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Sensitivity of larvae of *An. gambiae* to permethrin and deltamethrin (pyrethroids) in the town of Cotonou

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Abstract
The dynamism of the resistance of malaria vectors over time, in space and through the generation implies monitoring the susceptibility of available insecticides with respect to these vectors. This study aims to update the data on the susceptibility of *An. gambiae* to pyrethroids in the Cotonou city, a town endemic to malaria. Sensitivities tests for permethrin and deltamethrin were carried out at the CREC on stage 2, 3 and 4 larvae of the reference Kisumu, Kis-kdr and Wild strains according to the protocol recommended by WHO. The Kisumu strain served as a control. Mortalities were read 24 hours after exposure. Diagnostic doses were determined using the log-probit method of dose determination corresponding to proportion. The high doses are determined with larvae of the Wild and Kis-kdr strains. The weak ones refer to the reference Kisumu strain. However, the wild-type strain appears less sensitive than the Kis-kdr strain. The ratios determined are well above 2, for the larvae of the wild populations compared to the Kis-kdr strain, indicating the low sensitivity of the vectors to the pyrethroids tested, hence the limits of permethrin and deltamethrin in malaria control in areas of high resistance. Larvae of the wild strain *An. gambiae* appear to be less sensitive to permethrin and deltamethrin than to the laboratory strain Kis-kdr. These results show the involvement of other factors in the resistance of malaria vectors in Cotonou city which deserve to be elucidated.

Keywords: Pyrethroids, sensitivity test, resistant *An. gambiae s.s*, vector control

1. Introduction
Dieldrin, deltamethrin, lambda - cyhalothrin, alpha - cypermethrin and permethrin are insecticides recommended by WHO to control the nuisance of adult mosquitoes and the transmission of parasitoses for which they are responsible. These insecticides belong to the chemical family of pyrethroids and act on the sodium channel voltage dependent (CNa Vdp) disrupting the kinetics of inactivation of the sodium channel, resulting in paralysis and then death of the insect [1]. Pyrethroids are widely used in urban areas as fumigants and aerosols, for impregnating mosquito nets and curtains because of their rapid action on mosquitoes and practically safe for mammals with an LD_{50} for rats by ingestion of 135 to more than 5000 mg / kg [2]. In 2012, WHO reported the limits of modern malaria control to pyrethroids as a result of the increasing resistance of vectors to these products worldwide. This resistance is increasingly widespread and affects the majority of countries where malaria transmission persists. It concerns all major vector species and all classes of insecticides. India and sub-Saharan African countries are the regions most affected by this selection of resistant vector species with a major incidence of the disease. The selection of populations of resistant anophelines related to the intensive use of pyrethroids has led to the resurgence of malaria in several African countries [4, 5, 3, 6, 7]. Generally, data on levels of resistance of malaria vectors are limited according to the WHO (2012) reports and those that exist are difficult to consolidate because many countries have not yet systematically appropriate insecticide resistance tests. However, several studies have demonstrated the high frequency of the resistance allele of *An. gambiae* to pyrethroids in Benin [5, 8-11]. The vigorous resistance of vector populations over time and space requires studies to update data on the susceptibility of vectors to insecticides used after a period of control. Admittedly, the studies carried out by Agossa (2016) showed the resistance of *An. gambiae* to pyrethroids in the town of Cotonou in the South Benin. Although management strategies are proposed by WHO in this context [13], no alternative measures have been implemented in a tangible way.

Indeed, impregnated mosquito nets widely used by populations are impregnated with the same insecticides (permethrin and deltamethrin) although the efficiency limits are proved [14, 5, 9-11].

The aim of this study is to update the data on the susceptibility of *An. gambiae* to pyrethroids in Cotonou, a city of endemic malaria.

2. Material and methods

2.1 Study zone

The study was carried out in the town of Cotonou, Department of the Littoral (Benin), precisely in the district of *Zogbo, Ladji, Fifadji, Gbèdjromèdé, Fidjrossè, Houéyiho* 1 and 2, *Agla, Yénawa, Ménontin, Avotrou, Suru-Léré, Dandji*. This city has geographically featured by the proliferation of Culicidae. It has a flat sandy plain with a shallow groundwater table and is flush with the ground [15]. This lack of relief, combined with poor drainage of water, cause flooding during the rainy seasons and favors the multiplication of Culicidae deposits, including *An. gambiae* [15, 14]. In addition, the town of Cotonou is marked by land management and market gardening, which create real Culicidae deposits, including *An. gambiae*, the main vector of malaria [15, 16]. It is also due to the country's largest port, which imports and export containers in which other mosquitos' species could be hidden. These characteristics of the city create a very strong culicidal nuisance to its inhabitants and expose the populations to multiple diseases transmitted by the mosquitoes. It is one of the cities most affected by malaria in Benin [6, 7]. The study sites were chosen because of the frequency and the permanence of the breeding sites which cause the abundance and the strong aggressiveness of the mosquitos' species. There are puddles of water, temporary and permanent swamps, abandoned tires or stored in open garages and so on. All these reservoirs thus constitute potential deposits favorable to the proliferation of mosquitoes.

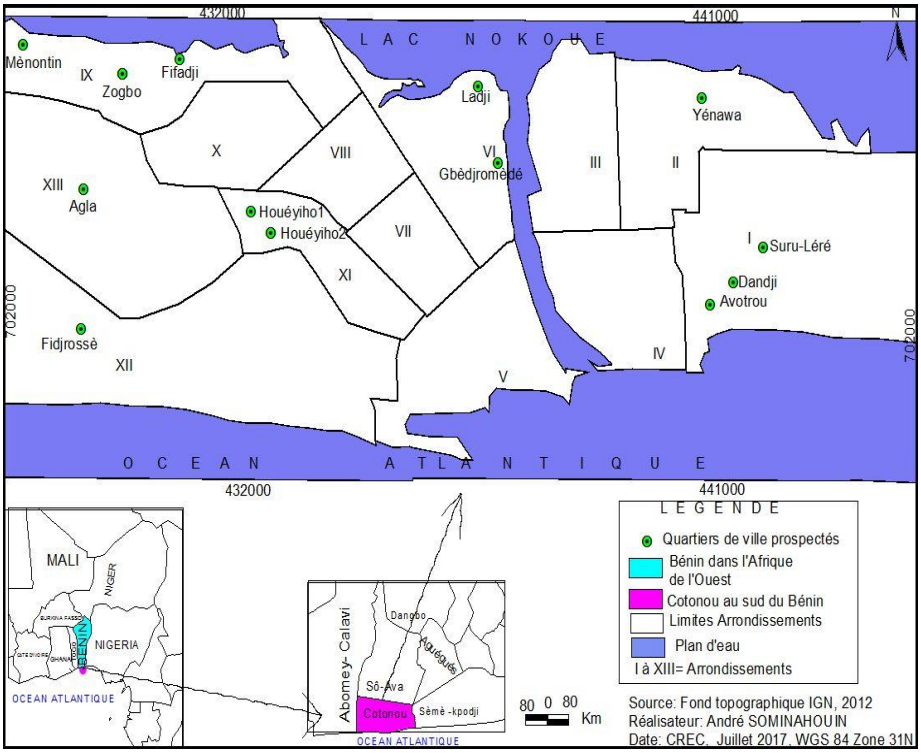

Fig 1: Location of Study Areas

2.2 Animal equipment

The tests were carried out on larvae of *An. gambiae* s.l. Three strains were used. This is the genotype SS "Kisumu" susceptible strain, which lacks the resistance allele, the Kis-kdr laboratory strain of RR genotype resistant to pyrethroids and organochlorines and then the wild populations characterized by a frequency of the resistance allele of the Kdr gene (>80%). The strains of *An. gambiae* "Kisumu" and Kis-kdr are raised at the Insectarium of the Center of Entomological Research in Cotonou (CREC). The insectarium consists of rooms where the pre-imaginary and imaginary stages of the different strains are separately conditioned. For a good multiplication of mosquitoes, temperature and humidity conditions must be met. For this purpose, the adults are constantly conditioned at a temperature of 26 ± 2 °C and a relative humidity of 72 ± 4% thanks to a permanently operating air conditioner. The temperature and the relative humidity of the air are measured every day thanks to a thermo hygrometer which allows monitoring the temperature and the humidity of the insectarium. Mosquitoes are spawned by blood meals. For this reason, three-day-old females are gorged for about 20 minutes by male rabbits immobilized on mosquito cages by a device developed accordingly (Figure 3). After the blood meal, the swollen females are continually fed honey juice to 10% in cotton deposited on the tulle mosquito net of the cages. This serves as food for pregnant females. The eggs of the different strains are harvested daily by removing the nesting boxes after the laying. These nests are systematically renewed after each withdrawal. The collected eggs are put into water (tap water) in bins for hatching. The larval breeding room is conditioned at a temperature of 29 ± 2 °C, favorable to the development of the pre-imaginary stages of mosquitoes, using a heating radiator that diffuses calories by radiation. Larvae obtained after hatching is fed with cat croquette from the first stage. Livestock water is renewed after 72 hours to ensure good larval growth. This allowed us to obtain larvae of stages 1, 2, 3, 4 and the pupae of the different strains necessary for the works realization.

Fig 2: Anopheline larvae breeding

Fig 3: Male rabbit immobilized on a cage for the gorging of anopheline females

2.3 Study of the sensitivity of larvae of *An. gambiae* to permethrin and deltamethrin

Sensitivity tests were carried out at CREC on stage 2, 3 and 4 larvae of the Kisumu reference strains, laboratory-resistant Kis-Kdr and Wild according to the protocol recommended by the World Health Organization [17-20]. Resistance of *An. gambiae* populations to pyrethroids has been confirmed on wild larvae and susceptible "Kisumu" with larval tests for deltamethrin and permethrin, which are widely used pyrethroids for impregnation of mosquito nets in malaria vector control in Benin.

Stock solutions of permethrin and deltamethrin were formulated in ethanol from which increasing concentrations were prepared. For each larval stage of the different populations of the mosquito strains tested, increasing concentrations were prepared from a stock solution plus controls with five cups per concentration, each containing 20 larvae. Sublethal concentrations were chosen to achieve at least a 100% mortality rate, so that some concentrations could induce a mortality rate between 50% and 100% and ranged from 5% to 50%. The room for insecticide tests on the larvae is conditioned at a temperature of 29 ± 2 °C using a heating radiator which diffuses calories by radiation. This temperature is required in real time for the development of pre-imaginary mosquito stages. Thus, temperature is not a limiting factor that can influence the results of the tests carried out. Larvae died after 24 hours of exposure to formulated insecticide products are counted for each dose applied. The results are expressed as a percentage of mortality which was determined by adding the numbers of dead larvae on the replicates of the exposure test in relation to the total number of mosquito larvae exposed as well as in the control batches according to the formula:

$$\text{Mortality during the test} = \frac{\text{Total number of dead larvae}}{\text{Total sample size} - \text{Number of pupae}} \times 100$$

When the mortality rate at the control level is greater than 20%, the test was invalided. If the mortality of the controls is greater than 5% and less than 20% the observed mortality was corrected by the Abbott formula:

$$\text{Corrected mortality} = \frac{\%\ \text{mortality in test} - \%\ \text{control mortality}}{100\%\ \text{control mortality}}$$

If the mortality at the control level was less than 5% it can be ignored and well, no correction was necessary.

For the determination of the diagnostic doses of the two pyrethroids on the different larval stages, the R Core Team software (Version 3.3.1-2016) was used. The method used is that of log-probit of determination of the dose corresponding to a given proportion.

For all the tests carried out, resistance of the wild-type strain to a test product was noted when the respective ratio (R) of the diagnostic doses of the different stages of the wild-type strain with respect to the reference sensitive strain is greater than 1 and / or equal to 2. Under these conditions, the product tested was capable for an integrated use in the fight against these resistant vectors. A ratio greater than 2 (R>2) indicated a high resistance to the product tested.

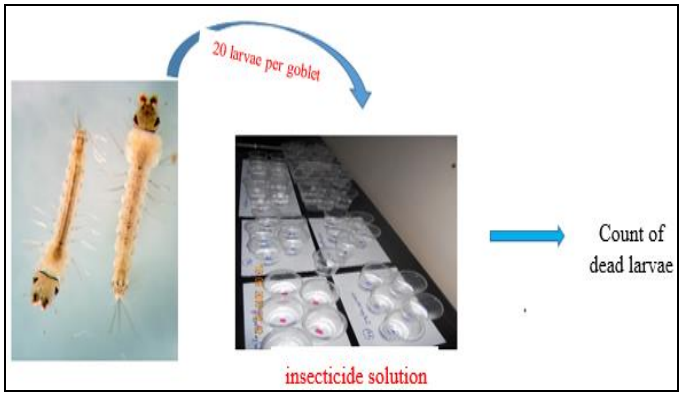

Fig 4: Bioassay technique

3. Results

Tables 1 and 2 summarize the level of susceptibility of larvae of *An. gambiae* populations in the town of Cotonou to permethrin and deltamethrin. The larvae exposed to permethrin showed differential sensitivity according to the strains. The reference "Kisumu" strain appeared more sensitive with LD99 of 1.05×10^{-4}; 1.048×10^{-4} and 2.89×10^{-4}

corresponding respectively to the larvae of stages 2, 3 and 4. The larvae of the wild populations appeared less sensitive to permethrin with the LD99 of 0.165; 0.250 and 0.613 corresponding respectively to stage 2, 3 and 4 larvae respectively. The ratio of the LD99 of the resistant strain Kis-kdr of genotype RR with the sensitive strain "Kisumu" of reference of genotype SS showed that for each stage a dozen times the LD99 of the susceptible strain induces 99% mortality of the larvae of the Kis-kdr strain at laboratory. For the ratios established with the Wild strain and the reference "Kisumu" strain, it appeared that hundreds of times the doses relative to the "Kisumu" strain have induced the same mortalities of the larvae of the wild populations.

As regards deltamethrin, the ratios of the Kis-kdr strain with the reference strain of reference also showed that for each larval stage there must be about ten times the LD99 of the susceptible strain to induce 99% mortality of larvae of the Kis-kdr strain at laboratory. Similarly, the doses of the "Kisumu" strain were also required hundreds of times to induce the same mortalities of the larvae of the wild populations.

In general, the ratios determined with these pyrethroids were well above 2. The lower sensitivity of larvae in wild populations has shown the limits of permethrin and deltamethrin in the mosquitoes' control in areas of the high resistance of vectors

Table 1: Determination of diagnostic doses of permethrin for larvae of stages 2, 3 and 4 of the wild and reference "Kisumu" mosquito populations and calculation of the corresponding ratios

	Presentation of the LD99 of larvae of stages 2, 3 and 4 of the Kisumu, Kis-kdr and Wild strains with permethrin							
Larval stages	**Kisumu**		**Kis-kdr**		**Wild**		**Ratio LD99 Kis-kdr/Kis**	**Ratio LD99 Wild/Kis**
	LD99	**CI95%**	**LD99**	**CI 95%**	**LD99**	**CI 95%**		
L2	1.05E-04	[7.371E-05 1.491E-04]	9.87E-04	[7.991E-04 1.220 E-03]	0.165	[0.134- 0.203]	9.421	1571.428
L3	1.048E-04	[7.371E-05 1.491E-04]	1.54E-03	[1.255E-03- 0.00189]	0.250	[0.208-0.299]	14.704	2385.496
L4	2.89E-04	2.586E-04 3.232E-04	1.80E-03	[1.5109E-03 2.136E-03]	0.613	[0.447-0.840]	6.212	2121.107

LD99: Diagnostic dose resulting in 99% mortality of a population; Ratio LD99 Kis-kdr / Kis: ratio of diagnostic dose of strain Kis-kdr to that of reference "Kisumu"; Ratio LD99 Wild / Sensitive LD99: Diagnostic dose ratio of wild strain to that of reference "Kisumu"; CI (95%) represents the 95% confidence interval;

Table 2: Determination of diagnostic doses of deltamethrin for larvae of stages 2, 3 and 4 of the wild and reference "Kisumu" mosquito populations and calculation of the corresponding ratios

	Presentation of LD99 of Stage 2, 3 and 4 larvae of Kisumu, Kis-kdr and Wild Deltamethrin strains							
Larval stages	**Kisumu**		**Kis-kdr**		**Wild**		**Ratio LD99 Kis-kdr/Kis**	**Ratio LD99 Wild/Kis**
	LD99	**CI95%**	**LD99**	**CI 95%**	**LD99**	**CI 95%**		
L2	1.41E-05	[9.787E-06 2.044E-05]	2.02E-04	[1.588E-04 2.580E-04]	0.016	[0.014-0.019]	14.314	1134.7517
L3	4.85E-05	[4.2E-05 5.595E-05]	4.01E-04	[3.152E-04 5.105E-04]	0.037	[0.032-0.041]	8.275	762.886
L4	4.85E-05	[4.2E-05 5.595E-05]	4.01E-04	[3.152E-04 5.105E-04]	0.037	[0.032-0.041]	8.275	762.886

LD99: Diagnostic dose resulting in 99% mortality of a population; Ratio LD99 Kis-kdr / Kis: ratio of diagnostic dose of strain Kis-kdr to that of reference "Kisumu"; Ratio LD99 Wild / Sensitive LD99: Diagnostic dose ratio of wild strain to that of reference "Kisumu"; CI (95%) represents the 95% confidence interval;

4. Discussion

Diagnostic doses for larvae of the laboratory Kis-kdr strain showed the influence of the Kdr gene on the efficacy of pyrethroids. These insecticides kill the larvae of the Kis-kdr strain in high doses compared to the reference "Kisumu" strain, which justifies the failure of the WHO target for interruption of malaria parasite transmission by intravenous spraying or by the use of pyrethroid-impregnated tissues (mosquito nets and / or curtains) [21-23]. Studies carried out in this context by Agossa (2016) in Benin, the aim of this study was to establish the link between the resistance of mosquitoes and the effectiveness of pyrethroid-impregnated materials. These works have shown a considerable decrease in the efficiency of pyrethroid-impregnated tools against malaria vectors and this correlates with a considerable increase in the resistance of vectors to the insecticides. The diagnostic doses determined on the larvae of the wild populations and those of the Kis-kdr strain in our study showed that the larvae of the wild populations were much less sensitive compared to the Kis-kdr strain, indicating the existence other mosquito adaptation factors to their environment outside the Kdr resistance gene which contributed considerably to the tolerance of high doses of these pyrethroids which should exert lethal pressure on exposed mosquitos when referred to sensitivity of the laboratory sensitive "Kisumu" strain. The resistance of mosquitoes to pyrethroids in southern Benin was therefore not only related to the frequency of the Kdr gene. Several authors have shown the involvement of detoxification enzymes in the mechanism of vector resistance to pyrethroids [24]. This resistance is characterized by the expression of the metabolic resistance genes CYP6M2, CYP6P3 and GSTe2. These enzymes were overproduced by mosquitoes resistant to sensitive mosquitoes and allowed them to metabolize or to degrade insecticide molecules before they exert a toxic effect on their target [25]. The strong production of enzymes was either linked to a modification of a regulatory gene which controls the activity of the structural genes determining the synthesis of these enzymes [26], or an increase in the number of copies of the genes that encode these enzymes [27-30]. This form of resistance was generally induced by the presence of natural and anthropogenic xenobiotics in the larval breeding sites of *An. gambiae* which is found in urban environments, which promotes anopheline density in polluted anthropogenic habitats [31-34, 12]. The selection of anopheline resistance strains was therefore mainly in the larval stage. The larval development environments in Cotonou would be a major factor in the strong resistance of *An. gambiae* to pyrethroids, which have been used since 1970 in Benin both in agriculture and insecticide-treated nets for the control of transmission of malaria. This intensive use of pyrethroids has therefore led to a high pressure on mosquitoes in their habitats with the pollution of breeding sites [12], which has led to a strong adaptation of *An. gambiae* to ecological factors [35]. Indeed, the work of Clements [36, 37] have shown that the larvae of mosquitoes were non-selective detritivores, incapable of discerning the food particles they ingested, which proved the strong capacity of these larvae in contact with the different pollutants in the lodges, to adapt to a toxic diet. These

observations gave rise to a hypothesis according to which the larvae would have diversified enzymatic equipment enabling them to neutralize the xenobiotics present in their diet. The resistance of the vectors to the pyrethroids was therefore multiple [38]. Other studies have also shown that exposure of larvae to environmental pollutants outside pyrethroids induced adult resistance of *An. gambiae* to pyrethroids [39, 12]. The lower susceptibility of larvae of *An. gambiae* to pyrethroids in the town of Cotonou was believed to be elucidated for new choices of alternative insecticides for vector resistance management, due to multiple factors that need. Vector control mainly through in-house spraying and impregnated mosquito nets became critical with the strong resistance of vectors to pyrethroids, which were the insecticides used in vector control. However, the involvement of environmental pollutants in vector resistance can be a handicap for larvicidal larval control. Indeed, pollutants, apart from their high susceptibility to detoxification enzymes, may also have properties which can interact with larvicides and neutralized the lethal effect of larviciding materials, which could render the larvicidal activity ineffective of certain products. It would therefore be useful to know the nature of the pollutants of the deposits and their chemical properties with regard to the larvicidal products envisaged.

5. Conclusion

In sum, the results of the sensitivity tests carried out in this study allowed us to understand the behavior of larvae of *An. gambiae* with respect to pyrethroids widely used for decades in ecosystems in Southern Benin. These results confirmed the resistance to

pyrethroids in the city of Cotonou. Management of vector resistance required the use of alternative means of control that were well suited to slow the spread of resistance to ensure control of malaria transmission in areas of resistance where transmission persisted in order to preserve populations from the recrudescence of *An. gambiae*.

6. Conflict of interests

The authors have not declared any conflict of interests.

7. Authors' contributions

Armand Akpo, Armel Djènontin and Martin Akogbéto conceived the study. Armand Akpo, Dieudonné Kpoviessi, Daniel Chougourou and Martin Akogbéto have participated in the design of the study. Armand Akpo, Luc Didolanvi, Armel Djènontin and Martin Akogbéto carried out the field activities and the laboratory analyses. Armand Akpo, Luc Didolanvi, Armel Djènontin, Dieudonné Kpoviessi, Daniel Chougourou and Martin Akogbéto drafted the manuscript Armand Akpo, Luc Didolanvi, Armel Djènontin, Dieudonné Kpoviessi, Daniel Chougourou and Martin Akogbéto critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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