https://www.scirp.org/journal/jbise
J. Biomedical Science and Engineering,
2025, Vol. 18, (No. 7), pp: 288-300

Resistance Profile Confirming the Presence of Three Knock-Down Mutations: *S*989*P*, *V*1016*G* and *F*1534*C* in *Aedes aegypti* in the Arrondissements of Abomey-Calavi and Ouedo in Benin

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Keywords: *Aedes aegypti*, Detoxification Enzymes, Pyrethroid Resistance, *kdr* Mutation, Abomey-Calavi, Ouèdo, Benin

Received: June 5, 2025 **Accepted:** July 22, 2025 **Published:** July 25, 2025

ABSTRACT

Context: The control of arboviruses such as Dengue focuses on the control of *Aedes* mosquitoes through the use of insecticides. Unfortunately, the effectiveness of this chemical control is influenced by the increasing frequency of insecticide resistance. The aim of this study was to assess the sensitivity of *Aedes aegypti* in the Abomey-Calavi district to pyrethroids, the frequency of *kdr* mutations and the level of expression of detoxification enzymes. Methodology: *Aedes* eggs were collected using 100 ovitraps in 04 districts of the Abomey-Calavi arrondissement and 96 ovitraps in Ouèdo. Female *Aedes* obtained were exposed to a diagnostic dose of permethrin, deltamethrin, alpha-cypermethrin and cyfluthrin according to the WHO tube test protocol. PCR was used to detect *kdr* mutations (*F*1534*C*, *S*989*P*, *V*1016*G*) in the voltage-gated sodium channel gene in exposed *Aedes* females. Finally, 65 females from the Abomey-Calavi district who had not been in contact with an insecticide were analyzed individually to assess the expression of detoxification enzymes. Results: The traps were highly positive, with a positivity rate of 77% in Abomey-Calavi and 85% in Ouèdo. The hatching and emergence rates

were 89% and 50% respectively in Abomey-Calavi, and 46% and 43% in Ouèdo. Identification of these adults yielded *Aedes aegypti* with a total of 1857 (99.73%), compared with 5 (less than 1% of the total) for *Aedes albopictus*. Sensitivity tests showed resistance to deltamethrin (86.53% in Zoundja and 88.95% in Zoca) and permethrin (45% Tokpa-Zoungo). In Ouèdo, on the other hand, the mortality rate of *Aedes* after exposure to permethrin, deltamethrin, alpha-cypermethrin and cyfluthrin was 100%. PCR genotyping of female *Aedes* DNA revealed the presence of *kdr* mutation alleles at very high frequencies. The *Aedes* mosquitoes analyzed had significantly higher median levels of oxidase and glutathione s-transferase (GST) expression than the insecticide-sensitive Rockefeller reference strain. Conclusion: The detection of deltamethrin resistance, several *kdr* mutations and overexpression of glutathione s-transferases and oxidases underscores the urgency of implementing alternative control strategies against *Aedes* mosquitoes. In addition, the implementation of a systematic monitoring of insecticide resistance in *Aedes* in Benin will enable a better understanding of susceptibility trends in space and time, and thus the development of better alternative control strategies to better control this vector.

1. INTRODUCTION

Vector-borne diseases are increasingly affecting populations, accounting for 17% of all infectious diseases. Among disease vectors, mosquitoes are the most formidable [1] and are mostly found in sub-Saharan Africa. Arboviruses are among these infectious diseases, causing 40,000 deaths worldwide every year. Yellow fever, dengue virus and Zika virus are arboviruses whose vector is *Aedes*, and whose disease burden is a source of major concern [1]. Between 2010 and 2019, cases of dengue fever were diagnosed in Benin, resulting in at least one death in the commune of Abomey-Calavi [2]. In the absence of effective vaccines or drugs, control relies mainly on vector control [3] through the use of chemical insecticides. Insecticides play a major role in mosquito control, and synthetic pyrethroids are the chemicals of choice because of their rapid and effective activity against insects, their low toxicity to mammals and their degradability in the environment [4, 5]. The WHO recommends the use of pyrethroids against adult mosquitoes and larvicides. Unfortunately, long-term intensive use of insecticides leads to the emergence of resistance in mosquito species under selection pressure, and this is one of the main obstacles to arthropod pest control [6, 7]. Many control programs are threatened by insecticide resistance. *Aedes aegypti* has been reported to be resistant to pyrethroids and organophosphates in various parts of the world, while little data is available on insecticide resistance in *A. aegypti* in Benin. The few studies that have been carried out show a decrease in the sensitivity of *A. aegypti* to a wide variety of active ingredients [8-10]. Two main mechanisms are involved in insecticide resistance in insects: secretion of detoxification enzymes and insensitivity of target sites [11]. The first mechanism involves overexpression or qualitative changes in the catalytic sites of enzymes such as non-specific esterases (NES), glutathione S-transferases (GST) and mixed-function oxidases (MFO). The importance of detoxification enzymes in *Aedes* resistance to different classes of insecticides has been reported in several previous studies in different parts of the world [12-14]. Target insensitivity is due to mutations that reduce the binding affinity between the insecticide and its physiological target. Pyrethroids and DDT directly target the sodium channel to cause nerve membrane depolarization [15] and insects develop resistance to these types of insecticides by substitution of one or more amino acids in the channel sequence [16]. These mutations in the sodium channel are known as "resistance *knockdown*" (*kdr*) and have been reported in *A. aegypti* in several African countries, including Benin, Ghana, Burkina Faso, Nigeria and Angola [8, 17].

In Benin, most data on insecticide sensitivity concern malaria vectors, and very little is known about Aedes. The lack of data on their sensitivity to insecticides used in public health is a growing obstacle for arbovirosis control programs. We assessed the susceptibility of *A. aegypti* adults to insecticides and the

mechanisms involved in four localities in the Abomey-Calavi district, in order to select the best insecticides to use in the event of an epidemic.

2. MATERIALS AND METHODS

2.1. Study Sites

The present study is a descriptive study that took place in the Ouèdo arrondissement from February 2022 to July 2022 and in Abomey-Calavi, more precisely in Tokpa-Zoungo from July to October 2022, then in Zoundja and Zoca from July to October 2023. Figure 1 shows the positions of the sites where the traps were set.

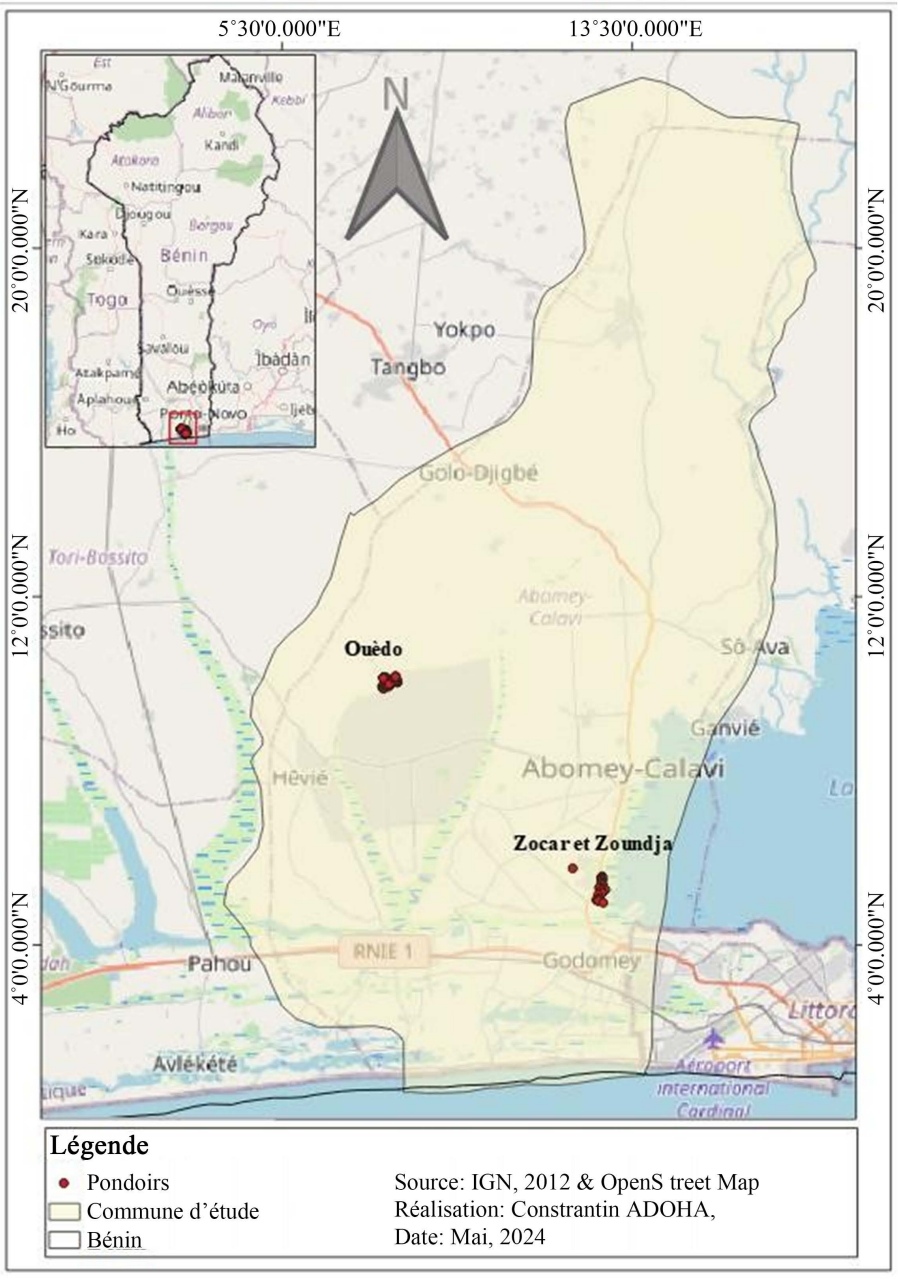

Figure 1. Trap positions at study sites.

2.2. *Aedes* Egg Collection and Adult Rearing at the Insectarium

The eggs were collected using *Aedes* ovitraps, due to the scarcity of *Aedes* mosquito breeding sites. We therefore used a total of 40 traps in each of the localities of Zoundja and Zoca during this collection period. In Ouèdo, a total of 96 traps were used, followed by 20 traps in Tokpa-Zoungo. These are black plastic pots that can hold half a liter of water, in which wooden egg-laying supports were immersed. The wooden egg-laying supports were removed from each pot after a week and transported to CREC. The water contained in the pots was received and transported to the CREC insectarium. Once at the Vector Bioecology Laboratory, eggs were counted using a binocular microscope and hatched according to standard insectary rearing procedures for Aedes species. Larval hatching rate was measured by visually counting the number of larvae per tray using a ladle. Adult emergence rate was measured using an aspirator.

2.3. Insecticide Sensitivity Tests

The insecticide resistance profile of *Aedes* populations in the field was assessed using the WHO tube method [18]. Given the number of *Aedes* adults available, *Aedes* females aged 2 - 5 days were exposed to diagnostic doses of Permethrin 0.75%; Deltamethrin 0.05%; Alphacypermethrin 0.05% and Cyfluthrin 0.15%. For each insecticide, four exposure tubes containing insecticide-impregnated papers and one control tube with untreated paper were used. 20 - 25 female *Aedes* mosquitoes were introduced into each tube, and the number of mosquitoes knocked down by the insecticide was counted every 15 min. After 60 min of exposure, the mosquitoes were transferred to tubes containing untreated paper, placed under observation (25˚C and 80% humidity) and fed on 10% honey juice. Mortality after 24 hours was recorded following WHO guidelines, with individuals considered dead if they were immobile or unable to stand upright.

2.4. Morphological Identification of *Aedes*

Adult *Aedes spp*. were identified using Fontenille's taxonomic keys [19] by microscopic visualization. *A. aegypti* and *Ae. albopictus* can be recognized by their characteristic white stripes on the legs. The thorax is then used to differentiate the two species. *A. aegypti* has two thin white median lines in the shape of a lyre, while *Ae. albopictus* has white median lines. *Ae. albopictus* has only one distinct white central line.

2.5. Genotyping for *kdr* Mutations

After grinding each mosquito in 200 µl of 2% CTAB, we then place in a Bain-Marie at 65˚C for 05 minutes. Then 200 µL of chloroform is added and centrifuged at 12,000 rpm for 05 minutes at room temperature after mixing by inversion at least 10 times. The supernatant collected in well-labeled tubes is mixed by inversion with 200 µl of isopropanol and centrifuged at 12,000 rpm at room temperature for 10 minutes. The isopropanol is drained off and centrifuged for 05 minutes at 12,000 rpm after adding 200 µl of 70% ethanol. After emptying the ethanol, the resulting DNA pellet is dried for 05 minutes in a speed-vac or for half a day on the bench. 40 µl of sterile H_2O is added to the DNA pellets in each tube, which are then left on the bench overnight or for half a day.

For *Kdr* genotyping, a random subset of both dead and alive mosquitoes from the WHO susceptibility bioassays was selected. This sampling strategy was applied to reduce selection bias and allow accurate estimation of allele frequencies. Allele-specific PCR (AS-PCR) was used to detect the presence of the *S*989*P*, *V*1016*G* and *F*1534*C* mutations according to the protocol of Li *et al.* 2015 [20]. Each mosquito was tested by AS-PCR twice, the first PCR used a primer specific for the susceptible and the second a primer specific for the mutant. The primers used for the genotyping were the following:

S989PF: 5'AATGATATTAACAAAATTGCGC3' and S989PR: 5'GCACGCCTCTAATATTGATGC; V1016GF: 5'GCCACCGTAGTGATAGGAAATC3' and V1016GValR: 5'CGGGTTAAGTTTCG TTTAGTAGC3'; and F1534CF: 5'GGAGAACTACACGTGGGAGAAC3' and F1534CR: 5'CGCCACTGAAATTGAGAATAGC3'.

2.6. Measurement of Detoxification Enzyme Activity

To quantify the activity of detoxification enzymes, biochemical tests were carried out only on 3- to 5-day-old female *Aedes* mosquitoes from Zoundja and Zoca not exposed to insecticides.

All mosquitoes were stored at −80˚C to avoid degradation of the enzymes prior to manipulation. All manipulations were carried out on ice. Mosquitoes were individually ground in 200 µl of distilled water. Grindings were centrifuged at 14,000 rpm for 2 minutes. For oxidases, 20 µl of the grindings were distributed in two wells of a microplate, and 10 µl in two replicates for the other enzymes. All plates must have two wells filled with 10 µl for background.

For oxidases, 80 µl of 0.0625M Potassium Phosphate buffer ($KHPO_4$) pH 7.2 was added to the 20 µl of shred and standard ranges in ascending order of concentration. Next, 200 µl of solution (composed of 12 mg of 3,3,5,5-tetra methyl Benzidine or TMBZ previously dissolved in 5 ml methanol and 18 ml of 0.25M Sodium Acetate Buffer pH 5.0) was added to the same replicates. After adding 25 µl of 3% hydrogen peroxide to each well, the plate was incubated for thirty minutes and the absorbance was read as an end point at 630 nm.

For non-specific esterases, 90 µl of 1% TBS (Triton Phosphate Saline) buffer was added to the 10 µl of shredded material from each mosquito and the standard ranges in ascending order of concentration. The plate was then incubated at room temperature for 10 minutes; after this, 100 µl of a solution (600 µl alpha-naphthyl acetate or beta-naphthyl acetate + 3 ml 1% Triton PBS buffer pH 6.5 + 6 ml H_2O) was added to each well and the plate was again incubated at room temperature for 30 minutes. Finally, we added 100 µl of a solution (10 mg Fast Garnett Salt dissolved in 12 ml distilled water) to each replicate and the plate was then incubated at room temperature with a lid on for 10 minutes. End-point absorbance was read on a spectrophotometer at 550 nm.

For total proteins, we added two 10 µl replicates of each mosquito grind to the plate and standard ranges in ascending order of concentration. Next, we added 200 µl of a solution (composed of 19.6 µl of Bicinchoninic Acid Solution and 380 µl of Copper Sulfate). End-point absorbance was read at 590 nm after holding the plate for 30 minutes at room temperature.

For glutathione-S-transferases, two 10 µl replicates of the crushed material were added to the plate. We then added 200 µl of a solution (composed of 20 ml 0.1M Sodium Phosphate buffer pH 6.5, 60 mg glutathione in reduced form (GSH), and 13 mg CDNB (-chloro-2,4-dinitrobenzene) all previously and fully dissolved in 500 µl methanol, respectively. Absorbance was read kinetically at 340 nm for 5 minutes.

3. DATA ANALYSIS

GPS coordinates of laying traps were recorded using the OSMTracker for AndroidTM application. Insecticide susceptibility test results were recorded and analyzed using Microsoft Excel 2019. Biochemical data were recorded through a computer connected to the microplate reader. Transformations from absorbance values to product quantity (µmol/min/mg protein) were automatically performed using GeneS.1 software, provided with the spectrophotometer. Statistical analyses were conducted using GraphPad Prism 5 software (version 5.00, San Diego, CA, USA). The Mann-Whitney test was chosen for comparison between Rockefeller (susceptible strain), and field mosquitoes. Statistical significance was determined if $p < 0.05$. All statistical analyses were performed in Stata/SE 17.0, including Pearson's chisquare test to investigate deviations from Hardy-Weinberg equilibrium.

4. RESULTS

4.1. Attractiveness of *Aedes* Traps

In this study, 196 traps were used, of which 159 were positive (*Aedes* females laid eggs in them) and 37 negatives, giving a positivity rate of 81% (Table 1). The total number of eggs obtained after collection was 8846.

Table 1. Trap-ponder positivity ratein the study areas.

Status of traps	Zoundja	Zoca	Tokpa zoungo	Ouedo	Total
Positive trap	35 (88%)	33 (83%)	09 (45%)	82 (85%)	159 (81%)
Negative trap	05 (12%)	07 (17%)	11 (55%)	14 (15%)	37 (19%)
Total	40 (100%)	40 (100%)	20 (100%)	96 (100 %)	196 (100%)

4.2. Hatching and Emergence Rates

After counting the eggs, we obtained a total of 6561 eggs for all the positive. After watering, hatching rates were 89% in Abomey-Calavi and 46% in Ouèdo. At the end, the rate of mosquitoes emerging was 50% in Abomey-Calavi and 43% in Ouèdo, as shown in Table 2.

Table 2. Hatching and emergence rates.

Abomey-Calavi district				
Locations	**Zoundja**	**Zoca**	**Tokpa-zoungo**	**Ouèdo**
Number of eggs	1015	954	269	4323
Number of larvae	960 (95%)	847 (89%)	180 (67%)	1998 (46 %)
Number of adults	453 (47%)	432 (51%)	110 (61%)	867 (43%)

4.3. *Aedes* Insecticide Test

Table 3 shows the results of 24-hour sensitivity testing of mosquitoes to insecticides. Analysis shows that *Aedes* strains from Ouèdo were 100% sensitive to permethrin, deltamethrin, alphacypermethrin and cyfluthrin within 24 hours. On the other hand, high resistance to deltamethrin and permethrin was observed in the *Aedes* populations of Zoundja, Zoca and Tokpa-zoungo, respectively.

Table 3. Results of mosquito sensitivity tests to insecticides.

Insecticides	Study site	Number of mosquitoes tested	Number of mosquitoes survived at 24 h	Number of mosquitoes dead at 24 hours	Mortality rate in 24 h	Status
Permethrin	Ouèdo	86	00	86	100%	S
	Tokpa-Zoungo	100	55	45	45%	R
Deltamethrin	Ouèdo	85	00	85	100%	S
	Zoundja	86	09	77	89.53%	R
	Zoca	81	13	68	83.95%	R
Alphacypermethrin	Ouèdo	87	00	87	100%	S
Cyfluthrin	Ouèdo	89	00	89	100%	S

Legend: S: Susceptible; R: Resistant.

4.4. Mosquito Identification

Following identification, the predominant species in the Abomey-Calavi and Ouèdo districts is *Aedes aegypti*, with a total of 1857 (99.73%), compared with 5 (less than 1%) for *Aedes albopictus.*

4.5. Identification of *kdr* Mutations

At the end of migration, the size of DNA fragments was visualized using ultraviolet light, by comparing the different bands obtained with the molecular weight marker. The size of PCR products for the detection of *kdr* mutations was 240 bp for (*S*989*P*), 284 bp for (*F*1534*C*), 348 bp (*V*1016*G*), while the size of products used as non-allele-specific external primers was 594 bp (*S*989*P*), 517 bp (*F*1534*C*), 592 bp (*V*1016*G*) (Figure 2).

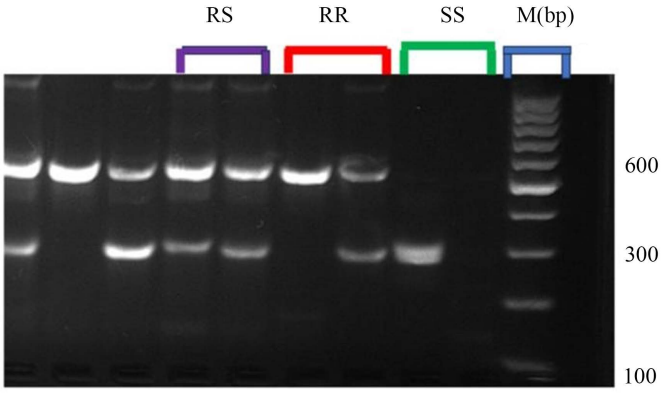

Figure 2. Gel electrophoresis bands of PCR products corresponding to *kdr* mutations. Image: A. Kotannou, 2023.

PCR was able to effectively distinguish between individual mosquitoes homozygous or heterozygous for the *S*989*P*, *F*1534*C* and *V*1016*G* mutations. The number of mosquitoes per genotype for the various mutations is summarized in Table IV below. Mutation frequency was calculated using the following formula [20] (Table 4):

$$\text{Fréquence } (kdr) = \frac{2RR + RS}{2(RR + RS + SS)} \times 100$$

Table 4. Frequency of *kdr* mutations.

kdr Mutation	Study Site	Mosquitoes Tested	Homozygote Mutation (RR)	Heterozygote Mutation (RS)	Homozygote Wild Type (SS)	Allele Frequency	
						R	S
*S*989*P*	Zoundja	14	06	06	02	0.64	0.36
	Zoca	09	04	03	02	0.61	0.39
	Tokpa-zoungo	46	00	01	45	0.01	0.99
	Ouèdo	46	00	07	39	0.08	0.92
*F*1534*C*	Zoundja	32	10	16	06	0.56	0.44
	Zoca	36	14	15	07	0.60	0.40
	Tokpa-zoungo	38	23	07	08	0.70	0.30
	Ouèdo	46	04	00	42	0.09	0.91

Continued

V1016G	Zoundja	28	05	11	12	0.38	0.62
	Zoca	31	10	08	13	0.45	0.55
	Tokpa-zoungo	45	04	02	39	0.11	0.89
	Ouèdo	46	00	00	46	00	1

4.6. Expression of Detoxification Enzymes

The *Aedes* mosquitoes analyzed showed significantly higher median levels of oxidases ($p > 0.0001$ at Zoundja and $p = 0.0002$ at Zoca) and glutathione s-transferases (GSTs) ($p = 0.0015$ at Zoundja and $p = 0.0055$ at Zoca) compared to the insecticide-sensitive Rockefeller reference strain. On the other hand, no significant difference was observed between the median levels of non-specific esterases ($p < 0.05$ in each study area) of the tested strain and those of the sensitive Rockefeller strain (Figures 3-5).

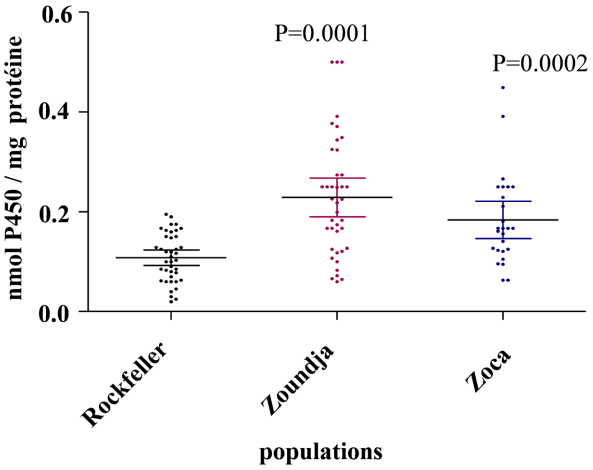

Figure 3. Expression of oxidases in *Aedes* mosquitoes at Zoundja and Zoca in the Abomey-Calavi commune.

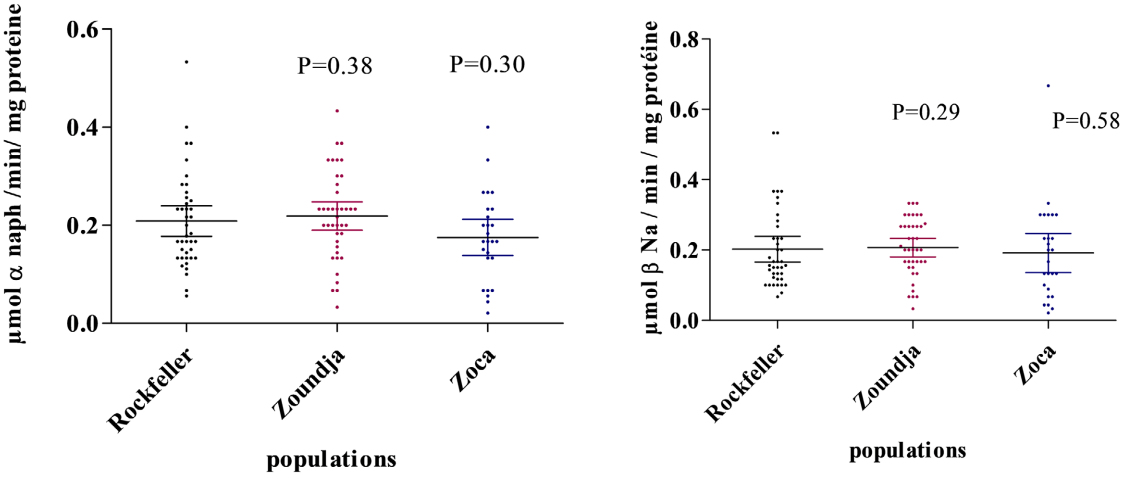

Figure 4. Expression of non-specific esterases (*α*-esterase on the left and *β*-esterase on the right) in *Aedes* mosquitoes at Zoundja and Zoca in the commune of Abomey-Calavi.

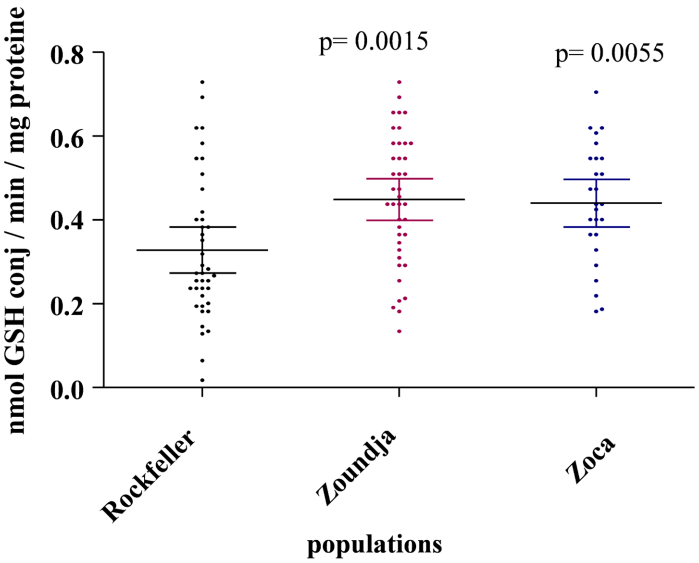

Figure 5. GSTs expression in *Aedes* mosquitoes at Zoundja and Zoca in the commune of Abomey-Calavi.

5. DISCUSSION

Insecticide resistance in arbovirus vectors is a major challenge in vector control. This study provides information on the current status of insecticide resistance, the presence of *kdr F*1534*C*, *S*989*P* and *V*1016*G* mutations and the expression of detoxification enzymes in *Aedes* populations in the Ouèdo arrondissement and three localities in the Abomey-Calavi arrondissement. The study revealed that the positivity of ovitraps was 85% in Ouèdo and 77% in Abomey-Calavi, testifying to the effectiveness of this method. This result shows that ovitraps are more attractive to gravid females and facilitate *Aedes* sampling. This confirms the results of previous studies, which reported that, in many epidemics, ovitraps showed positivity for the presence of *both A. aegypti and Ae. albopictus* [21, 22]. The low hatching rate of 46% observed in Ouèdo, on the other hand, may be due to the high proportion of unfertilized eggs, but also to the fact that the egg-laying trays were not left in the water long enough for all the eggs to hatch. As for the emergence rate, this is due to the density of the larval population. Larval population density increases larval mortality and lengthens larval development time, according to Pichon and Gayral in 1970 in three West African savannah villages (Dougoumato, Kongolekan and Koumbia) [23]. Sensitivity to permethrin, deltamethrin, alpha-cypermethrin and cyfluthrin was observed in the *Aedes* population of Ouèdo. These results also confirm those observed in Hêvié by Padonou *et al.* in 2020 [2], a neighbouring locality to Ouèdo, where a sensitivity of 100% to deltamethrin was observed. If, despite public health interventions in this locality, *Aedes* remains sensitive to insecticides, we can therefore affirm that insecticide resistance in vectors is due to agricultural practices.

On the other hand, resistance to deltamethrin and permethrin was observed in the *Aedes* population in the localities of Tokpa-zoungo, Zoundja and Zoca in the Abomey-Calavi arrondissement. Our results concur with those of Tokponnon *et al.*, 2024 [8] carried out in Cotonou and Abomey-Calavi and in Burkina-Faso by Sombié *et al.*, 2019 [24]. The reasons for these trends are not known, but a number of events may have contributed to them. This resistance to deltamethrin and permethrin is thought to be due to the unregulated use of insecticides for agricultural, domestic and public health purposes [25, 26].

After identification, *Aedes aegypti* and *Aedes albopictus* were the two *Aedes* species found in the Abomey-Calavi district. *Aedes aegypti* was in the majority, with a percentage of 99.73% (1857 *Aedes aegypti* and 5 *Aedes albopictus*). These results are close to those recorded in Hêvié in 2020 by Padonou *et al.* [2]. The abundance of *Aedes aegypti* in this locality is due to the poor storage of water in pots, buckets, used tires and domestic containers inside and outside houses as a result of livestock farming. In fact, water

used as a beverage for domestic animals remains stored for a long time in buckets, pots and other containers. Worn tires left by mechanics and extension workers are stacked one on top of the other, providing shelter for *Aedes* when it rains. This facilitates the proliferation of *Aedes aegypti*. There is a need to raise awareness among the local population of the need to change habits to avoid storing wastewater in households.

PCR genotyping revealed the presence of mutations *S*989*P*, *F*1534*C* and *V*1016*G* among the deltamethrin- and permethrin-resistant *Aedes* population. These mutations were first detected in Benin in 2024 by Tokponnon *et al.* [8]. The *F*1534*C* mutation has been described as widely distributed worldwide and associated with *Aedes* resistance to pyrethroids. The *S869P* mutation is thought to cause more potent resistance to insecticides when combined with *V*1016*G* or *F*1534*C* or both [27]. We detected the simultaneous presence of two mutations in 23 resistant *Aedes*, and all three mutations in 06 resistant *Aedes*. The co-occurrence of two or three *kdr* mutations has been reported in several countries, in Benin by Tokponnon *et al.* [8], in China by Li *et al.* [20], in Nigeria by Agbohun *et al.* [28] and in Malaysia by Zuharah *et al.* [29], and results in a higher level of resistance.

With regard to detoxification enzymes, we observed under-expression of esterases (α and β) and overexpression of GSTs and oxidases. Our results corroborate those reported by Konkon *et al.* [9] in Benin in 2023 and by Ngoagouni *et al.* [13] in the Central African Republic in 2016. On the other hand, in Saudi Arabia, Algamdi *et al.* [30] found a significant decrease in the activity of these enzymes. The metabolic mechanisms in which *GSTs* and oxidases are involved could confer the resistance to deltamethrin observed in *Aedes*. All comparisons were made with the insecticide-sensitive Rockefeller control strain. In addition, the insecticide resistance mechanisms observed in *Aedes* populations are characterized by the presence of all three mutations and the overexpression of GST and oxidases.

6. CONCLUSION

This study revealed a high sensitivity of *Aedes aegypti* to permethrin, deltamethrin, alphacypermethrin and cyfluthrin in Ouèdo, and high resistance to permethrin and deltamethrin in Zoundja, Zoca and Tokpazoungo. We also noted the presence of *Aedes albopictus*, an invasive mosquito in the Abomey-Calavi district of southern Benin. Mechanisms associated with this resistance included *kdr F*1534*C*, *S*989*P* and *V*1016*G* mutations and the expression of detoxification enzymes. Insecticide resistance can threaten the effectiveness of vector-borne disease control. The worldwide spread of deltamethrin resistance in *Aedes* mosquitoes and the overexpression of glutathione s-transferases and oxidases underscore the urgent need for additional monitoring studies. In this context, it is therefore necessary to understand which alternative insecticides would be most effective in controlling *Aedes* and how the resistance that has been detected can be effectively managed.

AUTHOR CONTRIBUTIONS

Conceptualization: T.F.T., Z.S.D., E.A.K.,O.K., D.G.H., and M.A.; data collection: T.F.T., Z.S.D., E.A.K., O.K., D.G.H., B.G, H.F., G.I., A.O., H.S; formal analysis: T.F.T., Z.S.D., E.A.K.,O.K., D.G.H., M.J.A., L.T., and R.O.; mobilization of funding: T.F.T., Z.S.D., E.A.K., O.K., D.G.H. and R.O.; methodology: T.F.T., R.O., and M.A.; project administration: T.F.T.; original draft preparation formal: T.F.T., Z.S.D., E.A.K., O.K., D.G.H. and M.A., supervision: T.F.T., L.B.M., and M.A. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work was partially supported by researchers from the Centre de Recherche Entomologique de Cotonou. Permission was requested and obtained before setting up the Aedes oviposition traps. All individuals mentioned in this section provided their consent for acknowledgment.

ACKNOWLEDGEMENTS

The observations and conclusions presented in this manuscript reflect only the opinion of the author(s). We express our gratitude to the Director of the Centre for Entomological Research of Cotonou, Mr. Germain Gil Padonou, and to his entire team, both in the field and in the laboratory, for their valuable support.

DATA AVAILABILITY STATEMENT

All data used in this study are included within the article.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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