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INVESTIGATING THE ROLES OF METABOLIC ENZYMES IN INSECTICIDE RESISTANCE IN THE *Culex pipiens sensu lato* POPULATION FROM GOMBE NIGERIA

[1,2]*ABUBAKAR N. and [3]HAMIDU S. K.

[1]Department of Biochemistry, Faculty of Sciences, Gombe State University.
[2]Molecular Laboratory, College of Medical Sciences, Gombe State University. [3]Department of [3]Human Anatomy, Faculty of Basic and Allied Medical Sciences, Gombe State University.

*Corresponding Author: abubakarnura@gsu.edu.ng

ABSTRACT

Despite the widespread reports of insecticide resistance in the arboviral vectors in Gombe state, the direct role of metabolic enzymes in such resistance is sparse. This study aimed to determine if some metabolic enzymes play a role in the resistance to insecticides in the population of *Cx pipiens* from Gombe State. The adult female *Cx pipiens* were picked randomly and categorized into "Exposed" and "Unexposed" groups. The Exposed group was treated with Fenitrothion or Bendiocarb insecticides for 30 min using a WHO bioassay tube while the Unexposed group was exposed to a plane paper using a similar method. The Glutathione-S-transferases (GSTs), alpha-esterases, and beta-esterases activities of 31 mosquitoes from both groups were determined using the biochemical assay protocol. The mean enzyme activity of Unexposed was slightly higher (35.64 $nmol^{-1}min^{-1}mg$) compared to the Exposed (34.89 $nmol^{-1}min^{-1}mg$) in the alpha-esterases but lower (34.69 $nmol^{-1}min^{-1}mg$) in beta-esterases than Exposed (35.93 $nmol^{-1}min^{-1}mg$). However, Unexposed (14.11$nmol^{-1}min^{-1}mg$) exhibited higher mean activity than the Exposed (12.93 $nmol^{-1}min^{-1}mg$) in GSTs but not to a significant extent. The insecticide exposure appeared to slightly elevate the activity of beta-esterases while moderately reducing the activity of alpha-esterases and GSTs in the Exposed group. The decrease in enzyme activity in the Exposed but not in the Unexposed group might indicate the involvement of these enzymes in the resistance to the 30 min insecticides exposure. Thus, although not to a significant level, alpha-esterases and GSTs appeared to be involved. Since no statistically significant difference ($P<0.05$) in enzyme activity between the two groups in all 3 enzymes was observed. We, therefore, conclude that the three metabolic enzymes do not seem to be majorly responsible for the insecticide resistance in these populations. we suggest that monooxygenases or target site mutations such as *kdr* L1014F may be involved.

Keywords: Metabolic enzymes, *Cx. pipiens*, Arbovirus, Insecticide resistance, Biochemical assay

INTRODUCTION

Arboviruses are transmitted by arthropod vectors such as mosquitoes and ticks. *Culex pipiens* complex is one of the major competent arboviral mosquito vectors that transmit West Niles Virus, Rift Valley Virus, St. Louis encephalitis, Japanese encephalitis, etc. Control of arboviruses through the control of the vectors using insecticides is recommended and has been applied in disease management (WHO, 2016). However, rampant insecticide resistance among mosquito vectors reduces the effectiveness of the control of diseases, for example, high level of insecticide resistance in the *Anopheles gambiae s.l.* from Gombe (Olatunbosun-Oduola *et al.,* 2019) and *Cx. quinquefasciatus* population from Jigawa

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Nigeria (Omotayoid *et al.,* 2022). The possible responsible mechanisms of resistance in the insecticide-resistant mosquito population could be established by directly determining the change in the activity of metabolic enzymes upon insecticide exposure.

The resistance mechanisms to the classes of insecticide in all insects have been established to be of four possible types. These mechanisms include increased insecticide excretion rates, decreased insecticide penetration rates, decreased target site sensitivity, and finally increased metabolism to non-toxic products. The last two types are the major contributors to the resistance. Three major metabolic enzymes in mosquitoes were found to be involved in the detoxification of insecticides: Glutathione-S-transferases (GSTs), monooxygenases, and esterases. These enzymes were shown to contribute to resistance to the WHO-recommended insecticide classes. Cytochrome P450-dependent (CYP450) monooxygenases are very important mechanisms in insects that are involved in the metabolism of xenobiotics and endogenous compounds such as insecticides/pesticides (Scott, 1999). Esterases were linked to insecticidal esters especially pyrethroids and organophosphates in mosquitoes (Hemingway and Ranson, 1997). GSTs were described as the dimeric multifunctional enzymes that catalyze the nucleophilic attack of reduced glutathione and thereby play a role in the detoxification of the variety of xenobiotics in insects (Prapanthadara *et al*., 1996).

Overexpression of two CYP450s, CYPM10 and CYP4H34 were shown to be involved in pyrethroids detoxification in a highly resistant JPal-per strain *Cx. quinquefasciatus* (Komagata *et al.,* 2010). Resistance to pyrethroid and DDT was shown to be linked to the overexpression of GSTs and CYP450. High metabolic resistance in permethrin-resistant *Ae. aegypti* was also been reported (Schluep & Buckner, 2021). Organophosphate (OPs) resistance was shown to be associated with the overexpression of carboxylesterases, although pyrethroid was likely to contribute to such resistance (Somwang *et al.,* 2011). Detoxification enzymes were demonstrated in conferring resistance in the field of *An. stephensi* (Enayati & Ladonni, 2006). High involvement of GSTs and CYP450 was shown in the resistant *An. coluzzi* population from the Niger-Delta region of Nigeria (Muhammad *et al.,* 2021).

Detoxification enzymes have also contributed to the resistant *An. gambiae* mosquito population from Kano Northern Nigeria (Mukhtar & Ibrahim, 2022). Deltamethrin resistance was demonstrated to be associated with the over-transcription of detoxification enzymes, while L1014F Knockdown resistance *(kdr)* mutation is linked to DDT and Permethrin resistance (Nardini *et al*., 2012). In *Cx. pipiens*, organophosphate resistance was linked to esterase and cytochrome p450s in combination with target site insensitivity of Acetylcholine esterases(*ace*) genes (Nikookar *et al*., 2019). Esterase was involved in the insecticide resistance in the Sebring *Cx. quinquefasciatus* population (Gordon & Ottea, 2012). *Cx. pipiens* population from our study area was highly resistant to deltamethrin, permethrin, and DDT and slightly resistant to dieldrin (Abubakar *et al*., unpublished)

However, despite the high resistance to multiple insecticides, no study has checked the direct involvement of metabolic enzymes in the resistant *Cx. pipiens* population from Gombe State through biochemical assay. The purpose of this study was to ascertain whether the esterases and GSTs are associated with insecticide

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resistance in the *Cx. pipiens* population from Gombe. Knowledge of the mechanisms responsible for resistance to the insecticide by mosquito vectors is the first very step towards management of the resistance problem. This can be possible by recommending the introduction of certain enzyme inhibitors in the insecticides used in vector control programs or adopting new insecticides that are not detoxified by the metabolic enzymes.

MATERIALS AND METHODS

Mosquito Collection and Identification

The culex mosquito larvae and pupae were collected at the breeding site along Bye pass road Gombe (Latitude: 10.264483; Longitude: 11.216911) in April 2022 and taken to the molecular laboratory, Gombe state university College of Medical sciences. Although the breeding sites contain other mosquito species such as Anopheles species, our study covered only the *Cx. pipiens* population. The collected mosquitoes were reared as described elsewhere (Abubakar *et al*., 2022). In brief, the larvae were fed with biscuit and yeast in a ratio of 3:1 while 10% sucrose wet on the cotton wool was used to feed the adults. The emerged adult mosquitoes were then identified using morphological key features (Kayedi *et al*., 2020).

Biochemical Assays and Sample Preparation

The adult female Culex mosquitoes were picked randomly from the cage and introduced into the prepared WHO-bioassay tube containing the insecticide papers for 30 min. The mosquitoes were exposed to either fenitrothion (OPs) or bendiocarb (Carbamates) and herein referred to as the "Exposed" (treated) group. Although some mosquitoes were knockdown, only those that resist the insecticide were collected as the exposed group. Some fresh insecticide-non-exposed female adult mosquitoes from the cage were randomly picked as the "Unexposed" (non-treated) group and subjected to the same method using plane paper. The enzyme activity level of GSTs and general esterases (alpha and beta esterase) in these mosquito groups were tested following the protocol (Hemingway, 1998) with very little modification. Briefly, to the 200 µL of double distilled water in a 1.5 mL microcentrifuge tube, individual adult mosquitoes were homogenized using a battery-powered mortar and pestle. The homogenates were used for the assays in the Microplate reader (AMR-100).

General esterases assay

Alpha-Napthyl acetate (1-NA) and Beta-Napthyl acetate (2-NA) were freshly prepared less than one hour prior to the assay. 20µL in a replicate of 31 mosquito homogenate was added to the 96well plate followed by 200µL of the 1-NA and 2-NA placed in the separate wells. The mixture was incubated for 15 minutes after being stained with Fast-blue. The absorbance was captured at the wavelength of 570nm as the endpoint using a microplate reader. The biochemical assay result was interpreted using visual and spectrophotometric analysis as described elsewhere (Hemingway, 1998).

Glutathione-S-transferase assay

Glutathione (GSH) and 1-Chloro-2,4-DiNitrobenzene (CNDB) solution was freshly prepared prior to use. To the 5µL of 31 mosquito homogenates in 96 well plates, 100µL of GSH/CNDB solution was added and read at 340nm as the endpoint with the aid of a microplate reader. The reading was done after 20 min of incubation at room temperature (Hemingway, 1998).

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Data Analysis

The data were presented as bar charts of mean ± standard deviation and the means were compared using T-test. 95% was set as the confidence level, and p-values less than 0.05 were considered significant. The analysis and visual were carried out using anaconda navigator Jupiter Notebook for python 3.10. The enzyme activity in nmol/min/milligram for the GSTs, alpha, and beta esterase employed in this study has been determined. In general esterase assay, the individual sample was read by the Bovine Serum Albumin curve and the value of 1-NA and 2-NA substrates was read from their various standard curves. GSTs activity for each sample was calculated assuming the activity followed Beer's lambert`s equation,

$$A = \varepsilon CL \qquad (1).$$

The value of GSTs was multiplied by 2 since the half volume of the homogenates (5µL instead of 10µL) and GSH/CNDB (100µL instead of 200µL) were used in the assay. The mean activity of these three enzymes was compared between the group of Exposed and Unexposed.

RESULTS

While the visual interpretation of GSTs assay is very hard since the absorbance is in the Ultraviolet range, the blue and red color was formed with 1-NA and 2-NA respectively a few minutes post-addition of stains in a few mosquito samples. This color formation may infer a higher enzyme activity in these mosquitoes. Other homogenates that retain pink in color may infer the lower activity (Figure 1).

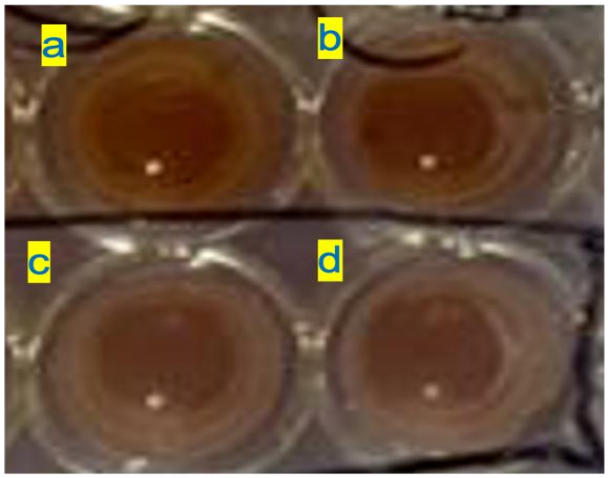

Figure 1: A microplate showing the esterase assays with 2-NA. a and b with red coloration indicate moderate to high enzyme activity *Cx. pipiens* while c and d with pink color may indicate lower ones.

Figure 2 displays the mean enzyme activities in $nmol^{-1}min^{-1}mg$ for alpha-esterases, beta-esterases, and GSTs of Exposed (n=18) and unexposed (n=13) *Cx. pipiens* population collected from Gombe. Clearly, the finding indicates only GSTs and alpha-esterases were slightly involved in the resistance to the insecticides. The mean enzyme activity of Unexposed was slightly higher (35.64 $nmol^{-1}min^{-1}mg$) compared to the Exposed (34.89 $nmol^{-1}min^{-1}mg$) in the alpha-esterases. In beta-esterases, the mean enzyme activity was also higher in the Exposed (35.93 $nmol^{-1}min^{-1}mg$) compared to the Unexposed (34.69 $nmol^{-1}min^{-1}mg$) population. However, the mean enzyme activity in GSTs was moderately higher in the "Unexposed" (14.11$nmol^{-1}min^{-1}mg$) compared to the exposed (12.93 $nmol^{-1}min^{-1}mg$) group. Fenitrothion and Bendiocarb might have insignificantly affected the activity of two esterases but moderately decreased GSTs'. The decrease in enzyme activity in the Exposed group might indicate their level of involvement in the resistance to the insecticides. Since the activity of

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these two enzymes was not significantly different between the Exposed and Exposed populations, they seem to be partially involved. In contrast, a study was shown that the activity of Acetylcholine esterase (AchE) was significantly inhibited by insecticide exposure, but GSTs activity was increased in the larvae of *Cx. quinquefasciatus* (Subahar *et al.*, 2022). However, in this study, GSTs and esterases were involved but not to a significant extent. The involvement of GSTs in conferring resistance in *An. coluzzi* population was shown in the study of Muhammad *et al.* (2021).

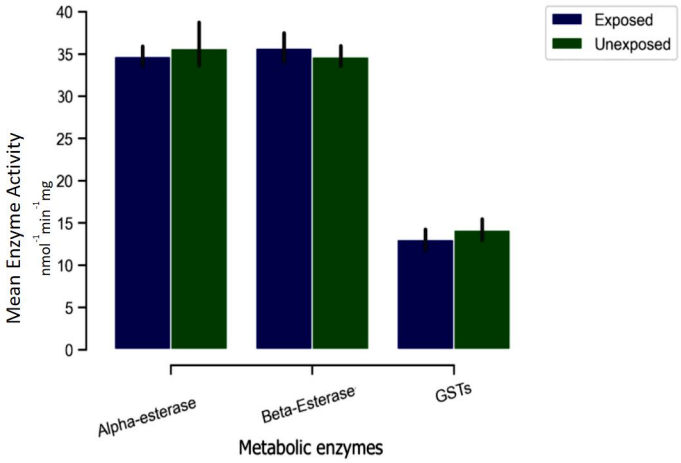

Figure 2: Mean activities of three major metabolic enzyme assessed in Adult *Cx. pipiens* population collected from Bye-pass Road Gombe. The mosquitoes treated with the insecticides (exposed) and not treated (Unexposed) groups.

DISCUSSION

The rampant insecticide resistance reported in the arboviral mosquito vector has led us to investigate its possible mechanisms. Metabolic enzymes were suspected to be associated with insecticide resistance in the resistant *Cx. pipiens* population in Gombe State. The findings, however, go against theory, alpha-esterases, beta-esterases, and GSTs appear not to be major mechanisms associated with insecticide resistance in the mosquito population from Gombe State. The involvement of monooxygenases and GSTs was shown in the resistant *An. coluzzi* population from the Niger-Delta region of Nigeria (Muhammad *et al.*, 2021). In addition, Alout *et al.*, (2008) have shown that the high insecticide resistance was due to the point mutation GGC to AGC in the *ace-1* gene which resulted in the substitution of Glycine by serine in the *Cx. pipiens* population. One possible conclusion for our result is that either monooxygenases or target-site insensitivity such as knockdown resistance *kdr* gene L1014F/or L1014S, *ace-1*/or *ace-2* mutation may be majorly responsible for the insecticide resistance in the *Cx. pipiens* population in Gombe.

A high frequency of L1014F *kdr* mutation was found and associated with the pyrethroids lambda-cyhalothrin in *Cx. pipiens* and hybrid form populations from Morocco (Bkhache *et al.,* 2016). Nevertheless, in the same *Cx. pipiens* larvae from Northern Italy, at least one of the three different point mutations investigated was found in the population at the frequency of 93.3%, 64.8%, and 10% for the I1043M, I1043L, and I1043F respectively (Fotakis *et al*., 2020). ATP-binding cassete (ABC) transporter appeared to be not associated with the insecticide resistance in the resistant *An. coluzzi* population from Auyo Jigawa Nigeria (Abulhassan *et al.*, 2019). 83.3% and 39% allelic frequencies of L1014F and L1014S *kdr* mutation were found in the resistant *An. gambiae* mosquitoes from Yamaltu Deba Gombe Nigeria (Ahmed-Yusuf *et al*., 2020).

Metabolic enzymes detoxify the insecticides rendering them ineffective in killing the mosquitoes. Metabolic enzymes were shown to be responsible for resistance in the permethrin-resistant *Ae. aegypti* (Schluep &

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Buckner, 2021). Moreover, overexpression of carboxylesterases was shown to confer resistance to OPs insecticides in the also *Ae. aegypti* strains from northern Italy (Somwang *et al.,* 2011). Metabolic resistance is one of the major challenges in the vector control of arthropod-borne diseases.

This study has taken a step in the direction of establishing the possible mechanism of insecticide resistance in *Cx. pipiens* collected within the Gombe State. It is possible of course that other areas with different environmental factors such as residential, industrial, or agricultural sites may produce entirely different results. Knowledge of the mechanism of resistance is vital in improving insecticide resistance management such as recommending the employing enzyme inhibitors in the insecticides used in vector control programs or introducing different insecticides with different targets for better vector control of arboviruses. Although the activity of two metabolic enzymes investigated in this study appeared to be slightly involved in the resistant population, their level of involvement was not significant. Therefore, these enzymes were not the major contributors to the resistance of insecticide in the *Cx. pipiens* population. The approach carried out in this study should be replicated in other areas in Gombe to have detailed evidence-based information on the mechanism of resistance in Gombe *Cx. pipiens* populations.

CONCLUSION

This study's finding reveals that GSTs and alpha-esterases but not beta-esterases appear to slightly play a role in the resistance to the insecticide although not to a significant extent. Therefore, all three enzymes investigated here seem to be not majorly responsible for the resistance to insecticides in the major arboviral vector, *Cx. pipiens* populations from Gombe. This result suggests the possibility of monooxygenases or other mechanisms such as target-site insensitivity may also contribute to the resistance. Further replication of the approach outlined in this study was highly recommended in different areas of Gombe State for a better understanding of the responsible mechanisms of insecticide resistance in the arboviral vectors in Gombe State.

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