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Status of organophosphate and carbamate resistance in *Anopheles gambiae sensu lato* from the Sudano Guinean area in the central part of Benin, West Africa

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***Anopheles gambiae*, which is the main malaria vector in Benin has developed high level of resistance to pyrethroid insecticides. This raises serious concerns to the future use of long- lasting insecticidal nets (LLIN) and indoor residual spraying (IRS). It is therefore important to seek better and effective resistance management strategies which will use organophosphates or carbamates as alternatives against pyrethroid resistant malaria vectors in the field. Larvae and pupae of *A. gambiae s.l.* mosquitoes were collected from the breeding sites in Dassa-Zoume and Zogbodomey districts. WHO susceptibility tests were conducted on unfed female mosquitoes aged 2-5 days old. WHO bioassays were performed with impregnated papers with fenitrothion 1%, pirimiphos-methyl 0.25%, and bendiocarb 0.1%. Polymerase chain reaction (PCR) techniques were used to detect species and *Ace-1* mutations. *A. gambiae* Dassa-Zoume populations were susceptible to bendiocarb 0.1% with mortality rate of 99%. *A. gambiae* Zogbodomey populations were susceptible to pirimiphos-methyl 0.25% and fenitrothion 1% with mortality rates of 98.96 and 99%, respectively. PCR assay revealed that 100% of mosquitoes tested were *A. gambiae s.s.* The frequencies of *Ace-1R* mutation in *A. gambiae* Dassa-Zoume and Zogbodomey were 0%. Carbamates (bendiocarb) and organophosphates (fenitrothion and pirimiphos-methyl) have maintained their efficiency against *A. gambiae* Dassa-Zoume and Zogbodomey populations. Carbamates (bendiocarb) and organophosphates (fenitrothion and pirimiphos-methyl) have proven to be powerful alternatives against pyrethroid resistant malaria vectors such as *A. gambiae* Dassa-Zoume and Zogbodomey populations. The use of any of these three compounds in the centre Benin would be successful in malaria vector control.**

Key words: *Anopheles gambiae*, *Ace-1*, resistance, fenitrothion, pirimiphos-methyl, bendiocarb, Benin.

INTRODUCTION

In Africa, vector control is very dependent on a single class of insecticides, the pyrethroids. The dramatic increase in reports of pyrethroid resistance in malaria vectors (Santolamazza et al., 2008; Coleman et al., 2006) over the past decade is therefore a great cause for concern. Pyrethroid-impregnated nets are now widely used to reduce malaria morbidity and mortality in tropical countries. Unfortunately, in West Africa, resistance to pyrethroids is widespread in *Anopheles gambiae s.s.* populations (Chandre et al., 1999), the major malaria

vector in sub-Saharan Africa. Current status of pyrethroid resistance in malaria vectors was recently studied in Benin (Djègbé et al., 2011; Aïzoun et al. 2013a; Aïzoun et al. 2013b).

Resistance management strategies are mainly based on the rational use of the compounds already available, especially in public health because the number of insecticides is very limited. An alternative strategy to maintain the global effectiveness of insecticide-treated nets should be the use of other insecticides such as carbamates (C) and organophosphates (OP). Carbamate and OP are the main alternatives for indoor residual spraying or larval treatments against mosquitoes in the case of pyrethroid resistance. These insecticides inactivate acetylcholinesterase (AChE). Acetylcholinesterase (AChE) is a synaptic enzyme that hydrolyzes the neurotransmitter acetylcholine to terminate nerve impulses. It is also involved in the development of the nervous system in vertebrates and invertebrates (Grisaru et al., 1999; Cousin et al., 2005). Organophosphates and carbamates (OP and C) insecticides are competitive inhibitors that irreversibly inhibit the AChE enzyme, blocking nervous transmission and leading to the death of the insect. So, acetylcholinesterase is a key enzyme in the nervous system, terminating nerve impulses by catalysing the hydrolysis of the neurotransmitter acetylcholine. It (AChE) is the major target for organophosphate (OP) and carbamate insecticides, which inhibit enzyme activity by covalently phosphorylating or carbamylating the serine residue within the active site gorge (Corbett, 1974).

Quantitative and qualitative changes in AChE confer resistance to insecticides (Fournier, 2005). Across all insect species there are two very distinct types of target site resistance, conferring (i) high carbamates and low OP resistance, or (ii) high OP with either equivalent or low carbamate resistance (Russell et al., 2004). In *A. gambiae* species, AChE1 insensitivity is due to the same Gly-to-Ser substitution at position 119 (Mas-soulié et al., 1992). *A. gambiae s.s.* displays resistance to organophosphates and carbamates due to a single amino-acid substitution in the AChE1 catalytic site G119S (Weill et al., 2003).

The Benin National Malaria Control Programme has implemented indoor residual spraying (IRS) campaign under the financial support of the PMI (President's Malaria Initiative) using bendiocarb in the north of the country since 2011 and pyrimiphos-methyl since 2013. Bendiocarb was also the product previously used to control *A. gambiae s.l.* populations from Oueme department in southern Benin (2008-2010). Permethrin was the insecticide used on OlysetNets that were distributed free by the NMCP in July 2011 across the entire country whereas deltamethrin was the insecticide used on Permanets 2.0 that were distributed free by the NMCP in Oueme department in October 2008 and May 2009 in the framework of President's Malaria Initiative of the U.S. Government in Oueme department. In addition, Aïzoun et al. (2013c) suggested that further studies are needed to show the current distribution of the *Ace-1R* mutation in other localities in the south-north transect Benin, which localities will be different from those already studied in the north and south of the country. According to Akogbéto et al. (2011), the IRS campaign in the department of Oueme was an initial experience and the plan was to implement IRS strategy in other parts of Benin if initial results were encouraging.

The current study was proposed to assess the resistance status of malaria vectors from Dassa-Zoume to bendiocarb and from Zogbodomey to fenitrothion and pyrimiphos-methyl in order to check if the insensitive acetylcholinesterase (*ace-1R*) detected in the north of the country (Aïkpon et al., 2013; Aïzoun et al., 2013c) was already widespread in *A. gambiae s.l.* from the central part of the country.

METHODOLOGY

Study area

The study was carried out in some localities; following a south-north transect, Benin. Two contrasting localities of Benin were selected for mosquito collection on the basis of variation in agricultural production, use of insecticides and/or ecological settings (Figure 1). The localities were: Lema, a rice growing area located in Dassa-Zoume district in Collines department, in the central part of the country and Cana, a cereal (maize, ground-nut and so on) growing area located in Zogbodomey district in Zou department, in the central part of the country too. The rice farm of Lema is located in the centre of Dassa-Zoume district. It is a small rice growing area about 5 ha with only one rice growing per year (July to November). According to some farmers of Lema, there has been no use of insecticide in this rice growing area (except fertilizers, weed-killers and threadworm-killers) (Akogbeto et al., 2005). The choice of the study sites took into account the economic activities of populations, their usual protection practices against mosquito bites, and peasant practices to control farming pests. These factors have a direct impact on the development of insecticide resistance in the local mosquito vectors. The central part of the country is characterized by a Sudano Guinean climate with two rainy seasons (April-July and September-November) with an average rainfall of 1,000 mm per year.

Mosquito sampling

A. gambiae s.l. mosquitoes were collected from April-July 2012 during the first rainy season in Zogbodomey district more precisely in Cana locality and in Dassa-Zoume district more precisely in Lema locality, both located in the central part of the

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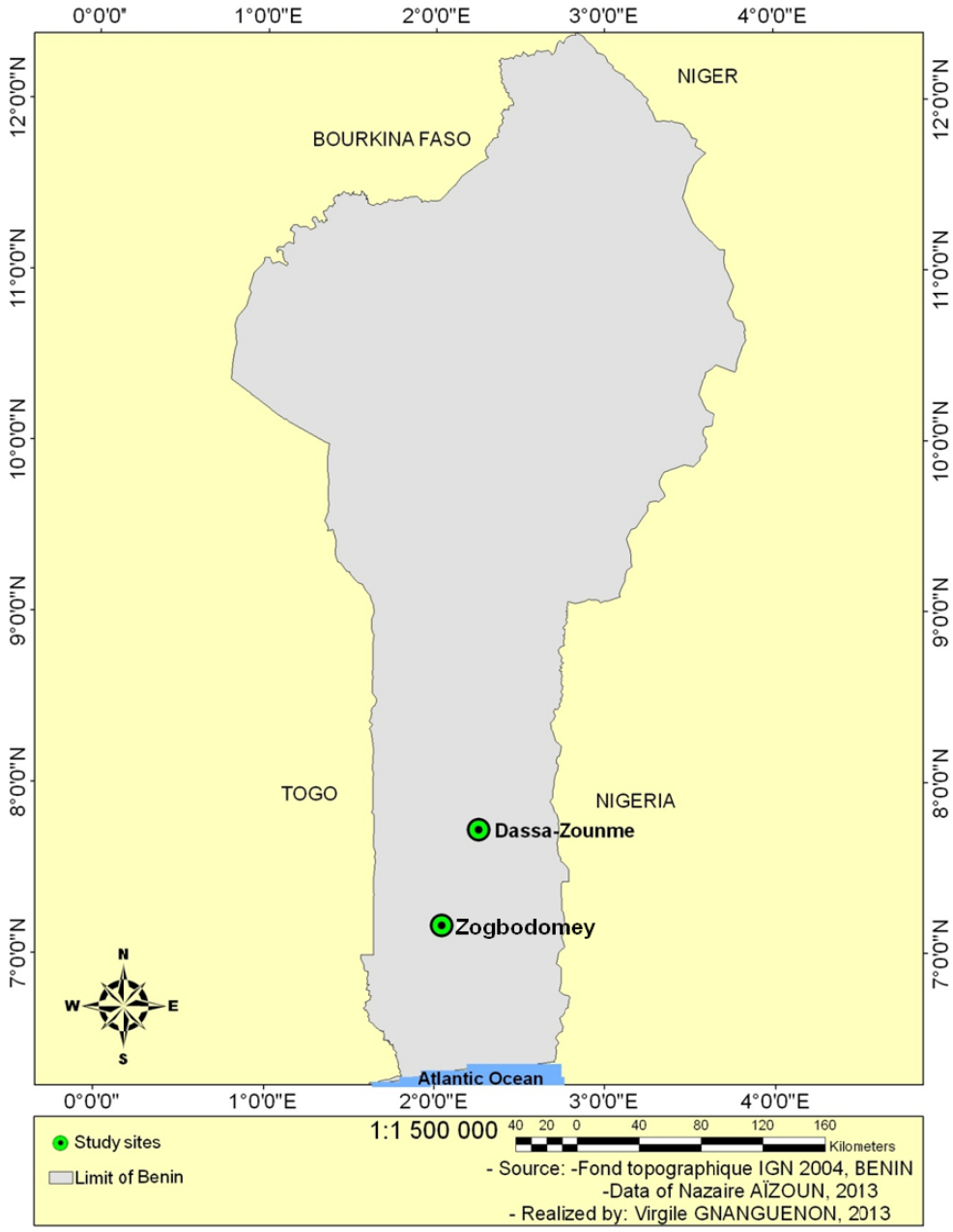

Figure 1. Map of the study area.

country. Larvae and pupae were collected in Cana locality within both padding and village using the dipping method on several breeding sites (brick pits, pools, marshes, streams, ditches, pits dug for plastering traditional huts, puddles of water, water pockets caused by the passage of cattle and gutters).

Anopheles pre-imaginal stages (L1 to L4 instars) were also collected via ladles within rice farms from Lema. Since the farms are irrigated, breeding sites are present throughout the year and we therefore assumed that the larvae collected in the study period were representative of the population that could be found during other periods of the year. Larvae collected from multiple breeding sites were pooled together then re-distributed evenly in development trays containing tap water. Larvae were provided access to powdered TetraFin® fish food, and were reared to adults under insectary conditions of 25+/-2°C and 70 to 80% relative humidity at Centre de Recherche Entomologique de Cotonou (CREC) located in Akpakpa, in Cotonou district. Larvae and pupae collected in Cana locality were also reared to adults under insectary conditions at CREC. *A. gambiae* Kisumu, a reference susceptible strain was used as a control for the bioassay tests. We used Kisumu more precisely to confirm the quality of treated or impregnated papers and then to calculate the resistance ratio. Susceptibility tests were done following WHO protocol on unfed females mosquitoes aged 2-five days old reared from larval and pupal collections. All susceptibility tests were conducted in the CREC laboratory at 25+/-2°C and 70 to 80% relative humidity.

Table 1. Percentage of dead *Anopheles gambiae* observed after 1 h exposure to WHO papers impregnated with bendiocarb in Dassa-Zoume district and with pirimiphos- methyl and fenitrothion in Zogbodomey district.

Population	Insecticide	Number tested	Mortality (%)	Resistance status
Kisumu (Control)	Fenitrothion	102	100	S
	Pyrimiphos-methyl	101	100	S
	Bendiocarb	101	100	S
Dassa-Zoume	Bendiocarb	99	99	S
Zogbodomey	Fenitrothion	100	99	S
	Pyrimiphos-methyl	94	98.96	S

Testing insecticide susceptibility

Females *A. gambiae* aged 2 to 5 days old were exposed to WHO diagnostic dosage of bendiocarb 0.1%, pirimiphos-methyl 0.25% and fenitrothion 1% according to the WHO protocol (WHO, 1998). Thus, an aspirator was used to introduce 20 to 25 unfed female mosquitoes into five WHO holding tubes (four tests and one control) that contained untreated papers. They were then gently blown into the exposure tubes containing the insecticide impregnated papers. After one-hour exposure, mosquitoes were transferred back into holding tubes and provided with cotton wool moistened with a 10% honey solution. The number of mosquitoes "knocked down" at 60 min and mortalities at 24 h were recorded following the WHO protocol (WHO, 1998). Dead and surviving mosquitoes were separately stored in individual tubes with silicagel and preserved at -20°C in the laboratory, for further molecular characterization. We used bendiocarb and pirimiphos-methyl because of the indoor residual spraying (IRS) campaign with these two compounds in progress in the north of the country. Fenitrothion, an insecticide of same class as pirimiphos-methyl, was used to check if there was cross-resistance to these two compounds in the central part of Benin.

PCR detection of species and *Ace-1* mutations

Specimens of *A. gambiae* from the WHO bioassay tests were subjected to the *A. gambiae* species specific PCR assays for species identification (Scott et al., 1993). The PCR-restricted fragment length polymorphism (PCR-RFLP) diagnostic test was used to detect the presence of G119S mutation (*ace.1R* gene) as described by Weill et al. (2003). Mosquito genomic DNA was amplified using the primers Ex3AGdir 5′GATCGTGGACACCGTGTTCG3′ and Ex3AGrev 5′AGGATGGCCCGCTGGAACAG3′according (Weill et al., 2003). One microlitre of total DNA extracted from a single mosquito was used as a template in a 25 µl PCR reaction containing Taq DNA polymerase buffer, 0.2 mM dNTP and 10 pmol of each primer. The PCR conditions were 94°C for 5 min and then 35 cycles of (94°C for 30 s, 54°C for 30 s and 72°C for 30 s) with a final 5 min extension at 72°C. Fifteen microlitres of PCR product were digested with 5U of AluI restriction enzyme (Promega) in a final volume of 25 µl. The PCR fragments were fractionated on a 2% agarose gel stained with ethidium bromide and visualized under UV light.

Data analysis

The resistance status of mosquito samples was determined according to the latest WHO criteria (WHO, 2013) as follows:

1. Mortality rates between 98-100% indicate full susceptibility
2. Mortality rates between 90-97% require further investigation
3. Mortality rates < 90%, the population is considered resistant to the tested insecticides.

Abbott's modified formula was not used in this study for the correction of mortality rates in test-tubes because the mortality rates in all control tubes was less than 5% (Abbott, 1987).

To compare the status of insecticide resistance, Fisher's exact test was carried out to determine if there was any significant difference between mortality rates of populations of *A. gambiae s.s.* of districts using Statistica 6.0. Allelic frequencies of G119S mutation were analysed using the version 1.2 of Genepop (Raymond and Rousset, 1995). To assess if the mutation frequencies were identical across populations, the test of genotypic differentiation was performed (Goudet et al., 1996).

The mortality times or lethal times for 50 and 95% of tested mosquitoes (LT_{50} and LT_{95}) were estimated using SPSS version 16.0 (SPSS Inc., Chicago, IL). The resistance ratio (RR_{50}) was determined relative to the Kisumu susceptible strain. This was obtained by dividing the LT_{50} of wild strain to the LT_{50} of the susceptible strain. The software R-2.15.2. (R Development Core Team, 2011) was used for the statistical analysis.

RESULTS

Susceptibility of *A. gambiae s.l.* populations to pirimiphos-methyl, fenitrothion and bendiocarb

Table 1 shows that Kisumu strain (control) confirmed its susceptibility status as a reference strain. We used Kisumu to confirm the quality of treated or impregnated papers and then to calculate the resistance ratio. The 24 h mortality recording shows that female mosquitoes of *A. gambiae* Kisumu which were exposed to WHO papers impregnated with bendiocarb 0.1%, pirimiphos-methyl 0.25%, and fenitrothion 1% were susceptible to these products with the mortality rates of 100%. Regarding *A. gambiae* Dassa-Zoume populations, they were also susceptible to bendiocarb 0.1% with the mortality rate of 99%. *A. gambiae* Zogbodomey populations were susceptible to pirimiphos-methyl 0.25% and fenitrothion 1% with the mortality rates of 98.96 and 99% respectively (Table 1).

Table 2. Resistance ratio of RR_{50} and RR_{95} with regard to *Anopheles gambiae* Dassa-Zoume and Kisumu populations susceptibility to bendiocarb.

Insecticide	LT50 Dassa-Zoume	LT50 Kisumu	RR50	LT95 Dassa-Zoume	LT95Kisumu	RR95
Bendiocarb	29.040	9.755	2.97	45.402	25.360	1.79

Table 3. Resistance ratio of RR_{50} and RR_{95} with regard to *Anopheles gambiae* Zogbodomey and Kisumu populations susceptibility to fenitrothion.

Insecticide	LT50 Zogbodomey	LT50 Kisumu	RR50	LT95 Zogbodomey	LT95Kisumu	RR95
Fenitrothion	68.840	183.512	0.375	86.449	309.229	0.27

Table 4. Resistance ratio of RR_{50} and RR_{95} with regard to *Anopheles gambiae* Zogbodomey and Kisumu populations susceptibility to pyrimiphos-methyl.

Insecticide	LT50 Zogbodomey	LT50 Kisumu	RR50	LT95 Zogbodomey	LT95Kisumu	RR95
Pyrimiphos-methyl	26.437	10.894	2.42	46.419	19.986	2.32

Table 5. *Ace-1* mutation frequency in *A. gambiae* populations issue from WHO bioassays tests.

Locality	Number tested	Species Ag	*Ace-1* mutation			
			RR	RS	SS	F(*Ace-1*)
Dassa-Zoume	47	47	0	0	47	0
Zogbodomey	49	49	0	0	49	0

Determination of resistance ratio (RR)

The resistance ratio (RR_{50}) of the wild populations of *A. gambiae s.l.* from Dassa-Zoume with regard to bendiocarb and from Zogbodomey with regard to pyrimiphos-methyl were higher than 1 (Tables 2 and 4). But the RR_{50} of the wild population of *A. gambiae s.l.* from Zogbodomey with regard to fenitrothion was lower than 1 (Table 3). The lethal time or mortality time (LT_{50}) of *A. gambiae s.l.* from Dassa-Zoume with regard to bendiocarb was 29.040 versus 9.755 min for *A. gambiae s.l.* Kisumu susceptible reference strain. The resistance ratio (RR_{50}) was 2.97. In similar way, the LT_{50} of *A. gambiae s.l.* from Zogbodomey with regard to pyrimiphos-methyl was 26.437 versus 10.894 min for *A. gambiae s.l.* Kisumu susceptible reference strain. The resistance ratio (RR_{50}) was 2.42. The same remark was made with LT_{95} values obtained with these same wild populations of *A. gambiae s.l* using these same insecticides. The resistance ratio (RR_{95}) obtained with *A. gambiae s.l.* from Dassa-Zoume with regard to bendiocarb was 1.79 whereas the resistance ratio (RR_{95}) obtained with *A. gambiae s.l.* from Zogbodomey with regard to pyrimiphos-methyl was 2.32. Conversely, the LT_{50} of *A. gambiae s.l.* from Zogbodomey with regard to fenitrothion was 68.840 versus 183.512 min for *A. gambiae s.l.* Kisumu susceptible reference strain. The resistance ratio (RR_{50}) was 0.375. The same remark was made with LT_{95} value obtained with this same wild population of *A. gambiae s.l* using this same insecticide. The resistance ratio (RR_{95}) obtained with *A. gambiae s.l.* from Zogbodomey with regard to fenitrothion was 0.27.

Species of *Anopheles gambiae* and *Ace-1* genotype

PCR revealed 100% of mosquitoes tested were *A. gambiae s.s.* The frequencies of *Ace-1R* in *A. gambiae* Dassa-Zoume and Zogbodomey were 0% (Table 5).

DISCUSSION

Anopheles gambiae, which is the main vector for malaria in Benin has developed high level of resistance to pyrethroid insecticides. This raises serious concerns to future use of long-lasting insecticidal nets (LLIN) and indoor residual spraying (IRS). In this context, one of the

pathways available for malaria vector control was the use of alternative classes of insecticides with different mode of action and which will be different for pyrethroids. The Benin National Malaria Control Programme distributes regularly pyrethroid-impregnated nets to the households. The IRS with carbamates (bendiocarb) was done in Atlantic department (Ouidah, Kpomassè and Tori districts) (2007-2009) in southern Benin and then in Oueme department (Adjohoun, Dangbo, Missérété and Sèmè districts) (2008-2010) in southern Benin. Both pyrethroid and carbamate compounds are currently in use in the country through recent free distribution of OlysetNets by the NMCP in July 2011 and through the (IRS) campaign under the financial support of the President's Malaria Initiative (PMI) using bendiocarb in the north of the country since 2011 and using pyrimiphos-methyl since 2013. The use of both compounds is in progress in the country in order to control *A. gambiae s.l.* populations, malaria vectors using integrated control.

In the current study, the LT_{50} and LT_{95} values obtained with *A. gambiae s.l.* Kisumu susceptible reference strain were lower than those recorded with the wild populations of *A. gambiae s.l.* from Dassa-Zoume. Therefore, *A. gambiae s.l.* Dassa-Zoume populations took more time to die when they were exposed to bendiocarb comparatively to Kisumu susceptible strain. The same remark was made with the wild populations of *A. gambiae s.l* from Zogbodomey with regard to pyrimiphos-methyl. However, a converse situation was observed with *A. gambiae s.l.* populations from Zogbodomey with regard to fenitrothion. In fact, the LT_{50} and LT_{95} values obtained with *A. gambiae s.l.* Kisumu susceptible reference strain were higher than those recorded with the wild populations of *A. gambiae s.l.* from Zogbodomey. So, the slow effect or action which characterizes organophosphates was observed with *A. gambiae s.l.* from Zogbodomey after their exposure to fenitrothion. But, this action or effect was not observed with these same *A. gambiae s.l.* populations when they were exposed to pyrimiphos-methyl, another organophosphate compound.

A. gambiae s.l. Dassa-Zoume populations were susceptible to bendiocarb. The susceptibility of *A. gambiae* to bendiocarb may be explained by the absence of individual homozygous RR in the central part of the country. This absence of resistance to bendiocarb has previously been documented in southern Benin (Akogbeto et al., 2010, Padonou et al., 2012). In addition, no insecticide product was generally used in rice growing area of Lema to control pests (Akogbeto et al., 2005). Aïzoun et al. (2013c) also recently found that *A. gambiae* Kandi and Malanville populations were still susceptible to bendiocarb in the northern Benin. Even if, certain *A. gambiae s.l.* populations from northern Benin, such as *A. gambiae s.l.* populations from Kouandé, Matéri, Natitingou, Péhunco and Tanguiéta were already resistant to bendiocarb (Aïkpon et al., 2013), those from the central part of the country were still susceptible to this product. So, bendiocarb has emerged as a promising insecticide for the control of vector populations that are resistant to pyrethroids (Akogbeto et al., 2011). According to Padonou et al. (2011), a possible alternative in the case of pyrethroid resistance in *A. gambiae*, is the use of bendiocarb, to which it was observed a good sensitivity. In addition, these authors mentioned that in case this product is held there is hope that malaria transmission will be reduced drastically.

A. gambiae s.l. Zogbodomey populations were susceptible to both fenitrothion and pyrimiphos-methyl, organophosphate compounds. The susceptibility of *A. gambiae* to these two products may also be explained by the absence of individual homozygous RR in the central part of the country. In addition, no insecticide product was generally used in cereal growing area of Cana to control pests. A recent study carried out by Aïzoun et al. (2013c) also showed that *A. gambiae* Kandi and Seme populations were still susceptible to fenitrothion in the northern and southern Benin respectively. According to Akogbeto and Yakoubou (1999), the difference between carbamate, organophosphate and pyrethroid insecticides can be explained by the emergence and widespread resistance of *A. gambiae s.l.* to pyrethroids.

In the current study, all *A. gambiae* specimens issued from WHO bioassays, were homozygous susceptible individuals. There were no homozygous resistant and heterozygote individuals. These results might be related to high fitness cost of the *ace-1R* mutation, resulting in death of the homozygous resistant mosquitoes (Weill et al., 2004, Asidi et al., 2005, Djogbenou et al., 2010). In *A. gambiae s.s.* populations, the *ace-1* mutation has been associated with a high fitness cost as the frequency of the *ace-1* mutation in mosquito populations declines rapidly after a few generations in the absence of selection pressure from organophosphates or carbamates insecticides (Labbé et al., 2007). In addition, no *ace-1* mutation has been found in *A. gambiae* Tchaourou and Savè populations susceptible to carbamates and organophosphates, both from the north-central Benin (data not shown).

There are no previous published studies on the resistance status of *A. gambiae* populations from Dassa-Zoume and Zogbodomey to carbamates and OPs until 2013. Our study was the first conducted for this purpose. Therefore, these populations of *A. gambiae* need to be monitored for insecticide resistance in this area.

Conclusion

Carbamates (bendiocarb) and organophosphates (fenitrothion and pirimiphos-methyl) have maintained their efficiency against *A. gambiae* Dassa-Zoume and Zogbodomey populations. The good efficiency of these three compounds against *A. gambiae* populations from the central part of Benin is clearly demonstrated in the

current study. The use of any of these three compounds in this part of the country would be successful for malaria control in this area.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Ethical approval

This study was approved by the Ministry of Health and the Center for Entomological Research of Cotonou.

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