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RESEARCH ARTICLE

EFFECTS OF SYNERGIST PIPERONYL BUTOXIDE (PBO) IN LAMBDACYHALOTHRIN TOLERANCE ON ANOPHELES GAMBIAE SENSU LATO LARVAE FROM TOVIKLIN DISTRICT IN COUFFO DEPARTMENT IN SOUTH-WESTERN REPUBLIC OF BENIN, WEST AFRICA

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ABSTRACT

Mosquito control programs are now threatened by the selection of mosquito populations resistant to the chemical insecticides. Thus, alternative vector control methods are necessary. The current study was aimed to study the effects of synergist piperonyl butoxide (PBO) on lambdacyhalothrin tolerance on *Anopheles gambiae* sensu lato larvae from Toviklin district in Couffo department in south-western Benin, West Africa**.** Larvae and pupae were collected from April to July 2024 during the great rainy season in Toviklin district. Larval bioassays were performed on these collected *Anopheles gambiae* s.l. larvae using lambdacyhalothrin as larvicide and piperonyl butoxide (PBO) as enzyme inhibitor or synergist. The results showed that mono-oxygenase enzymes played a role in *Anopheles gambiae* s.l. larvae tolerance to lambdacyhalothrin in Toviklin district. Synergist PBO contributes to improve the efficacy of insecticide when problems of resistance have arisen.

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INTRODUCTION

Globally, in 2023, the number of malaria cases was estimated at 263 million, with an incidence of 60.4 cases per 1000 population at risk. This is an increase of 11 million cases from the previous year and a rise in incidence from 58.6 cases per 1000 population at risk in 2022. The WHO African Region continues to carry the heaviest burden of the disease, accounting for an estimated 94% of malaria cases worldwide in 2023. The WHO Eastern Mediterranean Region has experienced a 57% increase in incidence since 2021, rising to 17.9 cases per 1000 population at risk in 2023 (World Malaria Report, 2024). Globally, in 2023, the number of deaths was estimated at 597 000, with a mortality rate of 13.7 per 100 000. The number of malaria deaths and the mortality rate steadily decreased from 622 000 and 14.9 deaths per 100 000, respectively, in 2020. The WHO African Region continues to carry the heaviest burden of mortality, with 95% of estimated malaria deaths worldwide (World Malaria Report, 2024). The mosquito *Anopheles gambiae* is the principal vector of malaria in Africa. According to the latest WHO statistics, this parasitic disease infects from 300 to 500 million persons per year in the world and kills more than a million and a half each year, mainly African children. Together with AIDS, malaria is one of the causes of mortality in the populations of African, South Asia and Latin America; it contributes a large part of the continued impoverishment of these populations. Most control strategies against *An. gambiae* target the adult stage of the vector (Killeen *et al,* 2017) and rely on pyrethroids, organochlorides, organophosphates and carbamates among other insecticides and against which the vector has developed resistance (Ranson *et al,* 2011). The resistance has adversely affected integrated mosquito vector control strategies (Ranson and Lissenden, 2016) and necessitated a search for alternative control agents, including phytochemicals, that target immature stages of the vector (White *et al,* 2011). This approach has largely been unexplored (Tusting *et al*, 2013; Worrall and Fillinger, 2011). Targeting the immature stages can perturb mosquito population dynamics, consequently contributing to reduced vectorial capacity and local malaria transmission (Kweka *et al,* 2015). Very few researches were published on lambdacyhalothrin tolerance in *Anopheles gambiae* sensu lato larvae from Toviklin district in Couffo department in south-western Benin. Therefore, there is a need to carry out new researches for this purpose. The goal of this study was to explore the detoxification enzymes mechanisms conferring

lambdacyhalothrin tolerance in *Anopheles gambiae* sensu lato larvae in Republic of Benin.

MATERIALS AND METHODS

Study area: The study area is located in Republic of Benin (West Africa) and includes the department of Couffo. Couffo department is located in the south-western Benin and the study was carried out more precisely in Toviklin district. The choice of the study site 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 an impact on resistance development in the local vector mosquitoes. We took them into account to determine the effects of synergist piperonyl butoxide (PBO) on lambdacyhalothrin tolerance in *Anopheles gambiae* sensu lato larvae from this district of the Couffo department. Couffo has a climate with four seasons, two rainy seasons (March-July and August-November) and two dry seasons (November-March and July-August). The temperature ranges from 25 to 30°C with the annual mean rainfall between 900 and 1100 mm.

Mosquito sampling: *Anopheles gambiae* sensu lato larvae were collected from April to July 2024 during the great rainy season in Toviklin district selected in south-western Benin. Larvae and pupae were collected in this district within both padding and town 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 gutters). Once, larvae and pupae collected, they were then kept in labeled bottles related to the localities surveyed. Otherwise, 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 under insectary conditions of 25+/-2°C and 70 to 80% relative humidity at Department of Sciences and Agricultural Techniques located in Dogbo district in south-western Benin. The larvae of *Anopheles gambiae* Kisumu, a reference susceptible strain was used as a control for the larval bioassays. All larval bioassays were conducted in the Laboratory of Pluridisciplinary Researches of Technical Teaching (LaRPET) of the Department of Sciences and Agricultural Techniques at 25+/-2°C and 70 to 80% relative humidity.

Preparation of stock solutions or suspensions and test concentrations: Stock solutions and serial dilutions were prepared following the protocol described in WHO guidelines (WHO, 2005). The volume of stock solution was 20 ml of 1%, obtained by weighing 200 mg of lambdacyhalothrin and adding 20 ml solvent to it. It was kept in a screw-cap vial, with aluminium foil over the mouth of the vial. Then, it was shacked vigorously to dissolve or disperse the lambdacyhalothrin in the solvent. The stock solution was then serially diluted (ten-fold) in ethanol (2 ml solution to 18 ml solvent). Test concentrations were then obtained by adding 0.1–1.0 ml (100–1000 µl) of the appropriate dilution to 100 ml or 200 ml distilled water.

Bioassays: Initially, the mosquito larvae were exposed to a wide range of test concentrations of lambdacyhalothrin and a control to find out the activity range of the larvicide under test. After determining the mortality of larvae in this wide range of concentrations, a narrower range (of 4-5 concentrations, yielding between 10% and 95% mortality in 24h or 48h) was used to determine LC50 and LC90 values (WHO, 2005). Batches of 25 fourth instar larvae were transferred by means of strainers, screen loops or droppers to small disposable test cups or vessels, each containing 100-200 ml of water. Small, unhealthy or damaged larvae were removed and replaced.

The depth of the water in the cups or vessels was remained between 5 cm and 10 cm; deeper levels may cause undue mortality. The appropriate volume of dilution was added to 100 ml or 200 ml water in the cups to obtain the desired target dosage, starting with the lowest concentration. Four replicates were set up for each concentration and an equal number of controls were set up simultaneously with tap water, to which 1 ml alcohol was added. Each test was run three times on different days. For long exposures, larval food was added to each test cup, particularly if high mortality was noted in control. The test containers were held at 25-28°C and preferably a photoperiod of 12h light followed by 12h dark (12 L: 12 D).

After 24 hours exposure, larval mortality was recorded. Moribund larvae were counted and added to dead larvae for calculating percentage mortality. Dead larvae were those that could not be induced to move when they were probed with a needle in the siphon or the cervical region. Moribund larvae were those incapable of rising to the surface or not showing the characteristic diving reaction when the water was disturbed. The results were recorded on the result form, where the LC50 and LC90 values, and slope and heterogeneity analysis were also noted. The form was accommodated three separate tests of six concentrations of lambdacyhalothrin, each of four replicates (WHO, 2005).

Biochemical assays using synergist: The presence of metabolic-based resistance mechanisms was investigated by exposing larvae to enzyme inhibitor prior to bioassays with lambdacyhalothrin. For that, tested samples that showed high tolerance to lambdacyhalothrin in *Anopheles gambiae* s.l. larvae from Toviklin district surveyed, were exposed to the effects of synergist piperonyl butoxide (PBO) (400 µg per test cup), which inhibits oxidase activity. The test allowed us to compare the obtained percentages of dead larvae before the addition of the synergist (s) to those obtained after the addition of the synergist (s).

STATISTICAL ANALYSIS

Data from all replicates were pooled for analysis. LC50 and LC90 values were calculated from a log dosage-probit mortality regression line using computer software programs. Bioassays were repeated at least three times, using new solutions or suspensions and different batches of larvae each time. Standard deviation or confidence intervals of the means of LC50 values were calculated and recorded on a form.

A test series was valid if the relative standard deviation (or coefficient of variation) was less than 25% or if confidence limits of LC50 overlap (significant level at $P < 0.05$). To appreciate the effects of synergists PBO on *Anopheles gambiae* s.l. larvae from Toviklin tolerance to lambdacyhalothrin, we used a Kruskal-Wallis test. LC50 and LC90 values were estimated using SPSS version 16.0 (SPSS Inc., Chicago, IL). The significance level was set at 5%.

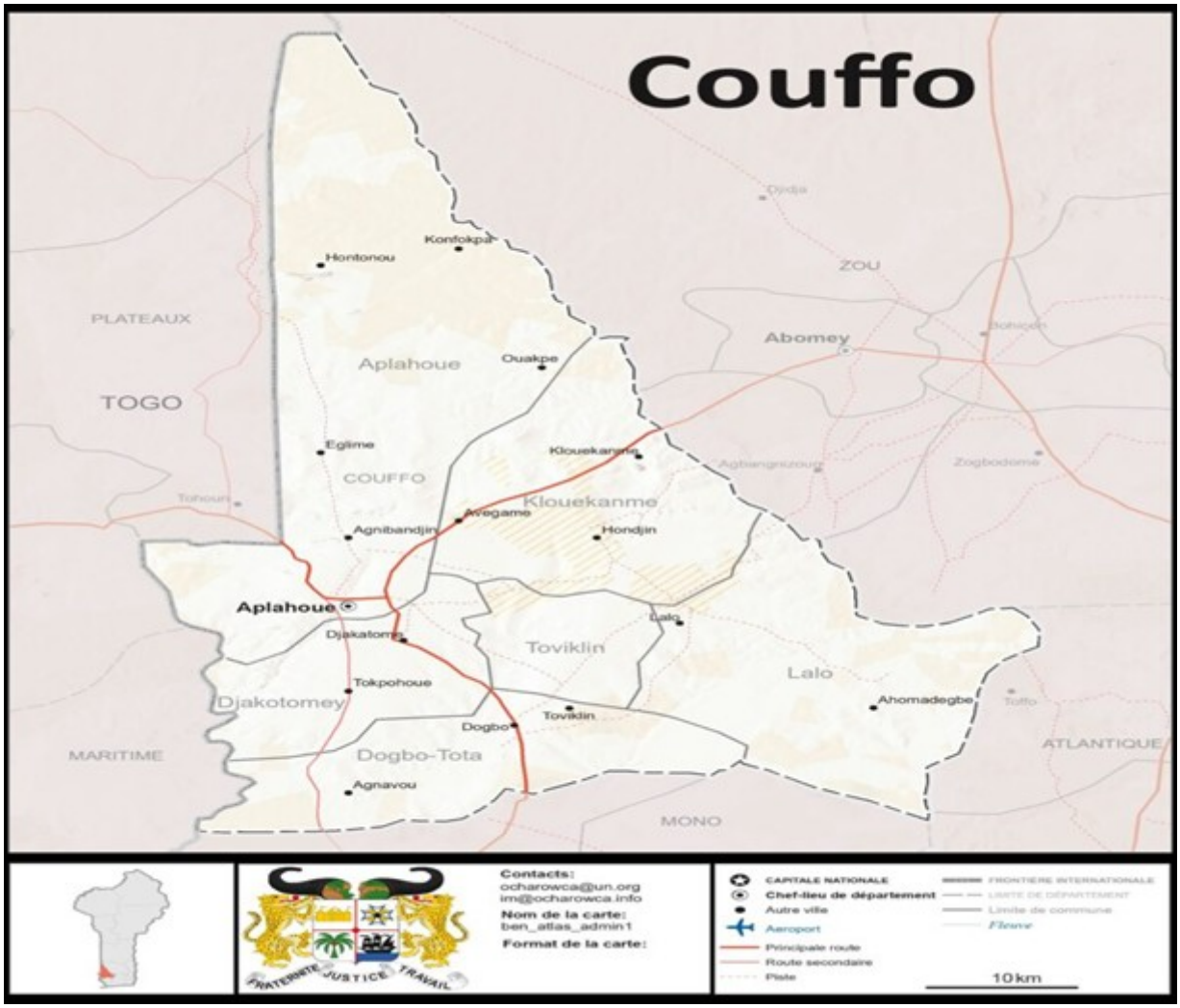

Figure 1. Map of Republic of Benin showing Toviklin district surveyed

Table 1. Determination of lethal concentrations LC50 and LC95

Populations	LC50 (mg/l)	LC95(mg/l)	RR50	RR95
Kisumu (Control)	0.003	0.005		
Toviklin	0.063	0.22	21	44

Table 2. Determination of lethal concentrations LC50 and LC95 and Synergism ratios SR50 and SR95 of *An. gambiae s.l.* larvae from Toviklin to lambdacyhalothrin + PBO

Population	Enzyme inhibitor	LC50 (mg/l)	LC95(mg/l)	SR50	SR95
	Without Synergist	0.063	0.22		
Toviklin	With PBO	0.015	0.049	4.2	4.48

Table 3. Advantages and disadvantages of the use of synergist

Advantages	Disadvantages
Synergist contributes significantly to improve the efficacy of insecticides, particularly when problems of resistance have arisen. Synergist inhibits detoxifying enzymes or increases target sensitivity, allowing lower doses and overcoming resistance. Synergist has to be exploited in vector-borne disease management programs for intention of resistance management in insects. Synergist can be used on mosquito's nets to improve bed's net effectiveness against mosquito's bites. Synergist can be used on insecticide for Indoor Residual Spraying programs in vector-borne disease control.	Increased toxicity to non-target organisms (humans, bees) (is the main disadvantage)
The use of synergists reduces the quantity of insecticide to be used.	

RESULTS

The analysis of Table 1 showed that *Anopheles gambiae s.l.* larvae from Toviklin district in Couffo department were highly resistant to lambdacyhalothrin (see Resistance ratios RR50 and RR95). The analysis of Table 2 showed that the underlying mechanism of the resistance pattern observed in this population was explored using a synergist assay. The synergist assay with PBO, an inhibitor of Cytochrome P450 monooxygenases, indicated that this enzyme family plays a role in this high **l**ambdacyhalothrin resistance observed in *Anopheles gambiae s.l.* larvae from Toviklin. The analysis of Table 3 showed that there are more advantages than disadvantages in the use of synergists

DISCUSSION

In the current study, *Anopheles gambiae* s.l. larvae from Toviklin district in Couffo department were highly resistant to lambdacyhalothrin. In addition, the underlying mechanism of the resistance pattern observed in this population was explored using a synergist assay. The synergist assay with PBO, an inhibitor of Cytochrome P450 monooxygenases, indicated that this enzyme family plays a role in this high **l**ambdacyhalothrin resistance observed in *Anopheles gambiae* s.l. larvae from Toviklin. Our results corroborated with those obtained by Aïzoun (2021) who had studied the effects of Synergist Piperonyl Butoxide (PBO) on deltamethrin tolerance in *Anopheles gambiae* sensu lato Larvae from Mono department in South-Western Benin. In fact, deltamethrin is an insecticide of same class as lambdacyhalothrin which is pyrethroid. In that study, larvae and pupae were collected from March to July and August to November 2018 during the rainy season in the locations of Athiémè, Grand Popo, Comè, Lokossa, Houéyogbé and Bopa. Larval bioassays were performed on these collected *Anopheles gambiae* sensu lato larvae using deltamethrin as larvicide and piperonyl butoxide (PBO) as enzyme inhibitor or synergist. The results showed that mono-oxygenases played a role in *Anopheles gambiae* sensu lato larvae tolerance to deltamethrin.

Previous studies showed that metabolic resistance mechanisms have been identified in adult vector populations for all major classes of insecticides currently used for vector control in Benin, including organophosphates, carbamates, pyrethroids and DDT (Aïzoun *et al,* 2013, 2014a, 2014b, 2014c). There is a need to overcome these resistance mechanisms by promoting for example Long Lasting Insecticidal Nets (LLINs) impregnated with synergists. But, these nets would not be effective in areas where Leu-Phe *kdr* mutation is the most important resistance mechanism. Another study carried out by Corbel *et al* (2007) have reported on multiple insecticide resistance mechanisms in *An. gambiae* Ladji populations from Cotonou district in southern Republic of Benin. Among these mechanisms, there were mixed function oxidase (MFO) and α-esterase with the presence of *Kdr* at high frequency (80%). However, even if the Leu-Phe *kdr* mutation is the most important resistance mechanism in these *An. gambiae* Ladji populations, metabolic resistance conferred by detoxifying enzymes is also an indication of phenotypic resistance to permethrin, which is a pyrethroid. In addition, Kacou *et al* (2024) had assessed the effects of three anthropogenic activities on the mosquito, *Anopheles gambiae* s.l. species composition and pyrethroid resistance mechanisms in Abidjan city, Côte d'Ivoire. In fact, Pyrethroid resistance and mechanisms for resistance for *Anopheles gambiae* sensu lato (s.l.) (Diptera: Culicidae) Giles, were assessed in three urban areas (vegetable farming, industrial and residential) of Abidjan. Susceptibility to pyrethroids (deltamethrin, permethrin and alphacypermethrin), with and without piperonyl butoxide (PBO) pre-exposure was evaluated. Their results showed that high resistance to deltamethrin, permethrin and alphacypermethrin was observed in Port-Bouet (vegetable farming) and Treichville (industrial site), whereas moderate resistance to deltamethrin and high resistance to alphacypermethrin and permethrin were found in Abobo (residential site). Pre-exposure to PBO with pyrethroid increased mortalities in all sites. In Treichville, pre-exposure to PBO restored susceptibility to deltamethrin, but not in Port-Bouet or Abobo. These data suggest that PBO + deltamethrin impregnated nets could aid malaria control, benefiting industrial areas of Côte d'Ivoire and other African cities. Regarding the advantages and disadvantages of the use of synergists, it is important to notice that there are more advantages than disadvantages in this use. In fact, as advantages: Synergist contributes significantly to improve the efficacy of insecticides, particularly when problems of resistance have arisen. Synergist inhibits detoxifying enzymes or increases target sensitivity, allowing lower doses and overcoming resistance. Synergist has to be exploited in vector-borne disease management programs for intention of resistance management in insects. Synergist can be used on mosquito's nets to improve bed's net effectiveness against mosquito's bites. Synergist can be used on insecticide for Indoor Residual Spraying programs in vector-borne disease control. The use of synergists reduces the quantity of insecticide to be used. However, as disadvantages: Increased toxicity to non-target organisms (humans, bees) is the main disadvantage.

CONCLUSION

The mono-oxygenase enzyme played a role in *Anopheles gambiae* sensu lato larvae tolerance to pyrethroids in Couffo department. This enzyme was implicated as mechanisms of pyrethroid resistance in *An. gambiae* s.l. from Couffo department in south-western Benin. However, further studies using a microarray approach followed by quantitative real-time RT-PCR validation are need to identify detoxification genes putatively involved in metabolic resistance. This will improve the implementation and management of future control programs against this important malaria vector particularly in Benin and in Africa in general.

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