US PRESIDENT'S MALARIA INITIATIVE ACTION TO REINFORCE MALARIA VECTOR CONTROL PROGRAM IN BENIN

CREC/Final Report 2020

"Entomological Monitoring Report Benin 2020"

Final report

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Abbreviations

Ace-1	Acetylcholinesterase
Ace-1R	*Ace-1R*: Acetylcholinesterase Resistant
DNA	Deoxyribonucleic acid
CREC	Centre de Recherche Entomologique de Cotonou/Entomological Research Center of Cotonou
CDC	Center for disease control
CS	Capsulated suspension
CSP	Circumsporozoite protein
EIR	Entomological Inoculation Rate
ELISA	Enzyme-linked Immunosorbent Assay
GST	Glutathion S-Transférase
HBR	Human Biting Rate
HLC	Human Landing Catch
HZ	Health zones
IRS	Indoor Residual Spraying
Kdr	Knock-down resistance
NMCP	National Malaria Control Program
PCR	Polymerase Chain Reaction
PSC	Pyrethrum Spray Catch
PMI	U.S. President's Malaria Initiative
SI	Sporozoite index
s.l.	Sensu lato
s.s.	Sensu stricto
USAID	United States Agency for International Development
WHO	World Health Organization

GENERAL INTRODUCTION

The Centre de Recherches Entomologiques de Cotonou (CREC) [English translation: Entomological Research Center of Cotonou] conducted three monitoring, evaluation, and research activities on behalf of Benin's National Malaria Control Program (NMCP) related to Indoor Residual Spraying interventions implemented in Benin by the PMI/VectorLink and the NMCP in 2020. The monitoring and IRS were supported by the US President's Malaria Initiative.

1. Mosquito behavior and malaria transmission in the Health Zone "Natitingou-Boukoumbe-Toucountouna" and in Pehunco district after the withdrawal of Indoor Residual Spraying.
2. Monitoring and Evaluation of the efficacy of the fourth year of Indoor Residual Spraying in Alibori and Donga, northern Benin, West Africa.
3. Quantification study and evolution of the insecticide resistance in *Anopheles gambiae* sensu lato in Benin – the implication of high vector resistance for vector control.

After six years of IRS implementation in the Atacora region (2011-2016), the National Malaria Control Program (NMCP), in agreement with various partners, decided to withdraw this intervention from some districts and relocate it to two other regions (Alibori and Donga). This decision falls within the framework of the implementation of the national insecticide resistance management plan and provides the opportunity for coverage in two high burden regions that have never benefited from this intervention.

After the withdrawal, continued entomological surveillance in 3 districts of the Atacora region (Natitingou, Boukombé, Toucountouna) was implemented to check if the withdrawal of the IRS is followed by a rebound in malaria transmission. This report relates data collected in 2020.

In 2017, IRS was extended in other regions (Alibori and Donga). This report also shows M&E results of the efficacy of the fourth year (2020) of IRS in these regions.

Since 2008, the M&E of IRS has been accompanied by a study on the evolution of vector resistance in various ecological areas. In 2020, we implemented a study to determine what could be the direct implications of the high intensity of insecticide resistance noted in Benin on vector control management. We have evaluated the response of mosquitoes that survived after exposure to high concentrations of insecticides and the combined insecticides + PBO to different types of LLINs. The results we obtained will serve as a database for the utilization of new generations Insecticide-Treated Nets in Benin.

1 Part I: Mosquito behavior and malaria transmission in the Health Zone "Natitingou-Boukoumbe-Toucountouna" and in Pehunco district after the withdrawal of Indoor Residual Spraying

1.1 Background

Malaria is endemic in Benin, with 1.5 million cases reported annually among a national population of 11.1 million. In this country, malaria vector control relies on the mass distribution of LLINs and IRS operations. From 2011 to 2016, an IRS program was implemented with funding from the US President's Malaria Initiative (PMI), in north Benin and targeted all houses in the Atacora region, an epidemic-prone area in Benin. During those 6 years of IRS implementation, two insecticides of two different classes were used in rotation: bendiocarb (a carbamate) and pirimiphos-methyl (an organophosphate), due to the emergence and expansion of resistance of Anopheles vectors to insecticides, especially pyrethroids[1]. After 6 years of implementation (2011–2016), IRS showed a significant reduction in malaria transmission in the Atacora region[2]. In 2017, the IRS program was withdrawn from some communes in Atacora and moved to two other regions (Donga and Alibori) in the north, with hopes that gains would relatively be sustained because of the seasonality of malaria transmission. To achieve this, only two districts (Pehunco and Kerou) continue to receive IRS until 2108. Since 2019, IRS has been withdrawn from the two remaining communes (Pehunco and Kerou), resulting in the total withdrawal of the intervention in Atacora. This decision not only falls within the framework of the implementation of the national insecticide resistance management plan, but it also offers the possibility to two high malaria prevalence regions that have never benefited from this intervention to be covered. Few entomological studies investigated vector control withdrawal and its implications on subsequent malaria transmission trends. Ten entomological indicators of malaria transmission such as vector abundance, human biting rate (HBR), and entomological inoculation rate (EIR) are parameters commonly used to assess the impact of vector control interventions and the intensity of malaria transmission. This final report presents the results of entomological monitoring in the communes of Natitingou, Toucountouna, Boukoumbé, and Pehunco, respectively 3 and 2 years after the withdrawal of IRS. The main objective was to assess the evolution of entomological indicators of malaria transmission

[1] 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.

[2] Akogbéto MC, Aikpon R, Azondekon R, Padonou G, Osse R, Agossa FR, et al. Six years of experience in entomological surveillance of indoor residual spraying against malaria transmission in Benin: lessons learned, challenges and outlooks. Malar J. 2015; 14:242.

in the withdrawal zones compared to a control commune under IRS intervention (Copargo in Donga region) with the same ecological characteristics as the latter.

In February, March, June, and August 2020, we proceeded with mosquito sampling to cross the nighttime socio-behavioral surveillance data of the household members (activity 4) with those of entomological parameters, for the dry season (hot period) (February and March 2020) and the rainy season (From June to August 2020) in northern Benin. For this reason, mosquito sampling was organized from 7:00 pm to 7:00 am. The *Anopheles* samples collected in February and March 2020 are not sufficient to make the correlation we expect. Indeed, we pooled the mosquitoes collected every hour for the whole of the areas to determine the trend in the hourly human biting rate for *Anopheles gambiae* (s.l.). However, data were not collected in April and May 2020 in these areas due to the restrictive measures taken by the government to limit the spread of COVID-19, these measures were taken at the time of mosquito collection in these localities.

1.2 Material and methods

1.2.1 Study area

- Health zone: Natitingou, Toucountouna, Boukoumbe (Atacora): where the IRS was withdrawn in 2017.
- Pehunco district (Atacora): where the IRS was withdrawn in 2019.
- Control (Copargo district): Located in Donga region, protected by the IRS since 2017, and has the same ecological characteristics as the areas withdrawn from the IRS (Fig. 1).

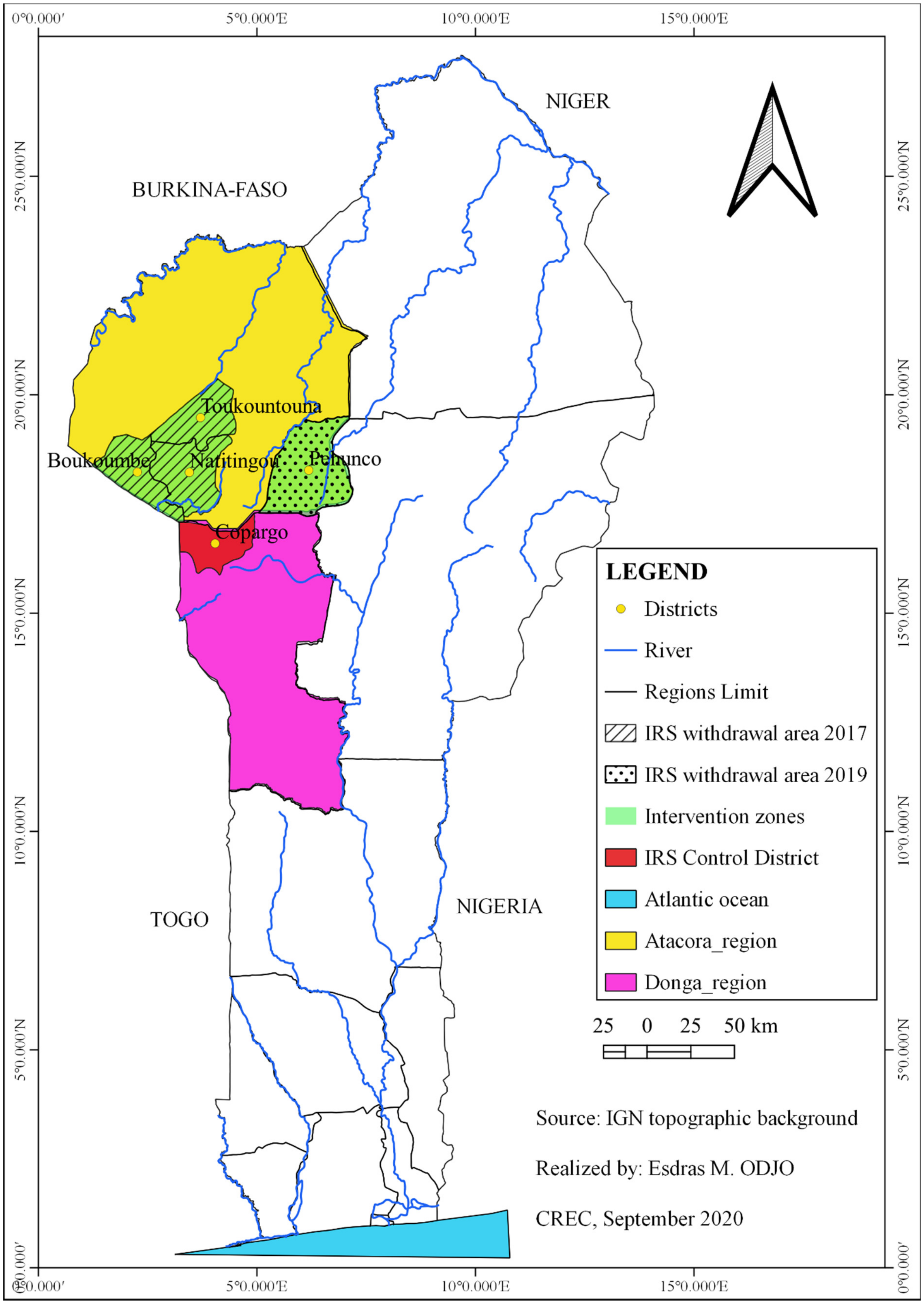

Figure 1. *Map showing the IRS withdrawal zones and control zone (under IRS)*

Atacora and Donga regions are located in the Sudano-Guinean climate zone. These two regions are dry savannah areas, with six months rainy season (mid-April to mid-October) and a dry season that spans the remainder of the year. Overall, average annual rainfall ranges between 1200–1300 mm in Atacora and Donga regions.

Figure 2 shows the rainfall and temperature trends in the Atacora and Donga regions. The rainiest months are from June to October in three regions with the rainfall peak occurring in August.

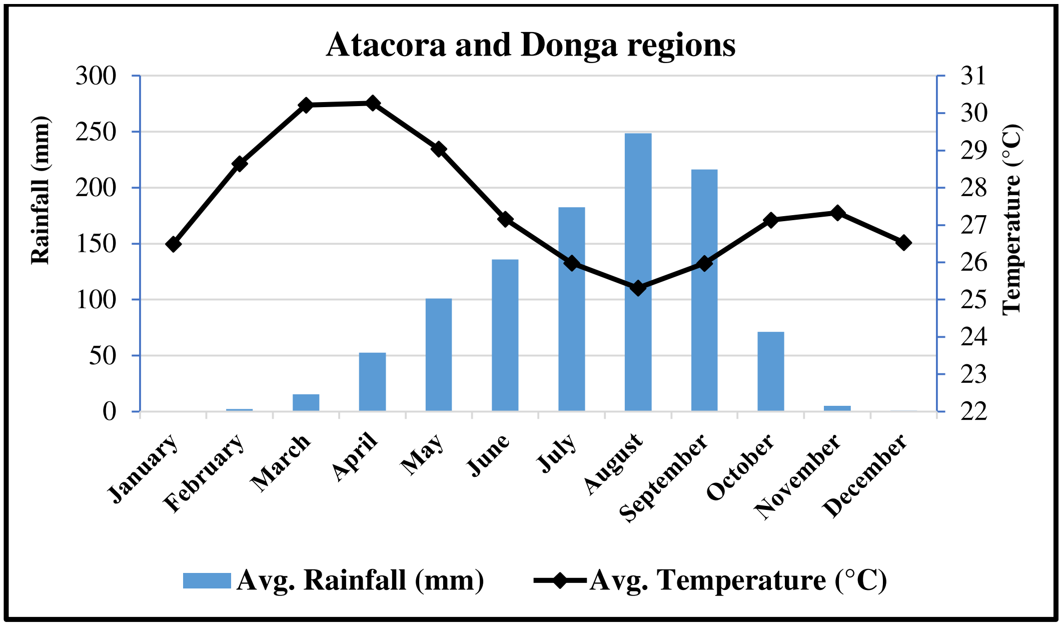

Figure 2. *Monthly climate data in the Atacora and Donga regions[3].*

Malaria is the leading cause of mortality among children under five years of age and morbidity among adults in Benin. Malaria accounts for 40% of outpatient consultations and 25 % of all hospital admissions. Malaria places an enormous economic strain on Benin's development. The incidence of uncomplicated and severe malaria in 2016 was 18.3% in the Atacora region and 25.5% in Donga.

1.2.2 Indicators measured

Activities planned between January to September 2020 provided data and information about the following entomological indicators required by PMI and the NMCP:

- Vector identification (species and molecular forms of *Anopheles gambiae*)
- The density of mosquitoes inside bedrooms
- Mosquito blood-feeding behaviors (endophagy, exophagy behaviors)
- Human Biting Rate (HBR)

[3] Climate data from World Bank Group, Climate Change Knowledge Portal (https://climateknowledgeportal.worldbank.org/country/benin/climate-data-historical)

- Entomological Inoculate Rate (EIR)
- Results of insecticide susceptibility tests
- Identification of mosquito genetic mutations that confer resistance (*Kdr, Ace-1*)

Table 1. *Summary of indicators by collection method*

Collection method	Indicator	Definition
	Indoor resting density	# mosquitoes / house / day
PSC	% of fed females	# fed mosquitoes / Total collected by PSC
	HBR	# bites /person/night
	Parity rate	Percentage of parous mosquitoes
	Exophagic rate	Percentage of mosquitoes biting outside
HLC	Endophagic rate	Percentage of mosquitoes biting inside

1.2.3 Organization of the report

Four visits were done from February to August 2020 to collect mosquitoes, conduct advanced laboratory testing on captured *Anopheles gambiae* species, and assess the susceptibility of mosquitoes and mechanisms involved in vectors.

1.2.4 Adult mosquito collections

Mosquito sampling was conducted in 5 districts selected for entomological monitoring: Natitingou, Toucountouna, Boukoumbe, and Pehunco (NTBP) where the IRS was withdrawn in 2017 & 2019 and 1 control (Copargo) under IRS since 2017.

Mosquitoes were collected by human landing catch (HLCs) in two villages per district, with one village located in the center of the district, and one village located at the periphery. Table 2 shows the mosquito sampling sites and their geographic coordinates. For each village, mosquitoes were collected in 2 houses by 4 mosquito collectors, 2 mosquito collectors indoors, and 2 outdoors. In total, 40 local mosquito collectors were used for one round of collection. Two rounds of sampling were done per month. Two teams of four mosquito collectors in each village worked inside and outside the selected dwellings, from 19:00 to 00:00 hours (7:00 PM to 12:00 AM) for the first team and from 00:00 to 07:00 hours (12:00 AM to 7:00 PM) for the second team. Mosquito collectors rotated through the different dwellings to avoid biases related to their trapping ability or individual attractiveness. The collection was done with the hemolysis tubes.

To estimate the indoor resting density of mosquitoes per room, 10 houses per village were selected[4]. The bedrooms were sprayed with pyrethrum (mixed with water) and a white canvas was placed on the floor to collect knocked-down mosquitoes. After 15 minutes, all fallen mosquitoes were collected from the floor and placed in Petri dishes, to determine the number of mosquitoes in the room and to determine the blood-feeding stage (unfed, fed, half-gravid and gravid).

Vector species that were collected and identified were transported to the Entomological Research Center of Cotonou (CREC) laboratory for ovary dissection using a microscope to determine the parous rates. The heads/thoraxes of the vector species were analyzed by the ELISA method to look for CSP antigens. Abdomens of female vector species were used for PCR analyses to identify sibling species and molecular forms.

Table 2. *Mosquito sampling sites and their geographic coordinates.*

District	Village	Area	Longitude/Latitude
Natitingou	Ourbouga 3	IRS Withdrawal area in 2017	10°14′40.72″N, 01°22′43.47″E
	Pouya	IRS Withdrawal area in 2017	10°16′57.15″N, 01°28′58.02″E
Toucountouna	Kpentikou	IRS Withdrawal area in 2017	10°29′55.01″N, 01°22′44.74″E
	Datakou	IRS Withdrawal area in 2017	10°32′56.82″N, 01°17′49.45″E
Boukoumbe	Kounadorgou	IRS Withdrawal area in 2017	10°10′25.10″N, 01°06′52.07″E
	Okouaro	IRS Withdrawal area in 2017	10°15′56.66″N, 00°59′47.90″E
Pehunco	Banikani	IRS Withdrawal area in 2019	10°13′35.35″N, 02°00′15.19″E
	Bouerou	IRS Withdrawal area in 2019	10°09′49.51″N, 01°57′15.18″E
Copargo	Toungouli	IRS area in 2020	09°50′19″N, 01°32′59″E
	Fowa	IRS area in 2020	09°53′49″N, 01°32′59″E

1.2.5 Mosquito identification and processing

1.2.5.1 Morphological identification of vectors species

After each collection, mosquitoes were counted and morphologically identified using the taxonomic key of Gillies & Meillon[5]. All *Anopheles* vectors captured through HLC were dissected to assess their physiological age[6]. Each specimen was then stored in a labeled Eppendorf tube containing silica gel and cotton for further molecular analyses.

1.2.5.2 Molecular analyses

Vector species identified were transported to the Centre de Recherche Entomologique de Cotonou (CREC) to detect the presence of *P. falciparum*. The heads/thoraxes of all females *An. gambiae* (s.l.) were

[4] These houses were different from the houses used in the HLC collection

[5] Gillies MT, De Meillon B. The Anophelinae of Africa south of the Sahara. S Afr Inst Med Res. 1968; 54:1–343.

[6] Detinova TS, Gillies MT. Observations on the determination of the age composition and epidemiological importance of populations of *Anopheles gambiae* Giles and *Anopheles funestus* Giles in Tanganyika. Bull World Health Organ. 1964; 30:23–8.

analyzed by ELISA CSP according to the protocol described by Wirtz et al[7]. The abdomens, legs, and wings of all specimens of *An. gambiae* (*s.l.*) captured through HLC was analyzed by PCR according to the protocol of Santolamazza et al[8], for molecular species identification. The same mosquitoes were genotyped for the *kdr* L1014F, *kdr* L1014S, and G119S *Ace-1* mutations, according to the protocols of Martinez-Torres et al[9], Ranson et al[10] and Weill et al[11], respectively.

1.2.6 Data analysis

Data were analyzed with the statistical R software, version 2.8. using the stats package[12]. The Chi-square test of comparison of proportions was also used to compare the proportion of *An. gambiae* (s.l.) indoors and outdoors, blood-feeding rate, sporozoite index, and parity rate of *An. gambiae* (*s.l.*). The Poisson test[13] was used to estimate the risk ratio (RR) and compare the confidence intervals of indoor vector density and EIRs of *An. gambiae* (s.l.) between IRS withdrawal area and IRS area.

1.3 Results

1.3.1 Mosquito composition in IRS withdrawal areas and IRS areas

During the four months of mosquito collection (February to August 2020), a total of 5,891 human-biting mosquitoes belonging to four genera (*Anopheles*, *Aedes*, *Culex*, *Mansonia*) and 13 species were collected in the IRS withdrawal areas (NTBP) and the control area (Copargo) (Table 3). Out of the 13 species, *Anopheles gambiae* s.l. was the second most abundant species collected (35.83% of the total of mosquitoes: 2,111 of 5,891) after *Culex quinquefasciatus* (60.58% of the total of mosquitoes; 3,569 of 5,891) (Table 3). The two major malaria vectors collected were *An. gambiae* s.l. and *An. funestus,* albeit at low frequency (1.07%: 63 *An. funestus* /5,891) in this period.

Overall, we noted a high abundance of all mosquito species between June to August 2020 compared to the February- March 2020 period. The highest proportion of *An. gambiae* s.l. was observed in Boukoumbe (56.14%) and the lowest in Toucountouna (24.10%). The relative abundance of *Culex*

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[7] Wirtz RA, Zavala F, Charoenvit Y, Campbell GH, Burkot TR, Schneider I, Esser KM, Beaudoin RL, Andre RG. Comparative testing of monoclonal antibodies against *Plasmodium falciparum* sporozoites for ELISA development. Bull World Health Organ. 1987; 65(1): 39-45.

[8] Santolamazza F, Mancini E, Simard F, Qi Y, Tu Z, della Torre A. Insertion polymorphisms of SINE200 retrotransposons within speciation islands of *Anopheles gambiae* molecular forms. Malar J. 2008;7:163.

[9] Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, Devonshire AL, Guillet P, Pasteur N. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol.1998; 7: 179–184.

[10] Ranson H, Jensen B, Vulule J, Wang X, Hemingway J, Collins F. 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.

[11] Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M, Raymond M. The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. *Insect* Mol Biol. 2004; 13: 1–7.

[12] R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (2018). Available online at https://www.R-project.org/.

[13] Rothman KJ. Epidemiology: an introduction. Oxford: Oxford University Press; 2012.

quinquefasciatus in Natitingou, Toucountouna and Pehunco may be due to the presence of larval habitats polluted (sewers, abandoned wells, and cisterns) with organic matter at this time of year (dry season and beginning of the rainy season) and in urban areas. Such breeding sites are preferred development sites for *Cx. quinquefasciatus* larvae.

Table 3. *Mosquito species composition (February to August 2020)*

Species	Natitingou	Toucountouna	Boukoumbé	Pehunco	Copargo	Total
An. gambiae s.l	467 (27.44)	94 (24.10)	599 (56.14)	602 (31.90)	349 (41.30)	35.83
An. funestus	8 (0.47)	3 (0.77)	20 (1.88)	29 (1.54)	3 (0.35)	1.07
An. pharoensis	0 (0.00)	0 (0.00)	1 (0.09)	0 (0.00)	1 (0.12)	0.03
An. ziemani	0 (0.00)	0 (0.00)	1 (0.09)	0 (0.00)	0 (0.00)	0.02
Cx. quinquefasciatus	1222 (71.80)	283 (72.56)	373 (34.96)	1237 (65.55)	454 (53.73)	60.58
Cx. nebulosus	0 (0.00)	3 (0.77)	2 (0.19)	2 (0.11)	18 (2.13)	0.43
Cx. tigripes	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	2 (0.24)	0.03
Cx. annulioris	0 (0.00)	0 (0.00)	1 (0.09)	0 (0.00)	0 (0.00)	0.02
M. africana	3 (0.17)	4 (1.03)	31 (2.91)	4 (0.21)	5 (0.59)	0.80
M. uniformis	0 (0.00)	0 (0.00)	8 (0.75)	0 (0.00)	0 (0.00)	0.14
Aedes aegypti	2 (0.12)	3 (0.77)	25 (2.34)	13 (0.69)	13 (1.54)	0.95
Aedes vitatus	0 (0.00)	0 (0.00)	3 (0.28)	0 (0.00)	0 (0.00)	0.05
Aedes longipalpis	0 (0.00)	0 (0.00)	3 (0.28)	0 (0.00)	0 (0.00)	0.05
Total	100.00	100.00	100.00	100.00	100.00	100.00

Nb: number of species; %: percentage

A total of 1,365 *An. gambiae* s.l. were analyzed by PCR for species identification: 1,135 (83.15%) were *An. gambiae* and 219 (16.04%) were *An. coluzzii* (X^2 = 1226.8; *df*= 1; *P* <0.001) (Fig. 3). However, a few specimens of *An. arabiensis* (0.51%: 7/1,365) and hybrids *An. gambiae*/*An. coluzzii* (0.29%: 5/1,365) were identified at a very low proportion. The two main sibling species were present throughout the dry and rainy seasons in the study area (Fig. 3). During the dry season (February to March), *An. coluzzii* predominated (74.09%). However, during the rainy season, we observed a predominance of *An. gambiae* (92.75%) in the study area (Fig. 3).

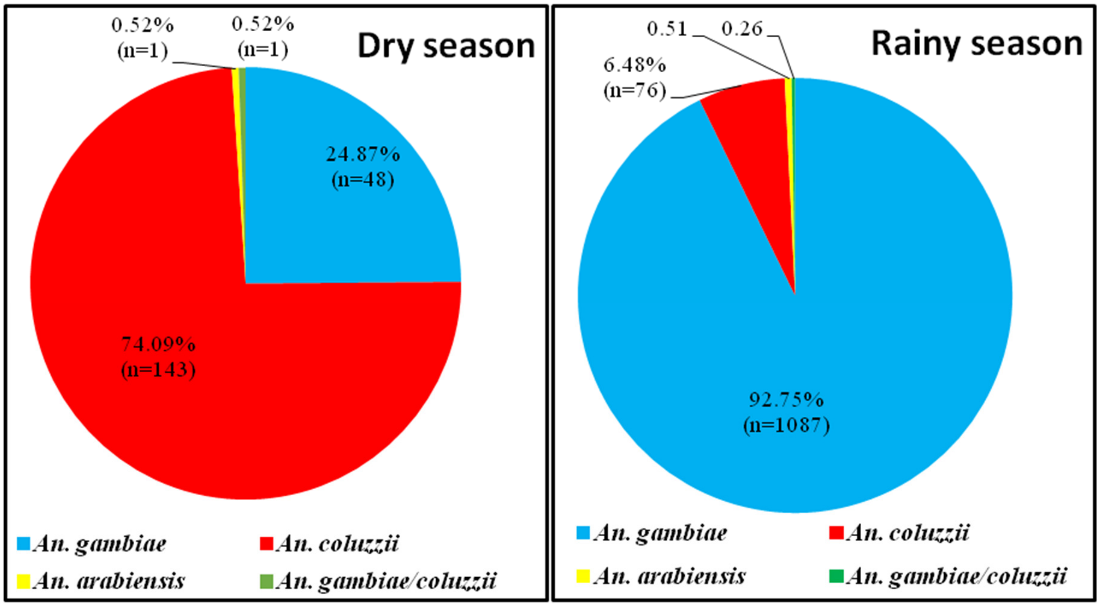


Figure 3. *Distribution of* An. gambiae *s.l. species in districts under IRS and control per season*

1.3.2 Mosquito blood-feeding behaviors

1.3.2.1 Human Biting Rate (HBR) of An. gambiae s.l. indoor versus outdoor in districts

A total of 2,111 *An. gambiae* s.l. were caught from February 2020 to August 2020 in the IRS withdrawal areas (NTBP) and the control area (Copargo). Table 4 shows the proportion of *An. gambiae s.l.* indoors compared to outdoors in the dry season and rainy season in these areas. During the dry season (period from February 2020 to March 2020), the density of *An. gambiae s.l.* was low compared to the period from June 2020 to August 2020. During this period (February to March 2020), the proportion of *An. gambiae* s.l. collected was higher outdoors compared indoors in some houses where IRS was withdrawn and similar in IRS area (Copargo). Globally, 60.11% (113/188) of *An. gambiae* s.l. were collected outdoors in houses where IRS was withdrawn compared to 39.89% (75/188) indoors (p<0.001). This higher trophic activity of *An. gambiae* outside the houses would be explained by the behavior of the human host population, which prefers to sleep outside during this time of the year in search of fresh air.

In contrast, during the rainy season (June 2020 to August 2020), the proportion of *An. gambiae s.l* collected was significantly higher indoors compared to outdoors in all sites except Toucountouna (Table 4). Similarly, in treated houses (Copargo), we recorded the same situation with higher biting rates indoors (Table 4). Globally, 53.52% (843/1575) of *An. gambiae s.l* was collected indoors in IRS withdrawal houses (NTBP) compared to 46.48% (732/1575) outdoors (X^2 = 15.36; *df*=1; *P*<0.001). Similarly, in treated houses (Copargo) (control), 57.14% (188/329) were collected indoor versus 42.86% (141/329) outdoors (X^2 = 12.86; *df*= 1; *P* =0.0003) (Table 4).

Tables 5 and 6 below present the details of the biting rate (HBR) of *An. gambiae s.l.* in the IRS withdrawal areas (NTBP) and the control area (Copargo)

Table 4. *Number and proportion of* An. gambiae *s.l. caught indoors and outdoors in the dry season (February- March 2020) and the rainy season (June-August 2020) in IRS withdrawal and IRS area.*

Districts	Dry season (Feb 2020-Mar 2020)			Rainy season (June 2020- Aug2020)		
	Indoors	Outdoors	p- value	Indoors	Outdoors	p- value
	nb (%)	nb (%)		nb (%)	nb (%)	
Natitingou	45 (36.29)	79 (63.71)	<0.001	153 (44.22)	193 (55.78)	0.003
Toucountouna	1 (50.00)	1 (50.00)	1.00	53 (57.61)	39 (42.39)	0.06
Boukoumbé	19 (61.29)	12 (38.71)	0.127	311 (54.95)	255 (45.05)	0.001
Pehunco	10 (32.26)	21 (67.74)	0.011	326 (57.09)	245 (42.91)	<0.001
IRS Withdrawal districts	75 (39.89)	113 (60.11)	<0.001	843 (53.52)	732 (46.48)	<0.001
IRS district (Copargo)	11 (55.00)	9 (45.00)	0.751	188 (57.14)	141 (42.86)	<0.001

Dry season: February and March; Rainy season: June and August; nb: number of *An. gambiae s.l.*; %: proportion of *An. gambiae s.l.*; p-value: p-value of comparison of the proportion of *An. gambiae s.l.* indoors and outdoors in the same district (Test used: Chi-square test)

Table 5. *Biting rates of* An. gambiae *s.l. indoor and outdoor in the IRS withdrawal districts (NTBP) and the IRS control area (Copargo)*

Districts	Position	Indicator	February 2020	March 2020	June 2020	August 2020	Dry season	Rainy season
Natitingou	Inside	Total Mosquitoes	2	43	59	94	45	139
		nb human catches	8	8	8	4	16	12
		HBR/night	0.25	5.38	7.38	23.50	2.81	11.58
	Outside	Total Mosquitoes	3	76	103	90	79	193
		nb human catches	8	8	8	4	16	12
		HBR/night	0.38	9.50	12.88	22.50	4.94	16.08
Toucountouna	Inside	Total Mosquitoes	1	NA	33	20	1	53
		nb human catches	8	NA	8	4	8	12
		HBR/night	0.13	NA	4.13	5.00	0.13	4.42
	Outside	Total Mosquitoes	1	NA	20	19	1	39
		nb human catches	8	NA	8	4	8	12
		HBR/night	0.13	NA	2.50	4.75	0.13	3.25
Boukoumbé	Inside	Total Mosquitoes	1	18	45	266	19	311
		nb human catches	8	8	8	4	16	12

Districts	Position	Indicator	February 2020	March 2020	June 2020	August 2020	Dry season	Rainy season
		HBR/night	0.13	2.25	5.63	66.50	1.19	25.92
	Outside	Total Mosquitoes	0	12	44	212	12	256
		nb human catches	8	8	8	4	16	12
		HBR/night	0	1.50	5.50	53.00	0.75	21.33
Pehunco	Inside	Total Mosquitoes	10	NA	194	132	10	326
		nb human catches	8	NA	8	4	8	12
		HBR/night	1.25	NA	24.25	33.00	1.25	27.17
	Outside	Total Mosquitoes	21	NA	177	68	21	245
		nb human catches	8	NA	8	4	8	12
		HBR/night	2.63	NA	22.13	17.00	2.63	20.42
Copargo (Control)	Inside	Total Mosquitoes	0	11	144	44	11	188
		nb human catches	8	8	8	4	16	12
		HBR/night	0	1.38	18.00	11.00	0.69	15.67
	Outside	Total Mosquitoes	1	8	110	31	9	141
		nb human catches	8	8	8	4	16	12
		HBR/night	0.13	1.00	13.75	7.75	0.56	11.75

NA: Not applicable; Dry season: February and March; Rainy season: June and August.

Table 6. *Global human biting rate (HBR) in the IRS withdrawal and the IRS area from February 2020 to August 2020.*

Districts	Parameters	February 2020	March 2020	Dry season	June 2020	August 2020	Rainy season
Natitingou	Total Mosquitoes	5	119	124	158	184	342
	nb human catches	16	16	32	16	8	24
	HBR/night	0.31	7.44	3.88	9.88	23.00	14.25
Toucountouna	Total Mosquitoes	2	NA	2	52	37	89
	nb human catches	16	NA	16	16	8	24
	HBR/night	0.13	NA	0.13	3.25	4.63	3.71
Boukoumbe	Total Mosquitoes	1	30	31	81	477	558
	nb human catches	16	16	32	16	8	24
	HBR/night	0.06	1.88	0.97	5.06	59.63	23.25

Districts	Parameters	February 2020	March 2020	Dry season	June 2020	August 2020	Rainy season
Pehunco	Total Mosquitoes	31	NA	31	371	200	571
	nb human catches	16	NA	16	16	8	24
	HBR/night	1.94	NA	1.94	23.19	25.00	23.79
IRS Withdrawal districts (NTBP)	Total Mosquitoes	39	149	188	662	898	1560
	nb human catches	64	32	96	64	32	96
	HBR/night	0.61	4.66	1.96	10.34	28.06	16.25
IRS district (Copargo)	Total Mosquitoes	1	19	20	254	75	329
	nb human catches	16	16	32	16	8	24
	HBR/night	0.06	1.19	0.63	15.88	9.38	13.71

NA: Not applicable; Dry season: February and March; Rainy season: June and August.

1.3.2.2 Hourly Human Biting Rate of An. gambiae (s.l.) during the night in the dry season (February-March 2020) (a) and in the rainy season (June-August 2020) (b)

This study was conducted to better understand the biting behavior of *An. gambiae s.l.* during the hot period (dry season) and in the rainy season. The biting cycle of *An. gambiae* observed is similar to other studies: a constant increase of biting from 7:00 PM to reach a peak between midnight (00:00 AM) and 3:00 AM followed by a gradual drop until early morning (Fig. 4 & 5). Outdoor bites are most noticeable early at night and early in the morning (Fig. 4 & 5). *An. gambiae* s.l. appears to be collected more outdoors in the middle of the night during the dry season, whereas the opposite situation was observed during the rainy season with more indoor biting. Figure 6 shows HBR per hour of *An. gambiae s.l.* collected during the two seasons.

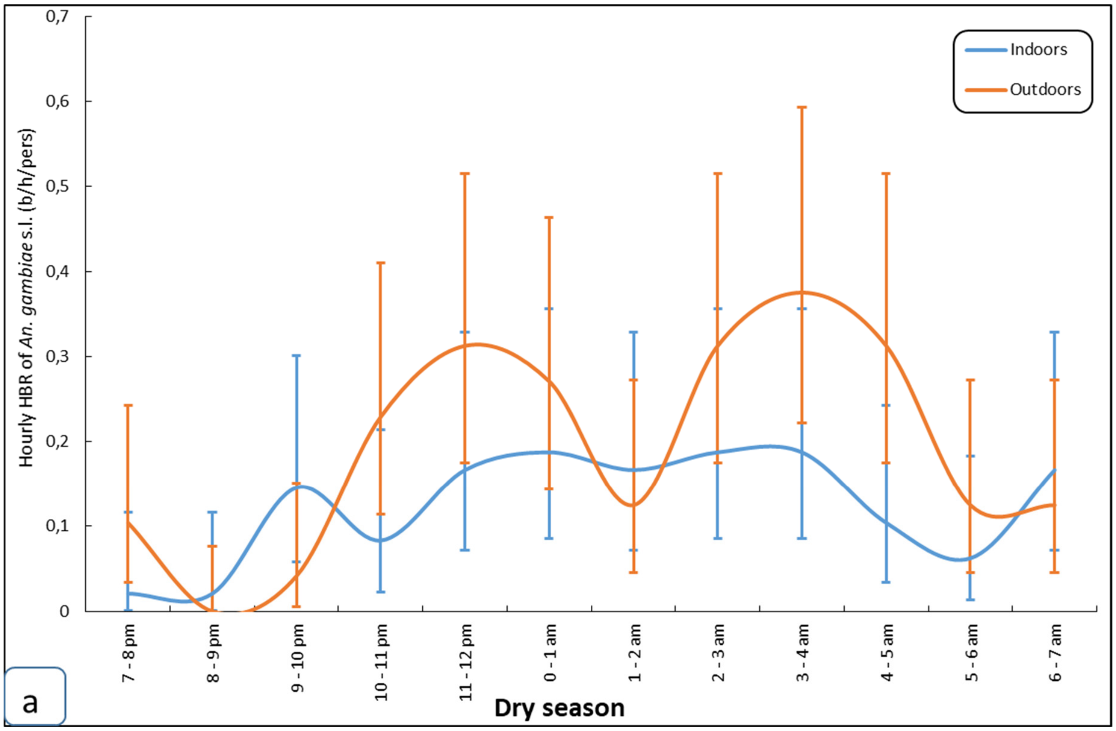


Figure 4. *Hourly HBR of* An. gambiae *s.l. in IRS withdrawal districts (NTBP) in dry (February-March 2020)*

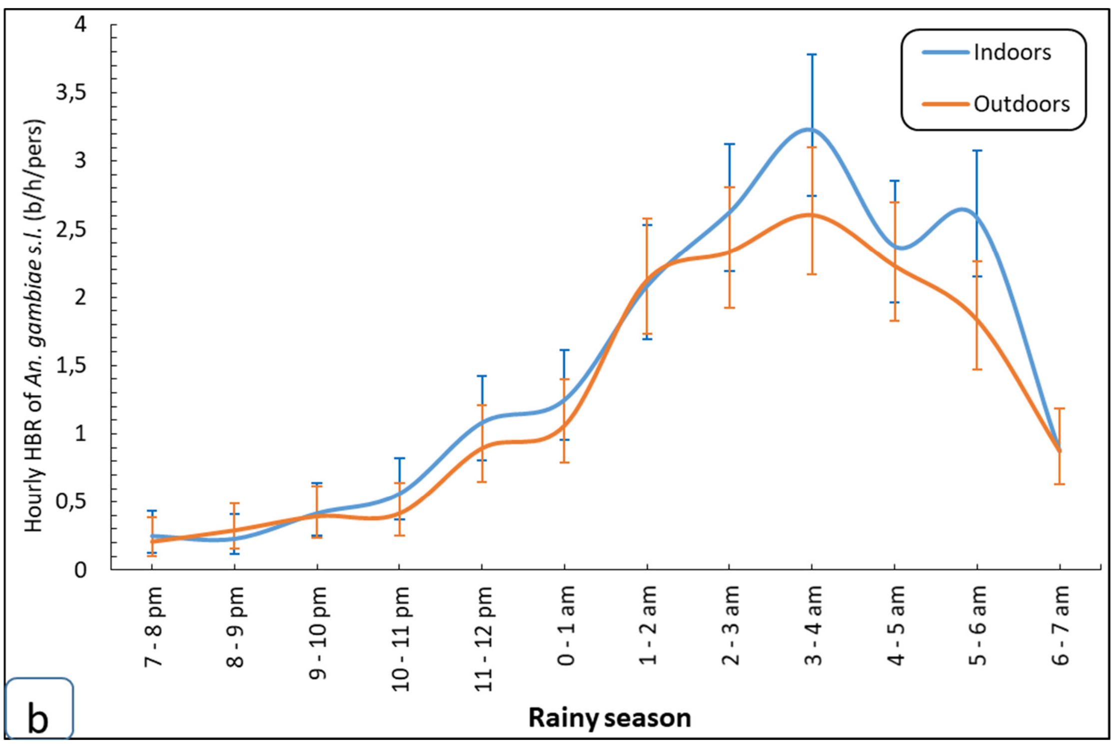


Figure 5. *Hourly HBR of* An. gambiae *s.l. in IRS withdrawal districts (NTBP) in the rainy season (June-August 2020).*

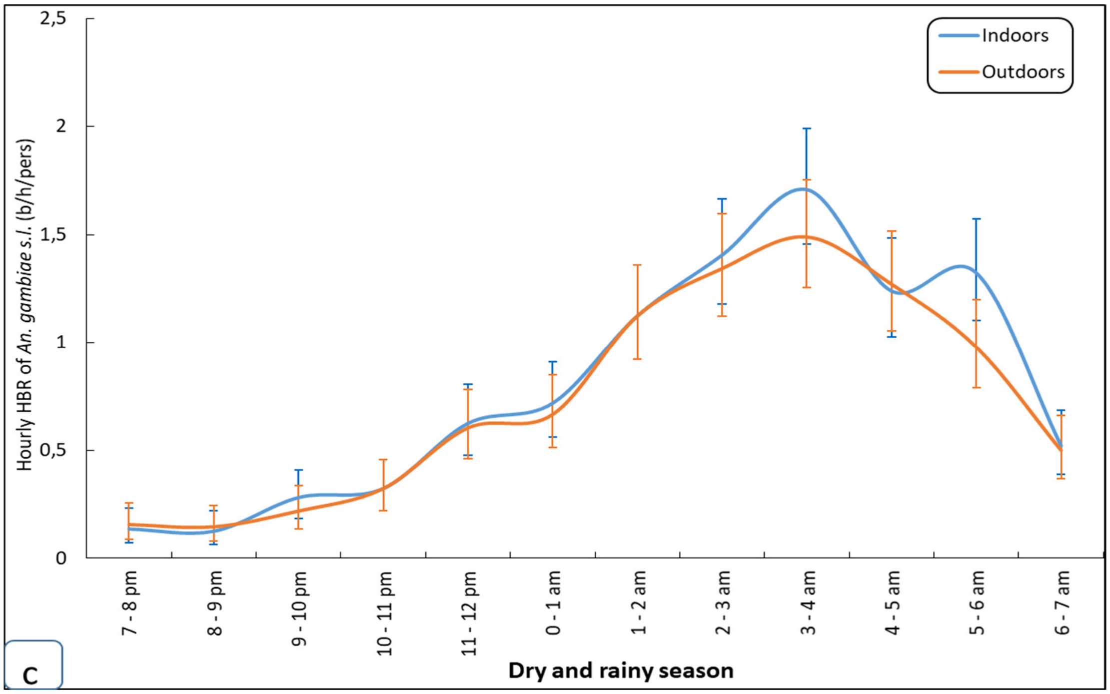


Figure 6. *Hourly HBR of* An. gambiae *s.l. in IRS withdrawal districts (NTBP) (Dry season and Rainy season) (February –August 2020)*

1.3.3 Indoor resting density and blood-feeding rate of An. gambiae s.l.

Three years after IRS withdrawal, approximately 0.96 *An. gambiae s.l* per room were collected early in the morning (7 AM - 9 AM) after PSCs in IRS withdrawal districts (NTBP), while 0.57 *An. gambiae* s.l. per room was collected in the IRS area (Copargo) (p<0.001) (Table 7). Indoor resting density was significantly higher in Natitingou and lower Toucountouna compared to Copargo (IRS district) (Control) p<0.001) (Table 7). However, this density was similar in the other localities compared to Copargo (Table 7). Globally, the blood-feeding rates of *An. gambiae* s.l. was also high in both the IRS withdrawal and the IRS areas (Copargo) (control) (Table 8). However, this rate was significantly higher in the IRS withdrawal areas compared to the control area (Copargo) (IRS area) (X^2 = 16.99; *df*= 1; *p<0.001*) (Table 8).

Table 7. *The indoor resting density of* An. gambiae *s.l. collected (PSCs data) in the IRS withdrawal areas (NTBP) and the IRS area (Copargo)*

Districts	Nb of rooms	Nb of *An. gambiae* s.l. collected	Indoor resting density	RR [95% CI]	P-value (Wald)
Copargo (Control)	98	56	0.57	1	-
Natitingou	80	157	1.96	0.29 [0.21 -0.40]	< 0.001
Toucountouna	60	14	0.23	2.44 [1.34 -4.76]	0.001
Boukoumbe	80	62	0.78	0.73 [0.50 -1.07]	0.115
Pehunco	60	37	0.62	0.92 [0.60 -1.44]	0.749
IRS withdrawal districts	280	270	0.96	1	-
IRS district (Copargo)	98	56	0.57	1.68 [1.26 -2.29]	< 0.001

RR: Rate Ratio; p (Wald): p-value of the Wald test; [95% CI]: 95% confidence interval

Table 8. *Blood feeding rates of* An. gambiae *s.l. collected (PSCs data) in the IRS withdrawal areas (NTBP) and the IRS area (Copargo)*

Districts	Nb of *An. gambiae* s.l. collected	Nb of blood feed	Blood Feeding rate (%)	P-value
Copargo (Control)	56	45	80.36	-
Natitingou	157	155	98.73	< 0.001
Toucountouna	14	13	92.86	0.475
Boukoumbe	62	59	95.16	0.027
Pehunco	37	33	89.19	0.397
IRS Withdrawal districts	270	260	96.30	-
IRS district (Copargo)	56	45	80.36	< 0.001

1.3.4 Parous rate observed in An. gambiae in the IRS withdrawal areas (NTBP) vs the IRS area (Copargo)

Table 9. shows the longevity of *An. gambiae* based on the proportion of mosquitoes that have laid at least once; mosquitoes from HLCs were used. Three years after IRS withdrawal, the parous rate of *An. gambiae* in IRS withdrawal districts (NTBP) was 88.10% (422/479) compared to 83.76% (98/117) in control districts (Copargo) (X^2 = 1.22; *df*=1; p=0.268). No significant differences were observed between the parous rates in different localities (Table 9).

Table 9. *Parous rate of An. gambiae s.l. in IRS withdrawal districts and control*

Districts	Nb of *An. gambiae* s.l. dissected	Nb of parous	Parous rate (%)	P-value
Copargo (Control)	117	98	83.76	-
Natitingou	195	177	90.77	0.094
Toucountouna	39	37	94.87	0.136
Boukoumbé	99	83	83.84	1.00
Pehunco	146	125	85.62	0.807
IRS Withdrawal districts	479	422	88.10	-
IRS district (Copargo)	117	98	83.76	0.268

1.3.5 Sporozoïte index (%CS+) of *Plasmodium falciparum* and entomological inoculation rate (EIR) of *An. gambiae* s.l. in IRS withdrawal areas (NTBP) vs. IRS area (Copargo)

Tables 10, 11, 12, and 13 summarize biting rates (HBR), sporozoïte index (SI), and entomological inoculation rate (EIR) recorded after IRS withdrawal in Natitingou, Toucountouna, Boukoumbe, and Pehunco vs IRS districts (Copargo) in dry and rainy seasons.

During the dry season (Feb-March 2020), a total of 188 head-thoraces of *An. gambiae s.l.* were analyzed by ELISA method to look for circumsporozoïte protein (CSP) antigens in IRS withdrawal areas (NTBP); this resulted in a sporozoïte positivity of 1.06% (2/188). Only in Boukoumbe, the two positive specimens were found. In the control districts (Copargo), the sporozoïte positivity was 5% (1/20). In this period, there was no statistically significant difference between sporozoïte positivity in IRS withdrawal areas and control districts (X^2 = 0.174; *df* =1; p=0.676) (Table 10). Similarly, the EIR was low in IRS withdrawal areas (NTBP) (0.63 infectious bites/pers/month) and in control (Copargo) (0.94 infectious bites/pers/month) (p=1) (Table 10). During the rainy season (June- August 2020), the EIR was 4.9 times higher in IRS withdrawal areas (NTBP) (30.63 infectious bites/pers/month) compared to the control district (Copargo) (6.25 infectious bites/pers/month), an increase of 79.59% of the EIR (Table 10).

Overall, the entomological inoculation rate (EIR) of *An. gambiae* (s.l.) was significantly higher in Boukoumbe (61.25ib/pers/month) and Pehunco (42.25 ib/pers/month) but low in Natitingou (7.50 ib/pers/month) (Tables 10).

Tables 11, 12, and 13 show the monthly biting rates (HBR), sporozoïte index (SI), and entomological inoculation rate (EIR) recorded indoors and outdoors in the IRS withdrawal areas (NTBP) and the IRS area (Copargo) (control).

Table 10. *Sporozoïte index (SI) of* Plasmodium falciparum *and entomological inoculation rate (EIR) of* An. gambiae *s.l. in the IRS withdrawal areas (NTBP) vs the IRS area (Copargo)*

Districts	Dry season			Rainy season		
	Period (Feb 2020-Mar 2020)			Period (June 2020- Aug2020)		
	SI	HBR/night	EIR/month	SI	HBR/night	EIR/month
Natitingou	0.00	3.88	0.00	0.02	14.25	7.50
Toucountouna	0.00	0.13	0.00	0.10	3.71	11.25
Boukoumbe	0.06	0.97	1.88	0.09	23.25	61.25
Pehunco	0.00	1.94	0.00	0.06	23.79	42.50
IRS Withdrawal districts	0.01	1.96	0.63	0.06	16.25	30.63
IRS district (Copargo)	0.05	0.63	0.94	0.02	13.71	6.25

Table 11. *Global sporozoïte index (SI) and entomological inoculation rate (EIR) of* An. gambiae *s.l. in IRS withdrawal areas (NTBP) vs IRS area (Copargo)*

Districts	Parameters	February 2020	March 2020	Dry season	June 2020	August 2020	Rainy season
Natitingou	Thorax	5	119	124	158	184	342
	Thorax +	0	0	0	3	3	6
	SI	0.00	0.00	0.00	0.02	0.02	0.02
	HBR/night	0.31	7.44	3.88	9.88	23.00	14.25
	EIR/night	0.00	0.00	0.00	0.19	0.38	0.25
	EIR/month	0.00	0.00	0.00	5.63	11.25	7.50
Toucountouna	Thorax	2	NA	2	52	37	89
	Thorax +	0	NA	0	8	1	9
	SI	0.00	NA	0.00	0.15	0.03	0.10
	HBR/night	0.13	NA	0.13	3.25	4.63	3.71
	EIR/night	0.00	NA	0.00	0.50	0.13	0.38

Districts	Parameters	February 2020	March 2020	Dry season	June 2020	August 2020	Rainy season
	EIR/month	0.00	NA	0.00	15.00	3.75	11.25
Boukoumbe	Thorax	1	30	31	81	477	558
	Thorax +	0	2	2	6	43	49
	SI	0.00	0.07	0.06	0.07	0.09	0.09
	HBR/night	0.06	1.88	0.97	5.06	59.63	23.25
	EIR/night	0.00	0.13	0.06	0.38	5.38	2.04
	EIR/month	0.00	3.75	1.88	11.25	161.25	61.25
Pehunco	Thorax	31	NA	31	371	200	571
	Thorax +	0	NA	0	20	14	34
	SI	0.00	NA	0.00	0.05	0.07	0.06
	HBR/night	1.94	NA	1.94	23.19	25.00	23.79
	EIR/night	0.00	NA	0.00	1.25	1.75	1.42
	EIR/month	0.00	NA	0.00	37.50	52.50	42.50
IRS Withdrawal districts (NTBP)	Thorax	39	149	188	662	898	1560
	Thorax +	0	2	2	37	61	98
	SI	0.00	0.01	0.01	0.06	0.07	0.06
	HBR/night	0.61	4.66	1.96	10.34	28.06	16.25
	EIR/night	0.00	0.06	0.02	0.58	1.91	1.02
	EIR/month	0.00	1.88	0.63	17.34	57.19	30.63
IRS district (Copargo)	Thorax	1	19	20	254	75	329
	Thorax +	0	1	1	3	2	5
	SI	0.00	0.05	0.05	0.01	0.03	0.02
	HBR/night	0.06	1.19	0.63	15.88	9.38	13.71
	EIR/night	0.00	0.06	0.03	0.19	0.25	0.21
	EIR/month	0.00	1.88	0.94	5.63	7.50	6.25

NA: Not applicable; **Dry season:** February and March; **Rainy season:** June and August; **Thorax:** mosquito thoraces tested; **Thorax +:** mosquito thoraces positive; **HBR:** human biting rate

Table 12. *Monthly sporozoïte index (SI), human biting rate (HBR), and entomological inoculation rate (EIR) of* An. gambiae *s.l. indoors and outdoors in IRS withdrawal areas (NTBP) vs IRS area (Copargo)*

Districts	Location	Indicators	February 2020	March 2020	June 2020	August 2020	Dry season	Rainy season
Natitingou	Inside	Total tested	2	43	58	94	45	152
		nb head Thorax +	0	0	1	2	0	3
		SI	0.00	0.00	0.02	0.02	0.00	0.02
		HBR/night	0.25	5.38	7.25	23.50	2.81	12.67
		EIR/night	0	0	0.13	0.50	0.00	0.25
		EIR/month	0	0	3.75	15.00	0.00	7.50
	Outside	Total tested	3	76	100	90	79	190
		nb head thorax +	0	0	2	1	0	3
		SI	0.00	0.00	0.02	0.01	0.00	0.02
		HBR/night	0.38	9.50	12.50	22.50	4.94	15.83

Districts	Location	Indicators	February 2020	March 2020	June 2020	August 2020	Dry season	Rainy season
		EIR/night	0.00	0.00	0.25	0.25	0.00	0.25
		EIR/month	0.00	0.00	7.50	7.50	0.00	7.50
Toucountouna	Inside	Total tested	1	NA	33	18	1	51
		nb head Thorax +	0	NA	4	0	0	4
		SI	0	NA	0.12	0.00	0.00	0.08
		HBR/night	0.13	NA	4.13	4.50	0.13	4.25
		EIR/night	0.00	NA	0.50	0.00	0.00	0.33
		EIR/month	0.00	NA	15.00	0.00	0.00	10.00
	Outside	Total tested	1	NA	19	19	1	38
		nb head Thorax +	0	NA	4	1	0	5
		SI	0.00	NA	0.21	0.05	0.00	0.13
		HBR/night	0.13	NA	2.38	4.75	0.13	3.17
		EIR/night	0.00	NA	0.50	0.25	0.00	0.42
		EIR/month	0.00	NA	15.00	7.50	0.00	12.50
Copargo (control)	Inside	Total tested	0	11	144	44	11	188
		nb head Thorax +	0	0	3	0	0	3
		SI	NA	0.00	0.02	0.00	0.00	0.02
		HBR/night	0.00	1.38	18.00	11.00	0.69	11.75
		EIR/night	NA	0.00	0.38	0.00	0.00	0.19
		EIR/month	NA	0.00	11.25	0.00	0.00	5.63
	Outside	Total tested	1	8	110	31	9	141
		nb head Thorax +	0	1	0	2	1	2
		SI	0.00	0.13	0.00	0.06	0.11	0.01
		HBR/night	0.13	1.00	13.75	7.75	0.56	8.81
		EIR/night	0.00	0.13	0.00	0.50	0.06	0.13
		EIR/month	0.00	3.75	0.00	15.00	1.88	3.75

NA: Not applicable; **Dry season:** February and March; **Rainy season:** June and August; **Thorax:** mosquito thoraces tested; **Thorax +:** mosquito thoraces positive; **HBR:** human biting rate

Table 13. *Monthly sporozoïte index (SI), human biting rate (HBR), and entomological inoculation rate (EIR) of An. gambiae s.l. indoors and outdoors in the IRS withdrawal areas (NTBP) vs the IRS area (Copargo)*

Districts	Location	Indicators	February 2020	March 2020	June 2020	August 2020	Dry season	Rainy season
Boukoumbe	Inside	Total tested	1	18	37	266	19	303
		nb head Thorax +	0	1	4	18	1	22
		SI	0.00	0.06	0.11	0.07	0.05	0.07
		HBR/night	0.13	2.25	4.63	66.50	1.19	25.25
		EIR/night	0.00	0.13	0.50	4.50	0.06	1.83
		EIR/month	0.00	3.75	15.00	135.00	1.88	55.00
	Outside	Total tested	0	12	44	211	12	255
		nb head thorax +	0	1	2	25	1	27
		SI	NA	0.08	0.05	0.12	0.08	0.11
		HBR/night	0	1.50	5.50	52.75	0.75	21.25
		EIR/night	NA	0.13	0.25	6.25	0.06	2.25
		EIR/month	NA	3.75	7.50	187.50	1.88	67.50
Ouassa-Pehunco	Inside	Total tested	10	NA	194	132	10	326
		nb head Thorax +	0	NA	6	10	0	16
		SI	0.00	NA	0.03	0.08	0.00	0.05
		HBR/night	1.25	NA	24.25	33.00	1.25	27.17
		EIR/night	0.00	NA	0.75	2.50	0.00	1.33
		EIR/month	0.00	NA	22.50	75.00	0.00	40.00
	Outside	Total tested	21	NA	177	68	21	245
		nb head Thorax +	0	NA	14	4	0	18
		SI	0.00	NA	0.08	0.06	0.00	0.07
		HBR/night	2.63	NA	22.13	17.00	2.63	20.42
		EIR/night	0.00	NA	1.75	1.00	0.00	1.50
		EIR/month	0.00	NA	52.50	30.00	0.00	45.00
Copargo (control)	Inside	Total tested	0	11	144	44	11	188
		nb head thorax +	0	0	3	0	0	3
		SI	NA	0.00	0.02	0.00	0.00	0.02
		HBR/night	0.00	1.38	18.00	11.00	0.69	11.75
		EIR/night	NA	0.00	0.38	0.00	0.00	0.19
		EIR/month	NA	0.00	11.25	0.00	0.00	5.63
	Outside	Total tested	1	8	110	31	9	141
		nb head Thorax +	0	1	0	2	1	2
		SI	0.00	0.13	0.00	0.06	0.11	0.01
		HBR/night	0.13	1.00	13.75	7.75	0.56	8.81
		EIR/night	0.00	0.13	0.00	0.50	0.06	0.13
		EIR/month	0.00	3.75	0.00	15.00	1.88	3.75

NA: Not applicable; **Dry season:** February and March; **Rainy season:** June and August; **Thorax:** mosquito thoraces tested; **Thorax +:** mosquito thoraces positive; **HBR:** human biting rate

1.3.6 Multiple insecticide resistance mechanisms in An. gambiae s.l. (Kdr, Ace-1) in IRS withdrawal areas (NTBP)

Data presented in tables 14, 15, 16, and 17 show the frequency and distribution of *kdr* L1014F and *Ace 1* resistance genes among *An. gambiae* complex species collected. *Kdr* mutation was detected in high frequency (85.59%) in all species of the *An. gambiae* complex within all the localities. It was significantly higher in Boukoumbe, Toucountouna, and Pehunco compared to Copargo (IRS district) (Table 14). *Kdr* varied from 53.38% in *An. coluzzii* to 100% in *An. arabiensis* (Table 15).

Table 14. *Frequencies Kdr L1014F of An. gambiae s.l. in the IRS withdrawal areas (NTBP)*

Localities	Number tested	RR	RS	SS	Freq. L1014F (%)	P-value
Copargo (Control)	80	52	16	12	75.00	-
Natitingou	454	307	67	80	75.00	1.00
Toucountouna	80	67	6	7	87.50	0.006
Boukoumbé	277	240	17	20	89.71	<0.001
Pehunco	554	481	37	36	90.16	<0.001
IRS Withdrawal districts	1365	1095	127	143	84.87	0.001
IRS districts (Coprago)	80	52	16	12	75.00	-

Table 15. *Distribution of Knock-down resistance (Kdr) frequencies between malaria vectors and localities*

Localities	Species	Number tested	RR	RS	SS	Freq. L1014F (%)
Natitingou	*An. gambiae*	302	243	30	29	85.43
	An. coluzzii	148	61	36	51	53.38
	An. arabiensis	1	1	0	0	100.00
	An. gam/col	3	2	1	0	83.33
Toucountouna	*An. gambiae*	72	61	6	5	88.89
	An. coluzzii	8	6	0	2	75.00
Boukoumbe	*An. gambiae*	251	225	12	14	92.03
	An. coluzzii	26	15	5	6	67.31
Pehunco	*An. gambiae*	510	453	30	27	91.76
	An. coluzzii	37	22	6	9	67.57
	An. arabiensis	6	5	1	0	91.67
	An. gam/col	1	1	0	0	100.00

SS = homozygous susceptible; **RS** = hybrid resistant and susceptible; **RR** = homozygous resistant; **F** = Frequency; ***An. gam/col*** = An*. gambiae/coluzzii*

The ace-1R mutation associated with carbamates and organophosphate resistance was identified in all sites but with very low frequencies (3.32%). It varied from 2.99 to 3.63% in *An. gambiae*, from 0 to 5.07% in *An. coluzzii* but zero in *An. arabiensis* and hybrids *An. coluzzii/gambiae* (Table 16 and 17).

Table 16. *Frequencies G119S Ace-1 of An. gambiae s.l. in IRS withdrawal districts*

Localities	Number tested	RR	RS	SS	Freq. G119S (%)	P-value
Copargo (Control)	80	0	5	75	3.13	-
Natitingou	454	0	35	419	3.85	0.824
Toucountouna	80	0	5	75	3.13	1.00
Boukoumbe	277	0	15	262	2.71	0.992
Pehunco	554	0	40	514	3.61	0.935
IRS withdrawal districts	1365	0	95	1270	3.32	0.987
IRS districts (Coprago)	80	0	5	75	3.13	-

SS = homozygous susceptible; **RS** = hybrid resistant and susceptible; **RR** = homozygous resistant; **F** = Frequency; ***An. gam/col*** = An. *gambiae/coluzzii*

Table 17. *Distribution of Ace-1 frequencies between malaria vectors and localities*

Localities	Species	Number tested	RR	RS	SS	Freq. G119S (%)
Natitingou	*An. gambiae*	302	0	20	282	3.31
	An. coluzzii	148	0	15	133	5.07
	An. arabiensis	1	0	0	1	0.00
	An. gam/col	3	0	0	3	0.00
Toucountouna	*An. gambiae*	72	0	5	67	3.47
	An. coluzzii	8	0	0	8	0.00
Boukoumbe	*An. gambiae*	251	0	15	236	2.99
	An. coluzzii	26	0	0	26	0.00
Pehunco	*An. gambiae*	510	0	37	473	3.63
	An. coluzzii	37	0	3	34	4.05
	An. arabiensis	6	0	0	6	0.00
	An. gam/col	1	0	0	1	0.00

SS = homozygous susceptible; **RS** = hybrid resistant and susceptible; **RR** = homozygous resistant; **F** = Frequency; ***An. gam/col*** = An. *gambiae/coluzzii*

1.4 Conclusion

After the IRS withdrawal from the Atacora districts, entomological monitoring made it possible to follow the evolution of entomological indicators of malaria transmission in this region. The results showed a significant increase in all indicators during the rainy season (June-August 2020) compared to the dry season (February-March 2020). This rebound of malaria transmission was also observed by comparing the entomological indicators in IRS withdrawal areas with those in the control area (Copargo) currently under IRS. It is recommended that new types of nets (i.e. PBO or dual AI ITNs) are deployed in these areas to address the high entomological transmission indicators.

1.5 Difficulties encountered

Mosquitoes were not collected in the two control areas (Toucountouna and Pehunco) in March 2020 due to the restrictive measures taken by the government to limit the spread of COVID-19, these measures were taken at the time of mosquito collection in these two localities.

2 Part II: Monitoring & Evaluation of the efficacy of the fourth year of Indoor Residual Spraying (IRS) in Alibori and Donga, northern Benin, West Africa

2.1 Background

With the support of the USAID/PMI, Benin's National Malaria Control Program (NMCP) has been extending indoor residual spraying (IRS) in the Alibori and Donga departments in northern Benin since 2017. Implementation of IRS in Alibori and Donga regions since 2017 was accompanied by a drastic reduction in Entomological Inoculation Rate (EIR), but the residual activity of the Actellic 300 CS used was short[14].

After three years of IRS with Actellic 300 CS, in April 2020, IRS was renewed in 6 districts: Donga region (Djougou, Copargo, and Ouake); Alibori region (Kandi, Gogounou, and Segbana) with Fludora ® Fusion, a new-generation insecticide with two different modes of action. This final report shows the results of the IRS entomological monitoring conducted in both regions. Thus, the impact of the strategy on key entomological indicators of malaria transmission as well as, the residual efficacy duration of Fludora ® Fusion on the different types of sprayed walls were evaluated.

The main objective of this evaluation was to collect data on mosquito behavior and malaria transmission in IRS districts and compare the results with those obtained in control districts (Bembereke and Bassila) during the pre-IRS period or baseline period (February to March 2020) and post-IRS period (June to December 2020).

To better assess the IRS impact on malaria transmission, we compared the entomological indicators not only between treated and control (untreated) areas but also between the pre-IRS period (February- March 2020) and post-IRS period (June –December 2020).

February to December 2020, we proceeded with mosquito sampling to cross the nighttime socio-behavioral surveillance data of the household members (activity 4) with those of entomological parameters, for the pre-IRS period (February- March 2020) (dry season) (hot period) (February- March 2020) and post-IRS (June- December 2020) in northern Benin. For this reason, mosquito sampling was organized from 7:00 pm to 7:00 am. The *Anopheles* samples collected in February and March 2020 were not sufficient to make the correlation we expected. Indeed, we pooled the mosquitoes collected every hour for the whole of the treated and control areas to determine the trend in the hourly human biting

[14] Salako AS, Dagnon F, Sovi A, Padonou GG, Aïkpon R, Ahogni I, Syme T, Govoétchan R, et al. Efficacy of Actellic 300 CS-based indoor residual spraying on key entomological indicators of malaria transmission in Alibori and Donga, two regions of northern Benin. Parasit Vectors 2019. 12:612

rate for *Anopheles gambiae* (s.l.). However, pre-IRS period (March 2020), data were not collected in the two control areas (Bassila and Bembereke) due to the restrictive measures taken by the government to limit the spread of COVID-19, these measures were taken at the time of mosquito collection in these two localities.

2.2 Material and methods

2.2.1 Study areas

The map below (Fig. 7) shows the two health zones (HZ) that will be protected by IRS in April 2020:

- HZ Djougou, Copargo, Ouake (Donga region)
- HZ Kandi, Gogounou, Segbana (Alibori region)

A total of 6 districts were used for entomological monitoring and evaluation (M&E) of the IRS intervention:

M&E sites:

- In the Donga department – the districts of Djougou and Copargo
- In the Alibori department – the districts of Kandi and Gogounou

Control sites:

- Bembereke district was selected because it was the closest to the district receiving IRS in Alibori department.
- Bassila district was selected because it was the closest to the district receiving IRS in Donga department.

However, mosquito collections were not conducted in March 2020 (pre-IRS period) in the two control areas (Bassila and Bembereke) and in all sites in May, September, October, and November 2020 for two main reasons:

- ✔ Restrictive measures were taken by the government to limit the spread of COVID-19, these measures were taken in March 2020 at the time of mosquito collection in some localities.
- ✔ Delay in the acquisition of funding until the end of the contract in September.

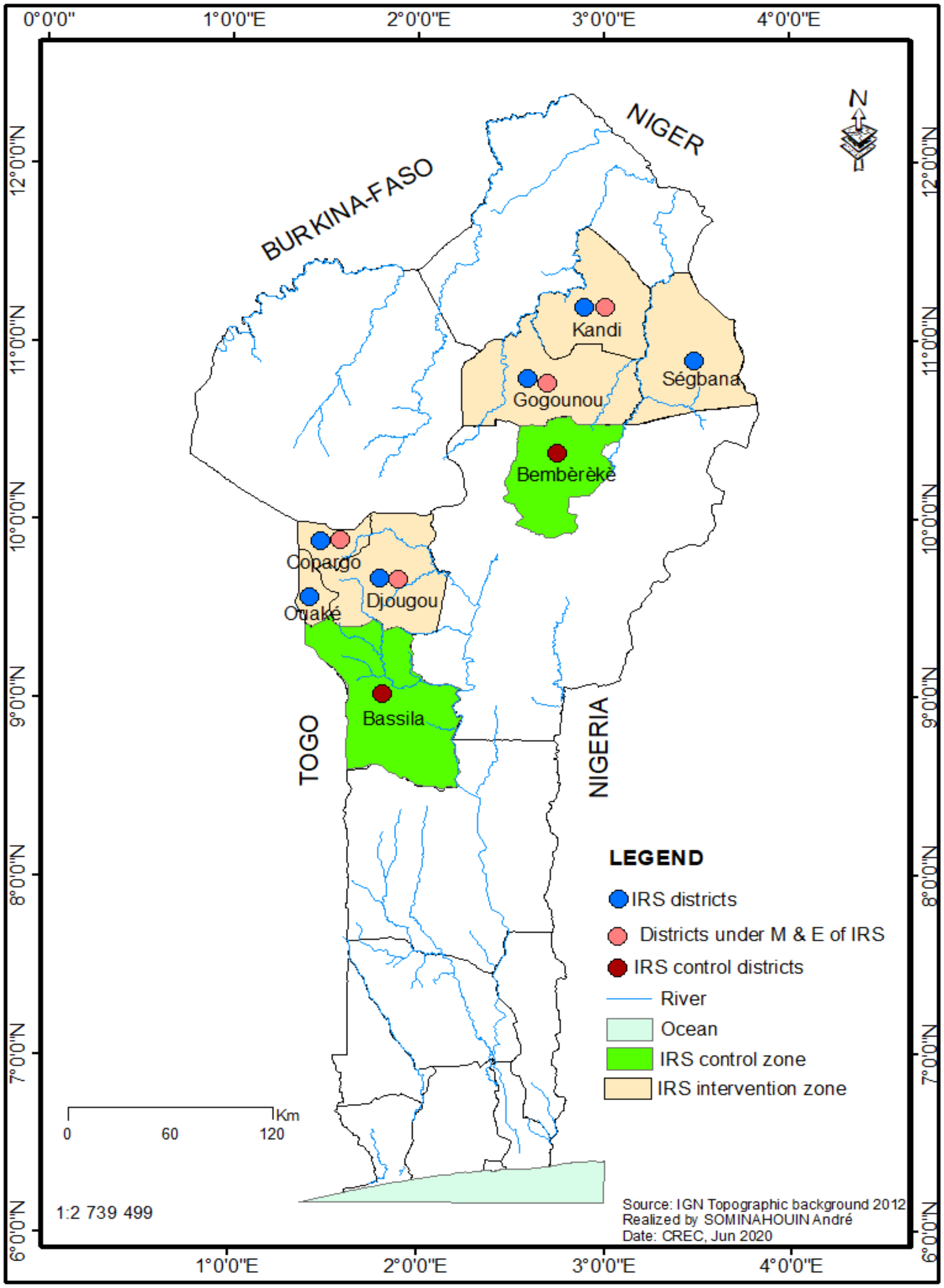


Figure 7. *Map showing the IRS evaluation areas*

The climate in the sites is Sudano-Guinean in the Donga and Sudanese in the Alibori regions. These two regions are dry savannah areas, with six months rainy season (mid-April to mid-October) and a dry season that spans the remainder of the year. Overall, average annual rainfall ranges between 700–1200 mm in Alibori and 1200–1300 mm in Donga.

Figure 8. shows the rainfall and temperature trends in the Alibori and Donga regions. The rainiest months are from June to October in both regions with the rainfall peak occurring in August.

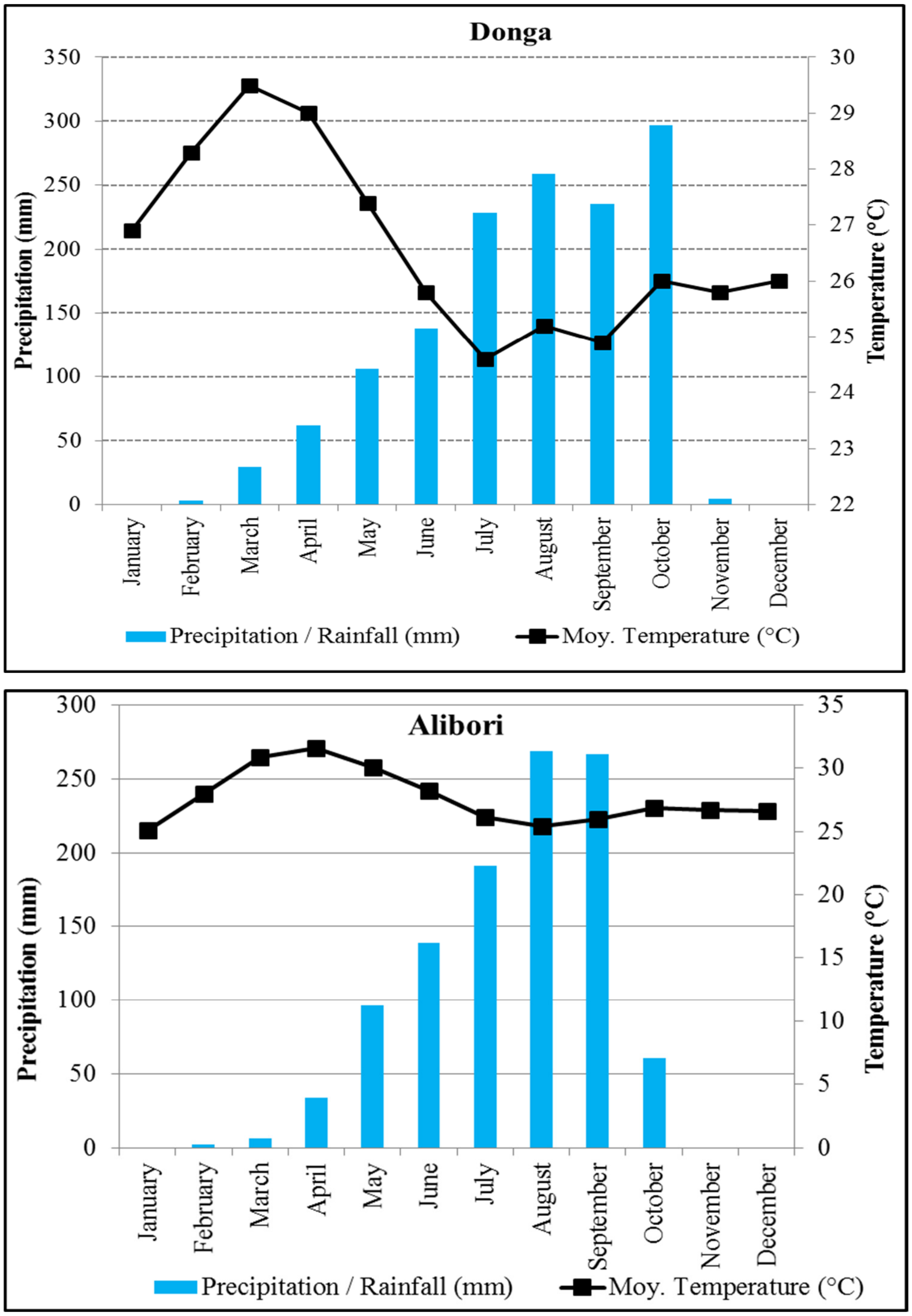


Figure 8. *Monthly climate data in the Alibori and Donga regions* [15].

[15] Climate data from World Bank Group, Climate Change Knowledge Portal (https://climateknowledgeportal.worldbank.org/country/benin/climate-data-historical)

2.2.2 Indicators measured

Activities planned between February and December 2020 provided data and information about the following entomological indicators required by PMI and the NMCP (Table 18):

- Efficacy control of the spraying: cone/wall bioassay.
- Residual activity of Fludora ® Fusion
- Mosquito composition
- Vector identification (species and molecular forms of *Anopheles gambiae*)
- Vector density
- Mosquito behavior: biting (endophagy or exophagy) and resting (endophily or exophily)
- Entomological Inoculate Rate (EIR)
- Vector resistance to insecticides and resistance mechanisms
- Altered target-site resistance: knockdown resistance (Kdr), acetylcholinesterase (Ace-1)

Table 18. *Summary of indicators by collection method*

Collection method	Indicator	Definition
PSC	Indoor resting density	# mosquitoes / house / day
	% of fed females	# fed mosquitoes / Total collected by PSC
HLC	HBR	# bites / person / night
	Parity rate	Percentage of parous mosquitoes
	Exophagic rate	Percentage of mosquitoes biting outside
	Endophagic rate	Percentage of mosquitoes biting inside

2.2.3 The WHO wall bioassays

A laboratory colony of *An. gambiae* s.s Kisumu strain which is fully susceptible to all insecticides was used for the bioassays. Among the districts chosen for the entomological monitoring of the 2020 IRS campaign, Djougou and Copargo were selected to evaluate the persistence of Fludora ® Fusion on cement and mud smooth walls. Sprayed houses were selected randomly. WHO wall bioassays[16] were conducted on treated walls of 40 houses randomly selected (20 treated houses/district) seven days (T0) after April 2020 IRS campaigns in Djougou and Copargo. This test allowed us to assess the quality of treatment in both districts. Every month, residual activity monitoring was carried out in the treated districts. This test allowed us to evaluate the persistence of the insecticide used on the wall surface. Using a mouth aspirator, 15 females of *An. gambiae* Kisumu aged 2–5 days old were carefully introduced into each cone, fixed at

[16] World Health Organization 2006 Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets(https://www.who.int/whopes/resources/who_cds_ntd_whopes_gcdpp_2006.3/en/)

four different heights (0.5 m; 1.0 m; 1.5 m; 2.0 m) of the treated walls. Mosquitoes were exposed to the sprayed walls for 30 min; then removed from the cones, transferred to labeled sterile cups, and provided with 10% sugar solution. Respectively, five cement walls and five smooth mud walls were selected in each of the four villages. Four untreated walls (02 cement and 02 mud walls) were also selected for control. Mosquitoes exposed to untreated wall surfaces were used as controls (Fig. 9). Based on the delayed effect of clothianidin on mosquito mortality as demonstrated in previous studies[17], mortality was recorded every 24 hours up to 120 hours post-exposure and observation at a temperature of 27 ± 2 °C and relative humidity of 80 ± 10 %. When the control mortality was between 5–20%, corrected mortality was calculated using Abott''s formula[18]. If mortality in the controls was >20%, the assay was repeated. If mortality was <5% no correction was needed.

Bioassays on the walls were done at the following time points:

- T0: 7 days after the spraying date (1week) (April 2020);
- T1: 1 month after the spraying date (May 2020);
- T2: 2 months after the spraying date (June 2020);
- T3: 3 months after the spraying date (July 2020);
- T4: 4 months after the spraying date (August 2020);
- T5: 5 months after the spraying date (September 2020);
- T6: 6 months after the spraying date (October 2020);
- T7: 7 months after the spraying date (November 2020);
- T8: 8 months after the spraying date (December 2020).

[17] Ngufor C, Fongnikin A, Rowland M, N'Guessan R. Indoor residual spraying with a mixture of clothianidin (a neonicotinoid insecticide) and deltamethrin provides improved control and long residual activity against pyrethroid resistant Anopheles gambiae s.l. in Southern Benin. PLoS One. 2017;12:e0189575.

[18] Abbott WSA. Method of computing of insecticide effectiveness. J Econ Entomol. 1925; 18:265–7.

Figure 9. *Exposure for 30 minutes to cement and mud walls treated with Fludora ® Fusion mortality reading after respectively 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours of observation*

2.2.4 Adult mosquito collections

Mosquito sampling was conducted in 6 districts selected for IRS M&E: Djougou, Copargo, Kandi, and Gogounou under IRS and 2 controls (Bassila and Bembereke).

Mosquitoes were collected by human landing catch (HLCs) in two villages per district, with one village located in the center of the district, and one village located at the periphery. Table 2 shows the mosquito sampling sites and their geographic coordinates. For each village, mosquitoes were collected in 2 houses by 4 mosquito collectors, 2 mosquito collectors indoors, and 2 outdoors. In total, 48 local mosquito collectors were used for one round of collection. Two rounds of sampling were done per month. Two teams of four mosquito collectors in each village worked inside and outside the selected dwellings, from 19:00 to 00:00 hours (7:00 PM to 12:00 AM) for the first team and from 00:00 to 07:00 hours (12:00 AM to 7:00 PM) for the second team. Mosquito collectors rotated through the different dwellings to avoid biases related to their trapping ability or individual attractiveness. The collection was done with the hemolysis tubes.

To estimate the indoor resting density of mosquitoes per room, 10 houses per village were selected[19]. The bedrooms were sprayed with pyrethrum (mixed with water) and a white canvas was placed on the floor to collect knocked-down mosquitoes. After 15 minutes, all knocked-down mosquitoes were collected from the floor and placed in Petri dishes to determine the number of mosquitoes in the room and to determine the blood-feeding stage (unfed, fed, half-gravid, and gravid).

Vector species that were collected and identified were transported to the Centre de Recherche Entomologique de Cotonou (CREC) laboratory for ovary dissection using a microscope to determine the parous rates. The heads/thoraxes of the vector species were analyzed by the ELISA method to look for CSP antigens. Abdomens of female vector species were used for PCR analyses to identify sibling species and molecular forms.

The central urban sites of Djougou and Kandi were not treated in 2020, due to the refusal of intervention observed in these large cities (Table 19).

Table 19. *Mosquito sampling sites and their geographic coordinates.*

District	Village	Treated or untreated site in April 2020	Latitude/Longitude
Djougou	Taïfa	Untreated	09°41′39″N, 01°41′57″E
	Serou	Treated	09°39′59″N, 01°41′57″E
Copargo	Toungouli	Treated	09°50′19″N, 01°32′59″E
	Fowa	Treated	09°53′49″N, 01°32′59″E

[19] These houses were different from the houses used in the HLC collection

District	Village	Treated or untreated site in April 2020	Latitude/Longitude
Kandi	Gansosso	Untreated	11°07’51”N, 2°55’36”E
	Koffoïssa	Treated	11°14’55”N, 2°59’55”E
Gogounou	Gbanin	Treated	10°50’06”N, 2°50’10”E
	Bantansouè	Treated	10°55’05”N, 2°51’33”E
Bembereke	Bembereke centre	Untreated	10°13’30”N, 02°40’05”E
	Gamia	Untreated	10°18’05.78”N, 03°29’11”E
Bassila	Bassila I	Untreated	9°12'54.7"N 1°58'07.7"E
	Penessoulou	Untreated	9°20'24.05"N 2°37'40.1"E

2.2.5 Mosquito identification and processing

2.2.5.1 Morphological identification of vectors species

After each collection, mosquitoes were counted and morphologically identified using the taxonomic key of Gillies & Meillon[20]. All *Anopheles* vectors captured through HLC were dissected to assess their physiological age[21]. Each specimen was then stored in a labeled Eppendorf tube containing silica gel and cotton for further molecular analyses.

2.2.5.2 Molecular analyses

Vector species identified were transported to the Centre de Recherche Entomologique de Cotonou (CREC) to detect the presence of *P. falciparum*. The heads/thoraxes of all females *An. gambiae* (*s.l.*) were analyzed by ELISA CSP according to the protocol described by Wirtz et al[22]. The abdomens, legs, and wings of specimens of *An. gambiae* (*s.l.*) captured through HLC was analyzed by PCR according to the protocol of Santolamazza et al[23], for molecular species identification. The same mosquitoes were genotyped for

[20] Gillies MT, De Meillon B. The Anophelinae of Africa south of the Sahara. S Afr Inst Med Res. 1968;54:1–343.

[21] Detinova TS, Gillies MT. Observations on the determination of the age composition and epidemiological importance of populations of *Anopheles gambiae* Giles and *Anopheles funestus* Giles in Tanganyika. Bull World Health Organ. 1964;30:23–8.

[22] Wirtz RA, Zavala F, Charoenvit Y, Campbell GH, Burkot TR, Schneider I, Esser KM, Beaudoin RL, Andre RG. Comparative testing of monoclonal antibodies against *Plasmodium falciparum* sporozoites for ELISA development. Bull World Health Organ. 1987; 65(1): 39-45.

[23] Santolamazza F, Mancini E, Simard F, Qi Y, Tu Z, della Torre A. Insertion polymorphisms of SINE200 retrotransposons within speciation islands of *Anopheles gambiae* molecular forms. Malar J. 2008;7:163.

the *kdr* L1014F, *kdr* L1014S, and G119S *Ace-1* mutations, according to the protocols of Martinez-Torres et al[24], Ranson et al[25] and Weill et al[26], respectively.

2.2.6 Species identification and insecticide susceptibility testing

Anopheles gambiae s.l. larvae were collected from natural breeding sites during the rainy seasons in districts under IRS (Djougou, Kandi, and Gogounou). The mosquito larvae collected were transported in well-labeled plastic bottles to the CREC insectary where they were maintained at 27 ± 2° C and 72 ± 5% relative humidity. The larvae were morphologically identified and separated for rearing. Adults obtained were provided with a 10% sugar solution on cotton wool. Unfed 2-5-day old *An. gambiae* s.l. adults were used for the WHO susceptibility test using various classes of insecticides. The susceptibility status of the population was graded according to the WHO protocol.

- Bioassays with a mortality rate between 98–100%, the mosquito population was considered susceptible to the tested insecticide.
- Bioassays with a mortality rate between 90–97%, the mosquito population was suspected of being resistant to the tested insecticide.
- Bioassays with a mortality rate below 90%, the mosquito population was considered resistant to the tested insecticide.

2.2.7 Data analysis

Data were analyzed with the statistical R software, version 2.8. using the stats package[27]. The Chi-square test of comparison of proportions was also used to determine if there was an association between the areas receiving IRS and the following indicators: proportion of *An. gambiae* (s.l.) indoors and outdoors, blood-feeding rate, sporozoite index, parity rate of *An. gambiae* (*s.l.*) and allelic frequencies of kdr L1014F and G119S Ace-1. The Poisson test[28] was used to estimate the risk ratio (RR) and compare the confidence intervals of indoor vector density and EIRs of *An. gambiae* (s.l.) between treated areas.

[24] Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, Devonshire AL, Guillet P, Pasteur N. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol.1998; 7: 179–184.
[25] Ranson H, Jensen B, Vulule J, Wang X, Hemingway J, Collins F. 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.
[26] Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M, Raymond M. The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. *Insect* Mol Biol. 2004; 13: 1–7.
[27] R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (2018). Available online at https://www.R-project.org/.
[28] Rothman KJ. Epidemiology: an introduction. Oxford: Oxford University Press; 2012.

We calculated the % reduction in EIR in IRS areas compared to control areas using Mulla's formula[29]:

$$\%R = 100 - [(\frac{C_1}{T_1}) \times (\frac{T_2}{C_2})] \times 100$$

where C_1 = pre-treatment EIR in unsprayed control area, C_2 = post-treatment EIR in unsprayed control area, T_1 = pre-treatment EIR in the sprayed area, and T_2 = post-treatment EIR in sprayed area.

2.3 Results

2.3.1 Residual effect of Fludora ® Fusion in-wall bioassays (2020 IRS campaign).

Fludora ® Fusion decay rates on treated cement and mud walls were monitored for 8 months after the 2020 IRS campaign. At T0 (1 week) (April 2020; 07 days after treatment of the walls), there was 100% mortality in *An. gambiae* s.s. Kisumu strain exposed to Fludora ® Fusion-treated walls irrespective of wall type (cement or mud) and wall height (Tables 20 & 21; Fig. 10 & 11). This suggests good quality of the treatment and the availability of the insecticide's lethal dose on the walls. Eight months after IRS 2020, mortality rates of susceptible mosquitoes were approximately 80% (WHO efficacy threshold) after 24 hours of observation on both cement and mud smooth walls in Djougou and Copargo (Tables 20 & 21; Figs. 10 & 11). However, there was an increase in mortality rates during the different observation periods, demonstrating the delayed lethal effect of Fludora ® Fusion as a function of time. This mortality varied between 80 and 82% after 24 hours of observation versus 95% and 97% after 120 hours of observation, eight months after the 2020 IRS campaign.

The detailed results showing the killing effect of Fludora ® Fusion against Kisumu by time are provided (Tables 20 and 21).

[29] Mulla MS, Norland RL, Fanara DM, Darwezeh HA, McKean DW. 1971. Control of chironomid midges in recreational lakes. J. Econ. Entomol. 64:300–307.

Table 20. *Efficacy of Fludora ® Fusion in Djougou represented by the 30-min knock-down (KD) rate and mortality rate (Mort.) after exposure to smooth cement and smooth wall substrate*

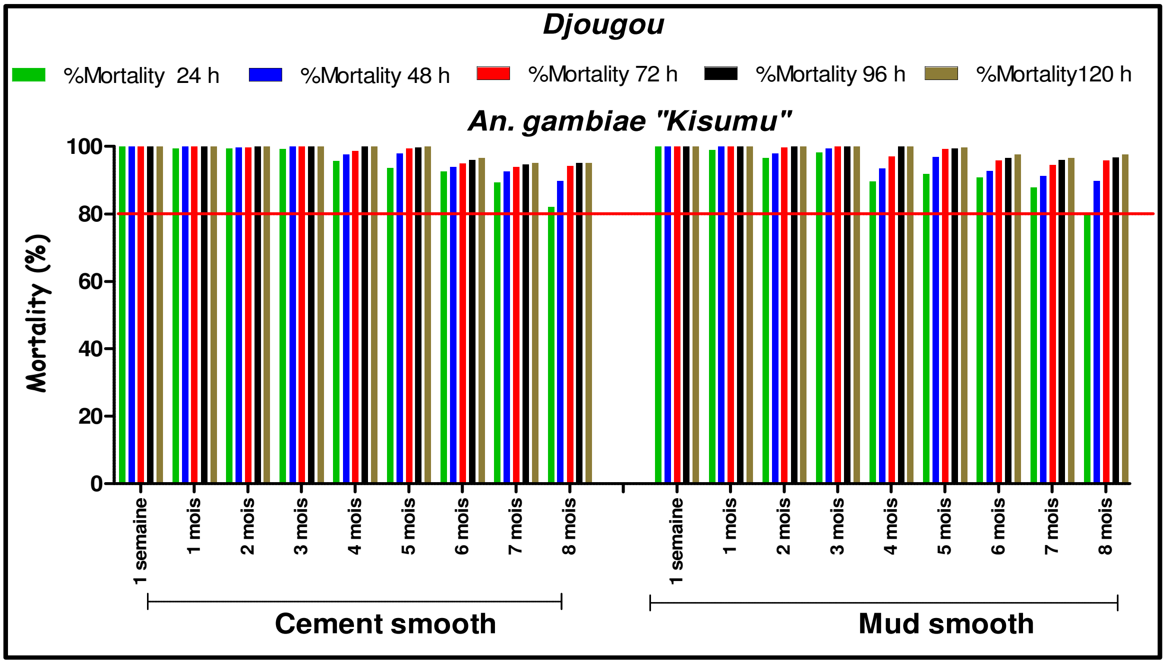
Type of walls	Period after IRS	%KD 30 min (n)	%Mort. 24 h (n)	%Mort. 48 h (n)	%Mort. 72 h (n)	%Mort. 96 h (n)	%Mort. 120h (n)	Total tested
Smooth cement	1 wk.	97.57 (441)	100 (452)	100 (452)	100 (452)	100 (452)	100 (452)	452
	1 mo.	84.59 (313)	99.46 (368)	100 (370)	100 (370)	100 (370)	100 (370)	370
	2 mos.	48.07 (224)	99.36 (463)	99.79 (465)	99.79 (465)	100 (466)	100 (466)	466
	3 mos.	34.96 (158)	99.34 (449)	100 (452)	100 (452)	100 (452)	100 (452)	452
	4 mos.	31.86 (151)	95.78 (454)	97.68 (463)	98.73 (468)	100 (474)	100 (474)	474
	5 mos.	12.9 (61)	93.66 (443)	97.89 (463)	99.37 (470)	99.79 (472)	100 (473)	473
	6 mos.	14.41 (69)	92.69 (444)	93.95 (450)	94.99 (455)	96.03 (460)	96.66 (463)	479
	7 mos.	13.74 (65)	89.43 (423)	92.6 (438)	93.87 (444)	94.71 (448)	95.14 (450)	473
	8 mos.	30.1 (62)	82.04 (169)	89.81 (185)	94.17 (194)	95.15 (196)	95.15 (196)	206
Smooth mud	1 wk.	93.69 (401)	100 (428)	100 (428)	100 (428)	100 (428)	100 (428)	428
	1 mo.	77.11 (320)	99.04 (411)	100 (415)	100 (415)	100 (415)	100 (415)	415
	2 mos.	47.66 (214)	96.66 (434)	98 (440)	99.78 (448)	100 (449)	100 (449)	449
	3 mos.	27.95 (128)	98.25 (450)	99.56 (456)	100 (458)	100 (458)	100 (458)	458
	4 mos.	33.15 (182)	89.62 (492)	93.44 (513)	97.09 (533)	100 (549)	100 (549)	549
	5 mos.	16.11 (58)	91.94 (331)	96.94 (349)	99.17 (357)	99.44 (358)	99.72 (359)	360
	6 mos.	20.82 (102)	90.82 (445)	92.86 (455)	95.92 (470)	96.53 (473)	97.76 (479)	490
	7 mos.	23.52 (107)	87.91 (400)	91.43 (416)	94.51 (430)	96.04 (437)	96.7 (440)	455
	8 mos.	20.93 (45)	80 (172)	89.77 (193)	95.81 (206)	96.74 (208)	97.67 (210)	215

KD 30 min: Knock down 30 min; **Mort.:** Mortality; **%:** Percentage

Table 21. *Efficacy of Fludora ® Fusion in Copargo represented by the 30-min knock-down (KD) rate and mortality rate (Mort.) after exposure to smooth cement and smooth wall substrate*

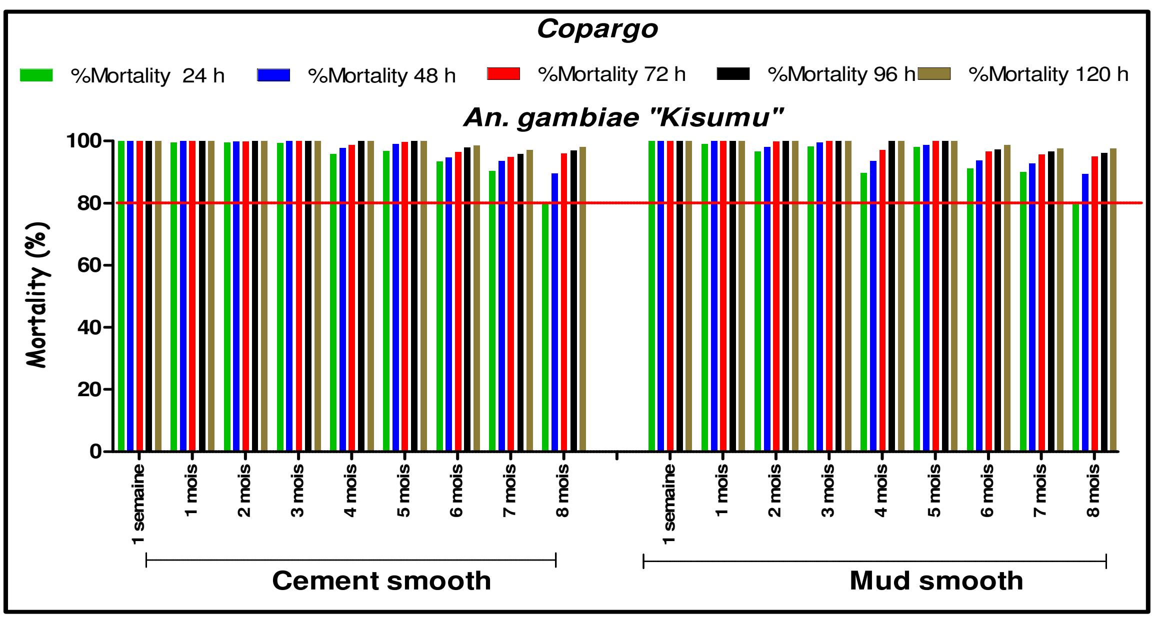
Type of walls	Period after IRS	%KD 30 min (n)	%Mort. 24 h (n)	%Mort. 48 h (n)	%Mort. 72 h (n)	%Mort. 96 h (n)	%Mort. 120h (n)	Total tested
Smooth cement	1 wk.	97.57 (441)	100 (452)	100 (452)	100 (452)	100 (452)	100 (452)	452
	1 mo.	84.59 (313)	99.46 (368)	100 (370)	100 (370)	100 (370)	100 (370)	370
	2 mos.	48.07 (224)	99.36 (463)	99.79 (465)	99.79 (465)	100 (466)	100 (466)	466
	3 mos.	34.96 (158)	99.34 (449)	100 (452)	100 (452)	100 (452)	100 (452)	452
	4 mos.	31.86 (151)	95.78 (454)	97.68 (463)	98.73 (468)	100 (474)	100 (474)	474
	5 mos.	33.33 (103)	96.76 (299)	99.03 (306)	99.68 (308)	100 (309)	100 (309)	309
	6 mos.	40.29 (193)	93.32 (447)	94.78 (454)	96.45 (462)	97.91 (469)	98.54 (472)	479
	7 mos.	13.74 (65)	90.27 (427)	93.45 (442)	94.93 (449)	95.77 (453)	97.04 (459)	473
	8 mos.	25.58 (88)	80.23 (276)	89.53 (308)	95.93 (330)	96.8 (333)	97.97 (337)	344
Smooth mud	1 wk.	93.69 (401)	100 (428)	100 (428)	100 (428)	100 (428)	100 (428)	428
	1 mo.	77.11 (320)	99.04 (411)	100 (415)	100 (415)	100 (415)	100 (415)	415
	2 mos.	47.66 (214)	96.66 (434)	98 (440)	99.78 (448)	100 (449)	100 (449)	449
	3 mos.	27.95 (128)	98.25 (450)	99.56 (456)	100 (458)	100 (458)	100 (458)	458
	4 mos.	33.15 (182)	89.62 (492)	93.44 (513)	97.09 (533)	100 (549)	100 (549)	549
	5 mos.	27.03 (110)	98.03 (399)	98.77 (402)	100 (407)	100 (407)	100 (407)	407
	6 mos.	40.33 (198)	91.24 (448)	93.69 (460)	96.54 (474)	97.35 (478)	98.78 (485)	491
	7 mos.	23.52 (107)	90.11 (410)	92.75 (422)	95.6 (435)	96.7 (440)	97.58 (444)	455
	8 mos.	16.29 (72)	79.64 (352)	89.37 (395)	95.02 (420)	96.15 (425)	97.51 (431)	442

KD 30 min: Knockdown 30 min; **Mort.:** Mortality; **%:** Percentage



The red line indicates the WHO efficacy threshold (mortality of 80%) of an insecticide

Figure 10. *Quality of the spray and residual effect of Fludora ® Fusion 8 months after the 2020 IRS campaign in Djougou.*



The red line indicates the WHO efficacy threshold (mortality of 80%) of an insecticide

Figure 11. *Quality of the spray and residual effect of Fludora ® Fusion 8 months after 2020 IRS campaign in Copargo*

2.3.2 Mosquito composition before and after 2020 IRS campaign

During the five months of mosquito collection (February to December 2020), a total of 18,146 human-biting mosquitoes belonging to four genera (*Anopheles*, *Aedes*, *Culex*, *Mansonia*) and 15 species were collected in IRS and control areas (Table 22). Out of the 15 species, *Anopheles gambiae* s.l. was the second most abundant species collected (29.60% of the total of mosquitoes: 5,372 of 18,146) after *Culex quinquefasciatus* (67.49% of the total of mosquitoes; 12,246 of 18,146) (Table 22). The two major malaria vectors collected were *An. gambie* s.l. and *An. funestus,* albeit at low frequency (0.46%: 84 *An. funestus* /18,146) in this period. *An. nili*, a local vector, was found only in Djougou.

Overall, we noted a high abundance of *An. gambiae* s.l. in the Donga region (Djougou: 50.54%; Copargo: 43.70%; Bassila: 35.10%) than in the Alibori region (Kandi: 21.24%; Gogounou: 17.42%) and Bembereke (21.80%).

The relative abundance of *Culex quinquefasciatus* may be due to the presence of larval habitats polluted (sewers, abandoned wells, and cisterns) with organic matters at this time of year (dry season and beginning of the rainy season) and in urban areas. Such breeding sites are choices of preference for the development of larvae of *Cx. quinquefasciatus*.

Table 22. *Mosquito species composition (February to August 2020).*

Species	Djougou	Copargo	Kandi	Gogounou	Bembereke	Bassila	Total
	% (Nb)	% (Nb)	% (Nb)	% (Nb)	% (Nb)	% (Nb)	
An. gambiae s.l.	50.54(1344)	43.70(610)	21.24(790)	17.42(468)	21.80(882)	35.10(1278)	29.60
An. funestus	1.17(31)	0.29(4)	0.05(2)	0.11(3)	0.02(1)	1.18(43)	0.46
An. nili	0.15(4)	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.02
An. pharoensis	0.00(0)	0.07(1)	0.05(2)	0.00(0)	0.30(12)	0.00(0)	0.08
An. ziemani	0.00(0)	0.00(0)	0.03(1)	0.00(0)	0.00(0)	0.03(1)	0.01
An. coustani	0.00(0)	0.07(1)	0.05(2)	0.00(0)	0.00(0)	0.00(0)	0.02
Cx. quinquefasciatus	45.20(1202)	50.00(698)	76.53(2846)	81.65(2193)	76.93(3112)	60.29(2195)	67.49
Cx. nebulosus	0.68(18)	1.65(23)	0.08(3)	0.26(7)	0.02(1)	0.80(29)	0.45
Cx. descens	0.00(0)	0.00(0)	0.00(0)	0.07(2)	0.07(3)	0.03(1)	0.03
Cx. tigripes	0.00(0)	0.21(3)	0.00(0)	0.00(0)	0.00(0)	0.03(1)	0.02
M. africana	0.98(26)	2.36(33)	1.64(61)	0.22(6)	0.12(5)	2.39(87)	1.20
M. uniformis	0.00(0)	0.14(2)	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.01
A. aegypti	1.28(34)	1.50(21)	0.30(11)	0.22(6)	0.67(27)	0.16(6)	0.58
A. luteocephalus	0.00(0)	0.00(0)	0.03(1)	0.04(1)	0.02(1)	0.00(0)	0.02
A. vitatus	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.02(1)	0.00(0)	0.01
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Nb: number of species; %: percentage

A total of 607 *An. gambiae* s.l. were analyzed by PCR for species identification: 327 (53.87%) were *An. coluzzii* and 280 (46.13%) were *An. gambiae* (p=0.008) (Fig. 12). Both sibling species were present throughout the dry and rainy seasons in both regions (Fig. 12). During the dry season (February to March), *An. coluzzii* predominated (88.61%) in both the Alibori and Donga regions. However, during the rainy season, we observed a predominance of *An. gambiae* (76.07%) in both regions (Fig. 12).

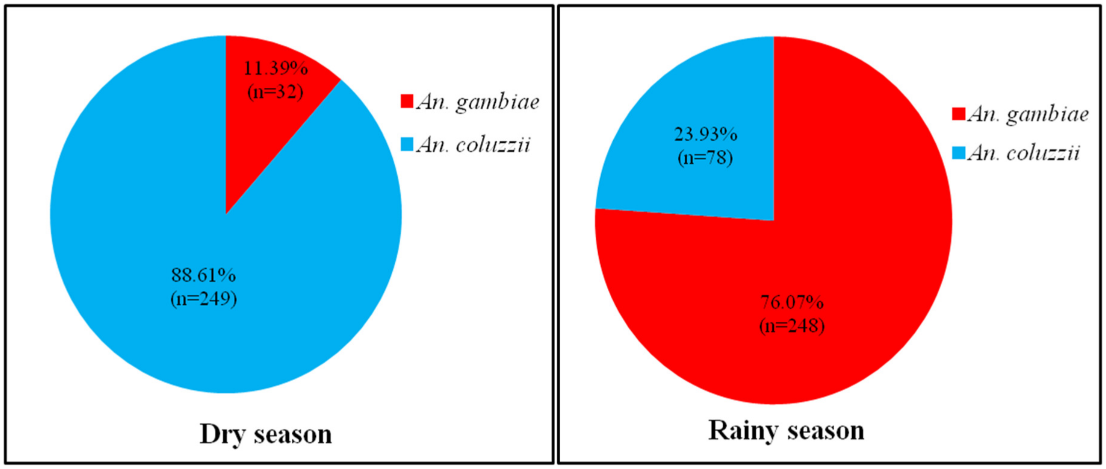


Figure 12. *Distribution of An. gambiae s.l. species in districts under IRS and control per season*

2.3.3 Mosquito blood-feeding behaviors

2.3.3.1 Human Biting Rate (HBR) of *An. gambiae* s.l. indoor versus outdoor in districts

A total of 5,372 *An. gambiae* s.l. were caught from February 2020 to December 2020 in treated districts (Djougou, Copargo, Kandi, and Gogounou) and control areas (Bembereke and Bassila). Table 23 shows the proportion of *An. gambiae s.l.* indoors compared to outdoors in these districts. Before the 2020 IRS campaign (period from February 2020 to March 2020), the density of *An. gambiae s.l.* was low compared to the period from June 2020 to December 2020. During this period (February to March 2020), the proportion of *An. gambiae* s.l. collected is similar indoors and outdoors in some houses designated for IRS treatment and control sites except Bassila (p>0.05). Globally, 51.34% (115/224) of *An. gambiae* s.l. were collected indoors in houses designated for IRS treatment compared to 48.66% (109/224) outdoors (P=0.636). Similarly, in houses designated as controls, 43.55% (27/62) were collected indoors versus 56.45% (35/62) outdoors. After the 2020 IRS campaign (June 2020 to December 2020), the proportion of *An. gambiae s.l* collected was significantly lower indoors compared to outdoors only in treated houses in Djougou, but similar indoors and outdoors in some treated houses in Gogounou and Copargo (Table 23). Similarly, in untreated houses (Bembereke), we recorded the opposite situation with higher biting rates indoors (Table 23), but higher outdoor biting rates were observed in Bassila (control). Globally, 47.86% (1430/2988) of *An. gambiae s.l* was collected indoors in treated houses compared to 52.14% (1558/2988) in outdoors (X^2 = 10.79; *df*=1; *P* =0.001). Similarly, in untreated houses, 48.43% (1016/2098) were collected indoor versus 51.57% (1082/2098) outdoors (X^2 = 4.02; *df*= 1; *P* =0.044) (Table 23).

Tables 24 and 25 below present the details of the biting rate (HBR) of *An. gambiae s.l.* indoors and outdoors in treated districts and control.

Table 23. *Number and proportion of* An. gambiae *s.l. caught indoors and outdoors before IRS intervention (February- March 2020) and after IRS intervention (June-December 2020) in treated and control districts.*

Districts	Pre-2020 IRS period (Feb 2020-Mar 2020)			Post-2020 IRS period (June 2020 - December 2020)		
	Indoors	Outdoors		Indoors	Outdoors	
	nb (%)	nb (%)	p-value	nb (%)	nb (%)	p-value
Djougou	19 (63.33)	11 (36.67)	0.07	557 (42.39)	757 (57.61)	<0.001
Copargo	11 (55.00)	9 (45.00)	0.751	288 (48.81)	302 (51.19)	0.449
Bassila (control)	13 (36.11)	23 (63.89)	0.033	493 (39.69)	749 (60.31)	<0.001
Kandi	53 (50.96)	51 (49.04)	0.889	377 (54.96)	309 (45.04)	<0.001
Gogounou	32 (45.71)	38 (54.29)	0.398	208 (52.26)	190 (47.74)	0.228
Bembereke (control)	14 (53.85)	12 (46.15)	0.781	523 (61.10)	333 (38.90)	<0.001
Total districts under IRS	115 (51.34)	109 (48.66)	0.636	1430 (47.86)	1558 (52.14)	0.001
Total (control)	27 (43.55)	35 (56.45)	0.208	1016 (48.43)	1082 (51.57)	0.044

nb: number of *An. gambiae* s.l.; **%:** proportion of *An. gambiae* s.l.; **p-value:** p-value of comparison of the proportion of *An. gambiae* s.l indoors and outdoors in the same district (Test used: Chi-square test)

Table 24. *Biting rates of An. gambiae s.l. indoor and outdoor in treated districts (Donga) and control (Bassila)*

Districts	**Position**	**Indicator**	**Feb 2020**	**Mar 2020**	**Jun 2020**	**Jul 2020**	**Aug 2020**	**Dec 2020**	**Pre-2020 IRS (Feb-Mar 2020)**	**Post-2020 IRS (Jun-Dec 2020)**
Djougou	Inside	Total Mosquitoes	1	18	274	255	23	5	19	557
		nb human catches	8	8	8	8	4	8	16	28
		HBR/night	0.13	2.25	34.25	31.88	5.75	0.63	1.19	19.89
	Outside	Total Mosquitoes	3	8	364	338	44	11	11	757
		nb human catches	8	8	8	8	4	8	16	28
		HBR/night	0.38	1.00	45.50	42.25	11.00	1.38	0.69	27.04
Copargo	Inside	Total Mosquitoes	0	11	144	91	44	9	11	288
		nb human catches	8	8	8	8	4	8	16	28
		HBR/night	0.00	1.38	18.00	11.38	11.00	1.13	0.69	10.29
	Outside	Total Mosquitoes	1	8	110	155	31	6	9	302
		nb human catches	8	8	8	8	4	8	16	28
		HBR/night	0.13	1.00	13.75	19.38	7.75	0.75	0.56	10.79
Bassila (control)	Inside	Total Mosquitoes	13	NA	269	200	24	0	13	493
		nb human catches	8	NA	8	8	4	8	8	28
		HBR/night	1.63	NA	33.63	25.00	6.00	0.00	1.63	17.61

Districts	Position	Indicator	Feb 2020	Mar 2020	Jun 2020	Jul 2020	Aug 2020	Dec 2020	Pre-2020 IRS (Feb-Mar 2020)	Post-2020 IRS (Jun-Dec 2020)
	Outside	Total Mosquitoes	23	NA	345	363	40	1	23	749
		nb human catches	8	NA	8	8	4	8	8	28
		HBR/night	2.88	NA	43.13	45.38	10.00	0.13	2.88	26.75

NA: Not applicable; **nb:** number; **Pre-2020 IRS period:** February, March; **Post-2020 IRS period:** June, July, August, and December.

Table 25. *Biting rates of* An. gambiae *s.l. indoor and outdoor in treated districts (Alibori) and control (Bembereke)*

Districts	Position	Indicator	Feb 2020	Mar 2020	Jun 2020	Jul 2020	Aug 2020	Dec 2020	Pre-2020 IRS (Feb-Mar 2020)	Post-2020 IRS (Jun-Dec 2020)
Kandi	Inside	Total Mosquitoes	44	9	63	190	113	11	53	377
		nb human catches	8	8	8	8	4	8	16	28
		HBR/night	5.50	1.13	7.88	23.750	28.25	1.38	3.31	13.46
	Outside	Total Mosquitoes	20	31	58	151	96	4	51	309
		nb human catches	8	8	8	8	4	8	16	28
		HBR/night	2.50	3.88	7.25	18.88	24.00	0.50	3.19	11.04
Gogounou	Inside	Total Mosquitoes	31	1	21	124	62	1	32	208
		nb human catches	8	8	8	8	4	8	16	28
		HBR/night	3.88	0.13	2.63	15.50	15.50	0.13	2.00	7.43
	Outside	Total Mosquitoes	33	5	20	87	75	8	38	190
		nb human catches	8	8	8	8	4	8	16	28
		HBR/night	4.13	0.63	2.50	10.88	18.75	1.00	2.38	6.79
Bembereke (control)	Inside	Total Mosquitoes	14	NA	175	307	39	2	14	523
		nb human catches	8	NA	8	8	4	8	8	28
		HBR/night	1.75	NA	21.88	38.38	9.75	0.25	1.75	18.68
	Outside	Total Mosquitoes	12	NA	121	195	16	1	12	333
		nb human catches	8	NA	8	8	4	8	8	28
		HBR/night	1.50	NA	15.13	24.38	4.00	0.13	1.50	11.89

NA: Not applicable; **nb:** number; **Pre-2020 IRS period:** February, March**;** **Post-2020 IRS period:** June, July, August, and December.

Figure 13 shows the dynamics of HBR from May 2016 to December 2020. The lowest HBR was observed during the dry periods (January 2017 to April 2017, November 2017 to March 2018, November 2018 to March 2019, December 2019 to March 2020, and December 2020) in both treated and control areas. After IRS implementation, lower monthly HBR was observed in the treated areas compared to the control areas between June and October 2017, 2018 & 2019, and June to August 2020, which equals 4 months of impact each year (Fig. 13).

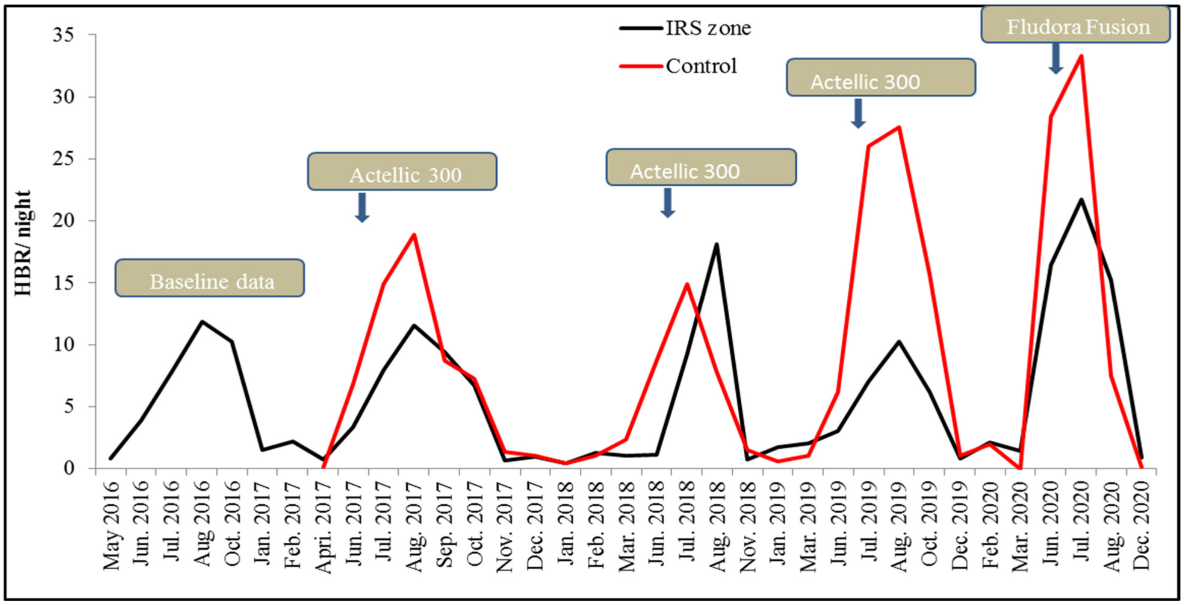


Figure 13. *Dynamic of the human biting rate in IRS and control areas from May 2016 to December 2020.*

2.3.3.2 Hourly human biting rate (HBR) of *An. gambiae* (s.l.) during the night before IRS intervention (February- March 2020) (a) and after IRS intervention (June-December 2020) (b).

This study was conducted to better understand the biting behavior of *An. gambiae s.l* before IRS intervention (February- March 2020) and after IRS intervention (June-December 2020). The biting cycle of *An. gambiae* observed is similar to what is mentioned in many papers: a constant increase of biting from 7:00 PM to reach a peak between midnight (00:00 AM) and 3:00 AM followed by a gradual drop until early morning (Fig. 14 & 15). Outdoor bites are most noticeable late at night and early in the morning (Fig. 14 & 15). *An. gambiae s.l.* appears to be collected more outside of the house in the middle of the night. This could be due to the excito-repellent effect of the deltamethrin contained in Fludora ® Fusion used to treat houses. Alternatively, *Anopheles gambiae* (s.l.) generally has a much more indoor-based biting behavior according to several studies. However, the presence of the vector depends on the presence of humans. This observed change in biting behavior of *An. gambiae* (s.l.) is thought to be related to the change in sleeping behavior of the population which prefers to spend part of the night outdoors in search of fresh air.

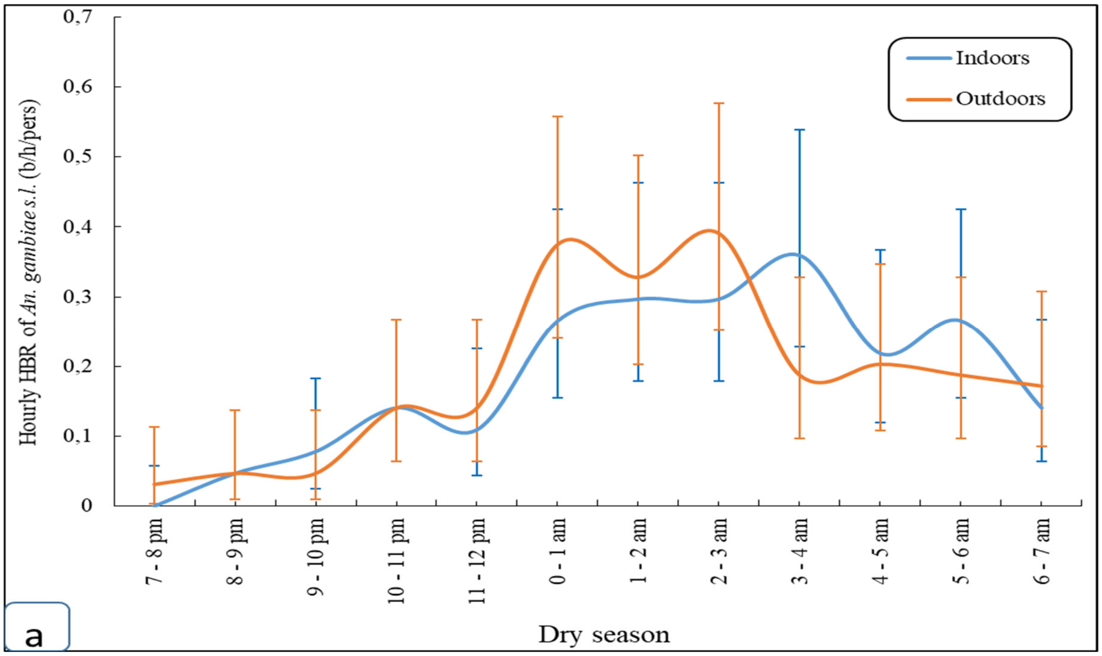


Figure 14. *Hourly HBR of An. gambiae s.l. in all treated districts) in the dry season (February-March 2020) (a).*

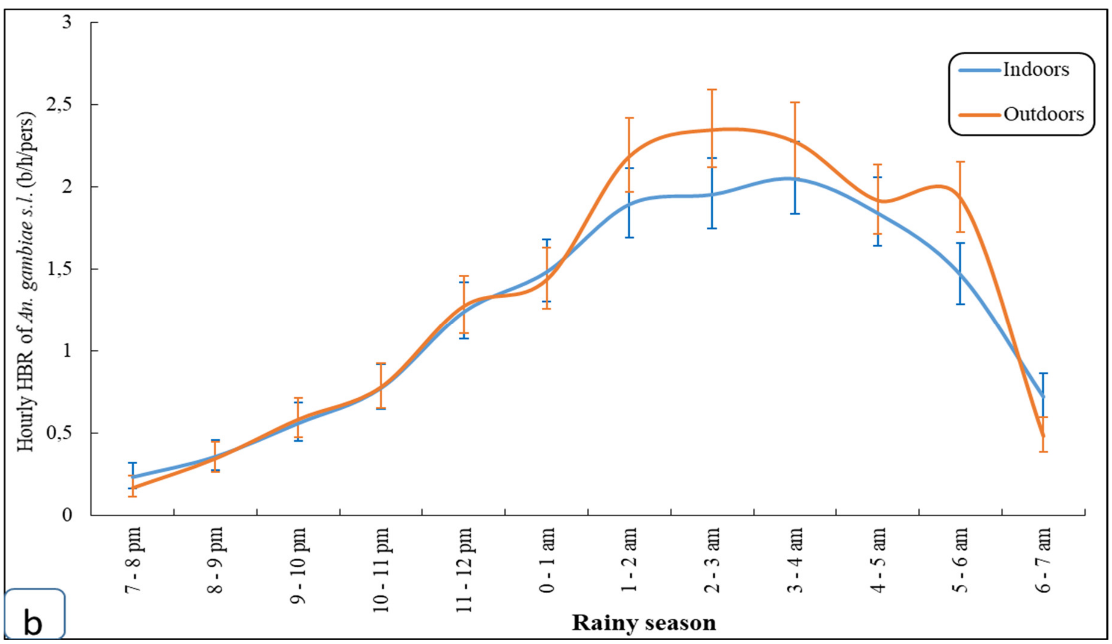


Figure 15. *Hourly HBR of* An. gambiae *s.l. in all treated and control districts after IRS intervention (June-December 2020) (b).*

2.3.4 Indoor resting density and blood-feeding rate of An. gambiae s.l.

Before the 2020 IRS campaign (February-March 2020), approximately 0.44 *An. gambiae s.l* per room were collected early in the morning (7 AM - 9 AM) after PSCs in IRS zone (Alibori and Donga), while 0.10 *An. gambiae* s.l. per room was collected in control areas (p=0.0006) (Table 26). The blood-feeding rates of *An. gambiae* s.l. were similarly high in treated (100%) and the control areas (71.43%) (p =0.501)

(Table 27). After the 2020 IRS campaign (June-August 2020), the density of *An. gambiae s.l* was significantly reduced in IRS areas compared to the control areas (Table 26). This density was 0.92 *An. gambiae* s.l./room in treated houses and 3.25 *An. gambiae* s.l./room in the control areas (p<0.001) (Table 29). Despite the reduction of room density observed in treated areas during this period (June-August 2020), the blood-feeding rates of *An. gambiae* s.l. remained high in treated (69.09%) and the control (69.23%) areas (X^2 = 7.64; *df*= 1; *p=1*) after the IRS campaign (Table 27).

Table 26. *The indoor resting density of* An. gambiae *s.l collected (PSCs data) before and after 2020 IRS intervention*

Period	Districts	Nb of rooms	Nb of *An. gambiae* s.l. collected	Indoor resting density	RR [95% CI]*	P-value (Wald)
Pre-IRS evaluation: February- March 2020	Bembereke (control)	20	2	0.10	1	-
	Kandi	40	44	1.10	0.1 [0.01 - 0.34]	< 0.001
	Gogounou	40	15	0.38	0.26 [0.03 - 1.14]	0.07
	Bassila (control)	20	2	0.10	1	-
	Djougou	40	10	0.25	0.4 [0.04 - 1.87]	0.358
	Copargo	38	1	0.03	3.8 [0.19 - 224.18]	0.274
	Total control districts	40	4	0.10	1	-
	Total treated districts	158	70	0.44	0.22 [0.06-0.6]	0.0006
Post-IRS evaluation: June-August 2020	Bembereke (control)	60	189	3.15	1	-
	Kandi	60	78	1.30	2.42 [1.85-3.19]	<0.001
	Gogounou	60	24	0.40	7.87 [5.13-12.59]	<0.001
	Bassila (control)	60	201	3.35	1	-
	Djougou	60	63	1.05	3.19 [2.39-4.30]	<0.001
	Copargo	60	55	0.92	3.65 [2.69-5.01]	<0.001
	Total control districts	120	390	3.25	1	-
	Total treated districts	240	220	0.92	3.54 [2.99-4.20]	<0.001

RR: rate ratio; p (wald): p-value of the Wald test; [95% CI]: 95% confidence interval; *the control sites (Bembereke and Bassila) are used as the numerator and IRS sites are used as the denominator in the calculations of the risk ratio (RR).

Table 27. *Blood feeding rates of* An. gambiae *s.l. collected (PSCs data) before and after the 2020 IRS intervention.*

Period	Districts	Nb of *An. gambiae* s.l. collected	Nb of blood feed	Blood feeding rate (%)	P-value
Pre-IRS evaluation: February-March 2020	Bembereke (control)	2	2	100	-
	Kandi	44	29	65.91	0.814
	Gogounou	15	13	86.67	1
	Bassila (control)	2	2	100	-
	Djougou	10	8	80	1
	Copargo	1	0	0	0.665
	Total control districts	4	4	100	-
	Total treated districts	70	50	71.43	0.501
Post-IRS evaluation: June- August 2020	Bembereke (control)	189	130	68.78	-
	Kandi	78	61	78.21	0.160
	Gogounou	24	14	58.33	0.424
	Bassila (control)	201	140	69.65	-
	Djougou	63	42	66.67	0.771
	Copargo	55	35	63.64	0.492
	Total control districts	390	270	69.23	-
	Total treated districts	220	152	69.09	1

2.3.5 Parous rate observed in An. gambiae before and after the 2020 IRS campaign

Table 28. shows the impact of the IRS on the longevity of *An. gambiae* based on the proportion of mosquitoes that have laid at least once; mosquitoes from HLCs were used. Before 2020 IRS intervention (February to March 2020), the parous rate of *An. gambiae* in the treated districts (Alibori and Donga) was 53.20% (108/203) compared to 47.37% (27/57) in controls districts (Bembereke and Kouande) (X^2 = 0.39; *df*=1; p=0.529). After the 2020 IRS campaign (June-July 2020), the parous rate of *An. gambiae s.l* was significantly reduced in Alibori IRS areas compared to the untreated area (Bembereke) (Table 28) (p<0.05). In contrast, the opposite situation was observed in the treated areas in Donga, with higher parous rates compared to the untreated district (Bassila) (p<0.05). Globally, in treated districts, the rate was 65.86% (382/580) while in the control areas, the rate was 60.09% (256/426) (X^2 = 3.27; *df*= 1; *P*= 0.07). Parous rates of *An. gambiae* (s.l.) appeared to be elevated in IRS areas after the intervention. This result could be due to the delayed effect of Fludora Fusion on mosquitoes, the insecticide used for IRS.

Table 28. *Parous rate of* An. gambiae *s.l. in IRS and control districts before and after the 2020 IRS campaign*

Period	Districts	Nb of *An. gambiae* (s.l.) dissected	Number of parous	Parous rate (%)	P-value
Pre-IRS evaluation: February-March 2020	Kandi	96	50	52.08	0.892
	Gogounou	64	29	45.31	1
	Bembèrèkè (control)	23	11	47.83	-
	Djougou	25	17	68.00	0.279
	Copargo	18	12	66.67	0.290
	Bassila (control)	34	16	47.06	1
	Total treated districts	203	108	53.20	0.529
	Total control districts	57	27	47.37	-
Post-IRS evaluation: June- July 2020	Kandi	153	70	45.75	0.003
	Gogounou	84	36	42.86	0.004
	Bembèrèkè (control)	179	112	62.57	-
	Djougou	170	129	75.88	0.0003
	Copargo	173	147	84.97	<0.001
	Bassila (control)	247	144	58.30	-
	Total treated districts	580	382	65.86	0.07
	Total control districts	426	256	60.09	-

Nb: Number; P-value: comparison of the parity rate of *An. gambiae* s.l. between the treated and control districts (Test used: Chi-square test).

2.3.6 *Sporozoite index (%CS+) of* **Plasmodium falciparum** *and entomological inoculation rate (EIR) of* **An. gambiae s.l.**

Tables 29, 30, 31, 32, and 33 summarize biting rates (HBR), sporozoite index (SI), and entomological inoculation rate (EIR) recorded before and after the 2020 IRS campaign in the treated and untreated districts.

Before the 2020 IRS campaign (February- March 2020), a total of 224 head-thoraces of *An. gambiae s.l.* were analyzed by *Plasmodium falciparum* CS-ELISA in the treated districts (Alibori and Donga); this resulted in a sporozoite positivity of 0.89% (2/224). No mosquito tested positive for the CSP antigen of *P. falciparum* in untreated districts (Bembereke and Bassila) (Table 29). However, the EIR was low in some houses designated for IRS treatment during this period (0.47 infectious bites of *An. gambiae* s.l. per human per month) (Table 29). After the 2020 IRS campaign (June- December 2020), the EIR in treated districts was 3.37 times lower in the treated districts (2.54 infectious bites/human/month) compared to control districts (8.57 infectious bites /human/month), representing a reduction of 70.37% of the EIR (Table 29).

Overall, like the parous rate, the entomological inoculation rate (EIR) of *An. gambiae* (s.l.) was relatively low in Kandi (0.05ib/pers/night) and Gogounou (0.04 ib/pers/night) districts but high in Djougou (0.11 ib/pers/night) and Copargo (0.14 ib/pers/night) (Tables 30 & 31).

Tables 32 and 33 show the monthly biting rates (HBR), sporozoite index (SI), and entomological inoculation rate (EIR) recorded indoors and outdoors before and after 2020 IRS intervention in treated and untreated districts. Figure 16 shows the dynamics of EIR in the treated area (Alibori, Donga) and in the control area (Bembereke, Bassila) from May 2016 to December 2020. From December 2019 to March 2020, EIR was relatively low in treated and control areas while from June 2020 to August 2020, we found a significant decrease in EIR in treated districts compared to controls localities.

Table 29. *Sporozoite index (SI) (%) of* Plasmodium falciparum *and entomological inoculation rate (EIR) of* An. gambiae *s.l. before and after 2020 IRS intervention*

Areas	Period before 2020 IRS intervention, Period (February- March 2020)			After 2020 IRS intervention, Period (June -December 2020)		
	SI (%)	HBR/night	EIR/month	SI (%)	HBR/night	EIR/month
IRS area	0.89	1.75	0.47	0.64	13.34	2.54
Controls	0.00	1.94	0.00	1.53	18.73	8.57

Table 30. *Monthly sporozoite index (SI), human biting rate (HBR), and entomological inoculation rate (EIR) of An. gambiae s.l. in treated areas (Alibori) and control (Bembereke).*

Districts	Indicators	Feb 2020	Mar 2020	June 2020	July 2020	Aug 2020	Dec 2020	Before IRS (Feb-Mar) 2020	After IRS (Jun-Dec) 2020
Kandi	SI	0.000	0.000	0.000	0.006	0.005	0.004	0.000	0.004
	HBR/night	4.00	2.50	7.56	21.31	26.13	12.25	3.25	12.25
	EIR/night	0.00	0.00	0.00	0.13	0.13	0.05	0.00	0.05
Gogounou	SI	0.000	0.000	0.000	0.005	0.007	0.005	0.000	0.005
	HBR/night	4.00	0.38	2.56	13.19	17.13	7.11	2.19	7.11
	EIR/night	0.00	0.00	0.00	0.06	0.13	0.03	0.00	0.04
Bembereke (control)	SI	0.000	NA	0.007	0.008	0.091	0.01	0.000	0.013
	HBR/night	1.63	NA	18.50	31.38	6.88	10.53	1.63	15.28
	EIR/night	0.00	NA	0.13	0.25	0.63	0.14	0.00	0.19

Table 31. *Monthly sporozoite index (SI), human biting rate (HBR), and entomological inoculation rate (EIR) of An. gambiae s.l. in treated areas (Donga) and control (Bassila).*

Districts	Indicators	Feb 2020	Mar 2020	June 2020	July 2020	Aug 2020	Dec 2020	Before IRS (Feb-Mar) 2020	After IRS (Jun-Dec) 2020
Djougou	SI	0.000	0.038	0.003	0.003	0.030	0.004	0.033	0.004
	HBR/night	0.25	1.63	39.88	37.06	8.38	22.46	0.94	23.46
	EIR/night	0.00	0.06	0.13	0.13	0.25	0.11	0.03	0.11

Copargo	SI	0.000	0.053	0.012	0.012	0.027	0.01	0.050	0.014
	HBR/night	0.06	1.19	15.88	15.38	9.38	10.53	0.63	10.53
	EIR/night	0.00	0.06	0.19	0.19	0.25	0.14	0.03	0.14
Bassila (control)	SI	0.000	NA	0.013	0.014	0.078	0.01	0.000	0.02
	HBR/night	2.25	NA	38.38	35.19	8.00	22.17	2.25	22.17
	EIR/night	0.00	NA	0.50	0.50	0.63	0.37	0.00	0.37

NA: Not applicable

Table 32. *Monthly sporozoite index (SI), human biting rate (HBR), and entomological inoculation rate (EIR) of* An. gambiae *s.l. indoors and outdoors in treated areas (Alibori) and control (Bembereke).*

Districts	Location	Indicators	February 2020	March 2020	June 2020	July 2020	August 2020	December 2020
Bembereke (control)	Inside	Total tested	14	NA	175	307	39	2
		nb Thorax+	0	NA	0	3	3	0
		SI	0.00	NA	0.00	0.01	0.08	0.00
		HBR/night	1.75	NA	21.88	38.38	9.75	0.25
		EIR/night	0.00	NA	0.00	0.38	0.75	0.00
		EIR/month	0.00	NA	0.00	11.25	22.50	0.00
	Outside	Total tested	12	NA	121	195	16	1
		nb Thorax+	0	NA	2	1	2	0
		SI	0.00	NA	0.02	0.01	0.13	0.00
		HBR/night	1.50	NA	15.13	24.38	4.00	0.13
		EIR/night	0.00	NA	0.25	0.13	0.50	0.00
		EIR/month	0.00	NA	7.50	3.75	15.00	0.00
Kandi	Inside	Total tested	44	9	63	190	113	11
		nb Thorax+	0	0	0	0	1	0
		SI	0.00	0.00	0.00	0.00	0.01	0.00
		HBR/night	5.50	1.125	7.875	23.75	28.25	1.375
		EIR/night	0.00	0.00	0.00	0.00	0.25	0.00
		EIR/month	0.00	0.00	0.00	0.00	7.50	0.00
	Outside	Total tested	20	31	58	151	96	4
		nb Thorax+	0	0	0	2	0	0
		SI	0.00	0.00	0.00	0.01	0.00	0.00
		HBR/night	2.5	3.88	7.25	18.88	24.00	0.50
		EIR/night	0.00	0.00	0.00	0.25	0.00	0.00
		EIR/month	0.00	0.00	0.00	7.50	0.00	0.00
Gogounou	Inside	Total tested	31	1	21	124	62	1
		nb Thorax+	0	0	0	1	0	0
		SI	0.00	0.00	0.00	0.01	0.00	0.00
		HBR/night	3.88	0.13	2.63	15.50	15.50	0.13
		EIR/night	0.00	0.00	0.00	0.13	0.00	0.00
		EIR/month	0.00	0.00	0.00	3.75	0.00	0.00
	Outside	Total tested	33	5	20	87	75	8
		nb Thorax+	0	0	0	0	1	0
		SI	0.00	0.00	0.00	0.00	0.01	0.00
		HBR/night	4.13	0.63	2.50	10.88	18.75	1.00
		EIR/night	0.00	0.00	0.00	0.00	0.25	0.00

Districts	Location	Indicators	February 2020	March 2020	June 2020	July 2020	August 2020	December 2020
		EIR/month	0.00	0.00	0.00	0.00	7.50	0.00

NA: Not applicable; **Thorax:** mosquito thoraces tested; **Thorax +:** mosquito thoraces positive; **HBR:** human biting rate

Table 33. *Monthly sporozoite index (SI), biting rate (HBR), and entomological Inoculation rate (EIR) of An. gambiae s.l. indoors and outdoors in the treated area (Donga) and control (Bassila).*

Districts	Location	Indicators	February 2020	March 2020	June 2020	July 2020	August 2020	December 2020
Bassila (control)	Inside	Total tested nb	13	NA	269	200	24	0
		Thorax+	0	NA	4	7	4	0
		SI	0.00	NA	0.01	0.04	0.17	0.00
		HBR/night	1.63	NA	33.63	25.00	6.00	0.00
		EIR/night	0.00	NA	0.50	0.88	1.00	0.00
		EIR/month	0.00	NA	15.00	26.25	30.00	0.00
	Outside	Total tested nb	23	NA	345	363	40	1
		Thorax+	0	NA	4	1	1	0
		SI	0.00	NA	0.01	0.00	0.03	0.00
		HBR/night	2.88	NA	43.13	45.38	10.00	0.13
		EIR/night	0.00	NA	0.50	0.13	0.25	0.00
		EIR/month	0.00	NA	15.00	3.75	7.50	0.00
Djougou	Inside	Total tested nb	1	18	274	255	23	5
		Thorax+	0	1	1	1	1	0
		SI	0.00	0.06	0.00	0.00	0.04	0.00
		HBR/night	0.13	2.25	34.25	31.88	5.75	0.63
		EIR/night	0.00	0.13	0.13	0.13	0.25	0.00
		EIR/month	0.00	3.75	3.75	3.75	7.50	0.00
	Outside	Total tested nb	3	8	364	338	44	11
		Thorax+	0	0	1	1	1	0
		SI	0.00	0.00	0.00	0.00	0.02	0.00
		HBR/night	0.38	1.00	45.50	42.25	11.00	1.38
		EIR/night	0.00	0.00	0.13	0.13	0.25	0.00
		EIR/month	0.00	0.00	3.75	3.75	7.50	0.00
Copargo	Inside	Total tested nb	0	11	144	91	44	9
		Thorax+	0	0	3	2	0	0
		SI	0.00	0.00	0.02	0.02	0.00	0.00
		HBR/night	0.00	1.38	18.00	11.38	11.00	1.13
		EIR/night	0.00	0.00	0.38	0.25	0.00	0.00

Districts	Location	Indicators	February 2020	March 2020	June 2020	July 2020	August 2020	December 2020
		EIR/month	0.00	0.00	11.25	7.50	0.00	0.00 0
	Outside	Total tested nb	1	8	110	155	31	6
		Thorax+	0	1	0	1	2	0
		SI	0.00	0.13	0.00	0.01	0.06	0.00
		HBR/night	0.13	1.00	13.75	19.38	7.75	0.75
		EIR/night	0.00	0.13	0.00	0.13	0.50	0.00
		EIR/month	0.00	3.75	0.00	3.75	15.00	0.00

NA: Not applicable; **Thorax:** mosquito thoraces tested; **Thorax +:** mosquito thoraces positive; **HBR:** human biting rate

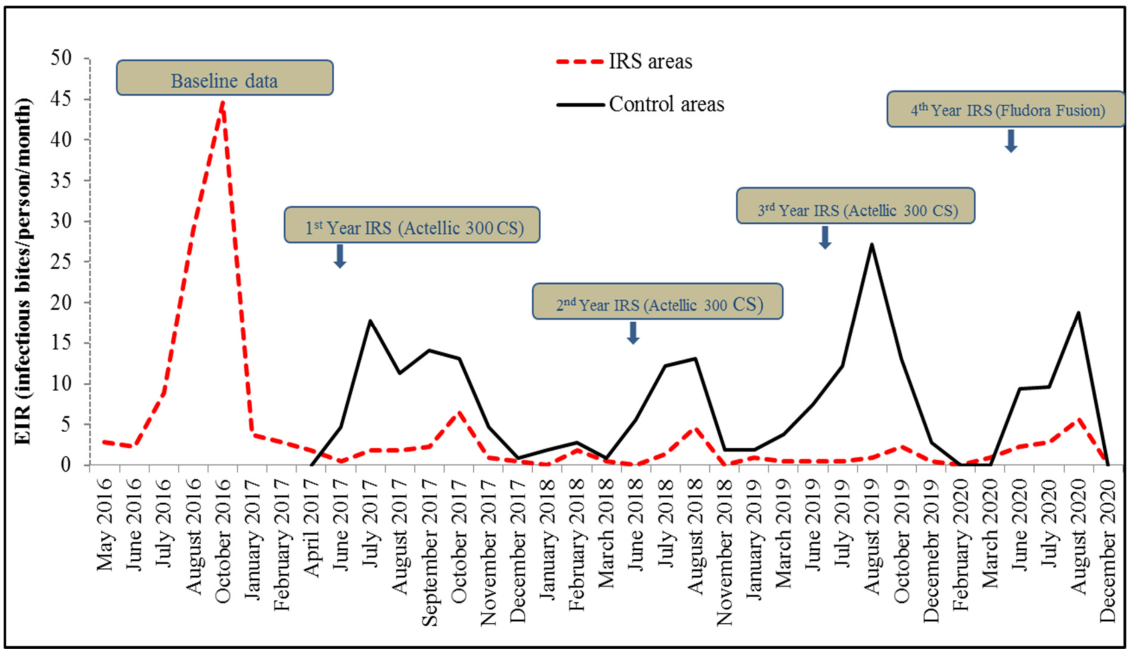


Figure 16. *Dynamics of EIR in the treated area (Alibori, Donga) and in the control area (Bembereke, Bassila) from May 2016 to December 2020.*

2.3.7 Insecticide susceptibility tests

Figure 17 summarizes the susceptibility level of local vectors to different insecticides (bendiocarb, pirimiphos-methyl, permethrin, and deltamethrin). All mosquito populations tested were susceptible to pirimiphos-methyl (mortality > 98%). However, Gogounou vectors populations showed resistance to bendiocarb (mortality < 90) but in Kandi, vectors are susceptible to Bendiocarb (100%). For pyrethroids (deltamethrin and permethrin), *An. gambiae* s.l. was resistant in all the districts (mortality< 90%) (Fig. 17).

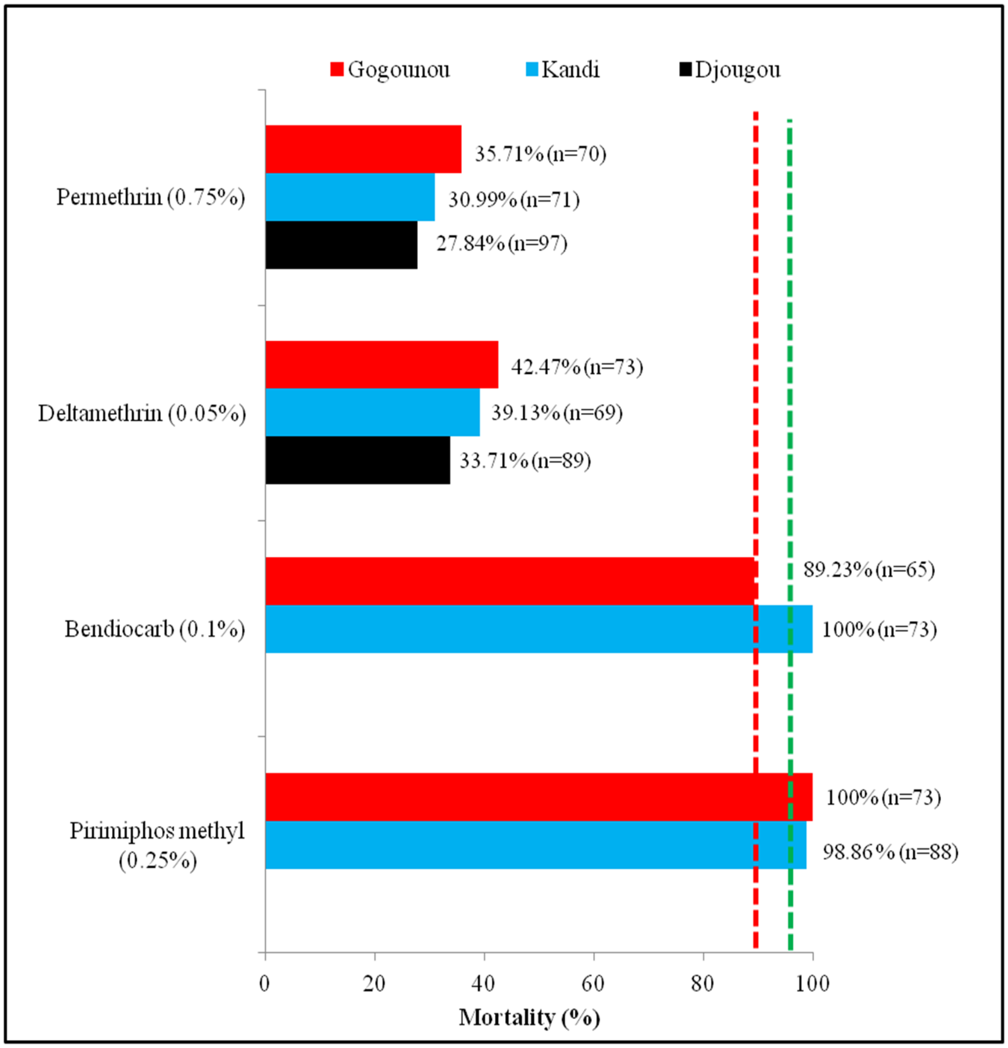


Figure 17. *Mortalities observed 24 hours after mosquito exposure to bendiocarb 0.1%, pirimiphos-methyl 0.25%, permethrin 0.75%, and deltamethrin 0.05% in four districts under IRS during period June -August 2020 (based on World Health Organization criteria the area below broken red lines indicates insecticide resistance; the area in between the broken red and green lines indicate possible resistance; the area above the green broken line indicates insecticide susceptibility)*

2.3.8 Multiple insecticide resistance mechanisms in *An. gambiae* s.l. (Kdr, Ace-1)

Data presented in Tables 34 and 36 show the frequency and distribution of *kdr* L1014F and *Ace 1* resistance genes among *An. gambiae* complex species collected. *Kdr* mutation was detected in high frequency in all species of the *An. gambiae* complex within all the localities. Globally, *kdr* L1014F mutation was detected at a significantly higher frequency in *An. gambiae* (77.70% in IRS districts and 86.73% in controls) compared to *An. coluzzii* (54.94% in IRS districts and 46.62% in controls) (Table 35). *Kdr* L1014F mutation frequency was similar between the IRS and control areas (p=0.386) (Table 34).

Table 34. *Frequencies Kdr L1014F of* An. gambiae *s.l. in IRS zone and control*

Localities	Number tested	RR	RS	SS	Freq. 1014F (%)	P-value
Kandi	224	115	43	66	60.94	-
Gogounou	111	62	21	28	65.32	0.309
Djougou	69	43	13	13	71.74	0.027
Copargo	80	52	16	12	75.00	0.001
Bembereke (control)	70	37	15	18	63.57	0.645
Bassila (control)	53	29	7	17	61.32	1
Total IRS districts	484	272	93	119	65.81	-
Total untreated districts	123	66	22	35	62.60	0.386

Table 35. *Distribution of knock-down resistance (Kdr) frequencies between malaria vectors and localities*

Localities	Species	Number tested	RR	RS	SS	Freq. L1014F (%)	P-value
Kandi	*An. gambiae*	80	50	12	18	70.00	0.004
	An. coluzzii	144	65	31	48	55.90	
Gogounou	*An. gambiae*	35	32	2	1	94.00	<0.001
	An. coluzzii	76	30	19	27	51.97	
Djougou	*An. gambiae*	51	35	9	7	77.45	0.021
	An. coluzzii	18	8	4	6	55.56	
Copargo	*An. gambiae*	65	45	12	8	78.46	0.061
	An. coluzzii	15	7	4	4	60.00	
Bembereke (control)	*An. gambiae*	33	25	6	2	84.85	<0.001
	An. coluzzii	37	12	9	16	44.59	
Bassila (control)	*An. gambiae*	16	14	1	1	90.63	0.0001
	An. coluzzii	37	15	6	16	48.65	
Total districts under IRS	*An. gambiae*	231	162	35	34	77.70	<0.001
	An. coluzzii	253	110	58	85	54.94	
Total districts control	*An. gambiae*	49	39	7	3	86.73	<0.001
	An. coluzzii	74	27	15	32	46.62	

SS = homozygous susceptible; RS = hybrid resistant and susceptible; RR = homozygous resistant; Freq. = Frequency.

The ace-1R mutation associated with carbamates and organophosphate resistance was identified in all sites but with very low frequencies (2.03 in untreated areas and 3.10 in IRS zone) (Table 36). It ranged from 3.06% in *An. gambiae* to 1.35% in *An. coluzzii* (Table 37).

Table 36. *Frequencies Ace-1R G119S of An. gambiae s.l. in IRS zone*

Localities	Number tested	RR	RS	SS	Freq. 119S (%)	P-value
Kandi	224	0	14	210	3.13	-
Gogounou	111	0	5	105	2.25	0.694
Djougou	69	0	6	60	4.35	0.671

Localities	Number tested	RR	RS	SS	Freq. 119S (%)	P-value
Copargo	80	0	5	75	3.13	1
Bembereke (control)	70	0	3	67	2.14	0.751
Bassila (control)	53	0	2	51	1.89	0.717
Total IRS districts	484	0	30	450	3.10	-
Total untreated districts	123	0	5	118	2.03	0.496

Table 37. *Distribution of Ace-1R frequencies between malaria vectors and localities*

Localities	Species	Number tested	RR	RS	SS	Freq. G119S (%)	P-value
Kandi	*An. gambiae*	80	0	6	74	3.75	0.776
	An. coluzzii	144	0	8	136	2.78	
Gogounou	*An. gambiae*	35	0	1	33	1.42	0.940
	An. coluzzii	76	0	4	72	2.63	
Djougou	*An. gambiae*	51	0	5	46	4.90	0.950
	An. coluzzii	18	0	1	14	2.78	
Copargo	*An. gambiae*	65	0	4	61	3.08	1
	An. coluzzii	15	0	1	14	3.33	
Bembereke (control)	*An. gambiae*	33	0	2	31	3.03	0.920
	An. coluzzii	37	0	1	36	1.35	
Bassila (control)	*An. gambiae*	16	0	1	15	3.13	1
	An. coluzzii	37	0	1	36	1.35	
Total districts under IRS	*An. gambiae*	231	0	16	214	3.46	0.660
	An. coluzzii	253	0	14	236	2.77	
Total districts control	*An. gambiae*	49	0	3	46	3.06	0.639
	An. coluzzii	74	0	2	72	1.35	

SS = homozygous susceptible; RS = hybrid resistant and susceptible; RR = homozygous resistant; Freq. = Frequency.

2.4 Conclusion

All entomological monitoring targets set during the deliverable covering the period from February 2020 to August 2020 were met. Monitoring and evaluation of the 4th year of indoor residual spraying campaign with Fludora ® Fusion carried out from February 2020 to December 2020 in Alibori and Donga has shown once more the impact of this strategy on the reduction of entomological indicators. From the evaluation of this campaign, we can note a significant difference between the entomological indicators of treated districts and those controls districts.

The low density of *An. gambiae* s.l. in all localities during this period (February to March 2020) is likely due to the harmattan season and the dry conditions which characterize it. Similar biting behavior of *An. gambiae* s.l. indoors and outdoors of treated houses during this period (February to March 2020) is likely due to the complete decrease in the effect of the insecticide used in May 2019.

Bioassays on treated walls have shown that Fludora ® Fusion remains effective with a mortality rate above 80% on the susceptible strain Kisumu, 8 months after the spraying date.
During this period of bio-efficiency of Fludora ® Fusion, we observed a spectacular reduction of some indicators like the indoors resting density, sporozoite index, and EIR, and strong exophagy of *Anopheles gambiae* in most treated districts compared to control areas. However, IRS impact is not so visible on some indicators such as the blood-feeding rate; this particular indicator appeared relatively high in treated and control districts.

Concerning vector susceptibility, *An. gambiae* s.l. was susceptible to pirimiphos-methyl in all sites but is experiencing resistance to bendiocarb in Gogounou and widespread resistance to pyrethroids in all localities.

2.5 Difficulties encountered and recommendations

- Mosquitoes were not collected in the two control areas (Bassila and Bembèrèkè) in March 2020 due to the restrictive measures taken by the government to limit the spread of COVID-19, these measures were taken at the time of mosquito collection in these two localities.

- The mosquitoes were not collected in May 2020 due to the restrictive measures taken by the government to limit the spread of COVID-19 and the temporary cessation of some activities required by a USAID decision.
- Since June when activities resumed, the different actors involved in field data collection have been complying with the measures required by the government of Benin to prevent the spread of COVID-19: masks and gloves are provided to the mosquito collectors by the Centre de Recherche Entomologique de Cotonou.
- During the period June -August 2020, the rarity of positive *Anopheles* larvae breeding sites in some treated localities was a handicap to carry out susceptibility tests in all localities and for all insecticide classes. The search for larvae will continue in these areas in July 2021.

3 Part III – Quantification study and evolution of the insecticide resistance in *Anopheles gambiae* s.l in Benin – the implication of high vector resistance in *Anopheles gambiae* s.l for vector control

3.1 Background

In September 2017 we implemented a study on the quantification of the intensity of vector resistance with the support of USAID. For two years, the exposition of various populations of *Anopheles gambiae s.l.* to doses of insecticides (permethrin, deltamethrin) 2 and 5 times higher than the diagnostic dose did not kill the total number of mosquitoes tested. On the other hand, the combination of the diagnostic dose with PBO has only partially abolished the resistance showing a very high resistance in *An. gambiae s.l.* in Benin. The second step we have investigated in 2020 is to determine what could be the direct implications of this high intensity of insecticide resistance on vector control management. We have evaluated the response of mosquitoes surviving after exposure to high concentrations of insecticides and the combined insecticides + PBO to different types of LLINs. The results we obtained will serve as a database for the utilization of LLINs of new generations in Benin.

3.2 Objectives

- Perform *An. gambiae* susceptibility to the diagnostic dose of permethrin, deltamethrin, and alphacypermethrin and doses 2 and 5 times higher using WHO and CDC bioassays;
- Determine the mechanisms associated with insecticide resistance within *An. gambiae* in the various ecological zones;
- Assess the efficacy of the CDC bottles or WHO papers with combined "pyrethroid + PBO" against resistant mosquitoes for each ecological zone with the mechanisms of insecticide resistance;
- Determine the response of *An. gambiae* survived after exposure to high concentrations of insecticides and combined insecticides + PBO to different types of LLINs

3.3 Study methods

In 2018, under implementation letter (IL)[30] #24, and in 2019, under IL #31, *Anopheles gambiae* s.l. collected in 13 districts was tested for susceptibility to permethrin, deltamethrin, and alphacypermethrin. To assess the survival rate of resistant mosquitoes after exposure, the same

[30] An agreement between PMI and CREC to achieve common objectives towards entomological monitoring of malaria vector in Benin in coordination with the NMCP for inform malaria control decision-making.

populations were exposed to diagnostic doses of the three insecticides; additionally, 2 and 5 times the diagnostic dose were also tested. The same populations of mosquitoes were exposed to the three insecticides combined with synergist piperonyl butoxide (PBO) using CDC bottles and WHO papers bioassays. Mosquitoes not killed after exposure to the high concentrations of insecticides or combination insecticide + PBO were exposed to different types of Long-Lasting Insecticide Nets (LLINs).

The three insecticides tested are those used to treat the majority of LLINs distributed in Benin: Olyset treated with permethrin, PermaNet 3.0 and PermaNet 2.0, and other nets treated with deltamethrin. Recently (2020), three types of LLINs (Royal Guard®, Interceptor®, and Interceptor G2 ®) were distributed in three communes in southern Benin as part of the mass distribution of the National Malaria Control Program and the New Nets Project (Unitaid Project) supported by Bill and Melinda Gates. These three types of nets are impregnated with alpha-cypermethrin combined or not with another product.

3.3.1 Study area

The study areas are chosen in various ecological zones (Fig. 18):

- **Cotton production area** (Kandi, N'Dali, and Parakou): this area is characterized by the high use of pesticides against cotton pests. Intensive cotton cultivation is practiced, combined with the use of several families of insecticides.
- **Rice growing area** (Malanville): Malanville rice area is about 70 hectares. Two rice crops are grown per year.
- **Urban vegetable production area**: characterized by high use of impregnated mosquito nets, various aerosol insecticides, smoke coils, and various pesticides against vegetable pests (Cotonou and Porto-Novo).
- **Cereal area:** districts of Missérété (Ouémé department), Bantè (Collines department), Ouidah and Allada (Atlantique department), Bohicon (Zou department)
- **Hilly area:** Dassa and Save in the central part (Table 38).

Table 38. *GPS coordinates of the thirteen study data collection sites*

	Departments	Communes	Latitude/ Longitude
Zone urbaine et maraîchère	Littoral	Cotonou	06°22'26.76"N, 02°27'32.4"E
	Ouémé	Porto-Novo	06°28'19.64"N, 02°37'24.632"E
Zone cotonnière	Alibori	Kandi	11°1'34.68"N, 02°55'37.2"E
	Borgou	N'Dali	09°52'21.108"N, 02°37'24.632"E
	Borgou	Parakou	09°33'47.633"N, 02°25'3026"E
Zone rizicole	Alibori	Malanville	11°51'9.781"N, 02°25'30.26"E
Zone céréalière	Ouémé	Missérété	06°38'17.275"N, 02°33'54.837"E
	Atlantique	Allada	06°38'9.92"N, 02°0'0"E
	Atlantique	Ouidah	06°19'29.279"N, 02°14'14.279"E

	Departments	Communes	Latitude/ Longitude
	Collines	Bantè	08°24’37.312”N, 01°53’11.38”E
Zone forestière	Zou	Bohicon	07°10’51553”N, 01°53’11.40”E
Zone des collines	Collines	Dassa	07°45’19.634”N, 02°11’40.261”E
	Collines	Savè	07°48’12.562”N, 02°35’2.953”E

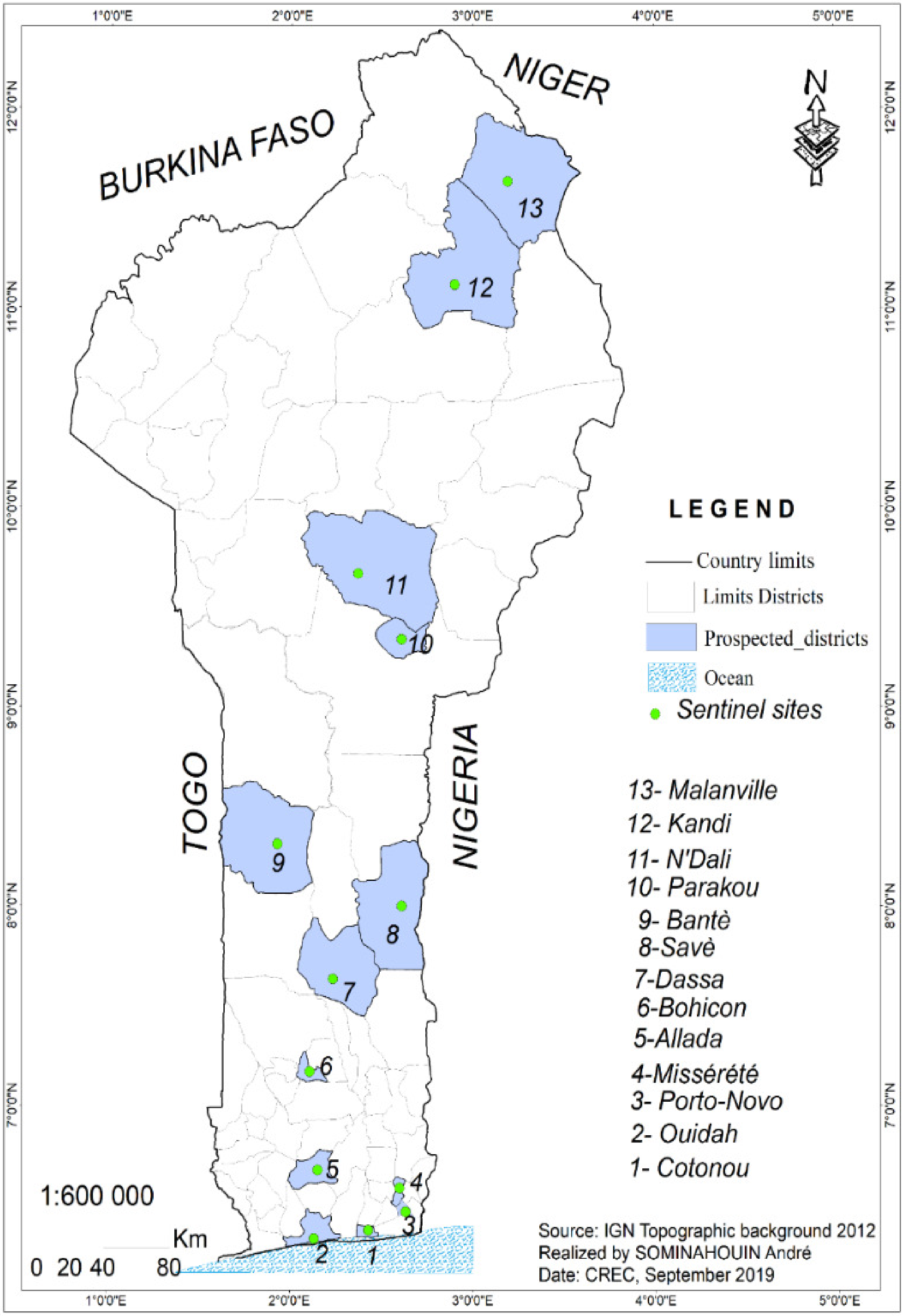


Figure 18. *Map of Benin showing the communes where larvae of* Anopheles gambiae *s.l. were collected to perform susceptibility tests.*

The study sites from northern Benin (Kandi, N'dali, Parakou, Malanville) are characterized by dry savannah areas, with six months rainy season (mid-April to mid-October) and a dry season that spans the remainder of the year. Overall, average annual rainfall ranges between 700–1200 mm in Kandi and Malanville region and 1200–1300 mm in Parakou and N'dali region.

The districts of Missérété (Ouémé department), Bantè (Collines department), Ouidah and Allada (Atlantique department), Bohicon (Zou department) are characterized by a long rainy season (March-July), a long dry season (December-February), a short rainy season (September-November) and a short dry season (August-September).

Malaria is the leading cause of mortality among children under five years of age and morbidity among adults in Benin. Malaria accounts for 40% of outpatient consultations and 25 %t of all hospital admissions. Malaria places an enormous economic strain on Benin's development. The incidence of uncomplicated and severe malaria in 2016 was 26.4% in the Donga region and 13.3% in Alibori.

3.3.2 Long-Lasting Insecticide Nets tested

Seven different nets were used for their efficacy against *Anopheles gambiae* characterized by a high intensity of vector control:

- PermaNet 2.0 (polyester + deltamethrin)
- PermaNet 3.0 (polyester + deltamethrin + PBO)
- Olyset Net (polyethylene + permethrin)
- Olyset Net Plus (polyethylene + permethrin +PBO)
- Alphacypermethrin Net (polyethylene + Alphacypermethrin +PBO)
- Yorkool (polyester + deltamethrin)
- Dawa (polyester + deltamethrin)
- Net not treated (control)

3.3.3 Insecticide resistance test with the WHO susceptibility assay and CDC bottle bioassay, and molecular and biochemical tests for insecticide resistance mechanisms

3.3.3.1 WHO insecticide susceptibility tests

The susceptibility tests are performed according to the WHO protocol[31]. At the end of the tests, live and dead specimens are used for species identification and determination of resistance mechanisms (*Kdr* L1014F and G119S *Ace-1R*) using PCR methods (protocols already described: see previous reports, IL 24 and IL 31).

[31] WHO. *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes.* Geneva: World Health Organization; 2013.

3.3.3.2 CDC bottle bioassay

The CDC bottle bioassay is conducted using *An. gambiae* s.l collected and the laboratory strain *An. gambiae* s.s Kisumu, according to the CDC protocol[32, 33](protocol already described: see IL 24 and IL 31).

3.3.3.3 Insecticide resistance mechanism tests

Three methods already described (see previous reports, IL 24 and IL 31) were used:

- Identification of the species of *An. gambiae* s.l. tested and molecular characterization of the Kdr *L1014F* and *G119S* Ace-1R resistance genes;
- Biochemical analyzes by spectrophotometry;
- Biochemical analyzes by pre-exposing *Anopheles gambiae* s.l. to synergist PBO.

3.3.4 The WHO cone test with insecticide-treated nets

Larvae and pupae of *Anopheles gambiae* s.l. were collected from several identified sites in four randomly selected villages per commune. Once the site was identified, larvae and pupae were collected from the surface of the water with a dipper and transported to the insectarium to be reared to the adult stage. The WHO cone test measures knockdown and mortality of mosquitoes in a standard WHO cone to a piece of treated netting for exposure time. Five non-blood-fed, 2–5-day-old female *Anopheles gambiae* s.l. mosquitoes of the F1 generation were exposed to netting materials (25 cm × 25 cm) for 3 minutes under standard WHO cones after which they were held for 24 h with access to the sugar solution. Five cones were used for each of the 5 surfaces of the net. Five mosquitoes were used for each cone and 2 replicates were carried out. In total, 50 mosquitoes are needed for one net. Mosquitoes exposed to untreated nets were used as the negative control. Bioassays were carried out at 27 ± 2 °C and 75% ± 10% relative humidity. Knock-down (KD) was recorded 60 minutes after exposure and mortality after 24 h. For susceptible mosquitoes, the net is declared efficacious when the KD rate is ≥ 95% after 60 minutes or there is a mortality rate ≥ 80% after 24 hours.

3.3.5 Statistical analysis

The resistance status of malaria vectors is determined according to WHO criteria (WHO, 2013). When the mortality rate of the *An. gambiae s.l.* population after 24 hours is between 98% and 100%, the *An. gambiae s.l.* the population is susceptible; when it is between 90% and 97%, the *An. gambiae* s.l. the

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[32] Brogdon WG, McAllister JC. Simplification of adult mosquito bioassays through use of time-mortality determinations in glass bottles. J. Am. Mosq. Control Assoc.1998 ; 14(2):159-164.

[33] Brogdon W, Chan A. Guidelines for Evaluating Insecticide Resistance in Vectors using the CDC Bottle Bioassay/ Methods in A nopheles research. Second edition. CDC Atlanta USA: CDC technical report. 2010; P 343.

population is suspected of being resistant and this resistance remains to be confirmed; finally, it is certified to be resistant when the mortality rate is less than 90%.

The one-way analysis of variance (ANOVA) tests coupled with Tukey's Multiple Comparison test allowed us to compare the mean values of enzyme activities (esterases, oxidases, and GST) between the field strains and between the reference Kisumu strain and the field strains. Mortality rates and allelic frequencies of the kdr-west and ace-1R genes were analyzed to assess their variability across different populations. Statistical analyses were performed using the R 2.15

3.4 Results

Susceptibility tests in WHO tubes and CDC bottles were performed on the thirteen (13) malaria vector populations (*An. gambiae* s.l.) collected in the different districts/communes: Cotonou, Malanville, Parakou, Kandi, Porto-Novo, Bohicon, Missérété, Allada, Ouidah, Savè, Dassa, Bantè, and N'Dali. The susceptibility results obtained, and the resistance status is summarized in the tables below.

3.4.1 Resistance status of *An. gambiae* s.l. to 1× and 2× doses of deltamethrin, permethrin, alpha-cypermethrin, and bendiocarb according to WHO and CDC test methods.

The results of susceptibility of *An. gambiae* s.l. using the WHO tube test method at 1 and 2 doses of permethrin, deltamethrin, and alpha-cypermethrin showed widespread resistance in the thirteen communes where larval surveys were carried out. When we associated PBO with these insecticides, a clear increase in the mortality rate of these vectors was observed, but resistance was still present. For Bendiocarb, only *Anopheles* from the cotton communes were found to be resistant. Most of the other communes tested were suspected to be resistant. The same trends were observed with CDC bottle tests. However, the addition of the synergist DEF to bendiocarb in the CDC bottled tests significantly increased mortality rates in all communes except in the commune of Misserete where we recorded a mortality rate of less than 90% (80.95%) despite the addition of this synergist (Tables 39 & 40 and Fig. 19, 20, 21, 22, 23 et & 24).

Table 39. *Mortality rate after exposure of thirteen populations of* An. gambiae *s.l. to multiples of diagnostic concentrations of pyrethroids with and without PBO and bendiocarb using WHO bioassays from different areas.*

Locality*	Delta 1×	Delta 1×+ PBO	Delta 2×	p-value	Per 1×	Per 1× + PBO	Per 2×	Alpha 1×	p-value	Bendio 1×
Kandi	17.34±7.5[a]	67.36±9.7[b]	46.53±9.4[c]	<0.0001	16.16±7.5[a]	46.80±10.1[bc]	34.73±9.6[bc]	31.76±9.9[abc]	<0.0001	92±5.3
Parakou	40.00±9.9[a]	90.81±5.7[b]	44.68±10.1[a]	<0.0001	23.95±8.5[a]	58.51±10[b]	38.04±9.9[a]	35.10±9.6[a]	<0.0001	96.84±3.5
Bohicon	29.89±9.1[a]	70.2±8.8[c]	45.45±9.8[b]	<0.0001	24.1±9[a]	55.32±10.1[b]	43.33±10.2[b]	45.83±10[b]	<0.0001	96.96±3.4
Cotonou	18.4±7.7[a]	76.24±8.3[c]	41.00±9.6[b]	<0.0001	10.4±6.1[a]	43.30±9.9[b]	35.22±10[b]	42.42±9.7[b]	<0.0001	73.52±8.6
Malanville	41.7±9.9[a]	77.01±8.8[b]	54.44±10.3[a]	<0.0001	31.9±9.4[a]	66.66±9.3[b]	39.02±10.6[a]	41.93±10[a]	<0.0001	91.48±5.6
Missérété	29.78±9.2[a]	82.65±7.5[b]	41.83±9.8[a]	<0.0001	23.86±8.9[a]	64.51±9,7[b]	48.91±10.2[b]	45.45±9.8[b]	<0.0001	97.72±3.1
Porto-Novo	21.11±8.4[a]	83.52±7.9[c]	60.20±9.7[b]	<0.0001	23.95±8.5[a]	79.56±8.2[b]	66.66±10.1[b]	33.67±9.4[a]	<0.0001	96.87±3.5
Allada	26.04±8.8[a]	85.85±6.9[c]	43.75±9.9[b]	<0.0001	15.00±7.2[a]	78.78±8.1[c]	36.26±9.9[b]	12.08±6.7[a]	<0.0001	97.95±3.3
Ouidah	20.00±7.8[a]	90.81±5.7[b]	47.00±10.2[a]	<0.0001	19.00±7.7[a]	92.00±5.3[c]	50.60±10.8[b]	21.00±8[a]	<0.0001	97.00±3.3
Bantè	44.56±10.2[a]	97.91±2.9[b]	48.38±10.2[a]	<0.0001	23.15±8.5[a]	58.00±9.7[b]	64.77±10[b]	26.66±9.1[a]	<0.0001	96.96±3.4
Dassa	51.25±11[a]	96.70±3.7[c]	67.90±10.2[b]	<0.0001	46.42±10.7[a]	85.55±7.3[c]	63.75±10.5[b]	34.04±9.6[a]	<0.0001	86.66±8.6
Savè	19.38±7.8[a]	82.65±7.5[b]	29.16±9.1[a]	<0.0001	15.30±7.1[a]	88.77±6.2[c]	58.82±10.5[b]	10.10±5.9[a]	<0.0001	93.20±4.9
N'Dali	30.00±9[a]	95.95±3.9[c]	46.73±10.2[b]	<0.0001	26.73±8.6[a]	89.53±6.5[c]	55.00±10.9[b]	28.12±9[a]	<0.0001	93.93±4.7

***Mortality rate (%) ± standard error; p-value**: comparison of the mortality rate between localities within in column; Within the insecticide type and locality (i.e. deltamethrin, permethrin and alpha-cypermethrin) the mortality rate with the same letter are not statistically different.

***Table 40.** Mortality rate after exposure of thirteen populations of* An. gambiae *s.l collected in seven districts to the diagnostic concentration of deltamethrin, permethrin with and without PBO, and bendiocarb with and without DEF using CDC bioassay.*

Locality*	**Delta 1×**	**Delta 1× + PBO**	**Per 1×**	**Per 1× + PBO**	**Bendio 1×**	**Bendio 1× + DEF**
Malanville	79.0±8.9^{a}	96.3±4.1^{b}	67.1±10.4^{a}	97.6±3.3^{b}	95.1±4.8^{a}	98.7±2,5^{a}
Porto-Novo	78.8±8.7^{a}	87.8±7.1^{a}	30.9±10.1^{a}	52.5±10.9^{b}	87.7±7.7^{a}	94±5.3^{a}
Kandi	38.3±17.1^{a}	86.7±7.8^{b}	51.9±15.3^{a}	97.5±3.5^{b}	96.5±4.0^{a}	100±0^{a}
Parakou	69.0±9.7^{a}	97.9±2.9^{b}	36.3±10.5^{a}	84.1±7.9^{b}	96.6±3.9^{a}	100±0^{a}
Misséreté	42.7±16.4^{a}	60.8±13.8^{b}	36.1±17.2^{a}	61.2±13.2^{b}	76.7±10.2^{a}	81.0±9.3^{a}
Cotonou	49.4±11.0^{a}	97.6±3.3^{b}	34.2±10.7^{a}	87.5±7.2^{b}	97.6±3.4^{a}	100±0^{a}
Bohicon	49.4±11^{a}	97.6±3.3^{b}	67.1±12.7^{a}	97.6±3.4^{b}	95.1±4.8^{a}	98.7±2.5^{a}
Allada	65.4±10.6^{a}	76.5±9.2^{a}	55.6±10.8^{a}	68.8±10.2^{a}	96.2±4.4^{a}	100±0^{a}
Ouidah	63±10.5^{a}	77.5±9.2^{a}	50.6±10.9^{a}	69.5±10^{b}	91.0±6.6^{a}	100±0^{b}
Dassa	39.2±10.8^{a}	67.5±10.3^{b}	37.3±10.4^{a}	66.7±10.3^{b}	87.8±7.6^{a}	96.3±4.2^{a}
Bantè	65.4±10.6^{a}	78.8±9.0^{a}	52.4±10.8^{a}	67.5±10.3^{a}	85.5±8.6^{a}	97.5±3.4^{b}
Savè	43.4±11.1^{a}	65.8±10.5^{b}	22±19.1^{a}	63.0±10.5^{b}	84.4±8.8^{a}	95.1±4.8^{a}
N'dali	61.2±10.4^{a}	80.2±8.4^{b}	38.8±10.7^{a}	72.2±9.9^{b}	85.5±8.2^{a}	96.2±4.3^{b}
p-value*	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

***Mortality rate (%) ± standard error**; **p-value**: comparison of the mortality rate between localities within in column; Within the insecticide type and locality (i.e. deltamethrin, permethrin, alpha-cypermethrin, and bendiocarb) the mortality rate with the same letter are not statistically different.

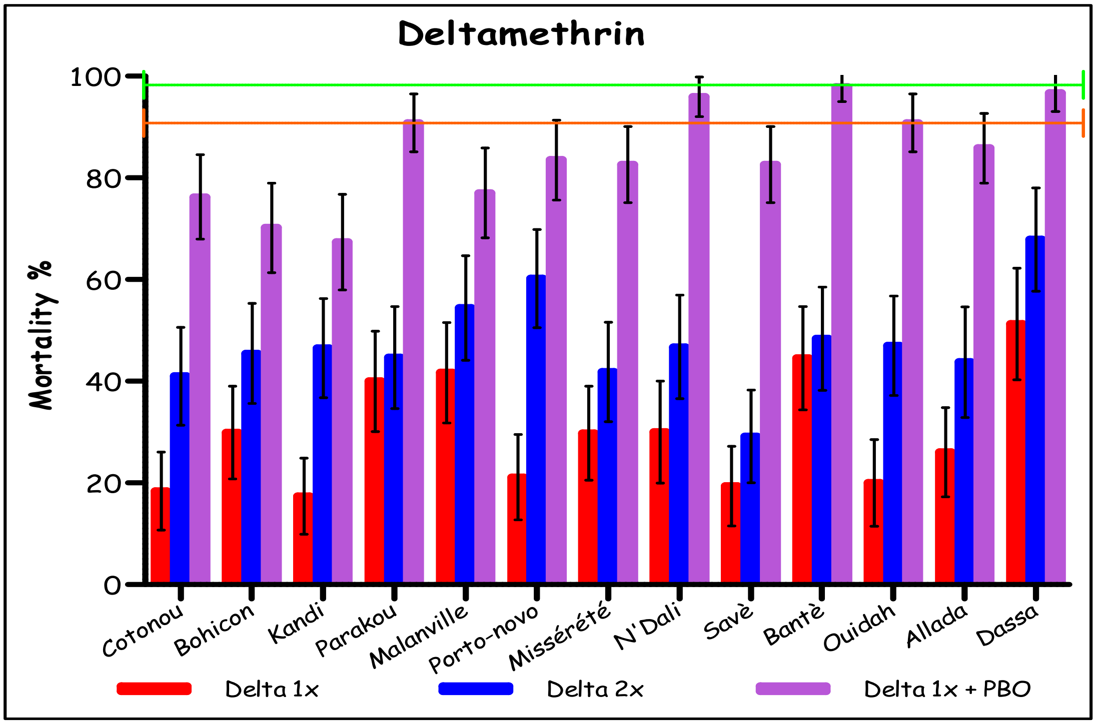

Figure 19. *Mortality rate after exposure of thirteen populations of An. gambiae s.l. to multiple diagnostic concentrations of deltamethrin with PBO using WHO bioassay from different areas.*

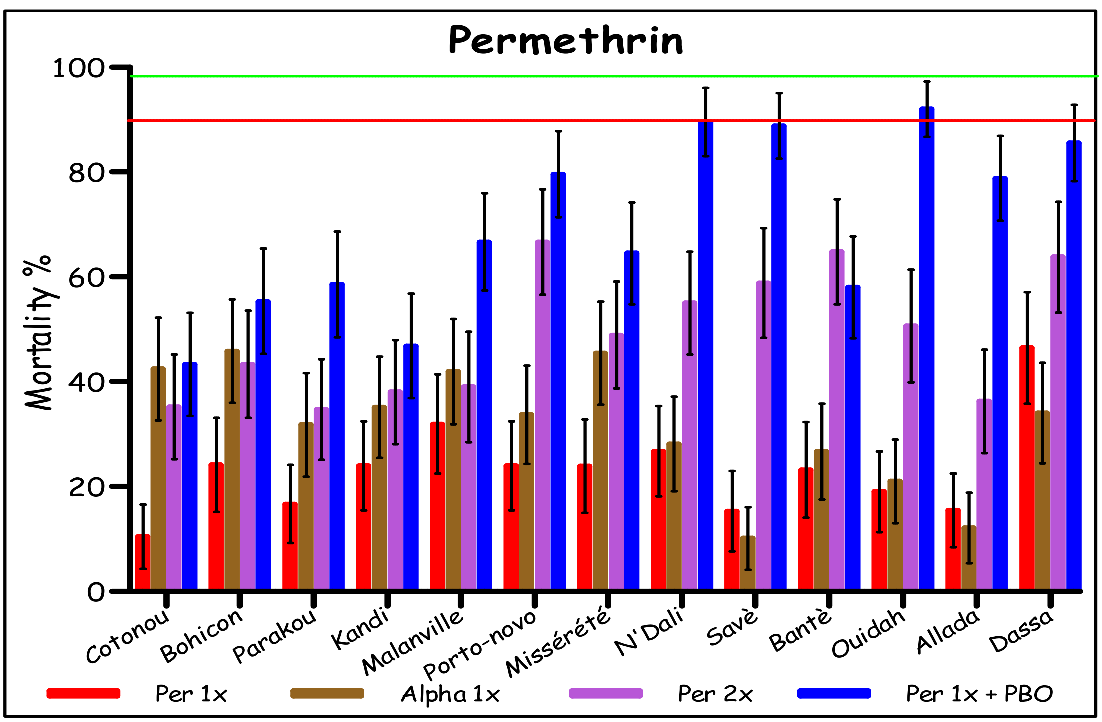

Figure 20. *Mortality rate after exposure of thirteen populations of An. gambiae s.l. to multiple diagnostic concentrations of permethrin and alpha-cypermethrin with PBO using WHO bioassay from different areas.*

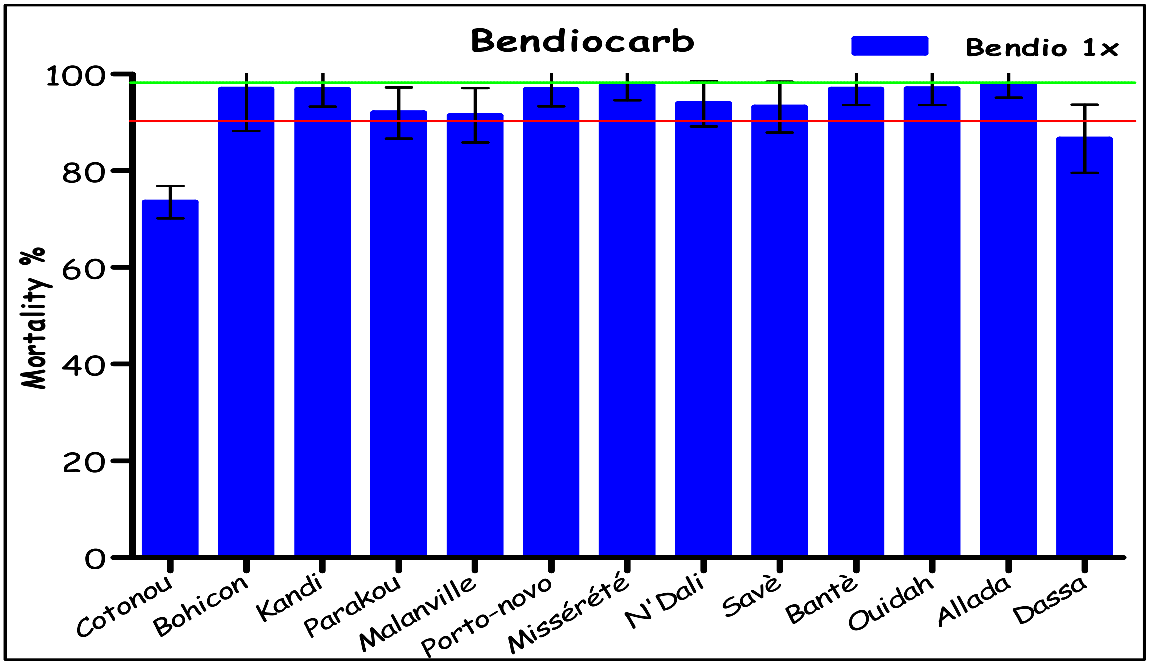

Figure 21. *Mortality rate after exposure of thirteen populations of* An. gambiae *s.l. to diagnostic concentrations of alpha bendiocarb using WHO bioassay from different areas****.***

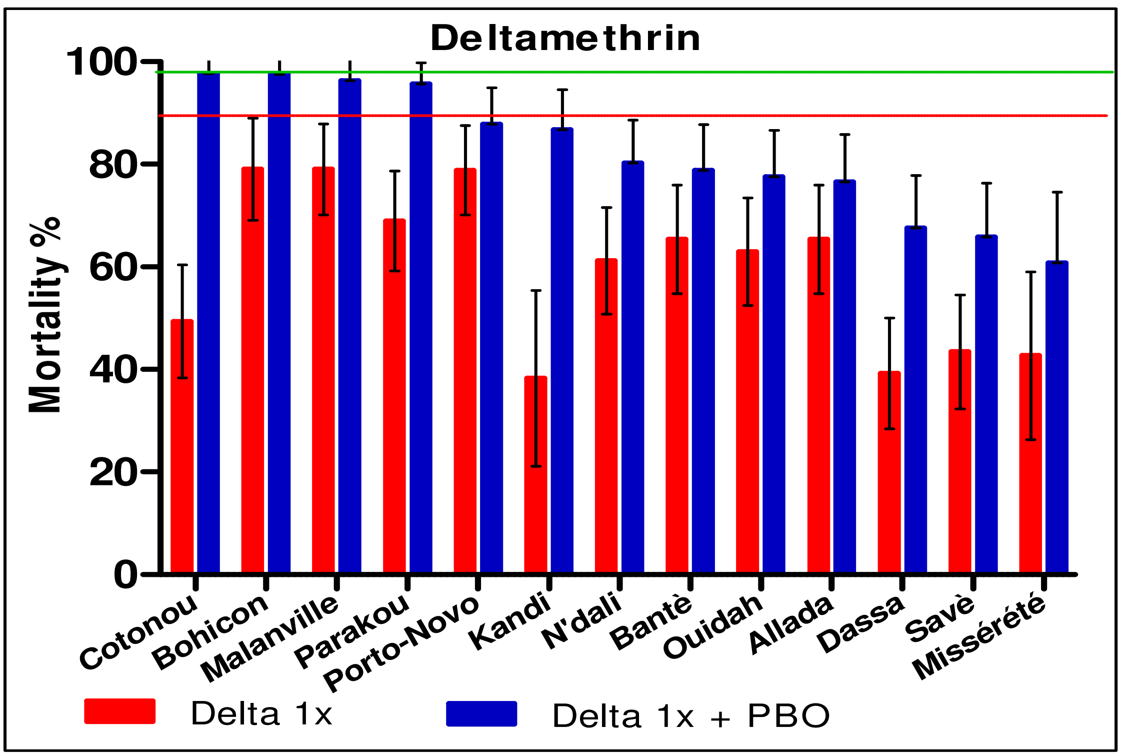

Figure 22. *Mortality rate after exposure of thirteen populations of An. gambiae s.l. to the diagnostic dose of deltamethrin associated with the PBO synergist using CDC bottle bioassays.*

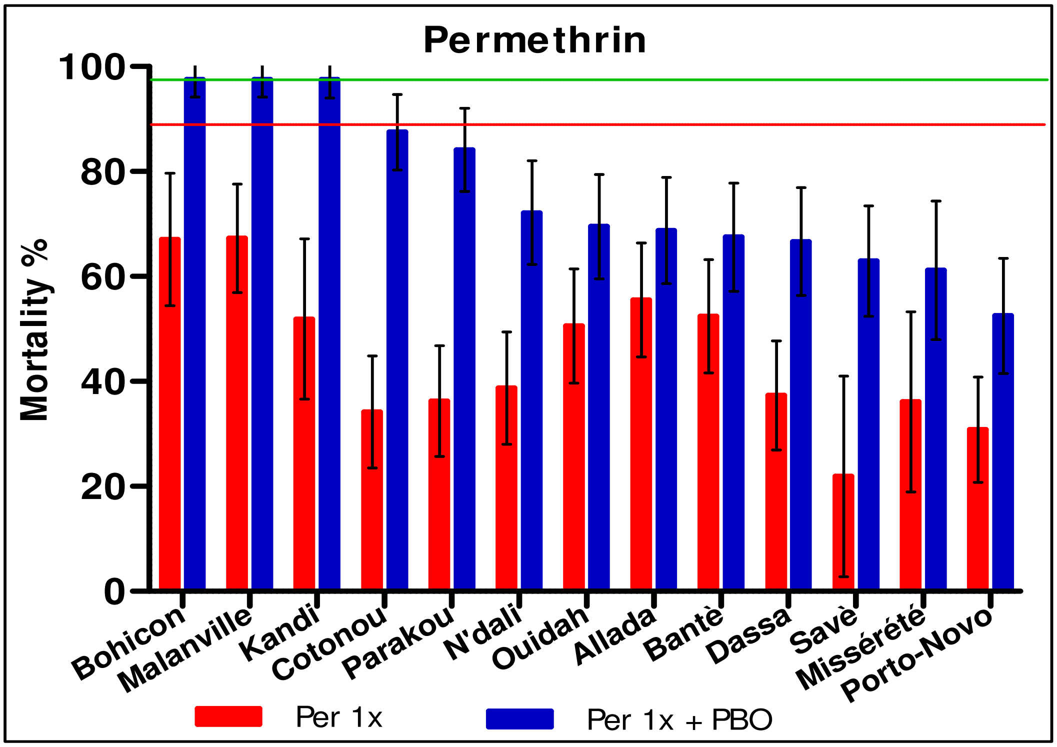

Figure 23. *Mortality rate after exposure of thirteen populations of* An. gambiae *s.l. to the diagnostic dose of permethrin associated with the PBO synergist using CDC bottle bioassays.*

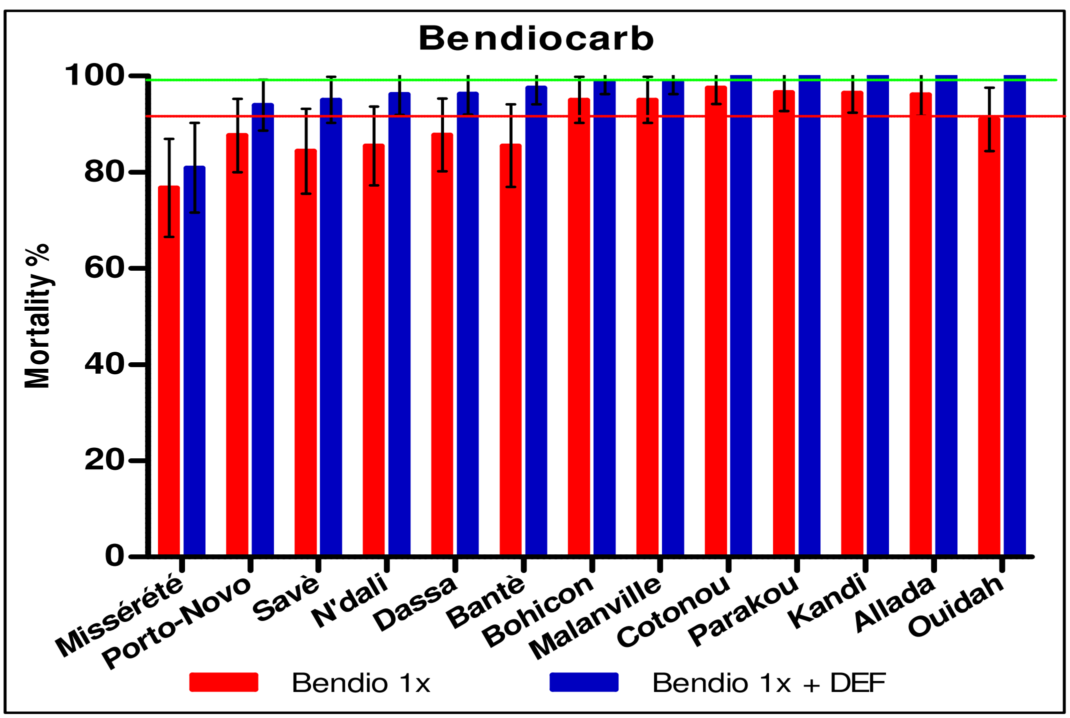

Figure 24. *Mortality rate after exposure of thirteen populations of An. gambiae s.l. to the diagnostic dose of bendiocarb associated with the DEF synergist using CDC bottle bioassays.*

3.4.2 Implication of *An. gambiae* s.l. resistance for impregnated materials (ITMs)

Efficacy tests carried out with seven types of mosquito nets (PermaNet 2.0, PermaNet 3.0, Olyset, Olyset Plus, Dawa, Yoorkol, and Aspirationel) on mosquitoes resistant to 1× and 2× doses of pyrethroids once again showed that these vectors are resistant to all the nets tested except the Olyset Plus and PermanNet 3.0. Olyset Plus and PermaNet 3.0 are new generation mosquito nets impregnated with pyrethroids associated with the PBO synergist. The recorded mortality rate varied from 91.04% to 100% for Olyset Plus and is 100% for PermaNet 3.0 in all the localities surveyed (Table 41 and Fig. 25 & 26). No dead mosquitoes were recorded after exposure of these resistant mosquitoes to the non-insecticide-treated net (control) (Table 41).

Table 41. *Mortality rate after exposure of thirteen resistance populations of* An. gambiae *s.l. to seven different net using cone bioassay from different areas.*

	LLNs test							
Locality	***Net not treated***	**Olyset Net**	**Olyset Net Plus**	**Permanet 2.0**	**Permanet 3.0**	**Yorkol**	**Dawa**	**Aspirationel**
Kandi	0.00%	83.33%	100%	07.14%	100%	39.21%	-	05.55%
Parakou	0.00%	76.92%	100%	03.63%	100%	38.46%	94.82%	03.70%
Bohicon	0.00%	66.15%	97.01%	42.10%	100%	03.70%	79.62%	86.20%
Cotonou	0.00%	96.66%	100%	08.77%	100%	14.54%	66.66%	43.39%
Malanville	0.00%	68.51%	91.07%	19.29%	100%	21.15%	25%	31.37%
Missérété	0.00%	35.29%	98.21%	14.43%	100%	26.41%	50%	23.52%
Porto-Novo	0.00%	46%	100%	12%	100%	29.41%	60.37%	13.72%
Allada	0.00%	60.00%	98.70%	12.69%	93.75%	71.21%	100%	85.13%
Ouidah	0.00%	75.00%	100%	34.48%	93.44%	76.78%	100%	72.00%
Bantè	0.00%	96.15%	100%	61.90%	100%	83.33%	93.33%	36.66%
Dassa	0.00%	42.00%	100%	26.31%	96.07%	40.67%	90.00%	38.33%
Savè	0.00%	31.03%	100%	13.69%	100%	21.21%	87.65%	28.75%
N'Dali	0.00%	91.54%	100%	31.66%	97.14%	29.62%	100%	43.54%

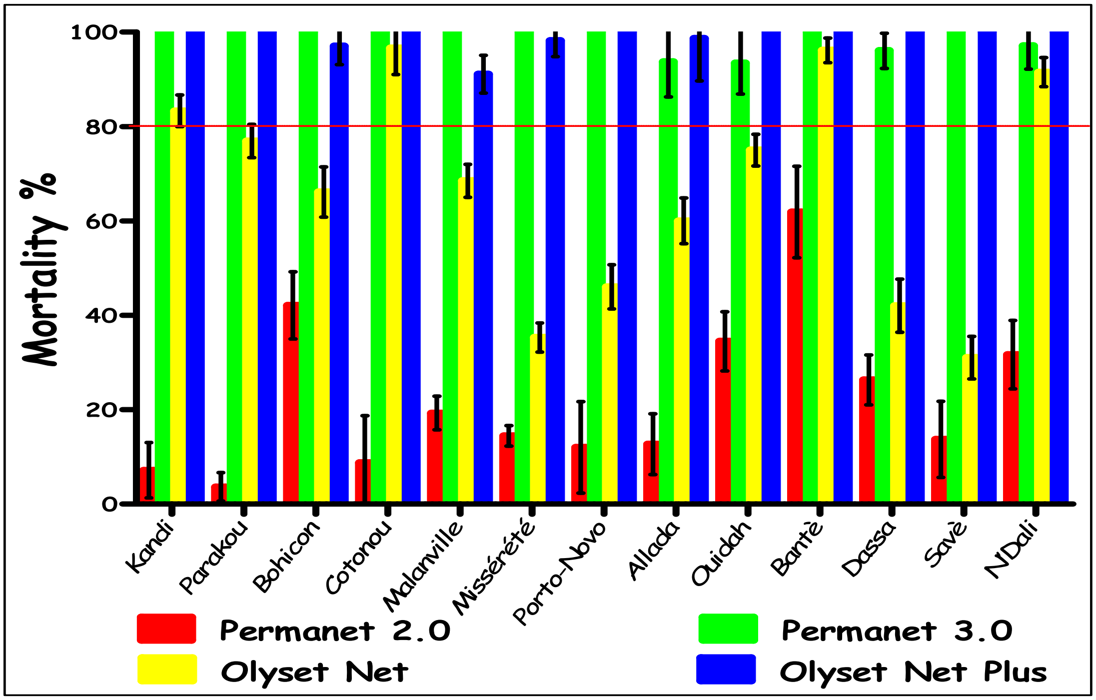

Figure 25. *Mortality rate after exposure of thirteen resistance populations of* An. gambiae *s.l. to Permanet 2.0. PermaNet 3.0. Olyset and Olyset Plus using cone bioassay from different areas.*

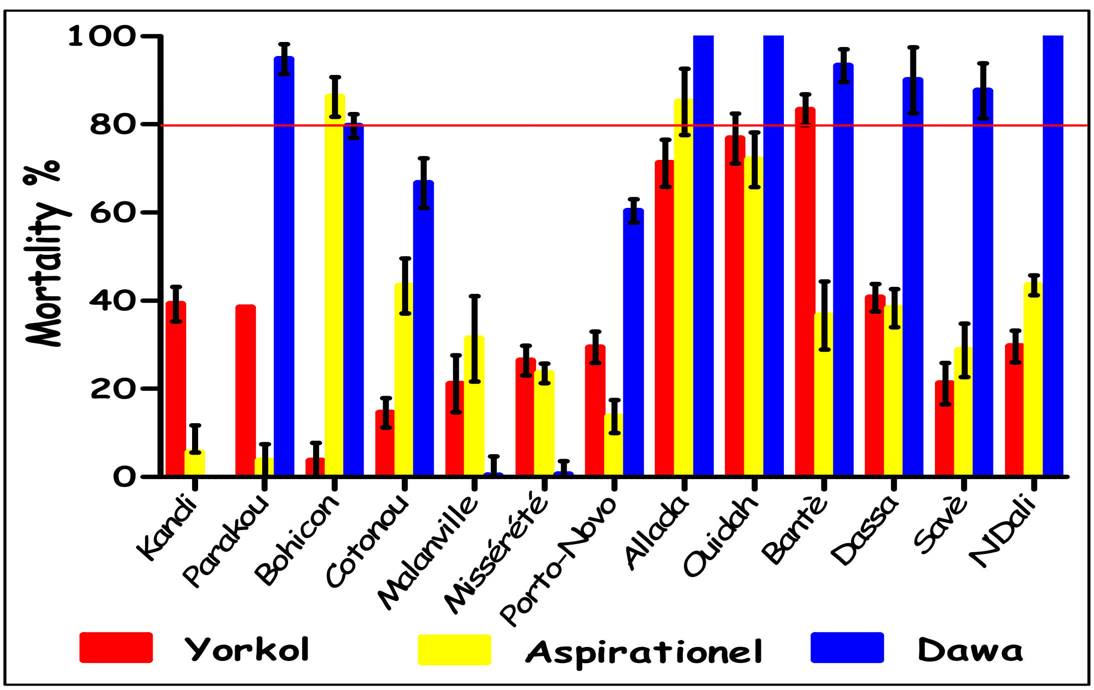

Figure 26. *Mortality rate after exposure of thirteen resistance populations of* An. gambiae *s.l. to Yorkool, Aspirational, and Dawa Plus using cone bioassay from different areas.*

3.4.3 Characterization of molecular forms and resistance genes (Kdr and Ace-1r) in the thirteen communes

The distribution of *An. gambiae* s.l. showed the presence of three main species: *An. gambiae. An. coluzzii* and *An. arabiensis* with very high proportions for *An. gambiae* in almost all localities except Malanville where all *Anopheles* analyzed were *An. coluzzii*. As for the distribution of resistance genes. the frequency of the L1014F allele of the *Kdr* gene was high in all localities. The lowest frequency of the *Kdr* resistance gene was observed in Malanville (0.57) and the highest in Cotonou (0.84). Unlike the *Kdr* resistance gene. the frequency of the G119S allele of the *Ace-1* gene was very low in all the localities surveyed (Table 42).

Table 42. *Distribution of resistance genes in An. gambiae. An. coluzzii and An. arabiensis in the thirteen communes.*

	Molecular form			Mutation *Kdr*					Mutation *Ace-1R*				
Locality	*An. arabiensis*	*An. coluzzii*	*An. gambiae*	RR	RS	SS	Freq. Kdr	P-value	RR	RS	SS	Freq. Ace1	P-value
Cotonou	0	0	50	36	12	2	**0.84[a]**		0	4	46	**0.04[a]**	
Porto-Novo	0	37	13	31	15	4	**0.77[a]**		0	1	49	**0.01[a]**	
Bohicon	0	33	17	31	15	4	**0.77[a]**		**0**	1	49	**0.01[a]**	
Misséréte	0	7	43	35	12	3	**0.82[a]**		0	6	44	**0.06[a]**	
Parakou	0	3	47	33	14	3	**0.80[a]**		0	2	48	**0.02[a]**	
Kandi	6	0	44	31	15	4	**0.77[a]**		0	0	50	**0.00[a]**	
Malanville	0	50	0	16	25	9	**0.57[b]**	**<0.0001**	0	2	48	**0.02[a]**	**0.091**
Allada	0	43	0	32	9	2	**0.84[a]**		0	2	41	**0.02[a]**	
Ouidah	0	46	0	31	9	6	**0.77[a]**		0	1	45	**0.01[a]**	
Savè	2	18	27	34	10	3	**0.82[a]**		0	1	46	**0.01[a]**	
Bantè	0	12	32	29	11	4	**0.78[a]**		0	1	45	**0.01[a]**	
Dassa	4	6	36	30	10	6	**0.76[a]**		0	1	45	**0.01[a]**	
N'Dali	1	13	32	23	13	10	**0.64[a]**		0	4	42	**0.04[a]**	

In the same column, the values indexed to the same letter are not significantly different.

3.4.4 Enzyme activities (MFO, esterase. and GST) between the thirteen wild populations and Kisumu

The activity of non-specific esterases (α and β esterases) is higher in the populations of Kandi. Parakou. Porto-Novo. Bohicon. Misséréte. Porto-Novo. Dassa. Bante. N'dali. Ouidah. and Cotonou than in the Kisumu strain ($p < 0.05$) (Table 43 & Fig. 27). Mixed-function oxidase activity is higher in the Cotonou populations than in those of Kisumu ($p < 0.0001$) (Table 43 & Fig. 28). The highest glutathione S-transferase (GST) activity was observed in the Cotonou. Kandi. Parakou. Bohicon. and Save populations compared to the Kisumu strain (p < 0.0001) (Table 43 & Fig. 29).

Table 43. *Mean (± SE) mixed-function oxidases. glutathione-S-transferases and esterases activities in* An. gambiae *s.l. populations.*

Strain	MFO	GST	α-Esterase	β-Esterase
	(nmol/P450/ min/mg protein)	(ɳmol/GSH conj/min/mg protein)	(µmol α Naph/ /min/mg protein)	(µmol β Naph /min/mg protein)
Kisumu*	0.060±0.008[a]	0.222±0.027[a]	0.101±0.010[a]	0.091±0.009[a]
Cotonou	0.116±0.011[b]	0.388±0.035[b]	0.129±0.027[a]	0.160±0.012[b]
Malanville	0.070±0.009[a]	0.305±0.044[a]	0.083±0.011[b]	0.084±0.012[a]
Porto-Novo	0.083±0.013[a]	0.255±0.025[a]	0.135±0.008[a]	0.123±0.008[a]
Misséréte	0.083±0.011[a]	0.341±0.028[a]	0.105±0.020[a]	0.131±0.022[b]
Bohicon	0.046±0.005[a]	0.410±0.037[b]	0.154±0.013[a]	0.159±0.015[b]
Parakou	0.057±0.007[a]	0.333±0.045[a]	0.141±0.012[a]	0.147±0.013[b]
Kandi	0.079±0.013[a]	0.405±0.054[b]	0.127±0.014[a]	0.133±0.016[b]
Dassa	0.061±0.009[a]	0.330±0.039[a]	0.114±0.013[a]	0.123±0.013[b]
Savè	0.051±0.007[a]	0.438±0.086[b]	0.067±0.007[b]	0.078±0.010[a]
Allada	0.060±0.012[a]	0.348±0.051[a]	0.084±0.010[b]	0.085±0.009[a]
Ouidah	0.044±0.008[a]	0.212±0.109[a]	0.093±0.014[b]	0.094±0.013[a]
Bantè	0.049±0.009[a]	0.313±0.037[a]	0.127±0.008[a]	0.130±0.008[b]
N'dali	0.072±0.012[a]	0.318±0.070[a]	0.110±0.010[a]	0.118±0.022[a]
One Way ANOVA	F=12.47; df=13. 574; P<0.0001	F=06.29; df=13. 574; P<0.0001	F=14.06; df=13. 574; P<0.0001	F=14.24; df=13. 574; P<0.0001

*Kisumu = susceptible *An. gambiae* ss colony; MFO= mixed-function oxidases; GST = glutathione-S-transferase; Mean followed by a different letter were significantly different. P<0.05. Tukey's test. *Significant increase in mean differences compared to the laboratory reference strain. P<0.05. t-test. In the same column, the values indexed to the same letter are not significantly different.

✔ **Non-specific esterase activity (α and β esterases)**

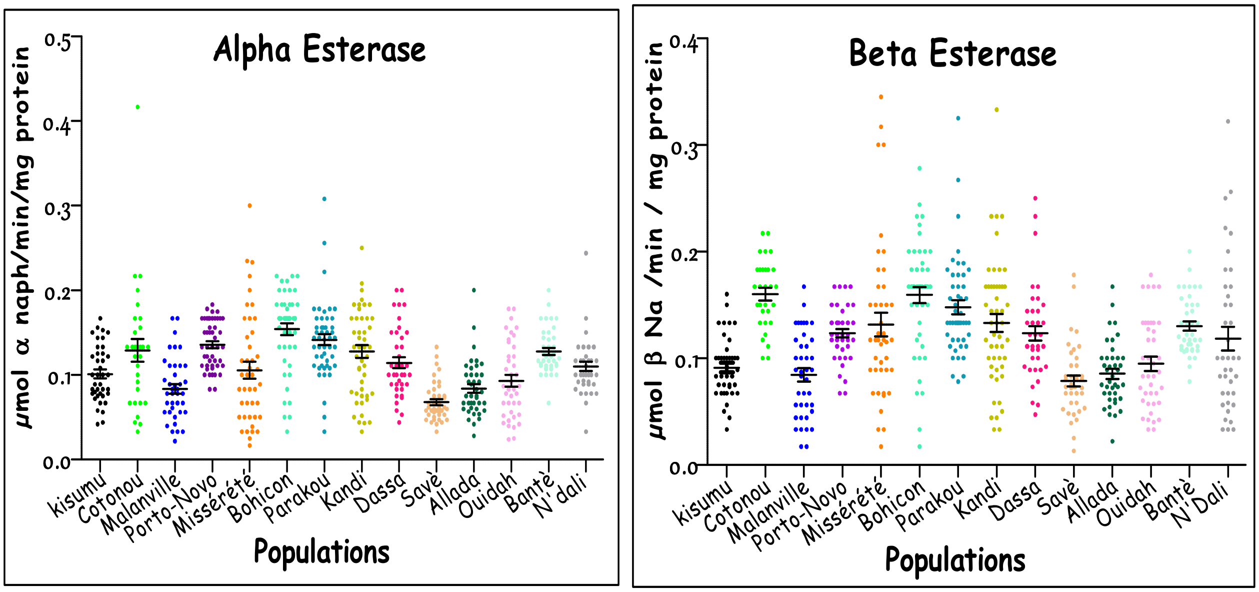

Figure 27. Graph showing results of biochemical test for non-specific esterase activity

✔ **Oxidase activity**

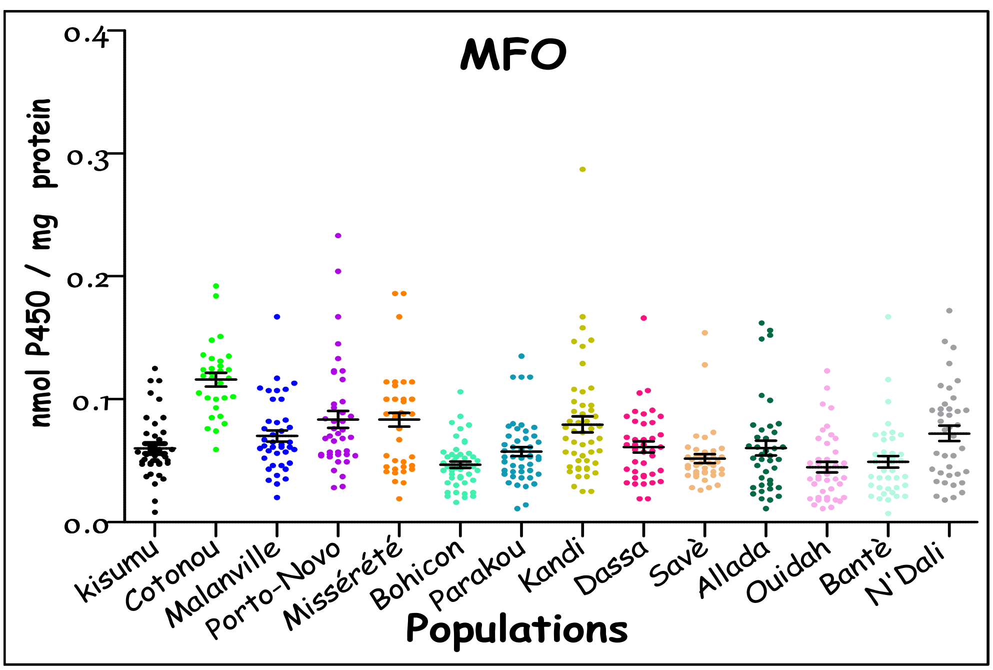

Figure 28. Graph showing results of biochemical test for oxidase (MFO) activity

✔ **Glutathione S-transferase activity (GST)**

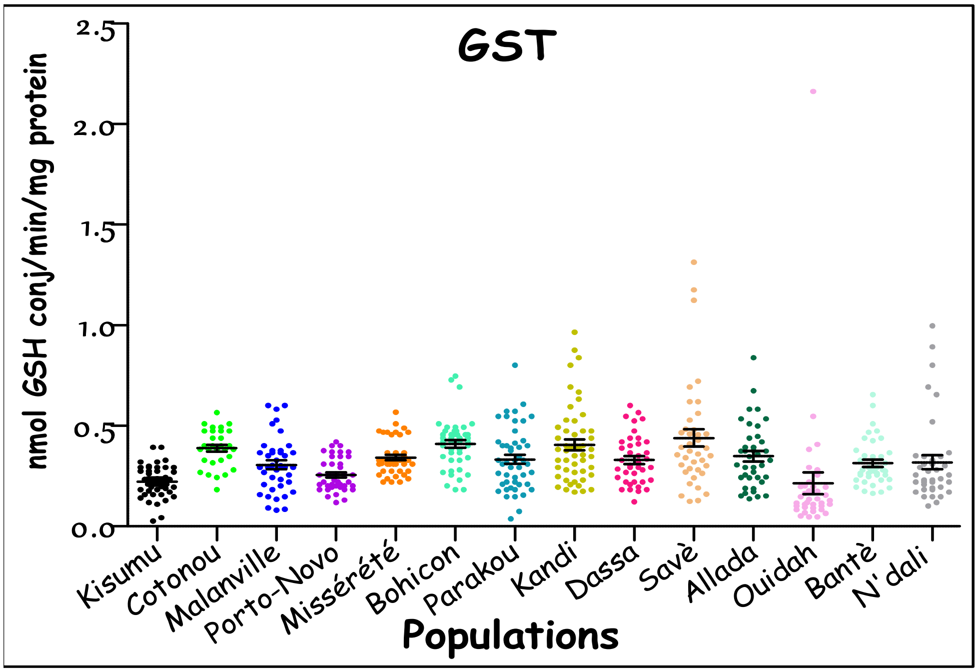

Figure 29. *Graph showing results of biochemical test for glutathione S-transferase (GST) activity*

3.5 Conclusion

Mosquito susceptibility to permethrin, deltamethrin, alphacypermethrin and bendiocarb followed the same trend in the thirteen different communes: strong resistance to these insecticides due to several mechanisms (i.e. *L1014F Kdr*, overexpression of oxidases, esterases, and glutathione S-transferase). However, mosquitoes resistant to these different insecticides were susceptible to pyrethroid-impregnated nets combined with PBO (PermaNet 3.0 and Olyset Plus). This result suggests that PBO ITNs are likely to perform better than standard ITNs which may sustain the gains of the NMCP's malaria control efforts.

3.6 Challenges

Our next challenge is to identify the other mechanisms involved in the resistance of these vectors including the level of expression of resistance genes through qPCR. The achievement of these results will allow the NMCP to define other vector resistance management strategies in the effective fight against malaria in Benin.