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

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Abstract

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

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

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

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

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

Background

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

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

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

Methods

Description of study sites

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

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

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

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

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

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

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

Mosquito collection

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

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

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

Insecticide susceptibility tests

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

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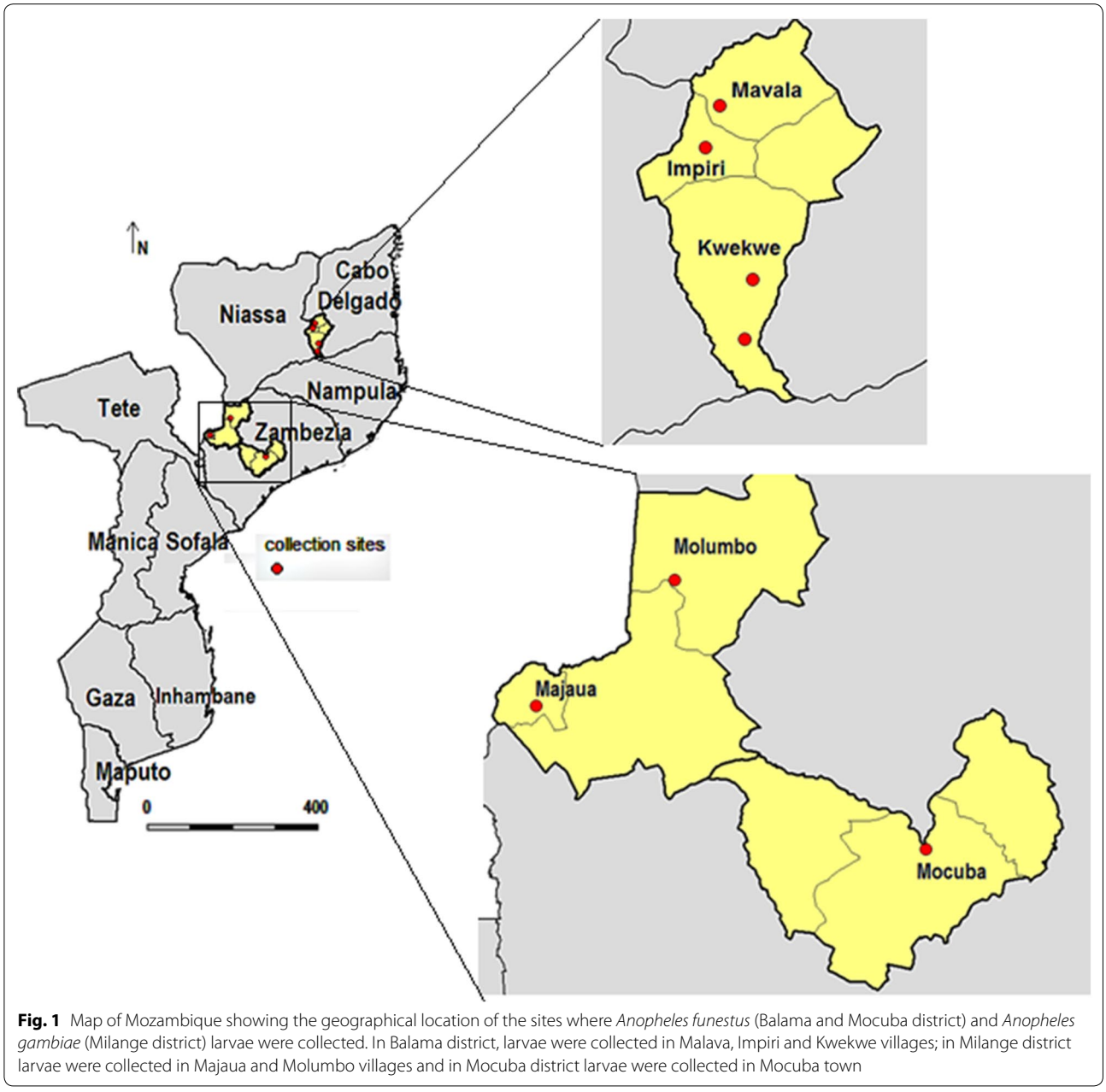

Fig. 1 Map of Mozambique showing the geographical location of the sites where *Anopheles funestus* (Balama and Mocuba district) and *Anopheles gambiae* (Milange district) larvae were collected. In Balama district, larvae were collected in Malava, Impiri and Kwekwe villages; in Milange district larvae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

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

Extraction and preparation of LLINs sub-samples

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

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

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

WHO cone bioassay

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

Chemical analysis for insecticide contents

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

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

Statistical data analysis

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

Ethical considerations

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

Results

Vector populations

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

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

Insecticide susceptibility

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

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

Bio-efficacy of LLINs against wild-caught vector populations

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

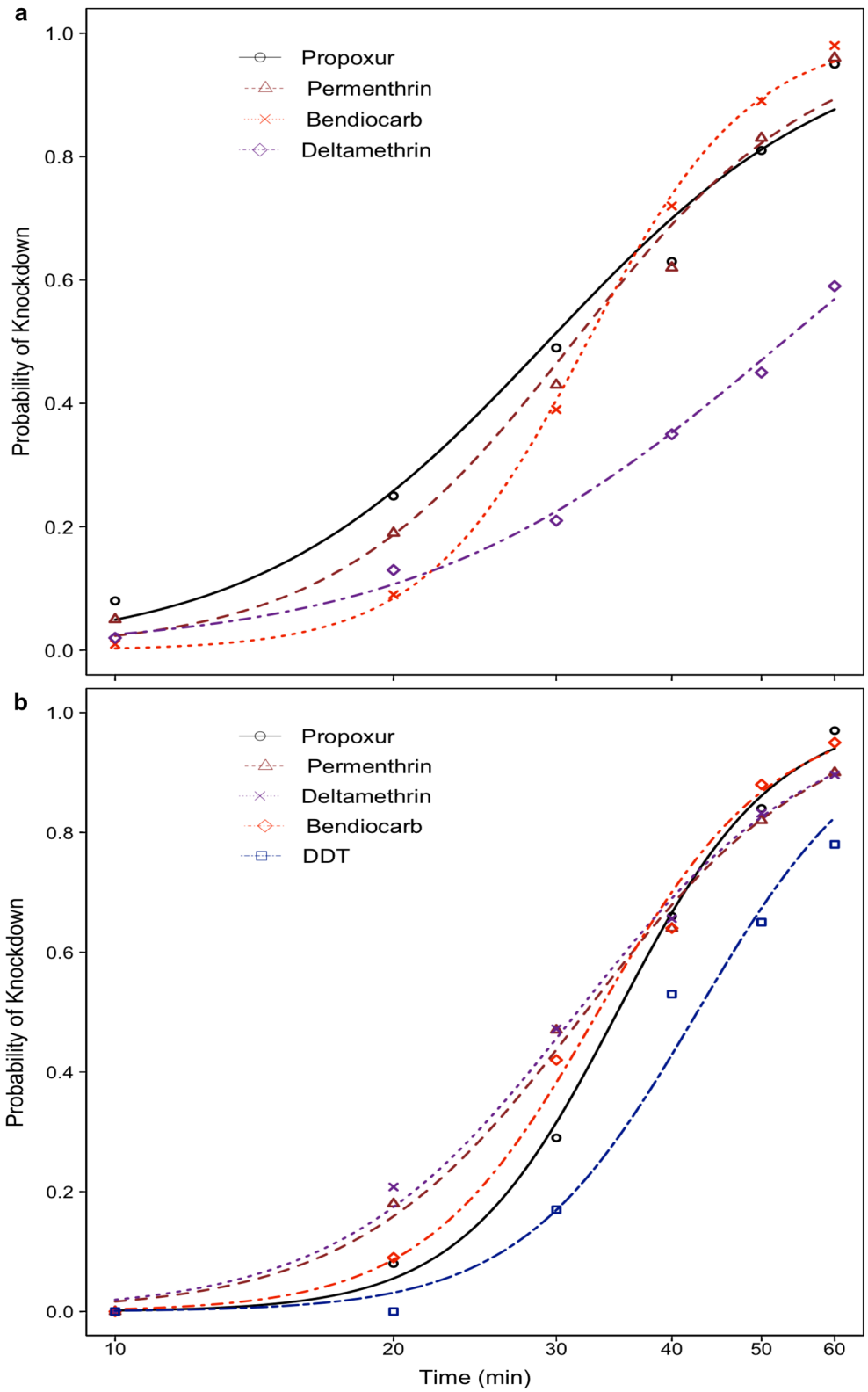

Fig. 2 Response curves showing the probability of knockdown of *Anopheles funestus* **a** from Balama distric and *Anopheles gambiae s.s* **b** from Mocuba district exposed to selected types of insecticides over 60 min exposure-time

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

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

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

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

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

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

(–) Not tested

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

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

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

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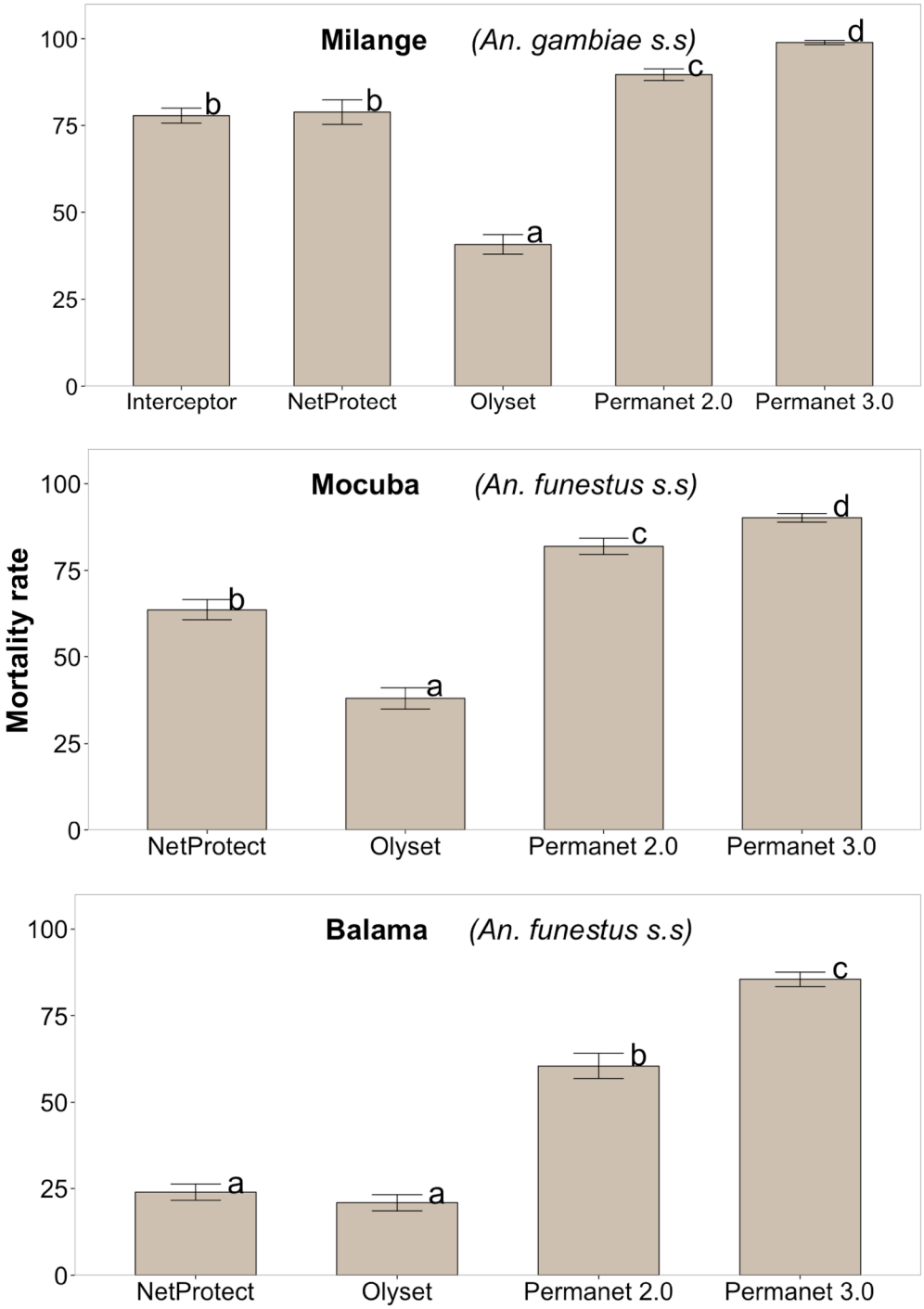

Fig. 3 Comparison of mortality rates of *Anopheles gambiae s.s* (Milange distric) and *Anopheles funestus* (Mocuba and Balama district) mosquito females exposed to different brands of LLINs. *Letters above each bar* display the significance of the difference of Mortality rates between pairs of bed nets,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

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

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

Bio-efficacy against colony susceptible vectors

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

Insecticide contents on the LLINs

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

Discussion

Vector susceptibility to insecticides

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

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

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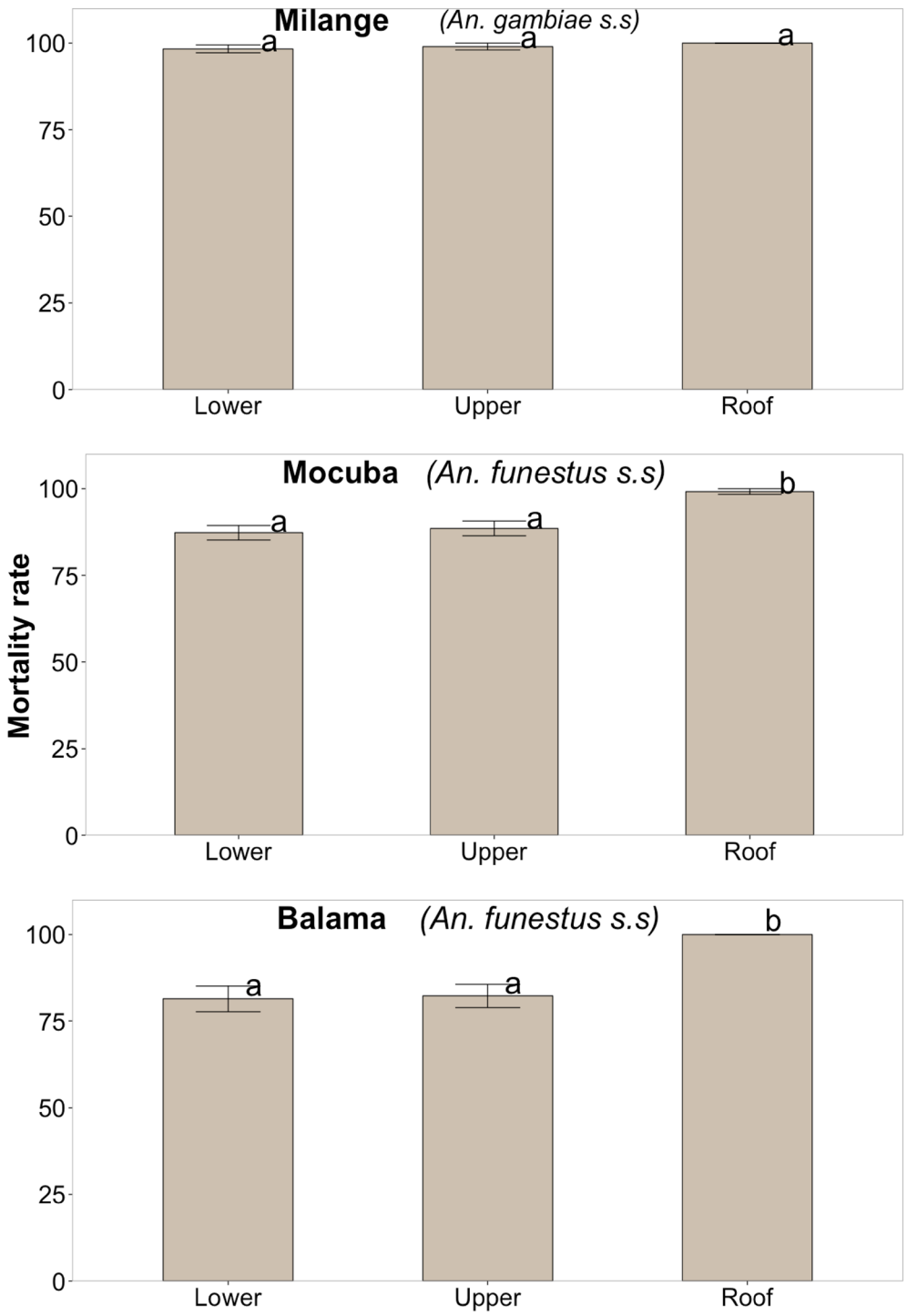

Fig. 4 Comparison of mortality rates of *Anopheles gambiae s.s* (Milange distric) and *Anopheles. funestus* (Mocuba and Balama district) mosquito females exposed to sides and roof of Permanet 3.0. *Letters above each bar* display the significance of the difference of Mortality rates between pairs of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

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

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

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

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

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

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

Bio-efficacy of pyrethroid-only LLINs

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

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

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

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

Bio-efficacy of combination Permanet 3.0®

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

Insecticide concentration on bed nets

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

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

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

Conclusion

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

Additional files

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

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

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

Authors' contributions

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

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Compliance with ethical guidelines

Competing interests

The authors declared that they have no competing interests.

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References

1. Snow RW, Lindsay SW, Hayes RJ, Greenwood BM. Permethrin-treated bed nets (mosquito nets) prevent malaria in Gambian children. Trans R Soc Trop Med Hyg. 1988;82:838–42.
2. Strode C, Donegan S, Garner P, Enayati AA, Hemingway J. The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African anopheline mosquitoes: systematic review and meta-analysis. PLoS Med. 2014;11:e1001619.
3. Millennium Development Goal (MDG) 6: Combat HIV/AIDS and other diseases. http://www.mz.one.in.org.
4. Programa Nacional de Controlo da Malária. Plano Estratégico da Malária. Maputo: Ministério da Saúde, República de Moçambique; 2012.
5. Norris LC, Norris DE. Efficacy of long-lasting insecticidal nets in use in Macha, Zambia, against the local *Anopheles arabiensis* population. Malar J. 2011;10:254.
6. Mouchet J. Mini review: agriculture and vector resistance. Insect Sci Appl. 1988;9:297–302.
7. Diabaté A, Baldet T, Chandre F, Akogbeto M, Guiguemde TR, Darriet F, et al. The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae s.l.* in Burkina Faso. Am J Trop Med Hyg. 2002;67:617–22.
8. Thompson R, Begtrup K, Cuamba N, Dgedge M, Mendis C, Gamage-Mendis A, et al. The Matola malaria project: a temporal and spatial study of malaria transmission and disease in a suburban area of Maputo, Mozambique. Am J Trop Med Hyg. 1997;57:550–9.

9. Mendis C, Jacobsen JL, Gamage-Mendis A, Bule E, Dgedge M, Thompson R, et al. *Anopheles arabiensis* and *An. funestus* are equally important vectors of malaria in Matola coastal suburb of Maputo, southern Mozambique. Med Vet Entomol. 2000;14:171–80.
10. Charlwood JD, Cuamba N, Tomas EV, Briet OJ. Living on the edge: a longitudinal study of *Anopheles funestus* in an isolated area of Mozambique. Malar J. 2013;12:208.
11. Charlwood JD, Macia GA, Manhaca M, Sousa B, Cuamba N, Braganca M. Population dynamics and spatial structure of human-biting mosquitoes, inside and outside of houses, in the Chockwe irrigation scheme, southern Mozambique. Geospat Health. 2013;7:309–20.
12. Cuamba N, Mendis C. The role of *Anopheles merus* in malaria transmission in an area of southern Mozambique. J Vector Borne Dis. 2009;46:157–9.
13. Brooke BD, Kloke G, Hunt RH, Koekemoer LL, Temu EA, Taylor ME, et al. Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: culicidae). Bull Entomol Res. 2001;91:265–72.
14. Casimiro S, Coleman M, Mohloai P, Hemingway J, Sharp B. Insecticide resistance in *Anopheles funestus* (Diptera: culicidae) from Mozambique. J Med Entomol. 2006;43:267–75.
15. Abilio AP, Kleinschmidt I, Rehman AM, Cuamba N, Ramdeen V, Mthembu DS, et al. The emergence of insecticide resistance in central Mozambique and potential threat to the successful indoor residual spraying malaria control programme. Malar J. 2011;10:110.
16. Casimiro S, Coleman M, Hemingway J, Sharp B. Insecticide resistance in *Anopheles arabiensis* and *Anopheles gambiae* from Mozambique. J Med Entomol. 2006;43:276–82.
17. WHO. Global plan for insecticide resistance management in malaria vectors. World Health Organization, Geneva, WHO Press; 2012.
18. The spatial distribution of *Plasmodium falciparum* malaria stratified by endemicity class map in 2010 in Mozambique. http://www.map.ox.ac.uk/explore/countries/MOZ/#!malaria-risk.
19. Giardina F, Kasasa S, Sie A, Utzinger J, Tanner M, Vounatsou P. Effects of vector-control interventions on changes in risk of malaria parasitaemia in sub-Saharan Africa: a spatial and temporal analysis. Lancet Glob Health. 2014;2:e601–15.
20. WHO. Manual on pratical entomology in malaria. Part II: Methods and Techniques. Geneva: World Health Organization; 1975.
21. Gillies MT, De Meillon B. The Anophelinae of the Africa south of the sahara (Ethiopian zoographical region). South Africa: South African Institute for Medical Research; 1968.
22. Koekemoer LL, Kamau L, Hunt RH, Coetzee M. A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. Am J Trop Med Hyg. 2002;66:804–11.
23. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am J Trop Med Hyg. 1993;49:520–9.
24. WHO. Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticide on treated surfaces. Geneva: World Health Organization; 1998.
25. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL, et al. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol. 1998;7:179–84.
26. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol. 2000;9:491–7.
27. Hemingway J, Ranson H. Insecticide resistance in insect vectors of human disease. Annu Rev Entomol. 2000;45:371–91.
28. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A, et al. Detection of knockdown resistance (kdr) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Malar J. 2007;6:111.
29. Permanet 3.0 Brochure. http://www.vestergaard.com/permanet-3-0.
30. Technical basis for deployment of PermanetNet 3.0 in areas with pyrethroid-resistant malaria vectors. http://www.vestergaard.com/images/pdf/PN3_Tech_Eng_2015.pdf.
31. Moores GD, Bingham G. Use of "temporal synergism" to overcome insecticide resistance. Outlook Pest Manag. 2005;16:7–9.
32. WHO. Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets. Geneva: World Health Organization; 2005.
33. Ouattara JP, Pigeon O, Spanoghe P. Validation of a multi-residue method to determine deltamethrin and alpha-cypermethrin in mosquito nets by gas chromatography with electron capture detection (GC-muECD). Parasit Vectors. 2013;6:77.
34. Collaborative International Pesticides Analytical Council (CIPA). http://www.cipac.org/cipacpub.htm.
35. Jenkins DW, Hensens A, Lloyd J, Payne M, Cizmarik P, Hamel S. Development and validation of a 'universal' HPLC method for pyrethroid quantification in long-lasting insecticidal mosquito nets for malaria control and prevention. Trop Med Int Health. 2013;18:2–11.
36. Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, Stevens MH, et al. Generalized linear mixed models: a practical guide for ecology and evolution. Trends Ecol Evol. 2009;24:127–35.
37. Bates D, Maechler M, Bolder B, Walker S. lme4: Linear mixed-effects models using Eigen and S4 (*arXiv* 2014, 1406:5823v1).
38. Hothorn T, Bretz F, Westfall P. Simultaneous inference in general parametric models. Biometrical J. 2008;50:346–63.
39. Bretz F, Hothorn T, Westfall P: Multiple Comparison Using R. Chapman & Hall/CRC; 2010.
40. R Core Team. A language and environment for statistical computing. Vienna: R Foundation for Statistical, Computing; 2015.
41. Feyereisen R. Insect P450 enzymes. Annu Rev Entomol. 1999;44:507–33.
42. Riveron JM, Yunta C, Ibrahim SS, Djouaka R, Irving H, Menze BD, et al. A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. Genome Biol. 2014;15:R27.
43. Amenya DA, Naguran R, Lo TC, Ranson H, Spillings BL, Wood OR, Brooke BD, et al. Over expression of a cytochrome P450 (CYP6P9) in a major African malaria vector, *Anopheles funestus*, resistant to pyrethroids. Insect Mol Biol. 2008;17:19–25.
44. Cuamba N, Morgan JC, Irving H, Steven A, Wondji CS. High level of pyrethroid resistance in an *Anopheles funestus* population of the Chokwe District in Mozambique. PLoS One. 2010;5:e11010.
45. Hemingway J. The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. Insect Biochem Mol Biol. 2000;30:1009–15.
46. N'Guessan R, Darriet F, Doannio JM, Chandre F, Carnevale P. Olyset net efficacy against pyrethroid-resistant *Anopheles gambiae* and *Culex quinquefasciatus* after 3 years field use in Cote d'Ivoire. Med Vet Entomol. 2001;15:97–104.
47. D'Alessandro U, Olaleye BO, McGuire W, Thomson MC, Langerock P, Bennett S, et al. A comparison of the efficacy of insecticide-treated and untreated bed nets in preventing malaria in Gambian children. Trans R Soc Trop Med Hyg. 1995;89:596–8.
48. Prevention and Management of Insecticide Resistance in Vectors of Public Heath Importance. http://www.irac-online.org/documents/irm-vector-manual/?ext=pdf.
49. Guillet P, N'Guessan R, Darriet F, Traore-Lamizana M, Chandre F, Carnevale P. Combined pyrethroid and carbamate 'two-in-one' treated mosquito nets: field efficacy against pyrethroid-resistant *Anopheles gambiae* and *Culex quinquefasciatus*. Med Vet Entomol. 2001;15:105–12.
50. Koudou BG, Koffi AA, Malone D, Hemingway J. Efficacy of PermaNet(R) 2.0 and PermaNet(R) 3.0 against insecticide-resistant *Anopheles gambiae* in experimental huts in Cote d'Ivoire. Malar J. 2011;10:172.
51. Awolola ST, Adeogun AO, Olojede JB, Oduola AO, Oyewole IO, Amajoh CN. Impact of PermaNet 3.0 on entomological indices in an area of pyrethroid resistant *Anopheles gambiae* in south-western Nigeria. Parasit Vectors. 2014;7:236.
52. WHO. Report of the Twelfth WHOPES Working Group Meeting. Geneva: World Health Organization; 2009.
53. Okia M, Ndyomugyenyi R, Kirunda J, Byaruhanga A, Adibaku S, et al. Bioefficacy of long-lasting insecticidal nets against pyrethroid-resistant populations of *Anopheles gambiae s.s.* from different malaria transmission zones in Uganda. Parasit Vectors. 2013;6:130.

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

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Abstract

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

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

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

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

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

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Background

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

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

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

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

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

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

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

Methods

Study design

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

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

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

Study sites

Malanville

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

Tanguieta

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

Abomey-Calavi

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

Cotonou

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

Porto-Novo

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

Parakou

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

Zangnanado

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

Larvae collection

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

Highlighting resistance mechanisms

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

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

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

Mosquito nets

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

Cone test

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

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

Data analysis

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

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

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

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

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

Results

Characteristics of the studied mosquito populations

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

Knock-down (KD) and mortality of laboratory strains

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

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

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

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

Inhibition of mortality conferred by the *kdr* resistance gene

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

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

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

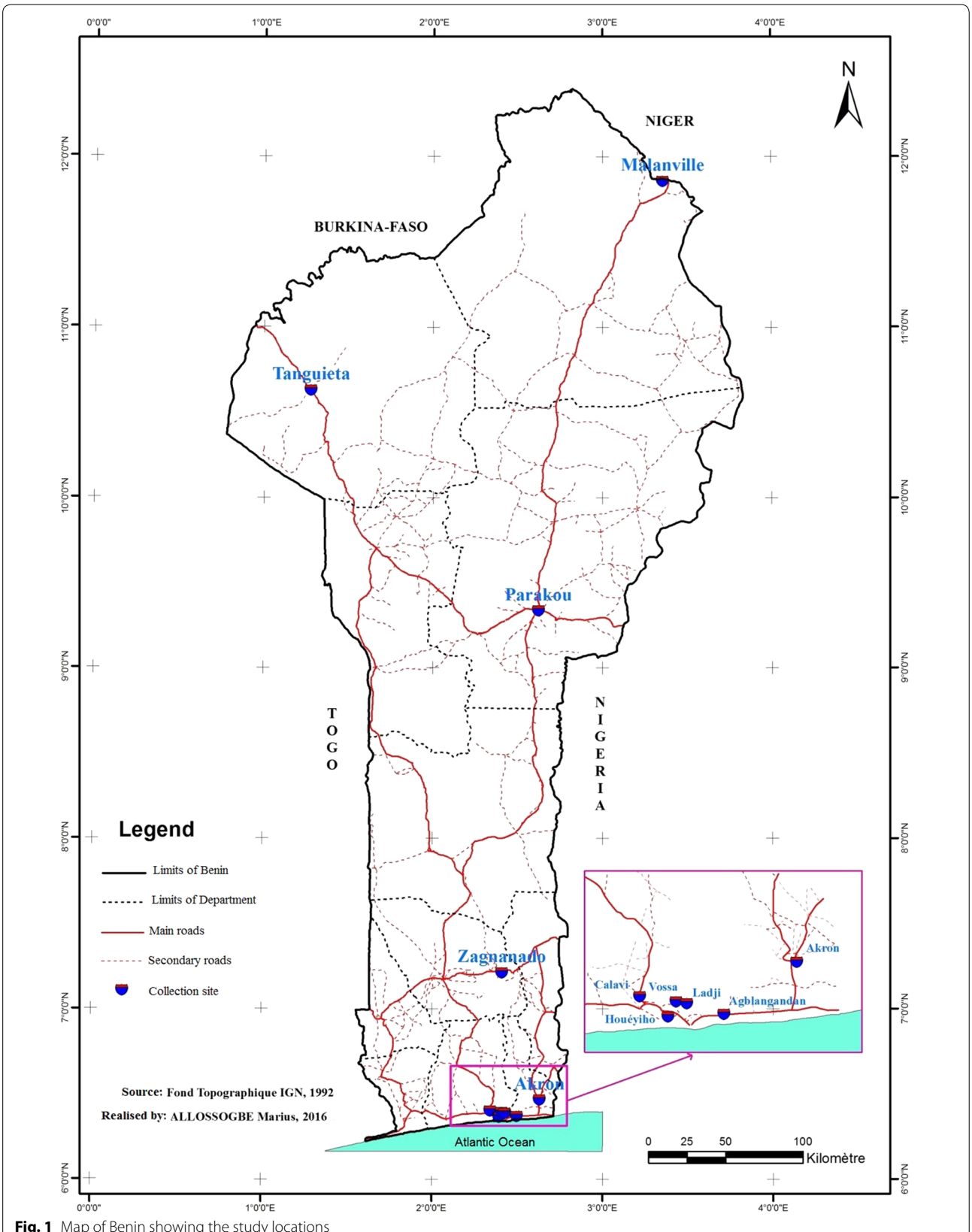

Fig. 1 Map of Benin showing the study locations

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

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

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

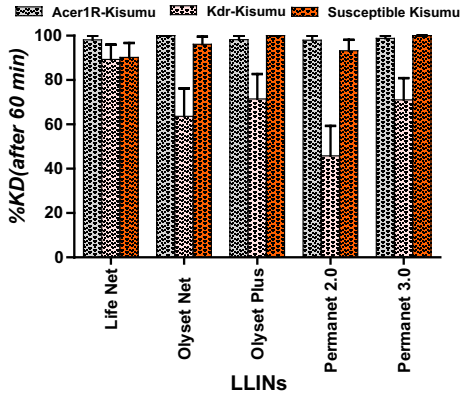

Fig. 2 Mosquito knock-down at 60 min post-exposure to LLINs

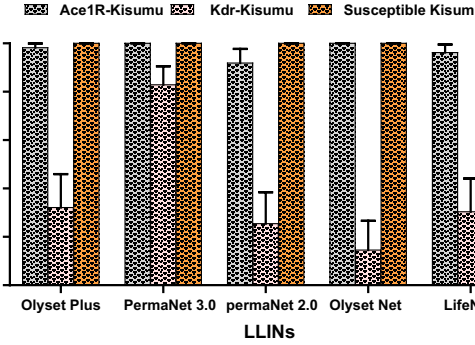

Fig. 3 Mosquito mortalities 24 h post-exposure to LLINs

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Inhibition of mortality in multi-resistant strains

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

Authors' contributions

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

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

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References

1. WHO. World malaria report 2015. Geneva: World Health Organization; 2015. http://www.who.int/malaria/publications/world-malaria-report-2015/report/en/. Accessed 15 Aug 2016.
2. Gimnig JE, Lindblade KA, Mount DL, Atieli FK, Crawford S, Wolkon A, et al. Laboratory wash resistance of long-lasting insecticidal nets. Trop Med Int Health. 2005;10:1022–9.
3. WHO. The Abuja declaration and the plan of action. An extract from the African Summit on Roll Back Malaria. Geneva: World Health Organization; 2003; p. 1–11. http://www.who.int/malaria/publications/atoz/whocd-srbm200346/en/. Accessed 15 Aug 2016.
4. Carnevale P, Robert V, Boudin C, Halna JM, Pazart L, Gazin P, et al. Control of malaria using mosquito nets impregnated with pyrethroids in Burkina Faso. Bull Soc Path Exot Filiales. 1988;81:832–46 **(in French)**.
5. Desfontaine M, Gelas H, Cabon H, Goghomou A, Kouka Bemba D, Carnevale P. Evaluation des pratiques et des couts de lutte antivectorielle à l'échelon familial en Afrique Centrale. II. Ville de Douala (Cameroun), juillet 1988. Ann Soc Belge Méd Trop. 1990;70:137–44.
6. Alonso PL, Lindsay SW, Armstrong JRM, de Francisco A, Shenton FC, Greenwood BM, et al. The effect of insecticide-treated bed nets on mortality of Gambian children. Lancet. 1991;337:1499–502.
7. D'Alessandro U, Olaleye BO, McGuire W, Thomson MC, Langerock P, Bennett S, et al. A comparison of the efficacy of insecticide-treated and untreated bed nets in preventing malaria in Gambian children. Trans R Soc Trop Med Hyg. 1995;89:596–8.
8. Aikins MK, Fox-Rushby J, D'Alessandro U, Langerock P, Cham K, New L, et al. The Gambian National Impregnated Bednet Programme: costs, consequences and net cost-effectiveness. Soc Sci Med. 1998;46:181–91.
9. Snow RW, Rowan KM, Lindsay SW, Greenwood BM. A trial of bed nets (mosquito nets) as a malaria control strategy in a rural area of The Gambia, West Africa. Trans R Soc Trop Med Hyg. 1988;82:212–5.
10. Karch S, Garin B, Asidi N, Manzambi Z, Salaun JJ, Mouchet J. Moustiquaires impregnées contre le paludisme au Zaire. Ann Soc Belge Méd Trop. 1993;73:37–53.
11. Nevill CG, Some ES, Mung'ala VO, Mutemi W, New L, Marsh K, et al. Insecticide-treated bednets reduce mortality and severe morbidity from malaria among children on the Kenyan coast. Trop Med Int Health. 1996;1:139–46.
12. Binka FN, Kubaje A, Adjuik M, Williams LA, Lengeler C, Maude GH, et al. Impact of permethrin impregnated bednets on child mortality in Kassena-Nankana district, Ghana: a randomized controlled trial. Trop Med Int Health. 1996;1:147–54.
13. Akogbeto PM, Nahum A. Impact of deltamethrin impregnated mosquito nets on the transmission of malaria in the coastal lagoon area, Benin. Bull Soc Path Exot. 1996;89:291–8 **(in French)**.
14. Mathanga DP, Campbell CH, Taylor TE, Barlow R, Wilson ML. Reduction of childhood malaria by social marketing of insecticide-treated nets: a case–control study of effectiveness in Malawi. Am J Trop Med Hyg. 2005;73:622–5.
15. Chandre F, Darriet F, Manguin S, Brengues C, Carnevale P, Guillet P. Pyrethroid cross resistance spectrum among populations of *Anopheles gambiae s.s.* from Côte d'Ivoire. J Am Mosq Control Assoc. 1999;15:53–9.
16. Akogbeto M, Yakoubou S. Resistance of malaria vectors to pyrethrins used for impregnating mosquito nets in Benin, West Africa. Bull Soc Pathol Exot. 1999;92:123–30 **(in French)**.
17. Akogbeto MC, Djouaka R, Noukpo H. Use of agricultural insecticides in Benin. Bull Soc Pathol Exot. 2005;98:400–5 **(in French)**.
18. Corbel V, N'Guessan R, Brengues C, Chandre F, Djogbenou L, Martin T, et al. Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. Acta Trop. 2007;101:207–16.
19. N'Guessan R, Corbel V, Bonnet J, Yates A, Asidi A, Boko P, et al. Evaluation of indoxacarb, an oxadiazine insecticide for the control of pyrethroid-resistant *Anopheles gambiae* (Diptera: Culicidae). J Med Entomol. 2007;44:270–6.
20. Djènontin A, Chabi J, Baldet T, Irish S, Pennetier C, Hougard JM, et al. Managing insecticide resistance in malaria vectors by combining carbamate-treated plastic wall sheeting and pyrethroid-treated bed nets. Malar J. 2009;8:233.
21. Yadouleton AW, Padonou G, Asidi A, Moiroux N, Bio-Banganna S, Corbel V, et al. Insecticide resistance status in *Anopheles gambiae* in southern Benin. Malar J. 2010;9:83.
22. Djogbénou L, Pasteur N, Akogbéto M, Weill M, Chandre F. Insecticide resistance in the *Anopheles gambiae* complex in Benin: a nationwide survey. Med Vet Entomol. 2011;25:256–67.
23. Gnanguenon V, Agossa FR, Badirou K, Govoetchan R, Anagonou R, Oke-Agbo F, et al. Malaria vectors resistance to insecticides in Benin: current trends and mechanisms involved. Parasit Vectors. 2015;8:223.
24. Henry MC, Assi SB, Rogier C, Yovo DJ, Chandre F, Guillet P, et al. Protective efficacy of lambda-cyhalothrin treated nets in *Anopheles gambiae* pyrethroid resistance areas of Cote d'Ivoire. Am J Trop Med Hyg. 2005;73:859–64.
25. Curtis CF, Jana-Kara B, Maxwell CA. Insecticide treated nets: impact on vector populations and relevance of initial intensity of transmission and pyrethroid resistance. J Vector Borne Dis. 2003;40:1–8.
26. Asidi A, N'Guessan R, Akogbeto M, Curtis C, Rowland M. Loss of household protection from use of insecticide-treated nets against pyrethroid-resistant mosquitoes, Benin. Emerg Infect Dis. 2012;18:1101–6.
27. Aïkpon R, Sèzonlin M, Ossè R, Akogbéto M. Evidence of multiple mechanisms providing carbamate and organophosphate resistance in field *An. gambiae* population from Atacora in Benin. Parasit Vectors. 2014;7:568.
28. Corbel V, Hougard J-M, N'Guessan R, Chandre F. Evidence for selection of insecticide resistance due to insensitive acetylcholinesterase by carbamate-treated nets in *Anopheles gambiae s.s.* (Diptera: Culicidae) from Côte d'Ivoire. J Med Entomol. 2003;40:985–8.
29. Kweka EJ, Lyaruu LJ, Mahande AM. Efficacy of PermaNet® 3.0 and PermaNet® 2.0 nets against laboratory-reared and wild *Anopheles gambiae* sensu lato populations in northern Tanzania. Infect Dis Poverty. 2017;6:11.
30. Brogdon WG, McAllister JC, Vulule JM. Association of heme peroxidase activity measured in single-mosquitoes identifies individuals expressing an elevated oxidase for insecticide resistance. J Am Mosq Control Assoc. 1997;13:233–7.
31. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, Devonshire AL, et al. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae s.s.* Insect Mol Biol. 1998;7:179–84.
32. Hemingway J, Hawkes N, Prapanthadara L, Jayawardenal KGI, Ranson H. The role of gene splicing, gene amplification and regulation in mosquito insecticide resistance. Philos Trans R Soc Lond B Biol Sci. 1998;353:1695–9.
33. Finney D. Review of probit analysis: a statistical treatment of the sigmoid response curve. J R Stat Soc. 1947;110:263–6.
34. Okia M, Ndyomugyenyi R, Kirunda J, Byaruhanga A, Adibaku S, Lwamafa DK, et al. Bioefficacy of long-lasting insecticidal nets against pyrethroid-resistant populations of *Anopheles gambiae s.s.* from different malaria transmission zones in Uganda. Parasit Vectors. 2013;6:130.

35. Yewhalaw D, Asale A, Tushune K, Getachew Y, Duchateau L, Speybroeck N. Bio-efficacy of selected long-lasting insecticidal nets against pyrethroid resistant *Anopheles arabiensis* from South-Western Ethiopia. Parasit Vectors. 2012;5:159.
36. Pennetier C, Bouraima A, Chandre F, Piameu M, Etang J, Rossignol M, et al. Efficacy of Olyset® Plus, a new long-lasting insecticidal net incorporating permethrin and piperonil-butoxide against multi-resistant malaria vectors. PLoS ONE. 2013;8:e75134.
37. Ranson H, Abdallah H, Badolo A, Guelbeogo WM, Kerah-Hinzoumbé C, Yangalbé-Kalnoné E, et al. Insecticide resistance in *Anopheles gambiae*: data from the first year of a multi-country study highlight the extent of the problem. Malar J. 2009;8:299.
38. Chouaibou MS, Chabi J, Bingham GV, Knox TB, N'Dri L, Kesse NB, et al. Increase in susceptibility to insecticides with aging of wild *Anopheles gambiae* mosquitoes from Côte d'Ivoire. BMC Infect Dis. 2012;12:214.
39. Akogbéto MC, Djouaka RF, Kindé-Gazard DA. Screening of pesticide residues in soil and water samples from agricultural settings. Malar J. 2006;5:22.
40. Yadouleton A, Asidi A, Djouaka R, Braïma J, Agossou C, Akogbeto M. Development of vegetable farming: a cause of the emergence of insecticide resistance in populations of *Anopheles gambiae* in urban areas of Benin. Malar J. 2009;8:103.
41. Diabate A, Baldet T, Chandre F, Akogbeto M, Guiguemde TR, Darriet F, et al. The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae* s.l. in Burkina Faso. Am J Trop Med Hyg. 2002;67:617–22.
42. Djouaka R, Irving H, Tukur Z, Wondji CS. Exploring mechanisms of multiple insecticide resistance in a population of the malaria vector *Anopheles funestus* in Benin. PLoS ONE. 2011;6:e27760.
43. Aïkpon R, Agossa F, Ossè R, Oussou O, Aïzoun N, Oké-Agbo F, et al. Bendiocarb resistance in *Anopheles gambiae* s.l. populations from Atacora department in Benin, West Africa: a threat for malaria vector control. Parasit Vectors. 2013;6:192.
44. WHO. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva: World Health Organization; 2013.
45. WHO. Guidelines for laboratory and field-testing of long-lasting insecticidal nets. Geneva: World Health Organization; 2013. p. 1–102.

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RESEARCH Open Access

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

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Abstract

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

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

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

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

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

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Background

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

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

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

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

Methods

Mosquito collections

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

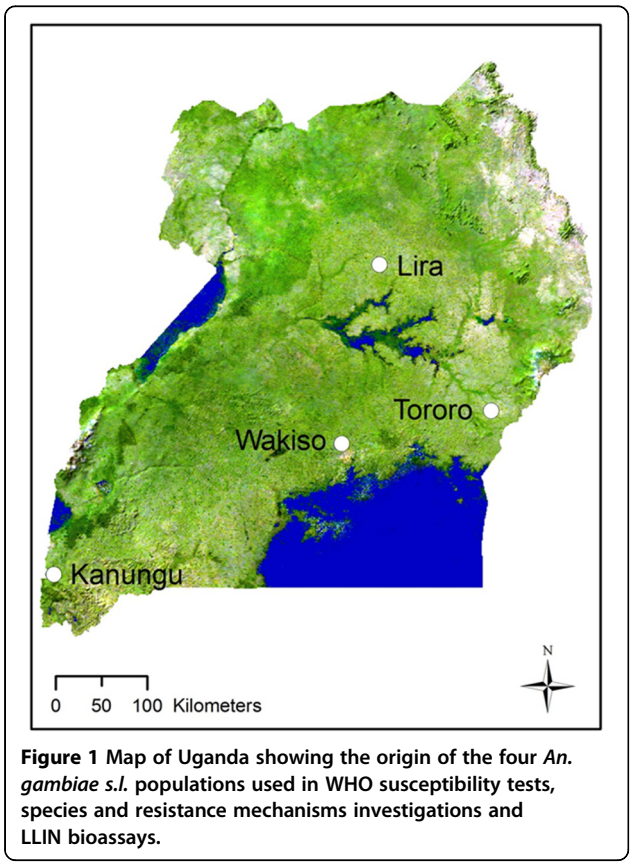

Figure 1 Map of Uganda showing the origin of the four *An. gambiae s.l.* populations used in WHO susceptibility tests, species and resistance mechanisms investigations and LLIN bioassays.

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

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

Insecticide susceptibility and molecular testing

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

LLIN samples

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

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

LLIN chemical analyses

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

LLIN bioassays

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

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

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

Data analyses

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

Results

Population characterisation and insecticide susceptibility

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

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

LLINs and bioassays

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

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

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

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

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

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

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

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

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

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

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

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

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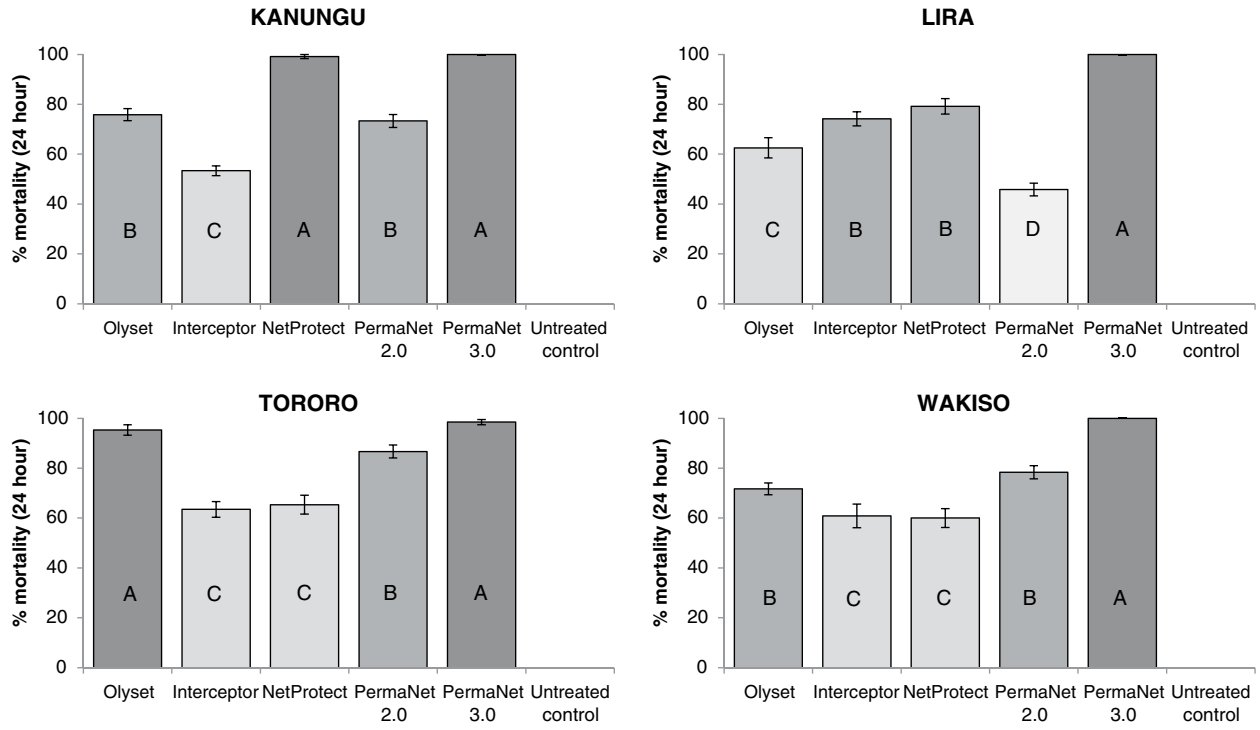

Figure 2 Bioefficacy (mean % 24-hour mortality) in cone bioassays of WHO-recommended LLINs against field-derived *An. gambiae* populations from four sites in Uganda. Vertical lines indicate standard error (n = 24). The same letter and shading within graphs indicates no significant difference (via Duncan's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

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

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

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

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

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

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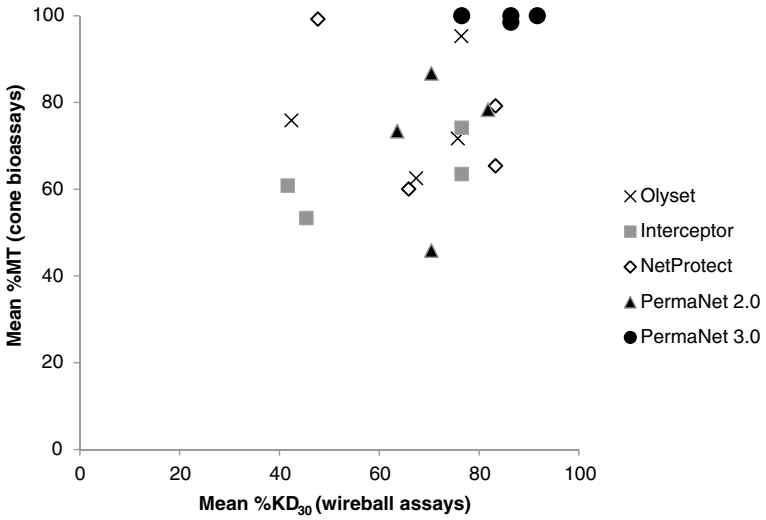

Figure 3 Comparative bioefficacy of different LLIN types as determined via wireball versus cone bioassays for four populations of *An. gambiae* from Uganda.

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

Discussion

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

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

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

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

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

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

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

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

Conclusions

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

Additional files

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

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

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

Competing interests

The authors declare no competing interests.

Authors' contributions

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

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References

1. WHO: *WHO World Malaria Report 2010.* Geneva: World Health Organization; 2010:136.
2. D' Alessandro U, Olaleye BO, Mcquire W, Langerock P, Bennett S, Aikins MK, Thomson MC, Cham MK, Cham BA, Greenwood BM: **Mortality and morbidity from malaria in Gambian children after introduction of a treated bed nets programme.** *Lancet* 1995, **345:**479–483.
3. Lengeler C: **Insecticide-treated bed net and curtains for preventing malaria.** *Cochrane Database Syst Rev* 2004, **2:**CDOO3363.
4. WHO: *Guidelines for Laboratory and Field testing of Long-lasting Insecticidal Mosquito Nets.* Geneva: World Health Organization; 2005. WHO/CDS/WHOPES/GDCPP/2005.11.
5. Zaim M, Aitio A, Nalasima N: **Safety of pyrethroid-treated mosquito nets.** *Med Vet Entomol* 2000, **14:**1–5.
6. Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V: **Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control?** *Trends Parasitol* 2010, **27:**91-98 doi:10.1016/j.pt.2010.08.004.
7. N'Guessan R, Corbel V, Akogbéto M, Rowland M: **Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin.** *Emerg Infect Dis* 2007, **13:**199–206.
8. Fane M, Cisse O, Traore CS, Sabatier P: ***Anopheles gambiae* resistance to pyrethroid-treated nets in cotton versus rice areas in Mali.** *Acta Trop* 2012, **122:**1–6.
9. Haji KA, Khatib BO, Smith S, Ali AS, Devine GJ, Coetzee M, Majambere S: **Challenge for malaria elimination in Zanzibar: pyrethroid resistance in malaria vectors and poor performance of long-lasting insecticide nets.** *Parasit Vectors* 2013, **6:**82.
10. Najera JA, Zaim M: *Malaria Vector Control: Decision Making Criteria and Procedures for Judicious Use of Insecticides.* Geneva: World Health Organization Pesticide Evaluation Scheme; 2005.
11. Bonabana-Wabbi J: *Dissertation, Assessing Factors Affecting Adoption of Agricultural Technologies: The Case of Integrated Pest Management (IPM) in Kumi District, Eastern Uganda.* Department of Agricultural and Applied Economics, Virginia Tech; 2008.
12. Bonabana-Wabbi J, Taylor BD: *Health and Environmental Benefits of Reduced Pesticide Use in Uganda: An Experimental Economics Analysis.* Orlando, Florida: Paper presented at the Joint Annual Meeting of the American Agricultural Economics Association and the American Council on Consumer Interests; 2008. http://ageconsearch.umn.edu/bitstream/6441/2/469143.pdf.
13. WHO: *Atlas of Insecticide Resistance of Malaria Vectors in the WHO African Region.* World Health Organization African Network for Vector Resistance, Regional Office for Africa; 2005:27pp.
14. Verhaeghen K, Van Bortel W, Roelants P, Backeljau T, Coosemans M: **Detection of the east and west African *kdr* mutation in *anopheles***

gambiae and *anopheles funestus* from Uganda using a new assay based on FRET/melt curve analysis. *Malaria J* 2006, **5**:16.
15. Rubaihayo J, Tukesiga E, Abaasa A: **Reduced susceptibility to pyrethroid insecticide treated nets by the malaria vector Anopheles gambiae s.l. in western Uganda.** *Malaria J* 2008, **7**:92.
16. Ramphul U, Boase T, Bass C, Okedi LM, Donnelly MJ, *et al*: **Insecticide resistance and its association with target-site mutations in natural populations of *Anopheles gambiae* from eastern Uganda.** *Trans R Soc Trop Med Hyg* 2009, **103**:1121–1126.
17. Morgan JC, Irving H, Okedi LM, Steven A, Wondji CS: **Pyrethroid resistance in an *Anopheles funestus* population from Uganda.** *PLoS One* 2010, **5**(7): e11872. doi:10.1371/journal.pone.0011872.
18. Verhaeghen K, Bortel WV, Roelants P, Okello PE, Talisuna A, *et al*: **Spatio-temporal patterns in *kdr* frequency in permethrin and DDT resistant *Anopheles gambiae s.s.* from Uganda.** *AmJTrop Med Hyg* 2010, **82**:566–573.
19. Santolamazza F, Calzetta M, Etang J, *et al*: **Distribution of knock-down resistance mutations in *Anopheles gambiae* molecular forms in west and west-central Africa.** *Malaria J* 2008, **7**:74.
20. Gillies M, Coetzee M: *A supplementary to Anophelinae of Africa South of the Sahara.* Johannesburg: South African Institute for Medical Research; 1998.
21. WHO: *Test Procedures for Insecticide Resistance Monitoring in Malaria Vectors, Bio-efficacy and Persistence of Insecticide on Treated Surfaces.* Geneva: World Health Organization; 1998. WHO/CDS/CPC/MAL/98.12.
22. Scott JA, Brogdon WG, Collins FH: **Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction.** *AmJTrop Med Hyg* 1993, **49**:520–529.
23. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, Devonshire AL, Guillet P, Pasteur N, Pauron D: **Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae s.s.*** *Insect MolBiol* 1998, **7**:179–184.
24. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH: **Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids.** *Insect MolBiol* 2000, **9**:491–497.
25. Moores G, Bingham G: **Use of 'temporal synergism' to overcome insecticide resistance.** *Outlook Pest Manag* 2005, **16**. doi:10.1564/16feb03.
26. Ahmad M, Denholm I, Bromilow RH: **Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethoid-resistant strains of *Helicoverpaarmigera* from China and Pakistan.** *Pest Manag Sci* 2006, **62**:805–810.
27. Yewhalaw D, Asale A, Tushune K, Getachew Y, Duchateau L, Speybroeck: **Bio-efficacy of selected long-lasting insecticidal nets against pyrethroid resistant *Anopheles arabiensis* from South-Western Ethiopia.** *Parasit Vectors* 2012, **5**:159.
28. Van Bortel W, Chinh VD, Berkvens D, Speybroeck N, Trung HD, Coosemans M: **Impact of insecticide-treated nets on wild pyrethroid resistant *Anopheles epiroticus* populations from southern Vietnam tested in experimental huts.** *Malaria J* 2009, **8**:248.
29. Tungu P, Magesa S, Maxwell C, Malima R, Masue D, Sudi W, Myamba J, Pigeon O, Rowland M: **Evaluation of PermaNet 3.0 a deltamethrin-PBO combination net against Anopheles gambiae and pyrethroid resistant Culexquinquefasciatus mosquitoes: an experimental hut trial in Tanzania.** *Malaria J* 2012, **9**:21.
30. Corbel V, Chabi J, Dabire RK, Etang J, Nwane P, Pigeon O, Akogbeto M, Hougard JM: **Field efficacy of a new mosaic long-lasting mosquito net (PermaNet 3.0) against pyrethroid-resistant malaria vectors: a multi-centre study in Western and Central Africa.** *Malaria J* 2010, **9**:113.
31. N'Guessan R, Asidi A, Boko P, Odjo A, Akogbeto M, Pigeon O, Rowland M: **An experimental hut evaluation of PermaNet® 3.0, a deltamethrin-piperonyl butoxide combination net, against pyrethroid-resistant *Anopheles gambiae* and *Culexquinquefasciatus* mosquitoes in southern Benin.** *Trans Roy Soc Trop Med Hyg* 2012, **104**:758–765.
32. Koudou B, Koffi AA, Malone D, Hemingway J: **Efficacy of PermaNet 2.0 and PermaNet 3.0 against insecticide-resistant Anopheles gambiae in experimental huts in Cote d'Ivoire.** *Malaria J* 2011, **10**:172.
33. Killeen GF, Okumu FO, N' Guessan R, Coosemans M, Adeogun A, Awolola S, Etang J, Dabire RK: **The importance of considering community-level effects when selecting insecticidal malaria vector control products.** *Parasit Vectors* 2011, **4**:160.
34. Takken W: **Do insecticide-treated bed nets have an effect on malaria vectors?** *Trop Med Int Health* 2002, **7**:1022–1030.
35. Hargreaves K, Koekemoer L, Brooke D: ***Anopheles funestus* resistant to pyrethroid insecticides in South Africa.** *Med Vet Entomol* 2003, **14**:181–189.
36. Sharp BK, Ridl FC, Govender D, Kuklinski J, Kleinschmidt: **Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea.** *Malaria J* 2007, **6**:52.
37. WHO: *The Technical Basis for Coordinated Action Against Insecticide Resistance:Preserving the Effectiveness of Modern Malaria Vector Control.* Geneva: World Health Organization Global Malaria Programme; 2010.
38. Okello PE, Bortelw V, Byaruhanga AM, Correwyn A, Roelants P, Talisuna A, D' Allessandro U, Coosemans M: **Variation in malaria transmission intensity in seven sites throughout Uganda.** *AmJTrop Med Hyg* 2006, **75**:219–225.
39. Proietti C, Pettinato DD, Kanoi BN, Ntege E, Crisanti A, Riley EM, Egwang TG, Drakeley C, Bousema T: **Continuing intense malaria transmission in Northern Uganda.** *AmJTrop Med Hyg* 2011, **84**:830–837.
40. WHO: *World Malaria Report 2009.* Geneva: World Health Organization; 2009.
41. Djenontin A, Clabi J, Baldet T, Irish S, Pennetier C, Hougard JM, Corbel V, Akogbeto M, Chandre F: **Managing insecticide resistance in malaria vectors by combining carbamate-treated plastic wall sheeting and pyrethroid-treated bed nets.** *Malaria J* 2009, **8**:233.
42. Steinhardt LC, Adoke Y, Nasr S, Wiegand RE, Rubahika D, Serwanga A, Wanzira H, Lavoy G, Kamya M, Dorsey G, Filler S: **The effect of indoor residual spraying on malaria and anaemia in a high transmission area of Northern Uganda.** *Am Trop Med Hyg* 2013, **88**:855-861 doi:10.4269/ajtmh.12-0747.

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RESEARCH Open Access

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

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

Abstract

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

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

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

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

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

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Background

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

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

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

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

Methods

Study area and period

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

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

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

Mosquito collections

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

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

CDC bottle assays

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

LLIN sample preparation and chemical assays

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

WHO cone bioassays

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

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Data analysis

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

Results

Bottle bioassays

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

Cone bioassays

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

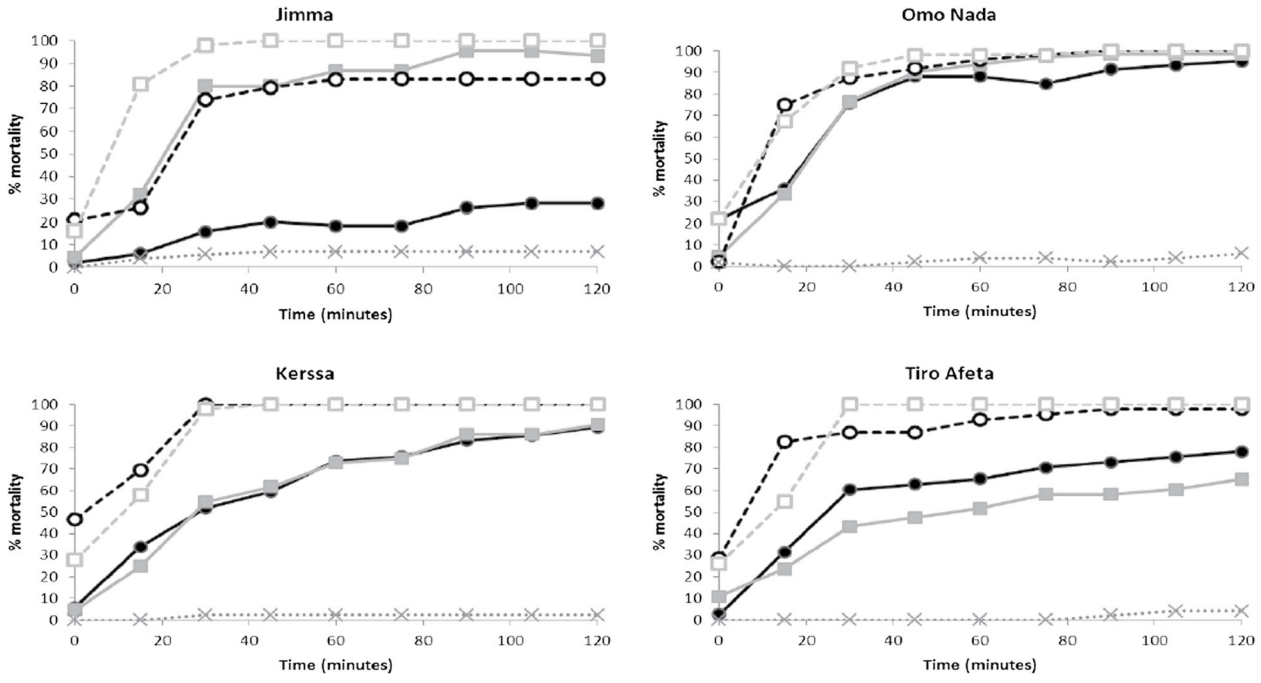

Figure 1 Susceptibility of populations of *An. arabiensis* adult female mosquitoes collected from four sites in Ethiopia to permethrin alone (black filled circle), permethrin following 60 mins pre-exposure to PBO (black unfilled circle), deltamethrin alone (grey filled square), and deltamethrin following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

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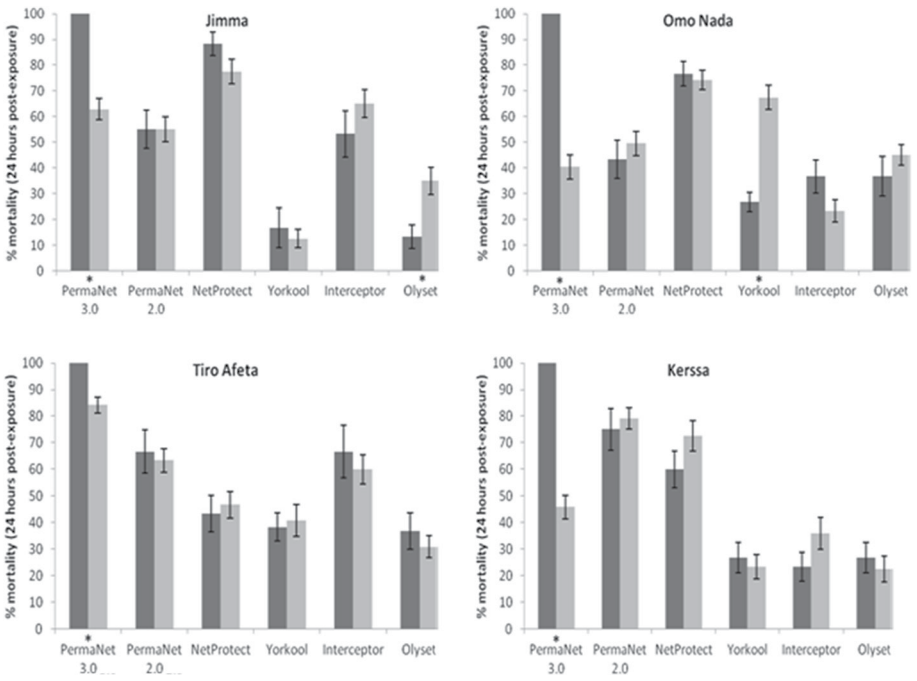

Figure 2 Bio-efficacy of roof (black rectangles) and side (grey rectangles) samples of six long-lasting insecticidal net types against *An. arabiensis* adult female mosquitoes collected from four sites in Ethiopia following 3-minutes exposure in standard WHO cone bioassays. Bars show mean percentage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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

Competing interests

Authors declare that they have no competing interests.

Authors' contribution

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

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References

1. Rafinejad J, Vatandoosta H, Nikpoor F, Abai MR, Shaeghi M, Duchen S, Rafi F: **Effect of washing on the bio-efficacy of insecticide-treated nets (ITNs) and long-lasting insecticidal nets (LLINs) against main malaria vector *Anopheles stephensi* by three bioassay methods.** *J Vector Borne Dis* 2008, **45:**143–150.
2. Gu W, Novak RJ: **Predicting the impact of insecticide –treated bed nets on malaria transmission: the devil is the detail.** *Malaria J* 2009, **8:**256.
3. World Health Organization: Malaria control today: *Current WHO recommendations*. Geneva, Switzerland: Working document Roll Back Malaria Department; 2005:75.
4. Lengeler C: **Insecticide-treated bed nets and curtains for preventing malaria.** *Cochrane Database of Systematic Reviews 2004, Issue 2* 2009, CD000363. doi:10./002/14651858.
5. Gunasekaran K, Vaidyanathan K: **Wash resistance of PermaNets in comparison to hand-treated nets.** *Acta Trop* 2008, **108:**154–157.
6. World Health Organization: *Guideline for laboratory and field testing of long-lasting insecticidal mosquito nets*. WHO/CDS/WHOPES/GCDPP/: World Health Organization; 2005:11.
7. Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V: **Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control?** *Trends Parasitol* 2011, **27:**91–98.
8. Coleman M, Sharp B, Seocharan I, Hemingway J: **Developing an evidence-based decision support system for rational insecticide choice in the control of African malaria vectors.** *J Med Entomol* 2006, **43**(4):663–668.
9. Hemingway J, Ranson H: **Insecticide resistance in insect vectors of human disease.** *Annu Rev Entomol* 2000, **45:**371–391.
10. World Health Organization: *World Malaria Report*. accessed on 23 March 2012; 2010. http://whqlibdoc.who.int/publications/2010/9789241564106 eng.pdf.
11. Yewhalaw D, Wassie F, Steurbaut W, Spanoghe P, Van Bortel W, Denis L, Tessema DA, Getachew Y, Coosemans M, Duchateau L, Speybroeck N: **Multiple insecticide resistance: an impediment to insecticide-based malaria vector control program.** *PLoS ONE* 2011, **6:**1. doi:10.1371/journal.pone.0016066.
12. Yewhalaw D, Van Bortel W, Denis L, Coosemans M, Duchateau L, Speybroeck N: **First evidence of high knockdown resistance frequency in *Anopheles arabiensis* (Diptera: Culicidae) from Ethiopia.** *Am J Trop Med Hyg* 2010, **83**(1):122–125.
13. Mathias DK, Ochomo E, Atieli F, Ombok M, Bayoh MN, Olang G, Muhia D, Kamau L, Vulule JM, Hamel MJ, Hawley WA, Walker ED: **Spatial and temporal variation in the kdrallele L1014S in *Anopheles gambiaes.s.* and phenotypic variability in susceptibility to insecticides in Western Kenya.** *Malaria J* 2011, **10:**10.
14. Gillies MT, Coetzee M: *A Supplement to the Anophelinae Africa south of the Sahara (Afrotropical Region)*. Johannesburg, South Africa: South African Institute for Medical Research; 1987:55.
15. Brogdon WC, McAllister JC: **Insecticide resistance and vector control.** *Emerg Infect Dis* 1998, **4:**605–613.
16. Center for Disease Control and Prevention: Evaluating mosquitoes for insecticide resistance. Atlanta, GA: *Center for Disease Control and Prevention*. 2012. http://www.cdc.gov/ncidod/wbt/resistance/assay/bottle/index.htm.
17. Abbott WS: **A method for computing the effectiveness of insecticides.** *J Econ Entomol* 1925, **18:**265–267.
18. Ahmed M, Denholm I, Bromilow RH: **Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethroid resistant strains of *Helicoverpaarmigera* from China and Pakistan.** *Pest Management Science* 2006, **62**(9):805–810.
19. Moores G, Bingham G: **Use of temporal synergism to overcome insecticide resistance.** *Outlooks on Pest Management* 2005, **16**(1):7–9.
20. Corbel V, Chabi J, Dabire RD, Etang J, Nwane P, Pigeon O, Akogbeto M, Hougard JM: **Field efficacy of a new mosaic long-lasting mosquito net (PermaNet 3.0) against pyrethroid-resistant malaria vectors: a multi-centre study in Western and Centeral Africa.** *Malaria J* 2010, **9:**113.
21. Etang J, Chandre F, Guillet P, Manga L: **Reduced bio-efficacy of permethrin EC impregnated bed nets against *Anopheles gambiae* strain with oxidase-based pyrethroid tolerance.** *Malaria J* 2004, **3:**46.
22. Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J: **Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiaes.s.* from southern Benin and Nigeria.** *BMC Genomics* 2008, **9:**538.
23. Muller P, Warr E, Stevenson BJ, Pignatelli PM, Morgan JC, Steven A, Yawson AE, Mitchell SN, Ranson H, Hemingway J: **Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolizes pyrethroids.** *PLoS Genet* 2008, **4:**e1000286.
24. Hardstone MC, Leichter CA, Scott JG: **Multiplicative interaction between the two major mechanisms of permethrin resistance, *kdr* and cytochrome P450-monooxygenase detoxification in mosquitoes.** *J EvolBiol* 2009, **22:**416–423.
25. Berticat C, Bonnet J, Duchon S, Agnew P, Weill M, Corbel V: **Costs and benefits of multiple resistance to insecticides for *Culexquinquefasciatus* mosquitoes.** *BMC EvolBiol* 2008, **8:**104.
26. World Health Organization: *The technical basis for co-ordinated action against insecticide resistance: preserving the effectiveness of modern malaria vector control*. Geneva, Switzerland: World Health Organization; 2011.
27. Trape GF, Tall A, Diagne N, Ndiath O, Ly AB, Faye J, Dieye-Ba F, Roucher C, Bouganali C, Badiane A, Sarr FD, Mazenot C, Toure-Balde A, Druile P, Puijalon OM, Rogier C, Sokhna C: **Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bed nets and artemesinin-based therapies: a longitudinal study.** *Lancet Infect Dis* 2011, **11**(2):925–932.
28. Van den Berg H, Zaim M, Yadav RS, Soares A, Ameneshewa B, Mnzava A, Hii J, Dash AP, Ejov M: **Global trends in the use of insecticides for vector-borne disease control.** *Environmental Health Perspectives* 2012, http://dx.doi.org/10.1289/ehp.11o4340.
29. Hunt RH, Fuseini G, Knowles S, Stiles-Ocran J, Verster R, Kaiser ML: **Insecticide resistance in malaria vector mosquitoes in four localities in Ghana, West Africa.** *ParasitVectors* 2011, **16:**107.
30. World Health Organization: *World Malaria Report*. Geneva, Switzerland: World Health Organization; 2011. http://www.who.int/malaria/world malaria report 2011/WMR.
31. Hunt RH, Edwardes M, Coetzee M: **Pyrethroid resistance in South African *Anopheles funestus* extends to Likoma Island in Lake Malawi.** *ParasitVectors* 2010, **3:**12.
32. Ranson H, Abdallah H, Badolo A, Guelbeogo WM, Kerah-Hinzoumbe C, Yangalbe-Kalnone E, Sagnon NF, Simard F, Coetzee M: **Insecticide resistance in *Anopheles gambiae*: data from the first year of multi-country study highlight the extent of the problem.** *Malaria J* 2009, **8:**299.
33. Vezenegho SB, Brooke BD, Hunt RH, Coetzee M, Koekemoer LL: **Malaria vector composition and insecticide susceptibility status in Guinea Conakry, West Africa.** *Med Vet Entomol* 2009, **23:**326–334.
34. President's Malaria Initiative: *Guidelines for entomological monitoring and insecticide resistance management*. President's Malaria Initiative; 2011:7.
35. Kolaczinski JH, Fanello C, Herve JP, Conway DJ, Carnevale P, Curtis F: **Experimental and molecular genetic analysis of the impact of pyrethroid and non-pyrethroid insecticide impregnated bed nets for mosquito control in an area of pyrethroid resistance.** *Bulletin Entomol Res* 2000, **90:**125–132.
36. Kulkarni M, Malima R, Mosha FW, Msangi S, Mrema E, Kabula B, Lawrence B, Kinung'hi S, Swilla J, Kisinza W, Rau ME, Miller JE, Schellenberg JA, Maxwell C, Rowland M, Magesa S, Drakeley C: **Efficacy of pyrethroid-treated nets against malaria vectors and nuisance-biting mosquitoes in Tanzania in areas with long-term insecticide-treated net use.** *Trop Med Inter Health* 2007, **12**(9):1061–1073.

37. Guillet P, N'Guessan R, Darriet F, Traore-Lamizana M, Chandre F, Carnevale P: **Combined pyrethroid and carbamate 'two-in-one' treated mosquito nets: field efficacy against pyrethroid resistant *Anopheles gambiae* and *Culexquinquefasciatus*.** *Med Vet Entomol* 2001, **5**:105–112.
38. Hougard JM, Corbel V, N'Guessan R, Darriet F, Chandre F, Akogbeto M, Baldet T, Guillet P, Carnevale P, Traore-Lamizana M: **Efficacy of mosquito nets treated with insecticide mixtures or mosaics against insecticide resistant *Anopheles gambiae* and *Culexquinquefasciatus* (Diptera: Culicidae) in Cote d'Ivoire.** *Bulletin Entomol Res* 2003, **93**:491–498.
39. Fane M, Cisse O, Sekou C, Traore F, Sabatier P: ***Anopheles gambiae* resistance to pyrethroid-treated nets in cotton versus rice areas in Mali.** *Acta Trop* 2011, **22**(1):1–6.

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