

Vector-Borne and Zoonotic Diseases

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Pyrethroid resistance intensity in *An. gambiae* s.l. from different agricultural production zones in Benin, West Africa

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	composed of <i>An. gambiae</i> s.s., <i>An. coluzzii</i> and <i>An. arabiensis</i> , with mean frequency of the L1014F kdr mutation > 75%. Overexpression of non-specific α and β esterases was observed in the cereal, cotton and urban market gardening areas, while an overexpression of mixed function oxidases (MFOs) was observed in the cotton and rice growing areas. Overall, <i>An. gambiae</i> s.l. showed high resistance intensity to both deltamethrin and permethrin. The synergist and biochemical tests performed, suggest that PBO LLINs may provide a greater control of pyrethroid-resistant mosquitoes.

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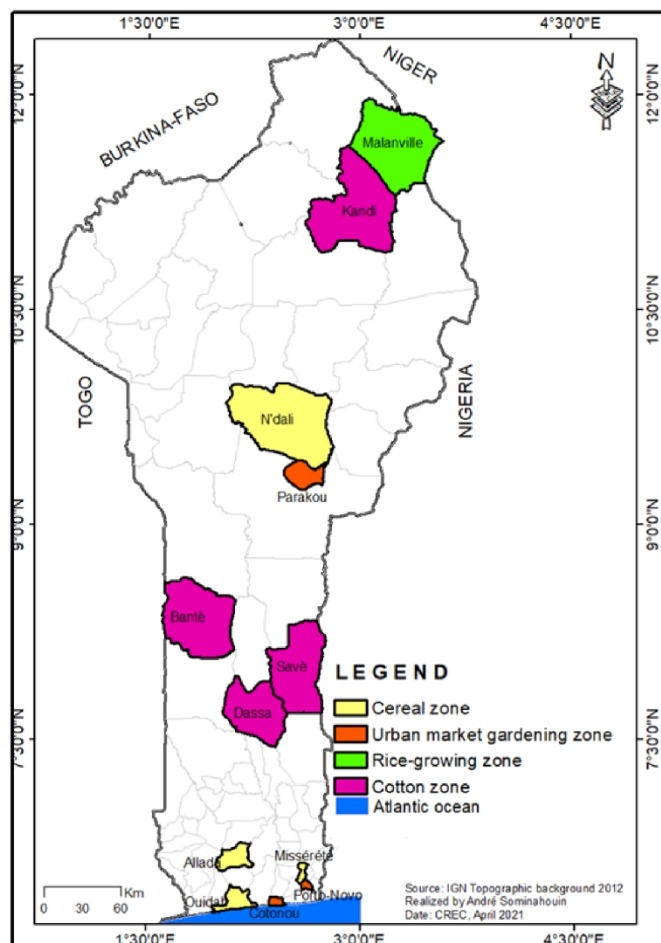


Figure 1: Map of the study area

210x297mm (200 x 200 DPI)

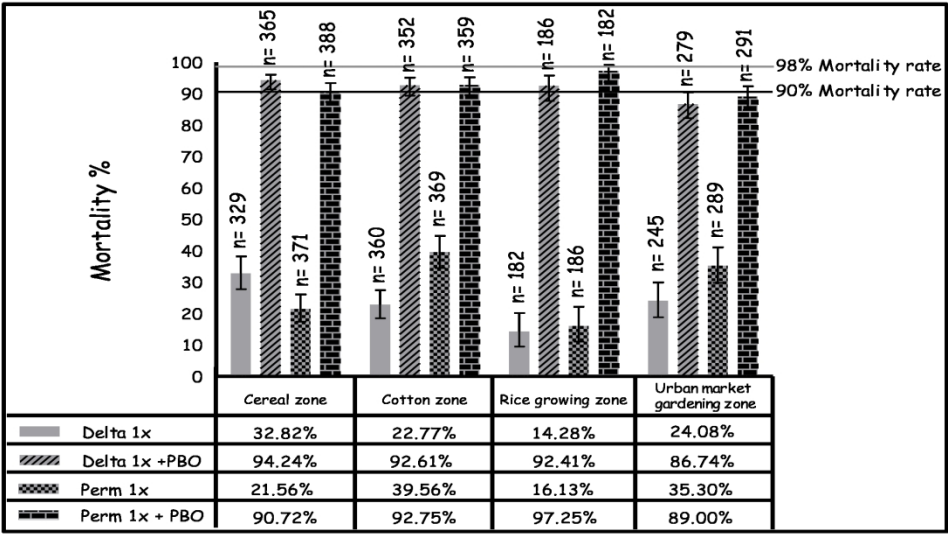


Figure 2: Mortality rates observed in *An. gambiae* s.l. after exposure to pyrethroids (permethrin or deltamethrin) alone and PBO + pyrethroids test (error bars represent the confidence intervals)

297x210mm (200 x 200 DPI)

Table 1: Pyrethroid resistance intensity assay results of *An. gambiae* s.l. collected from the 4 agricultural production zones

	Cereal zone	Cotton zone	Rice growing zone	Urban market gardening zone
Insecticide dose	% (95% CI), N	% (95% CI), N	% (95% CI), N	% (95% CI), N
Perm 1x	21.56 (17.48-26.10), 371	39.56 (34.54-44.75), 369	16.13 (11.15-22.21), 186	35.30 (29.79-41.10), 289
Perm 2x	45.26 (40.18-50.42), 380	66.30 (61.17-71.15), 362	46.15 (38.75-53.68), 182	52.15 (45.83-58.43), 255
Perm 5x	63.77 (58.45-68.84), 345	76.53 (71.79-80.83), 358	90.62 (85.58-94.34), 192	69.00 (63.43-74.19), 300
Perm 10x	88.36 (84.32-91.67), 318	88.74 (85.08-91.76), 373	97.89 (94.69-99.42), 190	96.44 (93.55-98.28), 281
Delta 1x	32.82 (27.77-38.19), 329	22.77 (18.54-27.46), 360	14.28 (09.55-20.23), 182	24.08 (18.87-29.94), 245
Delta 2x	47.50 (42.51-52.52), 400	48.47 (43.19-53.77), 359	67.04 (59.57-73.93), 176	49.44 (43.29-55.59), 267
Delta 5x	68.17 (63.04-72.98), 355	63.18 (58.00-68.15), 364	85.71 (79.77-90.45), 182	54.54 (48.57-60.41), 286
Delta 10x	92.00 (88.89-94.46), 400	84.15 (80.12-87.65), 385	95.70 (91.70-98.12), 186	88.61 (83.85-92.35), 237

N: Total number of mosquito tested, CI: confidence interval, Perm: Permethrin, Delta: Deltamethrin, x: Time

Table 3: Allelic frequencies of the *Kdr* L1014F mutation in dead and live specimens of *An. gambiae* s.l. collected from the 4 agricultural production zones

Agricultural production zones	Status	N tested	L1014F <i>kdr</i> genotypes			Freq (%)	95%IC
			1014F	1014L	1014L		
Cereal zone	Dead	100	60	24	16	72.00	65.23-78.10
	Live	100	70	24	6	82.00	75.96-87.06
Cotton zone	Dead	100	64	26	10	77.00	70.54-82.64
	Live	100	71	23	6	82.50	76.51-87.49
Rice growing zone	Dead	50	22	18	10	62.00	51.74-71.52
	Live	50	23	21	6	67.00	56.88-76.08
Urban market gardening zone	Dead	75	52	19	4	82.00	74.90-87.79
	Live	75	57	14	4	85.33	78.64-90.57

N: number, *kdr* : knock down resistance, Freq : frequency of the L1014F *kdr* mutation

Table 4: Measure of activity of metabolic enzymes in *An. gambiae* s.l. collected from the four agricultural production zones and in the Kisumu susceptible strain.

Strain / agricultural production zones	Activity of metabolic enzymes			
	α -Esterase ($\mu\text{mol } \alpha \text{ Naph/min/mg protein}$)	β -Esterase ($\mu\text{mol } \beta \text{ Naph/min/mg protein}$)	MFOs (nmol/P450/min/mg protein)	GST (nmol/GSH conj/min/mg protein)
Kisumu (Control)	0.097 ^a (0.091-0.103)	0.082 ^a (0.076-0.088)	0.048 ^a (0.044-0.053)	0.233 ^a (0.218-0.249)
Cereal zone	0.102 ^a (0.091-0.114)	0.118 ^b (0.104-0.132)	0.048 ^a (0.044-0.052)	0.352 ^b (0.322-0.382)
Cotton zone	0.141 ^b (0.125-0.152)	0.142 ^b (0.128-0.155)	0.067 ^b (0.061-0.074)	0.265 ^a (0.239-0.290)
Rice growing zone	0.112 ^a (0.100-0.124)	0.105 ^{ab} (0.098-0.112)	0.099 ^c (0.090-0.108)	0.200 ^a (0.175-0.225)
Urban market gardening zone	0.117 ^a (0.108-0.126)	0.127 ^b (0.117-0.138)	0.055 ^a (0.049-0.061)	0.302 ^b (0.268-0.336)

^{a,b}: Mean with different superscripts in the same column are significantly different ($p < 0.05$), MFOs: mixed function oxidases

Table 5: Effect of the L1014F *kdr* mutation, molecular species and agricultural production zones on the mortality of *An. gambiae* s.l. to deltamethrin 1x

Explanatory variables	Df	Deviance	Resid. Df	Resid. Dev	P (>Chi)
NULL	-	-	649	901.09	-
L1014F <i>Kdr</i> mutation	2	5.5211	644	895.57	0.06
Molecular species	2	4.1530	641	891.42	0.24
Zones	3	0.0000	646	901.09	1

Df: Degree of freedom, Resid. Df: residual degree of freedom, Resid. Dev: Residual deviance

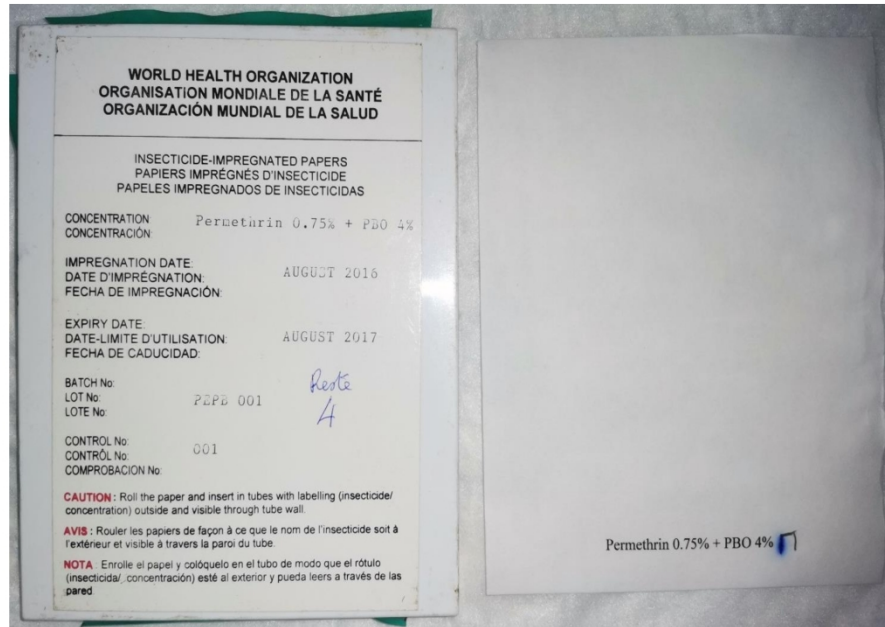


Fig 3 (WHO single Paper combining both PBO + insecticide)

279x215mm (200 x 200 DPI)

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2 **Pyrethroid resistance intensity in *An. gambiae* s.l. from different agricultural production zones in**
3 **Benin, West Africa**

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Abstract

Agricultural production activities usually occur in Benin with the use of a huge amount of insecticides including pyrethroids for pest control. It is therefore important to regularly monitor pyrethroid resistance intensity in *An. gambiae* s.l., the main malaria vector. The present study was conducted in cereal, cotton, rice growing and, urban market gardening areas throughout the country in 2018 and 2019. Females *An. gambiae* s.l. field-collected as larvae were exposed to deltamethrin 1x (0.05%), 2x (0.1%), 5x (0.25%) and 10x (0.5%) and, permethrin 1x (0.75%), 2x (1.5%), 5x (3.75%) and 10x (7.5%). Synergist assays were also performed using WHO papers combining PBO (4%) + deltamethrin 1x and, PBO (4%) + Permethrin 1x. Molecular species and L1014F *kdr* mutation were identified using PCR. Expression of metabolic enzymes was also assessed through biochemical tests. After exposure to permethrin and deltamethrin 10x, *An. gambiae* s.l. displayed mortality rates <98%. Synergist assays induced significantly higher mortality rates than pyrethroids alone ($p < 0.05$). *An. gambiae* s.l. complex was composed of *An. gambiae* s.s., *An. coluzzii* and *An. arabiensis*, with mean frequency of the L1014F *kdr* mutation > 75%. Overexpression of non-specific α and β esterases was observed in the cereal, cotton and urban market gardening areas, while an overexpression of mixed function oxidases (MFOs) was observed in the cotton and rice growing areas. Overall, *An. gambiae* s.l. showed high resistance intensity to both deltamethrin and permethrin. The synergist and biochemical tests performed, suggest that PBO LLINs may provide a greater control of pyrethroid-resistant mosquitoes.

Keyword index: *An. gambiae* s.l., pyrethroid resistance intensity, PBO, LLINs.

Background

For some years, Benin has based its vector control policy on the use of long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS). Distribution of LLINs incorporating pyrethroids, sole class of insecticide recommended by the World Health Organization (WHO) until recently for treating LLINs (WHOPES, 2014), generally occurs every 3 years. Moreover, insecticides recommended by WHO (WHOPES, 2015) and used for IRS in Benin since 2008, include carbamates (Bendiocarb) (Akogbéto et al., 2011) and organophosphates (Actellic 50 EC) (Aïkpon et al., 2014). It is therefore not surprising that malaria vectors develop resistance to these different classes of insecticides deployed in the country ~~is a reality~~. In Benin, malaria vectors include the *An. gambiae* complex that is the main one, followed by *An. funestus* group and, *An. nili* group (Osse et al., 2019). The *An. gambiae* complex is composed of a mixture of *An. gambiae* s.s., *An. coluzzii*, *An. arabiensis* and *An. melas* (Gnanguenon et al., 2015), while the *An. funestus* group and, *An. nili* group are composed of *An. funestus* s.s. and, *An. nili* s.s., respectively (Tchigossou et al., 2017, Osse et al., 2019). ~~Indeed, o~~ Over the last decade, the *An. gambiae* complexes ~~the main malaria vector in Benin~~, has developed a confirmed resistance to pyrethroids countrywide (Yadouleton et al., 2010; Djègbè et al., 2011, Padonou et al., 2012, Aïzoun et al., 2013, Gnanguenon et al., 2015, Salako et al., 2018, Sovi et al., 2020), and carbamates in specific sites (Aïkpon et al., 2013). This phenomenon affects also several other countries in West, East, Central and South Africa (World Malaria Report 2020). In Benin, commonly detected insecticide resistance mechanisms include the west *kdr* mutation (replacement of a leucine by a phenylalanine at position 1014 of the voltage-gated sodium channel), the *Ace-1^R* (substitution of a glycine by a serine at position 119), as well as the overexpression of metabolic enzymes such as esterases, oxidases and, glutathione-S-transferases (GST) (Salako et al., 2018). Although the community effectiveness of LLINs (Lengeler et al., 2004) and IRS (Pluess et al., 2010) is documented and beyond doubt, the emergence and widespread of vector resistance to insecticides might seriously undermine their efficacy.

According to Bhatt et al. (2015), LLINs are critically important as they contributed for up to 68% in the reduction of the number of malaria cases between 2000 and 2015 in Africa. Although pyrethroid resistance is widespread in Benin, it is unknown how intense this resistance is. It is therefore important to perform regularly, a nationwide monitoring of resistance intensity of *An. gambiae* s.l. to pyrethroid insecticides commonly incorporated to LLINs. Synergists (Piperonyl Butoxide: PBO, or DEF) bioassays are also important to perform as these products inhibit expression of metabolic

enzymes, thus help evaluating their potential involvement in vector resistance to insecticides (Brogdon et al., 1998). All these data have an important role to play in decision making regarding vector control products (PBO LLINs or others) that must be procured to improve the control of insecticide-resistant vectors. In addition, the agricultural production in Benin is highly dependent on the use of insecticide. Consequently, the present study was carried out in different agricultural production zones located throughout the country and where insecticides are highly used (Gouda et al. 2018). Data collected will guide National Malaria Control Program (NMCP) in refining its malaria control strategy. In addition, the pyrethroid resistance intensity of the populations of *An. gambiae* s.l., as well as their response after exposure to the PBO synergist were evaluated. In addition, the level of resistance mechanisms (target site modification and, biochemical) involved was also determined. Data collected will guide National Malaria Control Program (NMCP) in refining its malaria control strategy.

Materials and methods

Study area

Overall, Benin has 77 districts. The present study was carried out in 12 districts selected at random in the South, Centre and North of the country. These districts were subsequently grouped into 4 agricultural production zones, taking into account the specific crop types for each area. The four defined agricultural production zones are shown in Figure 1 and include:

- The cotton zone that consists of the districts of Kandi, Bantè, Dassa and Savè. This zone is characterized by a high use of insecticides (carbamates, organophosphates and, pyrethroids) against cotton pests. All districts are characterized by a dry season and a rainy season covering the periods from November to April and, May to October, respectively. The districts of Dassa, Savè and Bantè are characterized by a subequatorial climate with an average annual rainfall of 1100 mm while Kandi has a sudano-sahelian climate with an average annual rainfall of 1030 mm (INSAE, 2018).
- The rice-growing zone made up solely of the Malanville district that has a production perimeter of around 70 hectares. This area is characterized by a sudano-sahelian climate with a dry season covering the period from November to April and a rainy season ranging from May to October. The mean annual rainfall is about 750 mm per year. Malanville is an area that borders the Niger River and, is subject to flooding, which allows full-time rice production with a high use of pyrethroids and organophosphates (INSAE, 2018).

- The urban market gardening zone which includes the districts of Cotonou, Porto-Novo and Parakou where pyrethroids and organophosphates are widely used on market gardening perimeters and in domestic environments for protection against mosquito bites. The districts of Cotonou and Porto-Novo are characterized by a subequatorial climate, with an alternation of two dry seasons (August-September and, December-March) and two rainy seasons (April-July and, October-November). The average annual rainfall varies between 900 and 1500 mm. The Parakou district is characterized by a South Sudanese climate with one dry season (November to April) and one rainy season (May to October). The average annual rainfall is 1200 mm (INSAE, 2018).
- The cereal zone formed by the districts of Missérété, Ouidah, Allada and N'Dali. The crops produced there are mainly maize, yams, cassava, soybeans, peanuts and cowpeas with use of pyrethroids and organophosphates. Missérété, Ouidah and Allada are characterized by a subequatorial climate, with two dry seasons (August-September and, December-March) and two rainy seasons (April-July and, October-November). The annual average precipitation is 1500 mm. N'Dali has a sudano-sahelian climate with a dry season (November to March) and a rainy season (April to October). The average annual rainfall is 1030 mm (INSAE, 2018).

Mosquito larvae collections and rearing

Larvae collections were carried out in the 4 agricultural production zones, from January 2018 to January 2019, using the dipping technique (O'malley, 1995). Thus, the larvae were collected from the positive larval habitats using a ladle. They were transported to the insectary of the Center for Research in Entomology of Cotonou (CREC) for rearing. The pupae obtained were then grouped together in different cages (according to the 4 agricultural production zones) where they grew until the emergence of adult mosquitoes. After morphological identification of mosquitoes using the Gillies & de Meillon determination key, only specimens of *An. gambiae* s.l. were used for WHO susceptibility testing (Gillies & de Meillon, 1968).

WHO susceptibility tube testing

Specimens of females *An. gambiae* s.l. aged 2-5 days were used for WHO susceptibility tube testing. The tests were conducted by exposing for 60 minutes, batches of 20-25 female mosquitoes to Deltamethrin 1x (0.05%), 2x (0.1%), 5x (0.25%) and, 10x (0.5%) and, Permethrin 1x (0.75%), 2x (1.5%), 5x (3.75%) and, 10x (7.5%) to assess the intensity of resistance to these two insecticides.

Synergist assays were also performed by exposing populations of *An. gambiae* s.l. to single WHO papers combining both using WHO papers combining PBO (4%) ~~and~~ deltamethrin 1x (0.05%) ~~and~~ PBO (4%) and ~~permethrin 1x (0.75%)~~. This allowed assessing the involvement of mixed function oxidases (MFOs) in resistance to the two tested pyrethroid insecticides.

For all tests performed, the number of mosquitoes that fell under the shock of the insecticide was recorded every 15 minutes. Batches of 20-25 mosquitoes exposed to non-impregnated papers served as controls.

Post-exposure, the mosquitoes were transferred to the observation tubes where they were fed with a 10% sweetened juice for 24 hours. Mortality rates were determined 24 hours post-testing (WHO, 2016).

Polymerase Chain Reaction (PCR) for identification of sibling species and L1014F kdr mutation in *An. gambiae* s.l.

In each agricultural production zone, about 100-200 mosquito specimens that included both a subsample of dead and live ~~mosquitoes~~ obtained after exposure to deltamethrin 1x, were analyzed through PCR to determine the molecular species and, the presence of the L1014F *kdr* mutation following the protocols of Santolamazza et al. (2008) and, Martinez-Torres et al. (1998), respectively.

Bio-chemical assays

In each agricultural production zone, about 150 live females of *An. gambiae* s.l., aged 2 to 5 days, were used for the detection of metabolic enzymes (α -Esterase, β -Esterase ~~and~~, Oxidases and, GST) using the protocol described by Hemingway et al. (1998). Levels of expression of metabolic enzymes in *An. gambiae* s.l. collected from the four agricultural production zones were compared to the one of the Kisumu susceptible strain used as negative controls.

Statistical Analysis

Mortality rates recorded 24 hours post-resistance intensity testing were interpreted according to the World Health Organization criteria (WHO, 2016):

- Low resistance intensity (Mortality less than 90% at the 1x dose, but ranging 98-100% at the 5x dose);
- Moderate resistance intensity (Mortality less than 98% at the 5x dose, and ranging 98-100% at the 10x dose); and

- High resistance intensity (Mortality less than 98% at the 10x dose).

Involvement of metabolic mechanisms in resistance of *An. gambiae* s.l. to diagnostic dose of an insecticide is determined using the following criteria:

- Non-involvement (Insecticide-synergist mortality not greater than for insecticide alone)
- Partial involvement (Insecticide-synergist mortality less than 98%, and greater than for insecticide alone)
- Full involvement (Insecticide-synergist mortality superior or equal to 98%, and greater than for insecticide alone)

Allelic frequencies of the L1014F *Kdr* mutation were determined through this formula:

$$F = [2n.RR + n.RS] / [2(n.RR + n.RS + n.SS)]$$
 (n.= number of mosquito specimens having a given genotype).

The exact binomial test was used to calculate the confidence intervals for the mortality rates and the frequencies of the L1014F *kdr* mutation.

A logistic regression was performed to assess the impact of the L1014F *kdr* mutation, molecular species and agricultural production zones on the mortality of *An. gambiae* s.l. to deltamethrin 1x (0.05%).

The Mann-Whitney U test was used to compare the activity of the metabolic enzymes between the laboratory susceptible strain (Kisumu) and those collected from the four agricultural production zones. All statistical analyzes were carried out using R statistical software, version 3.6.2.

Results

Resistance intensity of An. gambiae s.l. from the 4 agricultural production zones to permethrin and deltamethrin

Table 1 shows results obtained after exposing *An. gambiae* s.l. from the 4 surveyed agricultural production zones to permethrin and deltamethrin at the 1x, 2x, 5x and 10x doses. Mortality rates of populations of *An. gambiae* s.l. exposed to permethrin 10x ranged from 88.36% (95% CI: 84.32-91.67) in the cereal zone to 97.89% (95% CI: 94.69-99.42) in the rice growing area. With deltamethrin 10x, *An. gambiae* s.l. showed a mortality rate varying from 84.15% (95% CI: 80.12-87.65) in the cotton zone to 95.70% (95% CI: 91.70-98.12) in the rice growing area. Overall, resistance intensity to both permethrin and deltamethrin was high at all surveyed zones. Moreover, increased mortalities are observed as the insecticide dose increases.

Synergist assays

Synergist assays performed using piperonyl-butoxide (PBO) allowed assessing the involvement of mixed function oxidases (MFOs) in resistance of *An. gambiae* s.l. to pyrethroids in the study area. Indeed, pre-exposure of *An. gambiae* s.l. to PBO induced significantly higher mortality rates compared to permethrin or deltamethrin alone ($p < 0.05$) in all four surveyed agricultural production zones, though full susceptibility was not reached (Figure 2). This means that mixed function oxidases (MFOs) were then partially implicated in phenotypic resistance of *An. gambiae* s.l. to permethrin and deltamethrin.

Frequency of molecular species and L1014F *kdr* mutation in *An. gambiae* s.l.

Table 2 displays the frequencies of identified molecular species and L1014F *kdr* mutation in the four surveyed agricultural production zones.

Overall, three molecular species were identified in the study area, with *An. gambiae* s.s. (58.61%, 381/650) being the predominant one, followed by *An. coluzzii* (38.77%, 252/650) and, *An. arabiensis* (2.62%, 17/650).

Depending on the agricultural production zone, frequency of the L1014F *kdr* mutation ranged from 79.2% (95% CI: 72.50-84.92) to 86.3% (95% CI: 80.95-90.18) in *An. gambiae* s.s., from 75% (95% CI: 19.41-99.37) to 76.7% (95% CI: 66.39-85.18) in *An. coluzzii* and, from 62.5% (95% CI: 24.48-91.47) to 100% in *An. arabiensis*. ~~For the whole study area, the mean frequency of the L1014F *kdr* mutation was 71.4% (95% CI: 67.26-75.34), 81.9% (95% CI: 78.96-84.56) and, 76.5% (95% CI: 58.82-89.25) in *An. coluzzii*, *An. gambiae* s.s. and, *An. arabiensis* respectively~~ The few specimens tested in some cases did not allow an accurate estimate of the frequency of this mutation (Table 2).

Irrespective of the agricultural production zone, similar frequencies of the L1014F *kdr* mutation were observed in dead and live specimens of *An. gambiae* s.l. obtained after exposure to deltamethrin 1x (Table 3)

Activity of metabolic enzymes in *An. gambiae* s.l.

Compared to the laboratory Kisumu susceptible strain, ~~overexpression of the~~ non-specific α -~~and~~ β esterases ~~were over-expressed in the cotton zone~~ ($p_{\alpha} < 0.0001$).

Regarding the non-specific β -esterases, an over-expression was observed in cereal ($p_{\alpha} = 0.152$, $p_{\beta} = 0.0008$), cotton ($p_{\alpha} < 0.0001$, $p_{\beta} < 0.0001$), and urban market gardening ($p_{\alpha} = 0.0021$, $p_{\beta} < 0.0001$) zones ~~as compared to the Kisumu susceptible strain~~. ~~In the rice growing zone, a similar expression of~~

~~these enzymes ($p_{\alpha}=0.215$, $p_{\beta}<0.0001$) was observed compared to the Kisumu susceptible strain~~
(Table 4).

For MFOs, an overexpression was observed in the cotton ($p<0.0001$) and the rice growing ($p<0.0001$) zones compared to the Kisumu susceptible strain. At the opposite, the expression of this enzyme in the cereal ($p=0.779$) and market gardening ($p=0.112$) zones was similar compared to that of the Kisumu susceptible strain (Table 4).

An overexpression of GST was observed in cereal ($p<0.0001$), urban market gardening ($p<0.0001$) and rice growing ($p=0.0011$) zones relative to the susceptible Kisumu strain (Table 4).

Impact of the L1014F kdr mutation, molecular species and agricultural production zones on the mortality of An. gambiae s.l. to deltamethrin1x

Table 5 shows the results obtained after the logistic regression performed to evaluate the impact of the L1014F kdr mutation, molecular species and agricultural production zones on the mortality of An. gambiae s.l. to deltamethrin 1x. Overall, no impact of the L1014F kdr mutation ($p=0.06$), the molecular species ($p=0.24$) and the agricultural production zones ($p=1$) on the mosquito mortality is observed (Table 5).

Discussion

Data from the present study show a widespread high pyrethroid resistance intensity in An. gambiae s.l. with the involvement of several resistance mechanisms in all agricultural production zones surveyed throughout the country.

For many years, pyrethroid resistance has been observed in Benin (Akogbeto &Yacoubou 1999). However, it is unclear how intense this phenomenon is in the malaria vector populations until the resistance intensity notion was included in the 2016 WHO testing guidelines (WHO, 2016). The high pyrethroid resistance intensity observed in the present study is reminiscent of works by Awolola et al. (2018) in Nigeria, Pwalia et al. (2019) in Ghana, Sovi et al. (2020) in Mali, Wat'senga et al. (2020) in Congo and, Tchouakui et al. (2021) in Uganda.

With the emergence and expansion of the resistance phenomenon, the fear of the main actors (funders, decision-makers, researchers) involved in the fight against malaria is that the effectiveness of LLINs is seriously jeopardized with operational failures recorded after deployment of this control tool. This fear is moreover justified insofar as, studies carried out in experimental huts (N'guessan et al., 2007) or, through a small scale community trial (Asidi et al., 2012) have revealed a decrease in the performance of pyrethroid only LLINs. Additionally, the same phenomenon is suspected to be

one of the causes at the origin of the stagnation observed in the reduction of the number of malaria cases globally, between 2015 & 2017 (World Malaria Report, 2018).

In the present study, the resistance intensity tests performed, showed that as the dose of insecticide increases (2x, 5x and 10x the diagnostic dose of pyrethroids), the mortality rate of *An. gambiae* s.l. increases. However, this increase did not eliminate all tested mosquitoes, even at 10x the diagnostic dose. These results indicate a high intensity of resistance of *An. gambiae* s.l. to both permethrin and deltamethrin in all surveyed agricultural production zones. This could be due to the high use of insecticides for both agricultural and public health (LLINs) purposes, in the study area. Indeed, in agricultural zones, *An. gambiae* s.l. generally lays its eggs, in natural or anthropogenic larval habitats, but polluted over insecticide treatments which exert a strong selection pressure on the larvae (Yadouléton et al., 2010, Yadouléton et al., 2011, Tia et al., 2017, Fodjo et al., 2018). Additionally, standard LLINs generally distributed every 3 years nationwide, exert a lethal effect on pyrethroid susceptible mosquito, thus selecting resistant individuals that continue to breed over several generations.

Overall, the *An. gambiae* s.l. complex consisted of a mixture of *An. gambiae* s.s., *An. coluzzii*, and *An. arabiensis* in which the L1014F *kdr* mutation is fixed. The relative proportion of the molecular species as well as their L1014F *kdr* mutation frequency are variable from an agricultural production zone to another one. However, the logistic regression analysis carried out, revealed that the molecular species, the L1014F *kdr* mutation as well as the agricultural production zones have no impact on the mosquito status (dead or alive), 24 hours after exposure to deltamethrin 1x (0.05%). These results corroborate those of Sovi et al. (2020) in the Plateau department, southern Benin.

The similarity of the L1014F *kdr* frequency between dead and live mosquitoes to deltamethrin 1x (0.05%), suggests the presence of a combination of pyrethroid resistance mechanisms. Indeed, testing with the PBO synergist + a pyrethroid insecticide (deltamethrin or permethrin) induced a mortality inferior to 98% but significantly higher than for pyrethroid alone, which indicates the partial involvement of mixed-function oxidases. The overexpression of this enzyme as well as esterases (enzyme conferring resistance to carbamates and organophosphates) and GST (enzyme conferring resistance to organochlorines and some organophosphates) was demonstrated by means of biochemical testing. These results are similar to those of Salako et al. (2018) in the Alibori and Donga regions, North Benin. It would have been also interesting to look for the presence of *Ace-1^R* and *kdr* N1575Y mutations as well as, the level of overexpression of the cytochrome P450 genes previously detected in other regions of the country

(Aikpon et al., 2014, Jones et al., 2012, Ngufor et al., 2015). Another limitation of the present study is the fact of not having assessed the resistance intensity for each detected molecular species (*An. coluzzii*, *An. gambiae* s.s. and *An. arabiensis*).

Although the PBO synergist bioassays did not induce full susceptibility, the large increase in mortality observed suggests that PBO LLINs could constitute a viable alternative for the control of pyrethroid-resistant mosquitoes, although the epidemiological impact is not guaranteed. Moreover, a cluster randomized controlled trial conducted in Tanzania showed that PBO LLINs induced a greater reduction in malaria incidence compared to standard LLINs (Protopopoff et al., 2018). However, the mortality rate required for bioassays with PBO to induce epidemiological impact still requires further investigation.

Conclusion

Overall, *An. gambiae* s.l. showed high pyrethroid resistance intensity in all agricultural production zones surveyed with involvement of several resistance mechanisms (L1014F *kdr*, MFOs, Esterases and GST). Data obtained through the synergist testing suggest that PBO LLINs may provide a better control of pyrethroid resistant mosquitoes. Indoor residual spraying performed with a non-pyrethroid insecticides to which mosquitoes are susceptible could also be a viable option.

Availability of Data and Materials

The data used and/or analyzed in this study are available from the corresponding author on reasonable request.

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Author’s Contributions

WHS, CDK, ARS, CA and MCA conceived the study. WHS, CDK, ARS, GGP, RO, CA and MCA has participated in the design of the study. Entomologic data was collected by WHS, CDK, ALS, CDA, BY, ID and AF. Bioassays and laboratory analysis was carried out by WHS, CDK and ABS. WHS, CA and MCA drafted the manuscript. ANS realized the map of the study area. Statistical data analysis by SC. RO, ARS, GGP, CA and MCA critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

Author Disclosure Statement

No conflicting financial interests exist

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Table 2: Allelic frequencies of the *Kdr* L1014F mutation in the molecular species of the *An. gambiae* s.l. complex collected from the 4 agricultural production zones

Agricultural production zones	Species	N tested (%)	L1014F <i>kdr</i> genotypes			Freq (%)	95%CI
			<i>1014F</i>	<i>1014L</i>	<i>1014L</i>		
			<i>1014F</i>	<i>1014F</i>	<i>1014L</i>		
Cereal zone	<i>An. arabiensis</i>	4 (2%)	2	1	1	62.5	24.48-91.47
	<i>An. coluzzii</i>	107 (53.5%)	68	26	13	75.7	66.38-81.29
	<i>An. gambiae</i> s.s.	89 (44.5%)	60	21	8	79.2	72.50-84.92
	Total	200 (100%)	130	48	22	77	72.56-81.04
Cotton zone	<i>An. arabiensis</i>	12 (6%)	8	3	1	79.1	57.84-92.87
	<i>An. coluzzii</i>	2 (1%)	1	1	0	75	19.41-99.37
	<i>An. gambiae</i> s.s.	186 (93%)	129	42	15	80.6	76.26-84.54
	Total	200 (100%)	138	46	16	80.5	76.27-84.27
Rice growing zone	<i>An. coluzzii</i>	100 (100%)	45	39	16	64.5	57.44-71.12
Urban market gardening zone	<i>An. arabiensis</i>	1 (0.66%)	1	0	0	100	15.81-100
	<i>An. coluzzii</i>	43 (28.66%)	27	12	4	76.7	66.39-85.18
	<i>An. gambiae</i> s.s.	106 (70.68%)	81	21	4	86.3	80.95-90.18
	Total	150 (100%)	109	33	8	83.6	78.98-87.66
Study area	<i>An. arabiensis</i>	17	11	4	2	76.5	58.82-89.25
	<i>An. coluzzii</i>	252	141	78	33	71.4	67.26-75.34
	<i>An. gambiae</i> s.s.	381	270	84	27	81.9	78.96-84.56
	Total	650	422	166	62	77.7	75.33-79.93

N: number, *Kdr*: knock down resistance, Freq: frequencies of the L1014F *Kdr* mutation, CI: confidence intervals