

Pilot study on the combination of an organophosphate-based insecticide paint and pyrethroid-treated long lasting nets against pyrethroid resistant malaria vectors in Burkina Faso

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ABSTRACT

A pilot study to test the efficacy of combining an organophosphate-based insecticide paint and pyrethroid-treated Long Lasting Insecticide Treated Nets (LLINs) against pyrethroid-resistant malaria vector mosquitoes was performed in a real village setting in Burkina Faso. Paint Inesfly 5A IGRTM, comprised of two organophosphates (OPs) and an Insect Growth Regulator (IGR), was tested in combination with pyrethroid-treated LLINs. Efficacy was assessed in terms of mortality for 12 months using Early Morning Collections of malaria vectors and 30-minute WHO bioassays. Resistance to pyrethroids and OPs was assessed by detecting the frequency of L1014F and L1014S *kdr* mutations and *Ace-1*^RG119S mutation, respectively. Blood meal origin was identified using a direct enzyme-linked immunosorbent assay (ELISA). The combination of Inesfly 5A IGRTM and LLINs was effective in killing 99.9–100% of malaria vector populations for 6 months regardless of the dose and volume treated. After 12 months, mortality rates decreased to 69.5–82.2%. The highest mortality rates observed in houses treated with 2 layers of insecticide paint and a larger volume. WHO bioassays supported these results: mortalities were 98.8–100% for 6 months and decreased after 12 months to 81.7–97.0%. Mortality rates in control houses with LLINs were low. Collected malaria vectors consisted exclusively of *Anopheles coluzzii* and were resistant to pyrethroids, with a L1014 *kdr* mutation frequency ranging from 60 to 98% through the study. About 58% of *An. coluzzii* collected inside houses had bloodfed on non-human animals. Combining Inesfly 5A IGRTM and LLINs yielded a one year killing efficacy against *An. coluzzii* highly resistant to pyrethroids but susceptible to OPs that exhibited an anthropo-zoophilic behaviour in the study area. The results obtained in a real setting supported previous work performed in experimental huts and underscore the need to study the impact that this novel strategy may have on clinical malaria and malaria exposure in children in a similar area of high pyrethroid resistance in South-Western Burkina Faso.

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1. Background

Malaria transmission occurs in 97 countries, putting about 3.4 billion people at risk (WHO, 2013). In Africa, it is estimated that

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in 2012 alone, about 165 million people suffered from malaria and about 562,000 people died from causes attributed to malaria. About 86% of those deaths were among children under 5 years of age (WHO, 2013). In addition to the devastating impact on human health, malaria also imposes an enormous economic burden, estimated at 1.3% of economic growth per year in sub-Saharan Africa (WHO, 2013). Primary prevention of malaria on a large scale is essentially achieved through vector control using mostly Long Lasting Insecticide Treated Nets (LLINs) and, to a lesser extent, Indoor Residual Spraying (IRS) (WHO, 2013). Between 2008 and 2010, 254 million LLINs were supplied to countries in sub-Saharan Africa (WHO, 2013). All currently recommended LLINs are treated

with pyrethroids. Protection using IRS reached 58 million people in Africa – representing 8% of the global population at risk – in 2012 as reported by National Malaria Control Programmes (WHO, 2013). In 2009, pyrethroids were estimated to account for about 75% of IRS coverage, while DDT was the second most widely used insecticide; carbamates and organophosphates (OPs) represented only small percentages of global usage (WHO, 2012). LLINs and IRS remain efficient and cost-effective tools for malaria control across a large number of settings (Lengeler and Sharp, 2003). The raised levels of pyrethroid resistance among malaria mosquito vectors (Chandre et al., 1999; Diabaté et al., 2004; Dabiré et al., 2012) and subsequent reports on reduced efficacy of pyrethroid-based vector control tools (Toé et al., 2014) are a source of concern. However, there is yet no final consensus on whether *kdr* based modifications reduce significantly the efficacy of insecticides operationally speaking (Briët et al., 2013; Hemingway, 2014). Furthermore, the use of LLINs is advocated because, when well used and intact, it will help reduce bloodfeeding thus increasing individual protection (Trape et al., 2014). Apart from the issue of pyrethroid resistance, there are operational obstacles surrounding LLINs (Toé et al., 2009; MCHIP/USAID/PNLP, 2013) and IRS (Najera and Zaim, 2001) potentially rendering these tools less operationally effective in protecting against malaria. To summarize, LLINs and IRS remain the cornerstone in malaria vector control but there is a growing need to find alternative malaria vector control strategies that can be added to the list of tools to choose from (Beier et al., 2008). The National Malaria Control Programme (“Programme National de Lutte contre le Paludisme”—PNLP) of the Ministry of Health in Burkina Faso, distributed more than 8 million nets were to a population of around 16 million targeted to the population at risk, children under 5 years old and pregnant women (MCHIP/USAID/PNLP, 2013). Thus, rather than departing from LLINs, the strategy implemented in this study enforced their use, in line with the PLNP efforts.

The LLINs in this study were PermaNet® 2.0 that had been distributed in the area by the PNLP in 2013 (MCHIP/USAID/PNLP, 2013) and were confirmed by the team to be well used by the population and intact. Insecticide paint Inesfly 5A IGR™ is a “cocktail” consisting of two OPs, chlorpyrifos and diazinon, and an insect growth regulator (IGR), pyriproxyfen. The paint was applied on plastic sheetings with no need of special equipment and placed in real houses, in a village in the Kou Valley, South-Western Burkina Faso, where there is high pyrethroid resistance among malaria mosquito vectors per the high frequency of the L1014F *kdr* mutation (Dabiré et al., 2008, 2009). Toxicology studies performed so far support the paint’s safety (Spanish Ministry of Health and Consumer Affairs (SMHCA), 1996; International Center of Training and Medical Investigations (ICTM), 2003; National Center of Tropical Diseases, 2004). Inesfly 5A IGR™ has been evaluated previously under experimental conditions in South America against the Chagas disease vector *Triatoma infestans* (Dias and Jemmio, 2008; Amelotti et al., 2009; Maloney et al., 2013).

Tests were also performed following the WHO Pesticide Evaluation Scheme (WHOPES) procedures (WHO, 1996) on Inesfly 5A IGR™ in the laboratory (Phase I), against 100% OP-resistant *Culex quinquefasciatus* (Mosqueira et al., 2010a), and in experimental houses in the field (Phase II), against wild pyrethroid-resistant populations of the main malaria vector, *Anopheles gambiae*, and pest mosquito, *Cx. quinquefasciatus* in Benin (Mosqueira et al., 2010b). In the laboratory, one year after treatment delayed mortality was 93–100% even against OP-resistant females on non-porous surfaces like hard plastic or softwood (Mosqueira et al., 2010a). Pyriproxyfen was added to the paint to confer and additional angle of attack against mosquito females once the OP effect diminishes over time. The effect of pyriproxyfen has been studied in the lab, where it was shown that pyriproxyfen had an effect on the fecundity, fertility and adult emergence of exposed adult females once the lethal effect of

OPs diminished over time even against OP-resistant mosquitoes (Mosqueira et al., 2010a). In the field, on porous surfaces made of cement, mortality rates were 90–100% against pyrethroid-resistant mosquito populations six months after treatment. Nine months after treatment, mortality rates in huts treated with two layers was still about 90–93% against *An. gambiae* and 55% against *Cx. quinquefasciatus*, both resistant to pyrethroids (Mosqueira et al., 2010b). In addition, a high spatial long term mortality (96–100%) was obtained for 12 months in the field on mosquitoes that were kept at distances of one meter overnight, never entering in direct contact with treated surfaces (Mosqueira et al., 2010b, 2013).

The objective of the present study was to assess the efficacy of Paint Inesfly 5A IGR™ in combination with pyrethroid-treated LLINs in real-life houses in a village setting. This pilot study supported the previous Phase II studies performed in experimental huts (Mosqueira et al., 2010b) and provided useful information on the method to apply the paint, perform the mosquito collections and mosquito populations, in preparation for the forthcoming large scale Phase III cluster randomized controlled evaluation to assess the impact of this combination strategy on the incidence of clinical malaria and malaria exposure in children aged from 6 months to 14 years old in a similar area of high pyrethroid resistance in South-Western Burkina Faso.

2. Methods

2.1. Study site and mosquitoes

The study was conducted in the Kou Valley, a rice growing area in South-Western Burkina Faso, West Africa. It is located at 30 km in the North of Bobo-Dioulasso (lat. 11°23′14″N and long. 4°24′42″W) and is composed of 7 villages with a total of 4470 habitants in 2013. The study was conducted specifically at the VK1 village (Fig. 1). Irrigation has existed in this area since 1972, and is now semi-permanent with two crops grown per year: from February to June during the dry season and from July to November during the rainy season. The study area was chosen because of its high malaria transmission, its high frequency of the L1014F *kdr* mutation, rendering local malaria vector populations highly resistant to pyrethroids and DDT (Dabiré et al., 2008, 2009). Both *An. gambiae* (former *An. gambiae* S form) and *Anopheles coluzzii* (former *An. gambiae* M form) coexist in sympatry in the study area, but *An. coluzzii* is preponderant within the rice field habitats. As part of the necessary background information, the exact species were determined molecularly (Santolamazza et al., 2008). The study was performed continuously for six months, from June to December 2013, and then again in June 2014, 12 months after treatment.

2.2. Insecticide paint and LLINs

Inesfly 5A IGR™ contains two organophosphates, chlorpyrifos (1.5%) and diazinon (1.5%), and an insect growth regulator (IGR), pyriproxyfen (0.063%), as active ingredients. The formulation is vinyl white-coloured paint with an aqueous base, with the active ingredients residing within CaCO₃ and resin microcapsules, allowing a gradual release of active ingredients. Microcapsules range from one to several hundred micrometers in size. The paint was applied on plastic sheetings with no need of special equipment, just a regular brush and gloves. Polypropylene plastic sheeting was bought at the local market and consisted of big plastic rolls cut and fit into the study houses. The plastic sheeting was used to homogenize test surfaces as some houses were made of adobe and some of cement. The plastic sheeting was then placed on the superior two thirds of interior house walls and ceilings. The lower part of all walls was left untreated for up to 1 m for all houses to reduce direct

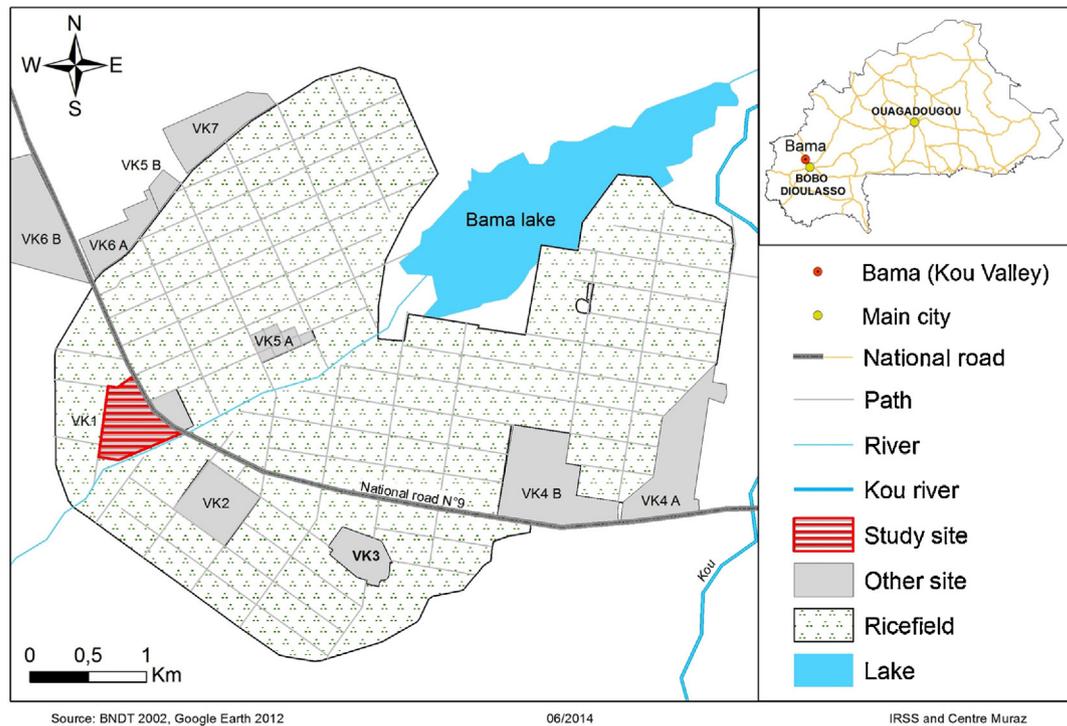


Fig. 1. Location of VK1 at Kou Valley in South-Western Burkina Faso.

exposure to both, babies and young toddlers. The LLINs in this study were PermaNet® 2.0, made of multifilament polyester netting (100 denier) factory impregnated with deltamethrin at 55 mg/m² in a wash-resistant binder system that had been distributed locally by the PNL in 2013. All nets were checked prior to the study and were found to be intact and correctly used by the owners.

2.3. Early morning collections (EMCs)

Inesfly 5A IGR™ was evaluated in 14 real-life village houses at VK1. The 14 houses at VK1 were chosen based on owners' wish to participate and equivalence in dimensions. The control houses consisted on plastic sheetings with no paint, but with intact LLINs. For the treated houses, paint was applied on plastic sheetings with one or two layers of insecticide paint at 1 kg commercial product/6 m², that is 0.51 g a.i. per m². Huts treated with two layers had the first layer diluted in 20% water following recommendations of the manufacturer. The ceilings of certain houses were also covered with painted plastic sheeting per the configuration below. The different configurations were treated in duplicate, in two houses each. Experimental hut studies commonly use one single hut per configuration (WHO, 2006). However, because this study was done using real houses that were similar but not identical to each other, we used two houses per configuration, collected for several nights in a row and performed the week of blank collections prior to treatment and rotated the volunteers. Configurations were designed to allow the evaluation of a potential volume effect and dose effect.

- (1) 2 houses = control sheeting with no paint + LLIN.
- (2) 2 houses = regular paint 1 layer + insecticide paint 1 layer on walls only + LLIN.
- (3) 2 houses = regular paint 1 layer + insecticide paint 1 layer on walls and ceiling + LLIN.
- (4) 2 houses = insecticide paint: 1 layer on walls only + LLIN.
- (5) 2 houses = insecticide paint: 1 layer on walls and ceiling + LLIN.

- (6) 2 houses = insecticide paint: 2 layers on walls only + LLIN.
- (7) 2 houses = insecticide paint: 2 layers on walls and ceiling + LLIN.

Mortality was the entomological indicator evaluated during this pilot study under real conditions. As there was no verandah, the exito-repellent effect generally assessed in Phase II WHOPES protocols, could not be implemented. Similarly, although house dimensions were similar, the number and size of openings (windows and doors) were too different to reliably evaluate the deterrent effect and bloodfeeding inhibition.

Before any treated sheetings were applied, mosquito collections took place for 1 full week just with LLINs to ensure there that there was no difference between houses in attractiveness to mosquitoes. Between June and December 2013 and again in June 2014, mosquito collections were performed nightly at VK1. The study was approved by the Ethics Committee of "Institut de Recherche en Sciences de la Santé" (IRSS) at Centre Muraz. Sixteen volunteers 18 years old or older were recruited from the population at VK1–2 volunteers served as back ups in case it was needed. After being informed about the study and discussing it, these volunteers provided an informed consent in writing or with a finger print if illiterate. The volunteers received training on mosquito collection procedures. At the first suspicion of malaria, volunteers were provided with the curative treatment recommended by the PNL in Burkina Faso. Furthermore, all houses were checked and had intact well used LLINs. Volunteers rotated houses each night to avoid bias while avoiding contamination between houses. The lower part of doors were covered with cloth to reduce the number of scavengers from entering houses. Houses were broomed every morning and every evening to remove scavengers that made it in through other openings. There was one volunteer sleeping per house. Mosquito collections were performed to assess mortality rates. Volunteers would enter their houses at 18:00 h, one volunteer per house, and sleep under LLINs until 5:30 h, when they would be awoken to close the windows (that had been left open during the night as it is commonly done in the area). Once windows were closed at 5:30 h, the volunteer

proceeded to collect mosquitoes within the house. After classifying mosquito females as dead or alive, they were put in observation for delayed mortality assessments after 24 h. All mosquitoes were then conserved in silica gel at -20°C to identify the species, resistance status and source of blood meal.

2.4. Residual efficacy tests

Thirty-minute standard WHO cone bioassays (WHO, 1998) were carried out using 2–4 days old unfed females of *An. gambiae* Kisumu, a reference strain susceptible to all insecticides reared at the IRSS/Centre Muraz insectarium. The local population identified molecularly as *An. coluzzii* and resistant to pyrethroids was reared at the insectarium from field caught larvae to the adult stage and was also tested in parallel to *An. gambiae* Kisumu. For each house, 10 females were introduced in 5 cones placed on five sides of the house (4 walls and ceiling) for 30 min. Cones were not placed on LLINs. Delayed mortality was observed 24 h later. Tests were performed monthly at T0, T1, T3, T6 and T12 after treatment.

2.5. Molecular analysis on resistance

The detection of *kdr* resistance genes was performed following protocols developed for the L1014F *kdr* mutation (Martinez-Torres et al., 1998), for the L1014S *kdr* mutation (Ranson et al., 2000), as well as the detection of the *Ace-1^RG119S* mutation (Weill et al., 2004). Testing took place each month for 5 months after treatment on *An. coluzzii* females collected in control houses and houses treated with 1 or 2 layers of insecticide paint on walls and ceiling.

2.6. Determination of blood meal source

Blood meal identification was performed using a direct enzyme-linked immunosorbent assay (ELISA) (Beier et al., 1988). The choice of antibodies tested was based on the animals that are more frequent in the study area. Six antibodies were tested: human, dog, sheep, donkey, cattle and pig. These antibodies, marked with peroxidase, were kept at $+4^{\circ}\text{C}$. Bloodfed *Anopheles* females collected during EMCs from June to December 2013 in control houses and houses treated with 1 or 2 layers of insecticide paint on walls and ceiling were tested. A total of 425 females identified molecularly as *An. coluzzii* were tested from each of those 3 configurations (>140 per configuration) to determine the source of the blood meal.

2.7. Statistical analysis

Results on mortality were compiled and analyzed using Epi-Info Version 6 to test for any significant difference in mortality rates between the different configurations via Chi square tests. A 95% confidence interval was applied. When mortality rates in control huts were between 5 and 20% Abbott's mortality correction formula was applied. Because bioassay tests are subject to variations, a 99% confidence interval was applied. The allelic frequency of each mutation (*kdr* and *ace-1R*) was calculated using the formula $F(R) = (2RR + RS)/2n$ where *n* is the total sample size, using GenePop version 4.

3. Results

3.1. Early morning collections (EMC)

No difference in house attractiveness was found prior to treatment. *An. coluzzii* (former *An. gambiae* form M) was the only *An. gambiae* s.l. species present in the study area as established from the molecular analysis performed during the study. Between June

Table 1

Mortality rates on wild populations of *Anopheles coluzzii* at VK1 using EMCs. Averages taken for each configuration, 2 houses per configuration. C = control with LLINs only; RP = regular Paint; IP = insecticide paint; T = time in months since treatment. EMCs = early morning collections. Numbers in the same column sharing a letter superscript do not differ significantly ($p > 0.05$).

% Mortality in <i>Anopheles coluzzii</i> collected via EMCs	T1	T3	T6	T12
C (LLINs)	9.5 ^a	5.2 ^a	8.9 ^a	7.6 ^a
RP/1 layer + IP/1 layer walls + LLINs	100 ^b	100 ^b	100 ^b	78.6 ^b
RP/1 layer + IP/1 layer walls + ceiling + LLINs	100 ^b	100 ^b	100 ^b	69.5 ^b
IP/1 layer walls + LLINs	100 ^b	100 ^b	100 ^b	78.9 ^b
IP/1 layer walls + ceiling + LLINs	100 ^b	100 ^b	100 ^b	79.9 ^b
IP/2 layers walls + LLINs	100 ^b	99.9 ^b	100 ^b	78.5 ^b
IP/2 layers walls + ceiling + LLINs	100 ^b	100 ^b	100 ^b	82.2 ^b

and December 2013 and June 2014, a total of 3903 females belonging to the *An. gambiae* complex identified molecularly as *An. coluzzii*, were collected in all houses combined. Full collections started one month after treatment (Table 1). For the first 6 months, the mortality rates observed in houses treated with the insecticide paint were 97–100%. Globally, 6 months after treatment, all houses treated with the insecticide paint, with 1 or 2 layers, on walls or on walls and ceiling, presented 100% mortality rates against wild populations of *An. coluzzii* whether they were bloodfed or not and were statistically significantly different from control ($p < 0.001$). By T12, mortalities were still high and significantly different from control ($p < 0.001$), but rates had slightly decreased to 69.5–82.2%. The highest mortality rates 12 months after treatment were observed in houses treated with 2 layers of insecticide paint and a larger volume (82.2%). No statistically significant differences were found between treated houses at T12. Mortality rates observed in control houses with no insecticide paint but with LLINs ranged from 5.2 to 9.5%, throughout the study (Table 1).

3.2. Residual efficacy tests

Thirty-minute standard WHO cone bioassays on *An. gambiae* “Kisumu” and local populations of *An. coluzzii* from VK1, yielded mortality rates of 98–100% in all houses treated with insecticide paint (Table 2) regardless of the configuration. Mortality in control houses was lower and significantly different from treated houses, but because mortality was over 5% (but always less than 20%), the Abbott formula was applied. Mortality rates were 100% at T6 against both *An. gambiae* “Kisumu” and local populations of *An. coluzzii* from VK1, in all treated houses. Mortality rates at T12 were still 98–100% in all houses against *An. gambiae* “Kisumu”. In the case of the local *An. coluzzii* from VK1, 12 months after treatment mortality rates were 97% in houses treated with 2 layers of insecticide paint on walls and ceiling, but slightly lower mortalities were observed in the other configurations. These differences were not statistically significant ($p > 0.05$). Mortality rates observed in control houses with LLINs only ranged from 1.7% to 10.9% (Table 2). Again, cones were only placed on walls and ceiling, not on LLINs.

3.3. Molecular Analysis on resistance

3.3.1. Allelic frequency of the L1014F and L1014S *kdr* mutations

All houses contained pyrethroid treated LLINs. Also, because the *Anopheles* females collected in treated houses were dead and around 89% to 94% of the females were alive in control houses, no comparisons could be done between dead and alive mosquitoes within each given configuration. Thus, comparisons were done overtime between control houses with LLINs and treated houses with LLINs and 1 or 2 layers of insecticide paint. Overall, *An. coluzzii* females at VK1 were pyrethroid resistant: the allelic frequency of the L1014F *kdr* mutation was high, ranging from 60 to 98% (Table 3)

Table 2
Residual efficacy tests on (A) *Anopheles gambiae* “Kisumu” and (B) *Anopheles coluzzii* VK1 using WHO test cones. Averages taken for each configuration, 2 houses per configuration. C = control with LLINs only; RP = regular paint; IP = insecticide paint; T = time in months since treatment. Numbers in the same column sharing a letter superscript do not differ significantly ($p > 0.05$). Molecular analysis on resistance Allelic frequency of the L1014F and L1014S *kdr* mutations *Anopheles coluzzii* VK1 (B).

% Mortality in <i>Anopheles coluzzii</i> using WHO test cones	<i>Anopheles gambiae</i> Kisumu (A)					<i>Anopheles coluzzii</i> VK1 (B)				
	T0	T1	T3	T6	T12	T0	T1	T3	T6	T12
C (LLINs)	10.9 ^a	7.9 ^a	6.1 ^a	5.6 ^a	6.9 ^a	1.7 ^a	2.6 ^a	2.9 ^a	2.1 ^a	2.1 ^a
RP + IP/1 layer walls + LLINs	100 ^b	100 ^b	100 ^b	100 ^b	99.0 ^b	100 ^b	100 ^b	100 ^b	98.9 ^b	90.9 ^b
RP + IP/1 layer walls + ceiling + LLINs	100 ^b	100 ^b	98.1 ^b	100 ^b	99.0 ^b	100 ^b	100 ^b	100 ^b	99.0 ^b	91.3 ^b
IP/1 layer walls + LLINs	100 ^b	100 ^b	98.0 ^b	100 ^b	99.0 ^b	100 ^b	100 ^b	100 ^b	100 ^b	85.0 ^b
IP/1 layer walls + ceiling + LLINs	100 ^b	100 ^b	100 ^b	100 ^b	98.1 ^b	100 ^b	100 ^b	100 ^b	100 ^b	81.8 ^b
IP/2 layers walls + LLINs	100 ^b	100 ^b	100 ^b	100 ^b	100	100 ^b	100 ^b	100 ^b	98.8 ^b	88.9 ^b
IP/2 layers walls + ceiling + LLINs	100 ^b	100 ^b	100 ^b	100 ^b	100	100 ^b	100 ^b	100 ^b	100 ^b	97.0 ^b

Table 3
Distribution of the frequency of L1014F and L1014S *kdr* mutations in *Anopheles coluzzii* in VK1. C = control with LLINs only; IP = insecticide paint; n = number of mosquitoes tested; T = time in months since treatment; F(*kdr*) = frequency of the mutation *kdr*; p (HW) = value for Hardy–Weinberg equilibrium hypothesis; “–” = non determinable.

Treatments	Month	n	SS	RS	RR	F(L1014F <i>kdr</i>)	p (HW)	SS	RS	RR	F(L1014S <i>kdr</i>)	p (HW)
C (LLINs)	T0	30	7	3	20	0.717	0.0001	30	0	0	0	–
	T1	30	0	0	30	0.98	–	30	0	0	0	–
	T2	30	11	2	17	0.6	0	30	0	0	0	–
	T3	31	8	3	20	0.694	0	31	0	0	0	–
	T4	30	7	0	23	0.767	0	30	0	0	0	–
	T5	25	5	0	20	0.8	0	25	0	0	0	–
IP/1 layer walls + ceiling + LLINs	T0	30	3	0	27	0.9	0.0001	30	0	0	0	–
	T1	28	1	0	27	0.964	–	30	0	0	0	–
	T2	30	4	3	23	0.817	0.002	27	3	0	0.05	1
	T3	31	6	1	24	0.79	0	30	1	0	0.016	–
	T4	29	3	6	20	0.793	0.066	24	5	0	0.086	1
	T5	30	4	7	19	0.75	0.048	23	7	0	0.117	1
IP/2 layers walls + ceiling + LLINs	T0	30	4	0	26	0.867	0	30	0	0	0	–
	T1	31	0	0	31	0.98	–	30	0	0	0.001	–
	T2	30	4	0	26	0.867	0	30	0	0	0	–
	T3	31	9	0	22	0.71	0	31	0	0	0	–
	T4	29	3	0	26	0.897	0.0001	29	0	0	0	–
	T5	30	4	10	16	0.7	0.378	20	10	0	0.167	0.563

with no significant difference between alive specimens collected from the control and dead specimens collected from the treated houses during the period tested, up to 5 months after treatment. Similarly, no increasing or decreasing trends were identified on the allelic frequency overtime. The L1014S *kdr* was not found in the samples collected in control houses with LLINs and was weakly detected in the heterozygous form in houses treated with 1 layer starting at T2, T4 and T5, and in houses treated with 2 layers, at T5, though only in the heterozygote form (Table 3).

3.3.2. Allelic frequency of the mutation *Ace-1R*

The *Ace1^R* mutation was detected at low allelic frequencies and was heterozygous. It was only randomly found at T0 and T5 in the control houses at frequencies of 8.3 and 4.0%, respectively (Table 4) and at no point in the treated houses.

3.3.3. Determination of the bloodfeeding origin

There were no statistical differences between control houses, houses treated with 1 insecticide paint layer, and houses with 2 insecticide paint layers (Table 5). The averages of all houses combined from T0 to T6, showed about 27% of females had fed on humans, about 58% on other animals and about 16% on both. All in all, the rate of zoophily was high (58%). Of the females having bloodfed on other animals (non human), about 45% of them had not blood fed on any of the domestic animals chosen as the most typical blood meal sources in the area. Of the identified domestic animals, cattle remained the most common blood meal source (Table 5).

4. Discussion

The study area was chosen based on parameters such as insecticide resistance and malaria transmission levels (Dabiré et al., 2008, 2009). In addition, the team was drawn by the population's interest on the paint and the efforts that home owners had previously undergone to try to paint the interior of their homes and the edges of windows and doors when their economic level allowed it. From that standpoint, the study area presented an optimal profile to perform a pilot study on the efficacy of combining an OP-based paint and LLINs. The fact that classical WHOPES Phase II experimental huts were not used posed some limitations on the measurement of certain entomological parameters (discussed throughout the text), but allowed the assessment of how the Phase III trial may be implemented. Furthermore, the results obtained in this pilot study supported previous findings observed during the WHOPES Phase I in the laboratory and Phase II study in experimental huts in the South of Benin using the same paint, Inesfly 5A IGRTM, in terms of entomological mortality rates, the porosity of materials and the notion of volume effect discussed below. In this pilot study, the combination of the insecticide paint Inesfly 5A IGRTM consisting of two different OPs with an IGR, and pyrethroid-treated LLINs was able to control *An. coluzzii* (former *An. gambiae* form M) populations yielding mortality rates of 100% for 6 months after treatment regardless of the treatment configuration in terms of volume (walls or walls + ceiling), dose of insecticide paint and number of layers. With time, however, houses with two layers of insecticide paint and a larger volume benefited with a higher long term efficacy. The mortality rates observed during mosquito collections on the pilot

Table 4

Allelic frequency and genotype of the *Ace-1^R* mutation in *Anopheles coluzzii* at VK1. C = control with LLINs only; IP = insecticide paint; n = number of mosquitoes tested; T = time in months since treatment; f(119S) = allelic frequency of the mutation ace-1 119S; p (HW) = value for Hardy–Weinberg equilibrium hypothesis; “–” = non determinable.

Treatment	Month	n	Genotypes			f(119S)	[95%CI]	p (HW)
			119G 119G	119G 119S	119S 119S			
C (LLINs)	T0	30	25	5	0	0.083	[0.00–0.18]	1
	T1	30	30	0	0	0	–	–
	T2	30	30	0	0	0	–	–
	T3	30	30	0	0	0	–	–
	T4	30	30	0	0	0	–	–
IP/1 layer walls + ceiling + LLINs	T5	23	21	2	0	0.04	[0.00–0.12]	1
	T0	30	30	0	0	0	–	–
	T1	30	30	0	0	0	–	–
	T2	30	30	0	0	0	–	–
	T3	30	30	0	0	0	–	–
IP/2 layers walls + ceiling + LLINs	T4	30	30	0	0	0	–	–
	T5	30	30	0	0	0	–	–
	T0	30	30	0	0	0	–	–
	T1	30	30	0	0	0	–	–
	T2	30	30	0	0	0	–	–
	T3	30	30	0	0	0	–	–
	T4	30	30	0	0	0	–	–
	T5	24	24	0	0	0	–	–

Table 5

Analysis of the blood source of bloodfed *Anopheles coluzzii* collected using EMCs at VK1. C = control with LLINs only; IP = insecticide paint; n = numbers of mosquitoes tested; T0–T6 = period from June to December 2013 when collected *Anopheles coluzzii* were pooled and randomly tested for bloodfeeding source. Numbers in the same column sharing a letter superscript do not differ significantly ($p > 0.05$).

Treatment	<i>Anopheles coluzzii</i> females tested (T0–T6)	Humans		Other animals						Mixed			
		n	%	Cattle	Sheep	Donkey	Pig	Dog	Other	n	%		
C (LLINs)	141	35	24.8 ^a	16	8	18	7	5	39	93	66.0 ^a	13	9.2 ^a
IP/1 layer walls + ceiling + LLINs	143	51	35.7 ^a	21	4	3	5	4	33	70	49.0 ^a	22	15.4 ^a
IP/2 layers walls + ceiling + LLINs	141	28	19.9 ^a	30	3	9	0	2	38	82	58.2 ^a	31	22.0 ^a
Total	425	114	26.8	67	15	30	12	11	110	245	57.6	66	15.5

study in VK1, in real houses, were also supported by the long-term residual tests using WHO cone tests. Mortality rates in all treated houses remained 98.9–100% for 6 months against both *An. gambiae* “Kisumu” (the insecticide-susceptible laboratory reference strain) and the pyrethroid-resistant *An. coluzzii* populations in VK1. Results obtained 12 months after treatment using WHO cones confirm that, in the long term, houses with two layers and a larger volume performed best. The results obtained using EMCs and WHO cones are in consistence with previous studies performed in an experimental field setting in Benin with the same paint (Mosqueira et al., 2010b), where huts treated with two layers of insecticide paint and, particularly, a larger volume had a longer lasting efficacy. The observed volume effect was in line with previous observations during the Phase II trial in the South of Benin (Mosqueira et al., 2010b) and a study performed in experimental huts on carbamate-treated plastic sheeting used concomitantly with nets treated with deltamethrin at 25 mg/m² (Djènontin et al., 2009). Overtime, starting mildly at T6 but becoming more evident by T12, the mortality rates observed in treated houses were higher on this study than those observed in experimental huts made of cement in Ladji, South of Benin (Mosqueira et al., 2010b). This was probably linked to the high porosity of cement compared to plastic sheeting used in VK1 as supported by Phase I studies exploring the effect that the porosity of materials have on the long term efficacy of insecticide treated surfaces (Mosqueira et al., 2010a). The treatment of plastic sheeting was sought as an interim decision to test the efficacy of the paint under optimal conditions while the manufacturer improves the sealing qualities of the paint so the paint is applied directly on walls.

Several studies have assessed vector mortality rates when combining sheetings or IRS with pyrethroid-treated nets: a study carried in experimental huts in the Kou Valley in Burkina Faso showed mortality rates of carbamate-treated plastic sheeting and LLINs were superior to sprayed carbamates via IRS and control using just LLINs (Djènontin et al., 2010). A study performed in experimental huts in Tanzania that tested several IRS compounds used concomitantly with LLINs showed IRS with DDT or pyrethroids did not confer additional value to LLINs alone, but showed IRS with OPs could be effective in preventing blood feeding and increasing vector mortality when combined with LLINs (Okumu et al., 2013). These studies suggest there may be value in adding a non-pyrethroid insecticide paint, insecticide treated plastic sheetings or IRS to LLINs.

Understanding the bio-ecology and spatio-temporal distribution of the malaria vector in the study area is important (Ferguson et al., 2010; The malERA Consultative Group on Vector Control, 2011; Sinka et al., 2012). During the study period, local wild populations were genomically identified as *An. coluzzii* (former *An. gambiae* form M) exclusively. The two reproductive units formerly referred to ‘M’ and ‘S’ molecular forms, are now officially recognised as *An. coluzzii* Coetzee & Wilkerson 2013 and *An. gambiae* s.s. Giles 1902 based on population genomic evidence (Coetzee et al., 2013). Whilst implementing vector control strategies, old or new, monitoring insecticide resistance is increasingly central (Enayati & Hemingway, 2010). *Anopheles coluzzii* in the study area showed high frequencies (ranging from 60 to 98%) of the target site L1014F *kdr* mutation that confers cross-resistance to pyrethroids and DDT (Martinez-Torres et al., 1998). There is a concern that concomitant

use of pyrethroids for IRS and LLINs could increase the pressure for resistance development in vector populations (WHO, 2011, 2012). The potential of this novel strategy for resistance development was assessed briefly during five months. Tests performed during the testing period showed the allelic mutation *kdr* L1014F did not vary significantly during the testing period. This was not the case for the mutation *kdr* L104S revealed in Burkina Faso in recent years (Dabiré et al., 2009). The distribution of the allelic frequencies of *kdr* L104S were low and heterozygous, but appeared 3 months after treatment in houses treated with insecticide paint and LLINs but not in control houses with LLINs alone. The above results provide only some indication that the combination of LLINs and the insecticide paint Inesfly does not select for this mutation. In order to properly assess this risk, a longer term full protocol will be developed and carried during the phase III study. With regard to the *ace-1^R* mutation, *An. coluzzii* in VK1 are considered to be susceptible to OPs as the distribution of the *ace-1^R* mutation is still low thus far (less than 10% overall) and in the heterozygous form.

The mortality rates observed in control houses with LLINs (no insecticide paint) were low. While it is acknowledged that the study design may have allowed for some limitations such as increasing the chances of having unwanted scavengers eat the dead mosquitoes thus underestimating the mortality, the low mortality rates observed in control houses with LLINs in the VK1 area in this study are supported by recent findings in the nearby VK7 village, also in the Bama area (Toé et al., 2014). Toé et al. (2014) study measured the efficacy of several pyrethroid-treated LLINs, including PermaNet 2.0 distributed by the PNL (such as the ones in VK1) against local populations of pyrethroid-resistant *An. gambiae* s.l. using WHO bioassays among other tests. PermaNet 2.0 used yielded mortality rates of about 20% against pyrethroid-resistant *An. gambiae* s.l. from VK7 in forced contact (Toé et al., 2014).

Assessing the impact that vector control tools have on blood feeding inhibition may yield misleading information as it cannot distinguish females entering houses to feed on humans, from females that have bloodfed outside (on either humans or animals, or both) and then enter the houses to complete their bloodfeeding and/or to rest. Analysis on the source of blood meals showed that an average of 58% of the *An. coluzzii* collected had bloodfed on other animals (non human) versus about 27% on humans, and about 16% had bloodfed on both other animals and humans. There were no differences between control and treated houses with regard to the rate of zoophily or anthropophily. It is worth noting that out of the 58% of females having blood fed on other animals (non human), about 45% obtained their blood meals on animals not identified as neither human nor any of the five chosen domestic animal antibodies. The surprisingly relatively low rate of anthropophily of *An. coluzzii* in this particular rice-field area had already been highlighted in previous studies and may be explained by the large mosquito densities and extensive livestock activities (Robert, 1989; Baldet et al., 2003). In this anthropo-zoophilic context, the insecticide paint consisting on OPs may have provided a more optimal coverage by decreasing the longevity of both, malaria vectors having bloodfed outside on humans or other animals and entering houses to rest, as well as malaria vectors entering houses to bloodfeed (Killeen et al., 2014).

To summarize: the advantages of combining Inesfly 5A IGRTM and LLINs could be many-fold in terms of the insecticides' mode of action as well as operational coverage: (a) combining different insecticides may help reduce the pressure for resistance development in vector populations (WHO, 2011, 2012); (b) the lethal effect of OPs coupled with pyrethroids' excito repellent effect may broaden the efficacy spectrum and thus increase protection to users; (c) the paint may provide protection before and after regular sleeping hours, when users are not yet under the net; (d) the paint may kill indoor resting as well as indoor bloodfeeding mosquitoes;

(e) whilst, IRS provides similar benefits, the application of the paint may lead to a perceived improvement of people's homes and requires no special equipment. IRS leaves a residue on walls and needs special equipment leading to some operational obstacles (Najera and Zaim, 2001). In fact, in the area where the study was performed, most owners had sought painting their homes and volunteers saw the study's paint as an added benefit.

Results obtained during this pilot study on the combination of Inesfly 5A IGRTM and LLINs in a real village in an area of high pyrethroid resistance were positive: the average mortality rates were well above the 80% threshold recommended by WHOPES as a criteria for an effective vector control tool for over 6 months in all six configurations of insecticide paint and LLINs. Houses with LLINs and where a larger volume had been treated still met the criteria after 12 months. The next phase is to test if clinical malaria incidence and malaria exposure are reduced when combining Inesfly 5A IGRTM and LLINs in children aged from 6 months to 14 years. The Phase III cluster randomized controlled study on the combination of Inesfly 5A IGRTM and LLINs will be conducted in South-Western Burkina Faso, where villages are being currently identified in an area similar to VK1, with pyrethroid-resistant malaria vectors and holoendemic malaria.

5. Conclusions

The combination of Inesfly 5A IGRTM and LLINs yielded a long-term mortality of 80% against *An. coluzzii* highly resistant to pyrethroids for about 12 months in houses where a larger volume was treated. The encouraging results obtained during this pilot study in a real village on malaria vector mortality sets the basis for the upcoming Phase III to study the impact of combining Inesfly 5A IGRTM and LLINs on clinical malaria incidence and malaria exposure in children aged 6 months to 14 years in a pyrethroid-resistant and holoendemic malaria area in South-Western Burkina Faso.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SMC, RKD, PC, TB, FF, AD and BM contributed to the design of the study. TB and RKD critically contributed to the implementation of the study. DDS, SP, MN conducted evaluations. The manuscript has been written by BM and has been revised by RKD, TB, FF and DDS. All authors read and approved the final manuscript.

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