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Effects of pyrethroids and carbamates on the immune system of *Culex quinquefasciatus*: Enzymatic activities and genetic mutations in the commune of Parakou

Yadouleton Anges[1*], Dramane Gado[1], Klotoe Jean Robert[2], Badou Yvette[3], Baba-Moussa Lamine[4]

[1] Department of Zoology, Natitingou Higher Normal School, National University of Sciences, Technologies, Engineering and Mathematics (UNSTIM), Benin

[2] Entomological Research Center, Cotonou, Benin

[3] Department of Zoology, Laboratory of Viral Hemorrhagic Fevers and Arboviruses of Benin, Benin,

[4] Department of Biochemistry and Cellular Biology, Faculty of Sciences and Techniques, University of Abomey-Calavi, Cotonou, Benin

Abstract

A cross sectional entomological study was carried out from January to December 2021 in the district of Parakou, East-Benin in order to evaluate the effects of pyrethroids and carbamates on the immune system of *Culex quinquefasciatus*. The study was based on assessing the susceptibility of adults of *Cx. quinquefasciatus* aged 2-5 days from larvae collected in two urban areas (Zongo and Tranza) and in two rural areas (Beyerou and Tourou). These mosquitoes were subjected to insecticide-impregnated papers (bendiocarb 0.1%, deltamethrin 0.05%, permethrin 0.75% and DDT 4%) following WHO testing protocol. The presence of G119S mutation in the acetyl cholinesterase-encoding gene (*ace-1*) and the knock down resistance (L1014F) were done with the surviving and dead mosquitoes post testing. Moreover, Mixed Function Oxidase (MFO), non-specific esterase (NSE) and glutathione-S-transferases (GST) activity in *Cx. quinquefasciatus* from each zone were evaluated and compared to the S-Lab (reference strain of *Cx. quinquefasciatus*).

Results from this study shows that *Cx. quinquefasciatus* populations were fully resistance to DDT (3% as a means of mortality), permethrin (15%), deltamethrin (22%) and bendiocarb (86%). The L1014F mutation was found both in urban and rural areas with 0.85 as mean of frequency. The *Ace-1* mutation was found at a very low frequency (≤ 5%). Moreover, Esterase, Glutathione-s-transferase (GST) and P450 monooxygenase activities were significantly higher in the wild population of *Cx. quinquefasciatus* populations from all the sites compared to the reference strain S-Lab ($P < 0,05$).

These findings showed that insecticide resistance status in *Cx. quinquefasciatus* in the district of Parakou is alarming. It's therefore crucial to develop Integrated Pest and Vector Management (IPVM) strategies for better control of this filariasis vector in this district.

Keywords: filariasis, vector control, *Culex quinquefasciatus*, resistance, *kdr*, ace^{-1R}, enzyme

Introduction

All living organisms, including animals and plants, have a defence system against any external aggression with biological mechanisms that allow the organism to develop an immune system capable of ensuring its integrity by recognising or tolerating what belongs to it and eliminating foreign substances [1].

In the fight against mosquitoes which in this case is *Culex quinquefasciatus*, the main vector of lymphatic filariasis, many insecticides such as pyrethroids (PY) and carbamates are used to defeat this mosquito [2]. In order to survive against these external attacks, mosquitoes are equipped with an immune defence weapon just after exposure to insecticides to better protect themselves against possible toxic products. These weapons include immune defence proteins and enzymes.

Resistance in *Cx. quinquefasciatus* has emerged in Africa since the 1960s. The first cases were recorded in Burkina with the appearance of resistance of *Cx. quinquefasciatus* to dieldrin, and a year later to DDT [3]. Faced with the resistance of malaria vectors to organochlorines (OC), pyrethroids (PYs) were introduced both in agriculture (against crop pests) and in public health (impregnation of mosquito nets, indoor spraying) because of their rapid action, their excito-repellent effect, their low toxicity dose and their good tolerance for humans [4].

Mosquitoes develop immunity to defend themselves against insecticides to which they are exposed in the wild, particularly in farmers' fields during insecticide treatments against crop pests and during house spraying. In fact, during insecticide treatments in crop fields against pests, pesticide particles come into contact with the larval breeding grounds. These particles exert either a lethal action on the larvae of certain insect populations or a pressure that progressively leads to the selection of resistance to PY and OC in *Cx quinquefasciatus* [5]. The knock down resistance (*Kdr*) mutation, one of the mechanisms of cross-resistance to PYs and OCs in *Cx quinquefasciatus* has been widely reported during the last decade in several African countries such as Côte d'Ivoire [6], Nigeria [7], Burkina Faso [8], Cameroon [9] and Benin [5]. It is likely that the immune system of *Cx. quinquefasciatus* may have developed a resistance mechanism to insecticides used in agriculture or public health. Alternatively, report from Benin on insecticide resistance in *Cx. quinquefasciatus* showed the presence of homozygous susceptible (ss) individuals in both live mosquitoes from PY and carbamate susceptibility tests, this

would suggest the existence of other resistance mechanisms such as enzymes within the Benin mosquito populations [10]. It is possible that the immune system of *Cx. quinquefasciatus* has developed enzymatic activities that enable it to resist xenobiotic factors in its ecological environment.

It is within this framework that the present work is being carried out in order to evaluate the effects of pyrethroids and carbamates on the immune system of *Culex quinquefasciatus*.

Materials and methods

Data collection areas

Data collection took place in the department of Borgou, more precisely in the commune of Parakou (9°20′13″N, 2°37′49″E). Four collection sites were chosen: two in urban areas (Zongo and Tranza), and two in rural areas (Beyerou and Tourou). The city of Parakou was chosen because of its rapid urbanisation and the absence of basic services and hygiene facilities, which constitute real breeding sites for *Cx. quinquefasciatus.*

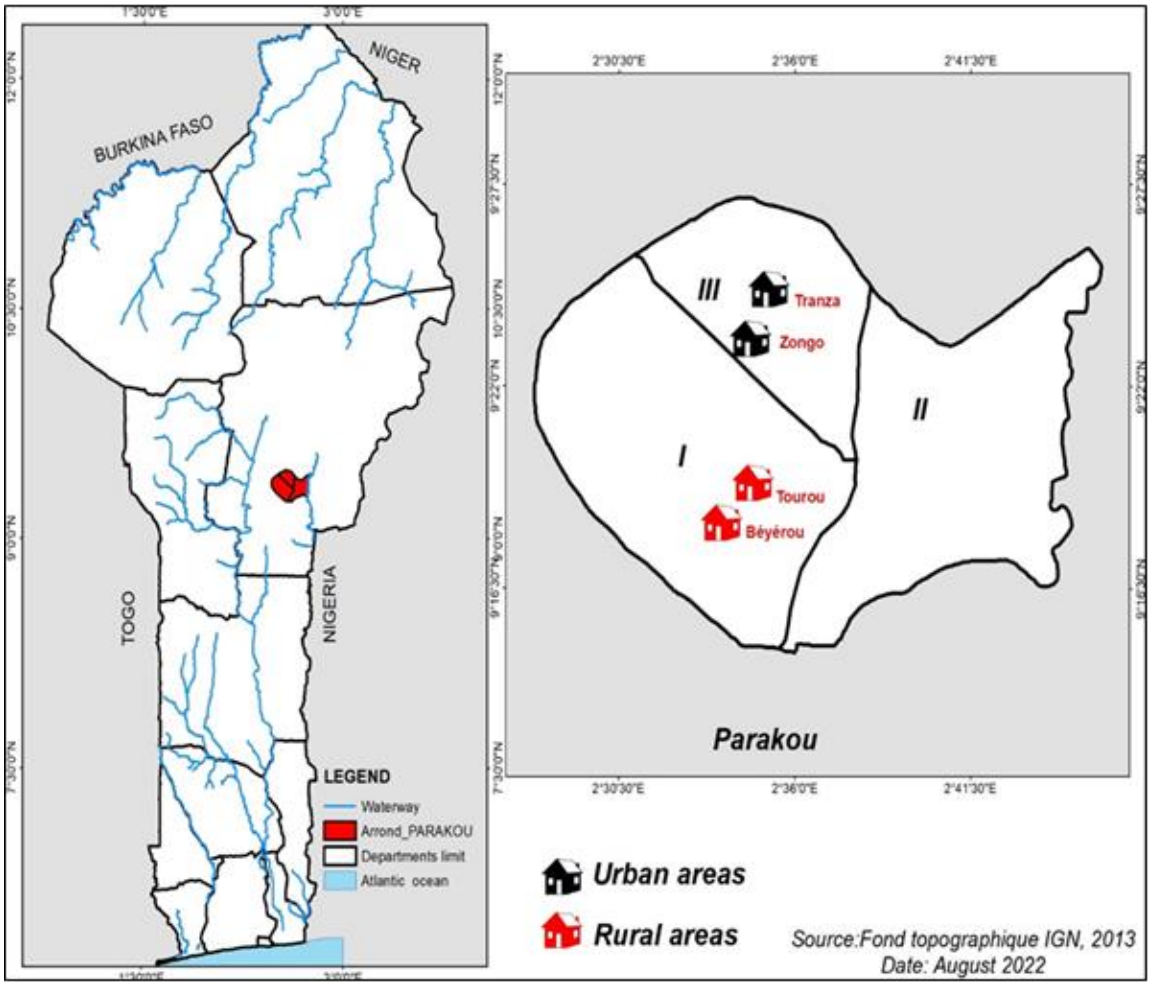


Fig 1: Map of the study area indicating the study sites

Larval collection

Several larval surveys were chosen for larvae collection. Once the site was located, larvae and pupae were collected and sent to the insectarium for rearing. Adults that emerged from the larvae collected from the field and caged were fed a 10% sucrose solution. The 2-5-day old adult females were isolated for susceptibility/resistance testing.

Susceptibility tests

Insecticide resistance bioassay was conducted following World Health Organization standard procedures WHO [11]. Insecticide papers treated with permethrin (0.75%), DDT (4%), deltamethrin (0.05%) and bendiocarb (0.1%) were used in this study in order to know the susceptibility of *Cx. quinquefasciatus* to the insecticides used in public health. Female aged 2–5 days were exposed to insecticide treated papers cited above for 60 min following. 20-25 female of *Cx. quinquefasciatus* were introduced into a cylinder tube and monitored every ten min the "knocked-down" mosquitoes. After the exposure time, mosquitoes were transferred into a new cylinder tubes and provided with cotton wool saturated with 10% honey solution. Mortality rate was recorded 24 hours later and death and alive mosquitoes were separated in Eppendorf tubes and placed in a freezer at -20°C for further diagnostic.

The susceptible strain SLAB was used as a control group.

Detection of the knock down mutation and the acetylcholinesterase mutation

100 *Cx. quinquefasciatus* females from each sites were used for searching the presence of the knock down mutation and the acetylcholinesterase using CTAB technique [12]. The genotype L1014F *kdr* mutation of the VGSC and G119S *ace-1* mutation were searched following the protocols developed respectively by Martinez-Torres *et al*. [13] and Weill *et al*. [14].

Enzymatic resistance

Biochemical analysis

100 adult females of the wild populations of *Cx. quinquefasciatus* from the 4 sites (Figure. 1) kept at -80 degrees were subjected to biochemical based on the methods decribed by Penilla *et al* [15] to compare the levels of activity of mixed function oxidases (MFO), non-specific esterases (NSE) using α-naphtyl acetate as a substrate and glutathione S-transferases (GST) to the laboratory S-Lab susceptible reference strain. Individual mosquitoes were homogenized in 200 µl ml distilled water. Each of 10 ml of the homogenate was used for monooxygenase, glutathion S-transferase and protein assay. The other 20 µl ml of homogenate was used for esterases assay.

Glutathione -S-transferase (GST) assay

10 µl of each homogenate was transferred to a microplate well followed by 200 µl of the GSH/CDNB working solution which was prepared by adding 0.060g of glutathione solution (GSH) in 20 ml of Phospahte sodium buffer 0.1M and 0.013gr (in 1mL of methanol) 1-chloro-2,4-dinitrobenzene (CDNB). The plates were read after 5 mins at a 340 nM.

Monooxygenase (Cytochrome p450)

Cytochrome P450 activity was determined using the heme-peroxidase assay according to the protocol described by Namountougou *et al* [17]. Following the protocol described by Penilla *et al*. [15]. This assay detects the elevation in the amount of heme, which is then converted into equivalent units of cytochrome P450. In addition to the protocol described by David *et al* [16], 80 ml of 0.625 M potassium phosphate buffer (pH = 7.2) were added to 20 ml of mosquito homogenate together with 200 ml Tetramethyl Benzidine solution (0.011 g of 3,3',5,5' Tetramethyl Benzidine in 5 ml of 70% methanol +15 ml sodium acetate buffer 0.25 M pH = 5.0); 25 ml of 3% hydrogen peroxide were then added and the mixture was incubated for 30 min at room temperature base on the protocol described by Namountougou *et al*[17]. The absorbance was read at 630 nm and values calculated from a standard curve of cytochrome C following the protocol described by David *et al*. [16].

- **Esterase assay**

20 µl of homogenate was placed into separate microplate wells. 200 µl of 0.3 mM Alpha/Beta napthyl acetate was added to each well. The plate was incubated at room temperature for 1 min and then 50 µl of fast garnet was added. After 30 minutes, enzyme activity was determined after the plate was read by microplate reader at a wavelength of 450 nm.

- **Protein assay**

Total protein of individual mosquitoes was determined using the BioRad Protein Assay Kit (Bio -Rad Laboratories).

Results

Insecticide resistance profile of *Cx. quinquefasciatus* for DDT, permethrin, deltamethrin and bendiocarb

These tests showed that: *Cx. quinquefasciatus* developed resistance to DDT with an average mortality rate of 3% for urban and rural area (Fig 2). With pyrethroids (permethrin, deltamethrin), this mosquito has developed resistance to these insecticides regardless of their area of origin. As a matter of fact, the average mortality rate is 15% both in urban and rural areas when *Cx. quinquefascitus* populations are exposed to permethrin papers (Fig 3). With deltamethrin, the average mortality is 22% (Fig4). This resistance was also observed with bendiocarb with 86% as an average mortality in urban and rural areas (Fig 5).

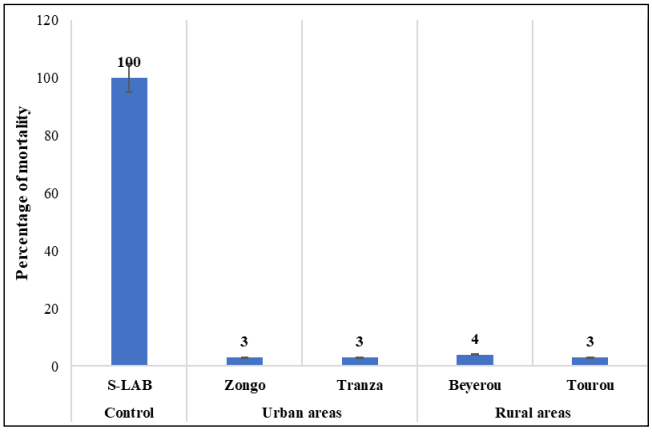


Fig 2: Mortality observed after exposure of *Cx. quinquefasciatus* populations to DDT (4%)

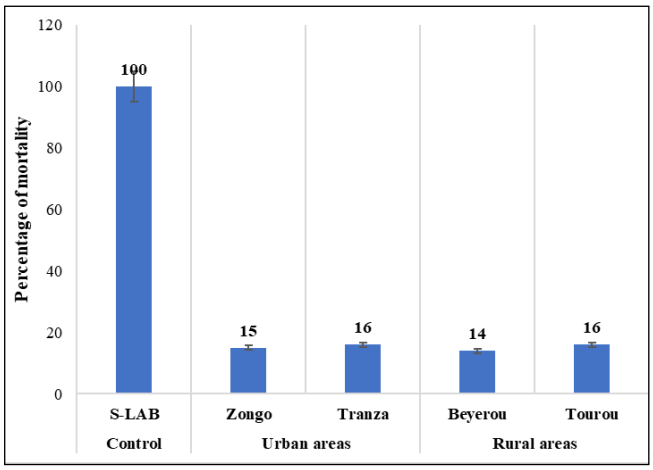


Fig 3: Mortality observed after exposure of *Cx. quinquefasciatus* populations to permethrin

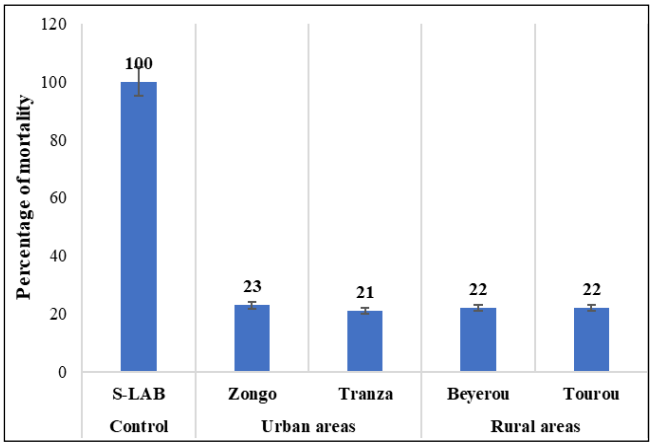


Fig 4: Mortality observed after exposure of *Cx. quinquefasciatus* populations to deltamethrin

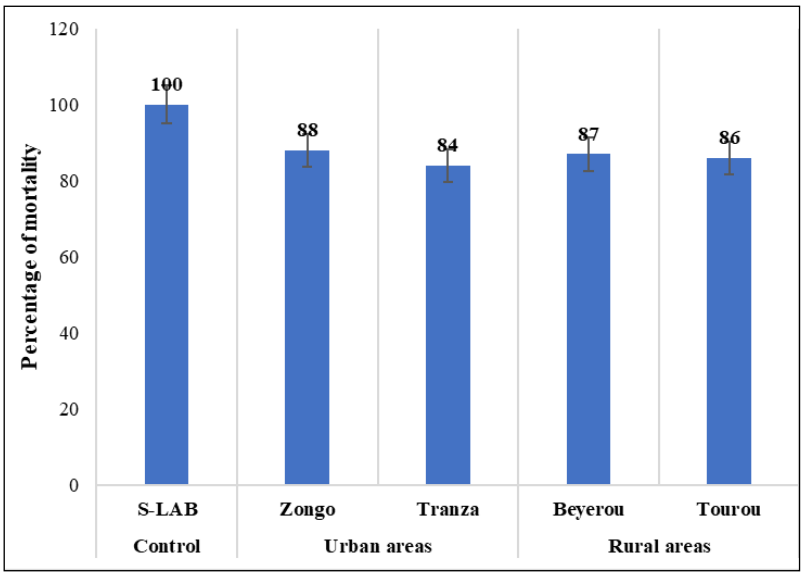


Fig 5: Mortality observed after exposure of *Cx. quinquefasciatus* populations to bendiocarb

Mechanism of resistance: PCR species, forms, *Kdr* and *Ace-1*

In each location, 100 mosquitoes were analysed for target modification mechanisms (*Kdr* and *Ace-1*). In rural and urban areas in Parakou, the *Kdr* gene appears to be the main resistance mechanism observed in these populations of *Cx. quinquefascitus* with a high frequency of 0.8 on average in rural areas (Table I). The *Ace-1* mutation was also found with a frequency of 0.25 and 0.27 in urban and rural areas respectively within the *Cx. quinquefasciatus* populations (Table I).

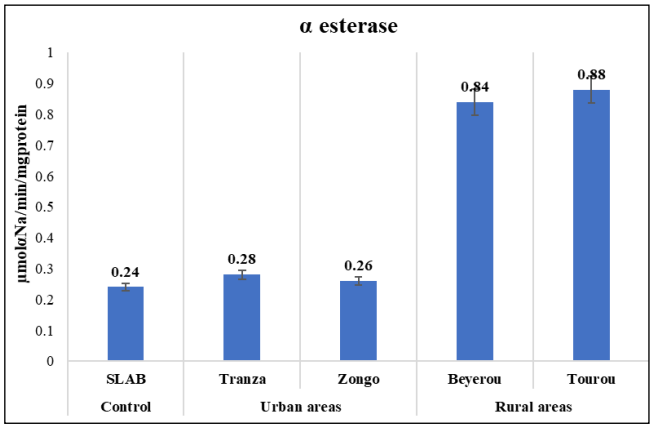
Table 1: Distribution of *Kdr* and *Ace-1* frequencies of *Cx. quinquefasciatus* from the study areas

		***Kdr* Mutation**				***Ace-1R* Mutation**			
Study sites	**Locations**	**SS**	**RS**	**RR**	**F(R)**	**SS**	**RS**	**RR**	**F(R)**
Urban areas	Zongo	3	21	78	0.88 [a]	72	18	18	0.25 [a]
	Tranza	4	24	74	0.84 [a]	72	18	18	0.25 [a]
Rural areas	Beyerou	4	30	68	0.81 [a]	60	16	18	0.27 [a]
	Tourou	4	32	66	0.80 [a]	90	24	0	0.27 [a]

NB. Values in the same column with superscript letters are not significantly different with Fisher's test (P >0.05)

Enzymatic resistance

Results of our research showed the presence of esterase activity (alpha (α) and beta (β) in populations of *Cx. quinquefasciatus* from our various study areas (Fig 6 A and B). These activities were very low in all mosquitoes and no significant difference was found in the esterase activities compared to of the control strain and (P >0.05).



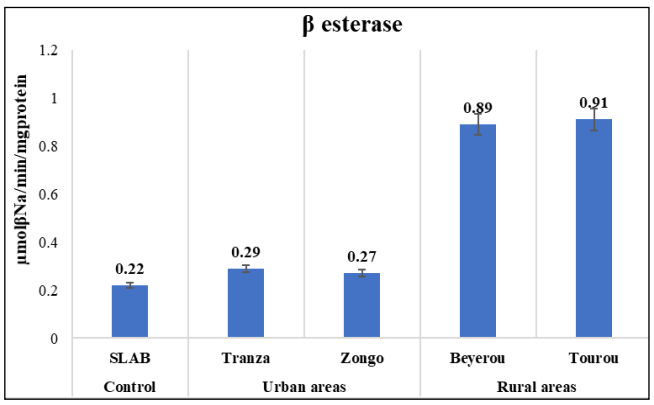


Fig 6: Alpha and beta esterase activities in *Cx. quinquefasciatus* populations from the study sites

- **P450 monooxygenase activities in mosquitoes**

Figure 7 shows that almost all populations of *Cx. quinquefasciatus* from the various study areas had high oxidase activities compared to the control strain SLAB (P<0.05).

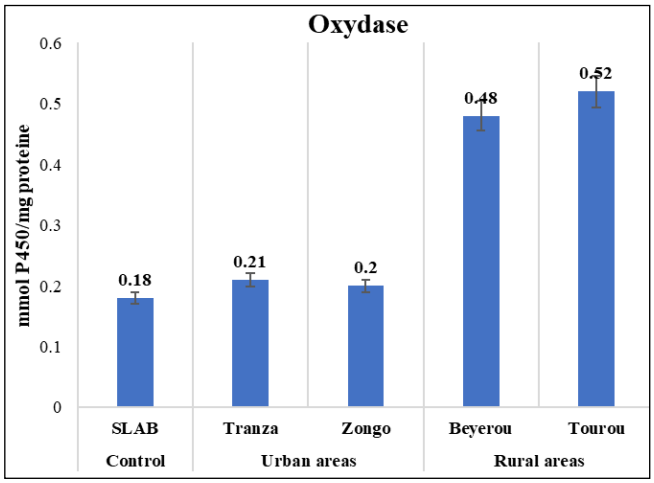


Fig 7: Oxidase activities in *Cx. quinquefasciatus* populations at the study sites

- **Glutathione-s-transferase activities**

GST was present in all populations of *Cx. quinquefasciatus* in the different study areas. However, there was a high GST activity in the wild populations from the different study sites compared to the susceptible strain SLAB (P <0.05) (Figure 8).

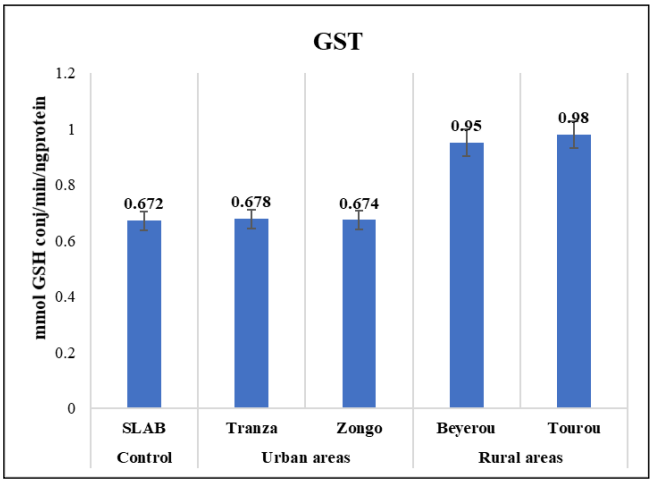


Fig 8: GST activity in *Cx. quinquefasciatus* populations from the study sites

Discussion

Culex quinquefasciatus, the main vector of urban Bancroft in Africa, has developed a strong resistance to insecticides in areas with high and low agricultural insecticide use (rural and urban) in the commune of Parakou. Farmers' practices in the use of insecticides in market gardening and especially in cotton production to control *Helicoverpa armigera* (the main cotton pest) and *Plutella xylostella* (the main cabbage pest) are a factor in the selection of resistant insects not only in crop pests but also in filariasis vectors [1]. After insecticide treatments, pesticide particles come into contact with the breeding sites. These particles exert either a lethal action on the larvae of certain insect populations or a selective pressure that progressively leads to the selection of insecticide resistance in certain mosquito populations, notably in *Cx quinquefasciatus* [10]. Yadouleton *et al* [20] have shown that many chemicals are used against pests in the agricultural areas mentioned above. These authors believe that the massive use of pesticides in agriculture in Benin is due to the deregulation of the input sector in Benin. In the 1960s, the selective role of agricultural treatments; organochlorine (OC) on the resistance of *Cx quinquefasciatus* was observed in Mali in areas that had never been subject to public health treatments but where these insecticides were widely used in agriculture. In Côte d'Ivoire and Burkina Faso, it was shown in the late 1990s that the level of resistance of vectors to pyrethroid insecticides increased during the cotton season [8].

In addition, our research work has also shown the resistance of *Cx. quinquefasciatus* to carbamates, particularly to bendiocarb. This observed resistance can be explained by the use of bendiocarb by the National Malaria Control Programme for indoor spraying since 2010. Although the use of this insecticide as an alternative to pyrethroids has reduced malaria transmission in this location (Parakou) and other places, but contributes to increase the level of insecticide resistance in Cx. quiquefasciatus populations [20]. This led also to the selection of the acetylcholinesterase mutation within *Cx. quinquefasciatus* populations. In addition, Yadouleton *et al* [20] showed that many farmers in cotton areas such as Parakou use Tihan, an insecticide of the carbamate family without respecting the doses and frequencies of application to control crop pests. Undeniably, the use of these various classes of chemical pesticides in agriculture and public health leads to physiological changes in the nerve cells of *Cx. quinuqefasciatus*, which could be the basis of the insecticide resistance observed in this insect. During insecticide treatments, insecticides penetrate the organism of *Cx. quinquefasciatus* and more rapidly at the cellular level the proteins and enzymes whose normal functioning they hinder. This malfunction manifests itself either by an overproduction of enzymes which will allow the insect to survive or by a decrease which leads to the death of the insect.

In the case of our study, we note an overproduction of these enzymes in *Cx. quinquefasciatus* towards these pesticides. However, the presence of high glutation transferase activities in all wild populations of *Cx. quinquefasciatus* from the various sites confirms the high resistance of this mosquito to DDT and confirms the work of Nchoutpouen *et al* [9]. Furthermore, the high P450 monooxygenase activity in all populations of *Cx. quinquefasciatus* is only a consequence of the high frequencies of the *Kdr* gene observed in *Cx. quinquefasciatus* in the collected samples (Table 1). This high oxidase activity also confirms of the work of Namountougou*et al* [17] explaining the high use of pyrethroids and carbamates in this commune of Parakou.

Conclusions

The study on the effects of insecticides on the central nervous system cells of *Culex quinquefasciatus* in the commune of Parakou in Benin revealed that the immune system within the populations of *Cx. quinquefasciatus* has developed resistance mechanisms towards organochlorines, pyrethroids and carbamates, thus enabling this mosquito to survive in its ecological environment despite the use of chemical pesticides.

In view of the high frequency of the *Kdr* gene mutation and the appearance of the *Ace-1R* mutation, it is essential that measures be taken for the rational use of chemical pesticides in both the agricultural and public health sectors. This will limit the selection of resistance through insecticide rotation, which will ensure the sustainable use of insecticide-treated nets against Culicidal pests.

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Competing interests
The authors declare that they have no competing interests

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