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Original Article

Importance of Rabbit's Blood Meal in the Assessment of Factors which Influence Vector Susceptibility to Insecticides

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Abstract *- The understanding of the factors that influence vector susceptibility to insecticides is important. The current study aimed at investigating the importance of rabbit's blood meal in the assessment of factors that influence vector susceptibility to Lambda-Cyhalothrin (Karate, as a commercial product name). Surveys were organized in the Couffo department, and larvae and pupae of Aedes aegypti mosquito populations were collected in the breeding sites from April to July 2025 during the great rainy season. The WHO susceptibility tests were conducted on unfed male and female mosquitoes aged 2-5 days old, and also conducted on unfed, blood-fed, and gravid female mosquitoes aged 2-5 days old. These susceptibility tests were also conducted on unfed, blood-fed, and gravid female mosquitoes aged 20 days old. The effects of synergist Piperonyl Butoxide (PBO) on lambda-cyhalothrin tolerance in Aedes aegypti larvae were also investigated. The results showed that Aedes aegypti females were more susceptible than the males. After females had taken their blood meals, vector susceptibility to Lambda-Cyhalothrin did not increase. Similarly, after females possess eggs in their abdomen, the results also showed that the old mosquitoes were more susceptible to Lambda-Cyhalothrin. Mono-Oxygenase enzymes may play a role in Aedes aegypti larvae tolerance to lambda-cyhalothrin in Djakotomey district. Many factors influence vector susceptibility to insecticides. The rabbit's blood meal is important in the assessment of factors which influence vector susceptibility to insecticides. The method which exposes human hands to mosquitoes in a cage to be fed is not recommended.*

Keywords *- Benin, Dengue, Lambda-Cyhalothrin, Rabbit's Blood Meal, Susceptibility to Insecticides.*

1. Introduction

Mosquitoes of the Aedes genus, particularly *Aedes aegypti*, are primary vectors of several emerging and re-emerging arboviral diseases worldwide, including Dengue, Chikungunya, and Zika (Poinsignon *et al*., 2025). Compounding the problem is the global emergence of insecticide resistance among *Aedes aegypti* and other mosquito species, which is threatening to jeopardise the operational effectiveness of vector control campaigns. The *Aedes aegypti* mosquito, a primary vector of arboviruses poses a significant public health threat worldwide. Its adaptability and genetic diversity complicate control efforts, enabling rapid resistance evolution (Hernandez and Pietrantonio, 2025).

The use of insecticides remains the predominant strategy for controlling adult female *Aedes* in case of epidemics. Insecticide Resistance (IR) to pyrethroids is found worldwide in *Aedes Aegypti* (L.). Populations are generally resistant to pyrethroids when laboratory insecticide resistance testing is conducted, though the field relevance of this resistance is often unclear (Gan *et al*., 2021; Zulfa *et al*., 2022). Pyrethroid insecticides, a cornerstone of vector control, target voltage-gated sodium channels, yet resistance driven by knockdown Resistance (*kdr*) mutations and detoxification mechanisms has undermined their efficacy (Hernandez and Pietrantonio, 2025). However, the emergence of resistance in Aedes populations in Africa (Bello SOT *et al*., 2024) threatens the effectiveness of vector control interventions.

Several mechanisms have been described by which mosquitoes develop resistance to insecticides. Among these are the knockdown resistance (*kdr*) point mutations on the Voltage-Gated Na+ Channel (VGSC), which confer resistance to both pyrethroids and DDT (Estep *et al*., 2025). The VGSC in *Aedes aegypti* is essential for the initiation and propagation of the action potential in neurons and other excitable cells. Another important mechanism of resistance is the enhanced detoxification of insecticides driven by elevated metabolic activity (Contreras-Perera *et al*., 2025). This is typically associated with the increased activity of three major enzyme families: esterases, mixed-function oxidases, and glutathione S-transferases. So, *Kdr* mutations and increased metabolic activity have been the most commonly reported mechanisms of pyrethroid resistance. However, modifications in the insect cuticle may also contribute to pyrethroid tolerance as the cuticle is a multifunctional structure that provides physical protection and structural support and serves as a barrier against desiccation, pathogens, and chemical agents. In mosquitoes and other arthropods, the cuticle plays a critical role in environmental adaptation and insecticide interaction. The structure and regulation of cuticle proteins are key determinants of resistance to insecticides, as they influence the composition and permeability of the cuticle, thereby limiting the penetration of toxic compounds (Ren *et al*., 2023).

To assess the factors that influence vector susceptibility to insecticides, researchers sometimes use a method that exposes human hands to mosquitoes to be fed. Ethically, this method is not well seen. For that, alternative methods are required. Very few researches were published on this topic. Therefore, there is a need to carry out new research for this purpose.

The aim of this study was to investigate the importance of the rabbit's blood meal in the assessment of factors that influence vector susceptibility to insecticides.

2. Literature Review

The Lambda-Cyhalothrin resistance affects differentially vector competence variables for Zika (ZIKV), DENV-2 (dengue), and Chikungunya (CHIKV) (Serrato *et al.,* 2022). The effectiveness of Lambda-Cyhalothrin insecticide against *Aedes aegypti* mosquitoes was assessed in the Pulang Pisau Port region of South Kalimantan, Indonesia, by analysing its knockdown and fatality rates. The results showed that the local *Aedes aegypti* population was deemed vulnerable to the pesticide, highlighting lambda-cyhalothrin's considerable efficacy as a vector control measure in the area (Suardie *et al.,* 2024). The insecticide resistance status and mechanisms in *Aedes aegypti* were assessed, and there was the presence of a varied and generalized resistance, enzymatic mechanisms, and the Val1016IIe mutation may be associated with the intensive use and possibly misuse of the different insecticides applied to control Aedes populations. These results highlight the need to develop a program for resistance management. Also, alternative approaches to mosquito control that do not involve insecticides should be explored (Carrera *et al.,* 2024). A novel perspective on the response of *Aedes aegypti* to lambda-cyhalothrin insecticide was studied. After long-term exposure, mosquitoes respond to insecticides by expressing different proteins. The authors propose a model that incorporates previously unexplored mechanisms found in response to prolonged insecticide exposure, along with the established and accepted insecticide resistance mechanisms such as *kdr* and metabolic resistance (Mejía *et al.,* 2025).

3. Materials and methods

3.1. Study Area

The study area is located in the Republic of Benin (West Africa) and includes the department of Couffo. The Couffo department is located in the south-western Republic of Benin, and the study was carried out in Djakotomey and Toviklin districts (Figure 1). The choice of the study sites took into account the economic activities of populations, their usual protection practices against mosquito bites, the Long-Lasting Insecticidal Nets, Permanets, and OlysetNets distribution frequently by the National Malaria Control Program in these localities, and peasant practices to control farming pests. These factors have a direct impact on the development of insecticide resistance in the local mosquito vectors. Four seasons characterized Couffo department; there are two rainy seasons (March-July and August-November) and two dry seasons (November-March and July-August). The temperatures range from 25 to 32°C, with the annual mean rainfall, which is between 900 and 1100 mm.

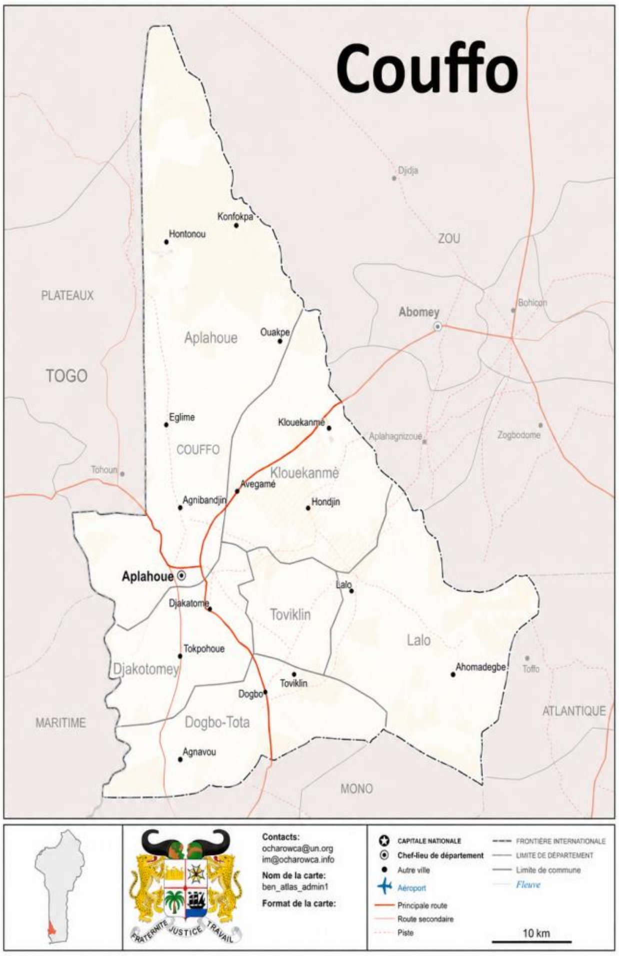

Fig. 1 Map of the study area showing Djakotomey and Toviklin districts

3.2. Mosquito Sample Collection

Surveys were organized across Djakotomey and Toviklin districts in Couffo department, and larvae and pupae of *Aedes aegypti* mosquito populations, which are responsible for dengue transmission to people, were collected in the breeding sites from April to July 2025 during the great rainy season in South-western Benin. Larvae and pupae were collected in breeding sites using the dipping method. They were then kept in separate, labeled bottles related to each locality. The samples were reared up to adult emergence at the insectary of the Department of Sciences and Agricultural Techniques located in Dogbo district in Normal High School of Technical Teaching (ENSET) of Lokossa. The rearing up to adult emergence was done in an insectary in some plastic containers, which contained tap water and were covered with cutting nets. After emergence, adults were transferred into mosquito cages for the assessment of susceptibility tests. *Anopheles gambiae* Kisumu, a reference susceptible strain, was used as a control for the bioassay tests. Susceptibility tests were carried out following the World Health Organization (WHO) protocol. All susceptibility tests were conducted in the laboratory of Pluridisciplinary Research of Technical Teaching (LaRPET) at 25+/-2°C and 70% to 80% relative humidity.

3.3. Obtaining Blood-Fed and Gravid Mosquitoes

After larvae and pupae of *Aedes aegypti* mosquito populations were collected in Djakotomey and Toviklin districts, they were reared up to adult emergence in an insectary. The female adult mosquitoes had been mated and given a rabbit's blood meal to become blood-fed mosquitoes. Then, male and female adult mosquitoes aged 5-7 days old were used in the reproduction to produce gravid mosquitoes.

3.4. Obtaining the Rabbit's Blood Meal

A rabbit was bought from a rearing farm in the study area. Then, it was carried out in the Department of Sciences and Agricultural Techniques located in Dogbo district in the Normal High School of Technical Teaching (ENSET) of Lokossa. Then, it was sheared with a razor and put in a mosquito cage to serve as a blood meal to mosquitoes. For that, the rabbit was fixed with cords in a mosquito cage in which were introduced adult *Aedes aggypti* mosquitoes. Once these mosquitoes were fed, they were used in the performance of the susceptibility tests.

3.5. Obtaining Old Female Mosquitoes

After larvae and pupae of *Aedes aegypti* mosquito populations were collected in Djakotomey and Toviklin districts, they were reared up to adult emergence in an insectary. Adult mosquitoes were provided with cotton wool moistened with a 10% honey solution until they were 19 days old. On Day 20, they were separated into two batches. The first batch was fed with rabbit's blood meal, and susceptibility tests were assessed the same day on blood-fed old female mosquitoes. On this same day (Day 20), the second batch containing unfed old female mosquitoes was also used in the assessment of the susceptibility tests.

3.6. Testing Insecticide Susceptibility

An aspirator was used to introduce 20 to 25 unfed female mosquitoes aged 2-5 days 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. So, four to five replicates were done simultaneously. After one hour of 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 minutes and mortalities at 24 hours were recorded following the WHO protocol (WHO, 1998). In this study, the insecticide tested was Lambda-Cyhalothrin (0.05%). We used lambda-Cyhalothrin, an insecticide of the same family as Permethrin (pyrethroids), which is an insecticide used on Long-Lasting Insecticidal Nets, OlysetNets distributed frequently by the National Malaria Control Program.

3.7. 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 Lambda-Cyhalothrin and adding 20 ml of solvent to it. It was kept in a screw-cap vial, with aluminum foil over the mouth of the vial. Then, it was shacked vigorously to dissolve or disperse the Lambda-Cyhalothrin 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 of distilled water.

3.8. Bioassays

Following the protocol described in WHO guidelines (WHO, 2005), initially, the mosquito larvae were exposed to a wide range of test concentrations of lambda-cyhalothrin 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. Batches of 25 third or 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 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 of 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 of 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 the 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 of exposure, larval mortality was recorded. Moribund larvae were counted and added to dead larvae for calculating the 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, slope, and heterogeneity analysis were also noted. The form was accommodated for three separate tests of six concentrations of lambda-cyhalothrin, each of four replicates.

3.9. Biochemical Assays using Synergist

The presence of metabolic-based resistance mechanisms was investigated by exposing larvae to an enzyme inhibitor prior to bioassays with lambda-cyhalothrin. For that, tested samples (from Djakotomey), which showed high tolerance to lambda-cyhalothrin in *Aedes aegypti* larvae, 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 percentage of dead larvae before the addition of the synergist to that obtained after the addition of the synergist.

3.10. Statistical Analysis

Stata 12 was used to analysis the data sets gathered from the two districts surveyed to compare for the tested insecticide, the mortality rates of *Anopheles gambiae* populations regarding mosquito sex, physiological status, and mosquito age. Data are presented with 95% confidence limits.

In addition, 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 the confidence limits of LC50 overlap (significant level at $P < 0.05$). To appreciate the effects of synergist PBO on *Aedes aegypti* larvae tolerance to lambda-cyhalothrin, 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%.

4. Results and Discussion

4.1. Comparison of Mosquito Susceptibility Regarding their Sex

The analysis of Figure 2 showed that both sexes of *Anopheles gambiae* Kisumu populations were fully susceptible to lambda-cyhalothrin when they were unfed and aged 2-5 days old. Regarding *Aedes aegypti* populations from Djakotomey and Toviklin, females were more susceptible than males when they were unfed and aged 2-5 days old ($P < 0.05$).

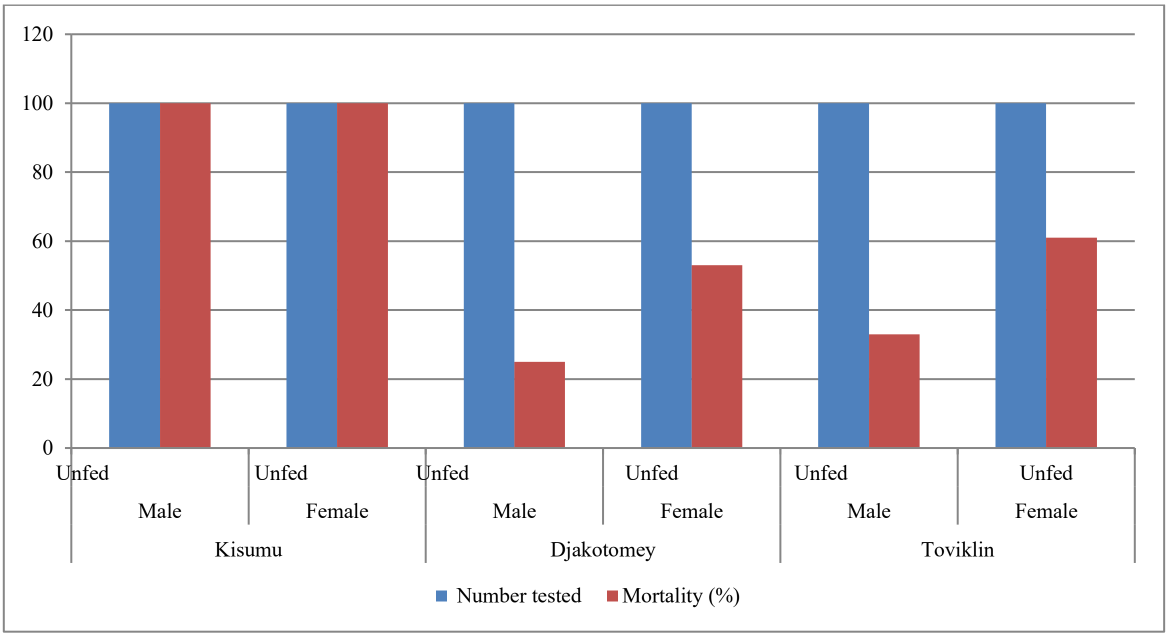

Fig. 2 Comparative susceptibility of *Aedes aegypti* populations aged 2-5 days old regarding their sex

4.2. Comparison of Aedes Aegypti Mosquito Susceptibility Regarding their Physiological Status

The analysis of Figure 3 showed, on the one hand, that female *Anopheles gambiae* Kisumu populations were fully susceptible to lambda-cyhalothrin when they were unfed, blood-fed, gravid, and aged 2-5 days old. The analysis of Figure 3 showed, on the other hand, that the mortality rates of blood-fed *Aedes aegypti* female populations from Djakotomey and Toviklin aged 2-5 days old were lower than those obtained when these females were unfed ($P < 0.05$). Regarding the mortality rates of gravid females from Djakotomey and Toviklin, they were lower and significantly different from those obtained when these females were unfed ($P < 0.05$).

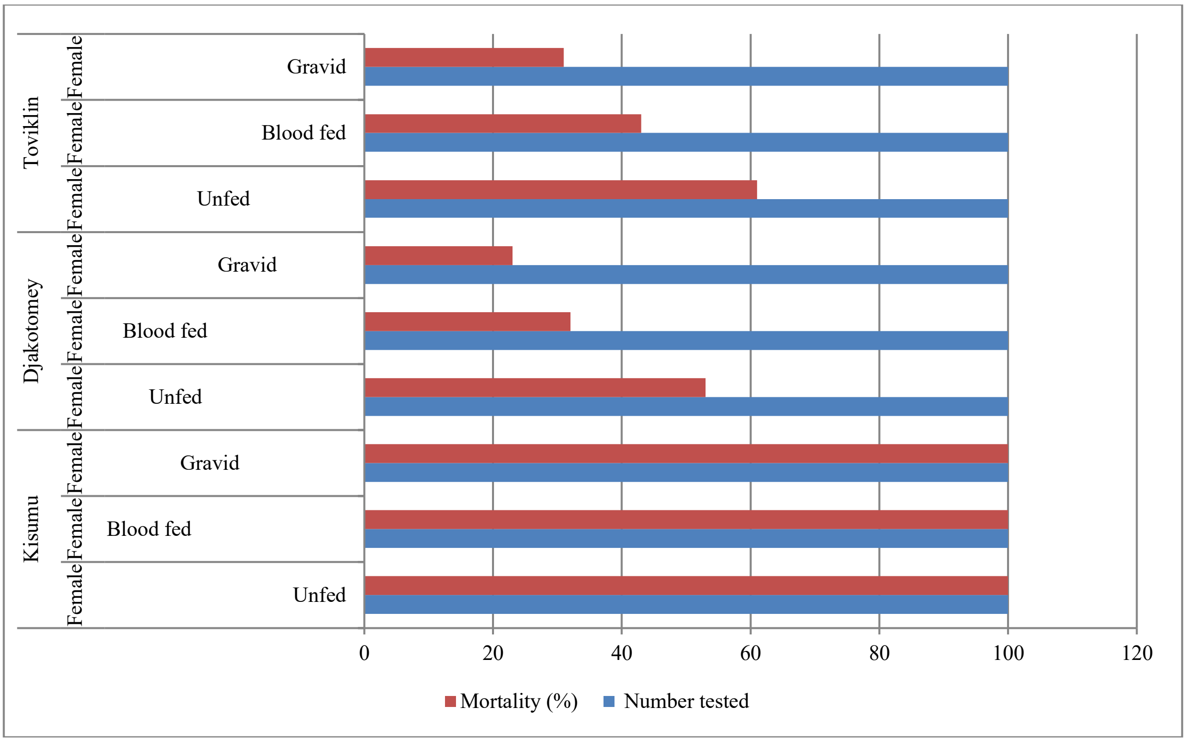

Fig. 3 Comparative susceptibility of *Aedes aegypti* populations aged 2-5 days old regarding their physiological status

4.3. Comparison of Mosquito Susceptibility Regarding their Age

The analysis of Figure 4 showed that, on the one hand, female *Anopheles gambiae* Kisumu populations were fully susceptible to lambda-cyhalothrin when they were unfed and aged 2-5 days old, and when they were unfed and aged 20 days old. The analysis of Figure 4 showed, on the other hand, that female *Anopheles gambiae* Kisumu populations were fully susceptible to lambda-cyhalothrin when they were blood fed aged 2-5 days old, and when they were blood fed and aged 20 days old. The analysis of this figure showed that the mortality rates of blood-fed and aged 20-day-old *Aedes aegypti* female populations from Djakotomey and Toviklin were higher than those obtained when these populations were blood-fed and aged 2-5 days old. A similar pattern was observed in *Aedes aegypti* female populations from Djakotomey and Toviklin when they were unfed and aged 20 days old comparatively to when they were unfed and aged 2-5 days old.

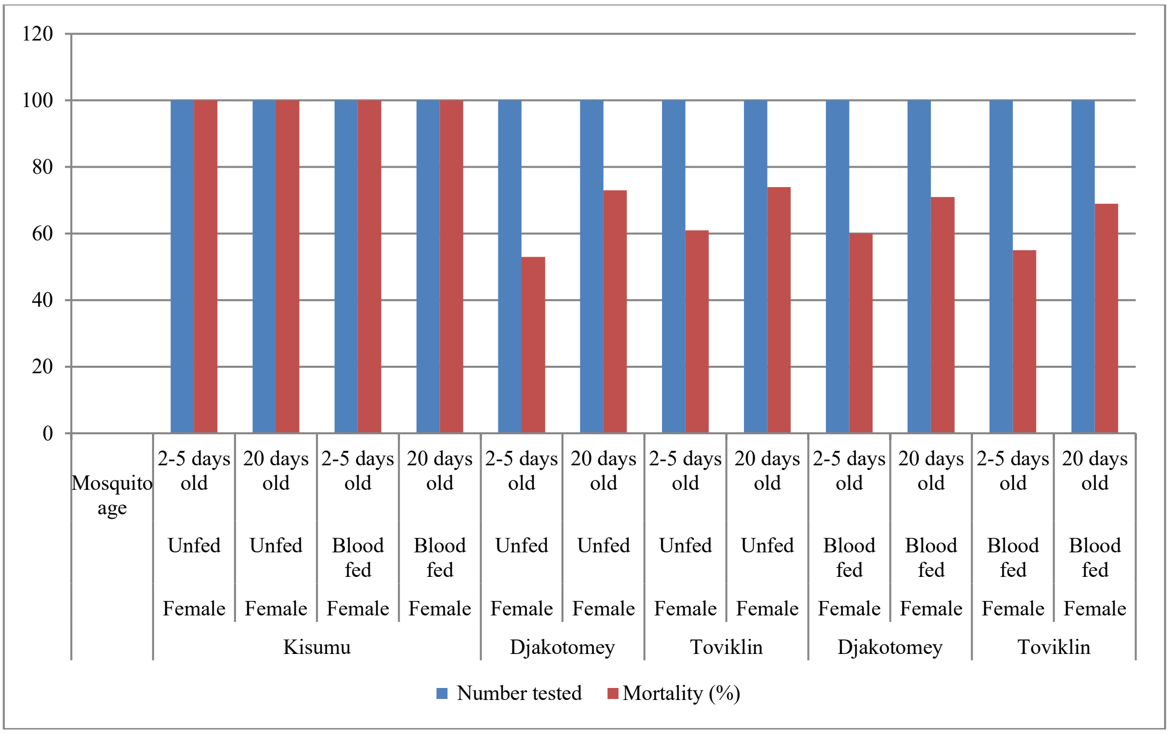

Fig. 4 Comparative susceptibility of *Aedes aegypti* populations regarding their age

4.3. Determination of Lethal Concentrations LC50 and LC95 and Synergism Ratios SR50 and SR95 of Aedes Aegypti Larvae from Djakotomey to Lambda-Cyhalothrin Alone and to Lambda-Cyhalothrin + PBO

The analysis of Table 1 showed that *Aedes aegypti* larvae from Djakotomey district in Couffo department were resistant to lambda-cyhalothrin. In fact, the resistance ratios RR50 and RR95 were all superior to one (1).

After the addition of synergist PBO in test cups, the LC50 value obtained with larvae from Djakotomey, which was 14.2 µg per liter was lower than that obtained with lambda-cyhalothrin alone, which was 16.2 µg per liter ($P < 0.05$). The Synergism Ratios (SR50) (before addition of PBO/after addition of PBO) were 1.14 (Table 2).

In the same way, the LC95 value obtained with larvae from Djakotomey, which was 49.6 µg per liter was lower than that obtained with lambda-cyhalothrin alone, which was 55.6 µg per liter ($P < 0.05$). The Synergism Ratios (SR95) (before addition of PBO/after addition of PBO) were 1.12 (Table 2).

Table 1. Determination of lethal concentrations LC50 and LC95

Populations	LC50 (mg/l)	LC95(mg/l)	RR50	RR95
Kisumu	0.0116	0.0465	_	_
Djakotomey	0.0162	0.0556	139	1.19

Table 2. Determination of lethal concentrations LC50 and LC95 and Synergism ratios SR50 and SR95 of *Aedes aegypti* larvae from Djakotomey to lambda-cyhalothrin + PBO

Population	**Enzyme inhibitor**	**LC50 (mg/l)**	**LC95(mg/l)**	**SR50**	**SR95**
Djakotomey	Without Synergist	0.0162	0.0556	_	_
	With PBO	0.0142	0.0496	1.14	1.2

In the current study, *Aedes aegypti* female populations from Djakotomey and Toviklin were more susceptible than males when they were unfed and aged 2-5 days old. However, it was not recommended to assess susceptibility tests with males because they tend to have higher control mortalities. In addition, the mortality rates of blood-fed *Aedes aegypti* female populations from Djakotomey and Toviklin aged 2-5 days old were lower than those obtained when these females were unfed. This result showed that there was no increase in vector susceptibility to lambda-cyhalothrin after females had taken their blood meals. Regarding the mortality rates of gravid females from Djakotomey and Toviklin, they were lower and significantly different from those obtained when these females were unfed. Also, there was no increase in vector susceptibility to lambda-cyhalothrin after females possess eggs in their abdomen. These results corroborated with those obtained in a recent study on the effect of sex and age on survival of adult *Culex tarsalis* from a susceptible laboratory strain exposed to Permethrin (Tsecouras *et al*., 2024). In fact**,** this study investigates the effect of mosquito sex and age on the survival and resistance determination of adult *Culex tarsalis* exposed to Permethrin, a pyrethroid commonly used for mosquito control, using the Centers for Disease Control and Prevention (CDC) bottle bioassay method. A permethrin-susceptible strain of *Culex tarsalis* (Bakersfield strain) was used in this study. Survival was compared for young adult females (2-5 days old) relative to older adult females (7-10 days old) and separately for female and male mosquitoes of the same age (2-5 days old). Mortality was slightly higher for males than for females during the first observation period (0-5 min) following permethrin exposure, and higher for older females relative to younger females from 5 to 10 min following permethrin exposure, with no differences in mortality by either sex or age for observation periods during the remainder of the diagnostic period. When evaluated over the full diagnostic period, survival varied with mosquito age but not sex. However, all mosquitoes, regardless of sex or age, died within the 30-min diagnostic period for this species, confirming their permethrin susceptibility per the CDC bottle bioassay. This research contributes valuable insight into the potential impact of sex and age on mosquito susceptibility to insecticides in the context of insecticide resistance determination.

In the current study, the mortality rates of blood-fed and aged 20-day-old *Aedes aegypti* female populations from Djakotomey and Toviklin were higher than those obtained when these populations were blood-fed and aged 2-5 days old. A similar pattern was observed in *Aedes aegypti* female populations from Djakotomey and Toviklin when they were unfed and aged 20 days old comparatively to when they were unfed and aged 2-5 days old. These results showed that the older the mosquitoes, the more they were susceptible to insecticides. Also, changes in mosquito physiology that are not specifically associated with insecticides but occur with senescence, such as an increase in the rate of cuticle permeability or a decrease in the rate of xenobiotic excretion, could also lead to an increase in susceptibility to insecticides. So, the mortality rate rose with age when mosquitoes were old.

For instance, a recent study showed that warmer temperatures accelerate reproductive senescence in mosquitoes. Warmer temperatures and aging decrease survival, delay oviposition, and reduce oviposition success, fecundity, and fertility. Importantly, warmer temperatures quicken the onset of the aging-dependent decline in fecundity and fertility, and at the warmest temperature of 32°C, mosquitoes are infertile. Warmer temperatures accelerate reproductive senescence, which has implications for disease transmission in this warming world (Martin *et al*., 2025).

In the current study, the underlying mechanism of the resistance pattern observed in mosquito populations from

Djakotomey was explored using a synergist assay. The synergist assay with PBO, an inhibitor of Cytochrome P450 mono-oxygenases, indicated that this enzyme family may play a role in this lambda-cyhalothrin resistance observed in *Aedes aegypti* larvae from the Djakotomey district. These results corroborate those recently obtained, which showed that there was the presence of metabolic resistance in *Aedes aegypti* resistance to Lambda-Cyhalothrin (Mejía *et al.,* 2025). In this study, the enzyme activity assays were performed to identify metabolic resistance in insecticide-pressured (AF13P), unpressured populations (AF13WP), and the Rockefeller reference strain. The enzymes assessed included Acetylcholinesterase (AChE), Alpha and Beta Esterases (α-EST and β-EST, respectively), Mixed-Function Oxidases (MFO), and Glutathione S-Transferase (GST). Forty female mosquitoes that had emerged within the last day were individually macerated in 300 µL of deionized water using a tissue grinder and a MicroPestle system until the sample was homogenized entirely.

Novelty of the Study

Comparatively to the previous studies, the originality of the current study remains in the method used. In fact, in the previous studies, the authors examined different factors that could influence the toxicity bioassays in *Aedes aegypti,* including sex and age in the context of resistance to pyrethroids. However, the current study is the first which has studied the importance of **r**abbit's blood meal in the assessment of factors which influence *Aedes aegypti* susceptibility to lambda-cyhalothrin, including their physiological status of abdomen as a factor.

Study Limitations

The comparison of the results obtained in the current study with studies using other blood sources is very scare in the literature. The current study was carried out in only one department of Benin, which has twelve departments. For that, further studies will be carried out in the future in more locations of the country by taking these factors or parameters into account.

5. Conclusion

Many factors influence vector susceptibility to insecticides. Among these factors, there are mosquito sex, physiological status, and mosquito age. The rabbit's blood meal is important in the assessment of factors which influence vector susceptibility to insecticides. The method which exposes human hands to mosquitoes to be fed is not recommended.

Recommendations

In order to guide future dengue vector control interventions in Benin, it is useful to report frequently the susceptibility status to pyrethroid or the pyrethroid resistance level in *Aedes aegypti* populations.

This will properly inform control programs of the most suitable insecticides to use and facilitate the design of appropriate resistance management strategies.

Conflicts of Interest

There are no conflicts of interest in regard to the publication of this paper.

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Ethics approval

The present study was approved and conducted in accordance with the guidelines of the Ethical Committee of the National University of Sciences, Technologies, Engineering and Mathematics, Abomey, Benin (EC approval 2025/11454) and after receiving approval.

Authorship Contribution

NA and DC: Conceptualization, Methodology, Investigation, GA and AY: conducted experiments, HT: Formal Analysis, NA: Writing of the Original Draft, DC: Review & Editing. All authors read and approved the final manuscript.

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