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Research Article

Enzyme profile of insecticide-resistant phenotypes of *Aedes aegypti* from Bagua, Peru

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ABSTRACT: Temephos and deltamethrin insecticides have been widely used to control *Aedes aegypti* in Bagua Grande district, Utcubamba, Amazonas, Peru. For this reason, the enzyme profile related to temephos and deltamethrin resistance in *A. aegypti* from Bagua Grande were evaluated. To determine the resistance status, bioassays were conducted with temephos using larvae and with deltamethrin using adults. Enzymes profile were evaluated by biochemical assays of αEST, βEST, GSTs, and remaining AChE in survived individuals to selective doses of both insecticides. Esterase patterns of larvae and adults were observed by native-PAGE. The population showed temephos susceptibility, with RR_{50} = 3.06 and 83.95% mortality, and deltamethrin resistance with a mortality equal to 1.21%. Enzyme assays revealed highly altered levels of GST and AChE in larvae and altered levels of αEST and highly altered levels of GST and AChE in adults. Native-PAGE only showed common bands to susceptible strain. Results suggest that the presence of GST and AChE do not improve temephos resistance, while αEST, GST and AChE mechanisms are involved in deltamethrin resistance in *A. aegypti* from Bagua Grande.

KEYWORDS: *Aedes aegypti*, deltamethrin, enzymes profile, insecticide resistance, temephos

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INTRODUCTION

Aedes aegypti is one of the main mosquito species associated with the transmission of the viral agents, which includes those who causes dengue, yellow fever, chikungunya and Zika diseases. These pathologies constitute a threat to global public health (Lwande *et al.*, 2020), with Dengue being the most prevalent, annually causing an estimated 96 million symptomatic cases and 40,000 deaths (WHO, 2020). Unfortunately, the geographic spread of the vector *A. aegypti* affected new areas, driven by the convergence of ecological, social and economic factors, putting the health of more than 3.9 billion inhabitants of tropical and subtropical areas of the world at risk (WHO, 2022).

In Peru, the presence of the vector *A. aegypti* was detected for the first time in 1852 (Cabezas, 2005). In 1958, the country obtained the certificate of eradication of the vector, however, it was reintroduced in 1984 (PAHO, 2001). In the Amazonas region, *A. aegypti* was detected for the first time in 1996 (Romaní *et al.*, 2014). According to the National Center for Epidemiology, Disease Prevention and Control (2022), at the beginning of the year, Peru presented a sustained increase in dengue cases, with 56,016 accumulated cases as of Epidemiological Week (EW) 28. These figures exceed the records of the last four years, placing Peru as the second country in the Region of the Americas with the highest number of dengue infections (PAHO, 2023). In 2021, the department of Amazonas was one of the 15 regions of the country declared in a health emergency due to dengue (Decreto Supremo Nº 029-2021-SA, 2021). This event was expected, so that at the beginning of the year the aedic index was 15.3, establishing a scenario of very high risk of transmission and maintenance of an outbreak (Vásquez, 2021). Currently, it is the fourth region with the highest fatality rate, with the city of Bagua Grande (Figure 1) concentrating the highest percentage of dengue cases reported up to EW 28 with 33.44% of 1277, similar to the previous year, which was 34.07% of 364 (Centro Nacional de Epidemiología, Prevención y Control de Enfermedades, 2022).

Worldwide, the management and reduction of diseases transmitted by *A. aegypti* is based solely on vector control, whose main strategy is the use of chemical insecticides.

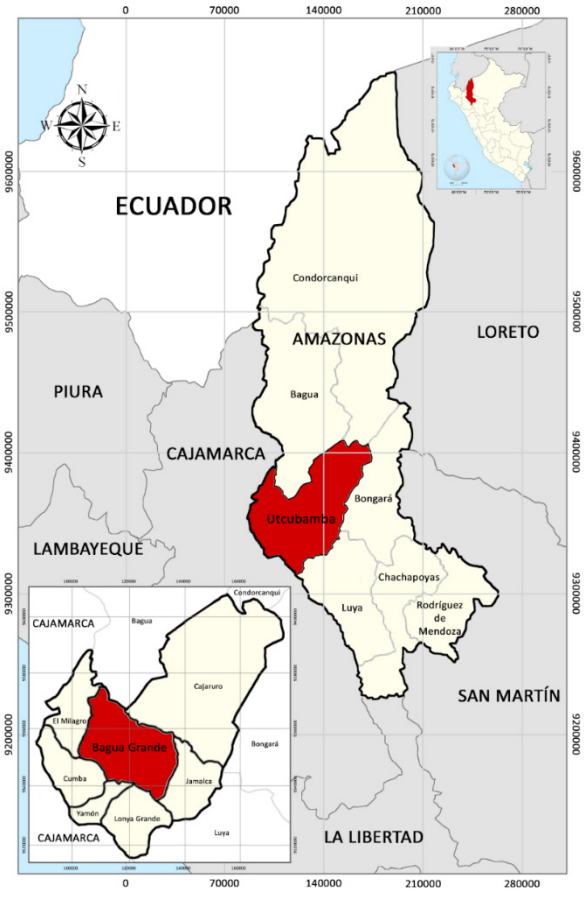

Figure 1. Map of Bagua Grande district, Utcubamba (Amazonas).

These compounds have turned out to be essential to achieve the reduction of the population density of the vector, due to its easy application in large areas, effectiveness, action time and residuality. In Peru, insecticides are continuously used operationally, however, ineffectiveness of chemical treatments has been observed, which according to Barrera (2015), this event may be suggesting the appearance of resistance to insecticides in mosquitoes (Barrera, 2015). The emergence of *A. aegypti* resistant to insecticides and their dispersion is the result of the selective pressure exerted by the prolonged and intensive application of insecticides used not only in public health, but also in the agricultural sector during pest management and control (Dusfour *et al.*, 2019). The MINSA of Peru, for more than 20 years, has controlled the population density of mosquito larvae through the application of the organophosphate Tempehos, but due to the resistance found in the vector, it was replaced by the growth regulator pyriproxyfen in various regions of the country as of 2016 (Pinto *et al.*, 2019). In the department of Amazonas, this rotation began to be implemented the following year (Gonzales *et al.*, 2017).

In this context, resistance to chemical insecticides can be attributed to two main types of mechanisms. The first is the molecular modification of the target site, as for example, in acetylcholinesterases and voltage-gated sodium channels; the second is a metabolism increase of the insecticide before it reaches its target site, through the overexpression of detoxification enzymes such as esterases, monooxygenases and glutathione S-transferases (Hemingway *et al.*, 2004). Insecticide resistance is one of the main operational obstacles facing the Ministry of Health of Peru in its efforts to control population densities of *A. aegypti*, whose persistence in almost all regions of the country favors the emergence and re-emergence of viral diseases. Currently, there are no reports about the susceptibility or resistance status of *A. aegypti* in the locality, as well as studies that describes the possible enzyme profile could be developing in the vector population, taking into account the prolonged use of chemical insecticides used in control. Therefore, this research aims to disseminate timely knowledge about the current state of resistance to insecticides and the enzymatic mechanisms involved, which can be used in the implementation of anti-vector strategies for the benefit of the local community.

MATERIALS AND METHODS

Type of study

A descriptive cross-sectional study was carried out, developed under laboratory conditions with the purpose of determining the enzyme profile associated with resistance to insecticides in the evaluated population.

Biological material

Mosquito larvae and pupae corresponding to the wild population of the parental generation (F0) from the Bagua Grande district of the Utcubamba province of the Amazonas department. Eggs of *Aedes aegypti* Rockefeller susceptible to insecticides were provided by the Institute for Research in Tropical Microbiology and Parasitology (INIMYPAT) of the School of Microbiology and Parasitology of the National University of Trujillo. The sample consisted of the evolutionary stages larva III late/IV early and adult. Adults of both sexes from 3 to 7 days of age were chosen, fed exclusively with sugar solution and physically intact.

Sampling place

The sampling was carrying out by convenience in the sectors of Gonchillo, Los Libertadores and Visalot, in the month of February 2022. Bagua Grande is a northeastern city, capital of the province of Utcubamba in the department of Amazonas. It has a tropical climate, with rainfall all year round, even in the driest month. The maximum and minimum temperatures are 30.3°C and 19.8°C, respectively; and the maximum and minimum Relative Humidity are 86.04% and 73.74%, respectively (ClimateData, 2022). It houses an estimated population of 58,380 inhabitants. Geographically it

is located at 5°45'17''S and 78°26'34''W on the banks of the Utcubamba river and at an altitude of 444 meters above sea level. (Instituto Nacional de Estadística e Informática, 2021).

Analysis unit

For each late III/early IV larva and adult stage of *A. aegypti* surviving exposure to temephos and deltamethrin, respectively, the activity of the following enzymes was analyzed: alpha-esterases, beta-esterases, remaining acetylconlinesterase and glutathione S-transferases.

Data collection instruments and record sheets

The digital record was made through photographs of the key anatomical structures for the taxonomic identification of adult mosquitoes from the wild population. Absorbance values were obtained by UV— visible spectrophotometry. PAGE-native gel scans with esterase activity bands from the wild population and the susceptible strain were recorded, so measurements of the migrated distances of the esterase bands and the running front through the use of digital vernier were obtained. For the record sheets, the number of live and dead individuals was obtained to determine the selection dose and diagnostic dose based on the Rockefeller strain against temephos and deltamethrin.

Procedures

For the establishment of the wild colony, wild mosquitoes and the susceptible strain were bred and maintained under laboratory conditions at a temperature of 26 ± 2°C, relative humidity of 61 ± 4% and a photoperiod of 12h light: 12h dark, in the INIMYPAT insectarium. The larvae were transferred to containers containing purified water and added as food crushed croquettes (Super cat®, Rintisa, Peru). The pupae were carefully transferred to small containers containing only purified water and placed inside a rearing box until imago emergence. The resulting adult mosquitoes were fed ad libitum with 10% sugar solution. The total number of parental mosquitoes was approximately 300.

The identification of the specimens comprised two stages. The first was carried out in the INIMYPAT insectarium and consisted of the presumptive identification of the *Aedes* genus, by simple inspection of the larval and adult stages. The larvae selection criteria were the barrel shape and short size of the respiratory tube, as well as the serpentine movements characteristic of the genus. The adult selection criteria were the horizontal rest position in relation to the surface, the presence of white dots on the abdomen and white-platinum bands at the base of the tarsi of the legs (León, 1997). The second stage of identification was carried out in the Protozoology laboratory of the Professional School of Microbiology and Parasitology of the National University of Trujillo, based on the stereoscopic observation of the morphological characteristics of twenty adult female mosquitoes, using the pictorial keys of Pratt and Stojanovich (2013) to identify belonging to the genus *Aedes* and Rueda (2004) to identify the species *A. aegypti*.

Egg laying by the mosquitoes

Adult females were fed with blood from albino rats (*Rattus norvegicus*) three times a week, and an ovitrap was placed inside the breeding box. Progeny obtained from both the field population and the Rockefeller strain were used in insecticide bioassays, enzymatic assays, and ester electrophoretic analysis. In all trials the first filial generation (F1) of the field population was used.

Larval bioassays

They were carried out based on the protocol formulated by the World Health Organization (1981).

Determination of lethal concentrations in the Rockefeller strain

Ten concentrations were evaluated in a range of 0.050 to 0.270 ppm of the larvicide. For this, a stock solution of the insecticide was prepared using acetone (J.T. Baker, Mexico) as solvent and temephos (PESTANAL, Sigma-Aldrich, Switzerland) as active ingredient. The bioassay consisted of four replicates for each insecticide concentration (PESTANAL, Sigma-Aldrich, Switzerland) with twenty stage III late/IV early (L3/L4) larvae in 120 ml of purified water containing the corresponding volume of insecticide stock solution. . In parallel, a solvent control was performed. The exposure time was 24 hours without food. At the end of the exposure period, the mortality of the larvae was recorded. Those larvae that were unable to ascend, descend, or did not react to the gentle agitation of the water were considered dead. The lethal concentration 90 was used as the selection dose, and double the lethal concentration 99 as the diagnostic dose. The lethal concentrations of the insecticides for the Rockefeller strain were calculated by performing a log-Probit (dose-response) analysis using the SPSS Statistics Version 26 program.

Selection of surviving larvae from the *Aedes aegypti* population

Ten replicates were carried out with twenty L3/L4 larvae in 120 ml of purified water at a final concentration of 0.159 ppm, corresponding to the selection dose (LC_{90}) determined in the susceptible strain. A solvent control was performed simultaneously. The exposure time was 24 hours without food. After exposure to the insecticide, the surviving larvae were placed to recover for a period of 24 hours in containers free of insecticide and provided with food. At the end of the recovery time, they were frozen at -20°C for up to 2 months for subsequent studies. Also, specimens of the susceptible

Rockefeller strain were evaluated under the same conditions, in order to use the survivors as a comparative group for enzymatic and electrophoretic analyses.

Evaluation of the resistance status of the population of *Aedes aegypti* from Bagua Grande

The diagnostic dose 0.468 ppm calculated in the susceptible strain was used. A total of eight replicates were carried out, distributed in three bioassays. For each replicate, between 20 and 25 L3/L4 larvae were evaluated in 120 ml of purified water at the mentioned dose. Simultaneously, a solvent control and a susceptibility control to the insecticide were carried out using the Rockefeller strain. The exposure time was 24 hours without food. At the end of the exposure period, the mortality of the larvae was recorded. Those larvae that were unable to ascend, descend, or react to the gentle agitation of the water were considered dead. In addition, in order to know in detail the degree of resistance of the immature stages of *A. aegypti* from Bagua Grande, it was decided to determine the Resistance Ratio (RR). For this, 12 concentrations of temephos were used in a range of 0.100 to 0.900 ppm. By concentration, three to four replicates were made with 20 to 25 L3/L4 larvae. In parallel, a solvent control was performed. The exposure time was 24 hours without food. Finally, the mortality of the larvae was recorded.

Bioassays in adults

It was carried out following the methodology of the Bottle Biological Assay of the Centers for Disease Control and Prevention - CDC (McAllister *et al.*, 2020).

Determination of lethal concentrations in the Rockefeller strain

Five concentrations of deltamethrin were evaluated in a range of 0.25 to 4.00 ppm. For this, a stock solution of the insecticide was prepared using absolute ethanol (Merck, Switzerland) as solvent and deltamethrin (Delta, Farmex, Mexico) as active ingredient. For each concentration, three to four replicates were performed. Each replicate consisted of the use of bottles impregnated with the corresponding concentration. In each bottle, 20 to 25 mosquitoes from 3 to 7 days of age were introduced, fed ad libitum with 10% sugar solution. In parallel, a solvent control was carried out, using only absolute ethanol. The mosquitoes were exposed for 30 mins. Once the exposure time was over, all the mosquitoes were transferred to containers free of insecticide provided with a 10% sugar solution; up to the 24-hr mortality reading. Mosquitoes that did not have coordinated movements, unable to fly and/or maintain an upright position were considered dead. Lethal concentrations 90 and 99 were used as selection dose and diagnostic dose, respectively.

Selection of surviving adults of *Aedes aegypti* population from Bagua Grande

A total of twelve replicates were carried out using the 0.721 ppm selection dose of deltamethrin, calculated in the Rockefeller strain. Between 20 and 25 adult mosquitoes of both sexes, 3 to 7 days old, were exposed and fed ad libitum exclusively with 10% sugar solution. In parallel, a solvent control was performed. The exposure time was 30 mins. Subsequently, they were carefully transferred to insecticide-free containers provided with a 10% sugar solution for 24 hours. At the end of the recovery time, the surviving mosquitoes were stored at -20°C for up to two months for subsequent studies.

Evaluation of the resistance status of *Aedes aegypti* population from Bagua Grande

14 replicates were carried out using 1,286 ppm of deltamethrin as a diagnostic dose, determined in the susceptible strain. For each replicate, 20 to 25 mosquitoes of both sexes, 3 to 7 days old and fed ad libitum exclusively with 10% sugar solution were evaluated. In parallel, a solvent control and a susceptibility control to the insecticide were carried out using the Rockefeller strain. The exposure time was 30 mins, after which the number of knocked down mosquitoes was recorded to find the knock down percentage (% KD). Subsequently, all mosquitoes were carefully transferred to insecticide-free containers provided with 10% sugar solution, until the mortality reading at 24 hours. The percentage of mortality of the larval stages was interpreted according to Diniz *et al.* (2014): 98-100%, susceptibility; 80-98% strength verification and ≤80% strength. To determine the resistance ratio, the RR_{50} and RR_{90} values were calculated by dividing the LC_{50} and LC_{90} of the Bagua Grande population by the LC_{50} and LC_{90} of the Rockefeller strain. The resistance ratio was interpreted according to the WHO (2016): RR<5, susceptible; 5<RR<10, moderately resistant and RR>10 highly resistant. Per cent mortality of adult mosquitoes was interpreted following CDC guidelines: 97-100% indicates susceptibility; 90-96% suggests the possibility of resistance; and <90% suggests resistance (McAllister *et al.*, 2020).

Enzyme profile

The activity levels of α-EST, β-EST, remaining AChE and GST of surviving individuals to temephos and deltamethrin were analyzed. The surviving individuals of the Rockefeller strain were also analyzed in order to establish cut-off values for each enzymatic group. The trials were performed following the instructions of the Brogdon (2015). The number of specimens analyzed corresponding to the Bagua Grande district were 83 larvae and 84 adults. Likewise,

a total of 20 larvae and 20 adults of the susceptible strain were analyzed. Duplicates of each individual were analyzed. Enzymatic tests were performed in 96-well microtiter plates (Brand, USA). Optical Density (OD) values were measured using the Multiskan GO spectrophotometer (Thermo Fisher Scientific, Finland).

Preparation of mosquitoes for enzyme assays

The surviving larvae and adults were individually distributed in microcentrifuge tubes, numbered, and placed on ice. Then, each specimen was homogenized in 30 µL of molecular grade water (MiliQ) and brought to a final volume of 300 µL. The homogenate was centrifuged at 14,000 rpm for 30", the supernatant was used for the EST and GST assays. Prior to centrifugation, four 25 µL aliquots were removed for AchE assay.

AChE assay

Two types of reactions were performed, one was to measure the total AChE activity, and the other was to measure the AChE activity remaining after the addition of an inhibitor. The inhibitor used was propoxur carbamate (Dr. Ehrenstorfer, UK). To 25 µl of homogenate was added 145 µl of triton phosphate buffer and 10 µl of 0.01 M 5,5'-dithiobis-2-nitrobenzoic acid [DTNB] (Sigma-Aldrich, USA) solution, followed by the addition of 25 µl of 0.01 M acetylcholine iodide solution for total AChE and 25 µl of 0.01 M acetylcholine iodide solution with 0.05 µl of 0.1 M propoxur for remaining AChE. Blanks were made for both reactions, using MiliQ water instead of the homogenate. It was allowed to react at room temperature for one hour. Finally, both reactions were read at 405 nm. Acetylcholinesterase activity in the presence of the inhibitor was expressed as percentage of AChE remaining. For this, the difference between the absorbance results in the presence (iAChE) and in the absence (AChE) of the propoxur inhibitor was calculated for each mosquito, which was divided by the AchE and multiplied by 100. It was not necessary to correct the values obtained by the total proteins nor by the volume of homogenate.

Non-specific esterase assay

Two types of reactions were performed: one to measure α-EST activity and the other to measure β-EST activity. To 20 µl of supernatant were added 200 µl of 30 mM α-naphthyl acetate (Sigma-Aldrich, USA) for α-EST and 200 µl of 30 mM β-naphthyl acetate (Sigma-Aldrich, USA) for β-EST. Blanks were made for both reactions, using MiliQ water in place of the supernatant. It was allowed to incubate at room temperature for 15 mins. After incubation, 50 µl of Fast Blue B salt dye solution (Himedia, India) was added to each well. The mixture was left in color for 5 mins. Finally, the measurement of the optical density values at 570 nm was performed. The activity of the esterases was calculated based on the standardization curves of the absorbances of known concentrations of α and β naphthol. Enzyme activities were expressed as nmol of α-naphthol or β-naphthol/mg protein/min. The values of these enzymes were represented in nmol/mg ptn/min. For this, the absorbance values obtained from the 20 µl aliquots were corrected to the total volume of homogenate (300 µl), multiplying the "bleached" absorbance by 15. Next, to transform into nmoles of substrate, it was divided by the conversion factor m = 0.3479 for α-EST and m = 0.4408 for β-EST, then the quotient obtained was divided by the total protein of each individual. The result was the total substrate (nmol) consumed per mg of protein of each individual in a time of 15 mins. To calculate the enzyme activity per minute, this result was divided by 15.

Determination of the standardization curve of α and β naphthol

Duplicates of successive concentrations were made in a range from 0 to 5 µg, starting from a 0.5 µg/ml α- or β-naphthol solution (Sigma-Aldrich, USA). To each duplicate was added 200 µl of 30mM α- or β- naphthyl acetate. It was allowed to incubate at room temperature for 15 mins. Subsequently, 50 µl of the Fast Blue B salt dye solution was added to each well. The mixture was left in color for 5 mins. Finally, the optical density values at 570 nm were measured. The conversion factors were obtained from the α/β-naphthol calibration curve, and the BSA calibration curve. For each curve, a graph was constructed locating the absorbances obtained on the ordinate axis; and on the axis of the coordinates, the known concentrations evaluated. Obtaining a least squares regression line with formula $y = mx + b$, where m is the conversion factor; as well as the correlation index (R2), this index supports the relationship between the observed and expected values.

GST assay

To 15 µl of supernatant was added 195 µl of the mixture of 1-chloro-2,4-dinitrobenzene [CDNB] (Sigma-Aldrich, USA) 63 mM and reduced glutathione [GSH] (Merck, Switzerland) 10 mM. Subsequently, it was incubated at room temperature for 10 mins, then the optical density at 340 nm was measured and the incubation was continued for another 10 mins. After the second incubation, the reading was performed again. Blanks were made using MiliQ water in place of the supernatant. GST activity was expressed as mmol GSH-CDNB/mg protein/min. To know the enzymatic activity, the amount of substrate used in the reaction was calculated. To do this, the difference between the "bleached" absorbance values obtained at the reading times of 20 min and 10 min was calculated. This result was multiplied by 20 in order to correct for the total volume of homogenate (300 µl). The product was divided between the extinction coefficient

(ε = 4.39), for the optical path equal to 0.6, and for the minutes of reaction between the reading times equal to 10. Finally, the result was divided between the total proteins of the individual correspondent. The final result was expressed in nmoles/mg ptn/min.

Protein assay

Due to the variety of sizes of the specimens analyzed, it was necessary to adjust the absorbances of the ESTs and GSTs assays to obtain the activity per mg of protein. For this, the protein concentration of each individual was used as a correction factor. The assay was carried out by mixing 200 µl of Bradford's reagent (BIORAD, USA) with 10 µl of supernatant. Then, it was left to incubate at room temperature for 5 mins. Finally, the measurement of the optical density values at 620 nm was performed. The absorbance values obtained were converted to µg of protein using the absorbance standardization curve of known concentrations of Bovine Serum Albumin (BSA). It was calculated for each individual. For this, the absorbance values obtained from the 10 µl aliquots were corrected to the total volume of homogenate (300 µl), multiplying the "bleached" absorbance by 30. Next, to know the amount of protein in mg, it was divided by the factor of conversion m = 0.0540, the quotient obtained was divided by a thousand.

Determination of the protein standardization curve

Duplicates of successive concentrations were made in a range of 0 to 10 µg and 15 µg, from a BSA solution (Sigma-Aldrich, USA) at 1 µg/ml for all cases except those 15 µg in which a 2 µg/ml BSA solution was used. To each duplicate, 200 µl of Bradford's reagent was added. It was allowed to incubate at room temperature for 5 mins. Finally, the measurement of the optical density values at 620 nm was performed.

Classification of enzyme profiles

The percentage of individuals from the wild population that presented enzyme activity higher than the 99th percentile of the corresponding enzyme of the susceptible strain was calculated. The percentages obtained were stratified according to the following criteria: 0 – 15, "unchanged" activity; 15 – 50, "altered" activity; and above 50, "highly altered" activity (Valle *et al.*, 2006).

Native polyacrylamide gel electrophoresis (Native PAGE)

This technique was carried out with the objective of separating the different isoforms of the esterases present in larvae and adults surviving to the treatment of temephos and deltamethrin, respectively. The methodology is similar to that of Muthusamy *et al.* (2011). To prepare the gels, a 30% acri/bis acrylamide solution was used, from which the 5% and 10% concentrator and developer gels were formulated in 1.5 M Tris-HCl buffer pH 8.8 and 0.5 Tris-HCl buffer. M pH 6.8, respectively. The running buffer used was Tris-Glycine. Four groups of four larvae and seven groups of four adults from the wild population were processed, as well as three groups of four larvae and two groups of four adults of the susceptible strain. Each group was homogenized in MiliQ water. The homogenates were centrifuged at 13,400 rpm for 90 seconds. An aliquot of each supernatant was diluted 2:1 with 3x sample buffer containing 1.05 mL of MiliQ water, 1.25 mL of 1.5 M Tris-HCl buffer pH 6.8, 7.5 mL of glycerol, and 0.2 mL of 0.5% xylenceanol. Subsequently, 20 µl of each mixture was transferred to the wells of the electrophoresis gel. The electrophoretic run was performed at 150 V for 1.5 hours at 4°C using an OmniPAGE Mini vertical camera (Cleaver Scientific, USA). At the end of the run, the gel was submerged in 20 ml of 20mM potassium phosphate buffer containing 0.04% of each of the α-naphthyl and β-naphthyl acetate substrates and incubated at 37°C for 15 mins. Then, to visualize the esterase bands, Fast Blue 0.6% developer in 3.5% SDS was added. After staining, the gel was scanned (Canon, MP250, Japan). Finally, for each of the different esterase bands present in the Bagua Grande population and in the susceptible strain, the relative mobility (Rm) was calculated by measuring the migrated distance of the band divided by the migrated distance of the run front; in order to identify those that were common or not to the Rockefeller strain. Dark-colored bands were classified as α-EST and currant-colored bands as β-EST, and they were assigned correlative numbering according to their location in the gel from the least anionic to the most anionic portion.

RESULTS AND DISCUSSION

Bioassays with insecticides

Table 1 shows that the larvae of the F1 generation of *Aedes aegypti* from Bagua Grande exposed to the diagnostic dose of 0.468 ppm of temephos presented a mortality of 83.95% at 24 hours of exposure, indicating verification of resistance in the population. Table 2 shows the resistance ratio values RR_{50} = 3.06 and RR_{90} = 3.57 times higher than that of the Rockefeller strain. According to the interpretation suggested by the WHO, the population is considered susceptible to the larvicide since the RR values were less than five.

Also, in Table 1, it is observed that the adult F1 mosquitoes of *A. aegypti* from Bagua Grande exposed to the diagnostic dose 1.286 ppm of deltamethrin presented a mortality of 1.21% after 30 minutes of exposure and 24 hrs of recovery. The percentage mortality indicates that the population is resistant to the pyrethroid deltamethrin. On the other hand, a KD ratio of 12.12% was observed.

Table 1. Status of resistance to the insecticides temephos and deltamethrin in larvae and adults of *Aedes aegypti* from the Bagua Grande district, Utcubamba province, Amazonas Region

Insecticide	DD (ppm)	N	% Mortality (±SD)	% KD (±SD)	Condition
Temephos	0.468	184	83.95 ± 4.60	n.a.	VR[a]
Deltamethrin	1.286	330	1.21 ± 2.94	12.12 ± 7.27	R[b]

Abbreviations: DD, diagnostic dose; N, total number of individuals evaluated; KD, knockdown ratio; n.a., data not applicable; VR, resistance verification; A, resistance. [a]Interpretation according to Diniz *et al.* (2014): 98-100%, susceptibility; 80-98% strength verification and ≤80% strength. [b]Interpretation according to CDC (2020): 97-100% susceptibility; 90-96% chance of resistance and <90% resistance.

Table 2. Rate of resistance to temephos in *Aedes aegypti* from the Bagua Grande district, Utcubamba province, Amazonas Region

Population	N	Slope	Lethal concentration (ppm)		RR*	
			LC_{50} (CI 95%)	LC_{90} (CI 95%)	RR_{50}	RR_{90}
Rockefeller	615	5.12	0.100 (0.092 – 0.107)	0.159 (0.149 – 0.172)	1	1
Bagua Grande	900	4.66	0.306 (0.288 – 0.323)	0.558 (0.521 – 0.604)	3.06	3.57

Abbreviations: N, total number of individuals evaluated; CI, confidence interval; RR, resistance ratio. *Interpretation: RR < 5, susceptibility; 5 ≤ RR < 10, moderate resistance; RR ≥ 10, high resistance.

Enzyme profile

The enzyme profile of *A. aegypti* specimens from Bagua Grande in stages L3/L4 surviving to temephos (Figure 2) shows that the activities of α-EST and β-EST are below the cut-off values (cv = 8.54463 nm/mg ptn/min and cv= 6.09286 nm/mg ptn/min) calculated for the Rockefeller susceptible strain for said enzymes, in contrast to the wide distribution of remaining GST and AChE activities that exceeded the cut-off values (cv = 0.92052 nm/mg ptn/min and cv = 73.52983%) calculated for the susceptible strain. The percentage of individuals whose enzymatic profile exceeded the 99th percentile of the susceptible strain showed unaltered α-EST (0%) and β-EST (0%) and highly altered GST (81.25%), and the remaining AChE (76.83%).

Enzyme profile of *A. aegypti* adult specimens from Bagua Grande that survived deltamethrin reveal in Figure 3 that the α-EST and β-EST activities are to a greater extent below the cut-off values (cv = 34.8503 nm/mg ptn/min and cv = 27.9409 nm/mg ptn/min) calculated for the insecticide-susceptible strain, in contrast with the wide distribution of the remaining AChE and GST activity that exceeded the cut-off values (cv = 86.3424% and cv = 0.9329 nm/mg ptn/min) calculated for the strain susceptible to such enzymes. The percentage of individuals whose enzymatic activities exceeded the 99th percentile of the Rockefeller strain showed unaltered β-ESTs (1.19%), altered α-ESTs (15.66%) and highly altered GSTs (80.95%), and remaining AChE (73.17%).

PAGE native

The analysis of the electrophoretic profile of esterases in the population of *Aedes* from Bagua Grande showed the presence of esterase bands that were designated as A or B, depending on the affinity of the enzymes in the reaction with the substrates α- or β-naphthyl acetate, and numbered correlatively based on relative mobility. Three esterase bands common to those of the susceptible strain were observed in the larvae of the wild population (Figure 4), named A1 (Rf = 0.05), A2 (Rf = 0.25) and A3 (Rf = 0.59). Four esterase bands were observed in adult mosquitoes from the wild population (Figure 5), named A1 (Rf = 0.15), B1 (Rf = 0.39), A2 (Rf = 0.50) and A3 (Rf = 0.64) and were common to the esterase bands of the susceptible strain.

The development of resistance to temephos as a consequence of the constant selective pressure exerted by the insecticide and over a long period of time has been documented by various researchers (Bisset *et al.*, 2007; Lima *et al.*, 2011; Lesmana *et al.*, 2022; Palomino *et al.*, 2022). Based on this and considering that, in the department of Amazonas, the larvicide temephos was used continuously from the detection of the vector in 1996 until 2017, it is possible that the immature stages of *A. aegypti* from the Bagua Grande district have developed resistance to the larvicide. However, toxicity tests revealed that the larval population of *A. aegypti* presents susceptibility to temephos with a value RR_{50} = 3.06. Given this finding, the possibility of a reversal of resistance in this population is suggested, due to the discontinuation

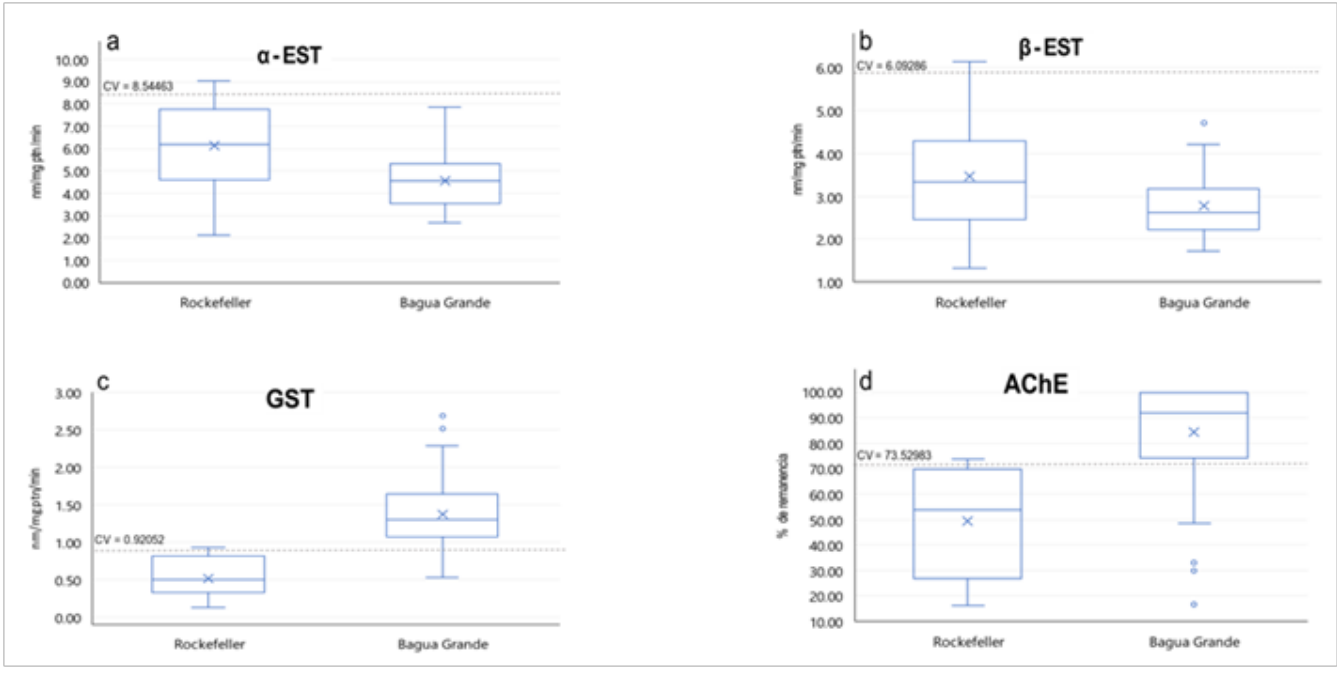

Figure 2. Box-and-whisker plot of the enzymatic profile of *Aedes aegypti* from the district of Bagua Grande, Amazonas. (a) alpha esterases (α-EST) (b) beta esterases (β-EST) (c) glutathione s-transferases (GST) (d) acetylcholinesterase insensitive (AChE). The dashed line indicates the cut-off value above which the field population could be considered with high activity of the respective enzymatic group. The Rockefeller strain is shown for comparison purposes.

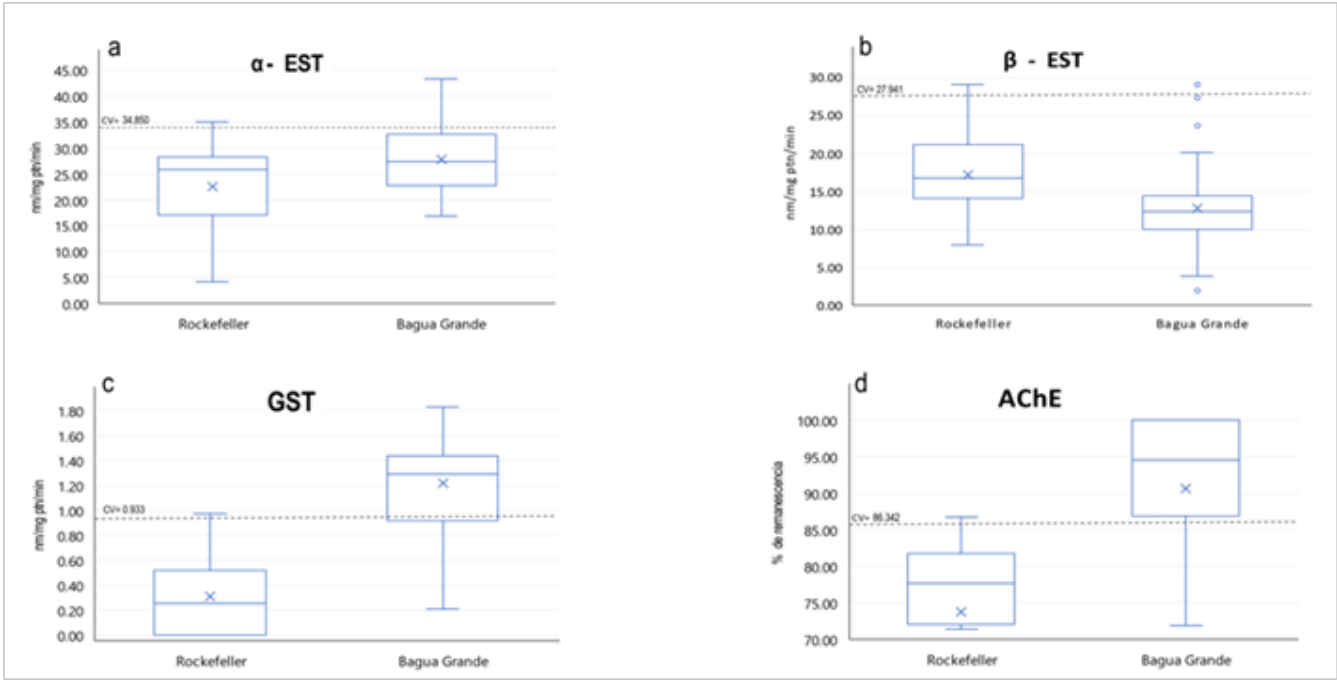

Figure 3. Box-and-whisker plot of the enzyme profile of adults of *Aedes aegypti* from the district of Bagua Grande, Amazonas. (a) alpha esterases (α-EST) (b) beta esterases (β-EST) (c) glutathione s-transferases (GST) (d) acetylcholinesterase insensitive (AChE). The dashed line indicates the cut-off value above which the field population could be considered with high activity of the respective enzymatic group. The Rockefeller strain is shown for comparison purposes.

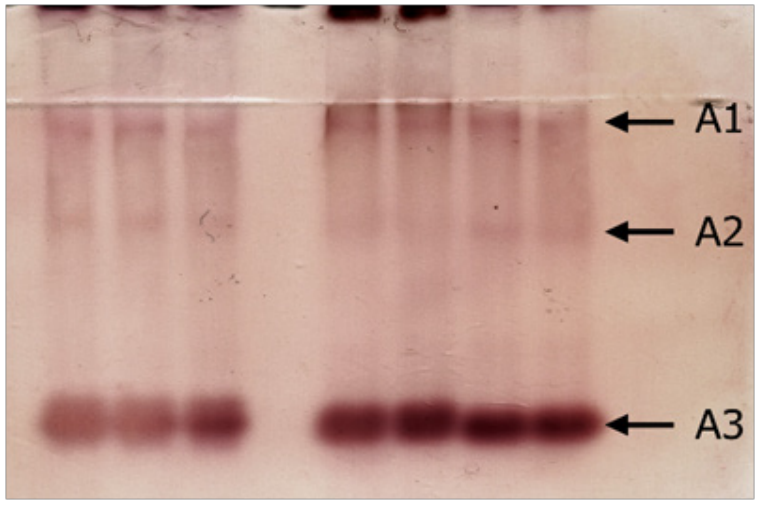

Figure 4. Electrophoretic profile of esterases in surviving L3/L4 larvae after exposure to temephos. Channels 1-3: Rockefeller; Channel 4: blank; Channels 5-8: Bagua Grande.

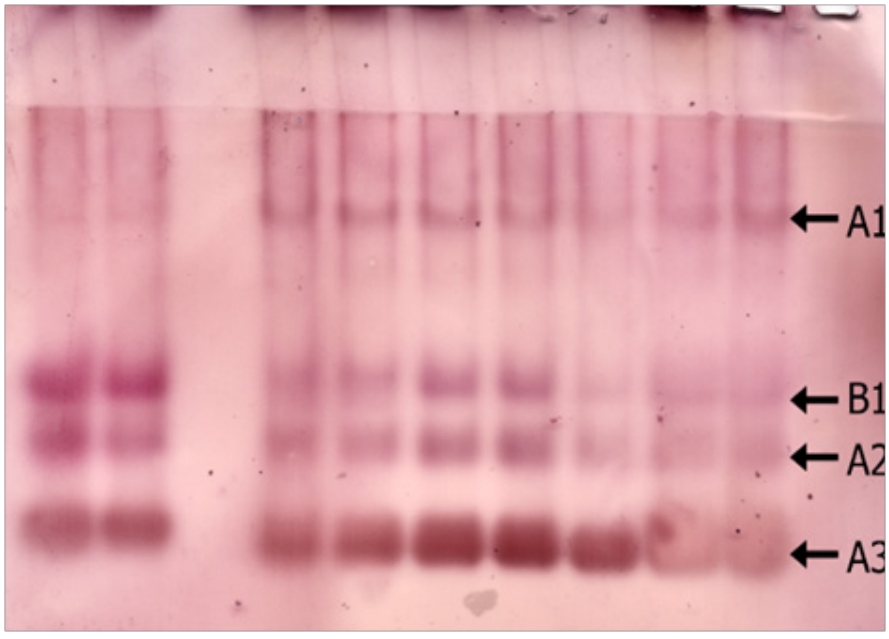

Figure 5. Electrophoretic profile of esterases in adult survivors of deltamethrin exposure. Channels 1-2: Rockefeller; Channel 3: blank; Channels 4-10: Bagua Grande.

of the use of temephos for six years, during which time it has been replaced by the growth regulator pyriproxyfen as a control strategy (Gonzales, 2017). Palomino *et al*. (2022), in their study, found similar results for populations of *A. aegypti* from 10 Peruvian cities corresponding to the departments of San Martín, Ucayali and La Libertad, whose RRs ranged between 2.1 and 4.9 (Palomino *et al.*, 2022). This finding of decreased resistance has also been reported in Brazil, where after seven years of replacing temephos with the biolarvicide Bti, they observed a decrease in RR from 10.4 to 7.2 (Lima *et al.*, 2011). In the same way, in two neighborhoods of Cúcuta, Colombia, where reducing the use of the larvicide for four years and strengthening alternative control measures, resulted in the attenuation of RR= 13.27 and RR= 11.48 to RR= 4.75 and RR= 5.61 (Conde *et al.*, 2014).

In Peru, since 1990, pyrethroids have been the main insecticides used to control dengue, malaria, leishmania and Chagas vectors. And, starting in 2000, the pyrethroid deltamethrin began to be used to control *A. aegypti*. The bioassays showed resistance to deltamethrin in the aedinos from Bagua Grande. In Peruvian territory, Chávez *et al.* (2005) found resistance to deltamethrin in *A. aegypti* from Piura, using the WHO methodology. Likewise, Pinto *et al*. (2019) found loss of susceptibility to deltamethrin and other pyrethroids in populations of *A. aegypti* from the districts

of Punchana (Loreto) and Piura (Piura), using the WHO methodology. Resistance to deltamethrin and other PYRs in populations of *A. aegypti* using the CDC methodology has been detected by various researchers such as Aponte *et al.* (2019) in Columbia, Morales *et al.* (2019) in Ecuador, Deming *et al.* (2016) in Mexico, Al-Amin *et al.* (2020) in Bangladesh, and Leong *et al.* (2019), in Selangor, Malaysia.

Esterases are enzymes that in an aqueous medium catalyze the hydrolysis of xenobiotic ester bonds, producing an acid and an alcohol as metabolites, thus eliminating its toxicity after preventing it from reaching the target site. Previous studies in *A. aegypti* have established that the overexpression of α/β-EST plays a key role in resistance to OPs and carbamates (CBs) (Adhikari & Khanikor, 2021; Bisset *et al.*, 2001; Polson *et al.*, 2011; Poupardin *et al.*, 2014). Mangas *et al.* (2017), mention that OPs insecticides are more reactive with esterases than with their target, therefore if the concentration of esterases is comparable to that of the insecticide, they can sequester/hide it from the insect's metabolic system and hydrolyze it in non-toxic products, and in this way the amount of active insecticide that reaches the target site is reduced. The enzymatic analysis of the surviving larvae to temephos showed that neither of the two esterases evaluated presented overexpressed levels of activity; which corresponds to the susceptibility observed in the population as a consequence of the discontinuation of the larvicide. Adhikari & Khanikor (2021) suggest that the low values of esterase activity at the spectrophotometric level may be due to the formation of an esterase-temefos complex during exposure to the larvicide, and since these are not overexpressed, very few esterases remain free to its detection. Bisset *et al.* (2019) found a similar event in larvae of a strain *A. aegypti* resistant to temephos, maintained for 19 years under the selective pressure of the insecticide. When the larvicide was suppressed, they observed a reversal of resistance and a decrease in esterase levels to such a degree that there was no significant difference with the Rockefeller strain. In resistance to PYRs, esterases participate to a lesser extent (Yang *et al.*, 2020b). Enzyme profile of adult mosquitoes showed that only α-ESTs were increased, so that it was similarly to the study by Contreras-Perera *et al.* (2020), where they found elevated α-EST in *A. aegypti* resistant to deltamethrin. Other studies carried out in various countries also report altered levels of ESTs and associate it with resistance to PYRs found in adult *A. aegypti* (Amelia-Yap *et al.*, 2018; Lee *et al.*, 2014; Lin *et al.*, 2013; Pareja-Loaiza *et al.*, 2020). On the other hand, regarding the zymographic profile of larvae and adults surviving exposure to temephos and deltamethrin, respectively, they only exhibited esterase bands common to those of the susceptible strain. In Peru, Bisset *et al.* (2007), in their study found that the population of *A. aegypti* from Tumbes that was susceptible to temephos, did not present A4 bands corresponding to α-EST.

Acetylcholinesterase is responsible for the degradation of acetylcholine, which is essential for the termination of cholinergic transmission in the insect nervous system (Liu, 2015). This enzyme is a target of OPs and CBs insecticides, which, when binding to the active site, results in an abnormal accumulation of acetylcholine that leads to overstimulation of the cholinergic system, causing paralysis and eventually death (Engdahl, 2017). Insensitivity at the catalytic site of this enzyme has been found to confer high resistance to these two groups of insecticides (Coleman & Heminway, 2007). This resistance is due to a structural change generated by the G119S mutation in the AChE gene (ace-1), which has been found in *Anopheles gambiae*, *Anopheles albimanus*, *Culex pipiens* (Weill *et al.*, 2004) and *Culex quinquifasciatus* (Amorim *et al.*, 2013). However, it has not yet been observed in *A. aegypti,* and it is hypothesized that it is very unlikely to occur spontaneously in this species. In our results we found that the larvae of *A. aegypti* from Bagua Grande presented highly altered acetylcholinesterase activity despite being susceptible to OP, similar to what was found by Leong *et al.* (2018). This finding can be explained considering the influence of the following factors: i) having used a standard concentration of propoxur determined in another genus of mosquito, so it would not be high enough to inhibit the unaltered acetylcholinesterases of *A. aegypti* (Perera *et al.*, 2008); ii) the decrease in propoxur concentration due to the intervention of alternative detoxification mechanisms such as ESTs, GSTs and MFOs, which would be sequestering or hydrolyzing the carbamate before it inhibits AChE, since the total extract of the mosquito was used where all the enzymes profile are present (Cuamba *et al.*, 2010). On the other hand, adult mosquitoes surviving deltamethrin exposure also had a high level of remaining AChE, as described by Granada *et al*. (2021), so that they mention that high levels of this enzyme have not been associated with resistance to pyrethroids, however, they found that *A. aegypti* from Colombia increased their AChE levels after a six-generation selection with PYRs. Badiou *et al.* (2008) demonstrated that the increase in AChE may be due to two mechanisms. The first is due to the overproduction of AChE to compensate for the increased release of acetylcholine as a consequence of exposure to deltamethrin. The second is due to the synthesis of new AChEs on the membrane surface to replace those eliminated during the conversion to its soluble form (Badiou *et al.*, 2008).

GSTs are a broad family of enzymes that participate in detoxification processes of endogenous and exogenous compounds. Mainly, the epsilon class of GSTs has been associated with organophosphate and pyrethroid resistance in *A. aegypti* (Amelia-Yap *et al.*, 2018. Resistance to OPs,

specifically to temephos, is possibly caused by defensive action against toxic endogenous compounds derived from exposure to the larvicide or by resistance to direct inhibition by the xenobiotic (Helvecio *et al.*, 2020). In our study, it was found that the population *A. aegypti* from Bagua Grande susceptible to temephos has highly altered activity of GSTs. Leong *et al.* (2018), also found similar results and suggests that there is no correspondence between enzyme activity levels and susceptibility status in *A. aegypti*. However, other studies indicate that there is a positive correlation between the reversal of resistance to temephos and the reduction of enzyme activity (Bisset *et al.*, 2019; Helvecio *et al.*, 2020; Strode *et al.*, 2012), with the exception of some GSTs, whose activity values remain in overexpression despite the fact that the population is susceptible to the insecticide (Strode *et al.*, 2012). According to Helvecio *et al.* (2020), this exception is due to the establishment and prevalence of a mutation in the GSTe2 gene, after the selective pressure exerted by temephos when applied continuously. Consequently, the process of reducing resistance to temephos is difficult and slow, requiring more time for its complete reversal (Lima *et al.*, 2011). Also, it is suggested that this overexpression of GSTs in the larval population may have been acquired in the adult stage of the mosquito and would be influencing both the mature and immature stages of the mosquito (Chavasse, 1997). On the other hand, in resistance to pyrethroids, the role of these enzymes is not fully elucidated, but it has been proposed that high GSTs reduce the mortality of resistant specimens, attenuating the lipid peroxidation induced by the pyrethroid (Vontas *et al.*, 2001), due to the predominantly peroxidase activity in the presence of PYRs (Lumjuan *et al.*, 2007) or by sequestering the insecticide (Kostaropoulos *et al.*, 2001). The results obtained showed that the population *A. aegypti* from Bagua Grande resistant to deltamethrin shows highly altered activity of GSTs, suggesting the participation of these enzyme profile in resistance to the pyrethroid insecticide. Lumjuan *et al.* (2011), demonstrated that the increased levels of GSTs are due to the presence of two gene polymorphisms (GSTe2 and GSTe7) of the enzyme, and are associated with the survival of *A. aegypti* to the pyrethroid deltamethrin. Likewise, previous studies also reported an increased activity of GSTs in *A. aegypti* resistant to pyrethroids (Aponte *et al.*, 2019; Leong *et al.*, 2018; Lima *et al.*, 2011; Lin *et al.*, 2013). On the other hand, the mortality result <2% of *A. aegypti* from Bagua Grande against deltamethrin and with a knockdown percentage of 12.12 suggests the presence of knockdown resistance (kdr) mutations. According to Francis *et al.* (2017), since when there is low mortality in adults exposed to pyrethroids, the main mechanism that confers complete protection are kdr mutations in the voltage-gated sodium channel gene, so that they modify the target site of pyrethroid insecticides (Francis *et al.*, 2017).

The limitations of traditional insecticide-based strategies, particularly the development of insecticide resistance, have resulted in significant efforts to develop alternative eco-friendly methods. Biocontrol strategies aim to be sustainable and target a range of different mosquito species to reduce the current reliance on insecticide-based mosquito control. Naturally occurring organisms that are pathogenic to mosquitoes can also be considered for biocontrol strategies (Benelli *et al.*, 2016). *Bacillus thuringiensis* var. *israelensis* (*Bti*) is currently the most common mosquito larvicide employed in European countries. *Bti* is a gram-positive, spore-forming bacterium that releases insecticidal toxins and virulence factors that selectively target the larval stages of insects (Becker, 1997). *Bti* has been successfully used in reducing the number of A. aegypti larvae (Novak *et al.*, 1985), and the use of *Bti* in large mosquito breeding sites in urban environments is now more demanding (Benelli *et al.*, 2016).

CONCLUSION

As conclusion, the population of *Aedes aegypti* from Bagua Grande is susceptible to the organophosphate larvicide temephos and resistant to the pyrethroid adulticide deltamethrin. Moreover, it presents unchanged α and β-esterase profile, while the acetylcholinesterase and glutathione-s-transferase perfil are highly altered. The adult stage of the population of *A. aegypti* presents alterations in the profile of α-esterases, glutathione S-transferases and acetylcholinesterases. The β-esterase profile is unchanged. In addition, the low mortality and low kill percentage suggest that the population may present mutations in the sodium channel. Finally, all esterase bands from larvae and adults of *A. aegypti* from Bagua Grande were common to those of the susceptible Rockefeller strain. It is also suggested that biological control strategies may be adopted to overcome insecticide resistance.

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