



# Gradual reduction of susceptibility and enhanced detoxifying enzyme activities of laboratory-reared *Aedes aegypti* under exposure of temephos for 28 generations

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## ABSTRACT

Temephos, an organophosphate insecticide, is widely accepted for the control of *Aedes aegypti*, vector of infectious diseases such as dengue, chikungunya, yellow fever, and zika. However, there are claims that repeated and indiscriminate use of temephos has resulted in resistance development in exposed mosquito populations. The present study attempts to evaluate the continuous performance of temephos on the *Ae. aegypti* population, in laboratory conditions, in terms of toxicity and the effect on marker enzymes associated with metabolic resistance. Results of the toxicity bioassay showed that after the initial exposure, toxicity increased till F4 generation by 1.65 fold, and continuous exposure resulted in a 7.83 fold reduction in toxicity at F28 generation. Percent mortality result showed a marked reduction in mortality with the passage of generations while using the same series of concentrations, viz. 2 ppm, which was 100 % lethal at the initial nine generations, could kill only 22.66 % at F28. Resistance to organophosphates is mainly governed by metabolic detoxifying enzyme families of esterases, glutathione-s-transferase, and cytochrome P450. Analysis of these metabolic detoxifying enzymes showed an inverse trend to toxicity (i.e. toxicity increased in early generations as enzyme activity dropped and then dropped as enzyme activity increased). At the initial exposure, enzyme activity decreased in 2–4 generations, however, repeated exposure led to a significant increase in all the metabolic detoxifying enzymes. From the toxicity level as well as marker enzyme bioassay results, it can be inferred that mosquitoes showed increased detoxification in generational time with an increase in enzymes associated with metabolic detoxification. In conclusion, repeated application of temephos led to resistance development in *Ae. aegypti* which may be associated with the increase in metabolic detoxifying enzyme activities.

## 1. Introduction

*Aedes aegypti* is a vector of infectious diseases such as dengue, chikungunya, yellow fever, and zika. Among them, dengue is the most prevalent disease worldwide that causes almost 390 million infections and thousands of deaths each year [1,2]. In India, with the first report of dengue in 1946 and the disease taking epidemic form in 1953–54 [3], the cases of dengue are continuously rising. There is no vaccine or specific therapeutics for the control of dengue, moreover, extensive vector control efforts have not been able to combat its emergence and

worldwide spread, and new cases from new regions are being recorded daily. *Ae. aegypti* show peak blood-feeding activity in the early morning and late afternoon when humans are active [4], so the use of bed nets is not recommended nor effective. Management of the breeding sites and use of botanical and synthetic chemicals individually and in combination forms the important control measure of the *Aedes* mosquito [5]. Today, botanical insecticides are widely experimented owing to their environment and user friendly nature [6–9]. *Ae. aegypti* can be best controlled at its immature stages because its breeding habitat is clean water, such as drinking water containers and rainwater harvesting

**Abbreviations:** PPM, parts per million; WHO, World Health Organization; GST, glutathione-s-transferase; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; LC50, lethal concentration 50; DMSO, dimethyl sulfoxide; °C, degree celsius; GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; TMBZ, 3, 3, 5, 5-tetramethyl benzidine; OD, optical density; BSA, bovine serum albumin; SPSS, statistical package for the social sciences; ANOVA, analysis of variance; IAEC, institutional animal ethical committee; CPCSEA, committee for the purpose of control and supervision of experiments on animals; SE, standard error; DEET, N,N-diethyl-meta-toluamide; MAPK, mitogen-activated protein kinases; OP, organophosphate; TPP, triphenyl phosphate; DEM, diethyl maleate; PBO, piperonyl butoxide.

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vessels. Scarcity of drinking water in some areas leads to storing water in vessels/containers, and also in reservoirs, for supply into households. These storage vessels form excellent breeding grounds for the mosquito. To kill these mosquitoes at larval stages, World Health Organization (WHO) has approved temephos use in water reservoirs at concentrations not exceeding 1 mg/l [10]. Temephos, an organophosphorus insecticide, is globally the most widely used insecticide for the control of *Ae. aegypti* at its larval stages [1]. Temephos is classified as 'U' under the WHO hazard classification, meaning that it is unlikely to cause an acute hazard in normal usage [11]. Almost 150 countries from five continents have reported dengue cases, and temephos use has been reported in the majority of these dengue-endemic areas. The extent of temephos use worldwide is also evident from the reports of resistant *Ae. aegypti* to temephos from across the globe [1,12,13]. Temephos acts as a covalent inhibitor of acetylcholinesterase, a central nervous system-associated enzyme that hydrolyzes the neurotransmitter acetylcholine. However, when temephos is present, it binds to acetylcholinesterase and inhibits its normal enzymatic function. This results in an abnormal accumulation of acetylcholine in the cholinergic synapses, which overstimulate the cholinergic system leading to hyperactivity, ataxia, paralysis, respiratory failure, and death [14–16]. As an efficient control agent, the use of temephos is extensive, which subsequently has led to resistance development in the exposed mosquito population [4,12,13,17,18].

Insecticide resistance is a growing problem that affects the success of vector control programs. Many chemicals (organochlorine, organophosphates, pyrethroid, carbamate, etc.) are being used, but the development of resistance by target mosquito renders these chemicals ineffective [12,19,20]. Resistance is a phenomenon whereby the toxicity of an insecticide gradually decreases and requires a higher concentration to kill the target pest populations on subsequent application. Reportedly, the insecticide exerts its effect on a genetic level and modifies the activity by upregulating the titer of detoxifying enzymes [21]. Insects develop resistance to insecticide through behavioral resistance, reduced cuticular penetration, resistance mediated by target site insensitivity, and metabolic resistance [22]. *Ae. aegypti* resistance to temephos is primarily modulated by metabolic detoxifying enzyme activity [18,23,24]. Metabolic resistance is mediated by three principal detoxifying enzyme families of esterase (a and b), glutathione-S-transferase (GST), and cytochrome P450 [13,25]. The importance of these enzymes in resistance has been established through transcription study [26,27] and synergists study [28]. Esterases, GST, and cytochrome p450 monooxygenases are the key enzymes that favor the survival of insects exposed to toxic metabolites [29]. Esterases are a particular group of hydrolases that hydrolyze the ester bond present in organophosphorus insecticide producing an acid and an alcohol as metabolites [30]. GST catalyzes the conjugation reaction in which electrophilic compounds are conjugated with the thiol group of reduced glutathione, rendering the compound amenable to excretion [31]. Cytochrome P450 detoxify xenobiotics by reductive cleavage of oxygen in presence of their obligatory electron donor NADPH-cytochrome P450-reductase to produce water and functional product [32].

Larvicidal potency of temephos is known, and that *Ae. aegypti* develop resistance upon continuous exposure has also been established [12,33,34]. However, a long-term toxicity assessment taking marker enzymes associated with metabolic resistance on *Ae. aegypti* was found inadequate. This study was conducted to (1) comprehend the toxicity of temephos on susceptible laboratory-reared *Ae. aegypti* for twenty-eight successive generations, in terms of median lethal concentration value (LC<sub>50</sub>), (2) to examine if continuous exposure of temephos has any effect on the metabolic detoxifying enzymes. This study will reveal the effectiveness of temephos use in water reservoirs where multiple generations of *Ae. aegypti* are likely to occur.

## 2. Methodology

### 2.1. Mosquito

The colony of *Ae. aegypti* was maintained in the Laboratory of Entomology Gauhati University since 2015, with no exposure to any insecticides [8,9,35]. Originally the susceptible, laboratory-reared egg strips of the mosquito were collected from the Indian Council of Medical Research, Dibrugarh. The mosquito colony was maintained as described in our earlier work [8]. Adults were kept in a wooden cage and provided with a 10 % sugar solution ad-libitum. In addition to the sugar solution, the adult females were offered a blood meal through an albino rat. Moist filter paper in the inner wall of a beaker was provided as a substrate for egg-laying. The eggs were collected and allowed to hatch in a water tray. A mixture of yeast powder and dog biscuit in a 1:3 ratio was used as a larval diet. When the larvae developed into pupae, they were collected in plastic cups containing three-quarters of water and put in the cage for adult development and continuation of life cycles. The culture was maintained at 12:12 light: dark cycle and temperature and humidity of 30 ± 2 °C and 75–85 % relative humidity.

### 2.2. Toxicity bioassay

Toxicity bioassay was carried out following the protocols described by WHO [36]. For this bioassay, the susceptible early fourth instar larvae were exposed to eight different grades of temephos (0.05 ppm, 0.1 ppm, 0.2 ppm, 0.25 ppm, 0.5 ppm, 1 ppm, 2 ppm, and 5 ppm). Dimethyl sulfoxide (DMSO) was used as an emulsifier. For each concentration, four replicas were set, with an equal number of controls, and in each replica, 25 larvae were introduced. Negative and no treatment controls were set with DMSO and water and water only, respectively. In each concentration, larval mortality was recorded at different time intervals- 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, and 24 h. All the experiments were set in a separate laboratory, away from the mosquito culture room, with sufficient precautions to minimize unforeseen errors. At a temperature and humidity described for the maintenance of *Ae. aegypti*, the development of the 4th instar larva requires a minimum of 24–48 h. So, only the early 4th instar larvae were selected and introduced into a selection pressure. Despite this precaution, if pupation occurred, the experiment was repeated. Furthermore, if death occurs more than 10 % in the control the experiment was repeated. If no mortality or mortality less than 10 % occurred, then the mortality percentage was calculated using Abbott's formula

$$\text{Mortality (\%)} = (X - Y) / X \times 100$$

Where, X = percentage of larvae that survived the selection pressure in control group

Y = percentage of larvae that survived the selection pressure in treated group

### 2.3. Calculation of LC<sub>50</sub>

From the experiment of toxicity bioassay, the median lethal concentration (LC<sub>50</sub>) was calculated at 24 h. The larva that showed no movement on touching with a fine brush was considered dead. The total dead and alive larvae were recorded, from this data, LC<sub>50</sub> was calculated. The LC<sub>50</sub> obtained from this bioassay was the LC<sub>50</sub> for F0 generation.

### 2.4. Selection of larvae in successive generations

From the susceptible mosquito colony, about 3000 fourth instar larvae were separated and treated with the median lethal concentration (LC<sub>50</sub>) determined as stated above for 24 h (F0). After 24 h, the larvae that survived the selection pressure were separated. Since the

concentration of temephos was LC<sub>50</sub>, it was expected that it would kill approximately 1500 larvae. The survived larvae (approximately 1500) were identified based on movement and were separated using a dropper. Even the knocked out larvae were probed with a fine brush to assess if they exhibit any movement. After separation, the larvae were placed in freshwater and food was provided. These larvae were allowed to grow into adults for continuing generations. The offspring that originated from those adults was the F1 generation. As the F1 larvae reached the fourth instar, the larvae were divided into two groups. From the first group, LC<sub>50</sub> was determined by applying a series of concentrations of temephos, as stated above. Then, the calculated LC<sub>50</sub> concentration was applied to the remaining group of 4th instar larvae of the F1 generation (almost 3000). Since the concentration was LC<sub>50</sub>, it was expected that it would kill 50 % of the population. After 24 h, the larvae that survived (nearly 1500) were separated and allowed to continue to the next generation, i.e., F2. The same procedure was followed to determine the LC<sub>50</sub> for succeeding generations and to select the remaining pool of larvae. The process was continued up to the F28 generation. The study period was from October 2018 to March 2020.

Simultaneously, enzyme activity was determined from some of the surviving F0 larvae that were exposed to LC<sub>50</sub> of the F0 generation. In the F1 generation, enzyme activity was again determined from some of the larvae that survived the LC<sub>50</sub> selection. For the rest of the F2-F28 generation, the same method was followed.

#### 2.4.1. Evaluation of the detoxifying enzyme activities in 28 consecutive generations

Alpha esterase, beta esterase, glutathione-s-transferase, and cytochrome P450 are the enzymes involved in the detoxification of xenobiotic compounds. The activity of these enzymes was examined to understand if continuous exposure to temephos has any effect. The bioassay was done following the method described by Safi et al. [37].

#### 2.4.2. Sample preparation

For the preparation of the samples, mosquito larvae were homogenized in 0.0625 M potassium phosphate buffer (pH 7.2). Samples were prepared from temephos selected larvae (as treatment group), DMSO in water (as negative control), and water only (as no treatment control). DMSO was used as an emulsifier to let temephos homogeneously dissolve in water. To see if DMSO has any effect on the enzyme activity of larvae, we prepared the sample from this group. For each group, four replicas were set, with an equal number of controls. In each replica, three larvae were homogenized in 900 µL of the phosphate buffer. The sample was cool centrifuged (at 4 °C) at 10,000 rpm for 10 min. The centrifuged supernatant served as the crude extract for all the enzymes and protein.

**2.4.2.1. Esterases.** For the esterase enzyme activity bioassay, alpha/beta naphthyl acetate served as the substrate. In an Eppendorf tube, 200 µL (30 mM) alpha/beta naphthyl acetate was taken, and 100 µL of the insect homogenate was added. The mixture was then allowed to stand for 30 min at room temperature. A fast blue stain was added to the mixture and then again allowed to stand for five minutes. The same procedure was followed for the negative control, and no treatment control, except for distilled water used in place of larval homogenate. The optical density of the sample was measured spectrophotometrically at 570 nm. Reference curves of alpha/beta naphthyl acetate were prepared by calculating the unknown concentration from the reference graph. The enzyme activity was recorded at the unit of µM of product formed/min/mg protein.

**2.4.2.2. Glutathione-s-transferase.** For the GST activity bioassay, 200 µL of 10 mM GSH and 3 mM 1-chloro-2,4-dinitrobenzene (CDNB) served as the substrate. A mixture of these two buffers is referred to as the cocktail buffer. 100 µL of cocktail buffer was added to 10 µL of larval homogenate and incubated for five minutes. The optical density of the sample was

taken at 340 nm. The GST enzyme activity was reported as mM of conjugate produced/min/mg protein using the extinction coefficient of CDNB corrected for the path length of the solution in the quartz cuvette.

**2.4.2.3. Cytochrome P450.** 3, 3', 5', 5'-tetramethyl benzidine (TMBZ) served as the substrate for the cytochrome P450 bioassay study. In an Eppendorf tube, 80 µL 0.625 M potassium phosphate buffer (pH 7.2), 200 µL TMBZ solution, 25 µL 3 % hydrogen peroxide, and 20 µL homogenate were mixed. The mixture was allowed to incubate at room temperature for 2 h. Negative and no treatment control bioassay was done following the same procedure, taking distilled water in place of larval homogenate. Optical density was taken at 450 nm and expressed as cytochrome P450/min/mg protein. The unknown concentration was calculated from the curve of cytochrome c.

#### 2.4.3. Enzyme activity calculation

**2.4.3.1. Esterase and cytochrome p450.** The calculation of enzyme activity was based on Safi et al. [37].

1 mg of alpha/beta naphthol dissolved in 1 mL buffer gives 0.0069 µmol. Again, 1 mg of cytochrome C dissolved in 1 mL buffer gives 0.00113 µmol.

From the OD of treatment as well as control group recorded for each generation, the enzyme was calculated from the standard alpha naphthol/beta naphthol for alpha/beta esterase and TMBZ for cytochrome p450. In a similar manner, unknown protein (in each generation) was calculated from the mean OD using standard BSA. The enzyme activity was calculated using the formula 
$$\frac{\text{Enzyme} \times 0.0069 (\text{esterase}) / 0.00113 (\text{cyt p450})}{\text{Incubation period}}$$

The product obtained is divided by protein estimated in each generation. This gives the enzyme activity.

**2.4.3.2. GST.** GST enzyme activity is calculated using the formula

$$C = \frac{A}{\epsilon l}$$

C = Concentration

A = Absorbance

ε = CDNB co-efficient

l = path length

The product obtained is divided by protein as discussed for esterase and p450

#### 2.4.4. Protein assay

Protein content was estimated from the whole larval homogenate prepared in a phosphate buffer. Four replications for treatment and controls were set up, and the OD was measured at 660 nm. Estimation was done following the procedure described by Lowry et al. [38], using Folin-Ciocalteu reagent and bovine serum albumin as the standards. Protein content was determined in each generation of treatment from the fourth instar larvae.

#### 2.5. Statistical analysis

Probit analysis [39] of SPSS software (version 20) and MINITAB software was used for the calculation of LC<sub>50</sub> values. One-way ANOVA was performed using Tukey's post hoc test for the determination of significance in difference.

#### 2.6. Ethical clearance

Albino rat was used in the experiment with due permission from the Institutional Animal Ethical Committee, Gauhati University, vide permission number- IAEC/ Per/2017/RF/2018-05. The CPCSEA guideline was followed for the healthy maintenance of rats. The rat was

obtained from the animal house facility, Gauhati University. After the blood meal was over, it was again shifted to the animal house and was kept in a hygienic environment with a healthy diet. The rat was exposed only during the daytime for 3–5 h.

### 3. Results

#### 3.1. Percent mortality

Percent mortality increased with the increase in the concentrations of temephos applied. The lowest concentration (0.05 ppm) had minimal mortality, while the highest doses (2 ppm and 5 ppm) had cent percent mortality up to F9 generation. Percent mortality of larvae skewed with the passage of generations, i.e., the efficacy of temephos decreased over generational time. A glimpse into the percent motility trend at 1 ppm concentration shows the initial low (F0-F3), which increased at F4 with the highest at F5 (97.33 percent), became quite steady at F6 - F9 generations. Gradually, the mortality percent decreased as the generations passed. At F28, it became very low (10.66 percent) compared to the initial trend. At 2 ppm concentration, the mortality was 100 % till F9, thereafter it gradually declined with the passage of generations, and by F28, it lowered to 22.66 % (Fig. S1).

#### 3.2. LC<sub>50</sub> dose

From figure, it is evident that till the F9 generation, the LC<sub>50</sub> value remained below the F0 LC<sub>50</sub> value. At the initial exposure, susceptibility increased till F4 generation as revealed in the LC<sub>50</sub>. After F4, the mosquito began to adapt. From the F10 generation onwards, the LC<sub>50</sub> began to rise above the initial value. The increase in LC<sub>50</sub> was found to be gradual. It took 11 generations for the LC<sub>50</sub> to become double (0.86 ppm at F12), than the original value (0.43 ppm at F0). While, in the later generations, the time required for tripling was just three generations (F15). Again after three generations the LC<sub>50</sub> quadrupled (F18). In this way, the time required for doubling became shorter upon continuous exposure, and at the last studied generation, the concentration became 7.83 fold higher (Fig. 1 and Table S1).

#### 3.3. Esterases

From the Fig. 2A, it is evident that the enzyme activity of the treated group decreased significantly up to F6 generation. During the F7-F10 generation, it remained almost at par with the negative control and no treatment control groups. Again, from F11 generation onwards, the alpha esterase enzyme activity of the treated group began to rise significantly. By F28 generation, the enzyme activity of the treated group increased 3.64 fold compared to the control groups. No significant difference between negative and no treatment control groups was found in any generations (Fig. 2A).

From the Fig. 2B, it is evident that the beta esterase activity of the treated group differed significantly compared to control groups in all generations. With the lowest enzyme titer at F2, it began to rise in the succeeding generations. By the F28 generation, beta esterase activity was found 3.63 fold higher compared to control groups (Fig. 2B). A significant difference between negative and no treatment control was not seen at any generation, so, only the negative control is mentioned.

#### 3.4. Cytochrome P450

From Fig. 3, it is evident that immediately after exposure (F0) to a discriminating dose of temephos, the cytochrome p450 monooxygenase activity remained at par with the control groups. However, From F1-F5 generation, the enzyme titer of the treatment group decreased significantly compared to control groups. Again at F6 and F7 generation, the enzyme titer remained at par with the control group. From the F8 generation onwards, it began to rise gradually and significantly. At F28 generation, cytochrome P450 activity was found to increase by 3.7 fold compared to control groups (Fig. 3). A significant difference between negative and no treatment control was not seen at any generation, so, only the negative control is mentioned.

#### 3.5. Glutathione-s-transferase

From Fig. 4, it is evident that from the initial exposure to temephos, the GST enzyme titer began to decrease significantly compared to the control groups. The lowest titer of the enzyme was recorded at F4 generation, after which it began to rise gradually in the later generations. From F21 generation onwards, the enzyme titer began to rise significantly. At F28, the GST enzyme titer increased by 1.53 fold compared to control groups. No significant difference between negative control and no treatment control groups was found in any generations, so, only the negative control is mentioned.

### 4. Discussion

*Aedes aegypti* control relies primarily on chemicals applied on the breeding sites and the use of adulticides/repellents. Pirimiphos-methyl and temephos are the organophosphorus larvicides used for controlling *Ae. aegypti* [40]. Insect growth regulators such as diflubenzuron, methoprene, novaluron, and pyriproxyfen are used at the larval stages [41]. Apart from these, spinosads and *Bacillus thuringiensis israelensis* are also used as larvicides. Aerosol or fog application consist of two classes of insecticides- organophosphorus (Fenitrothion, malathion and pirimiphos-methyl) and pyrethroids (including cyfluthrin, pypermethrin, permethrin etc.). Repellent compound DEET, IR3535 and Icaridin is used for household protection [40]. Temephos, an organophosphate, is globally the most commonly used insecticide for *Ae. aegypti* [1]. This chemical is used in the drinking water containers for controlling *Aedes*

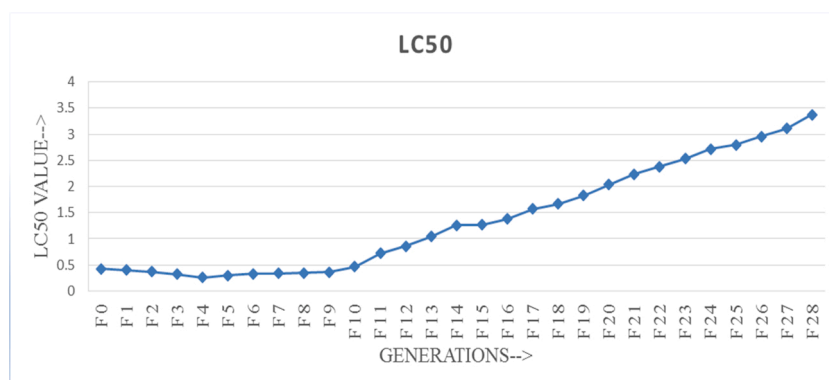
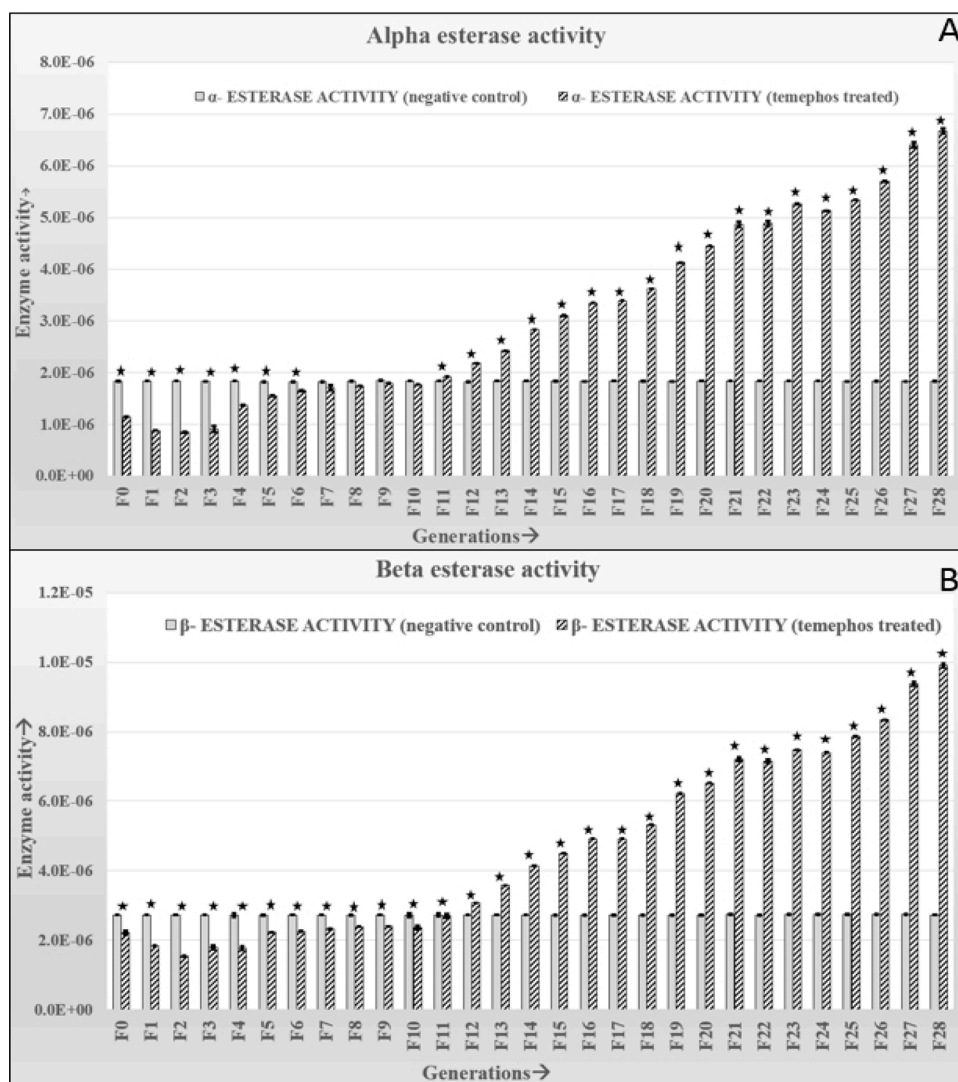
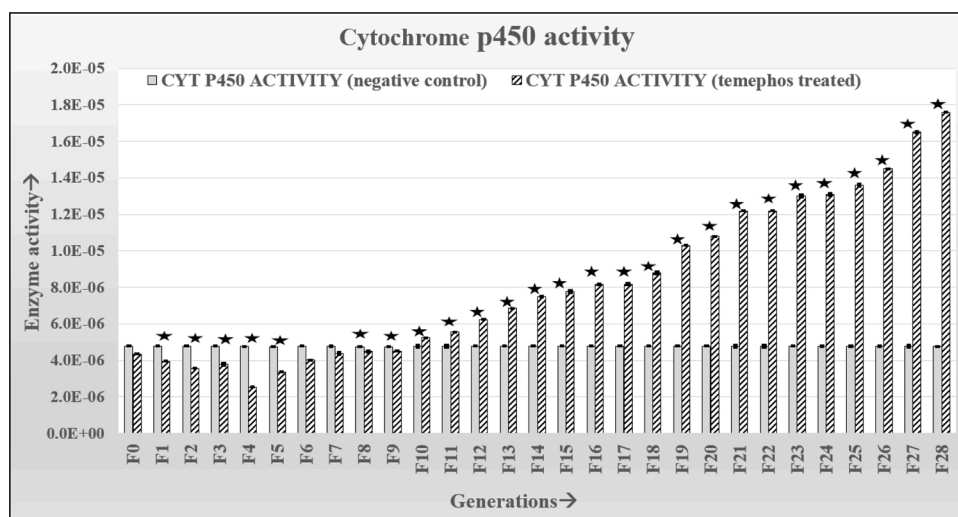


Fig. 1. Showing the trend of LC<sub>50</sub> of temephos on the larvae of *Ae. aegypti* when exposed continuously for 28 generations.





**Fig. 2.** Effect of temephos on (A) alpha esterase (B) beta esterase enzyme of *Ae. aegypti* exposed to temephos ( $\pm$ SE). The asterisks at the top of each bar represents the significance in difference among the experimental groups. The standard error is presented as a two sided error bar at the top of each generational enzyme bar. The enzyme activity is presented as a unit of  $\mu$ M of product formed/min/mg protein. \*. The mean difference is significant at the 0.05 level.

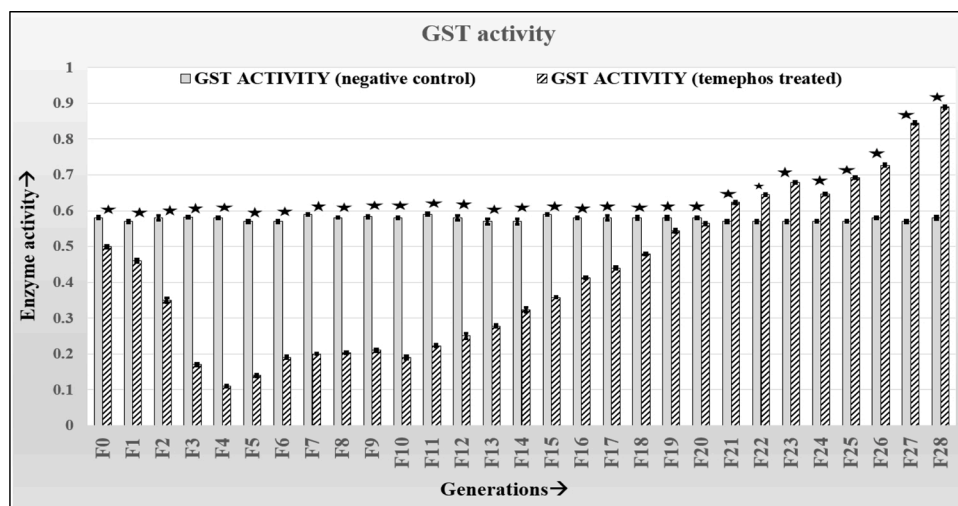


**Fig. 3.** Effect of temephos on the Cytochrome P450 enzyme system of *Ae. aegypti* exposed to temephos ( $\pm$ SE). The asterisks at the top of bar represents the significance in difference among the experimental groups. The standard error is presented as a two sided error bar at the top of each generational enzyme bar. The activity of cytochrome p450 monooxygenase is expressed at a unit of cytochrome P450/min/mg protein. \*. The mean difference is significant at the 0.05 level.

larvae. However, repeated exposure to temephos has resulted in the development of resistance in the said mosquito. The biochemical parameters associated with metabolic resistant associated enzymes are

worth studying because these enzymes favor the survival of mosquitoes against the insecticide.

In the present investigation, the dose-response study showed an



**Fig. 4.** Effect of temephos on the GST enzyme system of *Ae. aegypti* exposed to temephos ( $\pm$ SE). The asterisks at the top of bar represents the significance in difference among the experimental groups. The standard error is presented as a two sided error bar at the top of each generational enzyme bar. GST activity is expressed at a unit of mM of conjugate produced/min/mg protein. \* The mean difference is significant at the 0.05 level.

increased mortality with an increasing concentration of temephos. The lowest applied dose had minimal effect on the larvae of *Ae. aegypti*. However, with the increase in concentration of temephos, higher mortality was observed. During early generations, we observed larval mortality even at the lowest concentration, so the survival of the larvae was not due to reduced exposure to temephos. With the regular increase in concentrations, we observed higher mortality, which indicates a homogenous exposure of larvae. This dose dependent enhancement in mortality remained throughout the generations studied. However, over the generational time, mortality in each of the concentration decreased, which might be due to the development of resistance towards temephos. The present findings matches with the findings of Madhu et al. [42], where they reported increased mortality of *Culex quinquefasciatus* larvae with an increasing concentration of 9-oxoneoprocumamol and neoprocumamol. It also matches with the findings of Chapagain et al. [43], where they recorded increased mortality of *Ae. aegypti* with the increasing concentration of saponins derived from *Balanites aegyptiaca*. At the lowest concentration they recorded no mortality. Temephos is a lipophilic compound that easily gets absorbed through cuticular surfaces or spiracles [44]. When temephos is present in higher concentrations, it induces enhanced toxicity, which might be due to increased penetration through the larval cuticle. Temephos is a neurotoxic chemical that affects the acetylcholinesterase of the central nervous system [14]. Acetylcholinesterase (AChE) hydrolyzes the neurotransmitter acetylcholine. Alteration of AChE in treated larvae leads to failure of acetylcholine hydrolysis leading to over excitation of the nervous system, paralysis, and death.

Toxicity study revealed the rise in  $LC_{50}$  dose of temephos after the passage of F9 generation of selection. A total of eight concentrations were applied in which we observed a dose-dependent enhancement of mortality. The study revealed that a concentration lethal to half of the exposed populations in one generation could not kill the expected populations in the successive generation. This matches with the findings of Hidayati et al. [45], where they reported that the median lethal concentration determined for one generation was not median lethal for the subsequent generation. Their study reported a 52.7 fold increase in resistance ratio after 45 generations of malathion selection of *Ae. aegypti*. This result also matches with the findings of Hamdan et al. [46] in *Culex quinquefasciatus*, *Ae. aegypti*, and *Ae. albopictus*, a slow and gradual rise in resistance towards malathion, permethrin, and temephos. After 32 generations of exposure, the resistance ratio increased by 4.97, 64.2, and 51.0 fold to malathion, permethrin, and temephos, respectively in *Ae. aegypti*. Melo-Santos et al. [17] selected the already 7 fold temephos resistant field-collected *Ae. aegypti* mosquitoes for 17 generations and reported the increase in resistance ratio by 25.71 fold in terms of  $LC_{90}$

values. Rodríguez et al. [47] exposed the already resistant *Ae. aegypti* populations to temephos for six generations and reported an 11 fold increase in resistance in terms of  $LC_{90}$ . Wirth and Georghiou [48] presented four fold increases in resistance against temephos and permethrin after 14 generations in an *Ae. aegypti* colony named Tortola-Sel.

Esterases are enzymes that hydrolyze organophosphate insecticide into water and alcohol by breaking the ester bond [30]. The involvement of esterases in organophosphorus resistance in *Ae. aegypti* is established [18,49]. In resistant insect strains, esterase protein constitutes up to three percent of the total body protein compared to 0.4 percent in the susceptible strain [50]. This feat is achieved by rapid amplification of the esterase genes in response to insecticide [23]. In the study, we recorded an initial reduction in esterase activity till F10 generation, after which it began to rise. The rising trend continued, and by F28 generation, both alpha esterase and beta esterase activity increased by 3.6 fold compared to the susceptible counterparts. The initial decrease in the enzyme activity might have occurred due to the formation of a complex with temephos which left little free esterase for spectrophotometric detection. However, the subsequent exposure has resulted in the activation of the esterase gene in the *Ae. aegypti* genome that led to the overproduction of the enzyme. The increased activity of esterase might act as a savior of *Ae. aegypti* when exposed to selection pressure. The increased activity of esterases in temephos resistant *Ae. aegypti* have also been reported in the field [51,52]. Cao et al. [53] recorded a 4.54 fold increase in esterase activity in resistant *Aphis gossypii*. Our result contrasted with the findings of Rodríguez et al. [47], where they recorded a sharp increase in esterase activity in *Ae. aegypti* on treatment with temephos for six consecutive generations.

Cytochrome oxidases are the enzymes involved in the oxidation of xenobiotics. Insects neutralize organophosphorus compounds via sulfoxidation, where the compounds are converted to their corresponding sulfoxides [54]. There are reports of the involvement of cytochrome oxidases in the detoxification of organophosphorus compounds [55,18]. In the present study, we noticed an initial gradual decrease in enzyme activity, with the lowest recorded at F4. The initial decrease in the enzyme activity indicates the susceptibility of the larvae, which could not activate the cytochrome P450 enzyme system immediately. The initial decrease in P450 activity matches with the findings of Grigoraki et al. [56] where they showed the downregulation of P450 enzymes in *Ae. albopictus* after 12 generations of exposure. After the passage of F7 generation, the enzyme activity altered significantly compared to control groups, and by F28 generation, the activity increased 3.7fold. Continuous exposure to temephos has activated the P450 genes within the genomes, which in the latter generation led to overproduction of the detoxification enzymes. The increase in P450 activity in response to

resistance development upon continuous exposure has been reported by many earlier studies [57,58,33]. P450 s are not only involved in resistance development. They are also involved in numerous physiological processes such as the metabolism of important hormones like juvenile hormones, ecdysteroids, pheromones as well as fatty acids [59]. In this connection, alteration in P450 activity is directly related to survival in *Ae. aegypti* larvae.

Glutathione is a tripeptide compound consisting of cysteine, glycine and glutamic acid. Glutathione is present in a high amount in a cell [60]. GST is an enzyme that carries out the phase II reaction of detoxification of insecticides [54]. This enzyme adds reduced glutathione to the electrophilic substrate. The addition of glutathione to organophosphate results in its conversion into a product mosquito can readily excrete. In our study, we recorded the initial significant decrease in GST activity, and after the passage of F4 generation, it began to increase but remained significantly below the control group. After the F20 generation, the activity of GST began to rise compared to the control group, and by the F28 generation, it increased by 1.53 fold. The initial decrease in enzyme activity might be because of the involvement of enzymes in transferring glutathione to temephos, leaving little free GST for spectrophotometric detection. The initial decrease in GST activity is in conformity with the findings of Marcombe et al. [57], where they showed that in the Vauclin strain of *Ae. aegypti*, which is highly resistant to temephos, GST is the least involved. The study is also in conformity with the findings of Chen et al. [61] where they reported temephos resistant *Ae. albopictus* with mild GST activity. Muthusamy et al. [62] also reported decreased GST activity in temephos treated *Amsacta albistriga* caterpillar. However, in the latter generations (after F20), GST activity increased. The increased activity of GST in a resistant laboratory strain of *Ae. aegypti* has also been confirmed by the study of Vasantha-Srinivasan et al. [6], where they showed a significant increase in GST activity when treated with temephos. Melo-Santos et al. [17] also reported the increased activity of GST in temephos resistant *Ae. aegypti* after 17 generations under selective pressure of temephos. In addition to enzymatic activities, GSTs are involved in signal transduction pathways that modulate cell survival or apoptosis and regulate the activity of the MAPK pathway of signal transduction [63]. So, the alteration of the GST enzyme in the larvae may affect the survival and fitness of the larvae.

In the first eight generations, we observed increased mortality and the decreased activity of some detoxification enzymes. Prior to selection, the larvae were acclimatized to an insecticide-free environment. With the application of selection pressure, there was an abrupt switch from safe to stressed environment. Larvae that could not get adjusted in the stressed environment died. The vulnerability of the survived larvae increased till F4 generation and then gradually began to decrease with the continuation of selection. The increased vulnerability in early F0-F4 generations is expected to be associated with a decreased titer of detoxifying enzymes. As metabolism-associated processes play a key role in resistance development in *Ae. aegypti*, the normal titer of enzymes produced might have been used up in detoxifying temephos. Toxicity bioassay, as well as biochemical analysis, highlighted the increase in toxicity of temephos in response to a decrease in detoxifying enzyme activity. Again, with an increase in the activity of metabolic detoxifying enzymes, the toxicity of temephos decreased. From this correlation, it can be inferred that the metabolic detoxification enzymes modulate the susceptibility of *Ae. aegypti* to temephos. After the passage of the F9 generation, resistance began to develop, which was followed by the rise in cytochrome P450, alpha esterase, and beta esterase. In the experiment, we recorded reduced activity of GST in the early generations compared to esterase and Cyt p450. The cause of such a reduction is unknown. However, the more involvement of GST in conjugating glutathione to an electrophilic substrate and the initial exposure leading to oxidative stress that led to defense response, might have left little GST for spectrophotometric detection. Many earlier authors have reported similar reductions in GST enzyme activity. This is the case in permethrin exposed *Aedes aegypti* [64], pyrethroid exposed *Anopheles gambiae* [65],

and fungal stressed *Diaphorina citri* [66]. However, continuous exposure has resulted in an increase in GST activity after the F20 generation.

Temephos is ruling the market and is effective in controlling the populations of *Ae. aegypti* at larval stages. But, as reported in the present study, its prolonged use can lead to resistance development which is associated with increased production of metabolic detoxifying enzymes over generational time. However, for continuing its use, metabolic detoxification enzyme inhibitors (TPP, DEM and PBO) can be used in combination with temephos to increase its toxicity in resistant *Ae. aegypti* [67,68]. Apart from resistance development, organophosphates are also implicated with severe adverse effects such as reduced antioxidant capacity, generation of free radicals, and inhibition of acetylcholinesterase and bladder cancer [69,70]. Long-term use of OP has resulted in the accumulation of its residues in water, soil, food, cow milk, and other environmental components [71]. The residual effects of temephos last 22 weeks in laboratory conditions [72]. This property of temephos poses a threat to non-target organisms. Its uses have jeopardized the natural control system that prevails in the ecosystem [7]. It is time to shift from this harmful chemical to some eco-friendly alternatives. Among many other possible ways for checking *Ae. aegypti* problems, botanicals can be the outstanding candidates. Our recent work has presented the essential oil of *Allium sativum* as a potent ovicide against *Ae. aegypti*, which was found at par with the WHO-recommended synthetic insecticide temephos [35]. In another work, Sarma et al. [8] demonstrated the success of using combinations of plant-based terpene compounds against *Ae. aegypti*. They reported a combination of diallyl disulfide + limonene and carvone + limonene as the best larvicidal and adulticidal composition at a 1:1 ratio, respectively. The ethanolic extract of *Trichodesma indicum* is reported as an efficient larvicide against the third and fourth instar larvae of *Ae. aegypti*. It was found to be less harmful (mortality 43.47 %) to its larval natural predator, *Toxorhynchus splendens*, even at the highest tested concentration (1500 ppm) [7]. In a study where the wild strain of *Ae. aegypti* was found resistant to temephos, the same strain was found susceptible to the crude volatile oil of *Piper betle* [6]. The ethanolic seed extract of *Annona glabra* and *Annona crassiflora* can serve as an excellent larvicidal agent (4th instar) against *Ae. aegypti* at a median lethal concentration of 0.06 and 0.71  $\mu\text{g/mL}$ , respectively [73].

## 5. Conclusions

Temephos showed dose-dependent response and the mortality of larvae skewed with the passage of generations. At 2 ppm concentration, larval mortality was 100 % until F9, but then it gradually decreased until it was reduced to 22.66 % by F28. The LC<sub>50</sub> value of temephos showed a decreasing trend up to the F9 generation. However, at 28 generations, we observed an increase in LC<sub>50</sub> of 7.83 fold. Initially, alpha esterase, beta esterase, and cytochrome P450 enzymes also showed a decreasing trend. However, after 8–11 generations, the activity of these enzymes began to increase significantly and, by F28 generations, the activity of these enzymes increased by 3.64, 3.63, and 3.7 fold, respectively. The GST enzyme activity began to rise significantly from that of the control group in the F21 generation and, by F28, it had increased by 1.53 fold. Overall, it can be inferred that there exists an association between a rise in the LC<sub>50</sub> value with the rise in detoxification enzyme activities. In the case of susceptible larvae, temephos will initially remain effective for several generations, after which its effectiveness is affected by resistance development in the mosquito populations. The present study was conducted in laboratory conditions. Additional long-term studies will be required to clarify the pace of resistance development in *Ae. aegypti* in the field. Such a study will lead to a more improved pesticide design strategy that considers the possibility of breakdown by the pest's detoxification enzymes. Moreover, the present study considers only metabolic resistance; other modes of resistance development such as target site insensitivity and transcriptomic alteration due to repeated insecticide exposure must be examined to develop concrete knowledge



of the effect of long-term temephos exposure on the target pest.

## Data availability

No data was used for the research described in the article.

Data will be made available on request.

All data is within the manuscript and figure.

## Author contribution

**Kamal Adhikari:** Writing – Investigation, Original Draft, Writing - Review & Editing, Data Curation. **Bulbuli Khanikor:** Conceptualization, Resources, Supervision, Revision of the manuscript.

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## Conflict of Interest

The authors declare no conflict of interest.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.toxrep.2021.11.013>.

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