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Anopheles gambiae resistance to pyrethroid-treated nets in cotton *versus* rice areas in Mali

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ABSTRACT

The rise and spread of *Anopheles gambiae s.l.* (the major malaria vector sub-Saharan Africa) resistance to pyrethroids is of great concern owing to the predominant role of pyrethroid-treated nets in the WHO global strategy for malaria control. Use of pyrethroids for agricultural purposes may exert a strong selection pressure, favouring the emergence of insecticide resistance. The objective of this study was to evaluate the efficacy of alpha-cypermethrin treated nets in settings where insecticides are used against pests. This was assessed in two ways, i.e. under laboratory conditions using the WHO standard cones test technique and in experimental huts, on *Anopheles gambiae s.l.* collected in two Malian rural sites, Koumantou characterised by cotton crops and high insecticide use and Sélingué, a rice field area with low insecticide use. According to the WHO standard cones test technique, there was no difference between mosquitoes collected in the two sites; KD50 time was less than 3 min and the KD95 time below 30 min. Nevertheless, in the experimental huts with alpha-cypermethrin treated bed nets, the mosquito mortality rate was significantly lower in Koumantou (102/361, 28.2%) than in Sélingué (122/233, 52.3%) (RR: 0.65, 95%CI: 0.56–0.76) ($p < 0.001$). In addition, in Koumantou the percentage of unfed mosquitoes found in the veranda was much lower in the huts with untreated (26.0%, 33/127) than in those with treated nets (92.2%, 118/128) ($p < 0.01$) while in Sélingué there was no difference between huts with treated and untreated bed nets. Alpha-cypermethrin treated bed nets had a significant effect on mortality and repelling behaviour of *Anopheles gambiae s.l.* though in Koumantou treated bed nets were less efficacious, possibly due to the intense use of pesticide for agriculture.

Contents

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1. Introduction

Malaria is a leading cause of morbidity and mortality in Mali. In 2008, the Ministry of Health (MOH) reported more than 1.3 million clinical malaria cases, accounting for 37% of all outpatient visits, 42% for children under five (USAID, 2009). Thirty seven percent of all reported deaths were due to malaria, more than half of them among children under five. The National Malaria Control Program (NMCP) strategy is based on effective case management and the use of insecticide-treated nets (ITNs) targeted at vulnerable groups, i.e. children under five and pregnant women. ITN coverage has increased substantially after national distribution campaigns of free or highly-subsidized ITN. More recently, in December 2007, the Malian Ministry of Health (MOH) distributed for free 2.3 million long-lasting insecticide-treated nets (LLINs), attaining an estimated coverage of 82% in 2008 (World Health Organization, 2010).

Pyrethroids are the only insecticides currently recommended by the World Health Organization (WHO) for treatment of bed nets owing to their strong insecticidal activity at low concentrations and their low mammalian toxicity. Pyrethroid-treated nets are effective in reducing malaria morbidity and mortality and may also provide community protection through mass impact on the vector population, when coverage is high (Ilboudo-Sanogo et al., 2001; Binka et al., 1996; Nevill et al., 1996). Nevertheless, the emergence and spread of pyrethroid resistance is of concern. This is often due to mutations in the knockdown resistance (kdr) gene (Hemingway et al., 2004; Diabate et al., 2004). Both resistance to permethrin and decreased sensitivity to deltamethrin by *A. gambiae s.l.*, the main malaria vector sub-Saharan Africa, have already been observed in several African countries (Diabate et al., 2004; Chandre et al., 1999). In Bamako, Mali, the prevalence of the kdr mutation increased from 2.8% in 1987 to 62.1% in 2000 (Fanello et al., 2003). A similar trend was observed in other Malian sites (Tripet et al., 2007).

The resistance of mosquitoes to different pyrethroids underlines the importance of assessing vectors sensitivity to all insecticides belonging to the same family. The objective of this study was to evaluate the efficacy of alpha-cypermethrin treated nets, an insecticide not used in Mali for this purpose, on the control of malaria vectors in two areas characterized by different pattern of exposure to agricultural pesticides.

2. Material and methods

2.1. Study sites

The study was conducted in two sites in the region of Sikasso (Mali) differing by agricultural production, use of insecticides, and/or ecological settings (Fig. 1). The first site is located in the cotton crop growing area of Koumantou, where the Malian Company for Textile Development (CMDT) has a cotton factory. The climate is typical Sahelian (Sudano-Sahel), with a rainy season between May and November (annual rainfall 900–1000 mm) and a dry season between December and May. Extensive cotton crop growing (15,582 ha) is combined with widespread application of organophosphates and pyrethroids. The prevalence of the resistant kdr allele has been rising since 1996 to currently exceed 80%. To minimize the risk of resistance, CMDT uses a combination of insecticides (pyrethroid and organophosphore/carbamate) that are changed every 3 years.

The second site is located in a large flooded area of the hydro-agro-electrical dam of Sélingué, where rice is grown twice a year (more than 30,000 ha), i.e. (i) rainfall cultivation between July and November, (ii) post-rainfall cultivation between February and June. Malaria transmission is perennial with two peaks, one at the end of the rains and the other during the post-rainfall rice culture (Fondjo, 1996). *A. gambiae s.l.* is the major malaria vector. Use of pyrethroids is not as intense as in Koumantou and the prevalence of the resistant kdr allele is low (Fanello et al., 2003).

2.2. Insecticide-treated bed nets

Rectangular 100% polyester, white bed nets (12 m^2) were dipped into an insecticide solution (6 ml of alpha-cypermethrin (Fendona 6SC®, BASF) diluted in 480 ml of water (40 mg/m^2) and let dry before use. Control bed nets were untreated and protected against contamination. On each net, six holes (4 × 4 cm each) were purposely done, 2 on each width, 1 on each length, to simulate operational condition, measure insecticide efficacy and minimize the physical barrier of the net.

2.3. Mosquitoes

For the tests, wild *Anopheles gambiae* captured both in Koumantou (S-Koumantou) and Sélingué (S-Sélingué) where employed. A laboratory strain of *A. gambiae*, Kisumu strain (S-Kisumu), 100% susceptible to pyrethroids, imported from Centre Muraz of Bobo-Dioulasso, Burkina-Faso, was used as control strain for the cone test. For the laboratory study, F1 generation adults from the wild and laboratory strains were used. For the field study, mosquitoes were captured by collectors using flash lights and aspirators between 5.00 am and 7.00 am inside nets, huts and exit traps. Living mosquitoes captured in experimental huts were kept in separate cups corresponding to each collection's location: under bed net, around bed net or under veranda. A cotton wool swab soaked with 5% glucose solution was placed on the top of the cup covered by a piece of mosquito net.

2.4. Cone tests

The bioassays using WHO plastic cones (Koffi et al., 1998) were carried out at the Malaria Research and Training Center's field entomology laboratory in Sélingué. To perform one complete cones test, 4 pieces of 25 × 25 cm of both treated and untreated mosquito nets were used. For each mosquito strain, 5 unfed 2–5 days old mosquitoes were exposed to each bed net's piece for 3 min. The test was repeated 20 times, 160 times in total (80 for alpha-cypermethrin treated pieces and 80 for untreated pieces). After exposure, mosquitoes were removed from cones, grouped together in cardboard tumblers and fed by cotton soaked with 5% glucose solution. To evaluate KD50% and KD95%, the number of female knocked-down by insecticide was recorded immediately and thereafter every 10 min up to 1 h; the mosquito mortality rates were estimated at 24 h after exposure. The room for the tests located in Sélingué was air-conditioned, with glass windows and a adequate door. The technician controlled the room temperature so that all tests were carried out at 26 °C.

2.5. Experimental huts

Three experimental huts were used in each study village. Each hut (2.50 m × 1.75 m × 2.00 m) was a sleeping room, with cement plastered mud walls. The ground was cement, the carpentry in wood and the roof in corrugated iron. The room had: (a) 4 controlled windows that allowed mosquitoes entry but prevented their exit; and (b) a veranda-trap made with mosquito net rigid tulle (Darriet et al., 2002; Asidi et al., 2004). Each hut received either one treated or untreated net once a week. At the end of the week, huts were cleaned and aerated to avoid possible contamination.

Three adult men volunteers of similar age, after providing informed consent, were invited to sleep in the huts. In July 2008, mosquitoes were collected during 15 days, i.e. 45 hut-nights. Each night sleepers were randomly assigned to a different hut where he entered at 8:00 pm and slept on a mattress under either a treated or untreated net. At 5:00 am, the windows were closed and veranda curtains were rolled down to avoid new entrance of mosquitoes and movement from room compartments to the veranda. Each morning, the entomological team captured mosquitoes with aspirators under bed nets, around the bed and in the veranda. In addition, dead mosquitoes were collected. The living mosquitoes were placed under observation outside the huts to determine their mortality.

2.6. Data analysis

Mortality rates for the cones tests were calculated after angular transformation (arc sin $\sqrt{x}$) to alleviate the variation. The knock-down time was determined by graphical representation of the tested cones data with Excel 2003. Experimental huts data was analysed with EPI6-Info Version 6.04dfr. We studied the impact of alpha-cypermethrin treated nets on mortality rate, exophilic rate and repellent rate. Mortality rate was estimated by the percentage of dead mosquitoes found in the huts, exophilic rate by the proportion of blood-fed mosquitoes captured under the veranda over the total number of blood-fed mosquitoes captured under the veranda and around the bed net, and the repellent rate by the percentage of unfed mosquitoes captured around the bed and under the veranda. Pearson chi-square test was used to compare groups and calculate the 95% confidence interval.

Table 1
KD50 and KD95 time and mortality by mosquito strain.

Mosquito's strain	S-Koumantou	S-Sélingués	S-Kisumu
N	400	400	400
KD50 (min)	<3	<3	<3
KD95 (min)	26	21	26
Mortality after 24 h	100%	100%	100%

2.7. Ethical approval

This study received formal approval of the Malian Ministry of Health and Institutional Review Board of the Faculty of Medicine, Pharmacy and Dentistry, Bamako, Mali.

3. Results

3.1. Knock-down resistance in cone test

With the WHO standard cones test, there was no difference between S-Koumantou, S-Sélingué and S-Kisumu (Fig. 2). At 20 min, only one mosquito for S-Kisumu, compared to 12 for S-Sélingué and 21 for S-Koumantou had survived. Before the end of the exposure, more than 50% of mosquitoes, regardless of the strain, had been knocked-down. The KD50 time was below 3 min and the KD95 time below 30 min (Table 1). In the control cones with untreated net, mortality was between 0.5% and 1%.

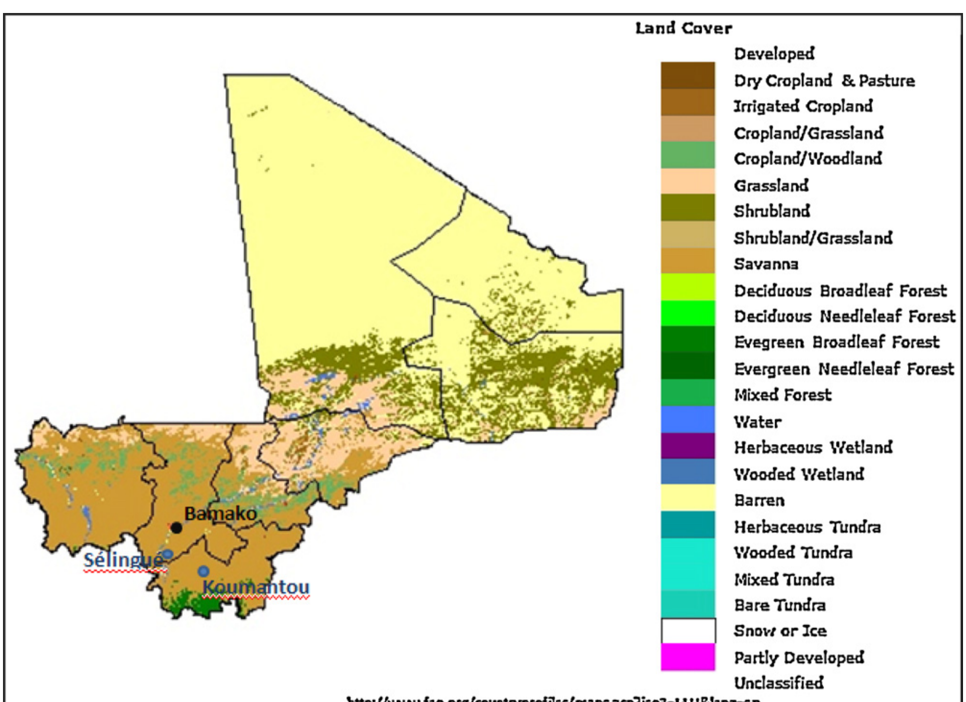

Fig. 1. Map of Mali: location of study sites in savanna area (FAO country profiles and mapping information, http://www.fao.org).

Table 2
Mortality of *A. gambiae s.l.* cohorts in experimental huts by sites (%).

	Experimental hut[a]		Under bed net	
	Treated net	Untreated net	Treated	Untreated
KoumantuMortality, n/N[b]	102/361 (28.2)	2/480 (0.4)	14/28 (50.0)	2/77 (2.6)
Sélingué Mortality, n/N[b]	122/233 (52.3)	100/583 (17.1)	17/20 (85.0)	4/114 (3.5)

[a] Total = All the captured mosquitoes in the site.
[b] N = Living + dead mosquitoes captured in the same location.

3.2. Mortality rate in experimental huts

A. gambiae s.l. represented 99% (841 mosquitoes) of malaria vectors in Koumantou and 100% (816 mosquitoes) in Sélingué. Few *A. funestus* (n = 9) were captured in Koumantou but not in Sélingué. In Koumantou, 43% (361/841) of all mosquitoes were collected in huts with treated bed nets, against 27% (233/816) in Sélingué. In the experimental huts with alpha-cypermethrin treated bed nets, the mosquito mortality rate was significantly lower in Koumantou (102/361, 28.2%) than in Sélingué (122/233, 52.3%) (RR: 0.65, 95%CI: 0.56–0.76) (p < 0.001) (Table 2).

The mortality pattern in the experimental huts, i.e. the place where most mosquitoes died, differed between Koumantou and Sélingué (Fig. 3). Among the mosquitoes collected under a treated bed net, mortality was significantly lower in Koumantou (14/28, 50%) than in Sélingué (17/20, 85%) (RR: 0.55, 95%CI: 0.35–0.86) (p = 0.01). In Koumantou, mortality rate was higher under treated (14/28, 50%) than under untreated bed nets (2/77, 3%), higher around treated (93/210, 44%) than around untreated bed nets (0/276, 0%), while it was similar in the verandas, regardless of the presence of a treated or untreated bed net. Similary in Selingué, mortality rate was higher under treated (17/20, 85%) than under untreated bed nets (4/114, 4%), higher around treated (24/60, 40%) than around untreated bed nets (2/176, 2%), but higher in verandas of huts with a treated bed net (85/153, 86%) than those with an untreated one (94/293, 32%). In Koumantou, most dead mosquitoes (102/361, 28.2%) in the huts with an insecticide-treated net where found under (14) or around the bed net (93) while in Sélingué, there were 17 dead mosquitoes under the insecticide-treated bed net, 24 around it, and 69.7% (85/122) were in the veranda (as compared to none in Koumantou). Mosquitoes having fed through an insecticide-treated bed net were unable to come out of the hut and were captured inside the hut. This phenomenon was more evident in Koumantou, where among the fed mosquitoes the large majority was captured in the huts (92.9%, 131/141), than in Sélingué (77.4%, 24/31).

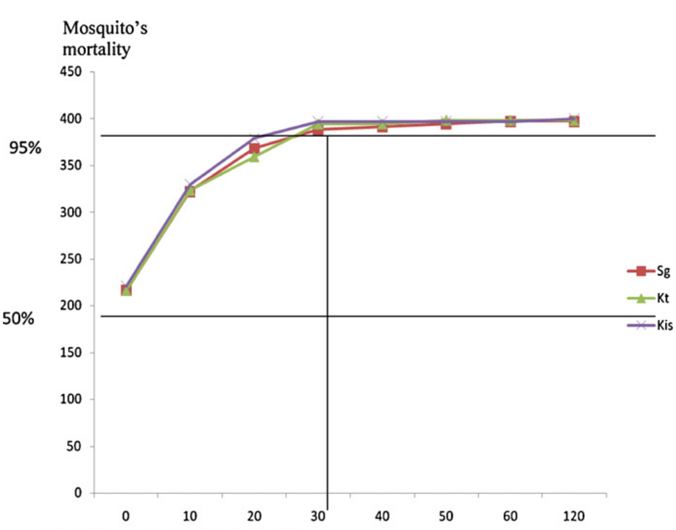

Fig. 2. Kinetics of mortality of wild strains of *A. gambiae s.l.* from Koumantou, Sélingué and of *A. gambiae* Kisumu.

3.3. Exophilic behaviour of fed mosquitoes in experimental huts

The percentage of fed mosquitoes captured in the veranda of huts with an untreated bed net was significantly higher in Koumantou than in Sélingué, indicating a more pronounced exophilic behaviour (Fig. 4). In Koumantu, such exophilic behaviour changed when there was a treated bed net as the percentage of fed mosquitoes caught in the veranda dropped. In Sélingué, the presence of a treated bed net did not change significantly the percentage of fed mosquitoes captured in the veranda.

3.4. Repellent effect of treated bed net in hut test

In Koumantou, the percentage of unfed mosquitoes was significantly lower in the huts with untreated (42.7%, 172/403) as compared to those with treated bed nets (62.8%, 209/333) (p < 0.001) (Table 3). Such difference was probably due to the repellent effect of the insecticide as the percentage of unfed mosquitoes found in the veranda was much lower in the huts with untreated (26.0%, 334/127) than in those with treated nets (92.2%, 118/128) (p < 00.1). Conversely, in Sélingué, there was no difference between huts with treated and untreated bed nets (Table 3).

4. Discussion

4.1. Killing effect

Alpha-cypermethrin treated nets, according to the results obtained with the standard cone tests, had similar effect on the S-Koumantou and S-Sélingué *A. gambiae s.l.* strains, the former from cotton crops area where insecticide use for agricultural purposes is probably high, and the latter from a rice crops area where insecticide use is low. Such a result was unexpected as the insecticidal selective pressure on the vector population in Koumantou was assumed to be higher. Indeed, in Ivory Coast, *A. gambiae s.l.* strains captured in cotton and rice crop growing areas had different insecticide susceptibility, with the former having a much lower mortality (Koffi et al., 1998). The similar susceptibility of the two Malian strains may be explained by the high dose of alpha-cypermethrine used in our test, 40 mg/m^2 as compared to 20 mg/m^2 used in Ivory Coast. The rapid effect of alpha-cypermethrine at 40 mg/m^2 on sensitive strains has already been reported, with a KD50% at 4 min, while in resistant strains this was at 10 min. Mortality among *A. gambiae* sensitive strains was 100% as compared to 94% in resistant ones (Hougard et al., 2003). A similar high killing effect of alpha-cypermethrin has been observed on *A. culicifacies* and *A. fluviatilis* in Northern India (Ansari & Razdan, 2003; Sahu et al., 2003).

4.2. Exophily and repellent effect

Exophiliy was defined as the proportion of blood-fed mosquito in the veranda of huts without a treated bed net, while in those

Table 3
Percentage of unfed *A. gambiae s.l.* by site of capture and insecticide treatment of bed nets (*n*/*N*).

	Treated bed nets			Untreated bed nets		
	Around bed	Veranda	Total	Around bed	Veranda	Total
Koumantou	44.4 (91/205)	92.2 (118/128)	62.8 (209/333)	50.4 (139/276)	26.0 (33/127)	42.7 (172/403)
Sélingué	67.9 (38/56)	95.6 (150/157)	88.3 (188/213)	68.2 (120/176)	92.5 (271/293)	83.4 (391/469)

with a treated bed net this proportion was related to both exophily and the repellent effect of the insecticide. In Koumantou, the high exophily of the local vector was modified by the presence of a treated bed net as the percentage of fed mosquitoes caught in the veranda dropped significantly. Conversely, in Sélingué treated bed nets did not affect the exophilic behaviour. This may be explained by the vulnerability of the mosquitoes in this site; possibly the mosquitoes able to feed were hit by the insecticide and died inside the hut, without being able to fly to the veranda. Unfortunately, we did not collect data on the fed mosquitoes found dead around the bed and no firm conclusion can be drawn.

Concerning the repellent effect of the insecticide, this was found in Koumantou but not in Sélingué, indicating that in the latter the mechanical barriers offered by the bed nets was sufficient to protect the sleeper. Nevertheless, in Koumantou, despite the lower mortality observed as compared to Sélingué, suggesting some degree of insecticide tolerance, alpha-cypermethrin was still having some effect on the local vector. Such difference may be attributed to the environmental exposure, i.e. to the more intense agricultural use of insecticides (Diarrassouba, 2002) that would have selected mosquitoes more tolerant to alpha-cypermethrin. Indeed, massive use of insecticides to fight against crop pests, representing 90% of worldwide use of insecticide (Akogbéto et al., 2006), and long-term exposure to sub-lethal doses of insecticide may select for the kdr mutation (Boyer, 2006). A significant reduction of *A. gambiae s.l.* susceptibility to pyrethroïds has been related to cotton crops insecticide's treatment in Cameroon (Chouaïbou et al., 2008). *A. gambiae* adult mosquitoes hatched from larvae sampled in cotton crops area better tolerated insecticides than those from non-cotton areas. Nevertheless, such phenomenon is not exclusively determined by the agricultural use of insecticides. In Burundi, during a 6-year vector control programme based mainly on indoor residual spraying, the prevalence of the kdr mutation increased steadily in sprayed valleys, confirming the selection pressure of pyrethroids (Protopopoff et al., 2008). In the same study, the prevalence of the kdr mutation increased also in sites far from sprayed areas, indicating a selection pressure other than that exerted by the vector control program.

In Mali, the kdr mutation was identified several years ago but only in the *A. gambiae* Savanna chromosomal form. Sensitivity tests to DDT and other pyrethroids carried out in the irrigated areas of Niono and Sélingué, and in the cotton crop growing areas of

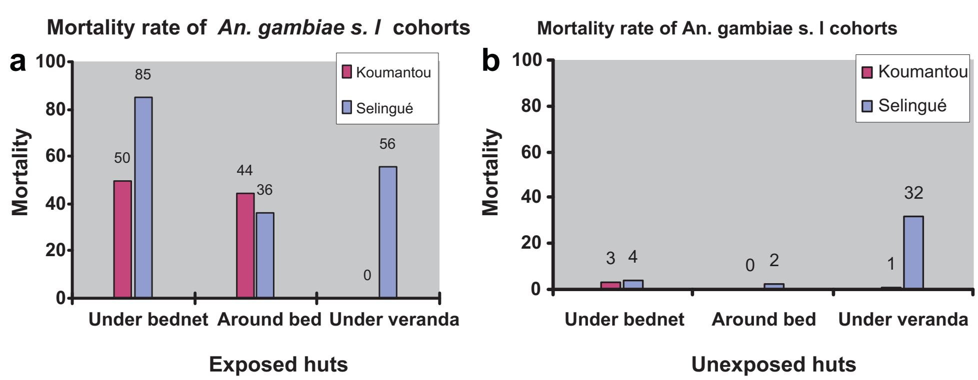

Fig. 3. Mortality rate of *A. gambiae s.l.* cohorts, under bed net, around the bed and under the veranda of experimental huts: (a) insecticide treated bed nets and (b) untreated bed nets.

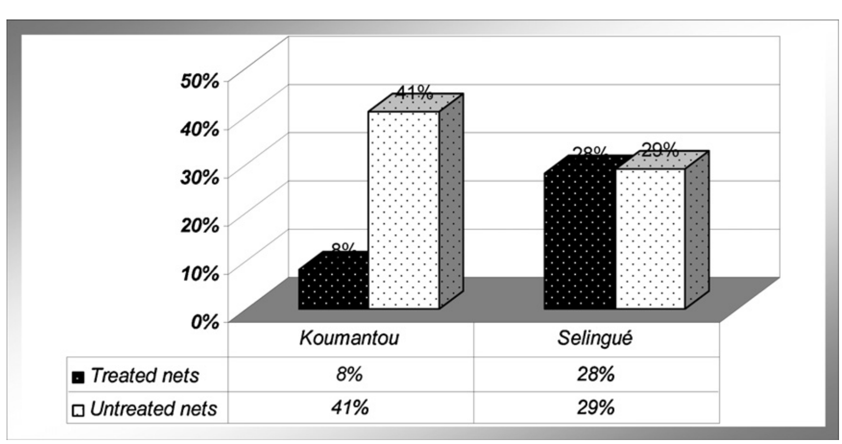

Fig. 4. Exophilic behaviour of *A. gambiae s.l.* cohorts (blood-fed mosquitoes in the veranda/(blood-fed mosquitoes in the veranda + blood-fed mosquitoes around the bed net) in experimental huts by sites (%).

Pimperena in the region Sikasso, revealed different degrees of insecticide resistance (Diarrassouba, 2002). In Banambani and Pimperena, wild *A. gambiae s.l.* were resistant to both permethrin and DDT but sensitive to deltamethrin and lambdacyhalothrin. The kdr gene was observed only in Pimperena in the Savanna chromosomal form of *A. gambiae s.s*. Conversely, wild *A. gambiae s.l.* and *A. funestus* caught in Niono and Sélingué were sensitive to all insecticides tested. In Sélingué, the good efficacy of insecticide-treated bed nets maintained over 6 months confirms the sensitivity of the local vector to permethrin.

In conclusion, alpha-cypermethrin treated bed nets had a significant effect on mortality and repelling behaviour of *Anopheles gambiae s.l.* though in Koumantou treated bed nets were less efficacious, possibly due to the intense use of pesticide for agriculture.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2011.11.013.

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