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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-

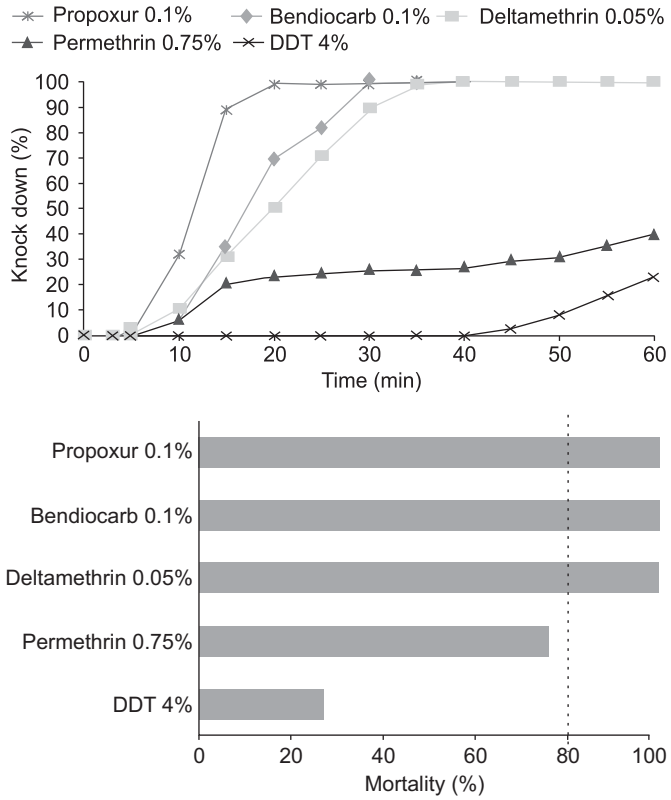

Fig. 1: Overview of the resistance status of *An. gambiae s.s.* from Kindele site. Lines represent mean percent knock down over 60 min of exposure to insecticide-impregnated papers in WHO susceptibility tests. Bars represent mean percent mortality after 24 h post-exposure for the same test mosquitoes.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

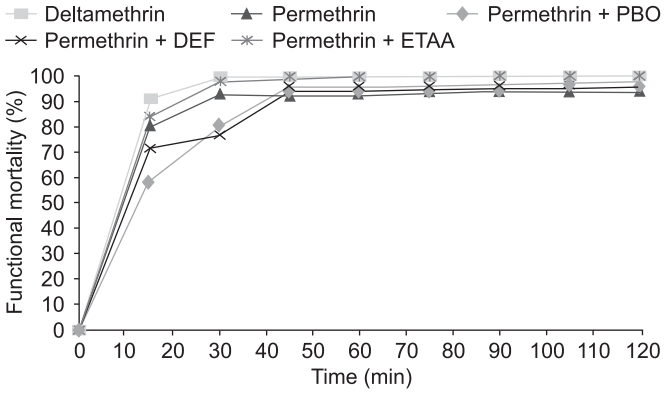

Fig. 2: Additional information on resistance status of *An. gambiae s.s.* from Kindele site. Lines represent mean percent functional mortality (as indicated by mosquitoes unable to rest) over 120 min exposure to deltamethrin- or permethrin-coated bottles in CDC bottle assays. 60 min pre-exposure to the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

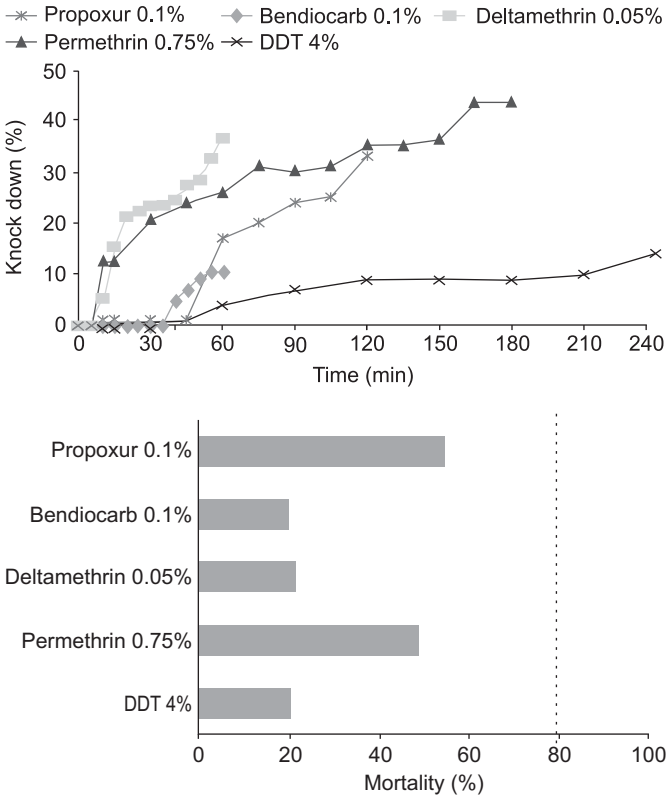

Fig. 3: Overview of the resistance status of *Culex* spp from Kimbangu site. Lines represent mean percent knock down over specified times of exposure to insecticide-impregnated papers in WHO susceptibility tests. Bars represent mean % mortality at 24 h post-exposure for the same test mosquitoes. Dotted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

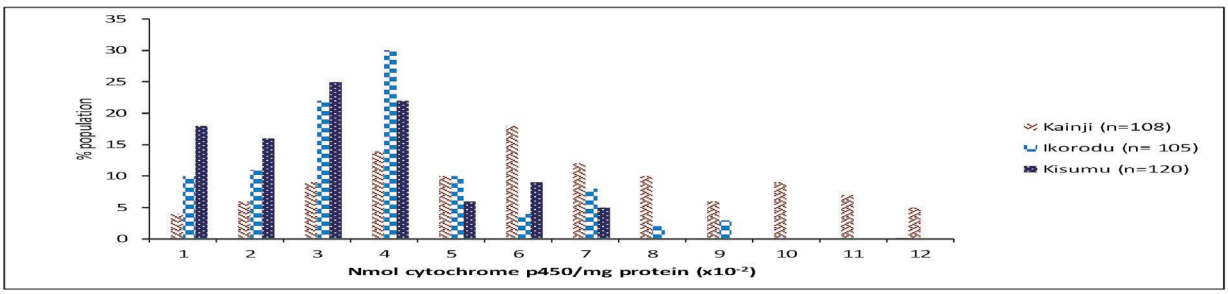

Figure 1: Frequency distribution of monooxygenase level detected in pyrethroid-resistant *An. gambiae s.s.* populations from Kainji and Ikorodu and in the susceptible *An. gambiae s.s.* Kisumu strain via biochemical assays.

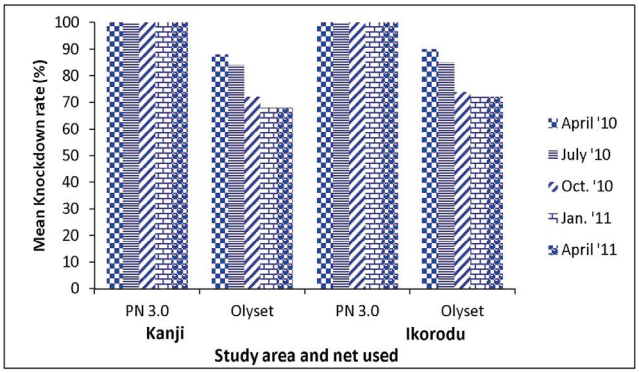

Figure 2: Mean knock down rates (KD) of pyrethroid-resistant laboratory strain of *Anopheles gambiae s.s.* (AGN) based on 3-minutes exposure to PermaNet 3.0 and Olyset nets in WHO cone bioassays prior to (April 2010) and following field usage for 3, 6, 9 and 12-months.

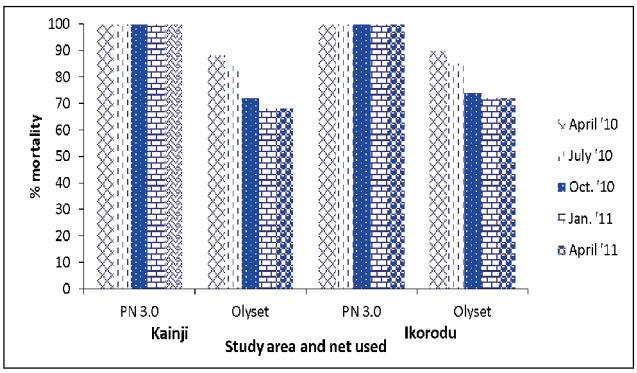

Figure 3: Bio-efficacy of PermaNet 3.0 and Olyset nets prior to and following field usage for 3, 6, 9 and 12-months based on % mortality in 3-minutes exposure in WHO cone bioassays using a pyrethroid-resistant laboratory strain of *Anopheles gambiae s.s.* (AGN).

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.

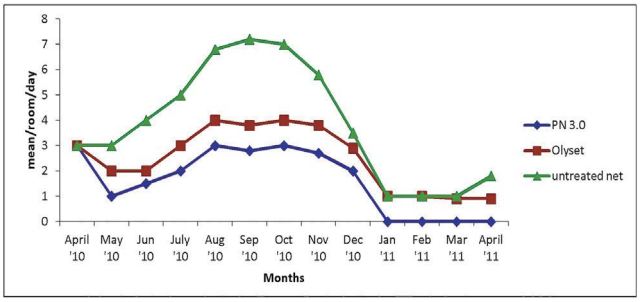

Figure 4: Mean number of *Anopheles gambiae s.s.* per room (pooled from monthly indoor resting, exit trap and floor sheet collections) at 10 houses with PN 3.0, Olyset and untreated nets at Kainji during the pre-intervention (April 2010) and intervention period (May 2010–April 2011).

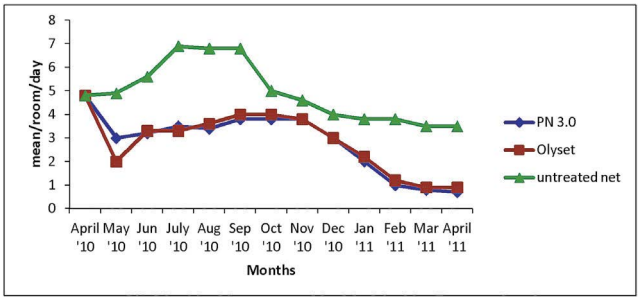

Figure 5: Mean number of *Anopheles gambiae s.s.* per room (pooled from monthly indoor resting, exit trap and floor sheet collections) at 10 houses with PN 3.0, Olyset and untreated nets at Ikorodu during the pre-intervention (April 2010) and intervention period (May2010–April 2011).

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

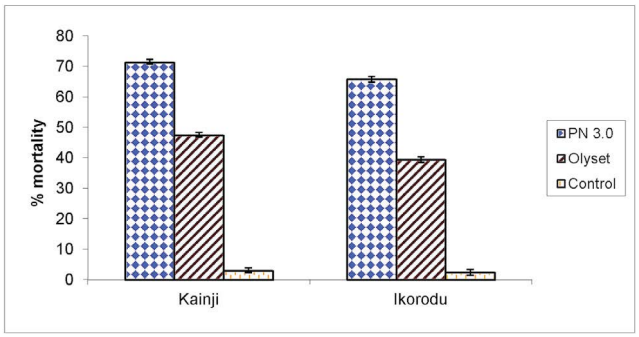

Figure 6: Mean mortality rate (%) based on immediate and delayed mortality of wild-caught female *Anopheles gambiae s.s.* (pooled from monthly indoor resting, exit trap and floor sheet collections) from 10 houses each in villages with PermaNet 3.0, Olyset nets or untreated nets at Kianji and Ikorodu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

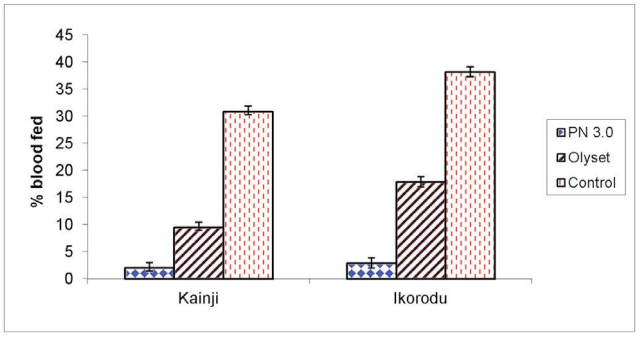

Figure 7: Mean proportion bloodfeeding (%) of wild-caught female *Anopheles gambiae s.s.* (pooled from monthly indoor resting, exit trap and floor sheet collections) from 10 houses each in villages with PermaNet 3.0, Olyset nets or untreated nets at Kainji and Ikorodu from May 2010 to April 2011.

(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.

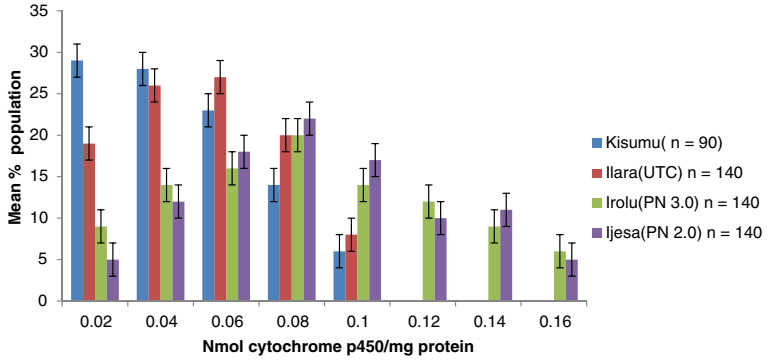

Figure 1 Mean level of P450 monooxygenase activity in pyrethroid resistant *Anopheles gambiae s.s.* from Ilara, Irolu and Ijesa in relation to the standard reference susceptible Kisumu strain of *Anopheles gambiae s.s.*

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

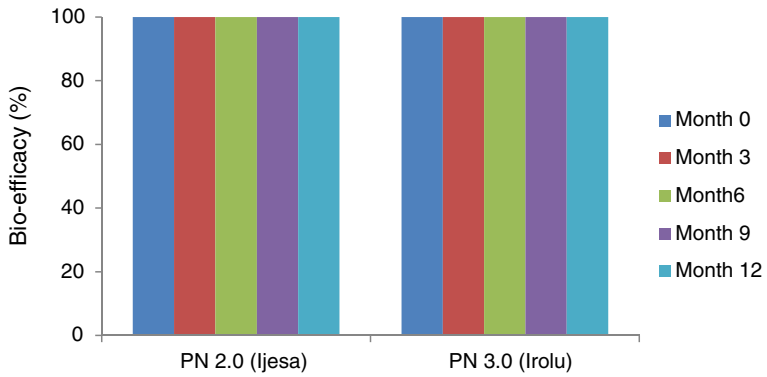

Figure 2 Bio-efficacy of PermaNet 2.0 and PermaNet 3.0 following 0, 3, 6, 9 and 12 months of field usage, as measured against a susceptible strain of *Anopheles gambiae s.s* (Kisumu) in WHO cone bioassays.

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

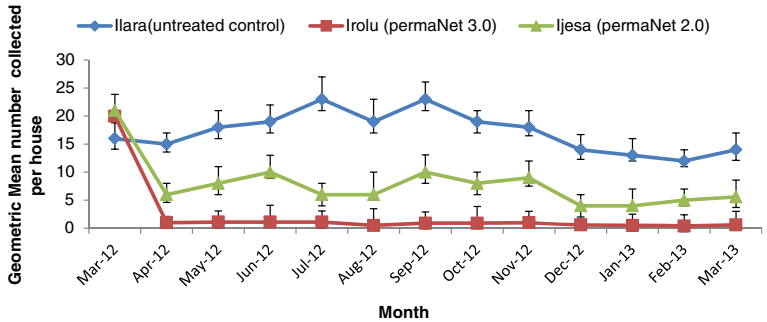

Figure 3 Geometric mean densities of *An. gambiae s.s.* collected per house at baseline (March 2012) and then monthly for 12-months post intervention at Ilara, Irolu and Ijesa.

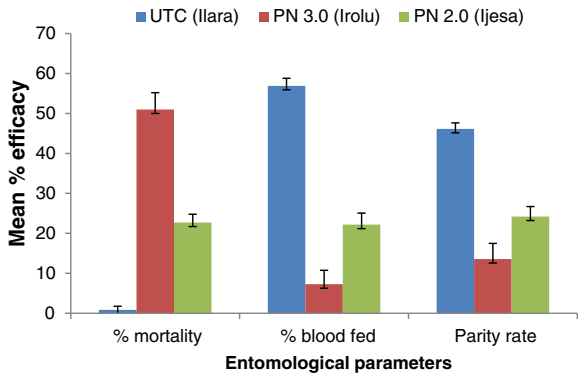

Figure 4 Geometric mean of percentage mortality, blood feeding and parity rate for *An. gambiae s.s.* collected indoor during 12-months post intervention at Ilara, Irolu and Ijesa.

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

FINAL REPORT

(20th September 2012)

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Contents

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.

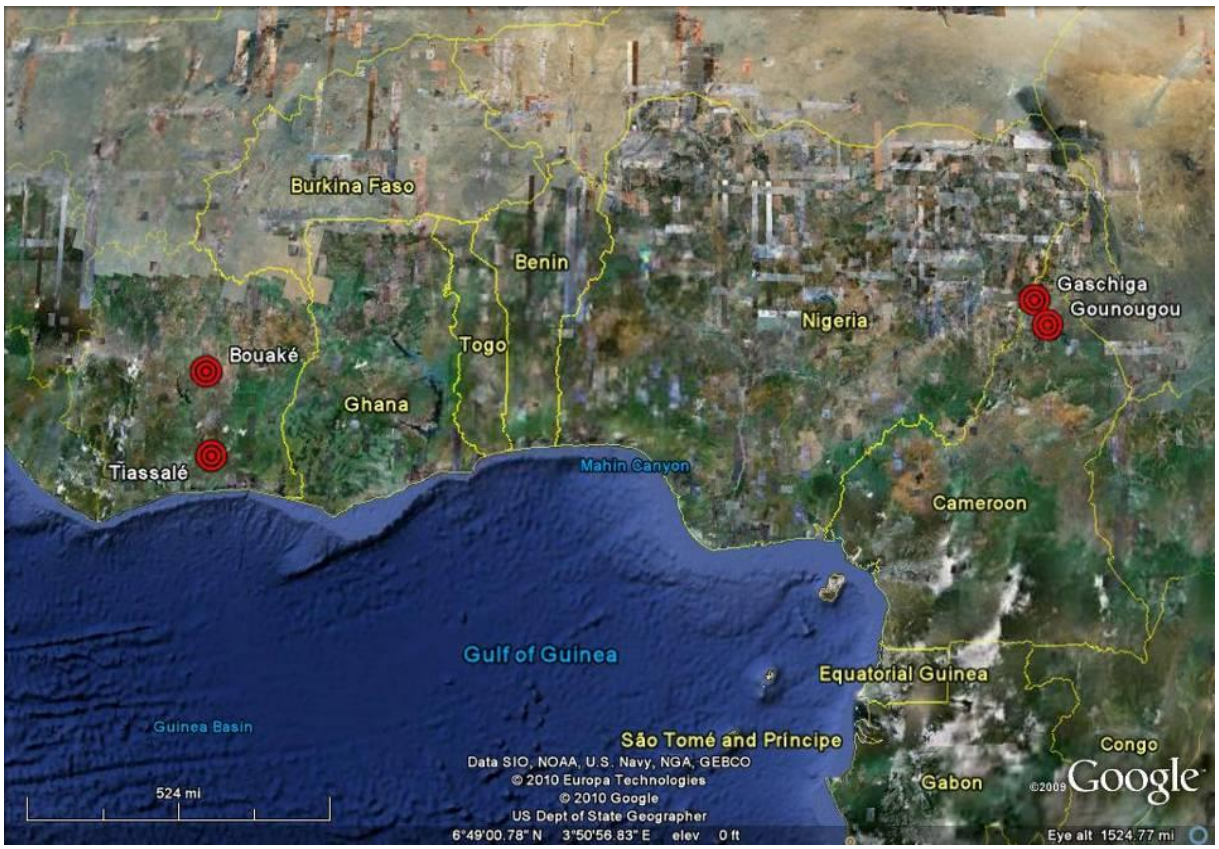

Figure 1: Google™ Earth map showing study site locations in Côte d'Ivoire and Cameroon

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).

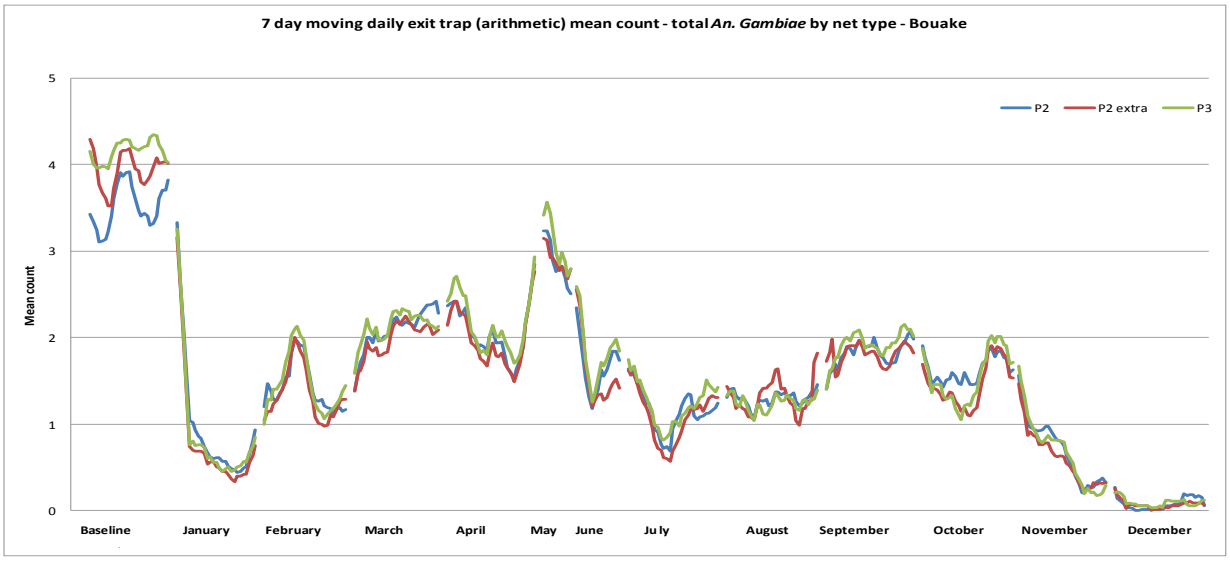

Figure 2. Monthly mean count of *An. gambiae s.s* collected in exit traps before and after net distribution in Bouaké from December 2009 (Baseline) to December 2010.

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.

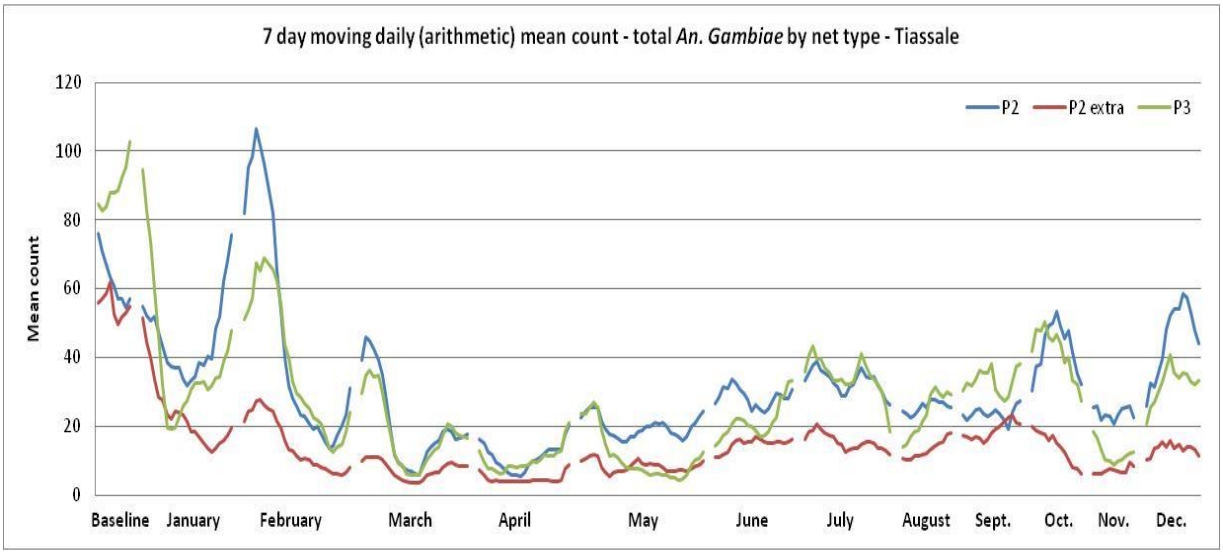

Figure 3. Monthly mean count of *An. gambiae s.s* collected in exit traps before and after net distribution in Tiassalé from December 2009 (Baseline) to December 2010.

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 ($p=0.031$).

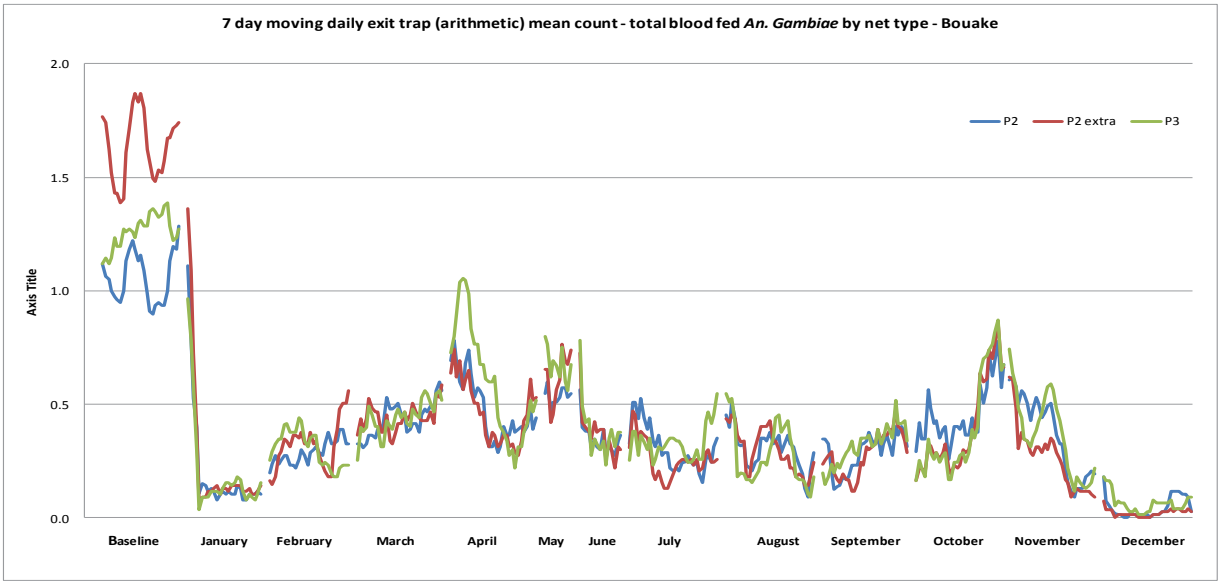

Figure 4. Monthly mean count of blood fed *An. gambiae s.s* collected in exit traps before and after net distribution in Bouaké from December 2009 (Baseline) to December 2010.

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).

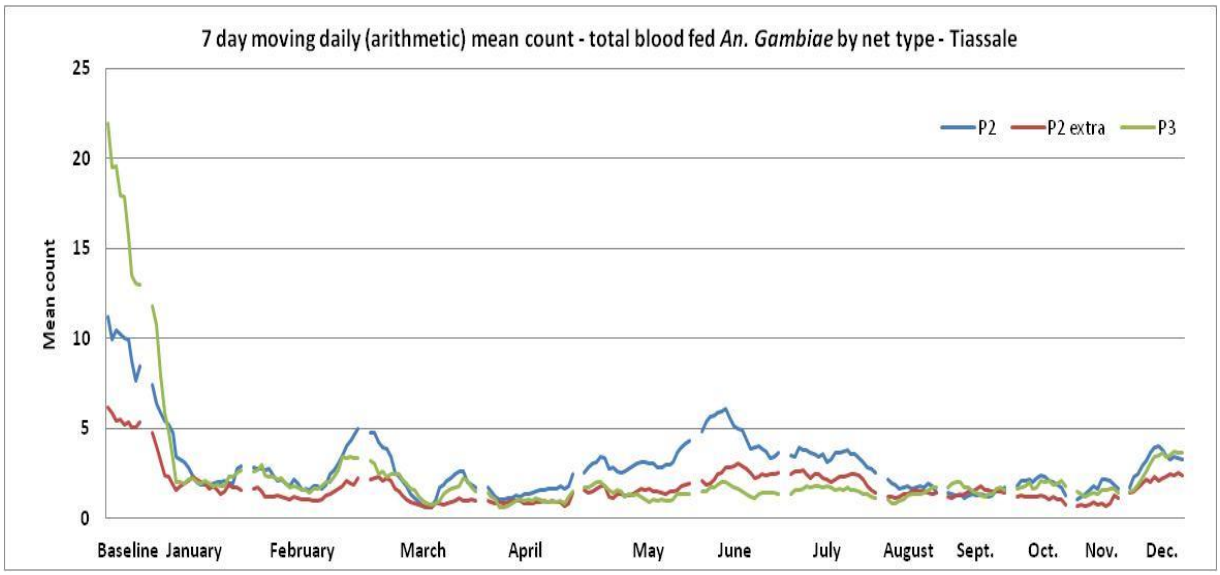

Figure 5. Mean monthly count of blood fed *An. gambiae s.s* collected in exit traps before and after net distribution in Tiassalé from December 2009 (Baseline) to December 2010.

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.

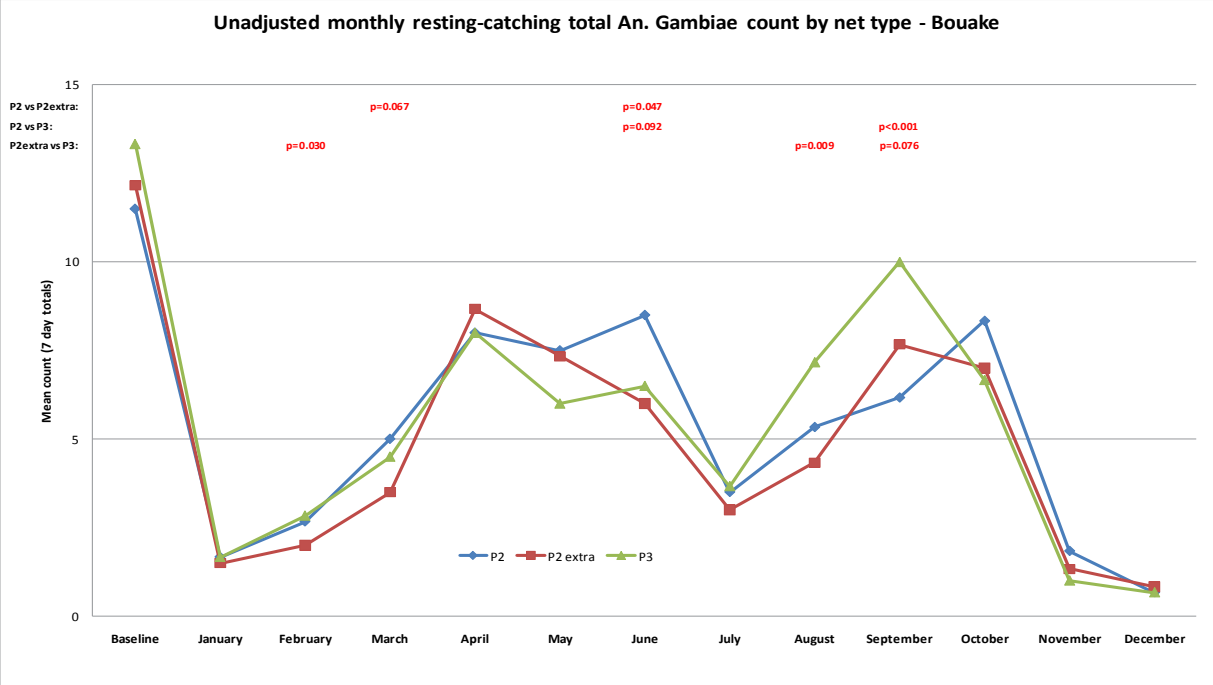

Figure 6. Mean monthly count of *An. gambiae s.s* collected in resting catches within houses before and after net distribution in Bouaké from December 2009 (Baseline) to December 2010.

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.

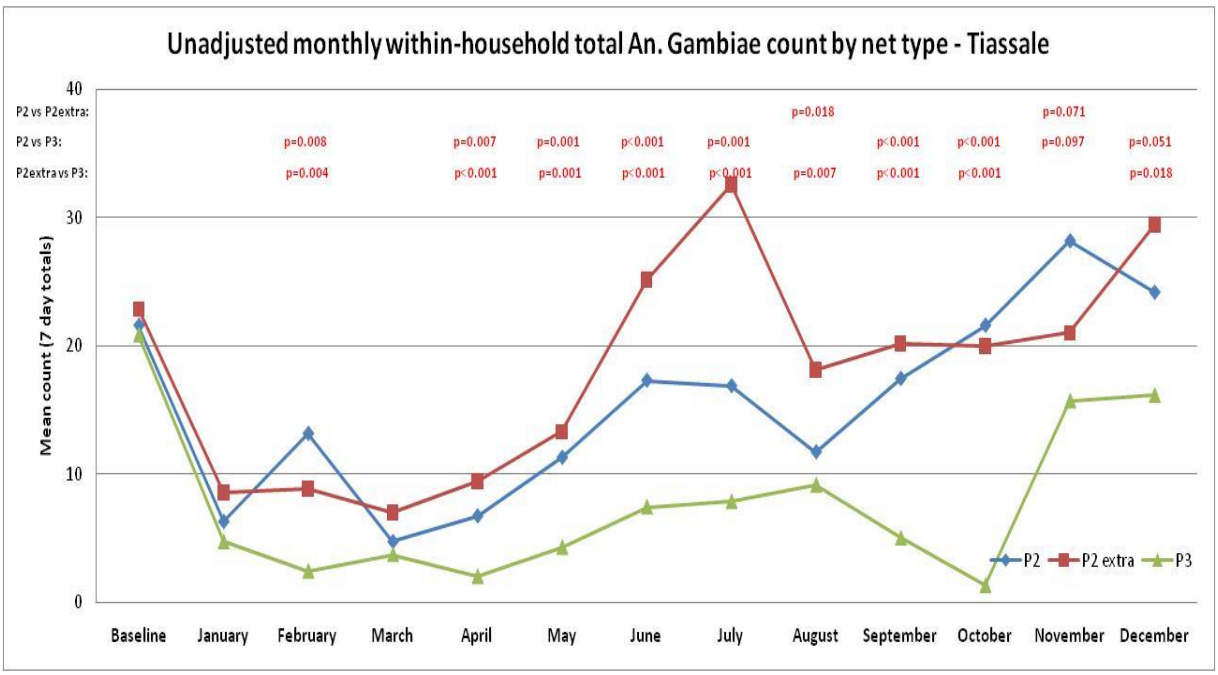

Figure 7. Mean monthly count of *An. gambiae s.s* collected in resting catches within houses before and after net distribution in Tiassalé from December 2009 (Baseline) to December 2010.

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.

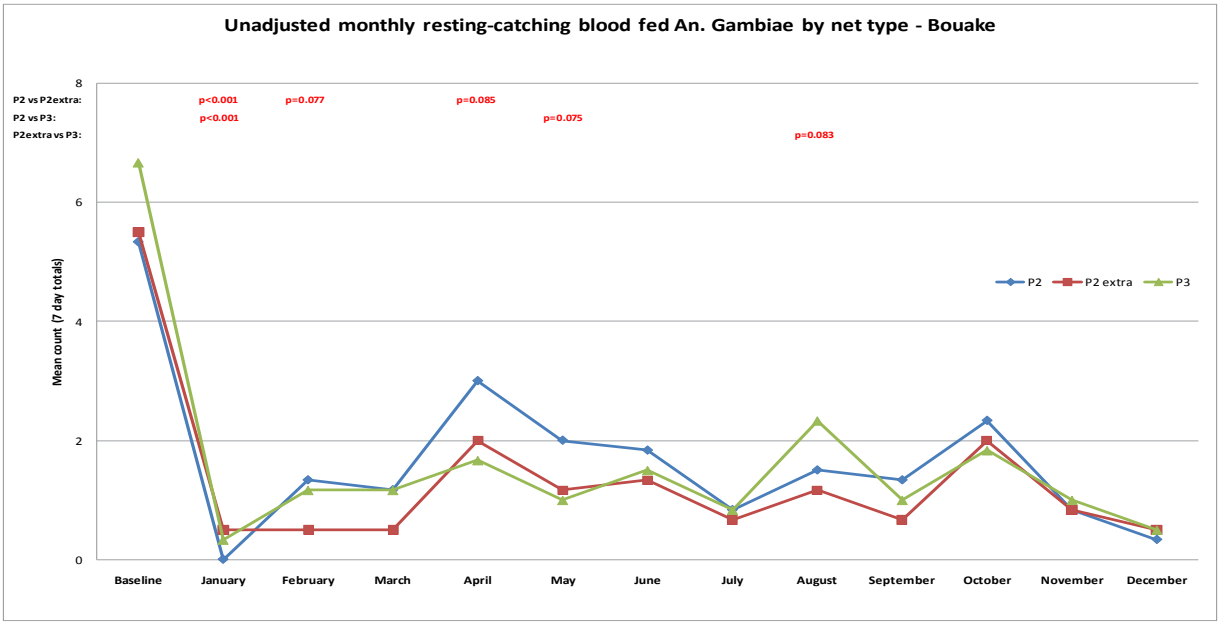

Figure 8. Mean monthly blood fed *An. gambiae s.s* collected within houses before and after net distribution in Bouaké from December 2009 (Baseline) to December 2010.

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.

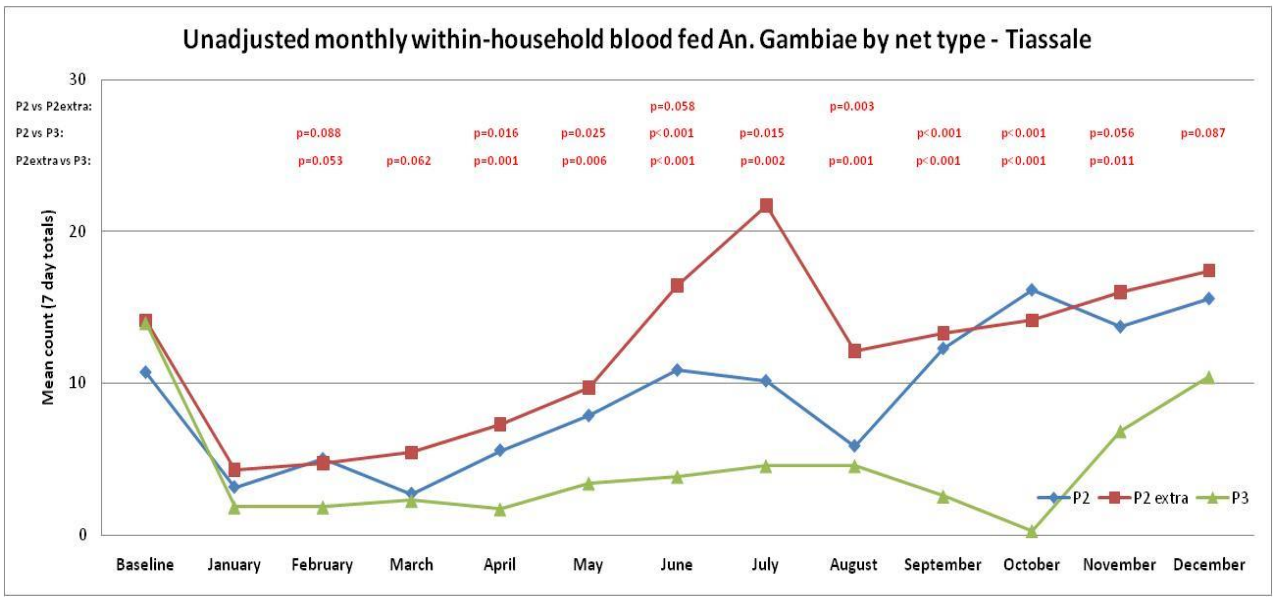

Figure 9. Mean monthly blood fed *An. gambiae s.s* collected within houses before and after net distribution in Tiassalé from December 2009 to December 2010.

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.

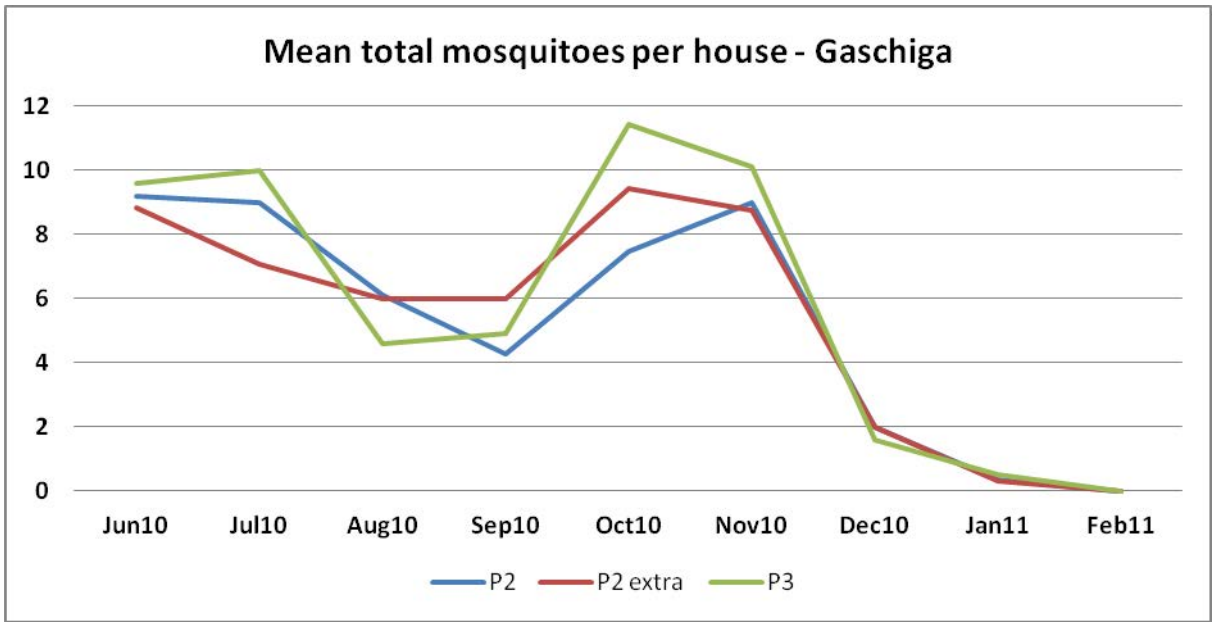

Figure 10. Mean monthly *An. gambiae s.s* collected in exit traps before and after net distribution in Gaschiga from June 2010 to February 2011.

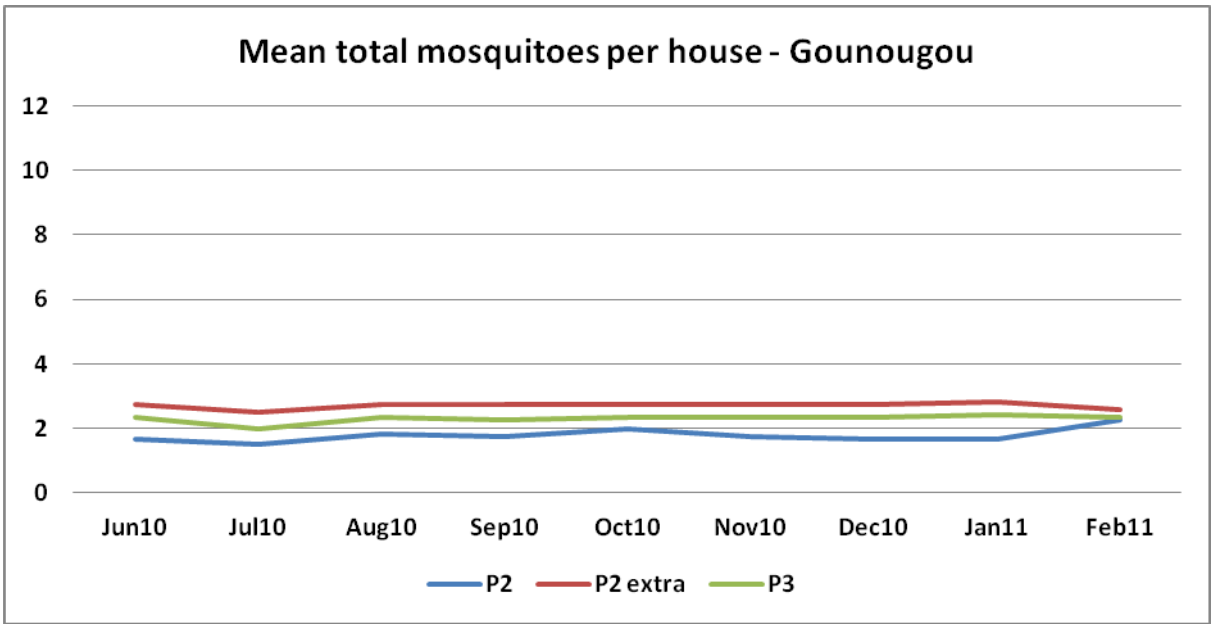

Figure 11. Mean monthly *An. gambiae s.s* collected in exit traps before and after net distribution in Gounougou from June 2010 to February 2011.

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).

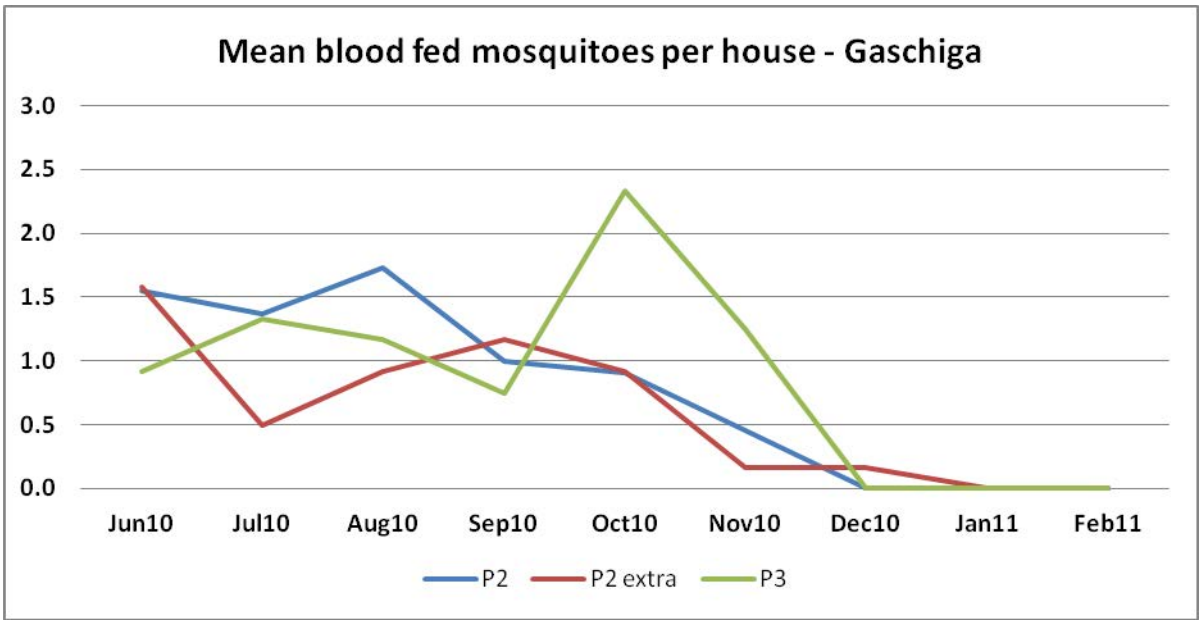

Figure 12. Mean monthly blood fed *An. gambiae s.s* collected in exit traps before and after net distribution in Gaschiga from June 2010 to February 2011.

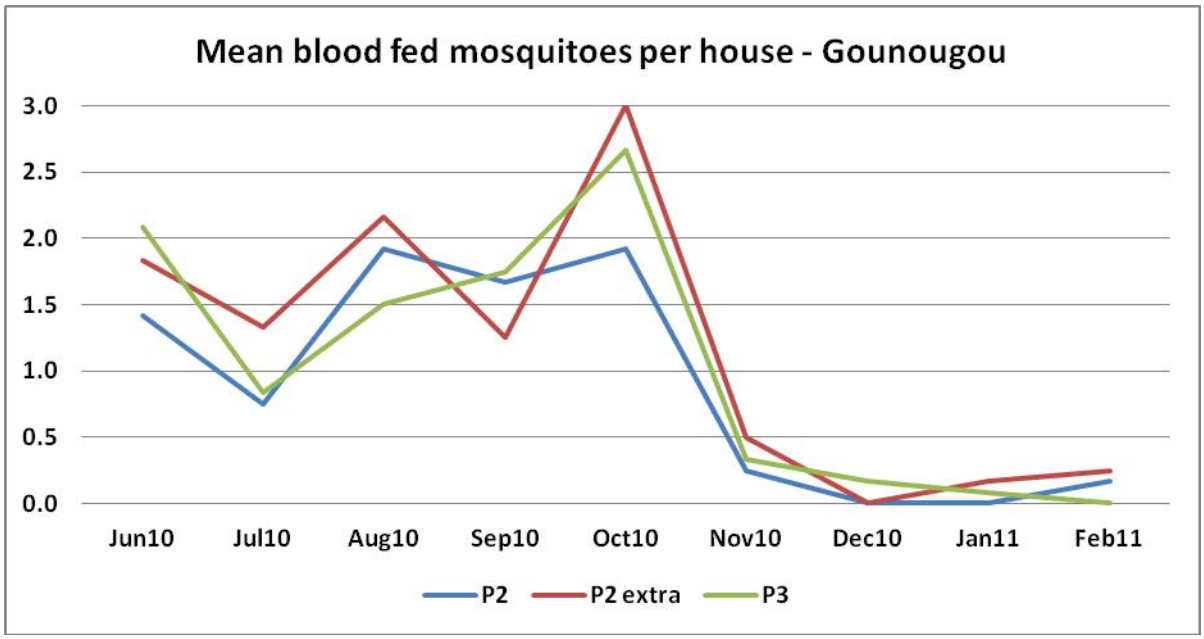

Figure 13. Mean monthly count of blood fed *An. gambiae s.s* collected in exit traps before and after net distribution in Gounougou from June 2010 to February 2011.

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.

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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

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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

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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]*

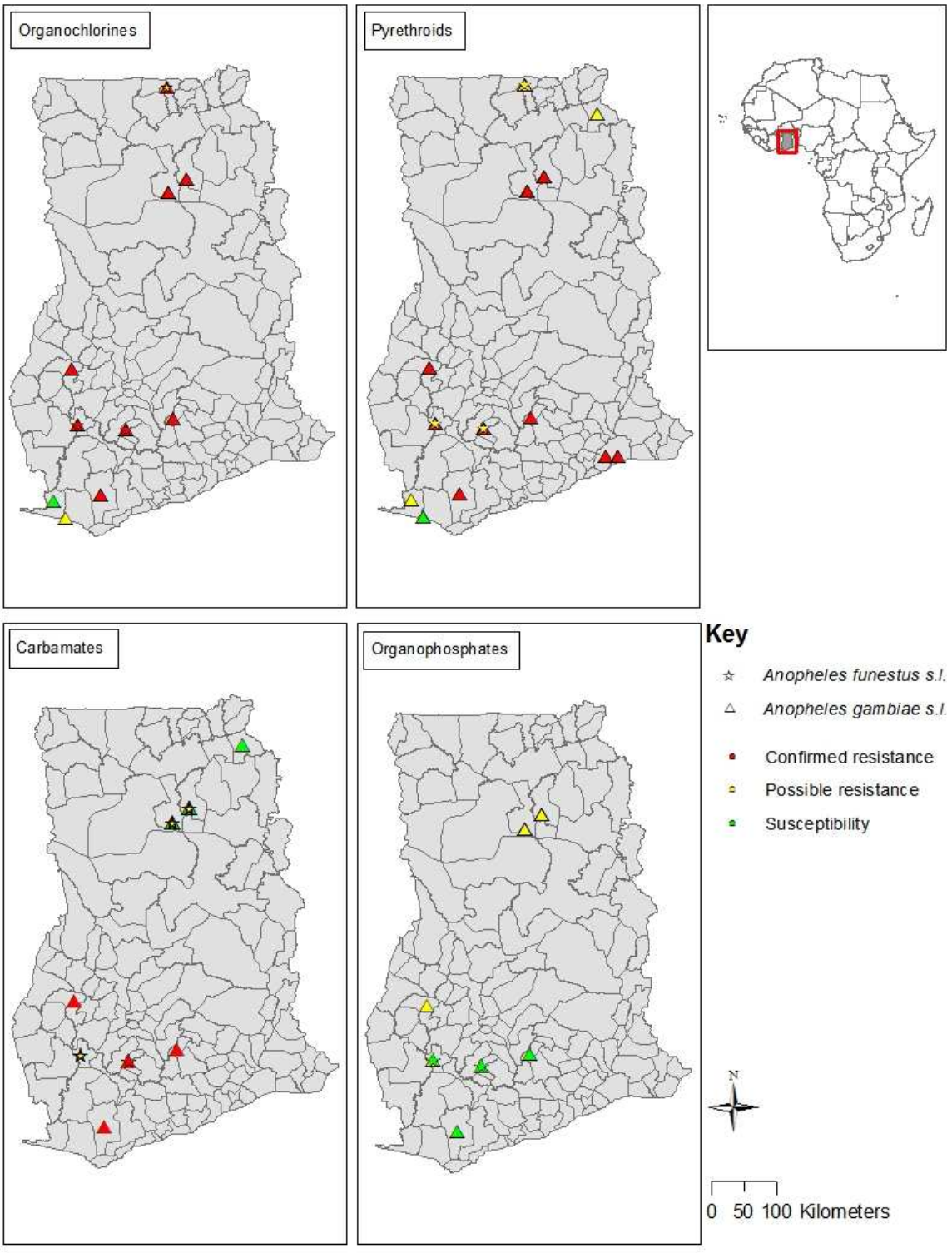
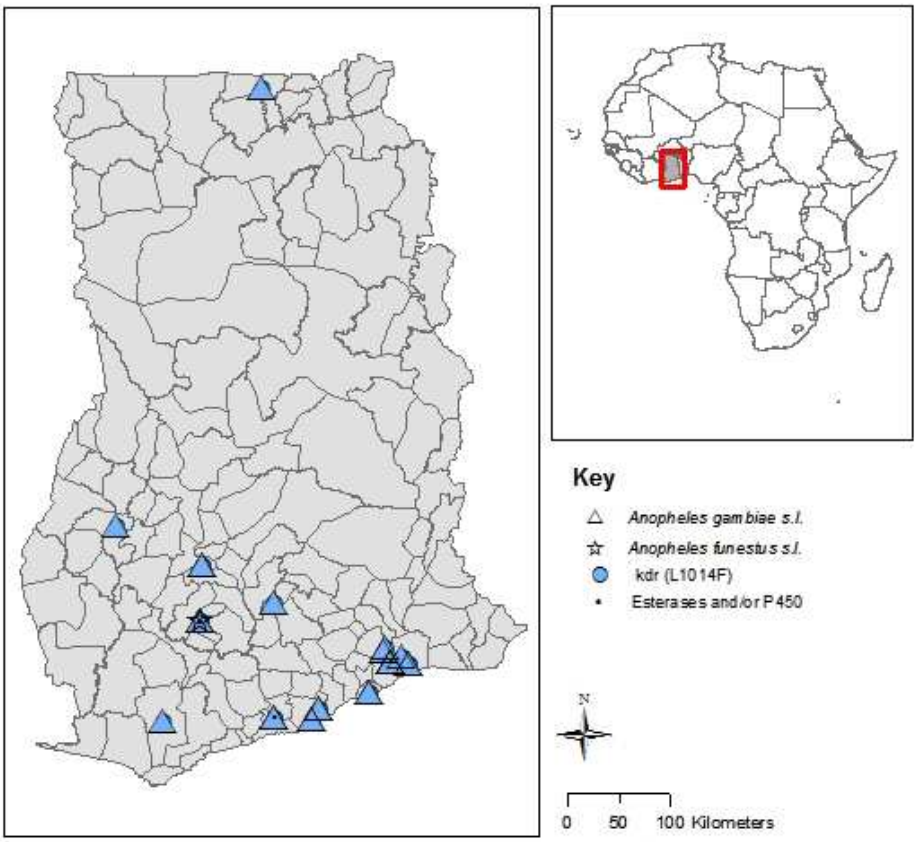


Figure 1. (B) Map of Ghana showing sites for which molecular / biochemical investigations of resistance mechanisms were conducted on **An. gambiae *s.l.*** *and* **An. funestus *s.l.*** *collected between 2002 and 2012. Data shown are for (a) kdr mutations (L1014F ●) and (b) Esterases and/or P450s (•). [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

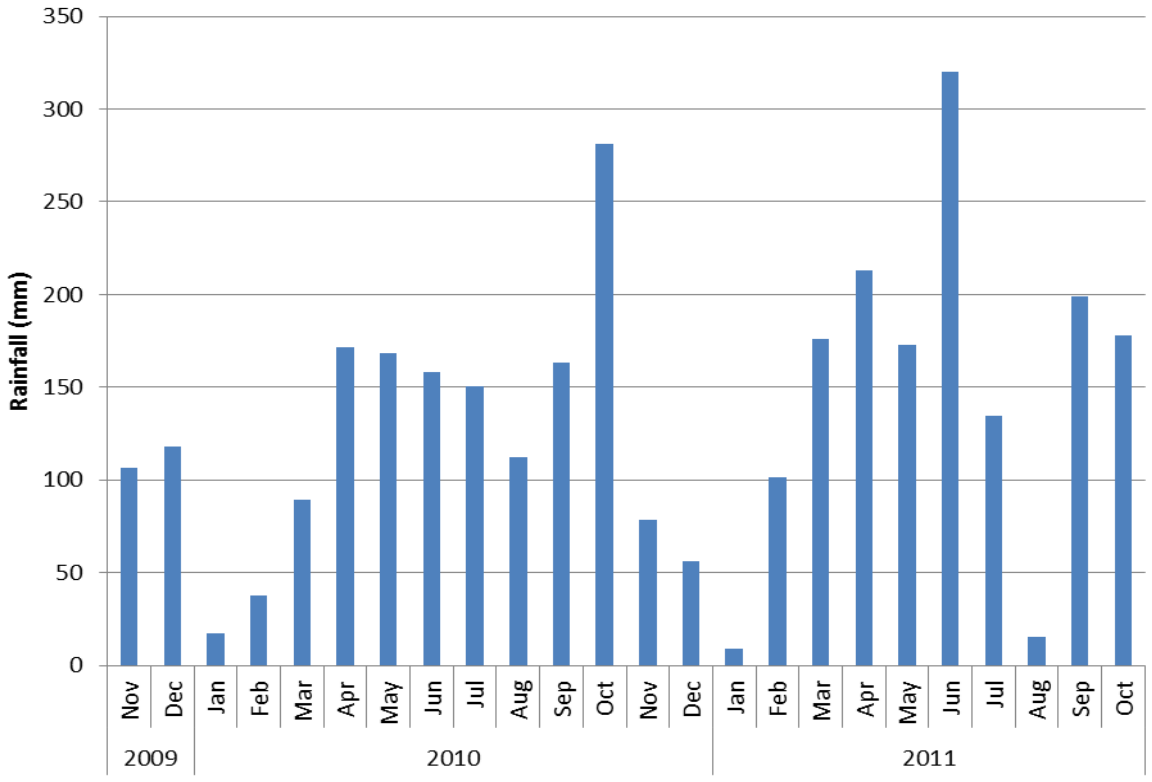
Study sites

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.

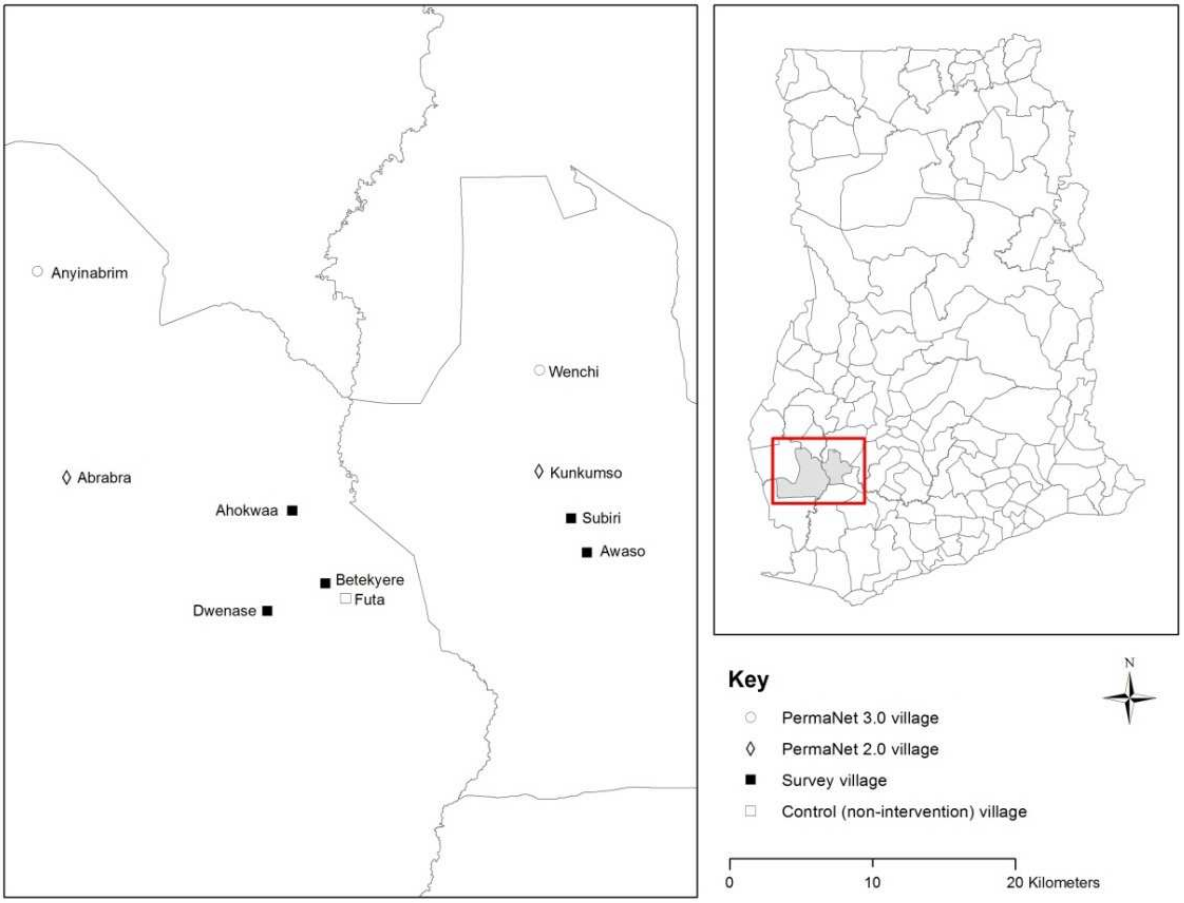


Table 1. WHO susceptibility test results on 2-5 day old F1 An. gambiae s.l. *collected from survey and study villages at baseline (November 2010- January 2011).*

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**

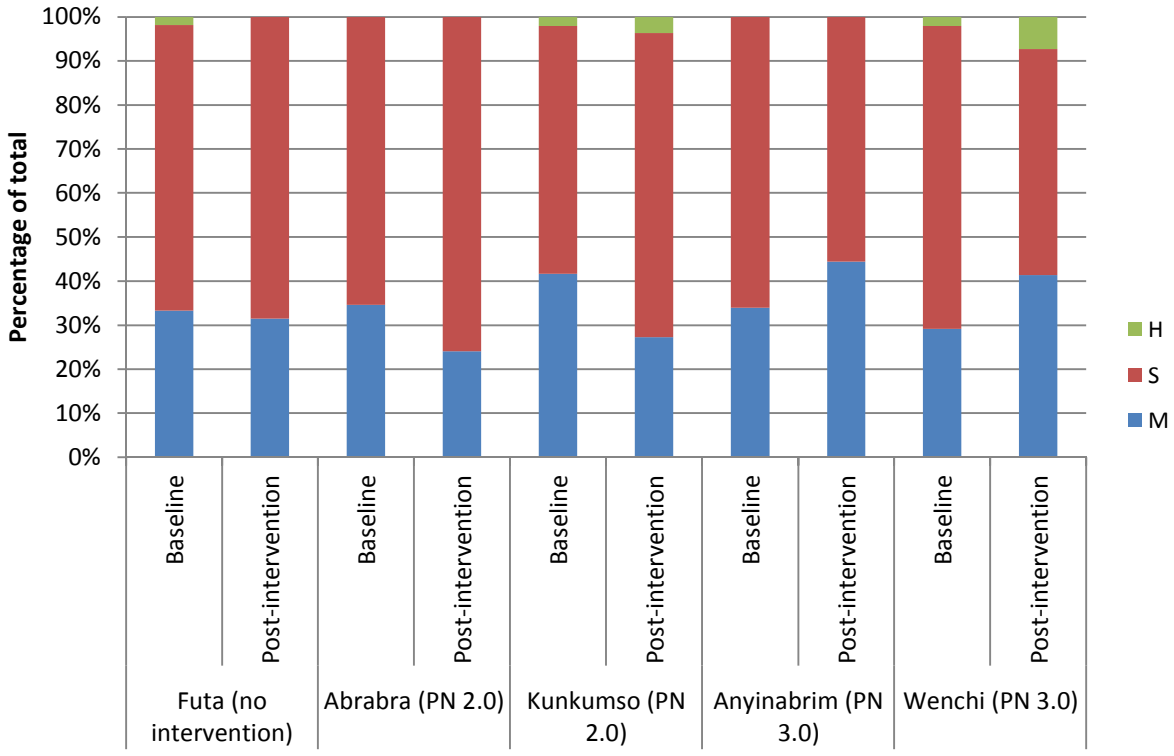
* Difference between mortality rates recorded at baseline and post-intervention, p value for χ^2 statistic with bold showing P < 0.05. Post-intervention survey conducted 18 months after LLIN distribution.

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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