 2.0 over the 12 month study duration. To further evaluate the migration dynamics and loss rates of PBO and pyrethroids from combination LLINs during field usage, extended field studies would need to be conducted.

Observations from the questionnaire surveys yielded insight into human behaviour in the study area. Human activities outside the home into the late evening hours are not common in the area. Therefore, with mosquitoes either reluctant to enter PN 3.0 households, or more likely to leave, and the absence of humans outdoors when the biting of *An. gambiae s.s* is at its peak, a considerable amount of *An. gambiae* s.s. blood meals were taken from alternative hosts such as cattle, as indicated in the post intervention blood feeding data from the PN 3.0 village. This is clearly a contributing factor to the reduction in malaria transmitting mosquitoes observed from the PN 3.0 village in the post-intervention period. Additionally, marginally fewer PN 3.0 had holes than PN 2.0, despite higher reported usage rates of PN 3.0. The greater proportion of householders reporting benefits of PN 3.0 compared to PN 2.0 is also consistent with studies conducted previously in Nigeria [12].

Overall, the results showed a significant impact of PermaNet® 3.0 on the mosquito population relative to that observed at the PermaNet® 2.0 village. This study is limited by the lack of replicates of each treatment arm, and the single point mosquito collection made at baseline. However, the results are consistent with similar work carried out in an area with *kdr* + metabolic based resistance mechanisms in malaria vector populations at other sites in Nigeria [12] and elsewhere in Africa [29-32] and supports increasing evidence indicating a reduction in efficacy of pyrethroid only LLINs against pyrethroid resistant malaria vectors [33,34].

Conclusion

The presence of pyrethroid resistant vector populations permitted the assessment of the impact of PN 3.0 on mass community protection against pyrethroid resistant malaria vectors. The use of PN 3.0 significantly reduced mosquito densities per house, which was coupled with an observation of changes in the bloodmeal origin, sporozoite rate and parity rate in the *An. gambiae* population resulting in a significant reduction in transmission indices. The trial confirmed that in the presence of *kdr* plus P450-based metabolic resistance, there was an increased efficacy of PN 3.0 compared to the pyrethroid-only LLIN (PN 2.0). The data presented in this study along with previous work in Nigeria suggests that the use of PN 3.0 will contribute towards a reduction in malaria transmission over time when compared to existing pyrethroid-only LLINs in areas with P450-based pyrethroid metabolic resistance.

Ethical approval

The study was approved by the Institutional Ethics Review Committee of the Nigerian Institute of Medical Research. All households in the three villages indicated their willingness to participate in the study and gave written consent.

Competing interests

The authors declared that they have no competing interests. Although the study was funded by Vestergaard Frandsen, the findings described in this manuscript are those of the authors and do not necessarily reflect views of Vestergaard Frandsen.

Authors' contributions

TSA designed the study protocols and drafted the paper. AOA, IOO and AOO coordinated and supervised the field collections, JBO coordinated laboratory work and analysed the data, CNA conceived the study and participated in the design. All authors read and approved the final version of the manuscript.

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Field efficacy and acceptability of PermaNet® 3.0 and OlysetNet® in Kinshasa, Democratic Republic of the Congo

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ABSTRACT

Background & objectives: Insecticide resistance in mosquitoes at Kinshasa may jeopardize the efficacy and usage of long-lasting insecticidal nets (LLINs). Entomological impact, user acceptance and bioefficacy of a combination LLIN (PermaNet® 3.0) and a standard LLIN (OlysetNet®) were evaluated at two sites in Kinshasa characterized by high densities of either *Anopheles gambiae s.s.* (Kindele) or *Culex* spp (Kimbangu).

Methods: Insecticide susceptibility (permethrin, deltamethrin, bendiocarb, propoxur and DDT) was determined via tube tests and bottle assays. Entomological impact of unwashed and washed LLINs and untreated nets was assessed via Latin square, based on rotation of nets and their users through selected houses at each site. User acceptability was evaluated through interviews using a questionnaire and net bioefficacy was measured via cone bioassays with field-derived *An. gambiae s.s.*

Results: The *An. gambiae s.s.* population from Kindele was resistant to DDT and permethrin with mortality rate of 27.3 and 75.8%, respectively, and *kdr* mutations (L1014F) plus suspected metabolic resistance. The *Culex* spp population was resistant to all five insecticides tested. No differences in entomological indices were observed for the five net treatments, but bioefficacy against *An. gambiae* was significantly higher for unwashed and washed PermaNet 3.0 (100 and 71% mortality) than for OlysetNet (56 and 36%). Householders reported a good sleep most often when using unwashed and washed PermaNet (94 and 88%) and least often with unwashed OlysetNet (46%).

Interpretation & conclusion: High bioefficacy via cone bioassays against an *An. gambiae s.s.* population with *kdr* and suspected metabolic resistance was observed with PermaNet 3.0 . Lower biting rates and a higher chance of a good night of sleep were reported when using PermaNet 3.0 compared to OlysetNet.

Key words Democratic Republic of the Congo; insecticide resistance; OlysetNet; PermaNet 3.0

INTRODUCTION

Although long-lasting insecticidal nets (LLINs) adequately circumvent the need for retreatment, insecticide resistance may be a major challenge to sustain their impact in certain areas. As the problem of insecticide resistance grows[1] and examples of reduced efficacy of control interventions are presented[2–4], there is increasing concern over preserving the effectiveness of insecticide-based vector control tools. New generation combination nets that utilise alternative or multiple classes of insecticides or other chemical synergists have or are being developed to address this problem.

One combination LLIN currently recommended by the World Health Organization (WHO) is PermaNet® 3.0. This net combines a pyrethroid (deltamethrin) with a synergist (piperonyl butoxide) in the roof structure to enhance bioefficacy against pyrethroid-resistant malaria vectors. Experimental hut trials in Vietnam, Burkina Faso, Benin, Cote d'Ivoire and Nigeria have indicated increased bioefficacy against pyrethroid-resistant malaria vectors relative to mono-treated deltamethrin or permethrin nets[5–8]. As with other insecticidal interventions, evaluations of PermaNet 3.0 to date have indicated that bioefficacy under field conditions will depend not only on the level of resistance and its underlying mechanisms but also on the behaviour of the specific vector population.

There is also evidence for increased personal protection of PermaNet 3.0 against nuisance mosquitoes, *Culex* spp. In experimental hut studies in Togo and Vietnam, a significant reduction in blood feeding was observed relative to a standard LLIN[9]. However, studies in Tanzania failed to detect an impact on *Culex* populations[10], although this could be a result of low *Culex* densities.

In the Democratic Republic of the Congo (DRC), malaria parasite transmission is maintained mainly by *Anopheles gambiae s.s.* and *An. funestus*[11–13] and is sea-

sonal with peaks during the rainy periods which differ depending upon the locations. In urban areas, the main nuisance mosquito problem is due to *Culex quinquefasciatus* while in rural areas the low mosquito nuisance observed is almost entirely due to two main *Anopheles* species. Published reports on insecticide susceptibility of mosquito species in DRC are scarce. Mulumba *et al*[14–15] confirmed the susceptibility of *An. gambiae s.l.* in Kinshasa to insecticides from all the four classes of insecticides recommended by the WHO for adult mosquito control. Although DDT resistance is reported, Webster *et al*[16] argued that both *An. gambiae* and *An. funestus* were thought to be sensitive to deltamethrin. More recently, evaluations of *An. gambiae s.s.* from four sites in DRC detected resistance to DDT at all sites and to pyrethroids (deltamethrin, permethrin and lambda-cyhalothrin) at three sites with resistance to an organophosphate (malathion) at one site[17]. The L1014F *kdr* allele, often associated with resistance to DDT and pyrethroids, was detected at all the sites albeit with various frequencies. This is of major concern for currently available control approaches which mainly use pyrethroids on nets or DDT, pyrethroids, carbamates or organophosphates sprayed onto the interior walls of houses.

The efficacy of LLINs against local mosquito populations is most commonly assessed in experimental hut trials as recommended by the WHO Pesticide Evaluation Scheme[18]. These follow a standard protocol using specific replicate housing structures in a latin square design, to allow for comparison of a candidate LLIN with a positive and negative control to determine the effect on deterrence, house entry, mortality and blood feeding of target vectors. In localities where such a testing facility does not exist, LLIN efficacy needs to be tested via an alternative protocol. This study was designed to investigate if an adapted latin square design could be applied in normal village households to evaluate comparative LLIN efficacy and acceptability. PermaNet 3.0, designed for increased bioefficacy against pyrethroid-resistant anopheline vectors, was evaluated against a standard LLIN (OlysetNet®) and an untreated net.

MATERIAL & METHODS

Study sites

The assessment was conducted at two sites in Kinshasa. Kindele in the peri-urban area (approx. at 20 km southeast of Kinshasa City Centre), with high densities of *An. gambiae s.s.* and Kimbangu (three in urban Kinshasa) with *Culex* spp nuisance. The study was conducted from January to May 2010 to coincide with the peak in the rainy season.

Study design

A baseline survey was carried out at each site to determine householder willingness to be included and to measure the relative density of mosquitoes in the selected households. Collections were done via overnight CDC light-traps. Based on the results, 20 households were selected randomly at each location with similar housing construction and approximately similar mosquito densities.

Treatment arms

The treated nets tested were: (a) PermaNet® 3.0 unwashed; (b) PermaNet® 3.0 washed 20 times; (c) Olyset Net® unwashed; (d) OlysetNet® washed 20 times; and (e) untreated polyester net. Each net type was assigned to four households per week at each of the sites for a total of 20 households per site. Sufficient nets of the specific type were provided to cover all persons in the household. At the end of each week, householders were asked to complete a simple questionnaire and existing nets were replaced with a net of a different treatment. Net types were coded such that householders and surveyors were not aware of treatment was being evaluated at each household.

Long-lasting insecticidal nets

PermaNet® 3.0 LLIN (Vestergaard Frandsen SA, Switzerland) and OlysetNet®LLIN (Sumitomo Chemical, Japan), have been approved by WHOPES[19]. The untreated net was a multifilament polyester (75 denier) fabric. The manufacturer-specified size of all nets was 160 cm wide × 180 cm long × 150 cm high. A standard procedure was used for washing nets (b) and (d) as per WHOPES Phase-II testing guidelines[18]. Nets were washed in clean water in aluminium bowls containing 10 L of well water with a small quantity of local soap. Nets were agitated for 3 min, left to soak for 4 min and re-agitated for 3 min. Agitation was conducted by hand at approx. 20 rotations per min. Nets were then dried vertically in the shade. For Olyset only, nets were then heated to 60°C for four hours in a regulated heater based on local regeneration time observations (F. Watsenga, Personal Communication). The subsequent wash for all the nets was then performed the following day.

Insecticide resistance testing

The insecticide susceptibility status of *An. gambiae s.s.* mosquitoes from Kindele and *Culex* spp from Kimbangu was determined using WHO discriminating doses and standard insecticide susceptibility kits[19]. CDC

bottle assays without and with synergists were also used for assessing *An. gambiae s.s.* susceptibility to selected insecticides as per the standard procedures[20]. Mosquitoes for assays were derived from larvae collected at each site which were reared to adults under standard conditions at the insectary of the University of Kinshasa. Unfed adult 2–3 day-old females were used in both WHO susceptibility tests and CDC bottle assays.

For the WHO susceptibility tests, DDT (4%), permethrin (0.75%), deltamethrin (0.05%), bendiocarb (0.1%) and propoxur (0.1%) were tested, for *Anopheles*[20] and *Culex*[21]. For CDC bottle assays, permethrin (21.5 µg/bottle) and deltamethrin (12.5 µg/bottle) were tested for *An. gambiae* only using standard procedures (CDC 2009). Assays were also conducted for permethrin following pre-exposure to piperonyl butoxide (PBO), s,s,s-tributyl phosphorotrithioate (DEF) or ethacrynic acid (ETAA) using standard dosages (CDC 2009). Negative controls without insecticide were assessed concurrently.

Specimens used in WHO susceptibility tests were assayed to determine species via polymerase chain reaction (PCR)[22], M and S molecular forms via restriction fragment length polymorphism PCR[23] and to detect *kdr* mutations via hot ligation oligonucleotide assay[24] as per standard procedures.

Entomological indices

CDC light-trapping[25] was conducted in selected households in both the study areas once per week from 1800 to 0600 hrs the following day. Standard procedures were followed with traps placed approximately 1.5 m from the ground, next to the mosquito net at the foot end of the bed. Specimens from each household were placed in labelled collection cups and transferred to the laboratory for sorting, species identification using keys[26], and enumeration.

User questionnaire

At the end of each week, the head of the household was issued a questionnaire to investigate for the net issued during the previous week: whether it was used, any observed health side effects, perceived benefits, and comparison to previously issued nets.

Net bioavailability

Standard WHO cone bioassays[18] were performed at the end of the field assessment on four nets from each of PermaNet 3.0 unwashed and washed, and OlysetNet unwashed and washed, using adults reared from *An. gambiae* larvae collected from Kindele site. For each net, subsamples (30 × 30 cm) were taken from the roof, lower side and upper side for PermaNet 3.0 or the roof and side for OlysetNet. Four cones were placed on each subsample and five non-blood fed, 2–3 day-old females were introduced and exposed for 3 min before being held for 60 min and observed for knock down then held for 24 h and observed for mortality. Mean knock down (KD_{60}) and mortality (MT_{24}) were calculated for each treatment group. Subsamples of untreated nets were assessed concurrently as negative controls.

Statistical analysis

For WHO susceptibility tests, CDC bottle assays and WHO cone bioassays, Abbott's adjustment was applied when the control mortality was >5% with assay results discarded if control mortality was >20%[19]. WHO susceptibility test and CDC bottle assay mortality data were used to define the resistance status of *Anopheles* and *Culex* for each insecticide using the standard criteria[20]. *kdr* allelic frequency was determined using genotyping calculation expressed by the formula: $F_{kdr} = 2N_{RR} + N_{RS} / 2(N_{SS} + N_{RS} + N_{RR})$.

Statistical software used for analyses of entomological impact, user acceptance and net bioefficacy data were Excel, SPSS and StatsDirect, with chi-square test and Fisher's Exact test used for assessing relationships resulting from contingency table. In addition, the Standard Normal Deviate (SND) test was used to compare the proportions between groups.

Ethical clearance and consent

Approval was obtained from the Ethics Review Committee of the University of Kinshasa. Informed and free consent was obtained from all the study participants. All the participants were offered chemoprophylaxis during and for one month after the study.

RESULTS

Insecticide resistance status

All the *Anopheles* spp specimens from Kindele and Kimbangu were identified as *An. gambiae s.s.* of M molecular form (n = 53). *Anopheles gambiae* from Kindele were found to be resistant to DDT and permethrin via WHO susceptibility tests, with low knock down rates and mortality < 80% (MT_{24} of 27.3 and 75.8%, respectively) (Fig. 1). Full susceptibility to deltamethrin, bendiocarb and propoxur was identified due to rapid knock down (KT_{50} of 17.2, 17.4, and 12.3 min and KT_{95} of 31.6, 28.9, and 18.4 min, respectively) and high mortality (MT_{24} of 100% for all). CDC bottle assays also indicated some resistance to permethrin but not to deltamethrin, with a maxi-

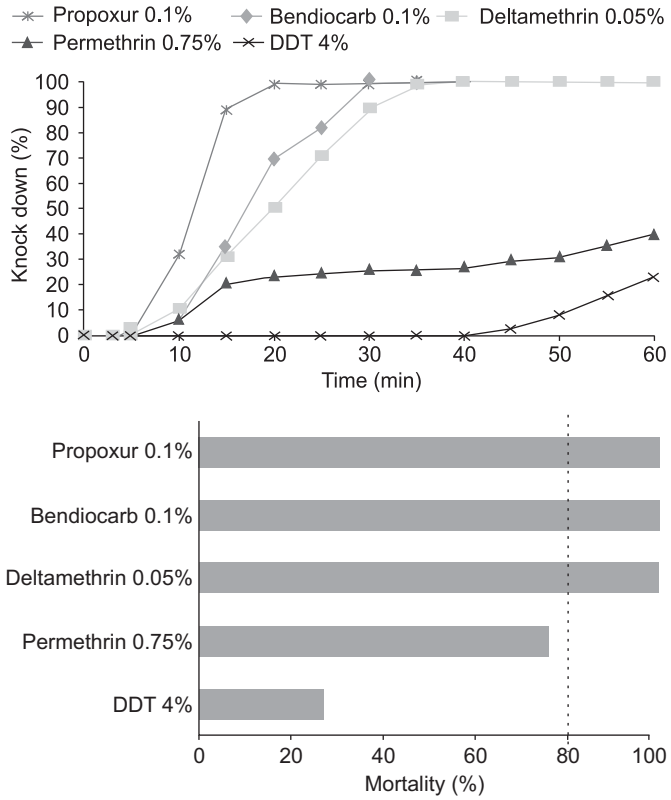

Fig. 1: Overview of the resistance status of *An. gambiae s.s.* from Kindele site. Lines represent mean percent knock down over 60 min of exposure to insecticide-impregnated papers in WHO susceptibility tests. Bars represent mean percent mortality after 24 h post-exposure for the same test mosquitoes. Dotted line (80%) is the WHO resistance threshold[19].

mum mortality of 93.9% reached after 75 min exposure to permethrin, whereas 100% mortality was observed after 30 min exposure to deltamethrin (Fig. 2). Pre-exposure to DEF and PBO did not significantly increase mortality due to permethrin (97.9 and 95.9% mortality after 120 min exposure, respectively). However, pre-exposure to ETAA yielded 100% mortality by 60 min post-exposure to permethrin, indicating the possible presence of elevated glutathione transferase activity in the *An. gambiae* population. *kdr* alleles were also identified in some specimens from both Kindele and Kimbangu, representing the first reports of the *kdr* mutation in *An. gambiae s.s.* from DRC. Very few specimens were available for processing (n = 7), with one homozygous and heterozygous each detected from Kindele and one homozygous from Kimbangu for overall allelic frequencies of 0.38 and 0.33, respectively.

Culex spp from Kimbangu were identified as resistant to all the five insecticides via WHO susceptibility tests, with low knock down rates over the duration of exposure and delayed mortality of <80%. Mortality was similarly recorded low against bendiocarb, DDT and

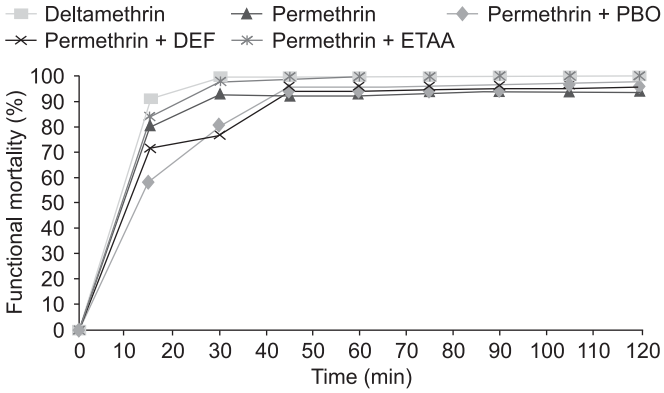

Fig. 2: Additional information on resistance status of *An. gambiae s.s.* from Kindele site. Lines represent mean percent functional mortality (as indicated by mosquitoes unable to rest) over 120 min exposure to deltamethrin- or permethrin-coated bottles in CDC bottle assays. 60 min pre-exposure to the synergists PBO, DEF or ETAA was also conducted prior to permethrin exposure.

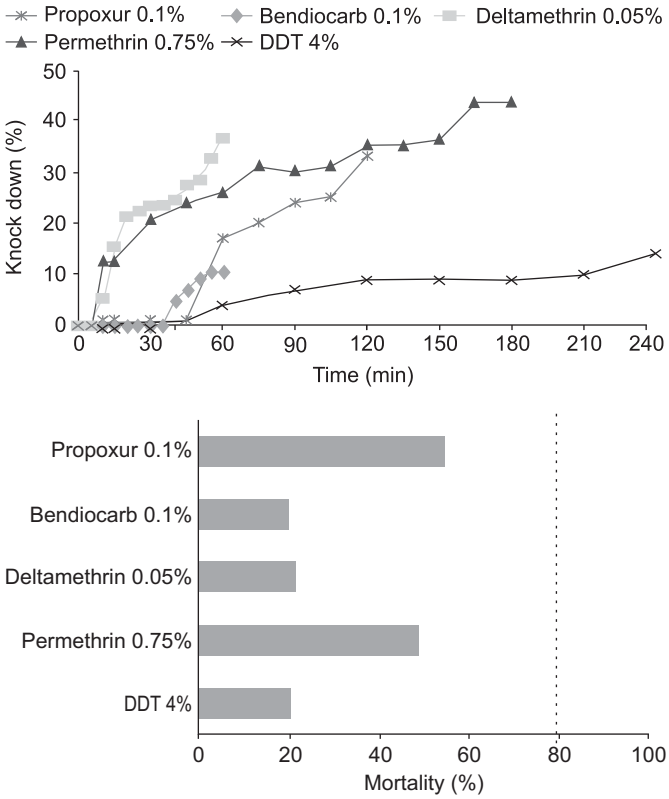

Fig. 3: Overview of the resistance status of *Culex* spp from Kimbangu site. Lines represent mean percent knock down over specified times of exposure to insecticide-impregnated papers in WHO susceptibility tests. Bars represent mean % mortality at 24 h post-exposure for the same test mosquitoes. Dotted line (80%) is the WHO resistance threshold.

deltamethrin (MT_{24} of 19.3, 20 and 21.2%, respectively), and was higher for permethrin (48.5%) and propoxur (54%) (Fig. 3).

Entomological impact

Entomological data were unavailable in cases of householders absence (n = 2) and were removed if householders had sprayed with insecticidal repellent within the previous week (n = 3). Remaining data were divided by site, and due to higher densities, detailed analyses were carried out for *An. gambiae* at Kindele and *Culex* spp at Kimbangu in order to determine the influence of household, week and net type on entomological parameters.

The number of *An. gambiae* and *Culex* spp differed significantly by site ($p = 0.0003$ and 0.0009, respectively). The total number of anophelines collected at Kindele was 681 and at Kimbangu was 125. A total of 99% of the collected anophelines were females, and of these, 5.5% were identified as blood-fed. The total number of culicines collected at Kindele was 188 and at Kimbangu was 19,501. Overall, 67.7% of the collected culicines were females, and of these, 9.5% were identified as blood-fed.

Anophelines at Kindele

The number of *An. gambiae* at Kindele did not vary between baseline and subsequent weeks ($p = 0.7442$) but did vary between households ($p < 0.0001$), ranging from 0 to 40 anophelines captured per house for a single sampling period after intervention (mean = 5.6, median 3). The net type was not found to influence the number of Anophelines for different weeks or households ($p = 0.3073$ and 0.0634, respectively). Similar findings were observed for females and the proportion of blood-feds. Therefore, the type of net did not have any significant influence on these parameters at Kindele ($p > 0.05$ for all).

Culicines at Kimbangu

Similar findings on relationships that were observed in Kindele for anophelines were also observed for culicines in Kimbangu except that the number of *Culex* spp at Kimbangu did vary significantly between weeks ($p = 0.0171$) with an increase from baseline (n = 2602) and a peak at Week 1 and 2 (n = 4739 and 4701, respectively) followed by a decrease in subsequent weeks ($n = \leq 2548$). There was significant variation in the number of culicines between households ($p = 0.0017$), with the number captured per household for a single sampling period after intervention ranging from 0 to 726 (mean = 174.2, median = 137). The type of net did not influence the number of culicines for different weeks or among households ($p = 0.4465$ and 0.3095, respectively). Similar relationships held for females and the proportion of blood-feds, such that the net type did not have a significant influence on these parameters at Kimbangu ($p > 0.05$ for all).

Net bioefficacy

Overall bioefficacy as measured via cone tests using wild-caught *An. gambiae s.s.* was significantly higher for PermaNet 3.0 than for OlysetNet (Table 1). Unwashed PermaNet 3.0 induced a significantly higher knock down and mortality than washed PermaNet 3.0 ($Z = 4.197$, $p < 0.001$ and $Z = 4.547$, $p < 0.001$, respectively). Similarly, unwashed OlysetNet had a higher bioefficacy than the same net washed 20 times ($Z = 2.27$, $p = 0.012$ and $Z = 2.153$, $p = 0.016$, respectively). However, even PermaNet 3.0 that had been washed 20 times had a significantly higher overall bioefficacy than unwashed OlysetNet (17.4% higher knock down and 15.2% higher mortality). Furthermore, approximately double the knock down and mortality was observed for washed PermaNet 3.0 relative to washed OlysetNet. While all the sections of unwashed PermaNet 3.0 induced 100% knock down and mortality for washed PermaNet 3.0, the roof had the highest bioefficacy followed by the lower sides and then the upper sides. There was no significant difference in bioefficacy between the roof and sides of unwashed and washed OlysetNet ($p < 0.05$ for all).

Table 1. Bioefficacy of PermaNet® 3.0 and OlysetNet® after field usage

Section	Unwashed (%)		Washed 20 × (%)	
	knock down (60 min)	mortality (24 h)	knock down (60 min)	mortality (24 h)
PermaNet® 3.0				
Roof	100 ± 0	100 ± 0	91.3 ± 6.3	88.6 ± 2.4
Side upper	100 ± 0	100 ± 0	63.3 ± 6.9	55.2 ± 3.5
Side lower	100 ± 0	100 ± 0	68.8 ± 3.8	68.8 ± 3.8
Total	100 ± 0	100 ± 0	74.4 ± 13.7	70.9 ± 14.6
OlysetNet®				
Roof	60.8 ± 4.4	55.7 ± 4.3	39.8 ± 3.4	38.5 ± 6.6
Side	53.2 ± 3.9	55.7 ± 3.1	23.8 ± 16	34 ± 2.7
Total	57 ± 5.6	55.7 ± 3.5	31.8 ± 13.7	36.2 ± 5.2

Mean (± standard deviation) knock down at 60 min and mortality at 24 h of *An. gambiae s.s.* from Kindele site after exposure in 3 min WHO cone bioassays on roof and side sections of unwashed and 20-times washed PermaNet® 3.0 and OlysetNet® LLINs.

User acceptance

Reported net usage did not differ significantly between the two sites ($p = 0.157$), with 84.3% of householders interviewed indicating that they slept under a net every night during the study (Table 2). However, householders were more likely to report mosquito bites in Kimbangu (19.8%) than in Kindele (5%) ($p = 0.004$). There was a significant association between net usage and lack of reported biting at each site ($p = 0.001$ for Kindele and $p = 0.004$ for Kimbangu), with nightly net

Table 2. Summary of entomological impact and user acceptance data for 20 houses each at Kindele and Kimbangu

Site/Mosquito species	Kindele *An. gambiae s.s.*	Kimbangu *Culex* spp
Entomological impact		
Total number collected	681	19,501
Percent females	99.8	67.4
Percent females blood-fed	2.5	9.6
Mean number per household	5.9	171.1
PermaNet 3.0 unwashed	4.6	132.6
PermaNet 3.0 washed 20×	4.7	216.9
Untreated net	8.6	202.1
OlysetNet unwashed	6.6	130.1
OlysetNet washed 20×	3.4	203.9
User acceptance		
Percent reporting net usage all nights	25	45
Percent reporting side effects	43.8	31.6

usage associated with low biting (reported by 15.1% of householders) and non-nightly usage associated with higher biting (reported by 60% of householders).

For the different net types, there was a significant difference in reported usage for both the sites ($p = 0.002$ at Kindele and $p < 0.001$ at Kimbangu). While >80% of the householders reported sleeping under PermaNet, unwashed OlysetNet or untreated nets every night, nightly usage was less common for washed OlysetNet at both Kindele (43.8%) and Kimbangu (45%). Furthermore, at Kimbangu biting was more commonly reported by the householders issued OlysetNet either washed (42.1%) or unwashed (27.8%) than for those issued an untreated net (15%) or PermaNet unwashed (5%) or washed (10.5%) ($p = 0.029$).

In terms of reported health side effects, a running nose and unpleasant odour were more commonly reported in Kimbangu (6.3 and 16.7% of householders, respectively) compared to Kindele (no reports of either). However, there was no noted difference between the sites in reports of other side effects such as sneezing, headache, nausea, burning sensation, and watery eyes (all $p > 0.05$). Overall, there was no significant difference in reported health-related concerns between net types (all $p > 0.05$).

The frequency of householders reporting a good sleep differed depending on the net type ($p < 0.001$). This was the highest for PermaNet unwashed and washed (94.1 and 87.5%), followed by unwashed OlysetNet (85.3%), untreated net (80.6%) and was the lowest for washed OlysetNet (45.5%). The nets remained in excellent condition throughout the study period, and were perceived as being new or clean by the householders. Although no significant preference was evident between nets, OlysetNet was reported as being too small or narrow by 27.8% (unwashed) or 66.7% (washed) of the householders.

When two net types were measured following washing, there was an overall shrinkage in the size of OlysetNet (97.5 ± 8.3% of the specified dimensions) and an overall increase in the size of PermaNet 3.0 (110.3 ± 5.3% of the specified dimensions). For separate dimensions, OlysetNet increased in height (108.4 ± 3) but decreased in length (92.4 ± 1.3) and width (91.7 ± 3.5%), whereas PermaNet 3.0 increased in height (112.1 ± 3.8), length (104.7 ± 2.7) and width (114.1 ± 3.6%).

DISCUSSION

This represents the first known study to compare the field efficacy of LLINs in existing housing structures in DRC, and also the first to use local field-derived mosquitoes to assess LLIN bioefficacy via cone tests in DRC. Although there was no difference detected in the impact on field entomological indices by net type, cone bioassays clearly indicated a significantly higher bioefficacy of PermaNet 3.0 compared to OlysetNet even after PermaNet 3.0 had been subjected to 20 washes. User surveys also indicated better performance of PermaNet 3.0, and unwashed OlysetNet were particularly associated with high reported biting rates and low reported frequency of a good night of sleep.

It is highly possible that the failure to detect differences in entomological impact despite significant difference in net bioefficacy may have been due to the study design. Many of the *p*-values observed during data analyses were close to 0.05, indicating that a larger or more robust study structure could potentially have yielded different conclusions. In contrast to the usual approach for such bioefficacy evaluations of LLINs, this study used human populations and local housing structures that were already in existence at the study sites. This would have introduced numerous sources of variation, such as: differences in the number of people under nets and thus acting as either attractants or blood meals for vectors; differences in housing construction such as the quality of material (e.g. metal or thatched roves) and number and size of windows/doors which could influence house attractancy and entry opportunities for vectors; and other human factors which could have influenced vector behaviour (e.g. time of entry and exit of humans from nets, cooking practices, etc). For these reasons, the WHO recommends using standardised experimental huts with a single sleeper per hut following set patterns of LLIN usage and rotation between houses to account for any differences in individual attractancy[18]. This design should

limit the differences between individual households and persons over time whilst revealing differences in mosquito parameters due to each treatment being tested. However, the establishment of such huts was not feasible in this case (nor was larger and longer field study), due to personnel and time limitations.

Differential susceptibility of the local *An. gambiae s.s.* population to deltamethrin versus permethrin would have contributed somewhat to the vast difference in bioefficacy of PermaNet 3.0 versus OlysetNet. WHO tube tests revealed full susceptibility to deltamethrin but confirmed resistance to permethrin (75.8% mortality) while CDC bottle assays also indicated susceptibility to deltamethrin but low level pyrethroid resistance (93.9% mortality) with potential glutathione-s-transferase (GST) activity. However, these levels of resistance translated into significant differences in susceptibility of the population to deltamethrin- versus permethrin-treated LLINs in cone bioassays. This emphasises the fact that insecticide susceptibility data from WHO tube tests cannot be directly interpreted to predict the susceptibility of a population to vector control formulations. Hence, the importance of bioefficacy tests such as cone bioassays using field-derived vectors. However, such bioefficacy evaluations also have limitations in predicting the impact of an intervention on a given vector population as those do not take into account vector behaviour and other extrinsic parameters. In a study in Mali[4], while no difference was detected in susceptibility of two *An. gambiae s.l.* populations to an alpha-cypermethrin LLIN, reduced efficacy was identified at one of the two sites during experimental hut studies. The somewhat tenuous link between insecticide susceptibility status of a population and the anticipated field impact of a particular vector control tool underscores the importance of field-based assessments of vector control candidates under local conditions where feasible.

The high level of resistance detected in *Culex* spp to all the five insecticides tested was not unexpected. Resistance to multiple insecticides has been detected previously in *Culex* spp from Kinshasa[16]. Although LLINs are not designed to target *Culex* or other nuisance mosquito populations, correct usage of intact nets with sufficiently small hole size provides protection from *Culex* bites even where insecticide resistance may be high. The importance of assessing the impact of nets on *Culex* populations is related to the perceived benefit of nets by users, rather than actual health benefits in areas where *Culex* are not the vector of any significant diseases. That is, if people perceive that nets are protecting them from mosquito bites (or even malaria), they may be more inclined to use the nets frequently and correctly[27–29], whereas if there is no perceived benefit they may be discouraged from using nets. However, such perception is difficult to document and warrants further investigation under different settings.

Other published semi-field studies for PermaNet 3.0 have compared this net to mono-treated LLINs in experimental hut structures in areas with pyrethroid-resistant malaria vectors. PermaNet 3.0 was shown to have increased bioefficacy relative to deltamethrin only, PermaNet 2.0 in areas with resistant malaria vectors in Kou Valley, Burkina Faso[5] and Akron, Benin[6], and against permethrin only OlysetNet in New Bussa, Nigeria[8]. In other areas, such as in Pitoa, Cameroon[5] and Yaokoffikro, Cote d'Ivoire[7] there was variable difference in bioefficacy compared to a mono-treated LLIN depending on net wash status. This is a clear indication that the relative increase in bioefficacy of this combination net will vary depending on the level and mechanism(s) of insecticide resistance present in the local mosquito population. This emphasises the importance of conducting comparative trials on such new tools designed for increased bioefficacy against pyrethroid-resistant malaria vectors, and defining robust alternative protocols for application in areas, where establishment of experimental huts is not feasible. Ideally, such studies should include an assessment of the age-structure of populations though this would need to be easily implementable in disease-endemic settings.

There has been some discussion in the literature on whether it is the higher dose of deltamethrin or the presence of PBO that increases the bioefficacy of the roof of PermaNet 3.0. The synergistic impact of piperonyl butoxide has been well-documented for various insect species, for which it has been shown to enhance the penetration of insecticide into the insects[30] and inhibit the metabolic enzymes used to sequester or break the insecticide[31]. Bingham *et al*[32] clearly demonstrated the synergistic impact of PBO when coupled with deltamethrin using net samples against a highly pyrethroid-resistant *Ae. aegypti* population from Vietnam. Both low and high dose of deltamethrin had little impact on the population (1 and 5% mortality respectively), whereas there was an increase to 98% mortality when PBO was incorporated into the sample along with a low dose of deltamethrin. However, the issue of whether increased bioefficacy is due to the concentration of deltamethrin or the presence of PBO on the surface of the net roof is less important than how the net is performing as a whole. Modelling of data from independent experimental hut studies with PermaNet 3.0 indicated consistently higher protection conferred versus a deltamethrin-only net when both personal and community protection were considered[33].

For the user acceptance evaluation, although there may have been some self-report bias this would have been minimised since householders were not aware of the particular type of LLIN they had been issued plus over the duration of the study they gave feedback on each net type. Unsurprisingly, nightly net usage was associated with fewer reports of biting than was less frequent net usage. Reported usage of washed OlysetNet (44–45%) was much lower than for all other net types (>80%), likely because of these nets being too small or narrow as reported by 67% of householders and as observed during net measuring. Lower usage rates of washed OlysetNet may have contributed to higher reported biting rates at Kimbangu though biting was also high with unwashed OlysetNet, which may indicate that the large mesh size of this LLIN type allowed access to mosquitoes. Such access would be more likely in the presence of reduced permethrin susceptibility, as was the case for *Culex* spp at Kimbangu (48% mortality). More frequent reports of a good night of sleep as associated with PermaNet 3.0 both unwashed and washed support the use of this LLIN in Kinshasa; such a perceived benefit is likely to be related to more frequent and correct usage which is especially important where reduced susceptibility to pyrethroids has been detected.

CONCLUSION

Anopheles gambiae s.s. (M form) from Kindele was resistant to DDT and permethrin but susceptible to deltamethrin, propoxur and bendiocarb. The west African *kdr* mutation was detected and susceptibility to permethrin was restored with pre-exposure to ETAA in bottle bioassays indicating the likely presence of elevated glutathione transferase enzymes. Although there were no detectable differences in *Anopheles* or *Culex* indices according to the net type or wash status, PermaNet 3.0 both unwashed and washed showed significantly higher bioefficacy against *An. gambiae s.s.* in cone bioassays and was associated with enhanced usage and perceived benefits compared to OlysetNet.

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Village scale testing of PermaNet® 2.0 and PermaNet® 3.0 to establish insecticide resistance breaking efficacy

FINAL REPORT

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1.0 EXECUTIVE SUMMARY

The efficacy of PermaNet® 3.0 was compared to PermaNet® 2.0 and PermaNet® 2.0 Extra against pyrethroid resistant *An. gambiae* (*Kdr* and metabolic mechanisms in Côte d'Ivoire, and only metabolic in Cameroon) at the household level in two study sites in Côte d'Ivoire (Tiassalé and Bouaké) and two study sites in Cameroon (Gaschiga and Gounougou). Mosquito collections were made at each site using sentinel rooms employing exit traps (window traps) and sleeping rooms for resting catches. Mosquitoes were analyzed for species composition, physiological status (unfed, fed, gravid) and resistance status. Sporozoite rates were assessed through ELISA technique. Species and molecular forms were assessed by PCR; *Kdr* and *AChE* genotyping were performed using the pyrosequencing method.

Prior to net distribution, extremely high allele frequencies of *Kdr* resistant homozygous (87.0%) and heterozygous (95.4%) individuals were recorded in Tiassalé and Bouaké, respectively. Biochemical analysis carried out with alive and dead *An. gambiae* specimens from both sites of Cameroon after exposure to insecticides confirmed the absence of *Kdr* mutations. Bioassay data collected for the study sites in both countries revealed high resistance to pyrethroids.

In Bouaké, Gaschiga and Gounougou, no difference in the monthly total number of unfed and blood fed *An. gambiae s.s* and *An. arabiensis* collected from each treatment arm was detected This may be due to extremely low numbers of mosquitoes being collected from these sites.

In Tiassalé, exit trap data showed that both PermaNet® 2.0 Extra and PermaNet® 3.0 performed significantly better than PermaNet® 2.0 in terms of reduction in the total number of blood fed mosquitoes collected, while the mean number of blood fed *An. gambiae s.s* collected within houses (in resting catches), showed that PermaNet® 3.0 was significantly more effective compared with PermaNet® 2.0 and PermaNet® 2.0 Extra, which had comparable efficacy.

In Tiassalé, of the mosquitoes collected within households, significantly lower sporozoite rates were recorded in the PermaNet® 3.0 households than in either the PermaNet® 2.0 or PermaNet® 2.0 Extra households, which showed comparable sporozoite rates. Over the course of the study, sporozoite rates in Bouaké and Gounougou were significantly reduced.

Considering the reduction in the number of blood fed *An. gambiae s.s*. in Tiassalé, it can be concluded that at this site PermaNet® 3.0 performed significantly better than PermaNet® 2.0 and PermaNet® 2.0 Extra. Therefore, at the Tiassalé area of Côte d'Ivoire, PermaNet® 3.0 would be the more effective tool for controlling resistant *An. gambiae s.s.* However, at the Bouaké site (Côte d'Ivoire) and at both of the Cameroon sites (Gaschiga and Gounougou), the very limited/ low monthly number of resistant *An. gambiae* meant that it was not possible to compare net performance in terms of personal protection. However, at all of these sites the infection rates of malaria vectors were significantly reduced during the months following distribution of nets.

2.0 Background

2.1 Introduction

PermaNet® 3.0 is a long lasting insecticidal net (LLIN) developed by Vestergaard Frandsen 'for use in areas with pyrethroid resistant malaria vectors'[1] . The net is constructed with two different fabric types; polyester, and polyethylene. The roof of the net is made with monofilament polyethylene (100D), incorporated with deltamethrin (4g/kg, equivalent to a minimum of 90 mg/m^2) and piperonyl butoxide (PBO) (25g/kg, equivalent to a minimum of 562.5 mg/m^2). The upper part of the net is made with multifilament polyester (75D) impregnated with deltamethrin (2.8g/kg, equivalent to 85mg/m^2) with the lower part of the net being made with multifilament polyester (75D) impregnated with deltamethrin (2.8g/Kg, equivalent to 115mg/m^2). The inclusion of piperonyl butoxide on the roof of the net is intended to act as a synergist and improve the performance of the net, against pyrethroid resistant mosquitoes.

In order to establish whether PermaNet® 3.0 has any selective advantage against pyrethroid resistant *An. gambiae* field populations, the efficacy of PermaNet® 2.0, PermaNet® 2.0 Extra (previously named PermaNet® 2.5) and PermaNet® 3.0 was observed and compared during studies conducted in two areas of Côte d'Ivoire and two areas of Cameroon.

PermaNet® 2.0, also manufactured and sold by Vestergaard Frandsen, is made of multifilament polyester (75D and 100D) impregnated with deltamethrin (1.8g/kg, equivalent to 55mg/m^2).

PermaNet® 2.0 Extra is also manufactured (but currently not marketed) by Vestergaard Frandsen. It is made of multifilament polyester impregnated with deltamethrin (2.8g/kg, equivalent to 85mg/m^2) which is the same insecticidal dose as the sides of PermaNet® 3.0 and was included in the study so as to compare the effect of using a higher dose of deltamethrin in the absence of synergist.

2.2 Objectives/ research questions

The study was implemented in order answer the following questions:

(i) Does PermaNet® 3.0 protect against pyrethroid resistant mosquitoes?

(ii) Where there is pyrethroid resistance, including metabolic and *kdr*-based resistance mechanisms, is there increased protection with PermaNet® 3.0 compared to PermaNet® 2.0 and PermaNet® 2.0 Extra?

3.0 Study sites

Two study sites in Côte d'Ivoire and two study sites in Cameroon (see figure 1) were chosen to reflect different types and levels of background pyrethroid resistance in the local malaria vector populations:

[1] according to manufacturer

1. Côte d'Ivoire: malaria vectors are highly resistant to pyrethroids with high levels of *kdr* mutation and low levels of metabolic resistance (Alou et al, 2010; Koudou et al, 2011; Edi et al, 2012).
2. Cameroon: malaria vectors show a history of mainly metabolic resistance, with very low levels or the absence of the *kdr* mutation (Chouaibou et al, 2008).

Resistance mechanisms in malaria vectors from all study sites were fully characterised during net distribution using the methodology shown in Appendix 1. A summary of the main features of the study sites and the resistance mechanisms present in the vector populations at each site is given in Table 1.

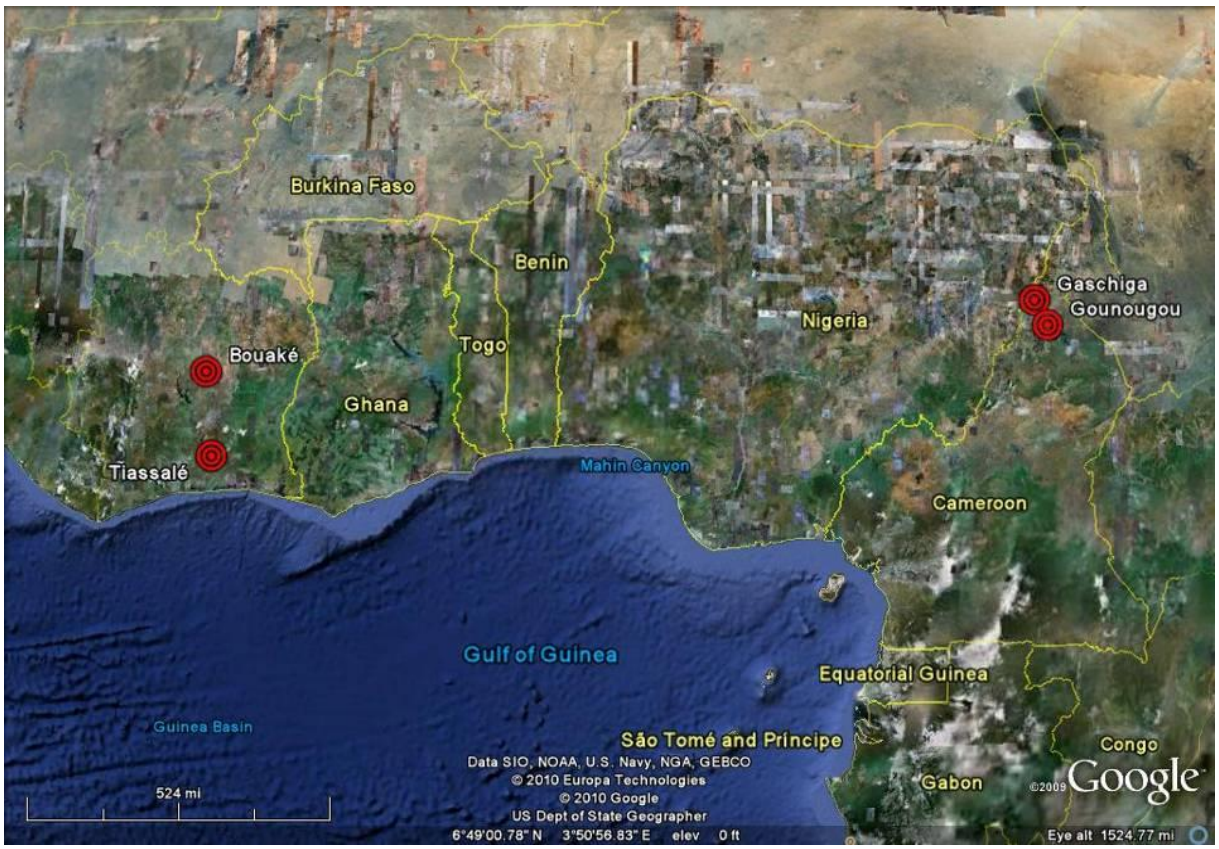

Figure 1: Google™ Earth map showing study site locations in Côte d'Ivoire and Cameroon

4.0 Methods

4.1. Study design: entomological parameters

The entomological parameters being examined by means of mosquito collections from households were:

- Exophily (the proportion of mosquitoes found in exit traps)
- Bloodfeeding
- Resting (the proportion of mosquitoes found resting inside houses)

For statistical analysis, the number of mosquitoes resting in the households, the numbers of mosquitoes that exit and the number that successfully blood fed were compared by species with the household as the repeat unit.

The primary criteria in evaluating different types of net were blood-feeding and density in the prominent malaria vectors at the study site. Thirty three to 39 sentinel rooms were selected per village for installation of exit traps (window traps). A further 18 to 21 sleeping rooms were selected per village for resting catches by sampling 4 to 5 houses in each village each week using the pyrethrum spray-catch method as described by WHO (WHO, 1975). Collected mosquitoes were analysed for species composition, physiological status (unfed, fed, gravid) and resistance status. The methods and results for the resistance characterization can be found in Appendix 1.

Entomological data was collected during a baseline period of one month and was continued for the duration of the study. Following the baseline period, distribution of bed nets started from the first house in the village and continued in a pre-determined systematic manner in which houses adjacent to a study house would be provided with the same type of net as the study house because we would like the study houses allocated different net types would not be adjacent to one another. But that was difficult because distance between adjacent houses was less than 150 meters. Same nets were allocated to each group of houses and all groups of houses could host 5 to 6 households. The following types of bed net were used for one year: PermaNet® 2.0 (“P2”), PermaNet® 2.0 Extra (“P2 extra”) or PermaNet® 3.0 (“P3”); for exit trap collections 13 houses and 11 houses were allocated to each bed net type in Tiassalé and Bouaké, respectively. In Cameroon, the number of houses allocated to each net was 12 in both districts. Concerning the pyrethrum spray sheet, in all the study sites of each country, for each net, every month, 7 houses were selected for mosquito collections early in the morning. In both countries, in each study site, we distributed approximately 125 PermaNet® 2.0 nets, 150 PermaNet® 2.0 Extra nets and 150 PermaNet® 3.0 nets.

During the baseline malaria transmission, parity ratio, blood feeding and repellency rates were assessed. In each study site, exposure to malaria parasites was assessed with resting catches collection method and exit traps, followed by detection of sporozoite in infected malaria vectors.

Protection against source of bias

Mosquito collector bias were reduced by using standard exit traps which do not rely on the ability of the fieldworkers to collect specimens and several experienced field technicians were involved to increase the ability of fieldworkers to collect as much as possible high number of specimens with the resting catches. Exit traps were examined by a different person blinded to the trap location.

4.2. Forms and species of malaria vectors

DNA was extracted from desiccated mosquitoes using the Livak protocol (Collins *et al*, 1987); the species and molecular forms of the *An. gambiae* complex were identified using the PCR method described by Fanello *et al* (2003). The results of species ID and forms are presented in the Table 1.

4.3. Bioassays

WHO cone bioassays (WHO, 2005) were performed on randomly selected nets during the study (at the beginning of the study, 6 months after net distribution and at the end of the study). Bioassays were performed on both the roof and sides of each net type and comparisons were made between each net type and location.

In both countries, WHO susceptibility tests were performed on 3-5 day old unfed wild-caught pyrethroid-resistant females reared from larval collections, using standard WHO test kits and protocols for adult mosquitoes. In brief, papers impregnated with 0.05% deltamethrin, 0.75% permethrin and 4% DDT were sourced from WHO. Batches of 20–25 females were exposed to impregnated papers in WHO test tubes for 1 h with at least four replicates per bioassay and concurrent negative controls with corresponding insecticide-free papers. Knockdown (KD) was recorded after 60 min and mosquitoes were transferred to holding containers with access to a 10% honey solution. Mortality was recorded after 24 h.

Additionally, in both sites of Cote d'Ivoire particularly, the same WHO susceptibility tests were performed with 0.05% deltamethrin + PBO, 0.75% permethrin + PBO and 4% DDT + PBO against wild resistant *An. gambiae s.s* and as previously the Knockdown (KD) was recorded after 60 min and and the mortality was recorded after 24 h.

Table 1: Summary of the main features of the study sites and the resistance mechanisms present in the vector populations at each site.

Study site	GPS coordinates	Species, molecular form	Climate	Topography	Phenotypic resistance (WHO susceptibility test): % mortality (% knockdown)			Resistance Profile	
					DDT	Permethrin	Deltamethrin	Target site[1]	Metabolic[2]
Tiassalé, Côte d'Ivoire	5°53'N, 4°49'W	*An. gambiae s.s.* - M form (100%) (n=184)	Humid; rainy season from Apr-Jul & Oct-Nov	Forest, irrigated rice fields from river & close to human habitation	10% (n=98) (7% KD)	2% (n=100) (3% KD)	8% (n=100) (21% KD)	87.0% *kdr* in M form only (n=180)	P450 = 21 GST = 5 COE = 3 ABC = 1
Bouaké, Côte d'Ivoire	7°44'N, 5°41'W	*An. gambiae s.s.* - S form (92%) - M form (8%) (n=180)	Dry & humid; long dry season	Savannah, non-irrigated rice fields, swamps and pools	3% (n=100) (3% KD)	11% (n=100) (3% KD)	49% (n=102) (68% KD)	95.4% *kdr* in both M & S forms (n=180)	GST = 1 ABC = 1
Gounougou, Cameroon	8°30'N, 14°00'E	*An. arabiensis* (95.9%) *An. gambiae s.s.* S form (4.1%) (n=122)	Very long dry season	Savannah, seasonal irrigated rice field, swamps and pools	96% (n=100) (100%)	39% (n=101) (56% KD)	23% (n=99) (48% KD)	0% *kdr* (n=122)	P450 = 13 GST = 3 COE = 1 ABC = 1
Gaschiga	9°21'N, 13°31'E	*An.arabiensis* (95.6%) *An. gambiae s.s*S form (4.8%) (n=124)	Dry	Savannah, site is crossed by a river, constituting main breeding sites	98% (96% KD)	25% (n=100) (48% KD)	42% (n=100) (60% KD)	0% *kdr* (n=124)	P450 = 3 GST = 5 COE = 1 ABC = 1

[1] *Kdr*, *AChE* and *Rdl* mutations were investigated; only *kdr* data is shown here at *kdr* relates to pyrethroid resistance. Refer to Appendix I for complete results of resistance characterisation.

[2] Number of genes differentially expressed; P450 = cytochrome P450/ oxidases; GST = Glutathione-S-Transferases; COE = Carboxyl-esterase; ABC = ABC Transporters (ATP-binding cassette genes)

4.4. Data analysis

During the baseline period and throughout the study period following net distribution, the total number of *An. gambiae* captured and the number of blood fed *An. gambiae* captured almost [3] daily in exit traps was recorded. Initially, the mean daily total and blood fed counts for each bed net type were plotted. However, it is difficult to identify anything other than large temporal patterns in these plots; and identifying important differences between the bed net types proved to be problematic using this approach. To improve these plots, moving averages across 7 consecutive days were computed, which when plotted provided clearer temporal patterns.

In order to perform meaningful statistical analyses, it was decided to compute the average total and blood fed counts for each house over the last 7 days of the baseline period and over the last 7 days of each month throughout the study period.

These counts should have followed a theoretical statistical Poisson distribution, but as is common with count data, the amount of variation in counts between the participating houses was greater than predicted by a Poisson model (*over-dispersion*). To overcome this problem, the counts were instead considered to follow a negative-binomial distribution, and the differences in average monthly counts between the three types of bed net were evaluated using negative binomial regression models, with robust methods applied to ensure that correct standard error and 95% confidence interval estimates were obtained for each average.

Both the Poisson and negative-binomial distributions are asymmetrical (i.e. are statistically slightly positively skewed). Nevertheless, arithmetic mean values were considered to be the appropriate average statistic for both distributions. Thus, the monthly mean total and blood fed *An. gambiae* counts (based on the last 7 days counts each month) were summarised in Tables for each bed net type separately (See Appendix II); graphical representations of these statistics are provided in the results section.

Levels of statistical significance (p-values) for differences in mean counts were computed not from the differences themselves but from the ratios of the means (often referred to as “incidence rate ratios” or “IRRs”). However, as IRR statistics are difficult to interpret, only means for each net type have been reported.

[3] The study protocol stated that daily catches in exit traps would be made. However, over the 12 month period of the study, there were incidences when not all exit trap catches were made every day.

5.0 Results

The differences between both total and blood-fed mean counts at baseline between the houses allocated to each bed net type in each village were not statistically significant, but were numerically large. The Tables and Figures in this report therefore show the mean values adjusted for the differences at baseline between the groups. These means are statistically more robust and are the preferred statistic for assessing statistical significance between the bed net types.

5.1. Bioassay results

In Cameroon, the mortality rates recorded (Table 2) with all the net types against wild *An. gambiae s.s* and *An. arabiensis,* assessed once during the month of nets distribution and once at the end of the trial (8 months after nets distribution), were very high (>92%). With PermaNet® 3.0 particularly, results recorded from roof and sides of nets showed high mortality rates.

In Côte d'Ivoire, at both study sites, with the exception of PermaNet® 2.0 nets, new nets were effective against *An. gambiae s.s* as mortality rates were above 80% (Table 3). When used and washed after 6 and 12 months, PermaNet® 2.0 and PermaNet® 2.0 extra were not effective against wild *An. gambiae s.s*. In contrast to PermaNet® 2.0 and PermaNet® 2.0 extra, PermaNet® 3.0 was not affected by washing and use after 6 months (July 2010) and 12 months (January 2011) with this net remaining effective against wild An*. gambiae s.s* at both study sites in Côte d'Ivoire.

The statistical analyses showed that new long lasting insecticide treated nets (PermaNet® 2.0, PermaNet® 2.0 extra and PermaNet® 3.0) remained efficient against wild *An. gambiae s.s* whatever the locality except for the PermaNet® 2.0 net in Bouaké, Côte d'Ivoire (χ-squared = 4.4064, df = 1, p-value = 0.03581). Indeed, when the number of dead mosquitoes from new PermaNet® 2.0 versus PermaNet® 2.0 washed are compared statistically, these show a significant difference (χ-squared = 9.7066, df = 1, p-value = 0.001836)**.**

In Côte d'Ivoire and Cameroon, although a low mortality was recorded when bioassays were carried out on the sides of the PermaNet® 3.0 net, the overall statistical analysis showed that PermaNet® 3.0 when used/washed or new, this net remains effective regardless of whether the mosquitoes are exposed on treated roof (χ-squared = 0, df = 1, p-value = 0.9954) or to the sides (X-squared = 0.8094, df = 1, p-value = 0.3683).

Regarding the added value of PBO, in Bouake, the results of the WHO susceptibility tests showed an increase of the mortality rate only with 0.05% deltamethrin + PBO (70%). With 0.75% permethrin + PBO and 4% DDT + PBO, we recorded 14% and 1% mortality rates, respectively, which are very weak. In Tiassale, we recorded the mortality rates of 75.7%, 21.3% and 0% when pyrethroids resistant *An. gambiae s.s* are exposed to 0.05% deltamethrin + PBO, 0.75% permethrin + PBO and 4% DDT + PBO, respectively. Thus, when combined with PBO, high mortality rate is recorded when wild pyrethroid

resistant *An. gambiae s.s* are exposed to 0.05% deltamethrin. These results support findings reported on cones bio-assays which confirm the fact that PermaNet® 3.0 performed significantly better than PermaNet® 2.0 and PermaNet® 2.0 extra as this net was impregnated with Deltamethrin in the sides and Deltamethrin + PBO in the top of the net.

Table 2. WHO cone bioassay results (% mortality) with PermaNet® 2.0, PermaNet® 2.0 Extra and PermaNet® 3.0 at the beginning (January 2010) and at the end of the trial (August 2010) against wild resistant *An. gambiae* in Cameroon.

Net type[4]	**% mortality in WHO cone tests [2]**	
	Cameroon	
	Gaschiga	Gounougou
PermaNet® 2.0: new	100 (88.4-100) (n=30)	100 (88.4-100) (n=30)
PermaNet® 2.0: used (8 months)	100 (89.1-100) (n=32)	100 (86.3-100) (n=25)
PermaNet® 2.0 Extra: new	96 (79.6-99.9) (n=25)	95 (75.1-99.9) (n=20)
PermaNet® 2.0 Extra: used (8 months)	92 (79.6-98.4) (n=40)	100 (86.3-100) (n=25)
PermaNet® 3.0: new	94 (80.3-99.3) (n=34)	97 (81.0-99.9) (n=27)
Roof	90 (55.5-99.7) (n=10)	100 (54.1-100) (n=6)
Sides	91 (70.8-98.9) (n=22)	95 (76.2-99.9) (n=21)
PermaNet® 3.0: used (8 months)	91 (82.3-96.8) (n=70)	90 (73.5-97.9) (n=30)
Roof	90 (55.5-99.7) (n=10)	89 (51.7-99.7) (n=9)
Sides	83.3 (71.5-91.7) (n=60)	90.5 (69.6-98.8) (n=21)

[4] Nets were used and washed traditionally and not as described in WHO protocol. Net users were asked if the net had been washed and how many times during collection of nets; a particular survey was not conducted to get this information.

Table 3. WHO cone bioassay results (% mortality) with PermaNet® 2.0, PermaNet® 2.0 Extra and PermaNet® 3.0 at the beginning (January 2010), six months after nets distribution (July 2010) and at the end of the trial (January 2011) against wild resistant *An. gambiae* in Côte d'Ivoire.

Net type [1]	% mortality in WHO cone tests [2]	
	Côte d'Ivoire	
	Tiassalé	Bouaké
PermaNet® 2.0: new	71 (55.4-82.1) (n=49)	99 (89.7-99.9) (n=52)
PermaNet® 2.0: used **(6 months)**	56 (41.40-69.08) (n= 54)	52 (38.03-65.34)(n= 56)
PermaNet® 2.0: used **(12 months)**	54 (39.2-68.6) (n=48)	32 (19.5-46.7) (n=50)
PermaNet® 2.0 Extra: new	94 (83.4-98.7) (n=50)	98 (89.3-99.9) (n=50)
PermaNet® 2.0 Extra: used **(6 months)**	63 (48.96-76.38) (n= 52)	68.5 (54.45-80.48) (n= 54)
PermaNet® 2.0 Extra: used **(12 months)**	60 (45.2-73.6) (n=50)	71 (56.5-84) (n=46)
PermaNet® 3.0: new	97 (86.3-99.5) (n=60)	99 (90.6-99.9) (n=57)
Roof	100 (83.2-100) (n=20)	100 (82.3-100) (n=19)
Sides	95 (83.1-99.4) (n=40)	97.3 (86.2-99.9) (n=38)
PermaNet® 3.0: used **(6 months)**	71.4 (54.45-80.48) (n= 56)	91.7 (57.79-82.71)(n= 60)
Roof	86.7 (59.54-98.34) (n= 15)	93.3 (68.05-99.83) (n= 15)
Sides	65.8 (49.40-79.92) (n= 41)	91.1 (78.78-97.52) (n= 45)
PermaNet® 3.0: used **(12 months)**	75 (61.5-84.5) (n=62)	93 (82.7-98) (n=56)
Roof	90 (68.3-98.8) (n=20)	95 (75.1-99.9) (n=20)
Sides	66.7 (50.4- 80.4) (n=42)	94.7 (81.3-99.3) (n=36)

Table 4. WHO Susceptibility Tests Data (% mortality) in *An gambiae* in May 2010

Insecticides	Tiassalé	Bouaké
DDT	9.72 (4.0- 19.01)	6.12 (2.28-12.85)
Deltamethrin	10.67 (4.72-19.94)	37.93 (29.08-47.41)
Permethrin	4.34 (1.20-1.076)	12.19 (6.99-19.31)

5.2. Exit trap results: Côte d'Ivoire

5.2.1. Monthly mean *An. gambiae s.s.* collected in exit traps in Bouaké

The statistical analysis revealed that from December 2009 to December 2010, there was no significant difference (except the months of February and June) in the monthly numbers of *An. gambiae s.s* collected in the exit traps installed in the windows of sleeping rooms between each treatment arm (Figure 2 below and Table II.I in Appendix II).

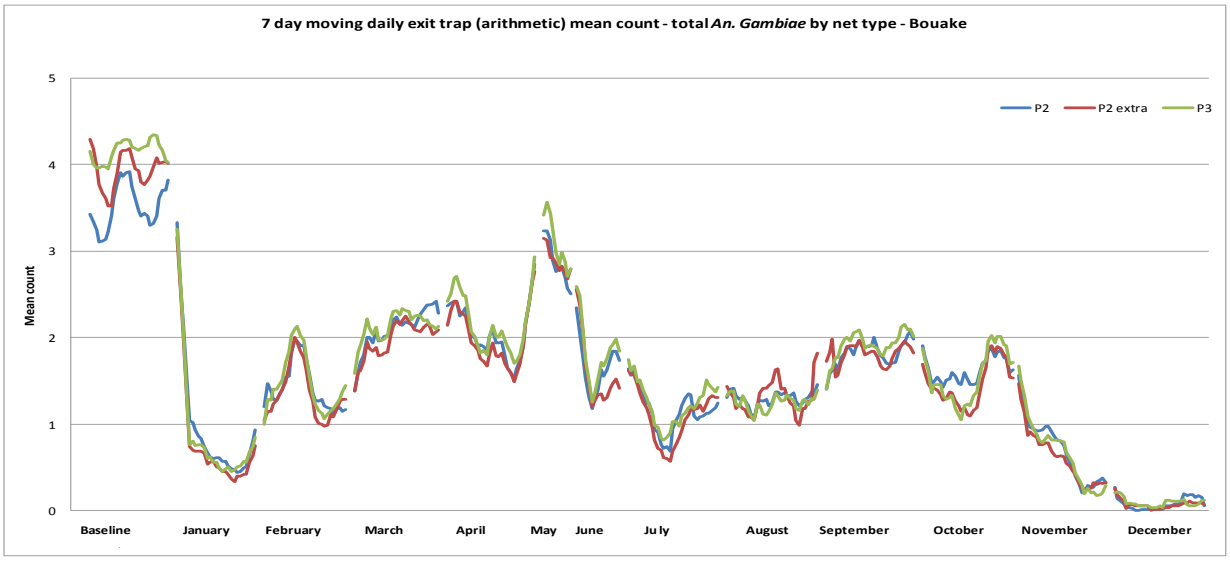

Figure 2. Monthly mean count of *An. gambiae s.s* collected in exit traps before and after net distribution in Bouaké from December 2009 (Baseline) to December 2010.

5.2.2. Monthly mean *An. gambiae s.s.* collected in exit traps in Tiassalé

The statistical analysis revealed that significantly fewer *An. gambiae s.s* were collected in the exit traps from households with PermaNet® 2.0 Extra compared with PermaNet® 2.0 for the period from January to July and from October to December 2010 (Figure 3 below). From January to April and in the last trimester of 2010, significantly fewer *An. gambiae s.s* were collected with PermaNet® 2.0 Extra compared with PermaNet® 3.0 (Table II.II in Appendix II). The performance of PermaNet® 2.0 and PermaNet® 3.0, as measured by the exit traps was similar except during the months of May and July 2010.

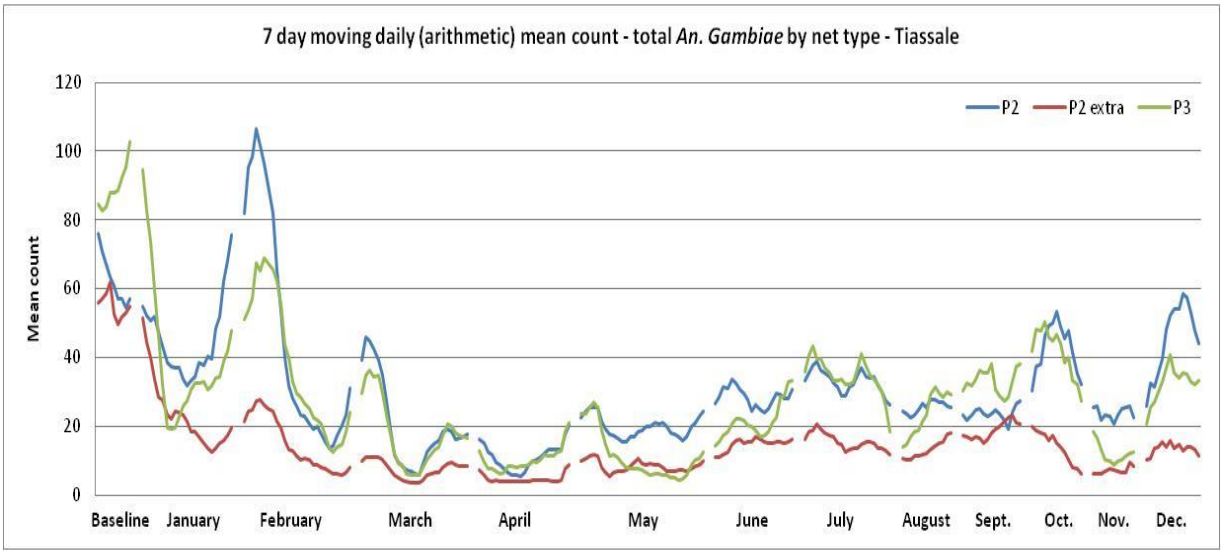

Figure 3. Monthly mean count of *An. gambiae s.s* collected in exit traps before and after net distribution in Tiassalé from December 2009 (Baseline) to December 2010.

5.2.3. Monthly mean blood fed *An. gambiae s.s.* collected in exit traps in Bouaké

Post-net distribution from December 2009 to December 2010, with the exception of the months of February and July, the efficacy of all nets against mosquito bites, as measured through the mean monthly blood fed from exit traps, was not significantly different (Figure 4 below; see also Table II.III in Appendix II). In February, the mean count of blood fed *An. gambiae s.s* collected with PermaNet® 2.0 was significantly lower than with PermaNet® 2.0 Extra ($p=0.048$) and significantly fewer *An. gambiae s.s* were collected with PermaNet® 2.0 Extra compared with PermaNet® 3.0 ($p=0.009$). In July and November 2010, the mean count of blood fed *An. gambiae s.s* collected with PermaNet® 2.0 Extra was significantly lower than for PermaNet® 3.0 ($p=0.031$).

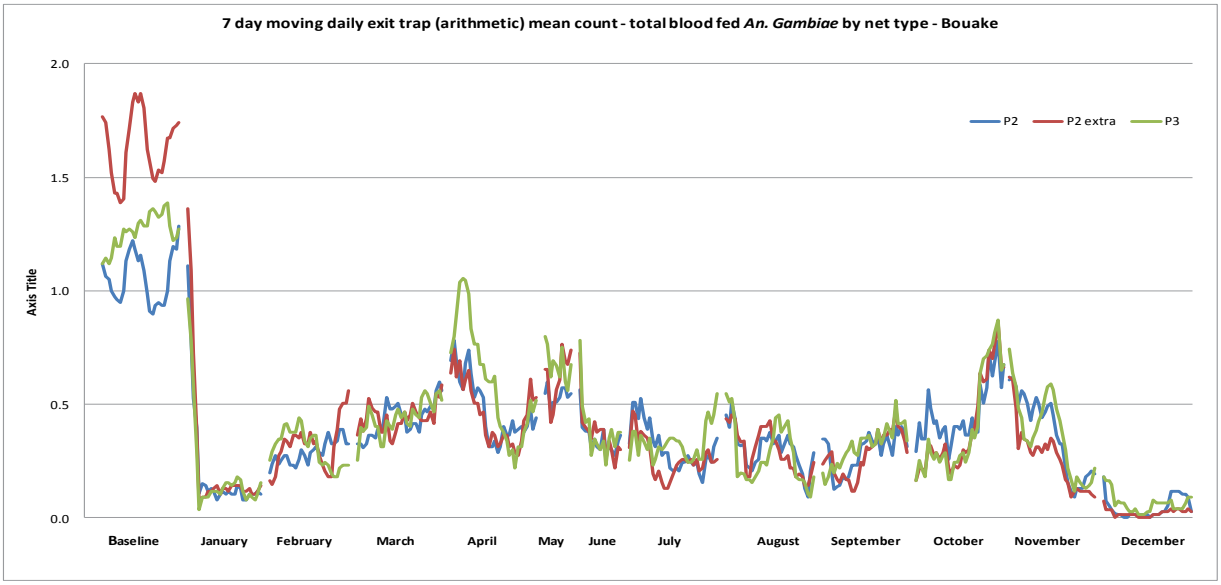

Figure 4. Monthly mean count of blood fed *An. gambiae s.s* collected in exit traps before and after net distribution in Bouaké from December 2009 (Baseline) to December 2010.

5.2.4. Monthly mean blood fed *An. gambiae s.s.* species collected in exit traps in Tiassalé

The statistical analysis revealed that a similar number of blood fed *An. gambiae s.s* were collected in the exit traps with each type of net, except from May to July 2010 when significantly fewer were caught with PermaNet® 3.0 than with PermaNet® 2.0 (see Figure 5 below and Table II.IV in Appendix II). The observed reduction rate of blood fed *An. gambiae* with PermaNet® 3.0 was very high (90%) according to the monthly number of blood fed *An. gambiae* collected from baseline (January 2010) to the end of the trial (January 2011).

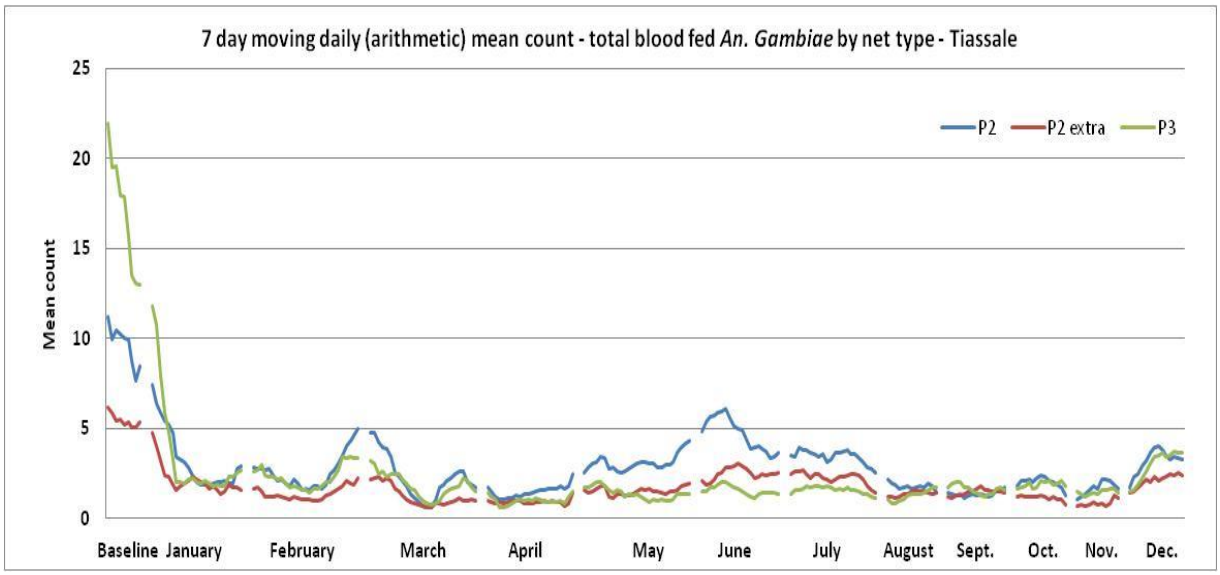

Figure 5. Mean monthly count of blood fed *An. gambiae s.s* collected in exit traps before and after net distribution in Tiassalé from December 2009 (Baseline) to December 2010.

5.3 Resting catch data: Côte d'Ivoire

5.3.1. Monthly mean *An. gambiae s.s* collected within households in Bouaké

The statistical analysis revealed that from December 2009 (Baseline) to December 2010, there was no significant difference between the monthly mean of *An. gambiae s.s* collected within households in sleeping rooms with each net type (see Figure 6 and Table II. V in Appendix II), except in February where significantly less *An. gambiae s.s* were collected with PermaNet® 2.0 Extra than PermaNet® 3.0 (p=0.003), in June where PermaNet® 2.0 Extra collected less *An. gambiae s.s* compared with PermaNet® 2.0 (p= 0.047) and in August 2010 where PermaNet® 2.0 Extra collected less than PermaNet® 3.0 (p= 0.009). Regarding this parameter, all nets performed equally.

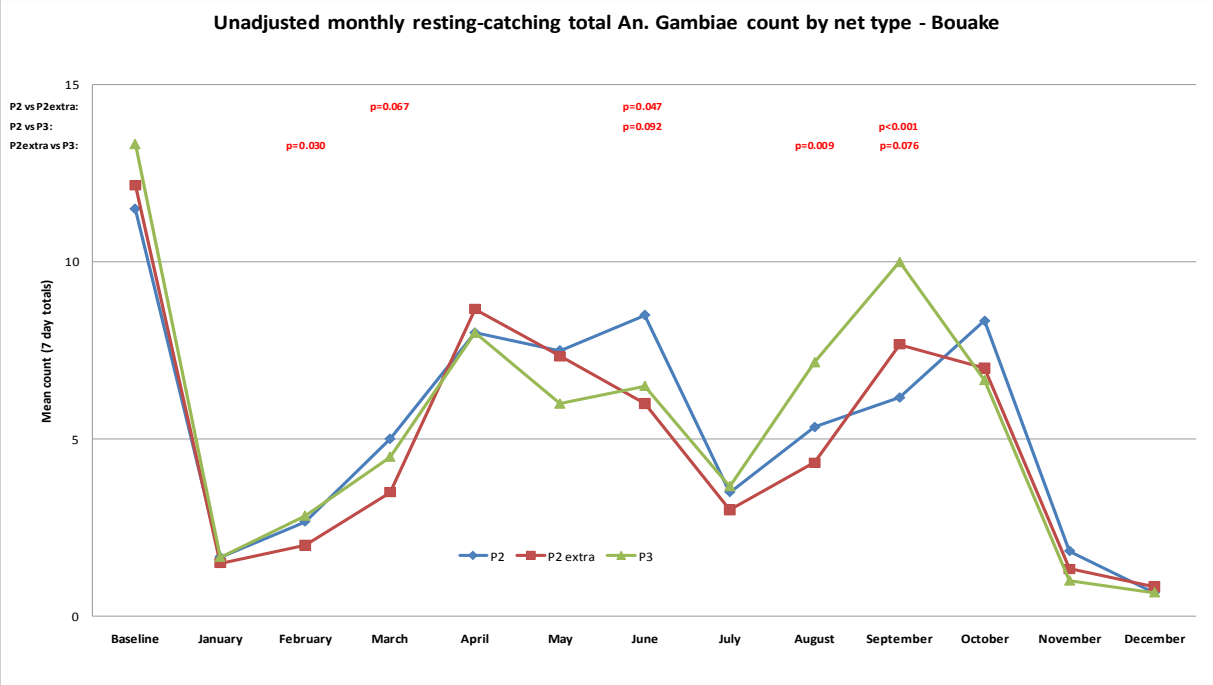

Figure 6. Mean monthly count of *An. gambiae s.s* collected in resting catches within houses before and after net distribution in Bouaké from December 2009 (Baseline) to December 2010.

5.3.2. Monthly mean *An. gambiae* collected within households in Tiassalé

From February to December 2010, significantly fewer *An. gambiae s.s* were collected within houses with PermaNet® 3.0 compared to PermaNet® 2.0 Extra and PermaNet® 2.0 (see Figure 7 below and Table II.VI in Appendix II). The performance of PermaNet® 2.0 and PermaNet® 2.0 Extra were not statistically different except the month of August 2010 (p= 0.018). Figure 7 shows the monthly mean number of *An. gambiae* collected within houses from December 2009 (Baseline) to December 2010

in Tiassalé. Thus, regarding, PermaNet® 3.0 performed significantly better compared to PermaNet® 2.0 Extra and PermaNet® 2.0.

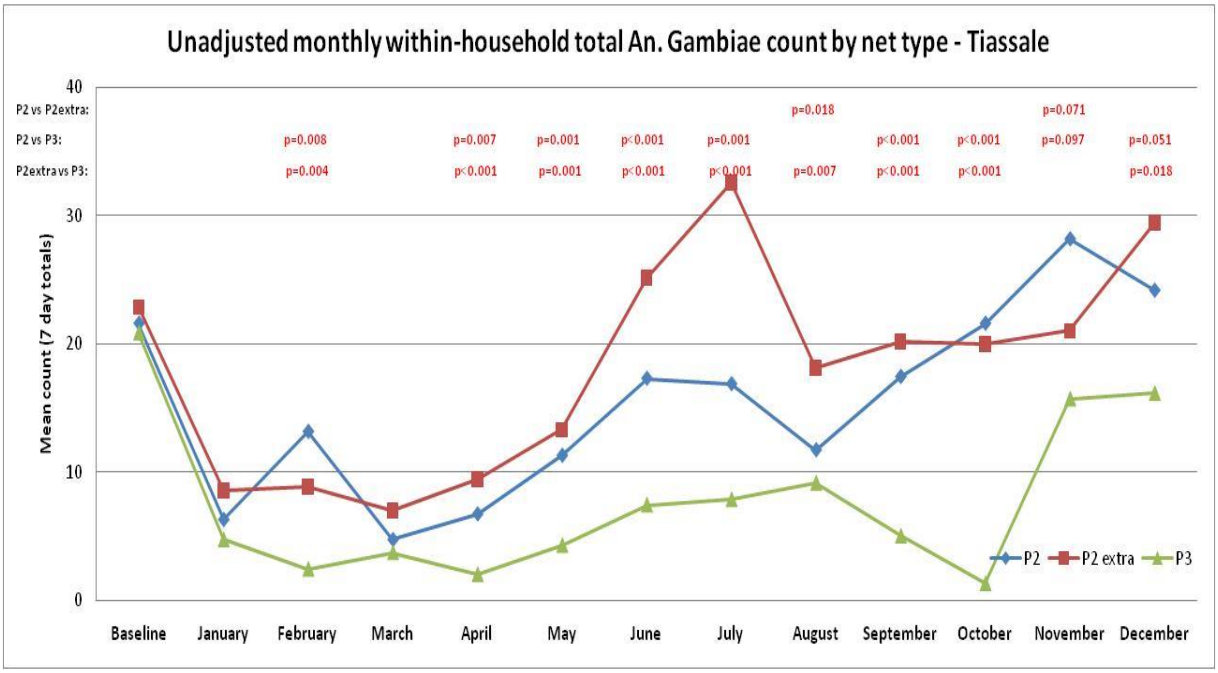

Figure 7. Mean monthly count of *An. gambiae s.s* collected in resting catches within houses before and after net distribution in Tiassalé from December 2009 (Baseline) to December 2010.

5.3.3. Monthly mean blood fed *An. gambiae* species collected within households in Bouaké

From December 2009 (Baseline) to December 2010, there was no difference between the monthly mean blood fed *An. gambiae s.s* collected within households with PermaNet® 2.0, PermaNet® 2.0 Extra and PermaNet® 3.0 (Figure 8 below) except in January when significantly less blood fed *An. gambiae s.s* were collected with PermaNet® 2.0 Extra and PermaNet® 2.0 than with PermaNet® 3.0 ($p< 0.001$). Figure 8 (also Table II.VII in Appendix II) shows the monthly mean number of bloodfed *An. gambiae* collected within houses from December 2009 (Baseline) to August 2010 in Bouaké. Thus, in Bouaké the three nets performed equally well even though fewer blood fed *An. gambiae* were caught in January 2010.

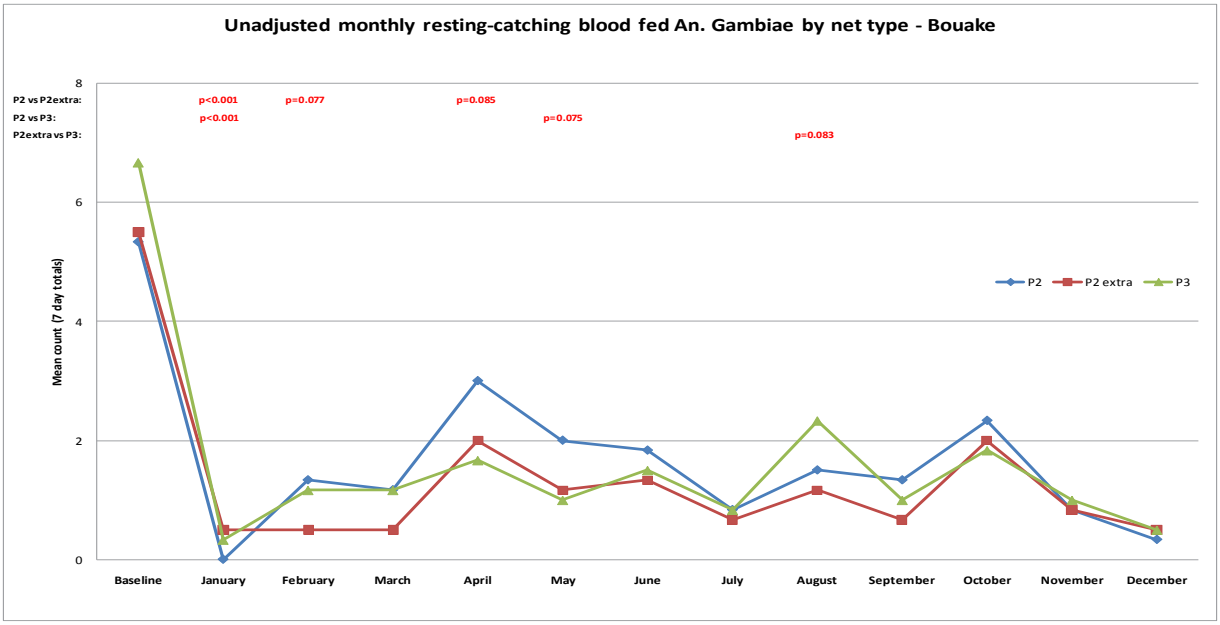

Figure 8. Mean monthly blood fed *An. gambiae s.s* collected within houses before and after net distribution in Bouaké from December 2009 (Baseline) to December 2010.

5.3.4. Monthly mean blood fed *An. gambiae s.s* collected inside households in Tiassalé

From February to December 2010, significantly less blood fed *An. gambiae s.s* were collected within houses with PermaNet® 3.0 compared with PermaNet® 2.0 Extra and PermaNet® 2.0 (Figure 9 below). The performance of PermaNet® 2.0 and PermaNet® 2.0 Extra were statistically similar except the months of June (p= 0.058) and August 2010 (p= 0.003). Figure 9 (also Table II.VIII in Appendix II) shows the monthly mean number of blood fed *An. gambiae* collected within houses from December 2009 (Baseline) to December 2010 in Tiassalé. Thus, as reported with the exit traps collection method, with the pyrethrum spray sheet collection method as well, PermaNet® 3.0 offered significantly better personal protection than PermaNet® 2.0 Extra and PermaNet® 2.0.

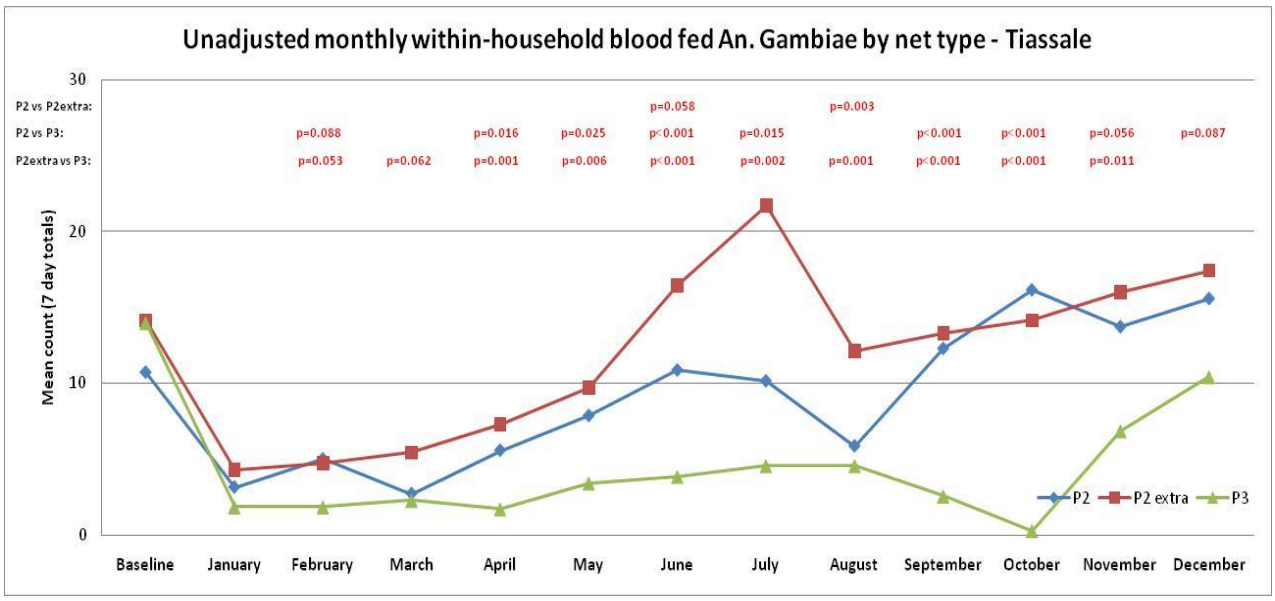

Figure 9. Mean monthly blood fed *An. gambiae s.s* collected within houses before and after net distribution in Tiassalé from December 2009 to December 2010.

5.4 Exit Trap results: Cameroon

5.4.1. Mosquitoes collected (*An. gambiae* and *An. arabiensis*) in exit traps in northern Cameroon

Extremely low monthly numbers of *An. gambiae* and *An. arabiensis* were collected in both study sites (Figures 10 and 11) in the exit traps, which affected possibility of finding any statistical difference between the monthly number of *An. gambiae* and *An. arabiensis* recorded with each net type.

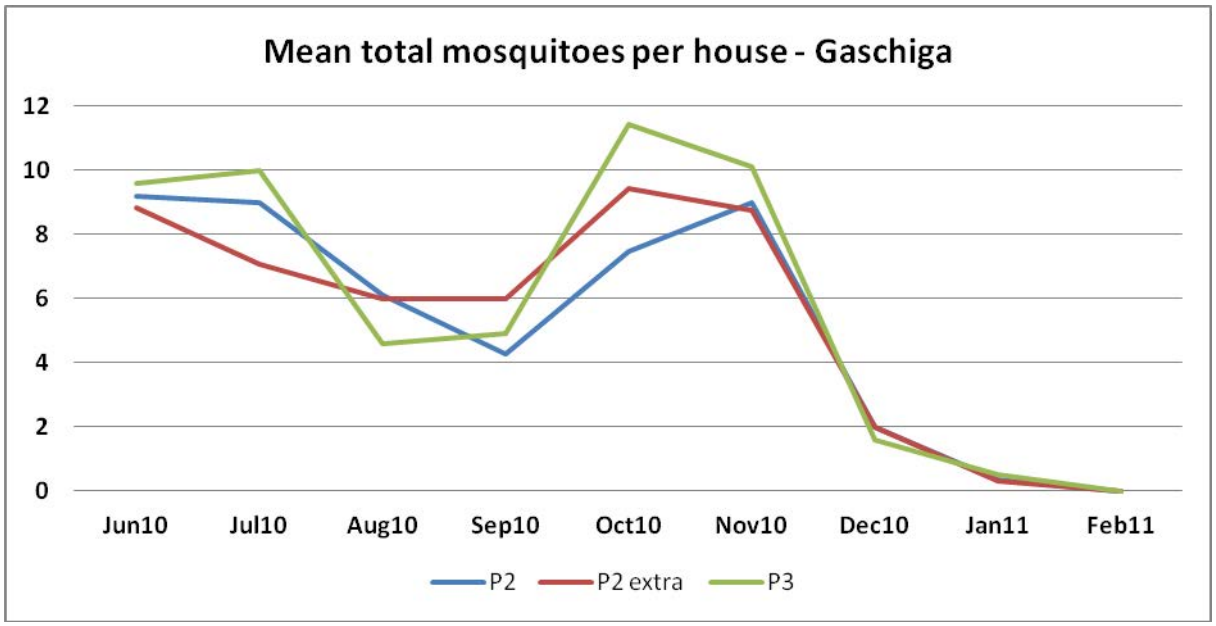

Figure 10. Mean monthly *An. gambiae s.s* collected in exit traps before and after net distribution in Gaschiga from June 2010 to February 2011.

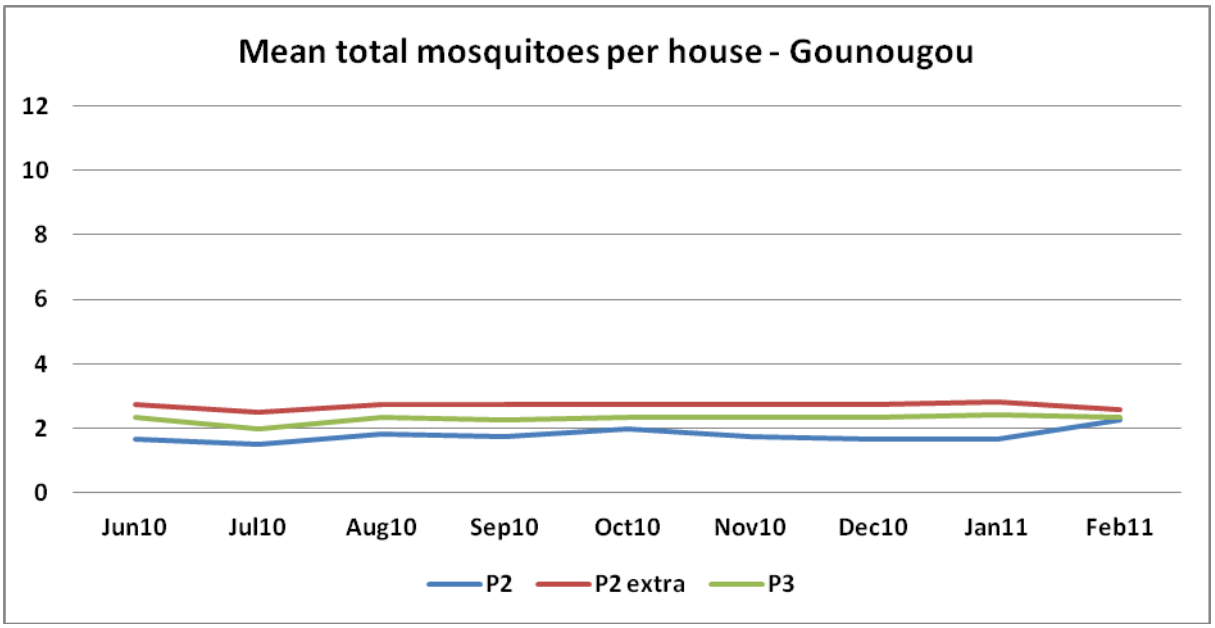

Figure 11. Mean monthly *An. gambiae s.s* collected in exit traps before and after net distribution in Gounougou from June 2010 to February 2011.

5.4.2. Mean Blood fed *An. gambiae* and *An. arabiensis* collected within households in northern Cameroon

Due to the extremely low monthly number of blood fed *An. gambiae* and *An. arabiensis* in both study sites in the exit traps, no statistical difference between the number of blood fed *An. gambiae* and *An. arabiensis* recorded with each net type could be seen (figures 12 and 13).

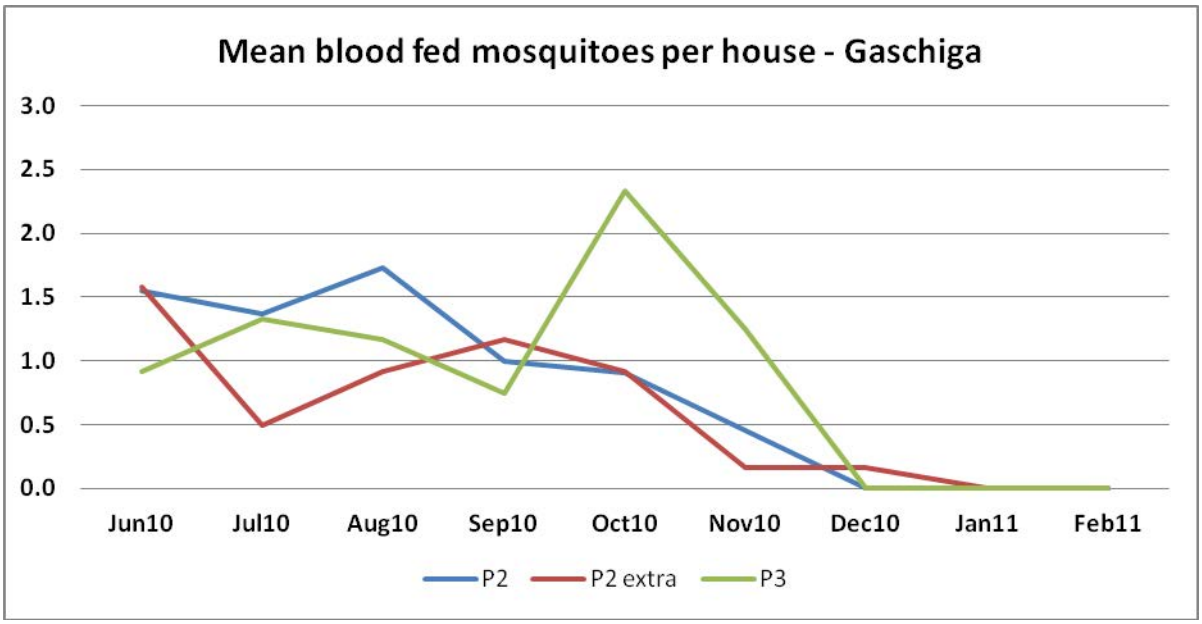

Figure 12. Mean monthly blood fed *An. gambiae s.s* collected in exit traps before and after net distribution in Gaschiga from June 2010 to February 2011.

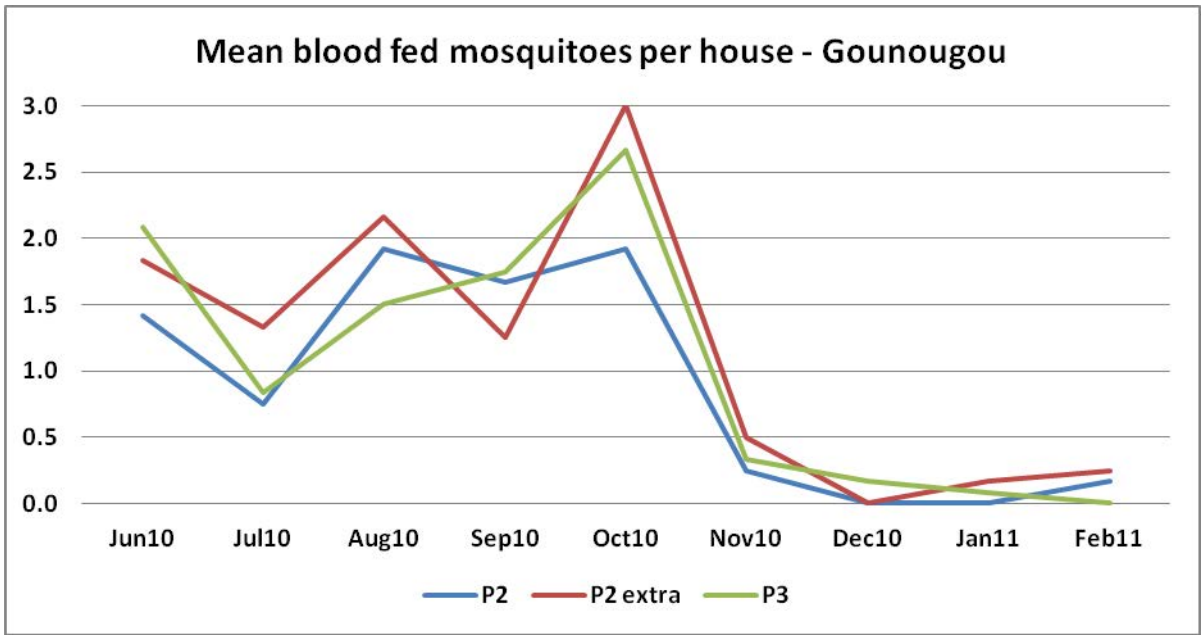

Figure 13. Mean monthly count of blood fed *An. gambiae s.s* collected in exit traps before and after net distribution in Gounougou from June 2010 to February 2011.

5.5. Sporozoite infection rates

Post net distribution, all net types in Bouaké (Côte d'Ivoire) and Gounougou (Cameroon) had significantly reduced the sporozoite rate in the main malaria vectors collected (Table 4). A statistically similar sporozoite rate was recorded between net types. However, in Tiassalé in households with PermaNet® 3.0 mosquitoes collected were found to have a significantly lower sporozoite rate when compared with households using either PermaNet® 2.0 or PermaNet® 2.0 Extra. No significant difference was found for the sporozoite rate between PermaNet® 2.0 or PermaNet® 2.0 Extra households. In Gaschiga, no statistically significant reduction of sporozoite rates was observed after distribution. The statistical significant difference recorded in the reduction of sporozoite rates could not be attributed to seasonal variation as that was observed in 3 out of 4 of the study sites. If in Bouake and Gounougou, the results showed clearly the community effect, in Tiassale, due to the high level of resistance to insecticide, the results suggest that only PermaNet® 3.0 performed well and was protective. Thus, even if the study was statistically under powered (as it was not possible to provide multiple, replicate study sites), reductions recorded in sporozoite rates can be attributed to the performance of the nets .

Before nets distribution very high sporozoite rates were recorded in all study sites. That could be explained by the high sensitivity of the new methodology used which is based on DNA extraction and sporozoite checking with RT – PCR machine in comparison to the old methodology based on ELISA technique.

Table 5. Overall sporozoite rates of *An. gambiae s.l.* recorded in Côte d'Ivoire and Cameroon before and after net distribution

Net types	Côte d'Ivoire		Cameroon	
	Bouaké	Tiassalé	Gaschiga	Gounougou
Before net distribution	13.7[a] (102)	18.4[a] (98)	15.3[a] (117)	15.4[a] (104)
PermaNet® 2.0	8.7[b] (126)	19.2[a] (104)	14.6[a] (143)	5.1[b] (138)
PermaNet® 2.0 Extra	6.2[b] (129)	15.5[a] (90)	10.5[b] (228)	5.4[b] (92)
PermaNet® 3.0	5.1[b] (174)	4.3[b] (184)	11.6[a] (146)	6.9[b] (129)

Note: Values along each row bearing the same superscript are not significantly different at the 5% level.

5.6. Kdr rates before and after net distribution in Côte d'Ivoire

Before net distribution in Bouaké, a very high frequency of *Kdr* was recorded with heterozygote *An. gambiae s.s*. S form (97.8%), which remained high and unchanged (Table 5) even following net distribution (98.4 – 100%).

Before net distribution in Tiassalé, an extremely high frequency of *Kdr* resistant homozygotes (75.5%) and low frequency of resistant heterozygotes (6.7%) were recorded. Post net distribution, the frequency of resistant homozygotes was significantly reduced with all net types. However, the frequency of resistant heterozygotes markedly increased for all nets (6.7% before net distribution vs 94.5% for PermaNet® 2.0, 96.4% for PermaNet® 2.0 Extra and 93.8% for PermaNet® 3.0 after net distribution). Thus, due to the design of the trial (randomized at the household), changes recorded in the rates of resistant homozygotes could not be attributed to a particular net.

Table 6. Overall *Kdr* rates (%) of *An. gambiae s.s.* in Côte d'Ivoire before and after net distribution

Net type	**% *Kdr* frequency (number tested)**					
	Bouaké			**Tiassalé**		
	SS	**RS**	**RR**	**SS**	**RS**	**RR**
Before net distribution	2.2[a] (90)	93.3[a] (90)	4.5[a] (90)	17.8[a] (90)	6.7[a] (90)	75.5[a] (90)
PermaNet® 2.0	1.6[a] (129)	98.4[a] (129)	0.0[a] (129)	4.0[a] (271)	94.5[b] (271)	5.1[b] (138)
PermaNet® 2.0 Extra	0.0[a] (135)	100[a] (135)	0.0[a] (135)	3.2[a] (279)	96.4[b] (279)	0.4[b] (279)
PermaNet® 3.0	0.6[a] (180)	99.4[a] (180)	0.0[a] (180)	3.3[a] (276)	93.8[b] (276)	2.9[b] (276)

Note: Values along each row bearing the same superscript are not significantly different at the 5% level

6.0 Discussion

One of the key and interesting findings of this study was that in Tiassalé, where the local malaria vector population has been shown to exhibit a high degree of resistance to pyrethroids and for which both *kdr* and metabolic resistance mechanisms have been detected, PermaNet® 3.0 provided significantly higher levels of protection than either PermaNet® 2.0 or PermaNet® 2.0 Extra, especially at times when the mosquito population was at its highest.

In Tiassalé , PermaNet® 3.0 performed significantly better compared to PermaNet® 2.0 and PermaNet® 2.0 Extra as significantly fewer blood fed and non blood fed (unfed, gravid and semi gravid) *An. gambiae s.s* were collected with PermaNet® 3.0 compared to PermaNet® 2.0 and PermaNet® 2.0 Extra. Thus, PermaNet® 3.0 offered better personal protection in Tiassalé against wild resistant *An. gambiae s.s* compared to the other nets. It should be noted that while PermaNet® 2.0 Extra has a higher dose of deltamethrin than PermaNet® 2.0, the deltamethrin dose of PermaNet 3.0 is the same as that for PermaNet® 2.0 Extra and the only differences between these two nets being the inclusion of piperonyl-butoxide and the use of monofilament polyethylene versus multifilament polyester on the roof of PermaNet® 3.0 .

In Bouaké, where pyrethroid resistance is widespread, but attributed only to *Kdr*, no significant differences in efficacy between the different net types was recorded. However, at this site, as well as at the sites in Cameroon, the low number of mosquitoes caught may have precluded the detection of any statistically significant differences in efficacy between net types.

With regard to the studies conducted in Cameroon, low rainfall/drought conditions likely contributed to the low numbers of mosquitoes collected from Gaschiga and Gounougou. The low mosquito populations were observed during the collection of baseline data, but it was hoped that the mosquito populations over the subsequent months would increase as and when the rains occurred. With hindsight a better choice may have been to stop the studies in Cameroon.

Post net distribution, all net types in Bouaké (Côte d'Ivoire) and Gounougou (Cameroon) had significantly reduced sporozoite rates in the main malaria vectors collected from the houses where entomological collections were made. However, for Tiassalé, in households with PermaNet® 3.0, mosquitoes collected were found to have a significantly lower sporozoite rate when compared with households using PermaNet® 2.0 or PermaNet® 2.0 Extra, and there was no difference found between the sporozoite rate between PermaNet® 2.0 and PermaNet® 2.0 Extra. Due to the design of the study (randomised at the household level) it is not possible to attribute overall effects on sporozoite rates to a single intervention, and as the CS ELISA is an indirect measure of sporozoite rate, the limitations of this method are recognized (Wirtz et al. 1987). However, assuming that the sporozoite rate for malaria vectors entering the homes with the different net types is the same, it would appear that PermaNet® 3.0 was having a greater impact on the proportion of sporozoite

infected mosquitoes than the other two net types. This might be an age related effect, if the sporozoite rate were to be considered as a proxy for the age structure of the mosquito population, as older mosquitoes are expected to show a higher degree of susceptibility than younger mosquitoes even where resistance is widespread (Chouaibou et al. 2012), then PermeNet® 3.0 may be more efficient in repelling older (sporozoite positive) mosquitoes than the other two net types being tested.

The performance of PermaNet® 2.0, PermaNet® 2.0 Extra and PermaNet® 3.0 in Tiassalé particularly is highlighted by the fact that there was a significant reduction of resistant (*kdr)* RR homozygotes, demonstrating an effect of the interventions at the community level. However, due to the study design that randomised at the household level, this effect cannot be attributed to one particular net type. It would have been helpful to have attempted to examine the resistance status (both *kdr* and metabolic mechanisms) amongst dead and surviving mosquitoes from the households with different net types particularly with regards metabolic mechanisms, however, the complexity of techniques required precluded its inclusion in this study. There would be value in further investigating this effect and relative fitness costs associated with varying mechanisms and degrees of resistance.

Cone bioassays were carried out on the nets using locally collected malaria vectors (collected as larvae and reared to adult in an insectary) at the time of net distribution and at the end of the trials. In both study sites in Cameroon the mortality rates both at the start and end of the trial, with all net types against *An. gambiae* and *An. arabiensis* were high >80%, although the lowest bioassay score (83%) was observed for the side of PermaNet 3.0® at the end of the trial.

In Bouaké, Côte d'Ivoire cone bioassay mortality rates with all net types against *An. gambiae* at the start of the trial were high (>90%) while at the end of the trial this was not the case, as both PermaNet® 2.0 and PermaNet® 2.0 Extra had reduced bioassay mortality rates. Mortality rates in cone bioassays for PermaNet® 3.0 at the start and end of the trial were similar for both the roof and sides of the net.

In Tiassalé, Côte d'Ivoire at the time of net distribution cone bioassays against *An. gambiae* was <80% for PermaNet® 2.0 but >80% for both PermaNet 2.0 Extra and PermaNet® 3.0 (sides and roof). At the end of the trial mortality in cone bioassays for PermaNet® 2.0, PermaNet® 2.0 Extra and the sides of PermaNet® 3.0 were reduced while for the PermaNet® 3.0 roof mortality remained high at 90%.

In Cameroon, PermaNet® 2.0 was efficient against vector as 100 % induced mortality were recorded either in Gaschiga or Gounougou at the beginning and end of the study. In general, this level of efficacy was detected in all type of LLINs used, as mortality rates were greater than 80%. In addition the decreased mortality rate in Gaschiga with PermaNet 3.0 is not significantly different to

Gounougou (X-squared = 0.0102, df = 1, p-value = 0.9196). Such observed efficacy could be related to the benefit linked to the combination deltamethrin-PBO (pyrethroid-synergist) in PermaNet 3.0. Indeed, PBO is a synergist that enhances the efficacy of pyrethroid insecticides by inhibiting enzymes that metabolise metabolic P450 (IRAC citation). It is clear from the study data that deltamethrin in combination with PBO reduced the deltamethrin tolerance level in all the species detected as previously documented (Fakoorziba et al. 2008). PBO itself is not in itself insecticidal but is added to insecticide formulations to increase the potential effect of insecticides and it has an important role in reducing the levels of resistance and, thus, insecticide application rates (Cetin et al 2010). The same observation was made with the Côte d'Ivoire bioassay results where evidence of decreased mortality was observed in *An gambiae*, with PermaNet® 3.0 in both Bouaké and Tiassalé.

7.0 Conclusions

PermaNet® 2.0, PermaNet® 2.0 Extra and PermaNet® 3.0 performed well in terms of the reduction of resistant *An. gambiae s.l* in all study sites in both countries.

In Tiassalé, where resistance to insecticide is mainly due to the metabolic gene P450 (as a recent published paper stated that there was no link between Kdr and resistance to pyrethroids), PermaNet® 3.0 performed better than PermaNet® 2.0 Extra and PermaNet® 2.0 which appeared to offer comparable efficacy in terms of personal protection. However, a clear limitation of the study has been the failure to collect sufficient numbers of mosquitoes from the study sites in Cameroon (which appears to have been due to unusual drought conditions) and there being too few study villages overall (a limitation for control of possible demographical and ecological confounders) and has resulted in part, statistical under powered. would be useful to replicate furthermore such studies in more clusters which will help to confirm the performance of Permanet 3.0.

In Bouaké, performance of the three net types was similar.

All nets resulted in a significant reduction in sporozoite rates post-net distribution. Regarding the detailed results of this study, it can be concluded that in areas where the local population of An. gambiae s.s are resistant to pyrethroid insecticides through both metabolic and kdr-based resistance mechanisms, as it is the case in Tiassalé, the PermaNet 3.0 performs significantly better and offer better personal protection compared to PermaNet 2.0 and PermaNet 2.0 Extra.

PermaNet® 3.0 performance compared to those of PermaNet® 2.0 and PermaNet® 2.0 Extra is illustrated by the proportion of blood fed *An. gambiae s.s*. caught, the overall number of resistant *An. gambiae s.s* caught, the sporozoite rates of *An. gambiae s.s* and bio-assays results, while in the *kdr*-based resistant area such as Bouaké the performance of the three different net types was not significantly different.

These studies show the challenge in demonstrating differences in efficacy between net types in the field where one net type is a modification of an existing WHOPES recommended net, as many factors may influence the measurement of net performance and the differences between the performances of the nets in the field may be subtle.

Though the present study was statistically under powered, the main results addressed the main research questions underlined in the background chapter:

(i) Does PermaNet® 3.0 protect against pyrethroid resistant mosquitoes? Yes, although this may depend on the resistance mechanisms involved and other factors; PermaNet 3.0 in this study provided significantly better protection against the mosquitoes with *kdr* and metabolic mechanisms (in Tiassalé) than PermaNet 2.0 and PermaNet 2.0 Extra (as measured by the number of indoor resting and blood fed *An. gambiae s.s.*).

(ii) Where there is pyrethroid resistance, including metabolic and *kdr*-based resistance mechanisms, is there increased protection with PermaNet® 3.0 compared to PermaNet® 2.0 and PermaNet® 2.0 Extra? Yes, the results of the study indicate that under certain circumstances PermaNet 3.0 affords better protection (again as measured by the number of indoor resting and blood fed *An. gambiae s.s.*) over PermaNet 2.0 and PermaNet 2.0 Extra against the mosquito populations with *kdr* and metabolic mechanisms.

Finally, it would be advisable to conduct a community randomised trial statistically powered which will aim to confirm the performance of PermaNet 3.0 reported in the current study.

8.0 References

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Appendix I: Resistance Characterisation

WHO susceptibility test results before net distribution in Ivory Coast and Cameroon

In both study sites In Ivory Coast, *An. gambiae s.s.* was highly resistant to the commonly used insecticides in public health (DDT and pyrethroids). Similarly in both study sites in Cameroon, high resistance levels were recorded with *An. gambiae s.s* and *An. arabiensis* species exposed to pyrethroids. However, the mortality rates recorded after exposure to DDT were extremely high (> 96.0%) (see Table 1 for full results).

kdr and AChE genotyping using the pyrosequencing method

The L1014F and L1014S *kdr* mutations were genotyped in a set of permethrin, deltamethrin and DDT resistant mosquitoes from Gaschiga and Gounougou in northern Cameroon using the pyrosequencing method (Wondji *et al*, 2007). Additionally, all live mosquitoes from bioassays were screened for the presence of the acetylcholinesterase target-site mutation G119S (*Ace-1*) and the Rdl mutation using the pyrosequencing method (software provided by Pyrosequencing AB).

The sequences for genotyping and the dispensation order for both reactions are indicated in Table I.I. The lower case of nucleotide "c" and "a" indicates the negative control that should not be incorporated in the target DNA. The PCR reaction contained forward and biotinylated reverse primers (10 pmol), 1X HotStarTaq buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 U HotStarTaq (Qiagen) and 10 ng genomic DNA. The parameters for amplification were: 1 cycle at 95 °C for 5 min; 50 cycles of 94 °C for 20 s, 57 °C for 30 s and elongation at 72 °C for 20 s; followed by 1 cycle at 72 °C for 5 min.

Pyrosequencing reactions were performed as described by [16] according to the manufacturer's instructions using the PSQ 96 SNP Reagent Kit (Biotage AB) and the sequencing primer shown in Table I.I. The genotype was determined using the SNP Software (Biotage AB).

Absence of Kdr L1014 West in Northern Cameroon

In Gaschiga, all of the 68 females of *An. arabiensis* and 6 females of *An. gambiae s.s*. S form species that were genotyped were homozygotes for the susceptible allele of the L1014 West and East *Kdr* mutations. They were also all homozygotes for the G119 susceptible allele of the Ace-1 gene (Table I.II). These results confirm the absence of *Kdr* mutations in *An. gambiae s.*s and *An. arabi*ensis from these areas.

In Gounougou, 65 female *An. arabiensis* and 5 female *An. gambiae s.s*. S form tested were homozygotes for the susceptible *Kdr* and *Ache* alleles; two *An. arabiensis kdr* heterozygotes were found. The absence of *Kdr* and *Ache* mutations in these malaria vectors strongly suggests the involvement of other resistance mechanisms such as metabolic resistance.

Table I.II. Frequencies of *Kdr, Ache* and *Rdl* in *An. arabiensis* and *An. gambiae s.s* from Gaschiga and Gounougou, in northern Cameroon

Study sites	Status	Insecticide tested	Number tested	*Kdr (%)*	*Ache (%)*	Rdl (%)	
						R	S
Gaschiga	Alive	DDT	4	0.0	0.0	0.0	100
		Deltamethrin	25	0.0	0.0	8.0	92.0
		Permethrin	25	0.0	0.0	0.0	100
	Dead	DDT	20	0.0	0.0	10.0	90.0
		Deltamethrin	25	0.0	0.0	0.0	100
		Permethrin	25	0.0	0.0	12.5	87.5
Gounougou	Alive	DDT	2	0.0	0.0	0.0	100
		Deltamethrin	25	0.0	0.0	8.0	92.0
		Permethrin	25	0.0	0.0	0.0	100
	Dead	DDT	20	0.0	5.0[h]	10.0	85.0
		Deltamethrin	25	0.0	10.0[h]	0.0	90.0
		Permethrin	25	0.0	0.0	12.5	87.5

h: heterozygote; R: resistant; S: susceptible

Characterisation of metabolic resistance in Deltamethrin resistant populations of Anopheles species from Ivory Coast and Cameroon

Clare Strode, Benjamin Koudou and John Morgan

LSTM

Introduction

Mosquitoes from the four study sites Tiassalé, Bouaké (Ivory Coast), Gounougou and Gaschiga (Cameroon) were scrutinised against a whole genome *Anopheles gambiae* microarray (~15,000 transcripts, Agilent technologies) to identify genes putatively involved in conferring deltamethrin resistance.

Methods

In order to minimise intra population variation 5 day old non-blood fed females characterised as *An. gambiae s.s.* M form, which had survived one hour of 0.05% deltamethrin exposure and were homozygous for the L1014F *kdr* allele were included in the microarray study involving the Tiassalé and Bouaké populations. Similarly 5 day old non-blood fed females from Gounougou and Gaschiga characterised as *An. arabiensis* which had survived one hour of deltamethrin exposure and were homozygous for the L1014F *kdr* allele were subjected to microarray analysis.

A lab-reared susceptible M-form colony of *An. gambiae,* Ngousso, which originated from Cameroon, was used for competitive hybridisation with the Tiassalé and Bouaké populations. In the case of the Gounougou and Gaschiga populations these were competitively hybridised with a laboratory susceptible *An. arabiensis* strain MOZ which originated from Mozambique. Three biological replicates of each population were screened along with accompanying dye swaps

Array data was subjected to a LOWESS normalisation. Following statistical analysis incorporating a student's t-test and Benjamini & Hochberg post hoc testing, genes were considered to be significantly differentially expressed between the field resistant and lab susceptible populations if they demonstrated both a $p<0.001$ and >2 fold change in expression in either direction.

Microarray Results

The *An. arabiensis* populations from Gounougou and Gaschiga showed the greatest number of differentially expressed transcripts compared to the susceptible strain (Table I.III). The increase in differential expression in the *An. arabiensis* populations compared with the *An. gambiae* mosquitoes from Tiassalé and Bouaké could be attributed to the fact that the microarray platform was designed using the *An. gambiae* genome (version AgamP3.6, source Vectorbase).

The Bouaké population demonstrated the least amount of differential expression across all four populations with only 68 genes involved. Table I.IV provides a summary of the number of differentially expressed genes from the 3 enzyme families associated with metabolic resistance (cytochrome P450s, glutathione transferases (GSTs) and carboxyl esterases (COEs)) and the ABC transporter family. Also included are genes associated with oxidative stress responses. With the exception of mosquitoes from Bouaké, the other three populations exhibited significant P450 activity, with Tiassalé mosquitoes in particular presenting the largest number of over expressed P450s. Less prominent were the number of GST and COEs involved in over expression in the resistant populations. A single detox gene, *GSTD1-4* was found to be over expressed in the Bouaké population. Overall the metabolic associated gene with the highest level of over expression was observed in the Gaschiga population with *GSTe2* (237 fold). *ABCB4* was the only gene to be over expressed in all four populations.

The majority of transcripts on the microarray did not have any annotation. In these cases Gene Ontology (GO) terms were derived from inputting the transcripts retrieved from BioMart into Blast2go® software.

Table I.III. Summary of the genetic characteristics of four Anopheline populations from West Africa including the number of significant genes differentially expressed compared with insecticide susceptible mosquitoes.

Population	Tiassalé (Ivory Coast)	Bouaké (Ivory Coast)	Gounougou (Cameroon)	Gaschiga (Cameroon)
Species	*An. gambiae*	*An. gambiae*	*An. arabiensis*	*An. arabiensis*
Molecular form	M (100%)	M (93%) S (7%)	95.4	83.0
kdr frequency (L1014F)	82%	100%	0.0	0.0
No. Significantly over expressed genes	406	42	563	737
No. Significantly under expressed genes	243	26	851	870
over expressed P450	21	0	13	3
over expressed GST	5	1	3	5
over expressed COE	3	0	1	1

Table I.IV. Details of the detoxification genes significantly over expressed (p<0.001) in deltamethrin resistant populations of *Anopheles* from Ivory Coast and Cameroon compared with susceptible mosquitoes. Fold change figures are given as absolute values and are presented in order of magnitude.

	Tiassalé		Bouaké		Gounougou		Gaschiga	
	Gene	Fold change	Gene	Fold change	Gene	Fold change	Gene	Fold change
P450	*CYP6P4*	16.58			*CYP9J5*	19.62	CYP9J5	6.91
	CYP6Z3	16.41			*CYP12F2*	8.56	*CYP6Z3*	5.36
	CYP6Z2	13.27			*CYP6M1*	6.37	*CYP12F2*	3.06
	CYP325F1	12.91			*CYP6N1*	4.73		
	CYP6M2	11.76			*CYP6M4*	3.79		
	CYP6N2	9.70			*CYP6Z3*	3.23		
	CYP6P3	8.71			*CYP6Z2*	3.10		
	CYP4H17	7.69			*CYP6P2*	3.06		
	CYP6P5	5.64			*CYP6AA1*	2.48		
	CYP6P2	4.72			*CYP6M3*	2.40		
	CYP4D22	4.39			*CYP4H24*	2.33		
	CYP314A1	3.79			*CYP306A1*	2.04		
	CYP6AA1	3.25			*CYP4H17*	2.04		
	CYP9L3	2.77						
	CYP9L1	2.74						
	CYP6P1	2.67						
	CYP6AH1	2.66						
	CYP6Z1	2.39						
	CYP6M3	2.30						
	CYP6AG1	2.28						
	CYP307A1	2.11						
GST	*GSTD1_4*	3.63	*GSTD1_4*	11.45	*GSTE4*	16.60	*GSTE2*	237.16
	GSTD3	2.90			*GSTE3*	11.49	*GSTE4*	47.56
	GSTD1_3	2.66			*GSTD1_5*	3.04	*GSTE3*	9.46
	GSTMS1	2.30					*GSTD1_5*	2.25
	GSTD7	2.20					*GSTD1_6*	2.04
COE	*COEAE6O*	6.08						
	COEBE4C	2.64			*COEunkn*	32.03	*COEJHE5E*	15.93
	COE13O	2.51						
ABC	*ABCB4A*	3.45	*ABCB4*	3.97	*ABCB4A*	8.02	*ABCB4*	7.22
					ABCC12	2.56		
Redox	*CAT1*	2.30			*TPX3*	14.61	*TPX2*	7.13
	Aldehyde_oxidase	2.35			*TPX2*	3.22	*TRX1*	5.86
	SOD3B	2.82			*SP11644*	2.51	*SP11644*	3.36
					PX16	2.09	*PX11*	2.78
					PX10	2.03	*TRX3*	2.04

Discussion

All four populations screened in this study showed a high level of deltamethrin resistance and expressed a high frequency of the West African *kdr* allele (L1014F). However the heterogeneity in the resistance level observed even in mosquitoes that are homozygous for the *kdr* allele indicated that additional resistance mechanisms are involved. This is supported by the microarray data which suggests a putative role for metabolic resistance and cellular removal of insecticides via ABC transporters in the Tiassalé, Gounougou and Gaschiga populations. The evidence for metabolic resistance in the Bouaké population is less tangible given the over expression of a single GST (*GSTD1-4*), although this gene was also observed to be over expressed in the Tiassalé mosquitoes. In the case of the Bouaké population this is the first time we have screened a resistant population by microarray and not found evidence of P450 over expression.

A number of the candidate genes identified in the Tiassalé populations have also been found in pyrethroid resistant population of *An. gambiae* from West Africa. For example, *CYP6P3* is > 8 fold over expressed in the Tiassalé population. This gene has been implicated in pyrethroid resistance in *Anopheles* sp. From Benin and Nigeria (Djouaka *et al*. 2008) and Ghana (Muller *et al*. 2008) and the enzyme has been shown to metabolise pyrethroids. Furthermore, the ortholog of *CYP6P3* in *An. funestus*, *CYP6P9,* has been genetically linked to pyrethroid resistance in this mosquito species (Amenya *et al*. 2008; Wondji *et al*. 2007). *CYP6M2,* over expressed > 11-fold in the current study is also a lead candidate pyrethroid resistant population of *An. gambiae* from West Africa. More recently this gene has been implicated with DDT resistance (Mitchell, unpublished). Also of note is the finding that three P450s, *CYP6Z1, CYP6Z2* and *CYP6Z3* were significantly expressed in the Tiassalé mosquitoes. Indeed *CYP6Z3* was observed in the two *An. arabiensis* populations. These three P450s are tightly clustered on chromosome 3R (Nikou, Ranson & Hemingway 2003) and have been genetically linked to pyrethroid resistance (Ranson *et al*. 2004).

All four populations demonstrated GST over expression. In fact the metabolic gene with the greatest fold change overall was *GSTe2* (Gaschiga). This gene is commonly associated to DDT resistance in mosquitoes (Fonseca-Gonzalez *et al*. 2011; Ortelli *et al*. 2003). Whilst GSTs are not believed to be directly involved in pyrethroid metabolism they offer protection against oxidative stress which can is an indirect consequence of insecticide activity (Vontas, Small & Hemingway 2001). GSTs may also play a passive role in sequestering pyrethroids, thereby reducing the circulating levels of the active insecticide (Kostaropoulos *et al*. 2001).

A cohort of genes involved in an oxidative stress response (eg. peroxidises, thioperoxidases and super oxide dismutase) were also differentially expressed in the Tiassalé and Gounougou mosquitoes. Insecticides cause oxidative stress in mosquito cells so it's unsurprising to see genes involved in counterbalancing the activity of reactive oxygen species to be on the list of significant genes.

The striking result from the study is that only one gene, *ABCB4,* exhibited over expression in all four populations regardless of the species or geographical location. *ABCB4* is a member of the ABCB subfamily of ATP-binding cassette genes (ABCs). ABCs encode primary active transporter proteins which bind and hydrolyze ATP the energy from which is used to pump compounds across the membrane or to flip molecules from the inner to the outer leaflet of the membranes (Dean & Annilo 2005). They are known to confer drug resistance in humans and parasites by actively pumping drugs out from the cell. In humans the *ABCB4* gene encodes the multi-drug resistant protein 3 (mdr3) (Smith *et al*. 2000).

An. gambiae houses 44 ABC transporter genes of which the number of members of the ABCB subfamily, which stands at five, is truncated compared with humans and *Drosophila* (Roth *et al*. 2003). The role of ABC transporters in insecticide resistance has not been fully explored. They have been linked to temephos and diflubenzuron resistance in the mosquito *Aedes caspius* (Porretta *et al*. 2008). ABC transporters have recently been associated with resistance to *Bti,* although in this instance the resistance is based on a mutation which prevents cry toxins from binding to the ABC which otherwise assists in its pore forming properties (Gahan *et al*. 2010). Based on the results from the microarray study we can speculate that *ABCB4* is linked to the removal of deltamethrin from mosquito cells.

For the vast majority of gene transcripts which lacked annotation, GO was determined by screening against other genome databases. Further *in silico* analysis will be required to identify other pathways that may be associated with resistance. In all mosquito populations putative members of the UDP-glucuronosyltransferases were observed to be over expressed. These genes belong to a family of enzymes involved in phase II detoxification of xenobiotics.

In summary, deltamethrin resistance in the West African populations of Anopheline mosquitoes appears to multifaceted. It cannot be attributed solely to *kdr* at least not for mosquitoes from Tiassalé, Gounougou and Gaschiga. Metabolic resistance appears to be a contributing factor in these populations which a particular emphasis on P450-based activity. Mosquitoes from Bouaké however do not appear to employ P450s as a defence mechanism. A potential role for transporter-based resistance is evident in all of the four deltamethrin resistant populations.

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Appendix II: Tabulated data and statistical analysis of exit trap and resting catch data

Table II.I. Unadjusted and adjusted total mean *An. gambiae s.s from* exit traps (aggregated over 7 days) by net type – Bouaké

Period	Mean values (95% confidence intervals)						p-values: raw (adjusted for baseline)		
	unadjusted			adjusted for baseline differences					
	P2	P2extra	P3	P2	P2extra	P3	P2 vs P2extra	P2 vs P3	P2extra vs P3
Baseline	26.73 (20.22 – 33.23)	28.09 (24.10 – 32.09)	28.18 (23.87 – 32.49)	---	---	---	0.735 (---)	0.724 (---)	0.976 (---)
January	6.55 (5.70 – 7.39)	5.27 (4.12 – 6.43)	5.91 (4.89 – 6.93)	6.57 (5.73 – 7.42)	5.26 (4.09 – 6.43)	5.88 (4.91 –6.85)	0.104 (0.101)	0.365 (0.323)	0.435 (0.432)
February	8.18 (6.90 – 9.46)	9.00 (7.87 – 10.13)	10.09 (8.84 – 11.34)	8.20 (7.07 – 9.32)	8.93 (7.88 – 9.98)	10.04 (8.74 –1.35)	0.362 (0.365)	**0.044 (0.043)**	0.213 (0.200)
March	16.00 (14.43 – 17.57)	14.64 (13.59 – 15.68)	14.91 (12.35 – 17.47)	15.91 (14.51 – 17.31)	14.67 (13.61 – 15.73)	14.92 (12.45 –17.39)	0.161 (0.153)	0.495 (0.550)	0.849 (0.843)
April	19.91 (17.09 – 22.73)	19.36 (18.00 – 20.73)	20.55 (17.31 – 23.79)	19.84 (17.16 – 22.51)	19.42 (17.94 – 20.90)	20.52 (17.53 –23.52)	0.737 (0.724)	0.776 (0.691)	0.511 (0.492)
May	17.55 (15.91 – 19.19)	19.55 (18.35 – 20.74)	19.55 (16.70 – 22.39)	17.52 (15.88 – 19.16)	19.55 (18.43 – 20.67)	19.55 (16.78 –22.33)	**0.064 (0.050)**	0.232 (0.230)	1.000 (0.995)
June	12.18 (10.00 – 14.36)	9.91 (8.41 – 11.41)	12.91 (10.23 – 15.59)	12.17 (10.08 – 14.26)	9.92 (8.39 – 11.45)	12.90 (10.29 –15.52)	**0.092 (0.070)**	0.686 (0.626)	**0.049 (0.050)**
July	8.73 (6.86 – 10.60)	9.18 (7.65 – 10.71)	10.00 (8.10 – 11.90)	8.65 (6.87 – 10.43)	9.23 (7.63 – 10.82)	9.98 (8.26 – 11.70)	0.720 (0.681)	0.363 (0.285)	0.518 (0.504)
August	10.18 (9.35 – 11.01)	10.00 (9.13 – 10.87)	9.73 (8.78 – 10.67)	10.19 (9.36 – 11.02)	10.00 (9.12 – 10.88)	9.72 (8.78 – 10.66)	0.773 (0.777)	0.490 (0.457)	0.685 (0.685)
September	13.91 (11.51 – 16.31)	12.73 (11.21 – 14.24)	14.09 (12.25 – 15.93)	13.89 (11.83 – 15.95)	12.72 (11.15 – 14.28)	14.09 (12.13 –16.05)	0.417 (0.350)	0.908 (0.970)	0.270 (0.261)
October	11.36 (9.59 – 13.13)	10.73 (9.72 – 11.74)	12.00 (9.69 – 14.31)	11.36 (9.63 – 13.09)	10.73 (9.71 – 11.75)	12.00 (9.69 – 14.31)	0.544 (0.510)	0.673 (0.715)	0.316 (0.276)
November	2.27 (1.26 – 3.28)	2.27 (1.09 – 3.46)	2.00 (1.29 – 2.71)	2.27 (1.25 – 3.30)	2.28 (1.09 – 3.46)	1.99 (1.27 – 2.71)	1.000 (0.988)	0.668 (0.682)	0.698 (0.693)
December	0.36 (0 – 0.88)	0.45 (0.07 – 0.84)	0.82 (0 – 1.75)	0.34 (0 – 0.75)	0.45 (0.05 – 0.85)	0.83 (0 – 1.82)	0.798 (0.612)	0.397 (0.387)	0.430 (0.416)

Table II.II. Unadjusted and adjusted total mean *An. gambiae s.s* counts (aggregated over 7 days) by net type – Tiassalé

Period	Mean values (95% confidence intervals)						p-values: raw (adjusted for baseline)		
	unadjusted			adjusted for baseline differences					
	PermaNet® 2.0	PermaNet® 2.0 Extra	PermaNet® 3.0	PermaNet® 2.0	PermaNet® 2.0 Extra	PermaNet® 3.0	P2 vs P2extra	P2 vs P3	P2extra vs P3
Baseline	400 (178-622)	383 (114-652)	718 (347-1088)	---	---	---	0.926 (---)	0.139 (---)	0.166 (---)
January	529 (128-930)	136 (66-206)	334 (94- 574)	345 (204-486)	168 (81-256)	181 (4-318)	**0.004** (0.110)	0.397 (0.142)	**0.051** (0.608)
February	216 (23-410)	55 (29- 81)	168 (72- 265)	147 (59-236)	63 (30- 96)	142 (33-250)	**0.010** (0.231)	0.648 (0.978)	**0.004** **(0.027)**
March	123 (56-190)	60 (35- 85)	116 (47- 186)	106 (69-143)	71 (33-108)	71 (30-111)	**0.046** (0.195)	0.897 (0.183)	**0.082** (0.792)
April	143 (34-252)	62 (14-110)	146 (59- 233)	117 (62-172)	79 (11-148)	100 (52-149)	0.139 (0.469)	0.967 (0.585)	**0.092** (0.389)
May	170 (60-281)	68 (46- 90)	88 (43- 133)	151 (90-213)	77 (46-107)	68 (25-112)	**0.016** **(0.025)**	0.124 **(0.040)**	0.422 (0.988)
June	214 (133-294)	114 (76-151)	234 (15- 453)	210 (148-271)	145 (79-211)	138 (50-225)	**0.016** **(0.087)**	0.864 (0.185)	0.163 (0.892)
July	183 (120-245)	82 (51-113)	129 (0- 259)	180 (130-230)	103 (49-157)	89 (37-141)	**0.003** **(0.039)**	0.530 **(0.010)**	0.426 (0.808)
August	177 (118-236)	125 (63-188)	204 (15- 392)	188 (122-255)	152 (59-246)	125 (27-222)	0.269 (0.347)	0.786 (0.280)	0.376 (0.637)
September	192 (48-335)	144 (73-215)	266 (0- 582)	174 (66-280)	210 (41-379)	152 (16-287)	0.544 (0.924)	0.655 (0.687)	0.363 (0.804)
October	226 (113-339)	43 (28- 57)	191 (47- 335)	221 (113-329)	45 (25- 65)	186 (36-337)	**<0.001** **(<0.001)**	0.724 (0.721)	**0.001** **(0.001)**
November	159 (69-248)	59 (43- 76)	87 (47- 128)	158 (70-246)	60 (48- 73)	83 (41-125)	**0.003** **(0.002)**	0.121 (0.113)	0.178 (0.294)
December	309 (187-431)	80 (57-103)	234 (100- 368)	296 (180-412)	82 (59-105)	232 (94-369)	**<0.001** **(<0.001)**	0.448 (0.522)	**0.002** **(0.002)**

Table II.III. Unadjusted and adjusted total mean blood fed *An. gambiae s.s* counts (aggregated over 7 days) by net type – Bouaké

Period	Mean values (95% confidence intervals)						p-values: raw (adjusted for baseline)		
	unadjusted			adjusted for baseline differences					
	P2	P2extra	P3	P2	P2extra	P3	P2 vs P2extra	P2 vs P3	P2extra vs P3
Baseline	9.00 (6.82 – 11.18)	12.18 (10.59 – 13.77)	8.91 (7.56 – 10.25)	---	---	---	**0.035** (---)	0.946 (---)	**0.003** (---)
January	0.73 (0.36 – 1.09)	1.00 (0.44 – 1.56)	1.09 (0.70 – 1.49)	0.75 (0.41 – 1.09)	0.81 (0.28 – 1.34)	1.18 (0.76 – 1.60)	0.419 (0.836)	0.209 (0.136)	0.804 (0.464)
February	2.27 (1.36 – 3.18)	3.91 (2.59 – 5.23)	1.64 (0.75 – 2.52)	2.30 (1.41 – 3.19)	3.69 (2.23 – 5.14)	1.70 (0.73 – 2.66)	**0.048** (0.181)	0.349 (0.360)	**0.009** **(0.031)**
March	3.91 (2.77 – 5.05)	4.09 (3.24 – 4.94)	3.64 (2.53 – 4.74)	3.96 (2.81 – 5.11)	3.97 (3.05 – 4.89)	3.69 (2.50 – 4.88)	0.808 (0.925)	0.742 (0.742)	0.541 (0.890)
April	3.09 (1.84 – 4.34)	3.73 (2.32 – 5.13)	3.64 (2.30 – 4.98)	3.14 (1.93 – 4.35)	3.37 (1.86 – 4.89)	3.86 (2.35 – 5.37)	0.516 (0.902)	0.569 (0.550)	0.929 (0.946)
May	3.82 (2.08 – 5.56)	5.18 (3.48 – 6.89)	4.73 (2.76 – 6.70)	3.98 (2.15 – 5.80)	4.27 (2.57 – 5.97)	5.15 (2.93 – 7.37)	0.299 (0.821)	0.508 (0.472)	0.741 (0.565)
June	2.55 (1.38 – 3.71)	2.09 (1.45 – 2.73)	2.64 (1.79 – 3.48)	2.39 (1.38 – 3.40)	2.27 (1.48 – 3.07)	2.53 (1.73 – 3.32)	0.494 (0.976)	0.904 (0.898)	0.317 (0.521)
July	2.45 (1.53 – 3.38)	1.82 (0.92 – 2.72)	3.82 (2.17 – 5.47)	2.45 (1.55 – 3.35)	1.82 (0.96 – 2.69)	3.81 (2.20 – 5.43)	0.356 (0.340)	0.139 (0.139)	**0.031** **(0.010)**
August	2.00 (1.29 – 2.71)	1.73 (0.72 – 2.74)	1.27 (0.91 – 1.64)	2.09 (1.40 – 2.78)	1.42 (0.55 – 2.30)	1.38 (0.97 – 1.78)	0.682 (0.293)	**0.058** **(0.061)**	0.369 (0.910)
September	2.18 (1.28 – 3.08)	2.00 (1.20 – 2.80)	2.36 (1.79 – 2.94)	2.18 (1.26 – 3.10)	2.01 (1.14 – 2.87)	2.36 (1.75 – 2.97)	0.772 (0.964)	0.750 (0.694)	0.494 (0.952)
October	4.73 (3.47 – 5.99)	4.64 (4.06 – 5.21)	4.73 (4.06 – 5.40)	4.76 (3.53 – 5.99)	4.55 (3.87 – 5.22)	4.78 (4.05 – 5.51)	0.900 (0.647)	1.000 (0.983)	0.844 (0.858)
November	1.36 (0.73 – 1.99)	0.64 (0.18 – 1.09)	1.55 (0.77 – 2.32)	1.34 (0.70 – 1.98)	0.66 (0.19 – 1.13)	1.51 (0.69 – 2.33)	**0.087** (0.112)	0.725 (0.725)	**0.052** (0.189)
December	0.18 (0 – 0.41)	0.18 (0 – 0.41)	0.64 (0 – 1.36)	0.19 (0 – 0.41)	0.16 (0 – 0.40)	0.67 (0 – 1.42)	1.000 (0.920)	0.157 (0.119)	0.157 (0.199)

Table II.IV. Unadjusted and adjusted total mean blood fed *An. gambiae s.s* counts (aggregated over 7 days) by net type – Tiassalé

Period	Mean values (95% confidence intervals)						p-values: raw (adjusted for baseline)		
	unadjusted			adjusted for baseline differences					
	PermaNet® 2.0	PermaNet® 2.0 Extra	PermaNet® 3.0	PermaNet® 2.0	PermaNet® 2.0 Extra	PermaNet® 3.0	P2 vs P2extra	P2 vs P3	P2extra vs P3
Baseline	59.3 (31.1-87.6)	37.5 (2.1-72.8)	91.1 (27.1-155.0)	---	---	---	0.404 (---)	0.331 (---)	0.147 (---)
January	20.3 (8.8-31.8)	11.1 (0 -22.4)	19.2 (9.3-29.0)	15.1 (9.8-20.4)	8.2 (5.0-11.3)	13.3 (5.5-21.1)	0.319 (**0.070**)	0.883 (0.648)	0.358 (0.172)
February	35.0 (9.6-60.4)	15.6 (8.1-23.1)	23.3 (12.7-33.9)	25.8 (11.7-39.9)	16.7 (10.0-23.4)	17.6 (10.6-24.6)	**0.075** (0.742)	0.361 (0.325)	0.244 (0.573)
March	12.3 (5.9-18.7)	7.1 (3.8-10.4)	10.5 (4.0-17.1)	10.8 (6.5-15.0)	7.4 (4.4-10.4)	9.1 (1.9-16.3)	0.128 (0.524)	0.713 (0.697)	0.324 (0.572)
April	17.4 (0.3-34.4)	9.9 (3.9-16.0)	10.5 (4.7-16.3)	13.3 (5.3-21.2)	10.5 (4.2-16.9)	7.6 (3.8-11.3)	0.351 (0.902)	0.386 (0.170)	0.902 (0.519)
May	30.5 (13.0-47.9)	13.5 (8.5-18.6)	9.8 (4.9-14.7)	27.5 (16.7-38.4)	14.8 (8.9-20.7)	8.7 (3.9-13.4)	**0.023** (0.117)	**0.004** **(0.001)**	0.314 (0.192)
June	25.4 (15.9-34.9)	17.8 (11.2-24.3)	9.6 (4.6-14.6)	24.0 (17.3-30.7)	19.6 (12.4-26.8)	8.5 (3.7-13.3)	0.190 (0.969)	**0.003** **(0.001)**	**0.064** **(0.034)**
July	17.9 (12.7-23.2)	10.0 (4.7-15.3)	7.8 (2.9-12.8)	17.3 (12.7-22.0)	10.8 (5.1-16.6)	7.2 (1.5-12.8)	**0.065** (0.471)	**0.024** **(0.035)**	0.572 (0.504)
August	10.0 (5.6-14.4)	10.0 (0 -20.0)	12.0 (4.8-19.2)	9.8 (6.0-13.6)	10.8 (0.1-21.5)	10.6 (3.1-18.2)	0.999 (0.679)	0.640 (0.826)	0.764 (0.996)
September	11.9 (3.6-20.2)	10.1 (0 -21.3)	11.1 (2.5-19.7)	10.7 (4.1-17.2)	12.3 (0 -25.6)	7.1 (2.4-11.9)	0.812 (0.531)	0.898 (0.396)	0.895 (0.464)
October	9.2 (4.1-14.4)	5.4 (0 -11.4)	12.8 (4.0-21.6)	9.0 (4.0-14.0)	6.4 (0 -13.5)	9.7 (5.5-13.9)	0.413 (0.779)	0.481 (0.807)	0.210 (0.498)
November	11.7 (6.8-16.6)	7.9 (3.7-12.0)	10.3 (5.8-14.7)	11.4 (6.9-15.9)	8.2 (3.8-12.6)	10.0 (5.4-14.7)	0.263 (0.408)	0.677 (0.689)	0.463 (0.612)
December	23.3 (20.0-26.6)	17.0 (9.0-25.0)	25.9 (19.7-32.1)	23.4 (20.6-26.1)	16.6 (8.5-24.7)	26.3 (19.8-32.8)	0.227 (0.191)	0.494 (0.406)	0.132 (0.152)

Table II. V. Unadjusted and adjusted total mean *An. gambiae s.s* collected within-household counts by net type – Bouaké

Period	Mean values (95% confidence intervals)						p-values: raw (adjusted for baseline)		
	unadjusted			adjusted for baseline differences					
	P2	P2extra	P3	P2	P2extra	P3	P2 vs P2extra	P2 vs P3	P2extra vs P3
Baseline	11.50 (7.15 – 15.85)	12.17 (8.73 – 15.61)	13.33 (8.68 – 17.99)	---	---	---	0.823 (---)	0.590 (---)	0.702 (---)
January	1.67 (1.29 – 2.04)	1.50 (0.89 – 2.11)	1.67 (1.29 – 2.04)	1.71 (1.23 – 2.19)	1.48 (0.98 – 1.97)	1.63 (1.24 – 2.02)	0.671 (0.623)	0.999 (0.970)	0.671 (0.841)
February	2.67 (1.78 – 3.55)	2.00 (1.54 – 2.46)	2.83 (2.28 – 3.38)	2.56 (1.76 – 3.36)	1.96 (1.62 – 2.29)	2.95 (2.16 – 3.73)	0.182 (0.139)	0.769 (0.697)	**0.030 (0.029)**
March	5.00 (3.87 – 6.13)	3.50 (2.49 – 4.51)	4.50 (1.86 – 7.14)	4.63 (4.00 – 5.26)	3.63 (2.07 – 5.19)	4.28 (1.95 – 6.60)	**0.067 (0.068)**	0.753 (0.943)	0.471 (0.424)
April	8.00 (6.10 – 9.90)	8.67 (7.09 – 12.14)	8.00 (5.78 – 10.22)	7.98 (5.95 – 10.02)	8.60 (7.18 – 10.03)	8.05 (5.86 – 10.24)	0.616 (0.599)	0.999 (0.993)	0.650 (0.742)
May	7.50 (6.13 – 8.87)	7.33 (5.56 – 9.10)	6.00 (4.33 – 7.67)	7.30 (6.18 – 8.42)	7.29 (5.48 – 9.09)	6.08 (4.53 – 7.63)	0.889 (0.959)	0.207 (0.281)	0.306 (0.358)
June	8.50 (6.50 – 10.50)	6.00 (4.61 – 7.39)	6.50 (5.30 – 7.70)	8.50 (6.29 – 10.71)	5.96 (4.60 – 7.31)	6.51 (5.60 – 7.42)	**0.047 (0.039)**	**0.092 (0.085)**	0.612 (0.365)
July	3.50 (2.89 – 4.11)	3.00 (2.08 – 3.92)	3.67 (2.47 – 4.86)	3.57 (2.84 – 4.30)	3.01 (2.05 – 3.97)	3.53 (2.47 – 4.60)	0.414 (0.434)	0.813 (0.972)	0.401 (0.471)
August	5.33 (3.69 – 6.98)	4.33 (3.58 – 5.09)	7.17 (4.88 – 9.45)	5.36 (3.74 – 6.99)	4.33 (3.64 – 5.02)	7.13 (4.75 – 9.51)	0.271 (0.223)	0.211 (0.222)	**0.009 (0.015)**
September	6.17 (5.31 – 7.02)	7.67 (5.72 – 9.61)	10.00 (8.78 – 11.22)	6.22 (5.45 – 6.98)	7.64 (5.84 – 9.43)	9.88 (8.50 – 11.27)	0.157 (0.140)	**<0.001 (<0.001)**	**0.076 (0.088)**
October	8.33 (6.82 – 9.84)	7.00 (4.99 – 9.01)	6.67 (3.87 – 9.46)	8.15 (6.64 – 9.66)	6.93 (5.11 – 8.74)	6.76 (3.98 – 9.54)	0.336 (0.371)	0.360 (0.435)	0.857 (0.924)
November	1.83 (0.98 – 2.69)	1.33 (0.45 – 2.22)	1.00 (0.08 – 1.92)	1.85 (0.99 – 2.71)	1.32 (0.47 – 2.17)	0.99 (0.07 – 1.90)	0.461 (0.418)	0.272 (0.251)	0.635 (0.619)
December	0.67 (0.07 – 1.26)	0.83 (0 – 1.69)	0.67 (0.07 – 1.26)	0.66 (0.07 – 1.24)	0.83 (0 – 5.02)	0.68 (0.04 – 1.32)	0.758 (0.728)	1.000 (0.989)	0.758 (0.743)

Table II.VI. Unadjusted and adjusted total mean *An. gambiae s.s* collected within-households counts by net type – Tiassalé

Period	Mean values (95% confidence intervals)						p-values: raw (adjusted for baseline)		
	unadjusted			adjusted for baseline differences					
	PermaNet® 2.0	PermaNet® 2.0 Extra	PermaNet® 3.0	PermaNet® 2.0	PermaNet® 2.0 Extra	PermaNet® 3.0	P2 vs P2extra	P2 vs P3	P2extra vs P3
Baseline	21.57 (15.31 – 27.83)	22.86 (12.73 – 32.99)	20.86 (13.98 – 27.73)	---	---	---	0.836 (---)	0.885 (---)	0.754 (---)
January	6.29 (2.89 – 9.69)	8.57 (3.13 – 14.01)	4.71 (1.98 – 7.45)	6.14 (2.53 – 9.74)	7.45 (3.72 – 11.18)	4.90 (1.60 – 8.21)	0.482 (0.695)	0.493 (0.502)	0.189 (0.332)
February	13.14 (1.27 – 25.01)	8.86 (5.96 – 11.75)	2.43 (0.54 – 4.32)	13.05 (1.26 – 24.83)	8.88 (5.91 – 11.86)	2.44 (0.55 – 4.33)	0.438 (0.674)	**0.008 (0.018)**	**0.004 (0.002)**
March	4.71 (1.41 – 8.02)	7.00 (3.31 – 10.69)	3.71 (1.52 – 5.91)	4.79 (1.32 – 8.26)	6.31 (4.01 – 8.61)	3.67 (1.30 – 6.04)	0.395 (0.503)	0.623 (0.614)	0.131 (0.145)
April	6.71 (2.64 – 10.79)	9.43 (4.28 – 14.57)	2.00 (0.81 – 3.19)	6.40 (2.46 – 10.33)	8.65 (5.50 – 11.80)	2.03 (0.75 – 3.31)	0.432 (0.415)	**0.007 (0.012)**	**<0.001 (<0.001)**
May	11.29 (9.13 – 13.44)	13.29 (8.90 – 17.67)	4.29 (1.99 – 6.58)	11.28 (9.13 – 13.44)	13.28 (9.03 – 17.53)	4.29 (1.98 – 6.60)	0.419 (0.392)	**0.001 (0.001)**	**0.001 (0.001)**
June	17.29 (14.49 – 20.08)	25.14 (14.20 – 36.08)	7.43 (5.02 – 9.83)	17.20 (14.65 – 19.75)	25.10 (15.06 –35.13)	7.17 (5.20 – 9.14)	0.127 **(0.097)**	**<0.001 (<0.001)**	**<0.001 (<0.001)**
July	16.86 (11.63 – 22.09)	32.57 (9.70 – 55.44)	7.86 (5.57 – 10.15)	17.75 (10.62 – 24.88)	27.47 (15.95 –38.99)	7.66 (5.31 – 10.01)	0.105 (0.157)	**0.001 (0.001)**	**<0.001 (<0.001)**
August	11.71 (9.04 – 14.39)	18.14 (13.37 – 22.92)	9.14 (5.50 – 12.78)	11.70 (9.02 – 14.39)	18.07 (13.25 –22.89)	9.18 (5.49 – 12.87)	**0.018 (0.009)**	0.308 (0.231)	**0.007 (0.009)**
September	17.43 (11.68 – 23.18)	20.14 (13.94 – 26.35)	5.00 (3.19 – 6.81)	17.15 (11.91 – 22.40)	20.36 (13.24 –27.47)	4.97 (3.22 – 6.72)	0.545 (0.530)	**<0.001 (<0.001)**	**<0.001 (<0.001)**
October	21.57 (16.16 – 26.98)	20.00 (15.70 – 24.30)	1.29 (0 – 2.80)	21.92 (15.67 – 28.17)	20.46 (14.11 –26.82)	1.20 (0 – 2.53)	0.666 (0.675)	**<0.001 (<0.001)**	**<0.001 (<0.001)**
November	28.14 (19.86 – 36.42)	21.00 (19.19 – 22.81)	15.71 (6.39 – 25.04)	28.23 (19.73 – 36.72)	20.95 (18.34 –23.57)	15.53 (6.40 – 24.66)	**0.071 (0.068)**	**0.097 (0.079)**	0.361 (0.351)
December	24.14 (19.48 – 28.80)	29.43 (19.44 – 39.42)	16.14 (10.69 – 21.59)	23.98 (19.87 – 28.08)	29.55 (19.06 –40.04)	16.13 (10.81 – 21.45)	0.338 (0.333)	**0.051 (0.033)**	**0.018 (0.018)**

Table II.VII. Unadjusted and adjusted mean of blood fed *An. gambiae s.s* collected within-household counts by net type – Bouaké

Period	Mean values (95% confidence intervals)						p-values: raw (adjusted for baseline)		
	unadjusted			adjusted for baseline differences					
	P2	P2extra	P3	P2	P2extra	P3	P2 vs P2extra	P2 vs P3	P2extra vs P3
Baseline	5.33 (2.73 – 7.93)	5.50 (3.61 – 7.39)	6.67 (3.51 – 9.82)	---	---	---	0.923 (---)	0.538 (---)	0.537 (---)
January	0	0.50 (0 – 1.11)	0.33 (0 – 0.71)	0	0.50 (0 – 1.08)	0.22 (0 – 0.58)	**<0.001 (<0.001)**	**<0.001 (<0.001)**	0.648 (0.472)
February	1.33 (0.45 – 2.22)	0.50 (0.10 – 0.90)	1.17 (0.45 – 1.88)	1.34 (0.50 – 2.18)	0.50 (0.11 – 0.89)	0.99 (0.32 – 1.65)	**0.077 (0.058)**	0.782 (0.511)	0.115 (0.210)
March	1.17 (0.45 – 1.88)	0.50 (0.10 – 0.90)	1.17 (0.45 – 1.88)	1.13 (0.44 – 1.82)	0.48 (0.09 – 0.88)	1.17 (0.50 – 1.84)	0.115 (0.113)	0.999 (0.861)	0.115 **(0.076)**
April	3.00 (1.87 – 4.13)	2.00 (1.54 – 2.46)	1.67 (0.47 – 2.86)	3.00 (1.81 – 4.18)	1.98 (1.53 – 2.43)	1.68 (0.49 – 2.86)	**0.085 (0.081)**	0.173 (0.162)	0.649 (0.749)
May	2.00 (1.35 – 2.65)	1.17 (0.45 – 1.88)	1.00 (0.35 – 1.65)	1.99 (1.29 – 2.70)	1.17 (0.43 – 1.91)	1.00 (0.36 – 1.63)	0.147 (0.115)	**0.075 (0.097)**	0.747 (0.824)
June	1.83 (1.28 – 2.38)	1.33 (0.58 – 2.09)	1.50 (0.89 – 2.11)	1.84 (1.40 – 2.29)	1.35 (0.56 – 2.14)	1.43 (0.81 – 2.05)	0.351 (0.320)	0.457 (0.294)	0.751 (0.844)
July	0.83 (0.54 – 1.13)	0.67 (0.07 – 1.26)	0.83 (0.12 – 1.55)	0.85 (0.53 – 1.16)	0.70 (0.03 – 1.37)	0.72 (0.08 – 1.37)	0.664 (0.670)	0.999 (0.727)	0.736 (0.896)
August	1.50 (0.73 – 2.27)	1.17 (0.45 – 1.88)	2.33 (1.34 – 3.33)	1.32 (0.60 – 2.03)	1.21 (0.31 – 2.10)	2.34 (1.36 – 3.31)	0.555 (0.577)	0.213 **(0.048)**	**0.083 (0.064)**
September	1.33 (0.45 – 2.22)	0.67 (0.07 – 1.26)	1.00 (0.35 – 1.65)	1.36 (0.35 – 2.37)	0.63 (0.09 – 1.17)	0.98 (0.42 – 1.54)	0.243 (0.248)	0.562 (0.578)	0.492 (0.279)
October	2.33 (0.82 – 3.84)	2.00 (0.47 – 3.53)	1.83 (0.66 – 3.00)	2.36 (0.86 – 3.85)	1.96 (0.41 – 3.51)	1.58 (0.52 – 2.64)	0.773 (0.772)	0.618 (0.388)	0.870 (0.576)
November	0.83 (0.12 – 1.55)	0.83 (0 – 1.80)	1.00 (0.08 – 1.92)	0.72 (0.09 – 1.36)	0.77 (0 – 1.75)	0.55 (0.06 – 1.03)	1.000 (0.885)	0.787 (0.670)	0.818 (0.607)
December	0.33 (0 – 0.93)	0.50 (0 – 1.11)	0.50 (0 – 1.11)	0.27 (0 – 0.73)	0.40 (0 – 0.79)	0.55 (0 – 1.31)	0.725 (0.549)	0.725 (0.682)	1.000 (0.913)

Table II.VIII. Unadjusted and adjusted mean blood fed *An. gambiae s.s* collected within-household counts by net type – Tiassalé

Period	Mean values (95% confidence intervals)						p-values: raw (adjusted for baseline)		
	unadjusted			adjusted for baseline differences					
	PermaNet® 2.0	PermaNet® 2.0 Extra	PermaNet® 3.0	PermaNet® 2.0	PermaNet® 2.0 Extra	PermaNet® 3.0	P2 vs P2extra	P2 vs P3	P2extra vs P3
Baseline	10.71 (8.45 – 12.98)	14.14 (8.88 – 19.40)	14.00 (6.62 – 21.38)	---	---	---	0.220 (---)	0.374 (---)	0.976 (---)
January	3.14 (0.92 – 5.36)	4.29 (1.76 – 6.81)	1.86 (0.16 – 3.56)	3.36 (1.31 – 5.40)	3.46 (1.58 – 5.34)	1.62 (0 – 3.25)	0.524 (0.868)	0.390 (0.263)	0.147 (0.194)
February	5.00 (1.64 – 8.36)	4.71 (3.36 – 6.07)	1.86 (0.25 – 3.46)	5.20 (1.91 – 8.50)	4.53 (3.04 – 6.03)	1.73 (0.11 – 3.34)	0.879 (0.699)	**0.088 (0.059)**	**0.053 (0.051)**
March	2.71 (0.42 – 5.01)	5.43 (1.93 – 8.92)	2.29 (0.93 – 3.64)	2.68 (0.39 – 4.97)	5.48 (1.91 – 9.05)	2.28 (0.92 – 3.64)	0.218 (0.255)	0.754 (0.771)	**0.062 (0.059)**
April	5.57 (1.88 – 9.26)	7.29 (3.60 – 10.97)	1.71 (0.61 – 2.81)	5.90 (2.06 – 9.75)	6.41 (3.04 – 9.77)	1.72 (0.57 – 2.87)	0.543 (0.885)	**0.016 (0.016)**	**0.001 (0.003)**
May	7.86 (5.71 – 10.01)	9.71 (6.71 – 12.72)	3.43 (1.23 – 5.63)	8.43 (6.12 – 10.74)	9.36 (6.48 – 12.23)	3.04 (1.29 – 4.79)	0.332 (0.471)	**0.025 (0.005)**	**0.006 (0.001)**
June	10.86 (8.37 – 13.34)	16.43 (10.77 – 22.08)	3.86 (2.78 – 4.94)	11.43 (9.21 – 13.65)	15.65 (10.17 –21.12)	3.61 (2.63 – 4.60)	**0.058** (0.181)	**<0.001 (<0.001)**	**<0.001 (<0.001)**
July	10.14 (5.85 – 14.43)	21.71 (3.45 – 39.98)	4.57 (2.52 – 6.62)	12.05 (6.82 – 17.27)	13.98 (6.96 – 21.00)	4.28 (1.84 – 6.72)	0.127 (0.996)	**0.015 (0.021)**	**0.002 (0.005)**
August	5.86 (3.71 – 8.01)	12.14 (8.80 – 15.49)	4.57 (2.41 – 6.74)	6.21 (3.75 – 8.66)	11.61 (8.68 – 14.54)	4.38 (2.30 – 6.47)	**0.003 (0.012)**	0.434 (0.379)	**0.001 (<0.001)**
September	12.29 (7.79 – 16.78)	13.29 (10.03 – 16.54)	2.57 (1.15 – 3.99)	11.57 (7.06 – 16.08)	13.85 (9.93 – 17.76)	2.50 (1.16 – 3.84)	0.737 (0.699)	**<0.001 (<0.001)**	**<0.001 (<0.001)**
October	16.14 (14.93 17.36)	14.14 (9.76 – 18.52)	0.92 (0 – 0.80)	15.87 (14.10 – 17.65)	14.26 (9.78 – 18.74)	0.29 (0 – 0.81)	0.433 (0.523)	**<0.001 (<0.001)**	**<0.001 (<0.001)**
November	13.71 (9.76 – 17.67)	16.00 (14.06 – 17.94)	6.86 (2.60 – 11.11)	12.05 (9.05 – 15.05)	16.94 (13.57 –20.32)	6.74 (2.35 – 11.13)	0.352 **(0.056)**	**0.056** (0.158)	**0.011 (0.009)**
December	15.57 (11.65 19.49)	17.43 (8.68 – 26.17)	10.43 (6.63 – 14.22)	16.27 (11.95 – 20.60)	16.96 (8.14 – 25.79)	9.98 (6.80 – 13.16)	0.705 (0.845)	**0.087 (0.032)**	0.118 **(0.095)**

Field Evaluation of PermaNet® 3.0 in controlling pyrethroid-resistant *Anopheles gambiae* in the Chirano Area, Western Region, Ghana

February 2013

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Executive Summary

The efficacy of insecticide treated and long lasting insecticidal nets (LLIN) in reducing human-vector contact, malaria morbidity and mortality has been shown in various epidemiological settings. PermaNet® 3.0 combination net is a new generation LLIN that was designed to give increased efficacy against pyrethroid-resistant malaria vectors. PermaNet® 3.0 (PN 3.0) contains a synergist, Piperonyl Butoxide (PBO) in the roof that works by inhibiting the metabolic enzymes the mosquito uses to sequester or break down the insecticide. This field trial was designed to evaluate the bio-efficacy of PN 3.0 against PermaNet® 2.0 (PN 2.0), a pyrethroid-only LLIN, under operational conditions by measuring *Anopheles* vector density, age structure and infection status in the study areas using human landing collections and indoor resting collections in sentinel houses. Resistance status in the study sites were evaluated using WHO susceptibility tests, biochemical assays and PCR for L1014F *kdr* genotypes for resistance to pyrethroids and DDT. Information on net use in each village was sampled using structured questionnaires prior to and immediately after LLIN distribution. Two intervention trial villages with similar ecological, demographic and insecticide resistance characteristics were selected for complete coverage with each LLIN type. Pre-trial baseline entomological information were collected two months prior to distribution and followed by six months post-intervention evaluation. Mortality rates of *Anopheles gambiae* tested in WHO susceptibility tests with pyrethroid insecticides increased significantly post-intervention in PN 3.0 sites compared to the baseline values. Mortality rates decreased or remained the same in PN 2.0 villages and no significant difference detected for the non-intervention control village. The L1014F kdr mutation was found in all villages at high frequency as well as overexpression of metabolic detoxifying enzymes; the control village and one PN 2.0 village showed elevated P450, GST and esterase activity, one PN 2.0 and one PN 3.0 village showed elevated P450 and esterase activity and one PN 3.0 village showed elevated GST and esterase activity. Significantly higher entomological impact was observed within PN 3.0 sites for *An. gambiae* post-intervention population than was found from the PN 2.0 sites in terms of human biting rate reductions and indoor resting catches. The reductions in parity rates, sporozoite rates and entomological inoculation rates at the PN 3.0 sites post-intervention were significantly higher than those for the PN 2.0 sites. This trial is the first to provide operational evidence on the improved bio-efficacy of PermaNet® 3.0 against pyrethroid-resistant *An. gambiae* field populations, over the conventional LLIN, PN 2.0. Further work on the operational impact of PN 3.0 on disease prevalence would be useful to determine the epidemiological/ health impact (i.e. disease prevalence estimations pre- and post-interventions) of such interventions in the presence of pyrethroid-resistant *Anopheles* vector populations.

Introduction

Background

The main malaria vectors in Ghana belong to the *Anopheles gambiae* complex, of which *An. gambiae s.s.* predominates. Other vectors such as *An. nili, An. funestus s.l.* and *An. melas* are important secondary vectors wherever found. Widespread resistance has been reported in populations of *An. gambiae s.l* and *An. funestus s.l.* to DDT, pyrethroids and carbamates (Anto *et al.* 2009; Klinkenberg *et al.* 2008; Adasi and Hemingway 2008; Stiles-Ocran, 2008; Muller *et al.* 2008; Coetzee, 2004; Yawson *et al.* 2004; Brooke *et al.* 2006; Coetzee *et al.* 2006; Koekemoer *et al.* 2006; Afrane *et al.* 2004; Kristan *et al.* 2003; Iyengar, 1963; Hunt 2004, 2011) (Figure 1A). The *kdr* mutation (L1014F) is present at relatively high frequency in *An. gambiae s.s.* (M & S forms) (Figure 1B) and the *Ace-1*^{R} mutation has also been reported in *An. funestus s.l.* from Obuasi. The *kdr* L1014S mutation has been assayed but not detected in *An. gambiae s.s.* (M & S forms). Metabolic resistance

mechanisms (esterases, oxidases and GSTs) have been documented at three sites in populations of *An. gambiae s.l.*

Year-round irrigation agriculture takes place in Ghana involving widespread use of pyrethroids (permethrin and lambda-cyhalothrin). These pyrethroids are also used in domestic sprays for mosquito and other pest control (Anto *et al.* 2009). Between 2009 and 2010, 5.6 million nets were procured and delivered to the country with an additional 8.5 million nets pledged in 2011. The National Malaria Control Strategic Plan targeted universal coverage with long lasting insecticidal nets (LLINs) by 2012 and the scale-up of indoor residual spraying (IRS) to a third of the population (170 districts) by 2015 (PMI, 2011). The universal coverage campaign commenced in December 2010 and ended in October 2012 with a total of 13.3 million nets distributed and coverage of 97% achieved. Prior to the development of the national strategic plan, IRS was implemented in a community-wide scale by mining companies (e.g. the Chirano Gold Mines Limited in the Sefwi-Chirano area and AngloGold Ashanti Ghana Limited in the Obuasi area) and the U.S. Agency for International Development (USAID) under the President's Malaria Initiative (PMI) in 5 districts in Northern Ghana. The PMI programme is now being extended to cover nine districts in the north. In collaboration with the Country Coordinating Mechanism (CCM) and the NMCP and with the help of the Global Fund, the AngloGold Ashanti malaria control programme in Obuasi is expected to be scaled-up to cover 40 more districts in Ghana by 2015. Pyrethroids (deltamethrin, lambdacyhalothrin and alphacypermethrin) have been used extensively in the past for vector control under the national malaria control strategy because their strong efficacy at low dosage, fast killing effect, low toxicity to humans, stability over time and relatively low cost of production (WHO, 2005). However, due to declining susceptibility by local vector populations, there are plans to switch from pyrethroids to organophosphates and carbamates in order to improve programme efficacy (PMI, 2011).

Synergists have been used commercially for about 50 years and have contributed significantly to improve the efficacy of insecticides, particularly when problems of resistance have arisen (Bernard and Philogène, 1993). The majority of synergists block the metabolic systems that would otherwise breakdown insecticides. Synergists have been used to overcome resistance to pyrethroids in several insect populations, for example PBO (piperonyl butoxide) inhibits P450s (oxidases) and esterases (Moores *et al.* 2005).

PermaNet® 3.0 is the first new generation long-lasting insecticidal net (LLIN) that was developed for use in areas with pyrethroid resistant malaria vectors, with a product claim of 'increased efficacy against pyrethroid-resistant malaria vectors'. PermaNet® 3.0 consists of a polyethylene roof incorporated with deltamethrin and PBO and polyester sides coated with deltamethrin. The efficacy of PermaNet® 3.0 will depend on the type and level of resistance mechanisms present in the target population. PermaNet® 3.0 has been tested in experimental huts against different field strains of vector species with a variety of resistance mechanisms. Results from these studies have shown a significantly improved efficacy when compared with mono-pyrethroid-treated long lasting nets and conventionally treated nets in terms of both mortality and/or personal protection in areas with *kdr* resistance and areas with metabolic based resistance (Adeogun *et* al, 2012; Corbel *et al.* 2010) although the increased efficacy in some areas declined after the nets had been washed 20 times (Koudou *et al*, 2011; N'Guessan *et al,* 2010).

Given the potential selection for pyrethroid resistance from exposure of mosquitoes to LLINs and/or agricultural insecticides, the present study sought to evaluate the efficacy of PermaNet® 3.0 versus PermaNet® 2.0 against the predominant malaria vector, *An. gambiae* s.s. and the community-wide impact on vector transmission indices.

Figure 1. (A) Maps of Ghana showing sites for which insecticide susceptibility tests were conducted on **An. gambiae *s.l.*** *and* **An. funestus *s.l.*** *collected between 2000 and 2012. Data is based on WHO susceptibility tests. ● = confirmed resistance; ● = possible resistance; ● = susceptibility. For sites for which multiple collections or insecticides were tested, the lowest susceptibility category is displayed [source: www.IRMapper.com]*

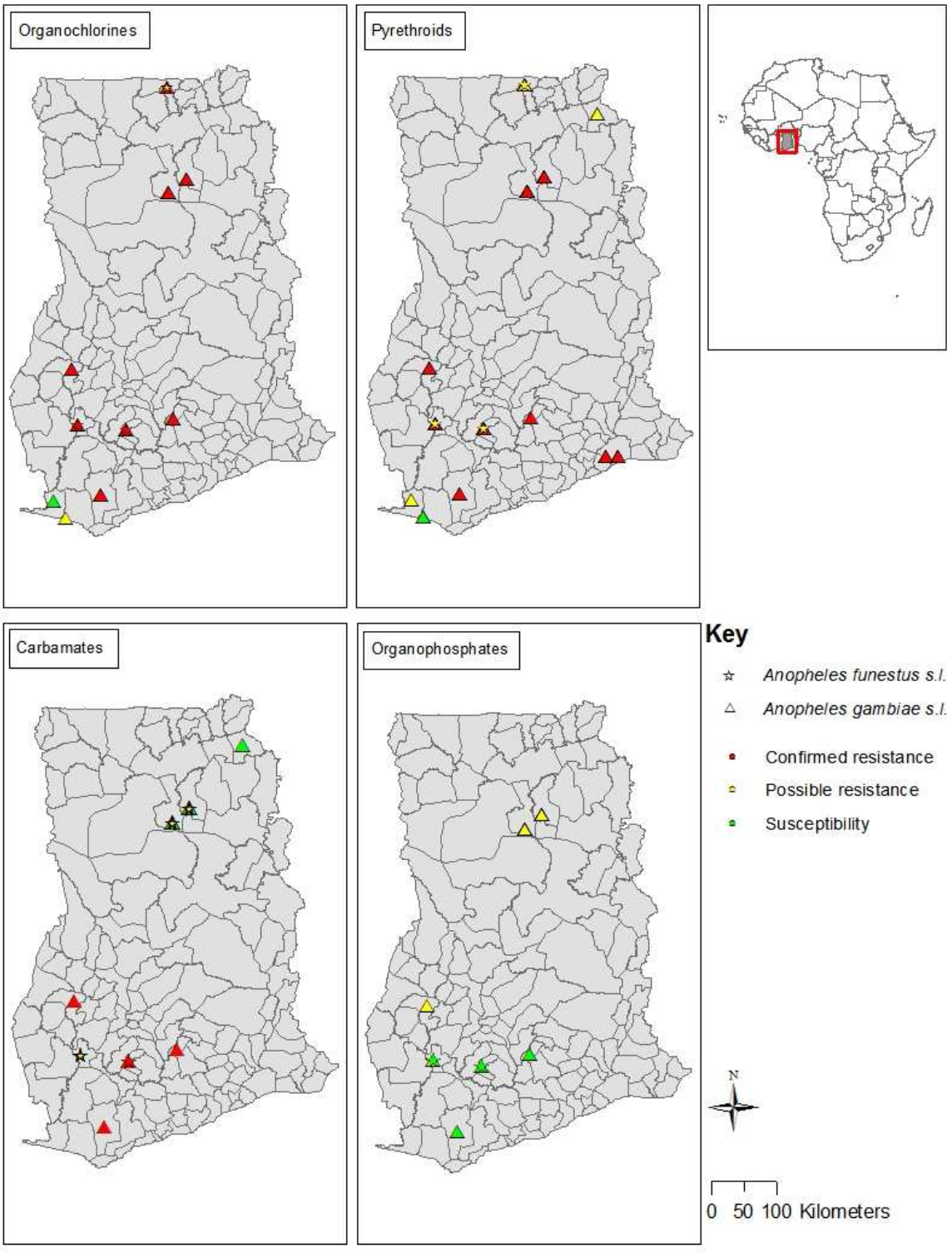

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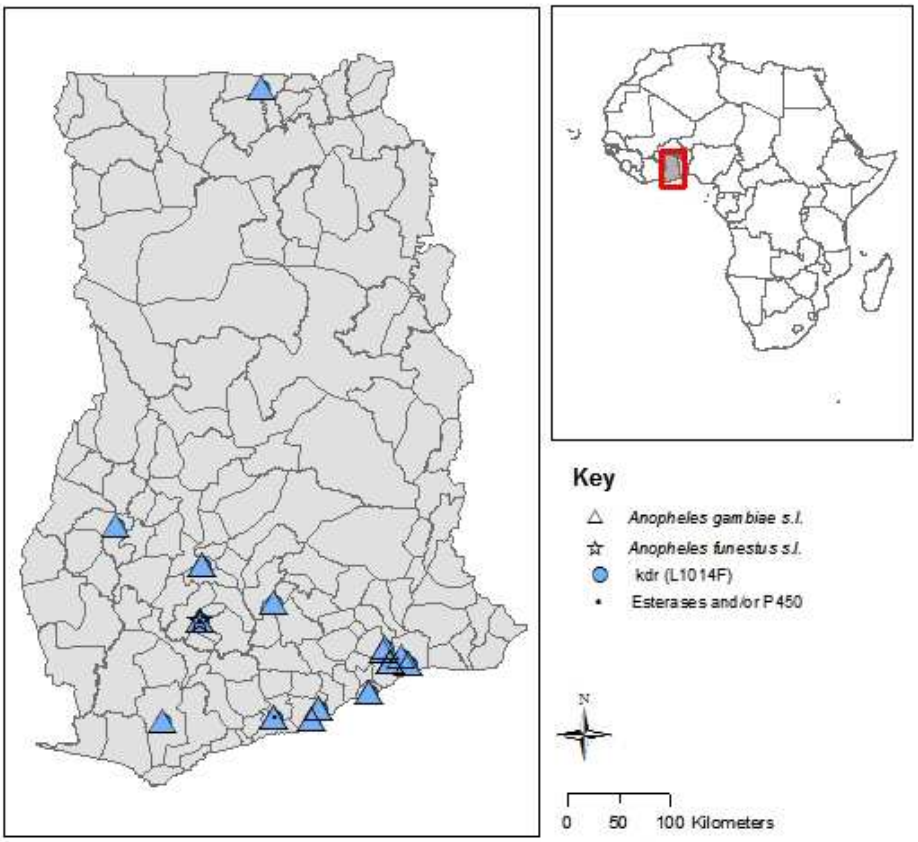
Figure 1. (B) Map of Ghana showing sites for which molecular / biochemical investigations of resistance mechanisms were conducted on **An. gambiae *s.l.*** *and* **An. funestus *s.l.*** *collected between 2002 and 2012. Data shown are for (a) kdr mutations (L1014F ●) and (b) Esterases and/or P450s (•). [source: www.IRmapper.com]*



Study Objectives

- Measure the operational impact of PermaNet® 3.0 (**PN 3.0**) and PermaNet® 2.0 (**PN 2.0**) on *Anopheles* vector and malaria parasite transmission indices (densities, blood feeding rates, vector age structure and sporozoite rates)
- Fully characterise the levels of phenotypic resistance in *Anopheles* populations as well as the resistance mechanisms
- Assess the impact of PN 3.0 and PN 2.0 with respect to the background resistance in the villages where those nets were tested

Materials and Methods

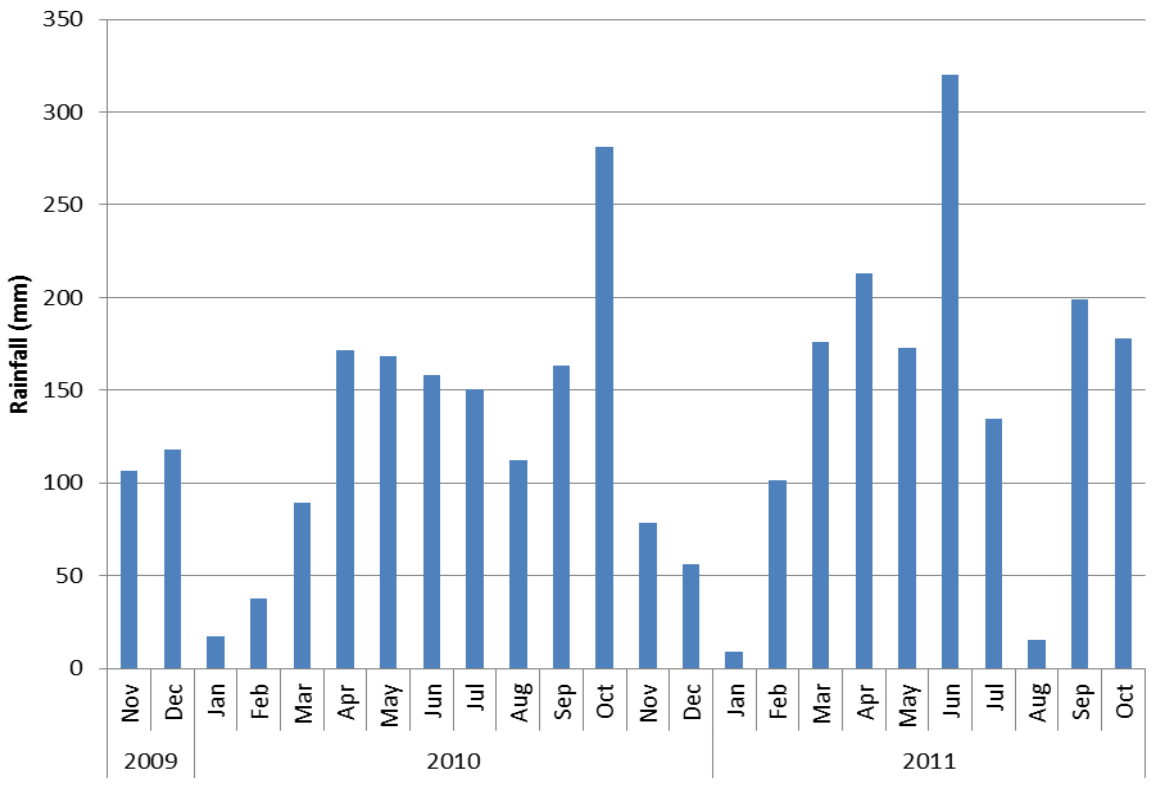
Study sites

The field evaluation was carried out in two districts in the Western Region of Ghana, Bibiani-Anhwiaso-Bekwai District (BABDA) and Sefwi Wiawso District (SWDA) where a pilot malaria control programme is being implemented by the Chirano Gold Mines Limited (a *Kinross Company*). BABDA is located in the equatorial climate zone and rain forest between latitude 6° N, 3° N and longitude 2° W, 3° W (Figure 2). The District is bounded on the West by the SWDA. The total land area of the

district is 873 km^2 and 2,634 km^2 respectively for BABDA and SWDA. Temperatures are uniformly high throughout the two districts and rainfall is heavy. The combination of the two translates into high relative humidity to support vector breeding and survival. The main malaria transmission season runs from March to December. The two main rainy seasons include the major rains from May to July and the minor rains from September to October; the trial was designed to capture these seasons for post-evaluation and baseline, respectively. December to March is usually dry, with rains usually commencing in mid-March to April; August is also usually dry (figure 2).

Baseline insecticide susceptibility studies were conducted in 10 villages using WHO tube tests with deltamethrin pre-treated filter papers at the discriminating dose (0.05%) (WHO, 1998) from November 2010 to January 2011. All mosquitoes tested were non-blood fed 2-5 day old females obtained via larval collection except for the mosquitoes from Ahokwaa which were first filial (F1) generation of indoor resting catches. Knockdown (KD) rates were measured after 5, 10, 15, 20, 30, 40, 50 and 60 minutes of exposure; KD rates observed for the wild mosquitoes at the end of the 60th minute ranged from 36% (95% CI: 27.3 – 44.1) at Wenchi to 88% (95% CI: 81.6 – 94.4) at Subiri. The non-overlap of the 95% confidence intervals estimated showed there were significant variations between the KD rates for these mosquito populations; KD rates from Anyinabrim, Wenchi, Abrabra, Futa, Kunkumso and Ahokwaa were lower than that of the Dwenase, Subiri, Awaso and Betekyere. One hundred percent KD rates were recorded for the reference susceptible *An. gambiae* s.s. Kisumu strain at the 50th minute. Mortality (M) rates for mosquito populations tested at baseline are shown in Table 1.

Figure 2. Rainfall (mm) recorded in the Chirano Area, Western Region, Ghana from 2009-2011.



Study Design

Two villages with relatively similar demographic and phenotypic resistance levels and located a minimum of 5km apart were selected from each of the two districts (Figure 3). Each of these villages were randomly assigned to an intervention arm and were provided with complete coverage with either of the LLINs PN 3.0[1] or PN 2.0[2]. The hang-up / installation of nets took place from mid-February to mid-March. Futa was selected to represent a non-treatment control arm to compensate for possible seasonal population fluctuations in vector indices under the influence of climate or natural declines. Futa was a community with no organized large scale malaria control intervention as applied to majority of communities within the two districts at the time as there were no immediate plans under the universal coverage for these areas from the NMCP level.

The baseline phenotypic resistance survey was carried out from November 2010 to January 2011; nets were then distributed in February 2011 and entomological field sampling was carried out in all trial villages every fortnight from March to August 2011 using HLC (*human landing catches*), IRC (*indoor resting catches*) and LC (*larval collections*) techniques. Field collectors were recruited from the trial villages, trained on entomological sampling techniques, and informed consent was obtained to undertake HLC, IRC and LC activities. Sentinel houses were selected for the HLCs and IRCs, based on data from initial baseline field collections. A power analysis was conducted to estimate exactly how many sentinel houses were required to carry out IRC to detect significant differences at the 5% level. GPS coordinates of all the study communities were recorded using a handheld GPS receiver (Garmin GPS MAP 96C).

Knowledge, attitudes and practices of residents within the four trial communities were studied prior to installation of the LLINs and two weeks after distribution to assess net use patterns. The National Malaria Control Programme was informed of the universal LLIN coverage in the study communities. Ethical clearance was obtained from the Noguchi Memorial Institute for Medical Research (NMIMR) for the trial in time for the baseline to start in November 2010.

Human Landing Catches

All night HLCs were carried out every fortnight within trial villages. Two matched houses were selected per village based on the attractiveness to *Anopheles* sp. from baseline studies. In addition, one adjacent house to each selected house was included to correct for variations in individual house attractiveness to mosquitoes. Indoor and outdoor HLCs were carried out in all four sentinel houses from 18:00 to 06:00. A one-man-indoor one-man-outdoor sampling approach was adopted and collectors were rotated hourly between indoor and outdoor within sentinel houses each night as well as between sentinel houses within trial villages to compensate for differences in individual attraction or repulsion for mosquitoes. Mosquitoes landing on the collectors were detected using a flashlight, aspirated and placed in paper cups covered by mesh screen (WHO, 1975). Collections were made for 50 minutes each hour (with 10 minutes changeover and stress release time). All collectors were screened weekly and given malaria treatment during the period of the study.

[1] PermaNet® 3.0 consists of a 100 denier polyethylene roof incorporated with deltamethrin (4g/kg) and PBO (25g/kg) and 75 denier polyester sides coated with deltamethrin (2.8g/kg)
[2] PermaNet® 2.0 consists of 100 or 75 denier polyester coated with deltamethrin (1.4 and 1.8g/kg respectively)

Indoor Resting Catches

IRCs were carried out by the field collectors in the early morning from 06:00 to 09:00 hrs in sentinel houses within each trial village once every fortnight using manual aspirators by searching on the walls and ceilings of rooms and any hanging material by a team of 2 for 10 minutes. The number of people who slept in those rooms in which mosquitoes were obtained the previous night before the survey was also recorded. In total, approximately 40 houses were sampled in each village. Mosquitoes were identified and scored as blood-fed or unfed (male mosquitoes were not recorded). All *An. gambiae* complex and *An. funestus* group species were preserved for molecular studies.

Figure 3. Map of Ghana showing Bibiani-Anhwiaso-Bekwai District (BABDA) and Sefwi Wiawso District (SWDA) where baseline WHO susceptibility tests were conducted in 10 villages in order to select 5 villages for the study (one non-intervention village [□]; two PermaNet® 2.0 villages [◊]; two PermaNet® 3.0 villages [○]; [■] represents all remaining villages included in the baseline survey.

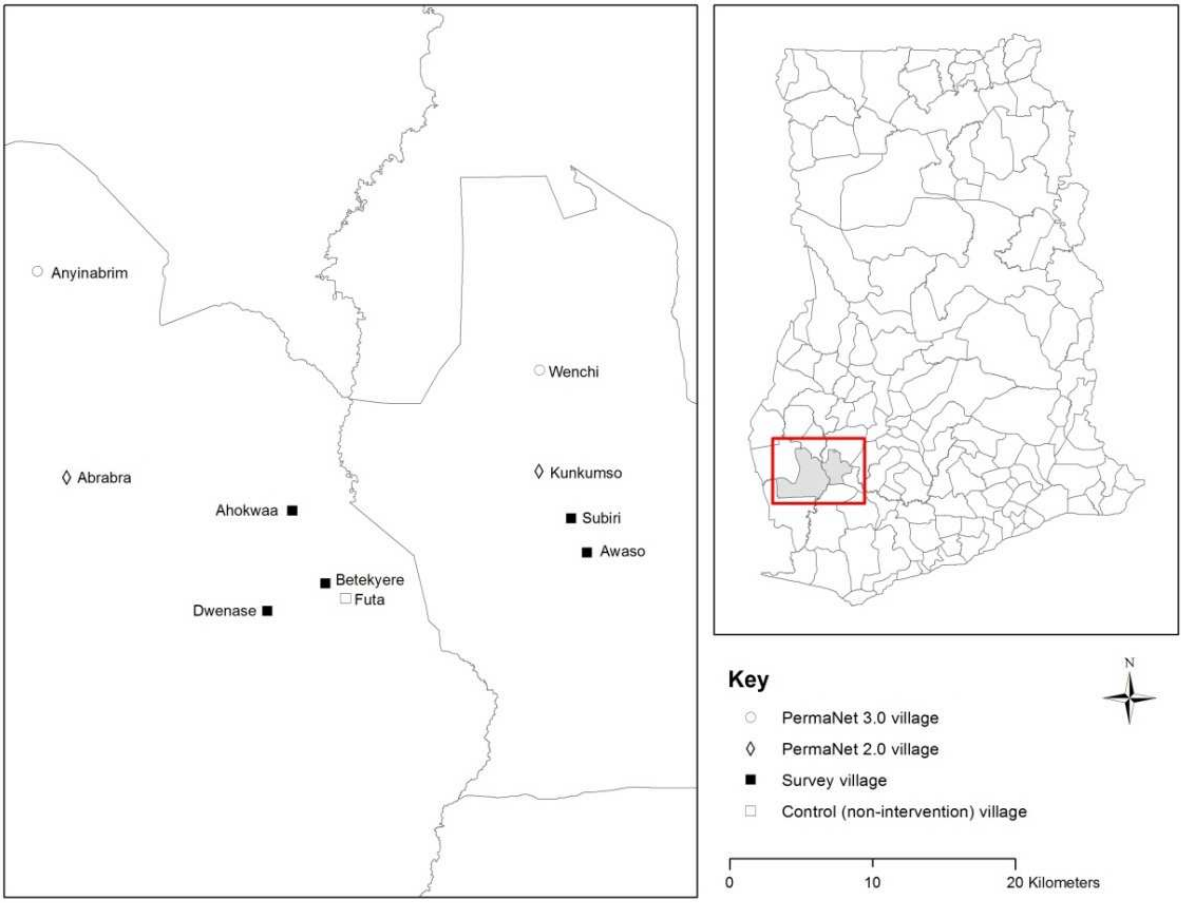


Table 1. WHO susceptibility test results on 2-5 day old F1 An. gambiae s.l. *collected from survey and study villages at baseline (November 2010- January 2011).*

Intervention	Population	Insecticide									
		Deltamethrin (0.05%)		Permethrin (0.75%)		DDT (4%)		Bendiocarb (0.1%)		Pirimiphos-methyl (0.9%)	
		M	*n*	M	*n*	M	*n*	M	*n*	M	*n*
	Kisumu	100	*25*	100	*25*	100	*25*	100	*20*	100	*20*
Control	Futa	33	*96*	21	*92*	3	*107*	98	*102*	96	*89*
PermaNet® 2.0	Abrabra	44	*126*	44	*109*	7	*96*	92	*64*	99	*73*
	Kunkumso	28	*109*	25	*85*	12	*59*	95	*96*	89	*88*
PermaNet® 3.0	Anyinabrim	53	*109*	34	*125*	9	*74*	94	*62*	95	*87*
	Wenchi	62	*126*	67	*132*	8	*91*	88	*117*	93	*91*
Survey village	Dwenase	92	*75*	-	-	-	-	-	-	-	-
	Betekyere	99	*75*	-	-	-	-	-	-	-	-
	Ahokwaa	93	*60*	-	-	-	-	-	-	-	-
	Subri	93	*100*	-	-	-	-	-	-	-	-
	Awaso	87	*100*	-	-	-	-	-	-	-	-

M, % mortality; *n, number tested*

Larval Collections

Mosquito larvae were collected fortnightly from available breeding sites within each study village using the dipper technique (WHO, 1975). The number of sites to be sampled was determined by the availability, size and density of breeding sites. The larvae obtained were transported to the insectary to be raised to adults, identified to species and used for insecticide susceptibility bioassays. A proportion of the *Anopheles* larvae obtained from the field were used for biochemical assays.

Vector Species identification

All female mosquitoes were identified morphologically using the keys of Gilles & de Meillon (1968), Gillies & Coetzee (1987) and Hervy *et al*. (1998). The newly developed species-specific polymerase chain reaction assays based on rDNA internal transcribed spacer 2 (ITS2) sequences of *Anopheles* species were used to determine cryptic species complexes and intra-species variations. Genomic DNA was extracted from single mosquitoes (Collins *et al.* 1988), for *An. gambiae* sibling species identification (Scott *et al*. 1993) and molecular form identification (Favia *et al*. 1997). Members of the An. *funestus* group were identified using the cocktail PCR assay (Koekemoer *et al.* 2002) with slight modifications (Cohuet *et al.* 2003).

Resistance characterization

Phenotypic resistance

WHO susceptibility tests were carried out with deltamethrin as already described, and subsequently with diagnostic doses of permethrin (0.75%), DDT (4%), bendiocarb (0.1%) and pirimiphos-methyl (0.9%%) during the baseline study prior to net distribution (November). Further testing was conducted with these five insecticides in June and July 2012 (16-17 months after LLIN distribution and 19-20 months after the baseline studies).

Detection of Kdr mutation

The PCR amplification method described by Martinez-Torres *et al* (1998) was used for detection of *kdr* mutations in the local *An. gambiae* s.s populations.

Biochemical determination of resistance mechanisms

The possible involvement of enzymes such as esterases, monooxygenases, and glutathione-S-transferases (GSTs) in insecticide metabolism were determined by the methods described by Penilla *et al*. (1998) and WHO (1998). Fourth instar larvae from field LCs or mixed F-1 progeny (4^{th} instars) of wild *An. gambiae* obtained from IRCs were assayed for monooxygenase (P450s), glutathione S-transferase (GST) and esterase (α-esterases) activity, as well as the presence of an altered acetylcholinesterase (AChE), following the protocols described by Polson *et al.* (2011) and El Kady *et al*. (2008). The Kisumu strain of *An. gambiae* was used as the susceptible control.

Measurement of malaria transmission indices

Entomological Inoculation Rate

The entomological inoculation rate (EIR) is considered a more direct measure of transmission intensity than incidence, prevalence or other traditional epidemiological estimates and is a commonly used metric that estimates the number of bites by infectious mosquitoes per person per unit time (Kelly-Hope and McKenzie, 2009). EIR is the product of the "human biting rate" – the number of *Anopheles* bites per person per day – and the fraction of vector mosquitoes that are infectious (the "sporozoite rate"). The human biting rate was calculated from the indoor human landing catches as a measure of the exposure that people get whilst indoors in bed/ asleep. The sporozoite rate was calculated using enzyme-linked immunosorbent assays (ELISA) of *Anopheles* mosquito heads and thoraces (i.e. from specimens obtained though indoor resting catches and human landing catches) (Burkot *et al*, 1984; Wirtz *et al*, 1987). Samples were prepared individually and assayed in batches of four with positive batches re-assayed as single mosquitoes. Insectary reared unfed female *Anopheles* mosquitoes were used as negative controls with the kit supplied CSP antigen as positive control. Samples were read by eye and on an ELISA plate reader (Multiskan® Spectrum, Thermo Scientific - UK) at 495 nm.

Vector Age structure

Parity was determined by dissection of all the unfed anophelines captured at each site through the human landing catch (Detinova, 1962). Parity rates in each village were determined in the baseline survey and then over the 6 month follow-up period, post-distribution.

Data Analysis

A mosquito population was classified as resistant based on the criteria which states that 98–100% mosquito mortality indicates susceptibility, 80–97% mortality implies potential resistance that needs to be confirmed through biochemical assays, and a mortality rate less than 80% suggests resistance (WHO, 1998a). For the wild mosquitoes, since all controls showed no mortality, there was no need for the use of the Abbott's formula. Also, there was no need for the use of the Abbott's formula to correct the mortalities recorded for the Kisumu reference although the mortality recorded for the control samples was 10%.

Data from biochemical assays followed very positively skewed distributions and were therefore all summarised using medians with their 95% confidence intervals. Statistical significance between the Kisumu reference strain medians and the remaining conditions was achieved by inspection of the confidence intervals. Genotype frequencies were tested using $\chi 2$ or Fisher's exact test.

For the baseline, data from November 2010 were used, representing the peak of the low rainy season. For the follow-up survey post-LLIN intervention, data from the period April- July 2011 was used, representing the peak in the long rainy season. Only November was used for the baseline as data collected in this month was much more homogeneous than in other baseline months; data from March included the period of hang up / installation. As statistical significance testing was confined to comparing sites at the post-intervention evaluation, the use of different time-frames being used at baseline and post-intervention was not an issue; however, to make the presentation of data consistent and to allow sensible informal comparisons to be made between the two time points, averages are presented per village and/or per person *per night.*

Human landing catches were assumed to have Poisson distributions, but were analysed using negative binomial regression models (with village as the only independent variables) to allow for extra variance. As there were replicate observations within each village, it was possible to analyse the indoor and outdoor counts separately. Statistical significance between the four "intervention" villages and the control village (Futa) was established by computing incidence rate ratios, but these are not reported as the actual means are more informative. The percentages of mosquitoes captured indoors within each house were assumed to follow a Normal distribution and were analysed using standard linear regression models (with village as the only independent variable). Statistical significance between the four "intervention" villages and the control village (Futa) was established by examination of the regression coefficients and their 95% confidence intervals.

For parity rate data, variables were assumed to have Poisson distributions, but were analysed using negative binomial regression models (with village and site (indoors / outdoors) as independent variables) to allow for extra variance. No statistically significant differences were observed between the indoor and outdoor counts for any of the variables, so statistical significance was determine using the total counts. Statistical significance between the four "intervention" villages and the control village (Futa) was established by computing incidence rate ratios, but these are not reported as the actual means are more informative.

The resting catch data were assumed to follow a Poisson distribution, but were analysed using negative binomial regression models (with village and time (baseline / post-intervention) as the independent variables) to allow for extra variance, and using the number of persons in households at each assessment as an offset. As there was only a single replicate observation in each village at baseline, no formal baseline adjustment was possible; for this same reason, 95% confidence intervals could not be computed for baseline). Statistical significance between the four "intervention" villages and the control village (Futa) was established by examination of the post-intervention 95% confidence intervals.

Results

During the baseline survey, 34.4% (n= 288) of the 837 respondents indicated that they used a bed net to prevent malaria transmission and 32.6% (n= 273) indicated specifically that they use insecticide treated nets. A post-intervention village survey established a net-use rate of 96.5% in the study area (1146/1188 of nets surveyed). Of the PermaNet® users, 92.9% (n= 566) indicated that they had slept under a net the night before the survey, 16.9% had washed their net (n= 100) with the average number of washed times since the hang-up being 1.2 times while 82.3% indicated they had not washed their net (n= 488).

Resistance characterization

Phenotypic resistance

Table 2 indicates mortality rates in WHO susceptibility tests carried out in each of the study sites before and after the intervention. Exposure of the Kisumu *An. gambiae s.s.* mosquitoes resulted in 100% mortality for all insecticides tested. There was high phenotypic resistance to deltamethrin, permethrin and DDT at each study site before the intervention (indicated by mortality rates less than 80%). After the intervention, deltamethrin resistance remained high and did not differ considerably from the baseline at Futa (non-intervention village) and Kunkumso (PN 2.0 village) but it increased significantly at Abrabra (PN 2.0 village) as demonstrated by a significant reduction in mortality rate. The mortality rate recorded after the intervention at Abrabra was 19.7% higher (95% CI: 5.7 – 33.7). At Anyinabrim and Wenchi (PN 3.0 villages), however, deltamethrin resistance reduced considerably as indicated by significant increase in mortality rates. The differences in mortality rates observed after the intervention at Anyinabrim and Wenchi were 38% (95% CI: 24.7 – 51.3) and 21.2% (95% CI: 10.3 – 32.1) respectively. Permethrin resistance remained high and did not differ significantly from the baseline at all the study sites except Anyinabrim where a significant reduction was observed. The mortality rate at Anyinabrim after the intervention was 57.7% (44.9 – 70.5) higher than the baseline. Lastly, DDT resistance was comparatively lower after the intervention at Futa ($p < 0.05$) and Wenchi ($p < 0.01$), although the resistance situation remained high at all the study sites. However, at Futa and Wenchi the mortality rates were 9.5% (95% CI: 2.0 – 17.0) and 19.0% (95% CI: 6.6 – 31.4) higher after the intervention respectively.

Resistance mechanisms: kdr mutation

A total of 581 female *An. gambiae* s.s. that survived exposure to either DDT or pyrethroids (0.05% Deltamethrin and 0.75% Permethrin) were tested for the presence of the L1014F *kdr* mutation. Of these, 272 female *An. gambiae* s.s. were from baseline insecticide susceptibility assays and 309 female *An. gambiae* s.s. from post-intervention assays. All successful PCR assays (96%, n=559) were found to be carriers of the *kdr* mutation with genotypic frequencies of 97% and 3% for the L1014F *kdr* (RR) and RS mutation, respectively. No homozygous susceptible (SS) were detected within the tested populations. The remaining 4% (n=22) female *An. gambiae* s.s. tested were unsuccessful for the PCR assays. Further molecular differentiation of the female *An. gambiae* s.s. tested were found to consist of 63.9% S-forms, 34.5% M-forms and 1.6% hybrids (M/S) (Table 3).

Table 2. Mortality rates in WHO susceptibility tests conducted in study sites before and after the intervention.

Intervention	Site	% Mortality (# tested)								
		Deltamethrin (0.05%)			Permethrin (0.75%)			DDT (4%)		
		Pre-	Post	*P* value*	Pre-	Post-	*P* value*	Pre-	Post-	*P* value*
No intervention	*Kisumu strain*	100 (25)	100 (25)	-	100 (25)	100 (25)	-	100 (25)	100 (25)	-
	Futa	33.3 (96)	37.7 (85)	0.544	20.7 (92)	22.2 (63)	0.814	2.8 (107)	12.3 (65)	**0.014**
PermaNet 2.0	Abrabra	43.7 (126)	23.9 (71)	**0.006**	44.0 (109)	35.4 (48)	0.312	7.3 (96)	15.0 (40)	0.164
	Kunkumso	28.4 (109)	28.2 (71)	0.964	24.7 (85)	26.2 (61)	0.834	11.9 (59)	15.4 (91)	0.544
PermaNet 3.0	Anyinabrim	53.2 (109)	91.3 (80)	**<0.001**	33.6 (125)	91.3 (103)	**<0.001**	9.5 (74)	14.3 (21)	0.525
	Wenchi	61.9 (126)	83.1 (130)	**<0.001**	67.4 (132)	69.3 (163)	**<0.001**	7.7 (91)	26.7 (45)	**0.003**

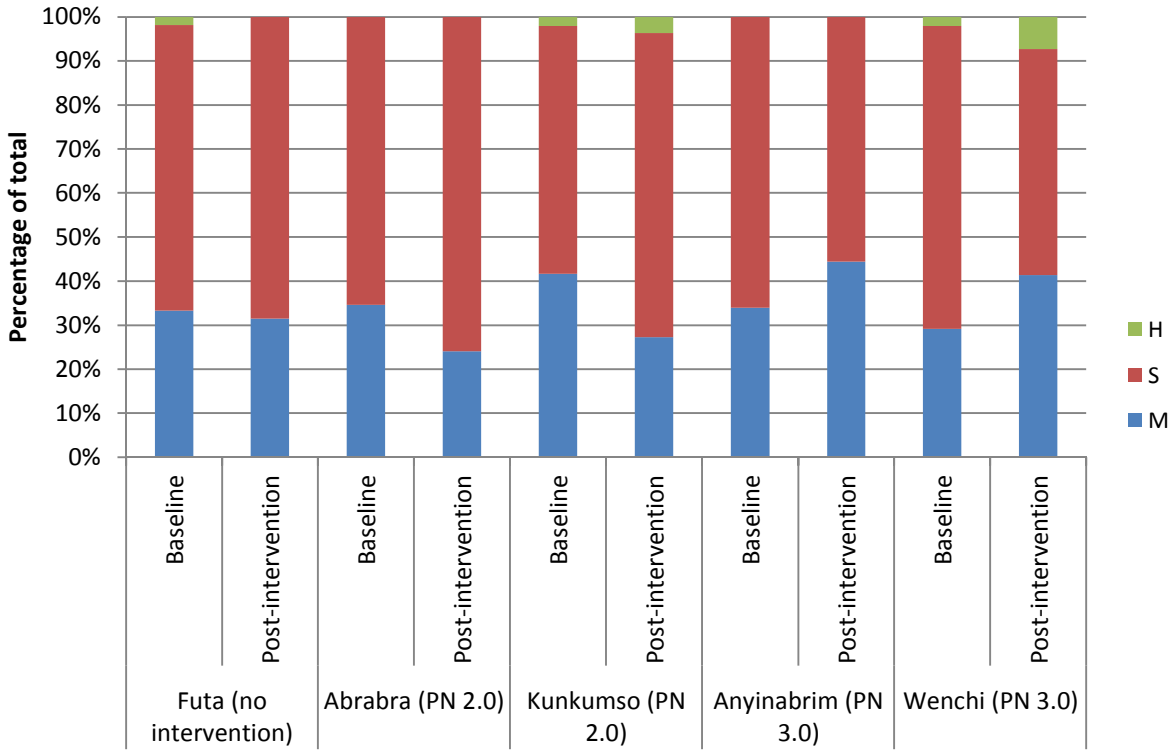
* Difference between mortality rates recorded at baseline and post-intervention, p value for χ^2 statistic with bold showing P < 0.05. Post-intervention survey conducted 18 months after LLIN distribution.

Table 3. The frequency of 1014F kdr alleles in An. gambiae s.s *M form (M), S form (S) and M and S hybrids (H) in the five study villages during baseline and post-intervention*

Intervention	Site	Species	Baseline		Post-intervention		p value
			N	Freq *1014F*	N	Freq *1014F*	*1014F*
No intervention	Futa	S	35	1.00	37	0.99	0.838
		M	18	0.97	17	0.94	
		H	1	1.00	-	-	
PermaNet 2.0	Abrabra	S	34	0.97	41	1.00	0.077
		M	18	0.97	13	0.96	
		H	0	-	0	-	
	Kunkumso	S	27	0.98	43	0.99	
		M	20	0.98	17	0.94	
		H	1	1.00	2	1.00	
PermaNet 3.0	Anyinabrim	S	37	0.99	35	1.00	0.058
		M	19	0.97	28	0.98	
		H	0	-	0	-	
	Wenchi	S	33	1.00	36	1.00	
		M	14	1.00	29	1.00	
		H	1	1.00	4	0.88	

Frequencies of the change in *1014F* from baseline to post-intervention were compared using Fisher exact test., with the level of significance set at P<0.10

Figure 4. Species identification within An. gambiae s.s. *for the five study sites during the baseline and post-intervention period. Data are presented as proportions of the total for each species,* An. gambiae *M form (M),* An. gambiae *S form (S) and the hybrid of M and S forms* by intervention period and by site. Sample sizes are a minimum of 48 per site for the baseline period and a minimum of 54 for the post-intervention period.



Differences in M and S form between baseline and post-intervention were analysed using negative binomial models. The trends observed in individual villages were no significant. However because of homogeneity between intervention sites, it was valid to combine sites by intervention for the analysis. There was no evidence of a significant change in the proportion of M and S forms in the non-intervention site between baseline and post-intervention. There was evidence of a significant change in the proportion of M and S forms in the PN 2.0 and PN 3.0 groups, with the proportion of M form significantly decreasing in the PN 2.0 villages and significantly increasing in the PN 3.0 villages post-intervention.

Resistance mechanisms: biochemical assays

P450

The activity of cytochrome P450 within field populations of *An. gambiae* and the reference Kisumu strain are indicated in Table 4. There were no significant differences in P450 activity between the Wenchi population (PN 3.0 Village) and the Kisumu before and after the intervention. Cytochrome P450 activity was significantly lower among the Futa population (non-intervention village) and higher among the Kunkumso population (PN 2.0 village) before and after the intervention. Lastly, the activity of this enzyme was considerably lower among the Abrabra (PN 2.0 village) and Anyinabrim (PN 3.0 village) population at post-intervention compared to the Kisumu.

Esterase

At Futa, the esterase activity was comparatively higher than the Kisumu strain during the baseline and follow-up period. There was no significant variation in esterase activity between the Kunkumso population and the Kisumu strain at baseline but the activity among the former was

considerably higher after the intervention. Among the Wenchi population, the activity of this enzyme was considerably higher before the intervention but became appreciably lower after the intervention. The activity of this enzyme among the Abrabra and the Anyinabrim population did not deviate significantly from that of the Kisumu strain after the intervention.

GST

The GST activity among the Futa and Kunkumso population did not differ considerably from that of the Kisumu strain at baseline. After the intervention, the activity of this enzyme was similar among the Futa population and the Kisumu strain but its activity became significantly higher among the Kunkumso population. Lastly, GST activity did not differ considerably between the Anyinabrim and the Kisumu strain but the activity of this enzyme was appreciably lower at Abrabra.

Acetylcholinestase (AChE)

There was higher AChE inhibition activity by propoxur among the Kunkumso (PN 2.0 village) and the Wenchi (PN 3.0 village) populations at baseline indicating the absence of an altered AChE responsible for conferring resistance to carbamates and organophosphate. After the intervention, the activity of this enzyme among the Kunkumso population remained significantly higher than the Kisumu strain whereas that of the Wenchi population became significantly lower. There was no significant difference between the Abrabra population and the Kisumu strain but the activity of this enzyme at Anyinabrim was significantly lower. Hence altered AChE were only found within the Wenchi and Anyinabrim (PN 3.0 villages) post intervention, demonstrated by their significantly lower AChE inhibition activity.

Entomological monitoring

The number of mosquitoes collected at any village at baseline ranged between 303 at Abrabra to 4,279 at Futa, while the numbers collected at post-intervention ranged from 724 at Anyinabrim to 3,040 at Futa (Figure 5). The proportion that were only *An. gambiae s.l.* was consistently high (93.0 - 99.7%) at Futa, Abrabra, Anyniabrim and Wenchi though was lower at Kunkumso (77.9 – 82.4%), with other Anopheles collected belonging to the *An. funestus* species group. Only *An. gambiae s.l.* were analysed in detail because this represented the vast majority of mosquitoes caught and the resistance characterisation was completed for this species.

For the human landing catches (HLC), mean numbers collected per person per village per night were calculated and analysed. In Futa (non-intervention site), mean numbers during the baseline period were much higher than in the PermaNet® intervention sites, making it inappropriate to directly compare the densities from the intervention sites with the control site. The data was therefore mathematically adjusted (Faragher, personal communication) to account for the differences observed between the baseline densities and allow direct comparisons to be made in the post-intervention period after adjusting for baseline differences. Significantly less *An. gambiae* were caught indoors per night post-LLIN distribution in Abrabra (PN 2.0 village), Anyinabrim and Wenchi (PN 3.0 villages) (Table 5). Slightly greater overall reductions in the mean number of *An. gambiae* caught indoors in PN 3.0 villages were observed compared to PN 2.0 villages. Similar trends were observed with the HLC samples obtained outdoors, where overall the reduction in mean numbers appeared to be slightly higher in PN 3.0 villages than in PN 2.0 villages. During the post-intervention

evaluation, the proportion of *An. gambiae* caught indoors was higher in PermaNet® intervention villages than in Futa (non-intervention village).

For the intervention villages, examination of the post-intervention 95% confidence intervals revealed significant reductions in female *An. gambia*e indoor resting densities in the PermaNet® villages and when the number of people in each of the households sampled was taken into account, significantly less *An. gambiae s.s.* man biting rates were observed in PN 3.0 villages than in either the PN 2.0 villages or the non-intervention village (Table 6).

Table 4. Comparisons of the average values for a range of biochemical assays between F1 An. gambiae s.s. *adults from trial villages and the* An. gambiae s.s. *insecticide susceptible reference strain (Kisumu).*

Mosquito population	Intervention	P450 (x 10^4)		GST (x 10^3)		α esterase (x 10^4)		AChE (x 10^3)	
		Median (95% CI) [n]	*p*-value	Median (95% CI) [n]	*p*-value	Median (95% CI) [n]	*p*-value	Median (95% CI) [n]	*p*-value
Kisumu reference strain		113 (102 : 128) [121]		112 (76 : 143) [99]		375 (347 : 416) [122]		14 (3 : 48) [58]	
Futa	Control Baseline	90 (28 : 115) [10]	***0.040***	25 (0 : 131) [4]	*0.062*	546 (244 : 1176) [10]	*0.045*	---	-
	Control follow-up	**42 (24 : 64) [58]**	***0.000***	**199 (111 : 352) [33]**	*0.750*	**515 (415 : 540) [58]**	***0.000***	---	-
Kunkumso	PermaNet® 2.0 Baseline	236 (89 : 357) [15]	***0.017***	94 (11 : 201) [10]	*0.431*	424 (259 : 496) [15]	*0.473*	**128 (98 : 145) [20]**	***0.000***
	PermaNet® 2.0 Post-intervention	**201 (138 : 312) [37]**	***0.000***	**264 (164 : 755) [25]**	***0.032***	**714 (542 : 855) [37]**	***0.000***	**400 (308 : 578) [27]**	***0.000***
Wenchi	PermaNet® 3.0 Baseline	121 (62 : 283) [36]	*0.619*	**553 (142 : 1382) [22]**	***0.019***	524 (325 : 853) [37]	***0.024***	**82 (4 : 190) [23]**	***0.012***
	PermaNet® 3.0 Post-intervention	68 (23 : 192) [22]	*0.095*	80 (26 : 3888) [17]	*0.499*	**252 (222 : 326) [23]**	***0.001***	**0 (0 : 0) [9]**	***0.000***
Abrabra	PermaNet® 2.0 Post-intervention	**87 (59 : 115) [83]**	***0.014***	**74 (47 : 97) [79]**	***0.046***	360 (296 : 409) [89]	*0.381*	11 (8 : 29) [43]	*0.406*
Anyinabrim	PermaNet® 3.0 Post-intervention	**48 (25 : 101) [51]**	***0.000***	98 (42 : 236) [42]	*1.000*	**318 (265 : 371) [56]**	*0.091*	**2 (1 : 3) [59]**	***0.000***

P450: mg cytochrome p450 produced/ min/ mg protein.
α esterase: mg α esterase produced/ min/ mg protein.
(95% CI): 95% confidence interval for median
GST: mMoles Glutathione-S-Transferase produced/ min/ mg protein.
AChE: mMoles Acetyl cholinesterase produced/ min/ mg protein.
[n]: number tested.

Figures in bold type denotes significant difference (p<0.05) compared to the Kisumu susceptible laboratory strain that may confer insecticide resistance.

Figure 5. Pie charts to show total number of Anopheles *species collected and species composition (%) in study sites at baseline and post-LLIN intervention. Key:* *A. gambiae s.l.;* *A. funestus s.l.*

Futa (non-intervention village)

Abrabra (PermaNet® 2.0 village)

Kunkumso (PermaNet® 2.0 village)

Anyinabrim (PermaNet® 3.0 village)

Wenchi (PermaNet® 3.0 village)

Table 5. Mean number of An. gambiae s.s. *caught per person per night per village via human landing catches (indoors and outdoors)*

Village	Intervention	Mean number of mosquitoes caught / night (95% CI)			% caught indoors
		Indoors	Outdoors	Total	
Futa	Baseline	230 (190 : 277)	186 (157 : 220)	415 (384 : 449)	55.0 (48.5 : 61.6)
	Post-intervention[a]	36 (30 : 45)	36 (28 : 46)	72 (57 : 91)	50.8 (43.1 : 58.5)
	Post-intervention[b]	*26 (18 : 37)*	*39 (22 : 69)*	*47 (28 : 79)*	52.0 (44.4 : 59.6)
Abrabra	Baseline	15 (11 : 21)	17 (14 : 21)	33 (27 : 40)	46.6 (40.0 : 53.1)
	PermaNet 2.0[a]	12 (11 : 14)	18 (15 : 22)	31 (28 : 33)	40.9 (33.2 : 48.6)
	PermaNet 2.0[b]	***14 (12 : 18)***	***17 (13 : 23)***	*38 (30 : 47)*	***39.2 (31.4 : 47.0)***
Kunkumso	Baseline	45 (31 : 66)	34 (24 : 48)	79 (55 : 115)	56.8 (50.3 : 63.3)
	PermaNet 2.0[a]	21 (17 : 26)	43 (36 : 51)	64 (57 : 72)	33.3 (25.6 : 41.0)
	PermaNet 2.0[b]	*23 (18 : 30)*	*41 (33 : 52)*	*73 (58 : 92)*	***35.1 (27.2 : 42.9)***
Anyinabrim	Baseline	47 (38 : 58)	51 (44 : 59)	98 (84 : 114)	47.7 (41.2 : 54.2)
	PermaNet 3.0[a]	8 (6 : 12)	11 (8 : 14)	19 (15 : 23)	43.1 (35.4 : 50.8)
	PermaNet 3.0[b]	***9 (6 : 12)***	***10 (8 : 14)***	***20 (16 : 26)***	***41.8 (34.2 : 49.4)***
Wenchi	Baseline	79 (51 : 123)	77 (50 : 117)	156 (103 : 234)	51.1 (44.6 : 57.6)
	PermaNet 3.0[a]	17 (15 : 20)	19 (17 : 21)	36 (32 : 41)	47.8 (40.1 : 55.5)
	PermaNet 3.0[b]	***18 (15 : 21)***	***19 (17 : 22)***	*36 (33 : 40)*	*47.7 (40.3 : 55.0)*

Figures in bold indicate statistical significant difference relative to control (Futa) post-intervention mean

a: unadjusted b: adjusted for baseline levels

Table 6. Mean number of An. gambiae s.s. *caught per village via indoor resting catches*

Site	Intervention	Mean total number caught per village (95% CI)	Mean total human biting rate (bite/ human/ month)* (95% CI)
Futa	Baseline	230	13.53
	Post-intervention[a]	79 (63 : 98)	0.39 (0.30 : 0.51)
Abrabra	Baseline	39	1.18
	PermaNet 2.0[a]	**36 (26 : 50)**	0.31 (0.24 : 0.39)
Kunkumso	Baseline	82	2.10
	PermaNet 2.0[a]	**45 (42 : 48)**	0.43 (0.34 : 0.54)
Anyinabrim	Baseline	77	1.64
	PermaNet 3.0[a]	**12 (7 : 19)**	**0.04 (0.03 : 0.06)**
Wenchi	Baseline	178	4.94
	PermaNet 3.0[a]	**15 (11 : 19)**	**0.07 (0.05 : 0.09)**

Figures in bold indicate statistical significant difference relative to control (Futa) post-intervention mean

a: unadjusted

*** Based on a calculation of the total number of people found sleeping in rooms where mosquitoes were collected**

Malaria Transmission Indices

Vector Age Structure

A total of 908 *An. gambiae s.l.* were dissected from Futa (control village), 482 from Abrabra (PN 2.0 village), 880 from Kunkumso (PN 2.0 village), 355 from Anyinabrim (PN 3.0 village) and 878 from Wenchi (PN 3.0 village). Overall, parous rates increased in Futa, Abrabra and Kunkumso, decreased in Anyinabrim and remained approximately the same in Wenchi (Table 7).

Table 7. Mean number of An. gambiae s.s. *per village analysed for parity status*

Site	Intervention	Nulliparous		Parous		Overall mean Parity rate
		Indoors	Outdoors	Indoors	Outdoors	
Futa	Baseline	56	50	72	57	54.9
	Follow up[a]	27	30	48	52	63.7
	Follow up[b]	*20*	*28*	*42*	*57*	*67.3*
Abrabra	Baseline	20	26	30	25	54.5
	PN 2.0[a]	11	18	21	35	65.9
	PN 2.0[b]	*15*	*21*	***25***	***35***	*62.5*
Kunkumso	Baseline	36	32	47	48	58.3
	PN 2.0[a]	24	38	51	37	58.7
	PN 2.0[b]	*28*	*38*	*47*	*65*	*62.9*
Anyinabrim	Baseline	11	16	25	24	64.5
	PN 3.0[a]	10	13	14	23	61.7
	PN 3.0[b]	*14*	*19*	***17***	***23***	*54.8*
Wenchi	Baseline	78	79	136	108	60.8
	PN 3.0[a]	19	26	30	40	60.9
	PN 3.0[b]	***11***	***16***	***25***	***34***	*68.6*

Figures in bold indicate statistical significant difference relative to control (Futa) post-intervention mean – as no significant differences between indoor and outdoor data, both sites combined for analysis.

a: unadjusted **b: adjusted for baseline levels**

Sporozoite Rates and Entomological Inoculation Rates (EIRs)

A total of 2114 *An. gambiae s.l.* were tested for presence of *Plasmodium falciparum* circumsporozoite protein from Futa (non-intervention village), a total of 483 from Abrabra and 1057 from Kunkumso (PN 2.0 villages) and a total of 623 from Anyinabrim and 985 from Wenchi (PN 3.0 villages). Sporozoite rates ranged from 4.7% in Anyinabrim prior to PN 3.0 distribution to 0.35% in Wenchi after PN 3.0 distribution. Sporozoite rates declined in all villages, except in Futa (non-intervention village), where an increase of 76.7% was observed (Table 7). The largest decrease was observed in Anyinabrim where the sporozoite rate decreased from 4.69% at baseline to 0.58% after PN 3.0 distribution constituting an 87.6% decrease. Significantly less mean sporozoite positive *An. gambiae s.s.* were detected in the PN 3.0 villages compared to PN 2.0 villages or the non-

intervention village (p<0.05). The EIRs were considerably lower after the intervention at all the study sites. The magnitude of reduction in EIRs was 38.1%, 72.3%, 78.9%, 93.9% and 97.9% at Abrabra, Futa, Kunkumso, Wenchi and Anyinabrim respectively.

Table 8. Sporozoite rates and Entomological Inoculation Rates calculated for study villages during the baseline and post-LLIN intervention

Village	Intervention	Indoor Human Biting Rate (bite/man/night)	# Tested	# sporozoite positive	Sporozoite rate	*p*-value	EIR
Futa	Baseline	230	*1238*	*20*	1.616	0.052	3.716
	Follow-up[a]	36	*876*	*25*	2.854		1.027
Abrabra	Baseline	15	*106*	*4*	3.774	0.654*	0.566
	PN 2.0[a]	12	*377*	*11*	2.918		0.350
Kunkumso	Baseline	45	*275*	*7*	2.545	0.103*	1.145
	PN 2.0[a]	21	*782*	*9*	1.151		0.242
Anyinabrim	Baseline	47	*277*	*13*	4.693	0.001	2.206
	PN 3.0[a]	8	*346*	*2*	0.578		0.046
Wenchi	Baseline	79	*408*	*5*	1.225	0.106*	0.968
	PN 3.0[a]	17	*577*	*2*	0.347		0.059

[a]unadjusted for baseline

*Abrabra: Yate's p-value = 0.896, Kunkumso: Yate's p-value = 0.180, Wenchi: Yate's p-value = 0.218. The p-values were obtained through the chi-square test for the homogeneity of proportions of the mosquitoes found to be infected with *P. falciparum*.

Discussion and Conclusions

This study investigated the immediate impact of two different LLINs on pyrethroid resistant populations of *An. gambiae s.l.* in Western Ghana. Determining the resistance profiles of populations in each study village was critical to enable a thorough analysis of the outcome parameters especially as density is not always sensitive enough indicator to measure changes following an intervention. The vast majority of *Anopheles* collected in the study villages were identified as *An. gambiae s.s.*, hence this species was analysed in detail in terms of resistance characterisation and entomological impact of the LLINs that were distributed. Although it should also be noted that approximately 25% of the *Anopheles* collected in Kunkumso belonged to the *An. funestus* complex, the data has not been analysed in this report.

Anopheles gambiae s.s. populations from all study villages were confirmed as pyrethroid resistant, both during the baseline study and post-LLIN intervention. Mortality rates in WHO susceptibility tests with deltamethrin were highly relevant, as this is the insecticide present in both PN 3.0 and PN 2.0. Rates did not change in the study villages, except in Abrabra where mortality rates with deltamethrin significantly decreased post-PN 2.0 distribution and in Anyinabrim and Wenchi, where mortality rates with deltamethrin and permethrin significantly increased post-PN 3.0 coverage. The L1014F *kdr* mutation was detected in all *An. gambiae s.s.* populations tested (Table 3 and 8) at a very high frequency, with a significant difference in the allelic frequency detected in each

molecular form between sites for the baseline and post-intervention period. Analysis of the proportion of molecular forms identified at each of the sites revealed no difference between the baseline and post-intervention period in Futa (non-intervention village), a significant decline in the proportion of M form in the PN 2.0 villages and a significant increase in the proportion of M form in the PN 3.0 villages. AA decrease in mortality rates in WHO susceptibility tests was detected only in Abrabra where the proportion of M form declined post-intervention, however in the other PN 2.0 site (Kunkumso) where a decline in the proportion of M form was also detected, no change in the mortality rates in WHO susceptibility tests were observed between baseline and post-intervention. It is unlikely that the shifts in the proportions of M form and S form at each site were due to seasonal effects, as there was no change in the non-intervention village (Futa) where a large seasonal effect in numbers was detected in this village; in addition a decline in the M form was noted in the PN 2.0 villages whilst there was an increase in the M form in the PN 3.0 villages. The clear and consistent findings in the PN 3.0 villages, which were geographically distant indicates a distinct and consistent trend that is more likely due to the intervention in these villages than a village-specific or seasonal effect. In *An. gambiae*, resistance appears to be higher in the S form rather than the M form, and evidence from Burkina Faso has suggested that the S form had a greater probability of surviving the insecticide (DDT or pyrethroid) (GPIRM, 2012). The data from this study suggests that more S form *An. gambiae* were killed in the PN 3.0 villages after PN 3.0 distribution, which resulted in a shift in the proportion of M and S forms in the post-intervention period.

Elevated P450s, GSTs and esterase activity were detected in Futa (non-intervention village) and Kunkumso (PN 2.0 village). Elevated P450s and esterases were detected in Abrabra (PN 2.0 village) and Anyinabrim (PN 3.0 village) and elevated GSTs and esterases were detected in Wenchi (PN 3.0 village) (Table 9). The involvement of an altered AChE which confers resistance to carbamates and organophosphates were observed only within the Wenchi and Anyinabrim (PN 3.0 villages) post intervention and demonstrated by their significantly low AChE inhibition activity. Future investigations using the microarray technique to investigate upregulated gene expression are recommended in order to complement the biochemical data and confirm the involvement of the different enzyme families in insecticide metabolism in these populations.

The study design used matched villages to compare the impact of PN 3.0 on the mosquito population and malaria transmission indices with PN 2.0, which meant that the baseline study was conducted at a different time of the year than the post-intervention survey. For some of the outcome measures, baseline adjusted values were calculated, which represent what would likely have occurred *ceteris paribus* (if everything had been started equal). During post-intervention evaluation, the overall mean parity rate significantly increased in Abrabra (PN 2.0 village) and significantly decreased in the PN 3.0 villages (Anyinabrim and Wenchi). Adjusting for baseline levels, no significant difference was observed in the human biting rates between Futa and the PN 2.0 villages, or Wenchi (PN 3.0 village), however human biting rates in Anyinabrim (PN 3.0 village) were significantly less than in Futa. For the resting catch data adjusted for baseline levels, significantly less *An. gambiae s.s.* were observed in the PN 3.0 villages compared to the control, although no significant difference was observed between PN 2.0 villages and the control.

An important seasonal effect was detected in Futa, (non-intervention village) where the Entomological Innoculation Rate (EIR) was significantly higher at baseline because of the extremely high human biting rates observed during the baseline period. During the follow-up survey, the sporozoite rate increased but mean biting rate significantly declined hence an overall reduction in EIR was observed. The largest reductions in sporozoite rates occurred in the PermaNet® 3.0 villages

(Anyinabrim and Wenchi) (Table 8). The significantly higher mosquito density found in Futa during the baseline period was completely disproportionate with the densities found in other villages in the same time period. A similar peak in mosquito activity was observed in Futa in June 2010, when human biting rates of over 210 *Anopheles* per human per night were recorded (CMCP [Chirano Malaria Control programme], unpublished data). Further investigation of potential sources of this peak activity (such as house design, surrounding vegetation type, and availability of temporary breeding sites) in this village is required. The inclusion of only one non-intervention village in this study therefore represents a major study limitation, which means that interpretation of post-intervention values in study villages is most appropriate.

Kunkumso (PermaNet 2.0 village) and Anyinabrim (PermaNet 3.0 village) were the two villages with the most similar baseline values, in terms of mosquito density (measured by human landing catch and indoor resting catch), as well as sporozoite rates. Significant reductions in all parameters were observed, including a larger decrease in parity rates in Anyinabrim than in Kunkumso, post-intervention. This was also reflected in a much larger reduction in EIR in Anyinabrim than in Kunkumso.

It is concluded that the reduction in EIR observed in the PermaNet® 3.0 villages was largely influenced by the combination of the reduction in human biting rate and sporozoite rate and it is interesting to note that a smaller reduction in EIR was noted in the PermaNet® 2.0 villages, where mortality rates in WHO susceptibility tests either decreased 16-17 months after LLIN coverage (Abrabra) or remained the same (Kunkumso). The decrease in the EIR and the increased mortality recorded in WHO susceptibility tests conducted 16-17 months after PN 3.0 coverage in Anyinabrim and Wenchi suggest that PN 3.0 was successfully killing the resistant mosquitoes although more of an effect was detected in Anyinabrim than Wenchi, whereas PN 2.0 had a lower impact on the resistant populations in Abrabra and Kunkumso. This trial is the first to provide operational evidence on the increased bio-efficacy of PermaNet® 3.0 against pyrethroid-resistant *An. gambiae* field populations over the conventional LLIN, PN 2.0. Further work on the operational impact of PN 3.0 on disease prevalence would be useful to determine the epidemiological/ health impact (i.e. disease prevalence estimations pre- and post-intervention) of such interventions in the presence of pyrethroid-resistant *Anopheles* vector populations. Future entomological evaluation over a wider time frame (2-3 years) may further reveal the operational impact of the LLINs on local vector entomological indices of transmission that could not be seen within the six months post evaluation period under this trial.

Table 9. Summary of findings from each study site at baseline and post-LLIN distribution

Site		**Futa**			**Abrabra**			**Kunkumso**			**Anyinabrim**			**Wenchi**		
Intervention		Baseline	Follow-up[a]	Follow-up[b]	Baseline	PN 2.0[a]	PN 2.0[b]	Baseline	PN 2.0[a]	PN 2.0[b]	Baseline	PN 3.0[a]	PN 3.0[b]	Baseline	PN 3.0[a]	PN 3.0[b]
WHO susceptibility test*	% mortality	33.3	37.7	na	43.7	***23.9***	na	28.4	28.2	na	53.2	***91.3***	na	61.9	***83.1***	na
	n	*96*	*85*	*na*	*126*	*71*	*na*	*109*	*71*	*na*	*109*	*80*	*na*	*126*	*130*	*na*
Mean Human Biting rate		415	72	47	33	31	38	79	64	73	98	19	***20***	156	36	36
Resting catches: mean total per person		13.53	0.39	na	1.18	0.31	na	2.1	0.43	na	1.64	***0.04***	na	4.94	***0.07***	na
Overall mean parity rate (%)		54.89	63.69	67.35	54.46	65.88	62.5	58.28	58.67	62.92	64.47	61.67	54.79	60.85	60.87	68.6
Overall mean sporozoite rate		1.1616	2.854	na	3.774	2.918	na	2.545	1.151	na	4.693	***0.578***	na	1.225	0.106	na
EIR		3.716	1.027		0.566	0.350		1.145	0.242		2.206	0.046		0.968	0.059	
Resistance mechanisms identified		*kdr*, P450s, GSTs, esterases			*kdr*, P450s, esterases			*kdr*, P450s, GSTs, esterases			*kdr*, P450s, esterases			*kdr*, GSTs, esterases		

Figures in bold indicate statistical significant difference relative to control (Futa) post-intervention mean (P<0.05)

a: unadjusted **b: adjusted for baseline levels** **na: not available**

*** WHO susceptibility test with 0.05% deltamethrin treated filter papers**

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