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Efficacy of pyriproxyfen-pyrethroid long-lasting insecticidal nets (LLINs) and chlorfenapyr-pyrethroid LLINs on mosquito feeding and resting behaviour in Benin, West Africa

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Abstract
Innovative mosquito nets combining pyrethroids with Pyriproxyfen or Chlorfenapyr significantly reduced Anopheles densities in Benin, but their impact on the feeding/resting behaviour of pyrethroid-resistant mosquitoes remains unstudied. Our study assessed the impact of pyriproxyfen-pyrethroid (Pyr-PPF) and chlorfenapyr-pyrethroid (Pyr-CFP) LLINs on the feeding/resting behaviour of pyrethroid-resistant mosquitoes in Benin, aiming to inform future mosquito control strategies amidst rising insecticide resistance. The study was conducted in the communes of Covè, Zangnanado, and Ouinhi in southern Benin, with 60 clusters randomly assigned to three treatment groups: Pyr-CFP LLINs, Pyr-PPF LLINs, and Pyr only LLINs as a control. Mosquitoes were collected using the Pyrethrum Spray Catch (PSC) method, their blood meals analysed using the Sandwich ELISA method, and the data analysed using a mixed-effect generalized linear model. A total of 6,925 mosquitoes were collected during the study, with *Anopheles* spp. (38%), *Culex* spp. (30.6%), and *Mansonia* spp. (28.9%). being the most common genera. Blood feeding rates (BFR) were high for *Anopheles* spp. (72.1% to 86.5%), with no significant differences between study arms after two years. Significant reductions in BFR for *Culex* spp. (BFR=15.0; 95%CI: 11.8-18.9) and *Mansonia* spp. (27.0; 23.1-31.3) were observed in the Pyr-PPF LLIN arm compared to Pyr only LLINs ([26.3; 22.2-30.8] and [41.9; 37.3-46.6] respectively). Indoor resting densities (IRD) of *Anopheles* spp. were significantly reduced in the Pyr-PPF LLIN and Pyr-CFP LLIN arms compared to the control arm ($p<0.05$). Human blood was the primary source of blood meals for all three mosquito genera. The study reveals a high prevalence of indoor *Anopheles*, *Culex*, and *Mansonia* mosquitoes, posing significant risks for malaria and lymphatic filariasis transmission. Pyr-PPF and Pyr-CFP LLINs reduced BFR for *Culex* and *Mansonia* and decreased indoor resting densities of *Anopheles*, but further vector control measures are needed.

Keywords: Pyrethroid-pyriproxyfen, pyrethroid-chlorfenapyr, LLINs, mosquito behaviour

Introduction
Malaria remains a major public health problem in Afro-tropical regions, accounting for around 93.6% of cases worldwide and 95.4% of deaths in 2022 [1]. In Benin, malaria is the primary cause of consultations and hospitalisation. In 2023, new malaria infections in the general population were 17% in adults and 39% in children under five [2].
The disease is transmitted from vector to host when the female mosquito takes a blood meal [3, 4]. The feeding and resting behaviour of malaria mosquitoes, particularly *Anopheles* mosquitoes, is a key factor influencing the transmission of the disease. Long-acting insecticidal nets (LLINs) have played a crucial role in reducing malaria transmission [5, 6] due to two main actions of the pyrethroids used to impregnate them: a repellent effect that keeps mosquitoes away from LLINs, thereby reducing mosquito-human contact and, consequently,

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infectious bites, and the lethal effect that kills mosquitoes into contact with the net.

The widespread distribution of mosquito nets has helped to reduce the spread of malaria throughout the world, by reducing populations of *Anopheles* mosquitoes, the vectors of the disease [5]. Despite the substantial progress made between 2000 and 2014 [5], the effectiveness of long-lasting insecticidal nets (LLINs) declined from 2015 due to the emergence and spread of vectors resistant to the pyrethroids used to impregnate these nets [7, 8].

In this context, recent innovations have led to the development of mosquito nets impregnated either with a pyrethroid insecticide and a synergist (Piperonil-Butoxide), or with two active substances combining a pyrethroid with a second insecticide (Pyriproxyfen or Chlorfenapyr) with distinct modes of action. Some of these nets have been evaluated in areas with pyrethroid-resistant vectors in Benin as part of community trials. The results showed reductions of 42% and 56% in the density of *Anopheles*, the malaria vectors, in the groups using Pyr-PPF LLINs (Alphacypermethrin and pyriproxyfen) and Pyr-CFP LLINs (Alphacypermethrin and chlorfenapyr) respectively, compared with the group using Pyr only LLIN (alphacypermethrin alone) [9]. However, our previous studies in the same area showed no evidence of a significant reduction in the density of non-*Anopheles* vectors such as *Culex* spp. and *Mansonia* spp. by these LLINs [10]. However, these studies did not address the feeding and resting behaviour of these pyrethroid-resistant mosquitoes.

Our study was therefore designed to assess the impact of Pyr-PPF LLINs and Pyr-CFP LLINs on feeding and resting behaviour of pyrethroid-resistant mosquitoes under field conditions. The results of this study will inform mosquito control strategies as insecticide resistance increases.

Materials and Methods

Study area

The study was carried out in the communes of Covè (07°13'08.0400" N, 02°20'21.8400" E), Zangnanado (07°16′00″ N, 02°21′00″ E) and Ouinhi (07°05′00″ N, 02°29′00″ E), in the Zou department, located in southern Benin (fig.1). The region is characterized by two rainy seasons (May to July and September to November), with annual rainfall ranging from 900 mm to 1,250 mm. A total of 123 villages, representing a population of approximately 220,000, were grouped into 60 clusters, each comprising approximately 200 households and 1,200 residents. Twenty clusters were randomly assigned to each of the three study arms: the alpha-cypermethrin and chlorfenapyr LLIN arm (LLIN Interceptor G2®), the alpha-cypermethrin and pyriproxyfen LLIN arm (LLIN Royal Guard®) and the alpha-cypermethrin only LLIN arm (LLIN Interceptor®) as a control [11]. The primary economic activities of the population include agriculture, fishing, hunting, and commerce [12, 13].

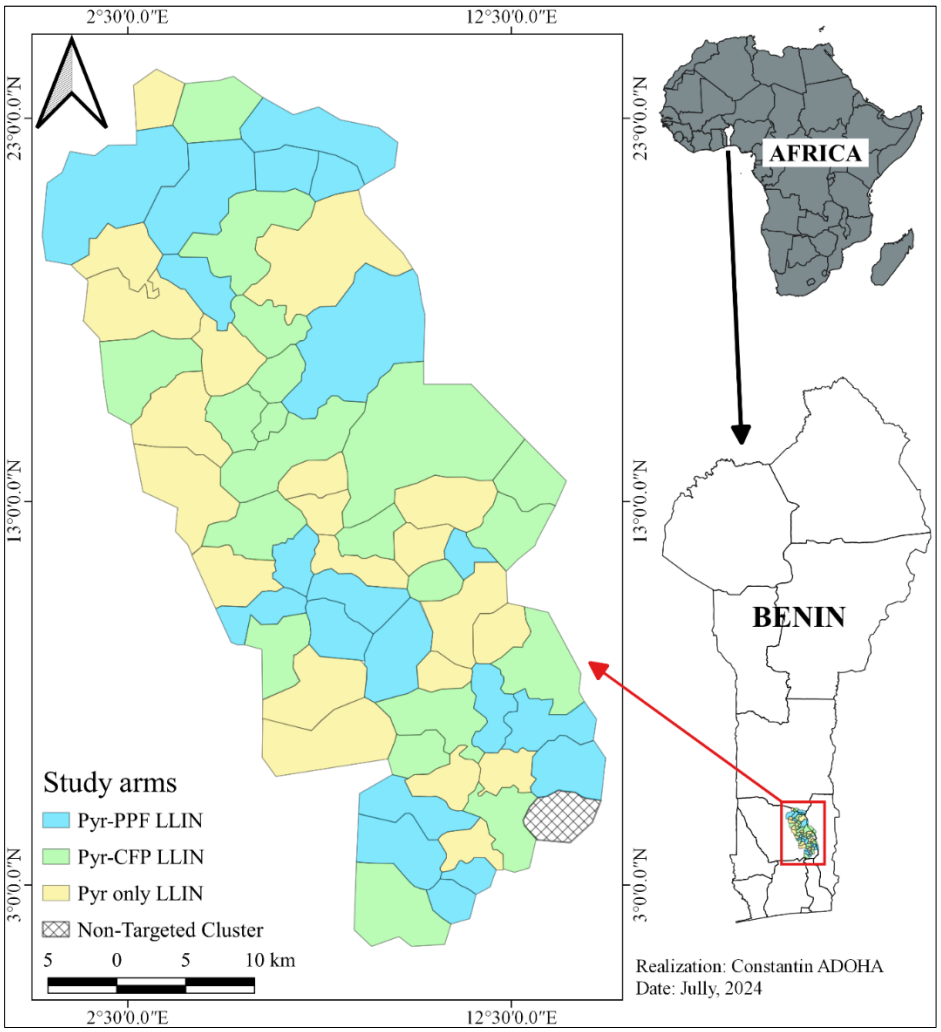

Fig 1: Map of the study area showing clusters and arms.

Mosquito sampling and processing

A round of collection was conducted between September-October 2019 before the net distribution, followed by eight post-intervention collection rounds between June 2020 and April 2022. The Pyrethrum Spray Catch (PSC) method was used between 6: 00 and 10:00 for mosquito collections in households [14]. In each cluster, one house was randomly selected from a census list, along with three additional houses located 15-20 meters away from the first one. In each house, a sheet was spread out to cover all the furniture and utensils. A collector sprayed the inside with a combination of 0.25% transfluthrin + 0.20% permethrin and the door was closed. After 10 to 15 minutes, the knockdown mosquitoes were collected. Mosquitoes were morphologically identified at genera level using a binocular loupe and the Gillies and Meillon [15] taxonomic key. Fed mosquitoes were stored in tubes with silica gel and kept under cool temperature for further laboratory analysis.

A sample of blood-fed mosquitoes from the *Anopheles*, *Culex*, and *Mansonia* genera were analysed to determine the origin of blood meals using the Sandwich ELISA protocol [16]. The blood samples were analysed for the presence of immunoglobulin G (IgG) from goat, human, pig, and cow.

Data analysis

Entomological surveillance data were entered twice into CS Pro 7.2 software and then cleaned using Stata 15.0 (Stata Corp., College Station, TX, USA).

At the household level, the mean proportion of blood-fed mosquitoes per night per house and the average number of indoor resting mosquitoes per night per house were calculated for *Anopheles* spp., *Mansonia* spp., and *Culex* spp. These metrics were compared between study groups using a mixed effect generalized linear model with a negative binomial distribution, incorporating collection rounds and clusters as random effects. The study arm was treated as a fixed effect. The analyses were performed with Stata 15.0 software (Stata Corp., College Station, TX, USA).

Ethical statement

Ethical approval was obtained from the ‘‘Comité National d'Ethique pour la Recherche en Santé du Bénin’’ (N°30/MS/DC/SGM/DRFMT/CNERS/SA, Approval n°6 of 04 March 2019) and the ethics committee of the London School of Hygiene and Tropical Medicine (16237-1). Informed written consent was obtained from household heads and mosquito collection volunteers.

Results

Mosquito species composition and relative abundance

The study collected a total of 6,925 mosquitoes across all species throughout the entire monitoring period. This included 2,091 mosquitoes collected at baseline and 4,834 mosquitoes collected at post-intervention (fig.2). At baseline, the most common mosquito genera collected were *Culex* spp., *Anopheles* spp., and *Mansonia* spp. These three genera accounted for 38%, 30.6%, and 28.9% of the collected mosquitoes, respectively. Other mosquito genera, including *Aedes* spp. and *Coquillettidia* spp., were found at a much lower frequency (<3%) (fig.2). The same trend was observed at post intervention (fig.2).

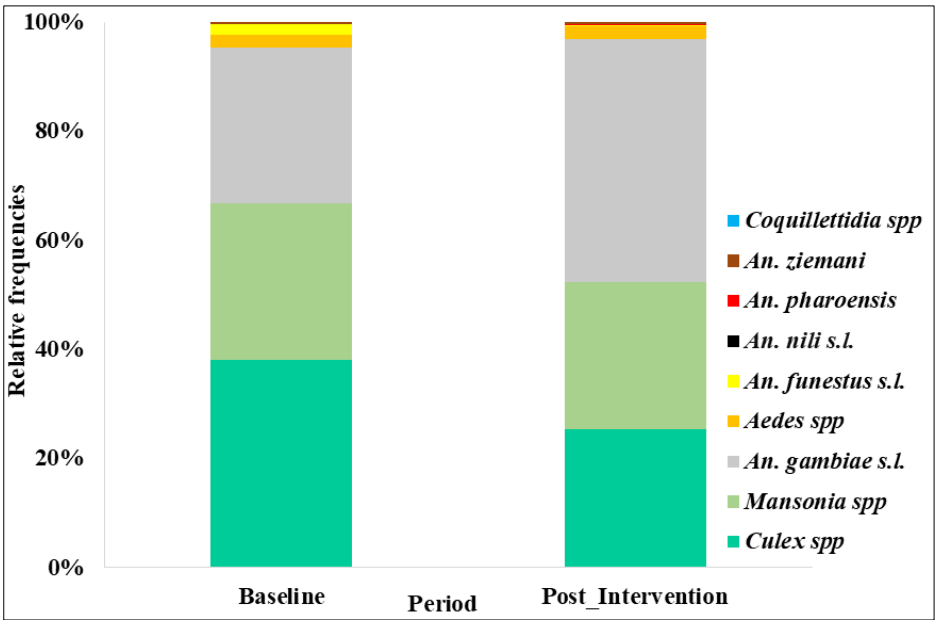

Fig 2: Mosquito species composition in the study area during before and after intervention (between September 2019 and April 2022).

Mosquitoes blood feeding behaviour in the study area

Blood feeding rates (BFR) were high throughout the study period (Baseline and Post-intervention) for *Anopheles* spp. (72.1% to 86.5%) in contrast to *Culex* spp. (08.1% to 44.2%) and *Mansonia* spp. (17.0% to 52.1%) (Table 1). BFR were similar in *Anopheles* spp. between the different arms after two years (Pyr LLIN [BFR=73.1; 95%CI 70.4-75.7], Pyr-PPF LLIN [72.9; 69.1-76.5] and Pyr-CFP LLIN [77.2; 73.1-80.8]) and throughout the study period.

For *Culex* spp. a significant reduction in BFR ($p<0.05$) was observed in the Pyr-PPF LLIN arm at year 1 (19.4; 14.8-24.9) and two years post-intervention (15.0; 11.8-18.9) compared to Pyr LLIN ([34.9; 29.1-41.2] and [26.3; 22.2-30.8] respectively) (Table 1). The same trend was observed with *Mansonia* spp. in Pyr-PPF arm (Year 1 [33.0; 27.7-38.7] and after two years [27.0; 23.1-31.3]) compared to P LLIN arm

(Year 1 [51.5; 46.0-57.0] and after two years [41.9; 37.3-46.6]) (Table 1).

Table 1: Blood feeding rate of *Anopheles* spp., *Culex* spp. and *Mansonia* spp

Period	Arms	*Anopheles* spp.			*Culex* spp.			*Mansonia* spp.		
		N	Fed	BFR (%)	N	Fed	BFR (%)	N	Fed	BFR (%)
Baseline	Pyr only LLIN	241	194	80.5[a]	258	114	44.2[a]	217	113	52.1[a]
	Pyr-PPF LLIN	222	192	86.5[a]	288	125	43.4[a]	260	117	45.0[a]
	Pyr-CFP LLIN	177	141	79.7[a]	249	95	38.2[a]	127	28	22.0[b]
Year 1_Post intervention	Pyr only LLIN	482	338	70.1[a]	252	88	34.9[a]	330	170	51.5[a]
	Pyr-PPF LLIN	258	186	72.1[a]	253	49	19.4[b]	294	97	33.0[b]
	Pyr-CFP LLIN	210	156	74.3[a]	217	62	28.6[a]	216	94	43.5[a]
Year 2_Post intervention	Pyr only LLIN	646	487	75.4[a]	163	21	12.9[a]	126	21	16.7[a]
	Pyr-PPF LLIN	326	240	73.6[a]	161	13	08.1[a]	176	30	17.0[a]
	Pyr-CFP LLIN	276	219	79.4[a]	179	35	19.6[a]	150	48	32.0[b]
Overall Post intervention	Pyr only LLIN	1128	825	73.1[a]	415	109	26.3[a]	456	191	41.9[a]
	Pyr-PPF LLIN	584	426	73.0[a]	414	62	15.0[b]	470	127	27.0[b]
	Pyr-CFP LLIN	486	375	77.2[a]	396	97	24.5[a]	366	142	38.8[a]

N=number collected; BRF=Blood feeding rates; Blood feeding rates with same letter superscript do not differ significantly ($p > 0.05$)

Mosquitoes resting behaviour in the study area

Overall, a significant reduction in indoor resting density (IRD) of *Anopheles* spp. was observed in the Pyr-PPF LLIN with 0.9 density/houses (d/h) and Pyr-CFP LLIN (0.8 d/h) arms compared to the Pyr only LLIN arm (1.8 d/h) (Table 2). A similar trend was observed in years 1 and 2 post-intervention. The Pyr-PPF LLINs (IDR=0.6; 95% CI 0.6-0.7) and Pyr-CFP LLINs (IDR=0.6; 95% CI 0.6-0.7) showed no evidence of significant reduction in indoor resting densities of *Culex* spp. compared to the standard LLIN (IDR=0.6; 95% CI 0.6-0.7) after two years. The same trend was observed throughout the study period (Table 2). The same trend was observed with *Mansonia* spp. with (0.7; 95%CI 0.7-0.8), (0.6; 95% CI 0.5-0.6) and (0.7; 95% CI 0.7-0.8) in Pyr-PPF LLIN, Pyr-CFP LLIN and standard LLIN respectively.

Table 2: Indoor resting density of *Anopheles* spp., *Culex* spp. and *Mansonia* spp

		Anopheles spp.			*Culex* spp.			*Mansonia* spp.		
Period	Arms	N	Nb HH	IRD	N	Nb HH	IRD	N	Nb HH	IRD
Baseline	Pyr only LLIN	241	80	3.0a	258	80	3.2a	217	80	2.7a
	Pyr-PPF LLIN	222	80	2.8a	288	80	3.6a	260	80	3.3a
	Pyr-CFP LLIN	177	80	2.2a	249	80	3.1a	127	80	1.6b
Year 1_Post intervention	Pyr only LLIN	482	320	1.5a	252	320	0.8a	330	320	1.0a
	Pyr-PPF LLIN	258	320	0.8b	253	320	0.8a	294	320	0.9a
	Pyr-CFP LLIN	210	320	0.7b	217	320	0.7a	216	320	0.7b
Year 2_Post intervention	Pyr only LLIN	646	320	2.0a	163	320	0.5a	126	320	0.4a
	Pyr-PPF LLIN	326	320	1.0b	161	320	0.5a	176	320	0.6a
	Pyr-CFP LLIN	276	320	0.9b	179	320	0.6a	150	320	0.5a
Overall_Post intervention	Pyr only LLIN	1128	640	1.8a	415	640	0.6a	456	640	0.7a
	Pyr-PPF LLIN	584	640	0.9b	414	640	0.6a	470	640	0.7a
	Pyr-CFP LLIN	486	640	0.8b	396	640	0.6a	366	640	0.6a

N=Number collected; Nb HH=Number of household; IRD=Indoor Resting Density; IRD with same letter superscript do not differ significantly ($p > 0.05$)

Identification of mosquito blood meal hosts in the study area

Of a sample of 431 blood-fed mosquitoes tested, *Anopheles* spp., *Culex* spp. and *Mansonia* spp. accounted for 69.6% (n=300), 12.5% (n=54) and 17.9% (n=77) respectively (Fig. 3). Among the 300 *Anopheles* analysed for blood-meal host identification, 224 (74.7%; 95%CI 69.3-79.4) contained only human blood, 3 (1%; 95%CI 0.3-3.1) contained blood from other animals (Goat and pork), and 73 (24.3%; 95% CI 19.7-29.7) were unidentified. For the 54 *Culex* spp. mosquitoes tested, 28 (51.9%; 95% CI 38.0-65.5) were found to have solely human blood meals, 10 (18.5%; 95% CI 9.7-31.9) contained blood from other animals (Cow, goat, and pork), and 16 (29.6%; 95% CI 18.4-43.8) were unidentified. Among the 77 *Mansonia* spp. tested for blood-meal host identification, 46 (59.7%; 95% CI 47.9-70.6) contained exclusively human blood meals, 18 (23.4%; 95% CI 14.8-34.7) had blood meals from other animals (Cow, goat, and pig), and 13 (16.9%; 95% CI 9.6-27.5) were unidentified (Fig. 3).

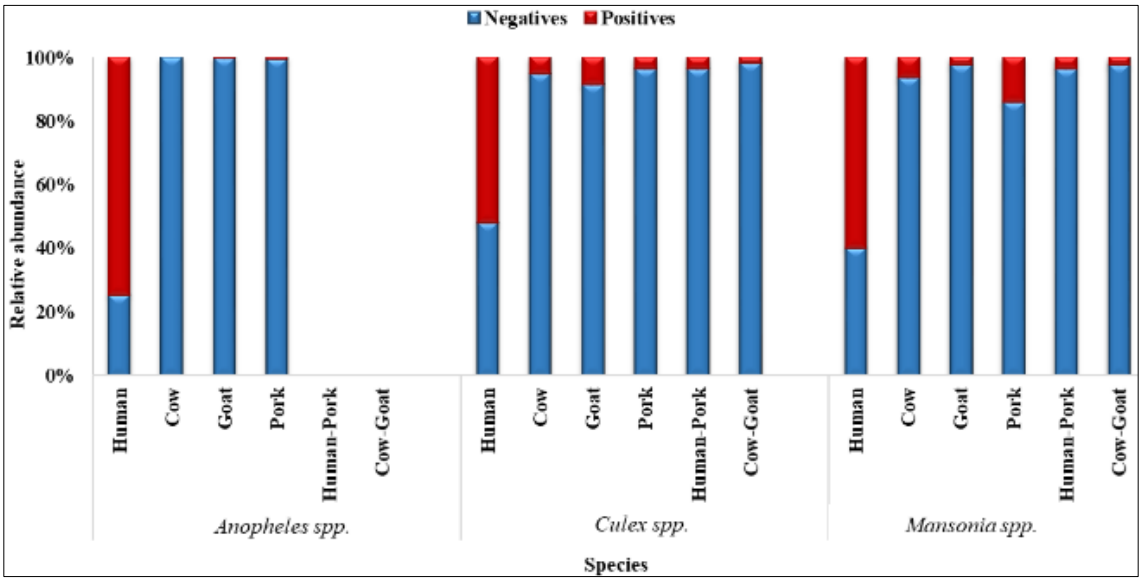

Fig 3: Blood meal of *Anopheles* spp., *Culex* spp. and *Mansonia* spp

Discussion

The present study focused on evaluating how mosquito nets impregnated with two different active ingredients (Pyrethroids-pyriproxyfen and pyrethroids-chlorfenapyr) could affect the feeding and resting behaviour of pyrethroids resistant mosquitoes. The results showed over 97% of indoor mosquito vectors were belonging to *Mansonia* spp., *Culex* spp., *Anopheles* spp. Significant reductions in the indoor resting density of *Anopheles* spp. were observed with Pyr-PPF and Pyr-CFP LLINs, but no significant reduction in their blood-feeding rate compared to standard nets. However, significant reductions in the blood-feeding rates of *Culex* spp. and *Mansonia* spp. were noted with Pyr-PPF LLINs, and high human-feeding rates were recorded for *Anopheles* spp., *Culex* spp., and *Mansonia* spp.

Overall, the mosquito vectors of the genera *Culex* spp., *Anopheles* spp. and *Mansonia* spp. were collected indoor using the PSC method, with a frequency of over 97%. In contrast, *Aedes* spp. and *Coquillettidia* spp. were collected in lower proportions (<3%). This finding corroborates the results of Yovogan *et al.* [17], obtained in the same study area using the human landing catches method. Mosquito species were found before and after the intervention, except for *Coquillettidia* spp. which were only observed pre-intervention. The predominance of *Anopheles*, *Culex*, and *Mansonia* mosquitoes indoor indicated a high transmission risk of diseases such as malaria and lymphatic filariasis to house members, particularly during periods when they are not under LLINs [18]. Consequently, additional methods of vector control, such as screen doors and windows, as a complement to LLINs, are required to limit mosquito-borne diseases transmission.

In the present study, the Pyr-PPF and Pyr-CFP nets showed no significant reduction in *Anopheles* mosquito BFR compared with the standard pyrethroid-only net, which had high values. These high rates for *Anopheles* spp. are of concern given their role as the main vectors of malaria [17, 19]. This finding is in contrast to that reported by Barreaux *et al.* [20] where Permanet and Olyset LLINs significantly reduced the rate of gorging in pyrethroid-resistant *Anopheles gambiae* s.l. However, a significant reduction in BFR was observed for *Culex* spp. and *Mansonia* spp. in the Pyr-PPF LLIN arm after two years of use ($p<0.05$), indicating the potential efficacy of this intervention on the blood-feeding behaviour of these species. This could be due to the known exophilic behaviour of these vectors.

A significant reduction in the indoor resting density of *Anopheles* spp. was observed in the Pyr-PPF LLIN (0.9 mosquitoes/house) and Pyr-CFP LLIN (0.8 m/h) arms compared to the Pyr alone LLIN arm (1.8 m/h). This reduction indicated the effectiveness of the interventions in reducing indoor *Anopheles* spp. populations, which could contribute to reducing the risk of malaria transmission. However, no significant reduction in indoor resting densities was observed for *Culex* spp. and *Mansonia* spp. suggesting that these interventions may be less effective against these mosquito species.

The high human-feeding rates of 74.7%, 51.9% and 59.7% for *Anopheles* spp., *Culex* spp. and *Mansonia* spp. respectively are relevant for assessing the risk of vector-borne disease transmission in the study area. These values indicated a strong likelihood of feeding on humans, which increases the probability of transmission of diseases associated with these mosquitoes. Indeed, *Anopheles* spp. are the main malaria vector species [21–24], and a high feeding rate on humans indicated a greater likelihood of transmission of this disease. *Culex* spp. transmit West Nile virus, lymphatic filariasis and Japanese encephalitis [25, 26]. *Mansonia* spp. were also a vector of lymphatic filariasis [26]. This strong preference for feeding on humans may be due to the availability of humans as hosts or to specific behavioural adaptations. Further research on environmental factors, human behaviour and the adaptations of mosquitoes that lead to feeding on humans is needed to better understand these preferences and to help develop more targeted control strategies.

The present study showed several limitations. The seasonality of mosquito species and their behaviour was not taken into account. The sampling method used did not allow for the collection of fed mosquitoes resting outdoors. The impact of environmental conditions, mosquito species and human behaviour was not considered. Furthermore, the resistance mechanisms of mosquitoes to the molecules used to impregnate the study nets were not addressed.

Conclusion

The study revealed a high prevalence of *Anopheles*, *Culex*, and *Mansonia* mosquitoes indoors, posing a significant risk for malaria and lymphatic filariasis transmission, and underscored the necessity of additional vector control measures beyond LLINs. While Pyr-PPF and Pyr-CFP nets did not significantly reduce *Anopheles* spp. BFR, they effectively reduced BFR for *Culex* spp. and *Mansonia* spp. after two years and decreased indoor resting densities of *Anopheles* spp. The high human-feeding rates of these mosquito species highlight the urgent need for ongoing research and integrated vector management to mitigate disease transmission risks.

Author Contributions

Conceptualization, C.J.A., G.G.P., B.Y., M.C.A. and C.A.; methodology, C.J.A., G.G.P., B.Y., M.C.A. and C.A.; software, C.J.A., B.A. and S.C.; validation, G.G.P., M.C.A. and C.A.; formal analysis, C.J.A., B.A. and S.C.; laboratory analysis, C.J.A., B.Y., A.S. and M.J.A. investigation, C.J.A., B.Y., E.M.O., B.Ad., Z.A., K.B., R.A., O.O.; resources, G.G.P. and M.C.A.; data curation, C.J.sA. and B.Y.; writing-original draft preparation, C.J.A.; writing-review and editing, G.G.P., B.Y., B.A., S.C., A.S., M.J.A., R.O., E.M.O., B.Ad., Z.A., K.B., R.A., O.O., M.C.A. and C.A.; visualization, C.J.A. supervision, G.G.P., M.C.A. and C.A.; All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Permission was obtained in writing from the heads of households after providing them with adequate information.

Data Availability Statement

The datasets used in this study are accessible upon reasonable request from the corresponding authors.

Competing Interests

The authors have declared no competing interests.

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