J Vector Borne Dis 51, June 2014, pp. 97–105

Differential expression of glutathione s-transferase enzyme in different life stages of various insecticide-resistant strains of *Anopheles stephensi:* A malaria vector

D. Sanil[1, 2], V. Shetty[1, 3] & N.J. Shetty[1]

[1]*Centre for Applied Genetics, Bangalore University, Bengaluru;* [2]*Yenepoya Research Centre, Yenepoya University, Mangalore;* [3]*Department of Biological Sciences, Poornaprajna Institute of Scientific Research, Bengaluru, India.*

ABSTRACT

Background & objectives: Interest in insect glutathione s-transferases (GSTs) has primarily focused on their role in insecticide resistance. These play an important role in biotransformation and detoxification of many different xenobiotic and endogenous substances including insecticides. The GST activity among 10 laboratory selected insecticide resistant and susceptible/control strains of *Anopheles stephensi* was compared using the substrates 1-chloro-2,4-dinitrobenzene (CDNB). The difference in the GST activities of different life stages of diverse insecticide resistant strains was compared and presented.

Methods: About 100 larvae, pupae, adult males, adult females and eggs (100 µg in total weight) were collected and used for the experiment. The extracts were prepared from each of the insecticide-resistant strains and control. Protein contents of the enzyme homogenate and GST activities were determined.

Results: Deltamethrin and cyfluthrin-resistant strains of *An. stephensi* showed significantly higher GST activity. Larvae and pupae of DDT-resistant strain showed peak GST activity followed by the propoxur-resistant strain. On contrary, the GST activity was found in reduced quantity in alphamethrin, bifenthrin, carbofuran and chloropyrifos resistant strains. Adults of either sexes showed higher GST activity in mosquito strain resistant to organophosphate group of insecticides namely, temephos and chloropyrifos.

Interpretation & conclusion: The GST activity was closely associated with almost all of the insecticides used in the study, strengthening the fact that one of the mechanisms associated with resistance includes an increase of GST activity. This comparative data on GST activity in *An. stephensi* can be useful database to identify possible underlying mechanisms governing insecticide-resistance by GSTs.

Key words *Anopheles stephensi;* glutathione s-transferase; insecticide-resistant strains; resistance management; vector control

INTRODUCTION

Anopheles stephensi Liston (Diptera: Culicidae) is the primary vector of urban malaria in the Indian subcontinent with distribution range extending from southern China to the Red Sea coast[1–2]. This species accounts for 15% of the total malaria incidences in India[3]. Efforts to control malaria have become more intricate because malarial parasites have become drug resistant and mosquitoes have become resistant to insecticides[4]. Mosquitoes have developed resistance to all major groups of insecticides, including biocides[5]. Genetics and intensive application of insecticides are responsible for the rapid development of resistance in many insects[6].

Glutathione s-transferases (GSTs) (GSTs; E.C. 2.5.1.18) belong to family of protein that are involved in the detoxification of a wide range of xenobiotics, protection from oxidative damage, intracellular transport of hormones, endogenous metabolites, and exogenous chemicals including insecticides[7–8]. They can metabolize insecticides by facilitating their reductive dehydrochlorination or by conjugating glutathione to xenobiotic compounds with electrophilic centers (e.g. drugs, herbicides and insecticides), converting them from reactive lipophilic molecules into water-soluble non-reactive conjugates that may easily be excreted[9–10]. The conjugation of glutathione to insecticides results in their detoxification via two distinct pathways. O-dealkylation pathway where, glutathione is conjugated with the alkyl portion of the insecticide, e.g. the demethylation of the tetrachlorvinphos in resistant houseflies[11] and O-dearylation pathway, where glutathione reacts with the leaving group, e.g. the detoxification of parathion and methyl parathion in the diamondback moth *Plutella xylostella*[12]. In addition, they contribute to the removal of toxic oxygen free radical species produced through the action of pesticides[13]. GSTs are expressed at high levels in multiple isoenzyme forms and in different patterns at various insect development stages[14].

Different insect GST forms are responsible for different insecticide specificities[15]. The objective of this study was to compare biochemical characterizations of GST activities expressed among different insecticide resistant strains of *An. stephensi*.

MATERIAL & METHODS

Mosquito rearing

Ten insecticide resistant strains of *An. stephensi* derived from different classes of insecticides maintained in the laboratory were used for the study. These strains were maintained at 25 ± 1ºC and 75 ± 5% relative humidity with 14 h photoperiods according to the procedure of Shetty[16]. The adults were fed on 10% sucrose in 8 × 8 × 8 inch iron cages covered with cotton net cloth. Plastic cup (33″ diam) containing clean water lined with filter paper was placed inside the cage for oviposition. The eggs were kept for 72 h to ensure complete hatching. The hatched larvae were transferred to enamel tray and reared. Powdered mixture of fish feed and dog biscuits were given as larval diet. The early IV instar larvae were subjected to insecticide susceptibility test using the diagnostic dose as recommended by WHO[17–18] at each generation so as to maintain its resistance and susceptibility status.

Development of insecticide resistant strains

Laboratory-induced resistant strains of *An. stephensi* were used in this study (Table 1). The said resistant strains have been established after continuous selection and inbreeding for several generations. WHO diagnostic dosages (Table 1) were selected and resistance tests were carried out according to the procedure of WHO[17–18]. The III instar larvae from the isofemales of resistant strains were exposed to their respective diagnostic doses in two replicates for 24 h. Larval diet was added to ensure none of the larval mortality occurs due to lack of feed. Mortality was recorded after 24 h moribund larvae (presenting weak, rigidity or mobility to reach water surface on touch, being in the state of inactivity or dying) were considered as dead. The surviving larvae after the treatment were maintained separately. The process of selective inbreeding was repeated until cent percent survival was reported at given diagnostic doses. Generation taken to attain cent percent survivability is listed in Table 1.

Susceptible batch of larvae which showed 100% mortality when exposed to diagnostic dose of insecticides was selected as control for the study. This susceptible/control was also obtained after several generation of inbreeding and selection.

Table 1. Insecticide resistant strains of *An. stephensi* used in the study

S. No.	Insecticide resistant strains of *An. stephensi*	Diagnostic dose mg/l (ppm)	Generation taken to attain 100% survivability
	Pyrethroids		
1.	Cyfluthrin-resistant strain (CYF-R)*	0.005	26
2.	Deltamethrin-resistant strain (DLM-R)[19]	0.004	20
3.	Alphamethrin-resistant strain (AM-R)[20]	0.12	27
4.	Bifenthrin-resistant strain (BIF-R)[21]	0.06	27
	Organophosphates		
5.	Temephos-resistant strain (TR-R)[22]	0.02	21
6.	Chlorpyrifos-resistant strain (CPF-R)[23]	0.2	23
	Carbamates		
7.	Propoxur-resistant strain (PR-R)[24]	0.01	16
8.	Carbofuran-resistant strain (CBF-R)*	0.5	17
	Organochlorine		
9.	DDT-resistant strain (DDT-R)[25]	3	19
	Plant extract		
10.	Neem-resistant strain (NM-R)[26]	0.43	36

*Unpublished data.

Enzyme preparation

About 100 larvae, pupae, adult males, adult females and eggs (100 µg in total weight) were collected and used for the experiment. The extracts were prepared from each of the insecticide-resistant strains and control. The samples were weighed as required and homogenized in 0.02 M phosphate buffer, pH 7.0. Homogenates were then centrifuged at 10,000 × g for 5 min at 5°C, and the supernatant was used for enzyme analysis.

Protein content assay

Protein contents of the enzyme homogenate were determined according to the method of Lowry *et al*[27] using bovine serum albumin as the standard. The measurement was performed with the wave length of 660 nm. Five replicates for each insecticide-resistant strains from each life stage were used for the assay and compared to control.

GST activities

Glutathione s-transferase activities were determined

using the model substrates 1-chloro-2,4-dinitrobenzene (CDNB) and reduced GSH as substrates according to Habig *et al*[28] with slight modifications. The non-enzymatic reaction of CDNB with GSH measured without homogenate served as control. The change in absorbance was measured continuously for 5 min at 340 nm and 37ºC in a Jenway UV/Visible (UK) spectrophotometer. Five replicates for each insecticide-resistant strain and controls for each life stage were used for the assay. Changes in absorbance per min were converted into nmol CDNB conjugated/min/mg protein using the extinction coefficient of the resulting 2,4-dinitrophenyl-glutathione: 9.6 nM/cm at 340 nm. GST activities among the resistant strains were observed at the same time against susceptible/controls.

Data analysis

Means of protein quantity, GST activities and specific GST activities were subjected to one-way ANOVA using Dunnett test in GraphPad Prism version 5.00 Windows, GraphPad software, San Diego California, USA.

RESULTS & DISCUSSION

Comparative protein content assay

Average µg protein/mg body weight in different life stages of insecticide-resistant strains is presented in Table 2. Maximum expressions of protein/mg weight in all the life stages of insecticide-resistant strains were compared to that of susceptible control. Eggs from propoxur-resistant strain of *An. stephensi* showed highest expression 57.95 ± 0.12 µg protein/mg weight in average, whereas least protein concentration was observed in the eggs of bifenthrin-resistant strains with 40.90 ± 0.21 µg protein/mg weight. Protein expression in larval stages was found to be more in cyfluthrin-resistant strains with an average of 134.20 ± 0.56 µg protein/mg weight. Larvae of chloropyrifos strain showed comparatively lower mean protein content of 96.30 ± 1.60 µg protein/mg weight among all the insecticide-resistant strains. Among pupa highest mean protein concentration was observed in alphamethrin-resistant strain with 103.60 ± 0.49 µg protein/mg weight, followed by the least in pupa of bifenthrin-resistant with 77.74 ± 0.57 µg protein/mg weight. Adult males of carbofuran-resistant strain showed highest expression of protein at an average of 123.15 ± 0.78 µg protein/mg weight. Least protein content was observed in temephos resistant strains with 97.64 ± 1.27 µg protein/mg weight. Approximately, 147.59 ± 0.989 µg protein/mg weight was the maximum observed in adult females of alphamethrin-resistant strain and least protein expression of 112.26 ± 0.64 µg protein/mg weight in adult females of bifenthrin-resistant strains. Level of proteins in all the life stages was less in susceptible control strains of *An. stephensi*.

Comparative assay of GST activities

Glutathione s-transferase activity per mg weight and

Table 2. Average protein (µg protein/mg weight) level in the different life stages of insecticide-resistant strains of *An. stephensi*

S.No.	Insecticide-resistant strains	Eggs (µg/mg)	Larvae (µg/mg)	Pupae (µg/mg)	Adult ♂ (µg/mg)	Adult ♀ (µg/mg)
1.	Susceptible control [CTRL]	30.80 ± 1.50	86.25 ± 1	58.33 ± 0.68	89.67 ± 0.75	97 ± 0.50
	Pyrethroids					
2.	CYF-R	46.23 ± 0.50*	134.20 ± 0.56*	91 ± 0.40*	112.69 ± 0.94*	124.80 ± 0.95*
3.	DLM-R	52.26 ± 0.88*	109.26 ± 0.65*	79.23 ± 0.48*	99.67 ± 0.89*	136.42 ± 1.27*
4.	AM-R	45.12 ± 0.35*	102.36 ± 0.59*	103.60 ± 0.49*	98.65 ± 0.85*	147.59 ± 0.98*
5.	BIF-R	40.90 ± 0.21*	132.36 ± 0.17*	77.74 ± 0.57*	105.98 ± 1.40*	112.26 ± 0.64*
	Organophosphates					
6.	TR-R	48.32 ± 0.59*	98.23 ± 0.49*	86.25 ± 0.98*	97.64 ± 1.27*	136.87 ± 1.36*
7.	CPF-R	46.30 ± 0.70*	96.30 ± 1.60*	87.90 ± 0.16*	116.75 ± 0.65*	118.69 ± 1.75*
	Carbamates					
8.	PR-R	57.95 ± 0.12*	127.57 ± 0.80*	97.95 ± 0.26*	102.81 ± 0.95*	112.36 ± 2*
9.	CBF-R	45.90 ± 1.10*	103.65 ± 1.20*	91.10 ± 1.20*	123.15 ± 0.78*	124.80 ± 1*
	Organochlorine					
10.	DDT-R	52.32 ± 0.95*	112.21 ± 1.02*	96.65 ± 0.98*	119.68 ± 0.85*	127.54 ± 0.99*
	Plant extract					
11.	NM-R	55.14 ± 0.54*	119.89 ± 0.91*	97.30 ± 0.62*	111.25 ± 0.90*	119.95 ± 1.50*

*Non-significant to control at $p < 0.05$.

Table 3. Comparison of the mean and specific GSTs activity in different life

Insecticide-resistant strains	Mean ± S.D.			
	Eggs		Larvae	
	Activity of GSTs (nmol/min)	Activity of GSTs (nmol/min/mg pro)	Activity of GSTs (nmol/min)	Specific activity of GSTs (nmol/min/mg pro)
Pyrethroids				
CYF-R	1.78 ± (0.0167)	0.117 ± (0.0011)	4.48 ± (0.0509)	0.103 ± (0.0011)
Control	2.66 ± (0.025)	0.111 ± (0.0007)	3 ± (0.036)	0.069 ± (0.0008)
DLM-R	1.67 ± (0.0165)	0.138 ± (0.0006)	3.42 ± (0.0278)	0.078 ± (0.0006)
Control	2.11 ± (0.0104)	0.109 ± (0.0010)	2 ± (0.0193)	0.062 ± (0.0005)
AM-R	1.74 ± (0.0166)	0.114 ± (0.0010)	2.65 ± (0.0236)	0.061 ± (0.0005)
Control	2.02 ± (0.0159)	0.142 ± (0.0015)	2.43 ± (0.0262)	0.057 ± (0.0003)
BIF-R	1.75 ± (0.0171)	0.114 ± (0.0011)	2.36 ± (0.0241)	0.054 ± (0.0005)
Control	2.22 ± (0.0099)	0.145 ± (0.0006)	1.86 ± (0.0035)	0.024 ± (0.0005)
Organophosphates				
TR-R	1.29 ± (0.0078)	0.084 ± (0.0005)	2.55 ± (0.017)	0.058 ± (0.0004)
Control	2.05 ± (0.0200)	0.147 ± (0.0010)	1 ± (0.0182)	0.023 ± (0.0005)
CPF-R	1.19 ± (0.0079)	0.078 ± (0.0005)	2.37 ± (0.0029)	0.054 ± (0)
Control	1.93 ± (0.0175)	0.174 ± (0.0016)	1.07 ± (0.0247)	0.023 ± (0.0004)
Carbamates				
PR-R	1.29 ± (0.0068)	0.085 ± (0.0004)	3.62 ± (0.0923)	0.083 ± (0.0021)
Control	2.16 ± (0.0231)	0.134 ± (0.0013)	1.04 ± (0.0229)	0.056 ± (0.0005)
CBF-R	1.14 ± (0.0034)	0.075 ± (0.0002)	2.58 ± (0.0229)	0.059 ± (0.0005)
Control	1.70 ± (0.0125)	0.132 ± (0.0010)	2.50 ± (0.0163)	0.045 ± (0.0004)
Organochlorine				
DDT-R	0.68 ± (0.0046)*	0.045 ± (0.0003)	5.92 ± (0.1259)*	0.136 ± (0.0029)
Control	1 ± (0.008)	0.065 ± (0.0005)	2.79 ± (0.0301)	0.064 ± (0.0006)
Plant extract				
NM-R	1.60 ± (0.0222)	0.105 ± (0.0014)	4.07 ± (0.0384)	0.093 ± (0.0009)
Control	2.25 ± (0.0155)	0.126 ± (0.0011)	2.73 ± (0.0218)	0.042 ± (0)

*Significant difference compared to control (p <0.05).

its specific activity were examined using CDNB as GST substrate for different life stages of different insecticide-resistant strains and susceptible control. Activity and specific activity of GSTs in different life stages of 10 insecticide-resistant strains are presented in Table 3. GST activity in the eggs of insecticide resistant strains ranged from maximum of 0.045 nmol CDNB conjugated/min/mg protein in deltamethrin-resistant strain to the least of 0.138 nmol CDNB conjugated/min/mg protein, in DDT- resistant strains. Although marginal variations in activity of GST were observed in eggs of insecticide-resistant strains when compared to that of susceptible control, it was found to be statistically significant (F = 43.65, p <0.05, df = 9, 150).

Overall range of GST activities in the larval stages showed maximum of 0.1365 nmol CDNB conjugated/min/mg protein in DDT resistant strains followed by the larvae of cyfluthrin-resistant strains with 0.1033 nmol CDNB conjugated/min/mg protein. Larvae-resistant to organophosphates namely, chloropyrifos and temephos showed comparatively less activity of GST with 0.0544 nmol CDNB conjugated/min/mg protein and 0.0586 nmol CDNB conjugated/min/mg protein, respectively. Among larvae-resistant to carbamate group of insecticides, propoxur-resistant strains showed more GST activity of 0.0834 nmol CDNB conjugated/min/mg protein. Pooled data among larvae of insecticide-resistant strains showed significant difference in activities of GST (F = 27.12, p <0.05, df = 9, 150).

GSTs assayed among the pupae of various insecticide-resistant strains also showed significant difference in activity level (F = 6.35, p <0.05, df = 9, 150). Pupae of carbofuran resistant strain showed higher GST activity

stages of diverse insecticide-resistant strains (mean ± S.D.) of *An. stephensi*

Mean ± S.D.					
Pupae		Adult males		Adult females	
Activity of GSTs (nmol/min)	Specific activity of GSTs (nmol/min/mg pro)	Activity of GSTs (nmol/min)	Specific activity of GSTs (nmol/min/mg pro)	Activity of GSTs (nmol/min)	Specific activity of GSTs (nmol/min/mg pro)
3.20 ± (0.0763)	0.127 ± (0.0013)	1.72 ± (0.0044)	0.050 ± (0)	3.01 ± (0.0058)	0.034 ± (0)
2.78 ± (0.0086)	0.066 ± (0.0004)	0.54 ± (0.011)	0.010 ± (0.0002)	1.95 ± (0.0117)	0.015 ± (0)
4.62 ± (0.1272)	0.129 ± (0.0035)	1.96 ± (0.0059)	0.034 ± (0.0001)	1.29 ± (0.0158)	0.017 ± (0.0002)
3.36 ± (0.0141)	0.094 ± (0.0004)	0.60 ± (0.0087)	0.008 ± (0.0002)	1.09 ± (0.0039)	0.015 ± (0.0001)
2.35 ± (0.0293)	0.089 ± (0.0021)	2.30 ± (0.0059)	0.030 ± (0.0001)	2.19 ± (0.016)	0.041 ± (0)
1.89 ± (0.0251)	0.078 ± (0.0002)	1.73 ± (0.0054)	0.034 ± (0.0001)	1.85 ± (0.0112)	0.030 ± (0)
2.46 ± (0.0219)	0.069 ± (0.0006)	3.14 ± (0.0113)	0.055 ± (0.0001)	3.05 ± (0.0072)	0.041 ± (0.0001)
1.94 ± (0.0170)	0.054 ± (0.0004)	1.95 ± (0.0111)	0.010 ± (0.0001)	1.16 ± (0.0145)	0.034 ± (0.0002)
3.39 ± (0.0366)	0.091 ± (0.0014)	4.35 ± (0.0216)	0.057 ± (0.0002)	3.92 ± (0.0202)	0.025 ± (0.0002)
2.38 ± (0.0176)	0.056 ± (0.0004)	2.02 ± (0.0131)	0.009 ± (0.0002)	2.02 ± (0.0118)	0.016 ± (0.0002)
3.18 ± (0.0396)	0.089 ± (0.0011)	3.76 ± (0.0151)	0.066 ± (0.0002)	4.66 ± (0.0155)	0.063 ± (0.0002)
2.27 ± (0.0135)	0.065 ± (0.0007)	1.98 ± (0.0055)	0.030 ± (0)	1.87 ± (0.0126)	0.030 ± (0)
4.20 ± (0.1293)	0.065 ± (0.0008)	3.10 ± (0.0118)	0.040 ± (0.0001)	1.05 ± (0.0151)	0.030 ± (0.0002)
2.41 ± (0.0264)	0.053 ± (0.0006)	1.92 ± (0.0120)	0.034 ± (0)	0.94 ± (0.0115)	0.015 ± (0.0001)
3.26 ± (0.0528)	0.144 ± (0.0055)	3.28 ± (0.0123)	0.067 ± (0.0001)	1.86 ± (0.0222)	0.056 ± (0.0001)
2.02 ± (0.0166)	0.067 ± (0.0007)	0.61 ± (0.0115)	0.030 ± (0)	1.11 ± (0.0146)	0.035 ± (0.0002)
5.15 ± (0.1994)*	0.117 ± (0.0036)	3.85 ± (0.0090)*	0.054 ± (0.0001)	4.11 ± (0.0123)*	0.014 ± (0.0002)
2.16 ± (0.0199)	0.063 ± (0.0003)	0.47 ± (0.0132)	0.035 ± (0.0002)	1.96 ± (0.0084)	0.013 ± (0.0001)
4.54 ± (0.0468)	0.095 ± (0.0010)	2.86 ± (0.0032)	0.076 ± (0.0004)	2.55 ± (0.0028)	0.053 ± (0.0002)
2.32 ± (0.0259)	0.060 ± (0.0005)	1.71 ± (0.0037)	0.033 ± (0.0002)	1.13 ± (0.0093)	0.033 ± (0.0002)

of 0.1443 nmol CDNB conjugated/min/mg protein followed by that of deltamethrin, cyfluthrin and DDT with GST activity of 0.1295, 0.1274 and 0.1178 nmol CDNB conjugated/min/mg protein, respectively. GST activity at an average of 0.09 nmol CDNB conjugated/min/mg protein was observed in pupae of chloropyrifos, temephos, alphamethrin, bifenthrin and neem-resistant strains.

Among the males of insecticide-resistant strains significant differences were observed in the GST activity level ranging from 0.0304 to 0.0763 nmol CDNB conjugated/min/mg protein ($F = 346.51$, $p < 0.05$, df = 9, 150). Males of neem-resistant strains showed higher GST activity of 0.0763 nmol CDNB conjugated/min/mg protein followed by the males of mosquitoes resistant to carbamate group, i.e carbofuran-resistant strains with GST activity of 0.0675 nmol CDNB conjugated/min/mg protein. Mosquitoes resistant to insecticides belonging to pyrethroid showed least GST activity with males of alphamethrin-resistant strains showing 0.0304 nmol CDNB conjugated/min/mg protein and deltamethrin-resistant strains showing 0.0345 nmol CDNB conjugated/min/mg protein. GST activities ranged from 0.0144 to 0.0637 nmol CDNB conjugated/min/mg protein in females of DDT and chloropyrifos-resistant strains. GST activity of 0.037 nmol CDNB conjugated/min/mg protein was the average activity recorded among the females of various insecticide-resistant strains with statistically significant difference ($F = 434.26$, $p < 0.05$, df = 9, 150).

Insect GSTs have been implicated in resistance to insecticides through direct metabolism of the insecticide[29], sequestration[30] or by protecting against secondary toxic effects, such as increase in lipid peroxidation, induced by insecticide exposure[31]. In this study, we compared quantitative expression of GST isozyme activity levels of dif-

ferent insecticide-resistant strains. Results showed that, although eggs, larvae, pupae and adults from insecticide-resistant strains presented higher activity of GST compared to that of susceptible control, this difference was more accentuated in larvae of insecticide-resistant strains. Since these insecticides act in larval stages and the selection process for resistance was based on larval exposure to this chemical, it is natural that higher expression of detoxifying enzymes is found at this life stage. Interestingly, in the present study, adults of bifenthrin, temephos and chloropyrifos-resistant strains were also reported with elevated activity for GST compared to that of larvae. Thus, higher GST activity in these insecticide-resistant adults possibly reflects natural differences in the expression of GST enzymes in different life stages[32].

The involvement of GSTs in resistance to insecticides other than DDT has been reported in the houseflies[15]. GSTs are also known as DDT hydrochlorinases because of their role in DDT metabolism[33]. GST activity levels observed in the present study in all the different life stages of insecticide-resistant strains of *An. stephensi* were found to be comparatively higher. Similar observations were also reported in DDT-resistant strains of the African malaria vector, *An. gambiae*[34]. In *Ae. aegypti*, elevated expression of GST-2, caused by a mutation in a transacting factor was found to be associated with insecticide resistance[35].

It is evident from earlier studies that pyrethroids do not serve as substrates for GST[32]. Conversely, we have reported elevated level of GST activity in the present study for *An. stephensi* resistant to pyrethroid insecticides namely, cyfluthrin, deltamethrin, bifenthrin and alphamethrin. Adult life stages expressed more of this detoxifying enzyme in alphamethrin and bifenthrin resistance, whereas, *An. stephensi* resistant to deltamethrin and cyfluthrin showed higher expression of GST in the larval stages. Elevated levels of GSTs have been found to bind molecules of many pyrethroid insecticides compromising effectiveness and toxicity by a sequestering mechanism in diamond black moth *P. xylostella* (L.) and coleopteran *Tenebrio molitor*[30, 36]. However, these reports on pyrethroid resistance suggest that the role of GST in insecticide resistance is as an antioxidant defense agent or binding protein[30–31, 37]. There is an example of direct metabolism of the pyrethroid tetramethrin by a non-insect GST[38].

GSTs have a more supportive or facilitating role against the pyrethroids and organophosphates[39–40]. We have shown elevated GST activity in temephos resistant strains of *An. stephensi*. Higher level of altered activity was found to be associated with temephos resistance in *Ae. aegypti* from Brazil[41]. Increased levels of GSTs were observed in Latin American population of *Ae. aegypti*-resistant to deltamethrin, temephos, chloropyrifos and cyfluthrin[42]. Few studies have also suggested the involvement of GSTs in temephos-resistance may be due to a cross-resistance to pyrethroids from a previous exposure to this insecticide[43–44]. This can be attributed to the possible synergistic effect of insecticides[45] or due to over production of esterases[40]. However, as the matter of fact, GST's tend to play a significant role in organophosphate resistance[46–48]. Strains of housefly-resistant to parathion, diazon and diazoxon were reported with increase in GST activity via de-ethylation of these insecticides[49–50]. Similarly, increase in GST activity via demethylation of tetrachlorvinphos was pragmatic in tetrachlorvinphos resistant houseflies[11, 51]. Glutathione conjugation was a major resistance mechanism for parathion and methyl parathion in diamondback moth[52] and *Lygus lineolaris* with resistance to malathion had significantly higher (1.5 fold) GST activity[53]. GST gene transcript has also been found to be elevated in resistant strain by 1.3 fold[54]. The role of GSTs as a secondary resistance mechanism in detoxication of the oxon analog of fenitrothion was reported in *An. subpictus*[55]. A higher level of GST has been associated with organophosphate detoxification in several other insect species[56].

The present study also reports increase in activity of GST with subsequent life stages in *An. stephensi* in most of the insecticide-resistant strains used with prominent observable in chloropyrifos and carbofuran resistant strains. Maximum activity of GSTs in larval and pupal stages followed by that in adult stages of *An. stephensi* was observed in propoxur-resistant strains in the present study. Higher GST activity was marked in *An. subpictus* resistant to carbamate insecticide propoxur[57]. Reports of GST in neem resistant mosquito being scarce, we have reported in our study elevated level of GST in larval and pupal stages of *An. stephensi* resistant to the plant extract neem (botanical insecticide). One factor that influences the expression levels of enzyme is the number of alleles of a resistance gene present. Large enzyme families have a degree of redundancy or overlap substrate specificities and thus it may be expected that metabolic mechanisms of insecticide resistance against a particular insecticide would differ between different populations of the same species[10]. In addition, regulation of GST expression is subject to a complex set of developmental, sex, and tissue-specific factors, as well as environmental and dietary parameters[9].

Insecticide resistance is an important man-made example of natural selection, and the factors governing the

origin and spread of resistance-associated mutations are both of academic and of applied importance[58–59]. Distribution of GSTs is known to be widespread in nature and there is no question about the importance of these enzyme systems for they may play critical role in explaining selective[60–61] as well as non-selective[62] toxicity and resistance mechanism among various organisms[63]. The detoxification function of these enzymes may achieve a particular significance in the insect world by contributing to the development of resistance to insecticides by catalyzing their degradation[14]. The biosynthesis of these enzymes seems to reflect a direct response to xenobiotics[60, 64]. Present study signifies the verity that GST activity is closely associated with insecticide resistance among *An. stephensi*. Our study strengthens the fact that one of the mechanisms associated with insecticide resistance found in many other insects includes an increase of GST activity, probably as a result of gene amplification.

In conclusion, the results presented here provide the first report of comparative GST activity in *An. stephensi* resistant to insecticides belonging to pyrethroid, organophosphate, organochlorine, carbamate and biocide group. The data amply demonstrate a predominant role of GSTs in conferring resistance in *An. stephensi*. This basic knowledge of GST activity may serve as a useful database and will be beneficial in unraveling the prevailing resistance mechanisms which in turn may pave way for the development of molecular marker for resistance detection. This may have an important implication in resistance management in the field and may vastly contribute in implementing effective mosquito control programmes in India.

ACKNOWLEDGEMENTS

DS and VS are grateful to the Poornaprajna Institute of Scientific Research (PPISR), Bengaluru, for research fellowships. The authors also acknowledge the Department of Science and Technology (DST) and University Grants Commission (UGC), Govt. of India, New Delhi for financial assistance.

.

REFERENCES

1. Rao TR. *The Anophelines of India*. Rev edn. Delhi: Malaria Research Centre 1984.
2. Glick JI. Illustrated key to female *Anopheles* of southwestern Asia and Egypt (Diptera: Culicidae). *Mosq Syst* 1992; *24:* 125–53.
3. Shetty NJ. The genetic control of *Anopheles stephensi:* A malaria mosquito. In: Raghunath D, Nayak R, editors. *Trends in malaria and vaccine research: The current Indian scenario.* New Delhi: Tata McGraw-Hill 2002; p. 44–79.
4. Brown SE, Severson DW, Smith LA, Knudson DL. Integration of the *Ae. aegypti* mosquito genetic linkage and physical maps. *Genetics* 2001; *157:* 1299–305.
5. Brogdon GW, McAllister JC. Insecticide resistance and vector control. *Emerg Infect Dis* 1998; *4:* 605–13.
6. Insecticide Resistance Action Committee-IRAC. Insecticide resistance: Causes and action. Available from: *http://www.irac-online.org/Resistance/Overview.asp.2007*.
7. Feng QL, Davey KG, Pang ASD, Primavera M, Ladd TR, Zheng SC, *et al*. Glutathione s-transferase from the spruce budworm, *Choristoneura fumiferana*: Identification, characterization, localization, cDNA cloning, and expression. *Insect Biochem Mol Biol* 1999; *29:* 779–93.
8. Enayati AA, Ranson H, Hemingway J. Insect glutathione transferases and insecticide resistance. *Insect Mol Biol* 2005; *14:* 3–8.
9. Hayes JD, Pulford DJ. The glutathione s-transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* 1995; *30:* 445–600.
10. Lumjuan N, McCarroll L, Prapanthadara L, Hemingway J, Ranson H. Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Ae. aegypti*. *Insect Mol Biol* 2005; *3:* 861–87.
11. Oppenoorth FJ, Van der Pas LJT, Houx NWH. Glutathione s-transferase and hydrolytic activity in a tetrachlorvinphos-resistant strain of housefly and their influence on resistance. *Pestic Biochem Physiol* 1979; *11:* 176–88.
12. Chiang FM, Sun CN. Glutathione transferase isozymes of diamondback moth larvae and their role in the degradation of some organophosphorus insecticides. *Pestic Biochem Physiol* 1993; *45:* 7–14.
13. Chen L, Hall PR, Zhou XE, Ranson H, Hemingway J, Meehan EJ. Structure of an insect δ-class glutathione s-transferase from a DDT-resistant strain of the malaria vector, *An. gambiae*. *Acta Crystallogr* 2003; *59:* 2211–6.
14. Yu SJ. Insect glutathione-s-transferase. *Zool Stud* 1996; *35:* 9–19.
15. Clark AG, Shamaan NA, Sinclair MD, Dauterman WC. Insecticide metabolism by multiple glutathione s-transferases in two strains of the housefly *Musca domestica* (L). *Pestic Biochem Physiol* 1986; *25:* 169–75.
16. Shetty NJ. Chromosomal translocation and semisterility in the malaria vector *Anopheles fluviatilis* James. *Indian J Malariol* 1983; *20:* 45–8.
17. *Instructions for determining the susceptibility or resistance of mosquito larvae to insecticide*. *WHO/VBC* 1981; p. 807.
18. *Guidelines for laboratory and field testing of mosquito larvicides*. *WHO/CDS/WHOPES/GCDPP/2005.13.* Geneva: World Health Organizaiton 2005; p. 8–12.
19. Rajashree BH, Shetty NJ. Genetic study of deltamethrin resistance in the malaria mosquito *Anopheles stephensi* Liston. *J Parasit Dis* 1998; *22:* 140–3.
20. Hariprasad TPN, Shetty NJ. Autosomal inheritance of alphamethrin, a synthetic pyrethroid, resistance in *Anopheles stephensi* Liston, a malaria mosquito. *Bull Entomol Res* 2013; *103:* 547–54.
21. Zin T, Minn ZM, Shetty NJ. Biochemical basis of bifenthrin resistance in *Anopheles stephensi* Liston 1901, a malaria mosquito. *J Myanmar Acad Arts Sci* 2009; *2:* 121–31.
22. Sanil D, Shetty NJ. Genetic study of temephos resistance (*tr*), an

organophosphate insecticide in the malaria mosquito, *An. stephensi* Liston. *J Cytol Genet* 2009; *11:* 15–22.
23. Chandrakala BN, Shetty NJ. Genetic studies of chloropyrifos resistance in the malaria mosquito *An. stephensi* Liston. *J Cytol Genet* 2006; *7:* 155–60.
24. Sanil D, Shetty NJ. Genetic study of propoxur resistance: A carbamate insecticide in the malaria mosquito, *An. stephensi* Liston. *Malar Res Treatment* 2010; doi: 10.4061/2010/502824.
25. Chandrakala BN, Shetty NJ. Genetic studies of DDT resistance in the malaria mosquito *An. stephensi* Liston. *J Cytol Genet* 2004; *5:* 185–90.
26. Zin T, Minn ZM, Shetty NJ. Estimation of proteins and enzymes in different developmental stages of neem susceptible and resistant strains of *An. stephensi* Liston 1901. *Universities Res J* 2008; *1:* 185–93.
27. Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with Folin-Phenol reagent. *J Biol Chem* 1951; *193:* 265–75.
28. Habig WH, Pabst MJ, Jakoby WB. Glutathione s-transferase. *J Biol Chem* 1974; *249:* 7130–9.
29. Wei SH, Clark AG, Syvanen M. Identification and cloning of a key insecticide-metabolizing glutathione s-transferase (MdGST-6A) from a hyper insecticide-resistant strain of the housefly *Musca domestica*. *Insect Biochem Mol Biol* 2001; *31:* 1145–53.
30. Kostaropoulos I, Papadopoulos AI, Metaxakis A, Boukouvala E, Papadopoulou-Mourkidou E. Glutathione-s-transferase in the defense against pyrethroids in insects. *Insect Biochem Mol Biol* 2001; *31:* 313–9.
31. Vontas JG, Small GJ, Hemingway J. Glutathione s-transferases as antioxidant defense agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochem J* 2001; *357:* 65–72.
32. Grant DF, Matsumura F. Glutathione s-transferase 1 and 2 in susceptible and insecticide resistant *Ae. aegypti*. *Pestic Biochem Physiol* 1989; *33:* 132–43.
33. Clark AG, Shamaan NA. Evidence that DDT-hydrochlorinase from the housefly is a glutathione s-transferase. *Pestic Biochem Physiol* 1984; *22:* 249–61.
34. Prapanthadara L, Hemingway J, Ketterman A. DDT-resistance in *An. gambiae* (Diptera: Culicidae) from Zanzibar, Tanzania, based on increased DDT-dehydrochlorinase activity of glutathione s-transferases. *Bull Entomol Res* 1995; *85:* 267–74.
35. Grant DF, Hammock BD. Genetic and molecular evidence for a transacting regulatory locus controlling glutathione s-transferase-2 expression in *Ae. aegypti*. *Mol Genet* 1992; *234:* 169–76.
36. Yu SJ, Nguyen SN. Insecticide susceptibility and detoxication enzyme activities in permethrin selected diamondback moths. *Pestic Biochem Physiol* 1996; *56:* 69–77.
37. Grant DF, Matsumura F. Glutathione-s-transferase in *Ae. aegypti* larvae: Purification and properties. *Insect Biochem* 1988; *18:* 615–22.
38. Smith IH, Wood EJ, Casida JE. Glutathione conjugate of the pyrethroid tetramethrin. *J Agric Food Chem* 1982; *30:* 598–600.
39. Liu N, Xu Q, Zhu F, Zhang L. Pyrethroid resistance in mosquitoes. *Insect Sci* 2006; *13:* 159–66.
40. Che-Mendoza A, Penilla PR, Rodriguez AD. Insecticide resistance and glutathione s-transferases in mosquitoes: A review. *Afr J Biotechnol* 2009; *8*(8): 1386–97.
41. Melo-Santos MAV, Varjal-Melo JJM, Araújo AP, Gomes TCS, Paiva MHS, Regis LN, *et al*. Resistance to the organophosphate temephos: Mechanisms, evolution and reversion in an *Ae. aegypti* laboratory strain from Brazil. *Acta Trop* 2010; *113:* 180–9.
42. Rodríguez MM, Bisset JA, Fernández D. Levels of insecticide resistance and resistance mechanisms in *Ae. aegypti* from some Latin American countries. *J Am Mosq Control Assoc* 2007; *23:* 420–9.
43. Rodriguez MM, Bisset J, Ruiz M, Soca A. Cross-resistance to pyrethroid and organophosphorus insecticides induced by selection with temephos in *Ae. aegypti* (Diptera: Culicidae) from Cuba. *J Med Entomol* 2002; *39:* 882–8.
44. Braga A, Lima JB, Soares SS, Valle D. *Aedes aegypti* resistance to temephos during 2001 in several municipalities in the states of Rio de Janeiro, Sergipe, and Alagoas, Brazil. *Mem Inst Oswaldo Cruz* 2004; *99:* 199–203.
45. Martin T, Ochou OG, Vaissayree M, Fournier D. Organophosphate insecticides synergize pyrethroids in the resistant strain of Cotton Bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) from West Africa. *J Econ Entomol* 2003; *96*(2): 468–74.
46. Hemingway J, Small GJ. Resistance mechanism in cockroaches: The key to control strategies. In: Wildey KB, Robinson WMH, editors. *Proceedings of the first international conference on urban pests*. Cambridge 1993; p. 141–52.
47. Simon JY. Insect Glutathione s-transferases. *Zoological Studies* 1996; *35*(1): 9–19.
48. Rivero A, Vezilier J, Weil M, Read AF, Gandon S. Insecticide control of vector-borne diseases: When is insecticide resistance a problem? *PLoS Pathogen* 2010; 6(*8*):e1001000.
49. Lewis JB. Detoxification of diazinon by subcellular fractions of diazinon-resistant and susceptible houseflies. *Nature* 1969; *224:* 917–8.
50. Lewis JB, Sawicki RM. Characterization of the resistance mechanisms to diazinon, parathion and diazoxon in the organophosphorus-resistant SKA strain of houseflies (*Musca domestica* L.). *Pestic Biochem Physiol* 1971; *1:* 275–85.
51. Oppenoorth FJ, Smissaert HR, Welling W, van der Pass LJT, Hitman KT. Insensitive acetylcholinesterase, high glutathione s-transferase, and hydrolytic activity as resistance factors in a tetrachlorvinphos-resistant strain of housefly. *Pestic Biochem Physiol* 1977; *7:* 34–47.
52. Kao CH, Sun CN. *In vitro* degradation of some organophosphorus insecticides by susceptible and resistant diamondback moth. *Pestic Biochem Physiol* 1991; *41:* 132–41.
53. Zhu Y, Snodgrass GL, Chen M. Comparative study on glutathione-s-transferase activity, cDNA and gene expression between malathion susceptible and resistant strains of the tarnished plant bug, *Lygus lineolaris*. *Pest Biochem Physiol* 2006; *87:* 62–72.
54. Pournourmohammadi S, Abdollahi M. Gene expression. In: Satoh T, Gupta RC, editors. *Acetylcholine esterase and pesticide: Metabolism, neurotoxicity and epidemiology*. Canada: Wiley Publications 2011; p. 175–86.
55. Hemingway J, Miyamoto J, Herath PRJ. A possible novel link between organophosphorus and DDT insecticide resistance genes in *Anopheles* supporting evidence from fenitrothion metabolism studies. *Pestic Biochem Physiol* 1991; *39:* 49–56.
56. Yu SJ, Huang SW. Purification and characterization of glutathione s-transferases from the German cockroach, *Blattella germanica* (L.). *Pest Biochem Physiol* 2000; *67:* 36–45.
57. Karunaratne SH. Insecticide cross-resistance spectra and underlying resistance mechanisms of Sri Lankan anopheline vectors of malaria. *Southeast Asian J Trop Med Public Health* 1999; *30:* 460–9.
58. French-Constant RH, Daborn PJ, Le Goff G. The genetics and genomics of insecticide resistance. *Trends Genet* 2004; *20:*

163–70.
59. Shetty NJ, Hariprasad, TPN, Sanil D, Zin T. Chromosomal inversions among insecticide-resistant strains of *An. stephensi* Liston, a malaria mosquito. *Parasitol Res* 2013; *112*(11): 3851–7.
60. Sívori JL, Casabé N, Zerba EN, Wood EJ. Induction of glutathione-s-transferase activity in *Triatoma infestans*. *Mem Inst Oswaldo Cruz* 1997; *92:* 797–802.
61. Low WY, Ng HL, Morton CJ, Parker MW, Batterham P, Robin C. Molecular evolution of glutathione s-transferases in the genus *Drosophila*. *Genetics* 2007; *177:* 1363–75.
62. Jowett T, Huang J, Murray S. Drug resistance in insects. In: Hayes JD, Wolf RC, editors. *Molecular genetics of drug resistance*. Langhorne, USA: Harwood Academic 1997; p. 175–220.
63. Frank MR, Fogleman JC. Involvement of cytochrome P450 in host-plant utilization by Sonoran Desert *Drosophila*. *Proc Natl Acad Sci* 1992; *89:* 11998–12002.
64. Poupardin R, Reynaud S, Strode C, Ranson H, Vontas J, David JP. Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito *Ae. aegypti:* Impact on larval tolerance to chemical insecticides. *Insect Biochem Mol Biol* 2008; *38:* 540–51.

Correspondence to: Dr N.J. Shetty (Professor Emeritus), Centre for Applied Genetics, Bangalore Universtiy, J.B. Campus, Bengaluru–560 056, India.
E-mail: shetty_nj@yahoo.co.in

Received: 13 July 2013 *Accepted in revised form:* 5 April 2014