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Detection of multiple insecticide resistance mechanisms in *Anopheles gambiae* s.l. populations from the vegetable farming area of Houeyiho, Southern Benin, West Africa

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Abstract

A study carried out in a vegetable farming area (Houeyiho) in southern Benin, showed that despite the benefits coming from this activity, its development contributes to the emergence of multiple insecticide resistance in malaria vectors.

To verify this hypothesis, we reared wild mosquitoes' larvae collected from a vegetable farming area during dry and rainy seasons. Bioassays were performed on the emerging adult mosquitoes to assess the susceptibility of malaria vectors to insecticide-impregnated papers (permethrin 0.75%, deltamethrin 0.05%, DDT 4%, and bendiocarb 0.1%) following WHOPEs guidelines. *An. gambiae* complex, knock down resistance (*kdr*) and acetylcholinesterase (*ace-1*) mutations were determined by Polymerase Chain Reactions (PCR).

Enzymatic mechanisms (Glutathione-S-Transferase, esterase, monooxygenase P450 and total protein) were also investigated from the F1 (the parental population after breeding) populations of *An. gambiae* s.l.

Results from this study showed that: a)-A wide range of pesticides is abusively used by farmers for crop protection which would have contributed to insecticide resistance in *An. gambiae* populations to organochlorines (average mortality rate = 1.19%) and pyrethroids (average mortality rate = 29%) but remains susceptible to carbamate (average mortality rate = 100%); b)-*kdr* mutation was found with a 0.95 frequency. The *Ace-1* mutation was also detected but at a very low frequency ($\leq 5\%$); c)-The presence of enzymatic activities was observed in the wild *An. gambiae* populations particularly GSTs and P₄₅₀ oxidase which had significantly elevated levels compared to the susceptible Kisumu strain.

Our research has shown that vegetable farming activity in Benin contributes to the emergence of multiple insecticide resistance in malaria vectors. The widespread pyrethroid resistance in malaria vectors reported in this study is a significant limitation to the use of LLINs (Long-Lasting Insecticidal Nets) as a malaria vector control strategy in many areas in southern Benin. Moreover, our findings clearly showed the presence of metabolic resistance mechanisms in malaria vectors. These findings suggested that metabolic resistance may be associated with resistance to different classes of insecticides

Keywords: Resistance, *Anopheles gambiae*, insecticides, vegetable farming, Benin

Introduction

In order to meet food security and poverty alleviation needs of a growing population in sub-Saharan Africa, peri-urban and urban agriculture is rapidly becoming a major economic activity in the cities [1]. In general, spare spaces (marshland, road edges, beaches etc) are turned into gardens where vegetable various flowers are cultivated.

In Cotonou, the economical capital of Benin, peri-urban agriculture consist of belts of vegetable farming every where in and around the city, involving the local production of exotic vegetables (lettuce, green beans, carrots, cabbages, cucumbers, beetroot etc.) all year round, and the adoption of manual watering of crops [2-3].

The advantages of urban agriculture are considerable. They contribute to the improvement of living conditions by supplying food, income and employment to urban populations [2-4]

The economic and social impact of urban and peri-urban agriculture is however limited by a number of factors including pest and disease problems, excessive and improper use of agrochemicals such as hazardous pesticides which have negative consequences on consumer health and environmental quality [5]. According to a study conducted by Akogbeto *et al.* [3] in Benin, vegetable pest control by the producers usually consists in intensive foliar sprays of broad-spectrum chemical pesticides particularly pyrethroids, which destroy the indigenous biodiversity required for natural pest control, raises personal and environmental hazards, and causes pesticide resistance in pest populations. Furthermore, the common pesticides (Pyrethroids) used in agriculture are of the same chemical classes and share the same targets and modes of action as those used for vector control, they have the potential to select for resistance in mosquitoes particularly in *Anopheles gambiae*, the main malaria vector in sub-Saharan Africa [6-7].

Given the pyrethroids resistance in *An. gambiae* populations, carbamate and organophosphate were introduced as alternatives to pyrethroids to strengthen vector control campaigns using Indoor Residual Spraying [8]. However, resistance to both classes of insecticides has already been documented in natural *An. gambiae s.l.* populations in northern Benin [9].

We suspect that the resistance of *An. gambiae* populations to the various chemical insecticides also involve metabolic activities such as monooxygenase, P450, esterases, and glutathion-s-transferases activities in *An. Gambiae* populations.

The present study proposes to assess multiple insecticide resistance mechanisms in *Anopheles gambiae s.l.* populations from the vegetable farming area of Houeyiho, an urban area located in Cotonou, Southern Benin, West Africa

Materials and Methods

Study area

The study was conducted in the Republic of Benin, from June to November 2016 in the vegetable farming area of Houeyiho (6° 45'N and 2°31'E) located in Cotonou in a highly populated suburb. It is a 14 hectares farm shared between five cooperatives, each managed by an elected cooperative president. Each cooperative approximately consists of 300 individuals making an estimated farmer population of not less than 2,000 persons. This area is characterized by two rainy seasons (March- July and October-November) and two dry seasons (December-March and August-September). The annual average rainfall is 1,500 mm in July, with a relative humidity (RH) of 70% ± 5 and a minimum/maximum temperature ranging between 23 and 32 °C.

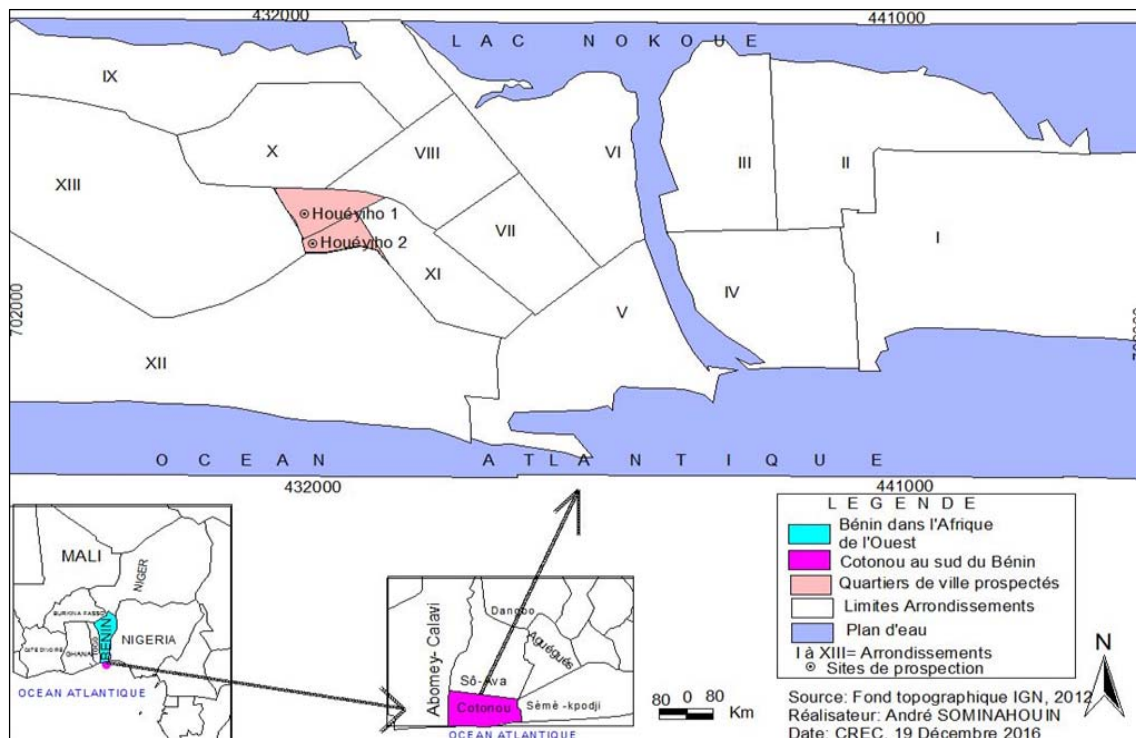


Fig 1: Map of Benin showing the study site

Mosquito collections

Anopheles larvae were collected from June to November 2016 from the vegetable area of Houeyiho (Fig 1) and were reared up to adult emergence at the CREC (Centre de Recherche Entomologique de Cotonou, Benin) insectary. All adults *Anopheles* females aged 2 – 5 days old were morphologically identified as belonging to the *An. gambiae* complex [10] after gDNA extraction [11] for further bioassay tests.

The Kisumu strain of *An. gambiae sensu stricto*, a reference strain susceptible to all insecticides, was reared simultaneously under the same conditions and used as a control for insecticide bioassays.

Insecticide susceptibility test

To assess the impact of agricultural pesticides on the selection of resistance in malaria vectors, non-blood-fed adult female mosquitoes aged 2-5 days old were exposed to insecticide-

impregnated papers, as described by the WHO testing protocol [12]. The susceptibility assays were carried out with four insecticides used in public health, the diagnostic doses recommended by WHO. Those insecticides included two pyrethroids (Permethrin 0.75%, Deltamethrin 0.05%), one carbamate (Bendiocarb 0.1%) and one organochlorine (DDT 4%).

For each assay, five test tubes (with batches of 20-25 mosquitoes) were used: one untreated paper as a control and four insecticide-treated papers to expose the mosquitoes. Control tubes contained filter papers impregnated with silicon oil (insecticide carrier).

For the bioassays test, mosquitoes were exposed for 1 hour to insecticide-treated papers. Control mosquitoes (Kisumu strain) were exposed for the same time to untreated filter papers. After exposure, mosquitoes were transferred into insecticide-free observation tubes and maintained on a 5% sucrose solution in take at a temperature ranging between 25 and 28 °C. Final mortalities in test and control mosquitoes were recorded 24 hours after exposure and the susceptibility status of the population was graded according to the WHO recommended protocol [12]. Dead and survived mosquitoes from this bioassay were separately kept in a Carnoy solution at -20 °C for further molecular characterization.

When control mortality was scored between 5 and 20%, mean observed mortality was corrected using Abbott's formula [13].

Species identification and target site mutation genotyping

Overall, 100 females *An. gambiae* samples from the WHO bioassays were processed for molecular. Species identification. They were identified to species and molecular form using PCR as described by Fanello *et al.* [14]. The last series of PCRs determined the presence of *kdr* mutations in *An. gambiae* ss populations, as described by Martinez-Torres *et al.* [15] and the presence of G119S mutation (*Ace-1* gene) as described by Weill *et al.* [16].

Biochemical analysis

Detoxifying enzymes activities were measured on single *An. gambiae* populations F1 from the parents collected in the study area. These populations (F1) were not exposed to any insecticide and were stored at -80 °C within 24 h from emergence (above). Each mosquito was ground on ice in 200 µl of distilled water and the homogenate was centrifuged at 1, 4000 rpm for 2 mins. Two 10 µl replicates of supernatant were transferred into two adjacent wells of a microtitre plate for non-specific esterases (NSEs), glutathione S-transferases (GSTs) and protein assays. Monooxygenases assays were performed with two 20 µl replicates of supernatant.

Proteins

Total protein content in 10 µl aliquots of mosquito homogenate was measured using the Bradford assay [17], in order to report detoxifying enzyme activity to protein value for each test mosquito. In each replicate well of the microtiter plate, 290 µl of Coomassie plus Protein Assay Reagent solution [18] were added to 10 µl of centrifuged mosquito homogenate and the mixture was incubated at room temperature for 5 min. The endpoint absorbance was read at 590 nm. Protein values were calculated using a standard curve of absorbance of bovine serum albumin and served as a correction factor for the enzyme analysis.

Non-Specific Esterases (NSEs)

Non-specific esterase activity was measured using α -naphthol acetate (α Na) and β -naphthol acetate (β Na) [19]. In each replicate well, 90 µl of phosphate buffer (PBS, pH=6.5) and 100 µl of 0.6 M α Na (or β Na) were added to 10 µl of centrifuged mosquito homogenate. After 30 min incubation, 100 µl of Fast Garnett BC solution (8 g Fast Garnett Salt + 10 ml distilled water) was added to stop the reaction. The concentration of the final product was determined at 550 nm as an endpoint calculated from standard curves of α - and β -Naphthol, respectively.

Glutathione-S-Transferases (GST)

To measure glutathione-S-transferase (GST) activity in the adult mosquitoes, 200 µl of GSH/CDNB working solution (100 µl of an extemporaneous solution of 0.6% weight/volume reduced glutathione in 0.1 M sodium phosphate buffer pH=6.5+0.013 g of 1-chloro-2, 4-dinitrobenzene diluted in 1 ml of 70% methanol) were added to each replicate of mosquito homogenate [19]. The reaction was read at 340 nm immediately as a kinetic assay for 5 minutes. An extinction coefficient of 5.76 mM⁻¹ (corrected for a path length of 0.6 cm) was used to convert absorbance values to moles of product. GST specific activity was reported as the rate of formation of GSH produced in mmol.min⁻¹.mg⁻¹ protein.

Oxydases (Cytochrome P450)

Cytochrome P450 activity was determined using the heme-peroxidase assay according to Brogdon *et al.* [18]. The assay detects the elevation in the amount of heme, which is then converted into equivalent units of cytochrome P450. Eighty µl of 0.625 M potassium phosphate buffer (pH=7.2) were added to 20 µl of mosquito homogenate together with 200 µl Tetramethyl Benzidine solution (0.011 g 3, 3',5,5' Tetramethyl Benzidine in 5 ml of 70% methanol +15 ml sodium acetate buffer 0.25 M pH=5.0); 25 µl of 3% hydrogen peroxide were then added and the mixture was incubated for 30 min at room temperature. Absorbance was read at 630 nm and values calculated from a standard curve of cytochrome C.

Data analysis

Mean absorbance values of replicate wells for each tested mosquito were converted into enzyme activity and divided by the protein values. The median enzymatic activity was calculated for each test mosquito population and the distribution of enzyme activities was compared between the Kisumu reference strain and the field populations using non-parametric Mann-Whitney tests.

Results

Resistance status

Results from the susceptibility tests showed that the mortality rate in the reference Kisumu laboratory strain was less than 5% requiring therefore no correction of test sample data. However, following the exposure of the wild female populations of *An. gambiae s.l* from the vegetable farming to the various impregnated papers cited above, these populations developed a high resistance to DDT (2% mortality rate), permethrin (22% mortality rate) and deltamethrin (35% mortality rate) but were fully susceptible to bendiocarb (Fig 2).

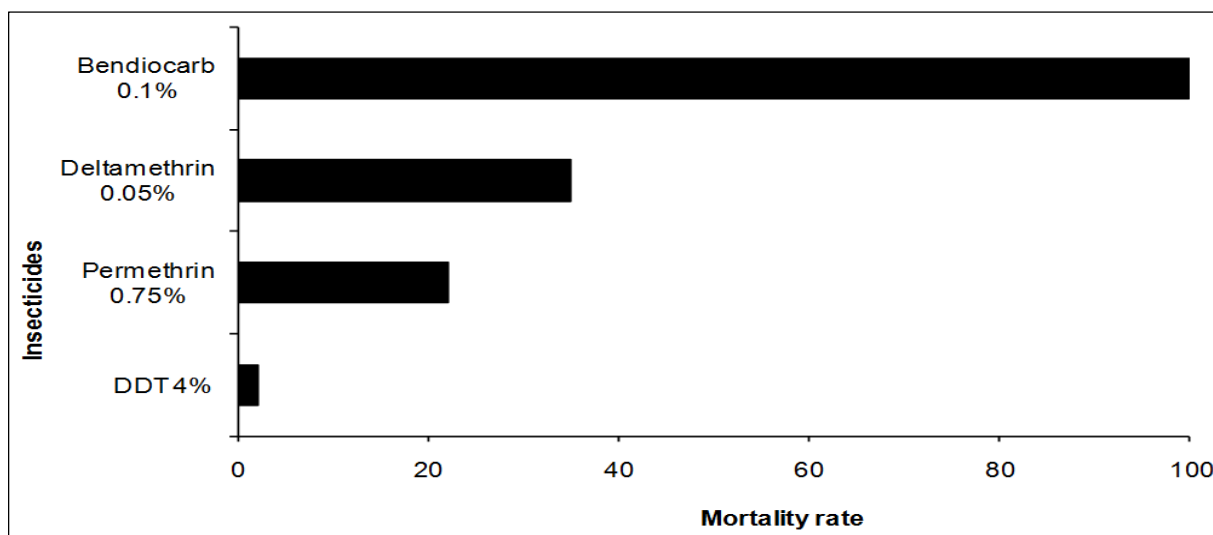


Fig 2: Susceptibility of *Anopheles gambiae* S.S. to insecticides at the study sites

Identification of molecular forms of *Anopheles gambiae* s.s

Overall, 100 mosquitoes from the study site were successfully processed for molecular forms and species identification. PCR revealed the presence of one sub-species of *An. gambiae*: *An. gambiae* s.s. where all were of M form).

Detection of resistance mutations by PCR

Table 1 showed allelic and genotypic frequencies at the *kdr* and *Ace-1* loci. The *kdr* 1014F resistant allele was found at the frequency of 0.95 in all *An. gambiae* populations collected from the study site. The *kdr* 1014S allele was not found in our samples. The *Ace-1* mutation was detected in *An. gambiae* populations but at a very low frequency (<0.05).

Biochemical assays

Enzyme activities are represented by clouds of dots in the figures 1, 2 and 3. Each dot of the cloud represents the activity recorded in a mosquito. The average of the enzyme activities of each population was obtained by dividing the sum of the enzymatic activities of the entire population by the total number of mosquitoes tested.

Esterase

The means of optical density values for esterase (α and β esterase) activity in the populations of mosquitoes from Houeyiho (Figure 3a and 3b) were not significantly higher compared to the reference susceptible Kisumu strain ($P>0.05$).

α esterase

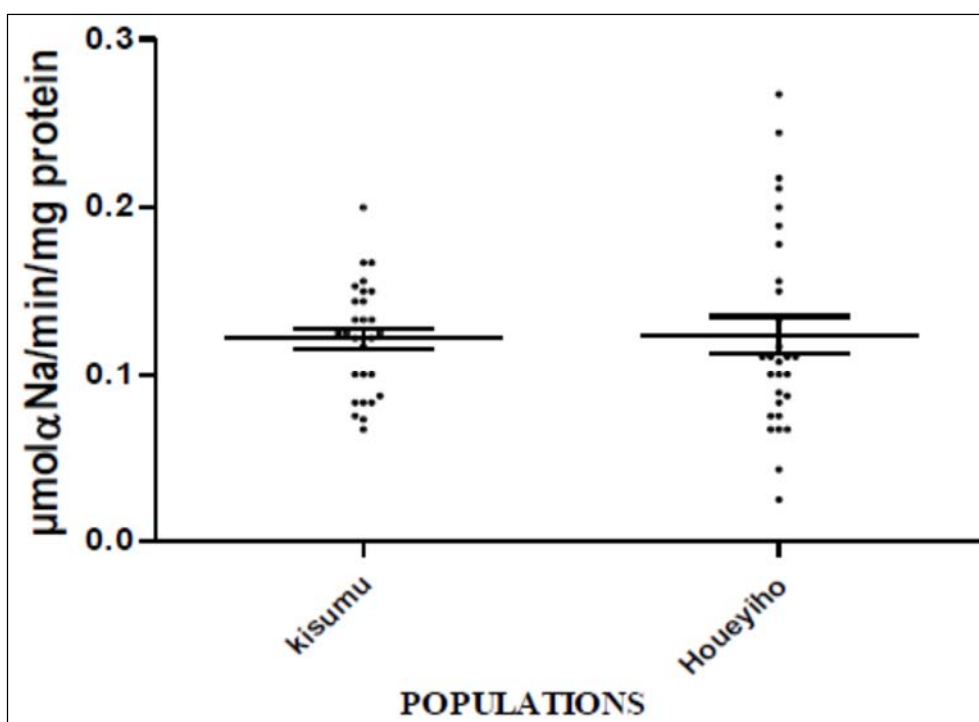


Fig 3a: Alpha-esterase activity of *Anopheles gambiae* populations from Houeyiho

β esterase

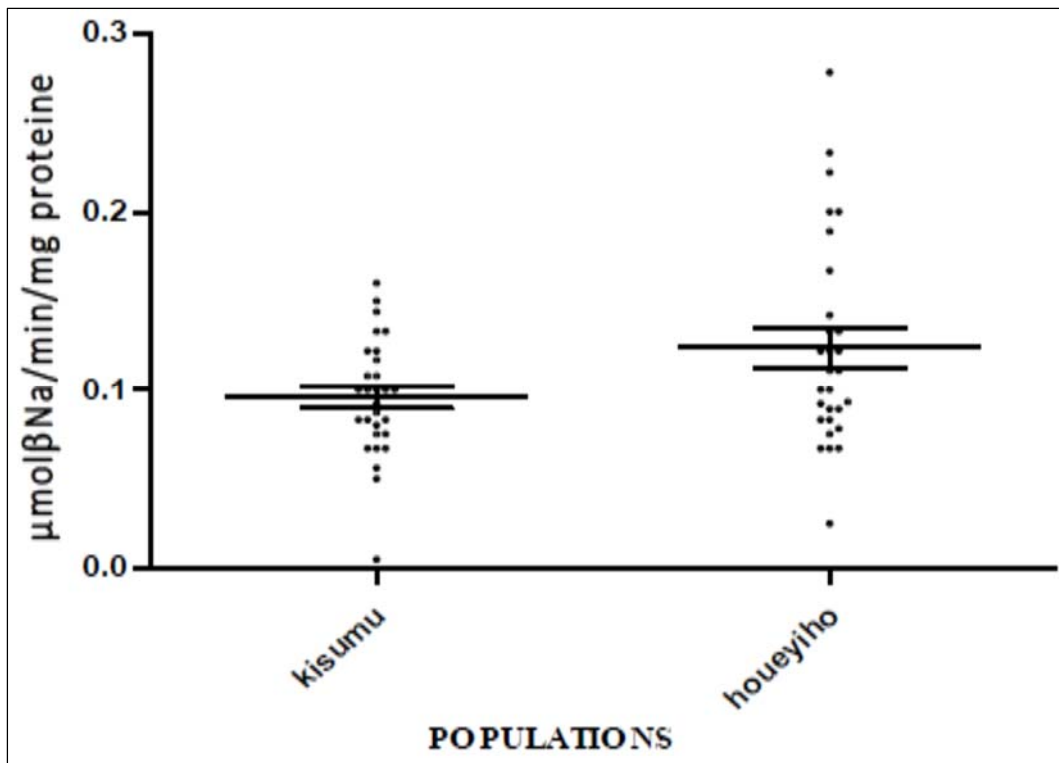


Fig 3b: Beta-esterase activity of *Anopheles gambiae* populations from Houeyiho

Oxidase and GST

For Mixed Function Oxidases (MFO) and Glutathione-S-Transferases (GST), the enzyme activity levels (Figure 4 and

5 respectively) were significantly higher in *Anopheles gambiae* populations from Houeyiho compared to the reference susceptible Kisumu strain ($P < 0.05$).

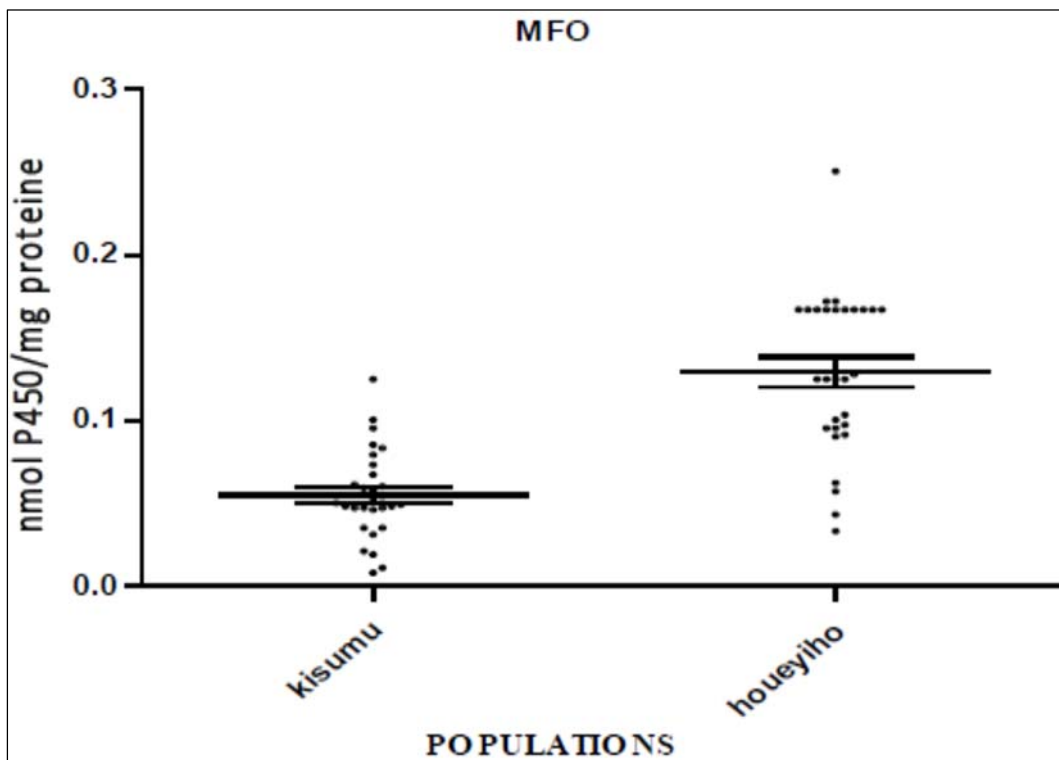


Fig 4: Mixed Function Oxidases activity of *Anopheles gambiae* populations from Houeyiho

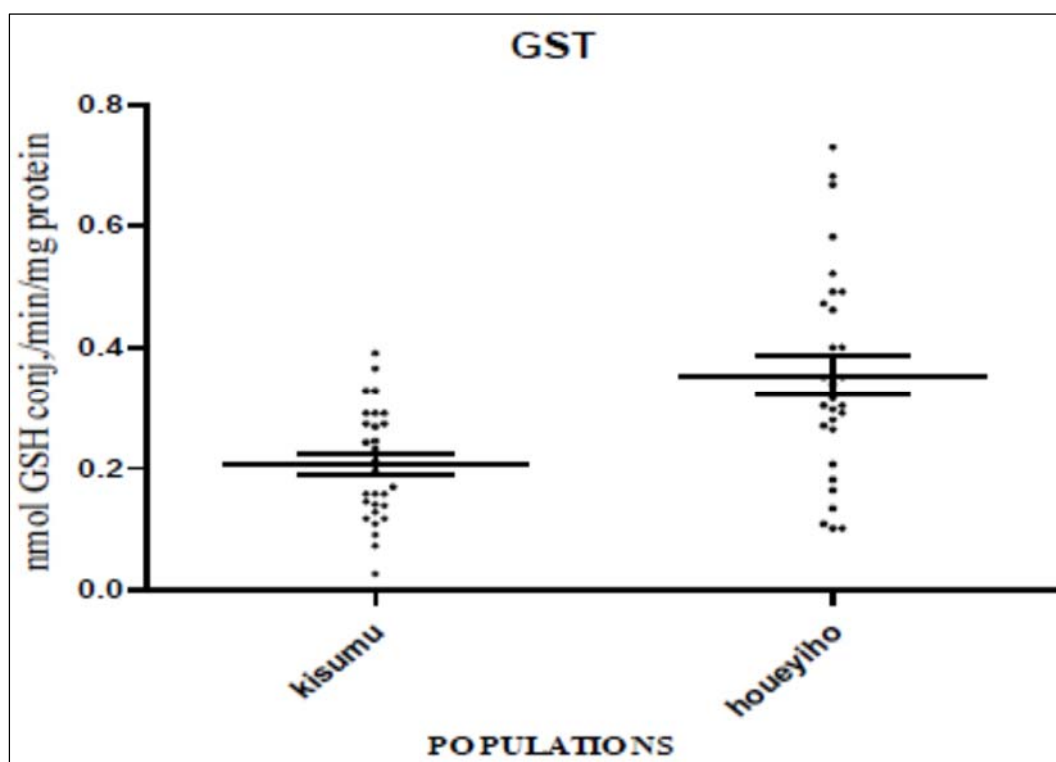


Fig 5: Glutathion activity of *Anopheles gambiae* populations from Houeyiho

Discussion

Results from studies conducted in many African countries [2-20-21] showed that despite the many advantages offered by urban agriculture, vegetable farming increases the intensity of malaria transmission in urban areas. Agronomic practices in vegetable farming create numerous trenches that retain rain and irrigation water. These stagnant bodies of water provide suitable breeding sites for mosquitoes, particularly for *Anophele gambiae*, the main malaria vector in Africa. Likewise, survey findings reported by Yadouleton *et al.* [2-3] and suggested that various families of insecticides were used for pests control management in agricultural settings particularly in vegetable farming ones. Unfortunately, many of these families of insecticides were not recommended for vegetable farming. This situation, coupled with the limited knowledge of most of the farmers had led to the use of insecticide in an improper manner to control vegetable pests, thus exerting a tremendous selection pressure on mosquito larval populations. It consequently resulted in the emergence of insecticide resistance in malaria vectors particularly to organochlorine (OC) and pyrethroids (PY).

The wide distribution of resistance to OC and PY in *An. gambiae s.l* reported in this study can be explained by a long-standing, massive use of DDT house-spraying in several districts of the country during the WHO malaria eradication programmes in the 1950s [21].

The high frequency of knock down resistance (*kdr*) mutation found in this study, suggested that this mutation is probably responsible for the emergence of resistance of *An. gambiae* populations to DDT and pyrethroids in Houeyiho. The *Ace-1* mutation found at a very low frequency ($\leq 5\%$) provided further evidence on the contribution of the *kdr* mutation to insecticide resistance in *An. gambiae* populations in Houeyiho.

The implication of metabolic mechanisms of resistance was not neglected in this study. Elevated levels of P₄₅₀ oxidases, NSEs and GSTs have been reported to be associated with insecticide resistance across all classes of insecticides [22]. In all of the processed mosquitoes from our study site, only GSTs, P₄₅₀ oxidase had significantly elevated levels compared to the reference susceptible Kisumu strain.

Reports from many scientists [22-23] have shown that elevation of NSEs and GSTs is related to organophosphate resistance, contradicting therefore the findings of this study. The general assumption is that insecticide resistance provides a selection pressure to all insecticides with similar mode of action. This is not true when it comes to metabolic resistance mechanisms, as some P450 enzymes show specificity for type I pyrethroids (such as permethrin) or type II pyrethroids (such as deltamethrin) [24]. This observation further confirms that metabolic resistance may be associated with resistance to different classes of insecticides.

Conclusion

Resistance to two classes of insecticide that are used for malaria vector control management has been identified in populations of *An. gambiae s.l* in the vegetable farming area of Houeyiho, located in Cotonou, southern Benin. The widespread pyrethroid resistance reported in this study could jeopardize the use of LLINs as a primary vector control tool in many areas of southern Benin. Finally, the findings of this study clearly show the presence of metabolic resistance mechanisms in malaria vectors, and suggested that metabolic resistance may be associated with malaria vectors' resistance to different classes of insecticides.

Competing interests

The authors declare that they have no competing interests.

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