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STUDY OF THE IMMUNE SYSTEM OF MOSQUITOES AGAINST INSECTICIDES: ENZYMATIC ACTIVITIES AND GENETIC MUTATION IN *ANOPHELES GAMBIAE* POPULATIONS AT NATITINGOU, NORTH-EAST OF BENIN

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ABSTRACT

In order to study the immune system of mosquitoes against the use of insecticides in their ecological environment, a study has been carried out on the enzymatic activities and genetic mutation in *Anopheles gambiae* populations in the district of Natitingou, northern Benin from January to May 2016. Bioassay tests to assess the susceptibility of malaria vectors were done where females of An. gambiae aged to 2-5 were subjected to insecticide-impregnated papers (permethrin 0.75%, delthamethrin 0.05%, DDT 4%, and bendiocarb 0.1%) following WHO testing protocol. The presence of knock down resistance (kdr) and acetylcholinesterase *(ace-1R)* mutations were determined by Polymerase Chain Reaction (PCR). Finally, biochemical analysis was done in order to detect Mixed Function Oxidase (MFO), non-specific esterase (NSE) and glutathione-S-transferases (GST) activity in individual 2–5 days old adult *An. gambiae* that had been not previously exposed to insecticides. This research showed that the improper manner of the use of insecticides by farmers contributed to the emergence of insecticide resistance in malaria vectors with a wide spread of resistance to DDT (3% as a means of mortality), permethrin (23%) and delthamethrin (30%) but fully susceptible bendiocarb. The knockdown resistance (kdr) mutation was the main resistance mechanism identified in these *An. gambiae* populations with 0.85 as frequency. The *Ace-1* mutation was found at a very low frequency (≤ 5%). The presence of enzymatic activity (Esterase, Glutathione-s-transferase (GST) and P450 monooxygenase) in the wild population of *An. gambiae* was significantly higher than the control strain ($P < 0,05$). This study provides clear evidence that the use of insecticides by local farmers for crop protection is one factor that impacts negatively the immune system of mosquito which has led to the emergence of insecticide resistance in malaria vectors. Therefore, the need to develop Integrated Pest and Vector Management (IPVM) strategies and management of insecticide resistance in malaria vectors seem to be very important for vector control.

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INTRODUCTION

More than 90% of recorded malarial deaths occur in Africa among the most vulnerable low immune response individuals, such as children under five years old and pregnant women (WHO, 2009). The National Malaria Control Programmes (NMCP) in African countries currently relies on strategies targeting mosquito vector control, which involve the use of long-lasting insecticidal nets (LLINs) and/or indoor residual spraying (IRS), the two most effective preventive measures. Both methods have shown to be effective against Anopheles mosquitoes (Aïkpon *et al*., 2013). In 2013 in Benin, a full coverage of LLINs countrywide couple with IRS in the district of Natitingou in northern Benin was done as a new tool in order to improve malaria prevention and control. However, the development of pyrethroid resistance in populations of *Anopheles gambiae* has become a serious threat to the

effectiveness of these two vectors control measures (Djenontin *et al*., 2009). N'Guessan *et al*. (2007) established a clear relationship between pyrethroid resistance caused by knock down resistance (kdr) and the failure of LLINs and IRS in experimental huts in south Benin. In the last decade, the emergence of resistance in populations of Anopheles to common classes of insecticides used in public health has been reported in many African countries (Fanello *et al.,* 2003; Diabate *et al.,* 2002; Czeher *et al*., 2008; Djouaka *et al*., 2011). In West Africa, the main mechanism involved in pyrethroid-resistance in *An. gambiae* is caused by target site insensitivity through a knockdown resistance (kdr L1014F) like mutation caused by a single point mutation (Leu-Phe) in the para-sodium channel gene (Weill *et al.,* 2004). The mechanisms involved in these resistances are due either to a mutation in the target of insecticides: *kdr* mutation (Czeher *et al*., 2008) and *Ace-1* G119S mutation (Yadouleton *et al*., 2011), or an increase of metabolic enzyme detoxification processes through the oxidase, esterases and Glutathione-s-transferases (David *et al*., 2013). In Benin, the National Malaria Control Program has implemented a comprehensive program of free distribution of LLINs at Natitingou. At the same time, an extensive campaign of indoor spraying (based on the use of the bendiocarb and pyrimiphos methyl) is underway. It is necessary to assess the effect of these two programs on the resistance of malaria vectors. Moreover, the expansion of cotton, vegetable farming cultivation in this district and the intensive and improper use of insecticides, mainly pyrethroids, but also organophosphates and carbamates, contributes to the selection of insecticide resistance in malaria vectors in this district. In addition, despite the extensive work on the resistance of vectors to insecticides in Benin (Djenontin *et al.,* 2009), not all sites are covered by this work, and we lack some data in the field of mechanisms of resistance and the relationship between the immune system of mosquitoes and insecticides. Therefore, the current study was designed to Study of the immune system of mosquitoes against insecticides: enzymatic activities and genetic mutation in *Anopheles gambiae* populations at Natitingou, North-East of Benin**.**

MATERIALS AND METHODS

Study sites: The study was carried out in 2 urban (Ourbourga and Kantaborifa) and two rural (Tigniti and Yimporima) areas located in the district of Natitingou (1°23 E, 10°18 N) in North East of Benin**.** (Figure 1). The choice of the city of Natitingou is justified by the fact that since 2010, the National Malaria Control Program used a various chemical pesticides in IRS activities to reduce the incidence of malaria in this district (Aïkpon *et al*., 2013).

Mosquito collections: Larvae and pupae were collected in the four points of the study site using the dipping on breeding sites and kept in separated labelled bottles related to each locality from January to May 2016. Larvae samples collected in the 4 points were reared up to adult emergence at the CREC (Centre de Recherche Entomologique de Cotonou, Benin) insectary for further bioassay tests.

Susceptibility test: Non blood-fed female mosquitoes aged 2 to 5 days, morphologically identified as *An. gambiae* s.l. were exposed to diagnostic doses of various insecticides for susceptibility testing using the paper impregnated with insecticides as described in the standard protocol of tests (WHO, 98). The following insecticides were tested: permethrin 0.075%, deltamethrin 0.05%, DDT 4% and, bendiocarb 0.1%. The emphasis was put on deltamethrin, because of a nationwide distribution of PermaNets by the NMCP. The use of DDT is justified by the detection of cross resistance between pyrethroids and organochlorine in Anopheles populations. The carbamate bendiocarb was one of the alternative insecticides to pyrethroids currently used for IRS in Benin (Akogbeto *et al*., 2010). For each treatment, five test tubes were used: one untreated paper as a control and four treated papers to expose mosquitoes. Control tubes contained filter papers impregnated with silicon oil (insecticide carrier) only, whereas treated papers were impregnated with diagnostic doses of insecticide plus carrier. An average of twenty-five mosquitoes was introduced into each tube. Females of *An. gambiae* used in this study were exposed for one hour to insecticide-treated papers and monitored at different time intervals (10, 15, 20, 30, 45, 60 minutes) to record the "knock-down" times. After one-hour exposure, mosquitoes were transferred into holding tubes and provided with cotton wool wetted with a 10% honey solution. Mortalities were recorded after 24 hours and the susceptibility status of the population was graded according to the WHO recommended protocol (WHO 98). Dead and survived mosquitoes from this bioassay were separately kept in Carnoy solution at -20°C for further molecular characterization

Detection of *Kdr* L1014F and *Ace-1* G119S mutations in *An. gambiae s.l.*: Mosquitoes from the susceptible test from each point were analyzed by Polymerase Chain Reaction to detect *Kdr* L1014F and *Ace-1* G119S mutations according respectively to the protocols by Martinez *et al.* 1998 and Weill *et al.* 2004. The resistance allele frequency at the kdr and Ace-1 locus was calculated using Genepop software (version 3.3) as described by Raymond and Rousset (1995).

Biochemical analysis: 60 adult females of the wild populations of *An. gambiae s.s.* from the study site were kept at -80 degrees and were subjected to biochemical based on the methods decribed by Hemingway *et al*. (2004) 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 Kisumu susceptible reference strain.

Data analysis: Biochemical assay data (enzymatic activity per mg protein, levels of MFO, NSE and GST between Kisumu and field populations *An. gambiae s.s.*) were compared using Mann-Whitney non-parametric ***U***-test (Statistica software).
The resistant status of mosquito samples was determined according to the WHO criteria:

- Mortality rates > 97%: the population was considered fully susceptible.
- Mortality rates ranged between 80 > × < 97%: resistance suspected in the population.
- Mortality rates< 80%, the population was considered resistant to the tested insecticides.

RESULTS

Resistance to insecticides: A total of 525 adult females of *An. gambiae* collected from the different exposed to papers impregnated with discriminating doses of permethrin (0.75%), deltamethrin (0.05%), DDT (4%) and bendiocarb (0.1%) showed that all populations of *An. gambiae* mosquitoes were

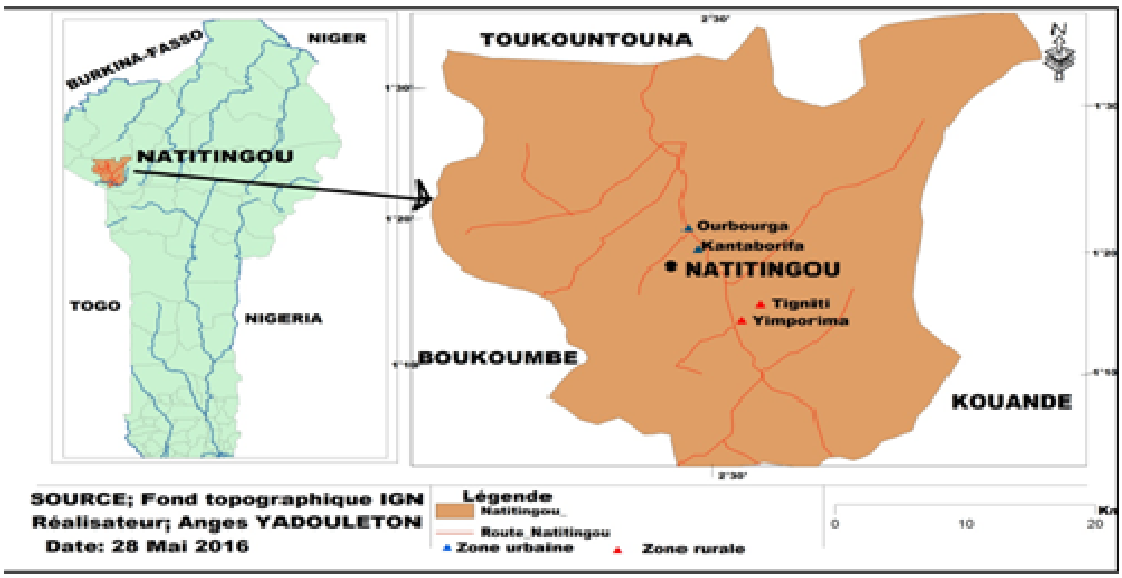

Figure 1. Map of Benin showing the study site

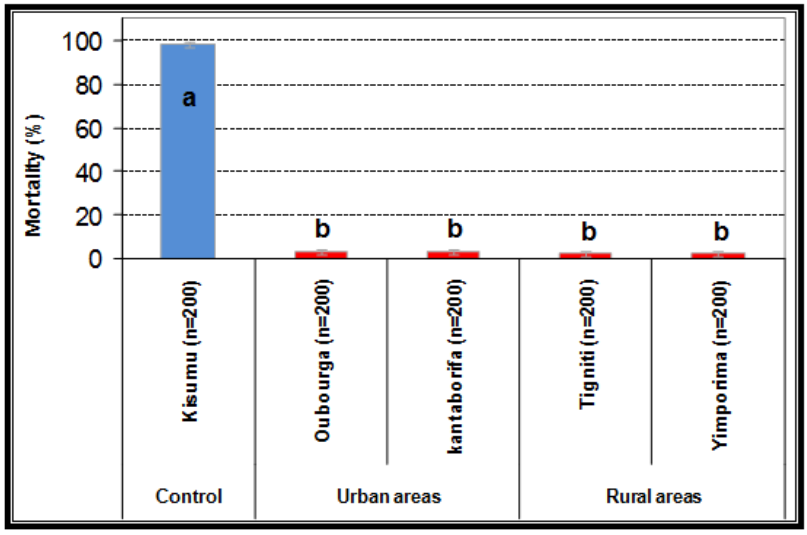

Figure 2. Mortality rates of *Anopheles gambiae* s.l exposed to DDT 4 %

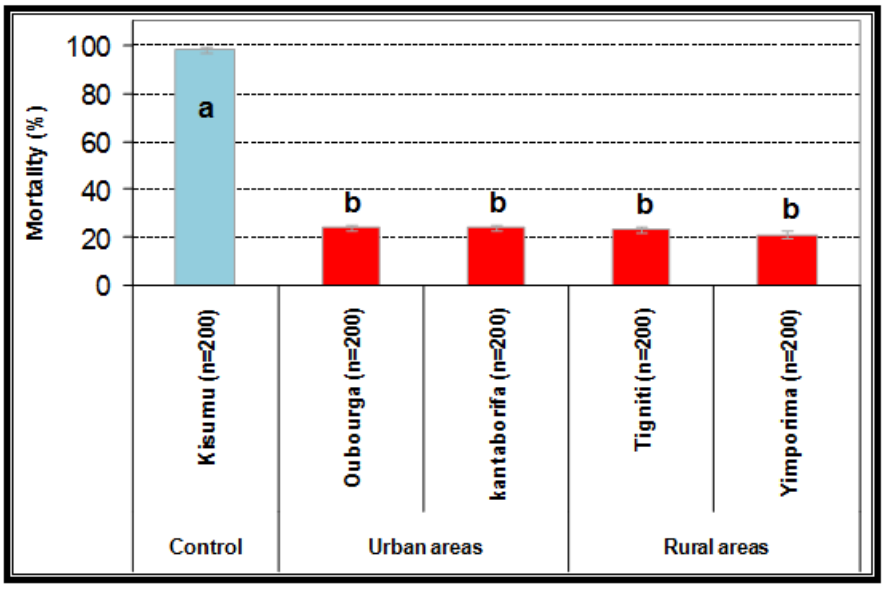

Figure 3. Mortality rates of *Anopheles gambiae* s.l exposed to permethrin 0.75%.

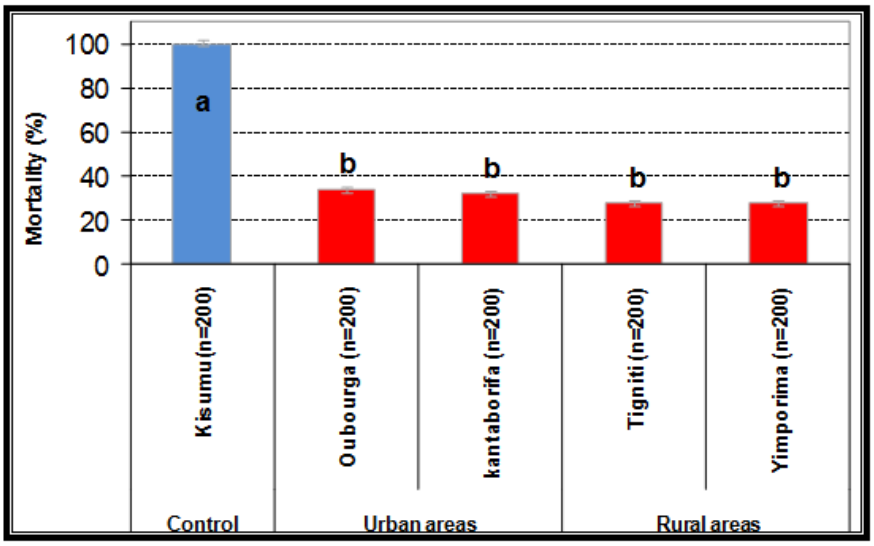

Figure 4. Mortality rates of Anopheles gambiae s.l exposed to deltamethrin 0.05%

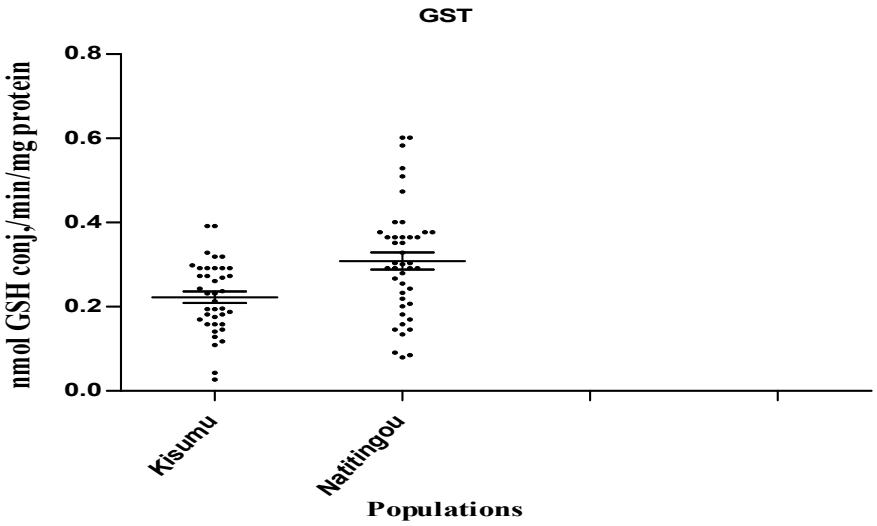

Figure 5. Gluthation activity of *Anopheles gambiae* populations from Natitingou

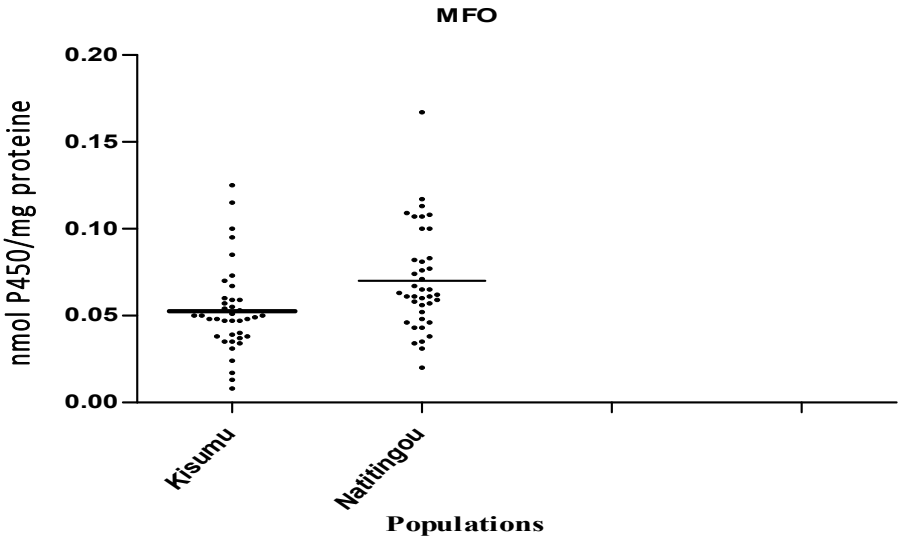

Figure 6. Mixed Function Oxidases activity of *Anopheles gambiae* populations from Natitingou

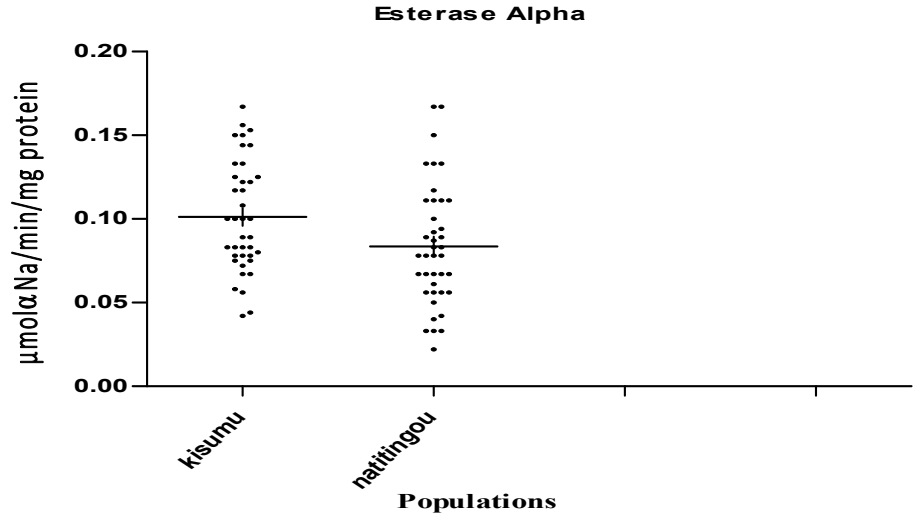

Figure 7a . Alpha-esterase activity of Anopheles gambiae populations from Natitingou

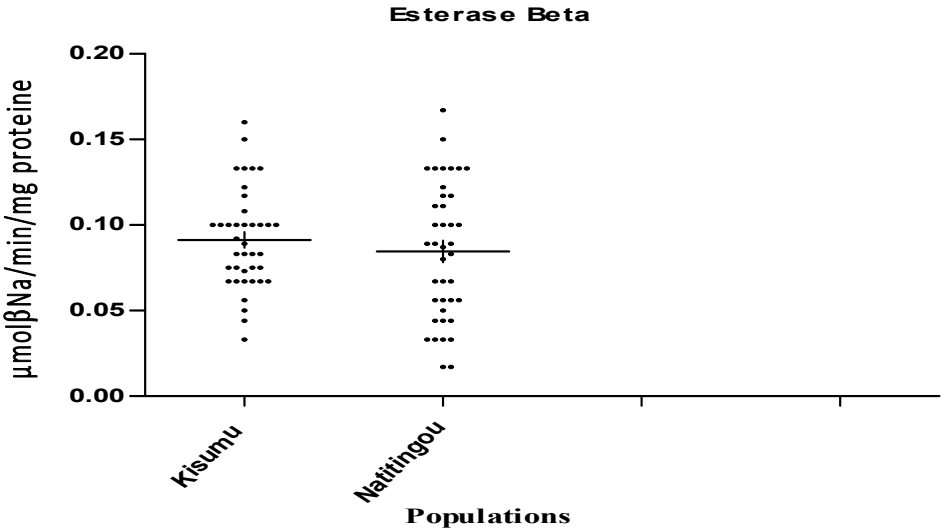

Figure 7b. Beta-esterase activity of Anopheles gambiae populations from Natitingou

Table 1. The frequency of Kdr and Ace-1R mutations in Anopheles gambiae s.s. from the district of Natitingou

		Kdr Mutation				*Ace-1* Mutation			
Study site	Localities	SS	RS	RR	F(R)	SS	RS	RR	F(R)
Urban areas	Ourbourga	7	45	40	0.68	75	05	0	0.03
	Kantaborifa	15	25	50	0.69	86	04	0	0.02
Rural areas	Tigniti	10	36	54	0.72	92	08	0	0.04
	Yimporima	6	34	40	0.71	90	10	0	0.05

resistant to DDT, permethrin and deltamethrin with an average of 3%, 23% and 30% of mortality respectively (Figure 2-4). However, the same populations of *An. gambiae* from the four points of the study site were fully susceptible to bendiocarb.

Resistance mutations: Results from this study showed that the kdr mutation was present in all *An. gambiae* populations collected from the different points of the study site. The average of kdr frequency is 0.68 in urban and 0.71 in rural areas (Table 1). The *Ace-1* mutation was found in *An. gambiae* populations collected from the different points but at very low frequency (from 3% to 5%) (Table 1).

Enzymatic resistance in *Anopheles gambiae* population: Results from biochemical assay showed a significantly higher level of GST activity from the wild populations of *An. gambiae* from the study site compared to susceptible Kisumu strain (Mann-Whitney test, P>0.05) (Figure 5). The same trend was observed with Monooxygenase (Cytochrome p450). (Figure 6). For esterase activity (α and β esterase), the means of optical density values in the populations of mosquitoes from Natitingou (Figure 7a and 7b) were not significantly higher compared to the reference susceptible Kisumu strain (*P*>0.05).

DISCUSSION

Results from this study conducted at Natitingou showed that *An. gambiae* populations have developed strong resistance against organochlorine (OC) and pyrethroids (PY). In fact, the district of Natitingou is agricultural city where farmers

produce cereals, vegetables and cotton. Agronomic practices for these crops 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 (Yadouleton *et al*., 2009). Likewise, survey findings reported by Yadouleton *et al*. (2009; 2010) suggested that various families of insecticides were used for pests control management in agricultural settings. Unfortunately, many of these families of insecticides were not recommended for agriculture (Etang *et al*., 2003; Yadouleton *et al*., 2011; Kabula *et al*., 2014). 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 agriculture 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 OC and PY (Awolola *et al.,* 2003; Nkya *et al*., 2014).

In Benin, PY have been extensively introduced in agriculture since 1980s (Akogbeto *et al*., 2005). This factor is probably one of the causes of the selection of strong resistance in *An. gambiae* to PY and OC. Based on recent results, many authors (Protopopoff *et al.,* 2013; Nkya *et al.,* 2014) have reported that past and current agricultural use of DDT then pyrethroids for crop protection have led to the selection of resistant mosquitoes through insecticide residues accumulated in breeding sites in agricultural areas. This hypothesis was recently confirmed by Akogbeto *et al.* (2006) showing indirectly the presence of pesticide residues in soil and water from vegetable farms and other agricultural activities in Benin that delay or reduce the emergence rates of mosquito larvae. 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 (Aïzoun *et al.,* 2013; Corbel *et al*., 2007). 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 (Aïzoun *et al*., 2013; David *et al.,* 2013) 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) (David *et al*., 2013). This observation further confirms that metabolic resistance may be associated with resistance to different classes of insecticides.

Conclusion

This study provides clear evidence that the use of insecticides by local farmers for crop protection is one factor that impacts negatively the immune system of mosquito which has led to the emergence of insecticide resistance in malaria vectors. Therefore, the need to develop Integrated Pest and Vector Management (IPVM) strategies and management of insecticide resistance in malaria vectors seem to be very important for vector control

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