Glutathione S Transferase Activity in Indian Vectors of Malaria: A Defense Mechanism Against DDT

K. GUNASEKARAN, S. MUTHUKUMARAVEL, S. S. SAHU, T. VIJAYAKUMAR, AND P. JAMBULINGAM
Division of Vector Biology and Control, Vector Control Research Centre (Indian Council of Medical Research), Indira Nagar, Pondicherry-605 006, India


ABSTRACT Glutathione S transferases (GSTs) are multifunctional enzymes involved in detoxification of xenobiotic compounds in majority of the insect groups. Significance of insect GSTs is their elevated level of activity in association with insecticide resistance. This investigation was to explore the metabolic status of GSTs in two Indian DDT-resistant malaria vectors, Anopheles culicifacies and Anopheles annularis, and one DDT-susceptible vector, Anopheles fluviatilis. Malkangiri and Koraput districts of Orissa State, endemic for falciparum malaria and having a long insecticide spraying history, were the study areas. F1 progeny was raised from wild-caught females of the three vectors and used for biochemical assays to detect the GST-mediated DDT resistance mechanism. Results of the enzyme assay showed a significant 3-fold increase in GST activity in DDT-resistant An. annularis compared with its susceptible population. In DDT-resistant An. culicifacies, the median GST activity (71.8 μmol/min/mg) was almost the same as estimated in the DDT-resistant An. annularis (74.6 μmol/min/mg), suggesting that the GST activity estimated in An. culicifacies could be an elevated level for detoxification of DDT. Furthermore, the GST activity in DDT-resistant An. culicifacies and An. annularis was significantly higher than that in the DDT-susceptible An. fluviatilis, which had a GST activity of 20.0 μmol/min/mg. Also, the GST-mediated DDT detoxification was confirmed by comparing GST activity in wild-caught females with that in their F1 progeny.

KEY WORDS glutathione S transferases (GSTs), DDT resistance, An. culicifacies, An. annularis, India

Glutathione S transferases (GSTs; E.C.2.5.1.18) are multimeric, multifunctional, and multigene family enzymes found ubiquitously in aerobic organisms and involved in various interlinked cellular metabolisms, including detoxification of xenobiotic compounds. Detoxification is one of the key processes in the cellular system, in which GST enzymes get activated in the presence of reduced glutathione (GSH) and bind to the toxic molecules to convert them into nontoxic lipophilic compounds (Habig et al. 1974). Xenobiotic compounds are conjugated with charged glutathione, sulfate, glycine, or glucuronic acid, which are catalyzed by a large group of broad-specificity transferases. Insect GSTs have been classified into six classes viz., Delta (δ), Epsilon (ε), Omega (ω), Sigma (σ), Theta (θ), and Zeta (ζ), based on their differing catalytic activities to accommodate the wide range of substrate specificities (Chen et al. 2003). Significance of insect GSTs is focused on their role in insecticide resistance. In insects such as Helicoverpa armigera, a Lepidopteran polyphagous pest (Benuka et al. 2003), and Drosophila melanogaster, a dipteran fruit fly (Sawicki et al. 2003), the elevated level of GST activity has been associated with insecticide resistance. It was reported to be true with DDT resistance in African malaria vectors also (Ranson et al. 1997).

In India, of the six principal malaria vector species, Anopheles culicifacies and Anopheles stephensi have shown widespread resistance to insecticides such as DDT, benzene hexachloride (BHC), and Malathion (Kumari et al. 1998). Recently, An. culicifacies has been reported to show resistance to synthetic pyrethroids also (Singh et al. 2002). Another vector species, Anopheles annularis has developed resistance to DDT and BHC (Bansal and Singh 1996). Resistance status of these malaria vectors to DDT and other insecticides has been detected based on bioassay of field mosquito populations using the conventional World Health Organization (WHO) kits (WHO 1981). The conventional method allows only detection of resistance, but not determination of its level or the underlying mechanism of resistance. Also, it may not detect resistance phenotypes at low frequencies (Brogdon et al. 1997). The type of resistance mech-
anism selected by a vector species to a particular insecticide, and consequently the spectrum and levels of resistance conferred to other compounds, need to be understood to formulate appropriate resistance management strategies. Even though various reports summarize the gravity of the problem of DDT resistance developed by malaria vectors in India and studies conducted elsewhere implicated GST's role in the mechanism (Ranson et al. 2000), no such information is available for Indian vector species of malaria. As DDT is still being used in India for the control of disease vectors, it has become imperative to generate information on this line.

The objectives of this study were therefore to explore the metabolic status of GSTs in two Indian DDT-resistant malaria vectors, An. culicifacies and An. annularis. A DDT-susceptible vector, Anopheles fluviatilis, was also included in the study for comparison. The outcome of the study would clarify the involvement of GSTs in conferring resistance to DDT in malaria vectors. As elucidated in Anopheles gambiae, the African malaria vector, the responsible GST classes (among the six) for DDT resistance in An. culicifacies and other malaria vectors can be identified and based on this; expression status of the corresponding gene(s) can be measured, and that would lead to development of a molecular tool for screening DDT-resistant populations in field.

Materials and Methods

Study Area. This study was carried out on populations of the three vector species in Malkangiri and Koraput district of Orissa State (India), which is a high-risk malaria transmission area with Plasmodium falciparum predominance. Malkangiri district, the southernmost part of Orissa State (17°45’N to 18°40’N and 81°10’E to 82°00’E), is hilly and forested with numerous streams and rivers (altitude: 150–200 m above mean sea level). Koraput district is situated along the Eastern Ghats (17°40’N to 20°7’N and 81°24’E to 84°2’E) at an altitude of 600–900 m above mean sea level. The terrain of this district is hilly and a highly undulating one covered with forests. Several perennial and seasonal streams and canals are present. Places around two Primary Health Centres (PHC), viz., Korkonda and Borigunguma of Malkangiri and Koraput district, respectively, were randomly selected for collecting mosquito samples. The Korkonda PHC has been highly endemic for P. falciparum malaria with >90% of the total malaria cases belonging to it and is transmitted mainly by An. fluviatilis. Annual parasite incidence in the PHC ranged from 26.5 to 61.0 during 2000–2008 (source: Korkonda PHC). Cerebral malaria and death as a result of malaria are common, particularly among tribes. Incidence of malaria was the maximum during July to December (rainy and postrainy months). The PHC Borigunguma has also been highly malariaous and P. falciparum is the predominant species, contributing >90% to the total malaria cases. In this PHC, malaria incidence peaks during two seasons, one during July to September (rainy) and the other during November to December (cool postrainy). An. fluviatilis is the incriminated major malaria vector (Gunasekaran et al. 2005). Annual parasite incidence of this PHC ranged from 15.1 to 20.9 during 2000–2008 (source: Borigunguma PHC).

Insecticide Use in the Study Areas. DDT has been used for indoor residual spraying (IRS) in the entire state of Orissa since 1953 to till date, except for the period from 1970 to 1997, during which in some districts/PHCs, it was replaced with hexachlorocyclohexane (HCH). However, in view of the ban on this insecticide for public health use, DDT was reintroduced in 1998. Recently, synthetic pyrethroids have been introduced for IRS. Apart from these, in 2004, synthetic pyrethroid-treated mosquito nets were also distributed at district level for malaria control.

Since 1957 to till date, DDT has been the insecticide used for IRS in Borigunguma PHC of Koraput district. Synthetic pyrethroids have not yet been used in this PHC for IRS. In Korkunda PHC of Malkangiri district, DDT has been used for IRS since 1953, but with a break between 1970 and 1997, when HCH was the insecticide used for IRS. Consequent to the ban on HCH, DDT was brought back in 1998, and two rounds of IRS using DDT are still continued. During 2007–2008, a total of 12,500 synthetic pyrethroid-treated mosquito nets was distributed to a population of ≈25,500 (of a total population of 125,000) residing in this PHC.

Target Species and Their Insecticide Resistance Status. An. fluviatilis, An. culicifacies, and An. annularis are the incriminated malaria vectors, widely distributed in Orissa State including in the present investigation sites. An. culicifacies (comprising sibling species B and C) and An. annularis have developed resistance to DDT and BHC (Sahu et al. 1990), whereas An. fluviatilis remains susceptible to DDT (Gunasekaran et al. 2005).

Mosquito Samples for Susceptibility Tests and Biochemical Assay. Indoor resting female mosquitoes of An. culicifacies, An. fluviatilis, and An. annularis were collected from randomly selected Tekaguda and Nuaguda villages of Korkonda PHC and Bondaguda, Kendhuguda, and Deulaguda villages of Borigunguma PHC. Mosquito collections were done using oral aspirator and flashlight during dawn hours both in human dwellings and cattle sheds. Mosquitoes were identified to species on the basis of morphological characteristics.

The DDT-susceptible strain of An. annularis was collected from Deorgoan PHC, Tinsukia district of Assam state, and used as a reference strain for comparison with the DDT-resistant population of this species present in the study areas. In the case of An. culicifacies, as a result of its widespread resistance to DDT, susceptible field population to this insecticide was not available to use as a reference for comparison with the resistant populations.

WHO Insecticide Susceptibility Tests. Susceptibility/Resistance status (phenotype expression) of the vector populations to DDT was determined following the standard procedure (WHO 1981). Ten to 20 blood-fed wild-caught female mosquitoes were ex-
posed to 4% DDT, the diagnostic dosage, by releasing them into WHO susceptibility test kits containing the insecticide impregnated paper (Whatmann filter paper impregnated with mixture of 4% DDT in Risella oil). Tests were carried out in triplicates, and parallel controls (mosquitoes in the test kits were exposed to filter paper impregnated only with Risella oil) were maintained for comparison. Mortality recorded in the tests was corrected to the control mortality, if any, using Abbott’s formula (WHO 1981). The tests that showed >20% mortality in control were discarded.

**Categories of Mosquito Samples Used for Biochemical Assay.** It is known that the field-caught mosquitoes exposed directly to pesticides are not suitable for biochemical assays, as the enzyme level will have been seriously reduced and, hence, the results will be meaningless (Hemingway and Brogdon 1998). Therefore, in the current study, as the study areas have been under insecticide use for malaria control since many years, F₁ progeny was raised in the laboratory from the wild-caught female mosquitoes, and F₁ unfed females (category I) were used for biochemical assays.

To confirm the involvement of GSTs in detoxification of DDT and to determine its resistance threshold in the DDT-resistant populations of *An. culicifacies*, four more categories of mosquito samples of this species were obtained and subjected to GST assay. These categories were as follows: category II, F₁ progeny (1-d-old unfed females) raised from wild-caught females that survived the 1-h exposure to DDT (4%) impregnated paper and 24-h holding; category III, F₁ isofemales (1-d-old unfeds) raised from wild-caught females that survived the 1-h exposure to DDT (4%) impregnated paper and 24-h holding (isofemales were used to verify variations in GST activity, if any, between resistant families); category IV, wild-caught fully fed females; and category V, wild-caught fully fed females that survived the 1-h exposure to DDT (4%) impregnated paper and 24-h holding. The first three categories dealt with F₁ progeny, whereas the last two dealt directly with field-collected females.

Mosquitoes were kept in eppendorf tubes, five to 10 in each tube, and stored in liquid nitrogen. The mosquito samples in liquid nitrogen cylinder were brought to the laboratory at Vector Control Research Centre (Pondicherry, India), transferred to −70°C deep freezer, and stored till biochemical assay was done.

**GST Assay.** Mosquitoes were homogenized individually in 200 µl of distilled water by placing the sample tube on ice. The homogenates were spun at 14,000 rpm for 30 s in a microfuge. GST assay was performed using the supernatant of individual mosquito homogenate (Hemingway and Brogdon 1998). To each of the replicates containing 10 µl of the supernatant, 200 µl of GSH/1-chloro-2,4-dinitrobenzene (CDNB) working solution (10 mM GSH prepared in 0.1 M phosphate buffer, pH 6.5, and 63 mM chlorodinitrobenzene diluted in methanol) was added. Two blanks were prepared for each microtitre plate with 10 µl of sterile distilled water and 200 µl of GSH/CDNB working solution. Enzyme levels were measured at 340 nm for 20 min. The GST activity for individual sample was recorded as µmol CDNB conjugated/min/mg protein using known extinction coefficients (Hemingway and Brogdon 1998) corrected for the path length. For protein estimation, 200 µl of Lowry’s reagent (Solution A and B) was added to 20 µl of the homogenate and incubated at room temperature for 30 min. Sterile distilled water was used for blank preparation. After incubation, diluted Folin reagent was added to each sample. The reaction was read at 755 nm after 30-min incubation. Protein values in mg/ml were calculated for individual sample from a standard curve of absorbance of known concentrations of bovine serum albumin (Lowry et al. 1951).

**Data Analysis.** The median is one of the measures of central location, and it is more appropriate when the results are with wide range. The median GST activity was therefore calculated for the mosquito samples tested to understand the higher and lower half values. In addition, to understand and compare the distribution of phenotype-resistant and susceptible individuals by their GST activity, cumulative frequency distribution and cumulative percentage were plotted.

The results on GST activity were divided into intervals, and then the number of results in each interval was counted and frequency distribution table was prepared. Cumulative frequency, which is used to determine the number of observations that lie above (or below) a particular value in a data set, was calculated by adding each frequency from the frequency distribution table to the sum of its predecessors. The last value will always be equal to the total for all observations, because all frequencies will already have been added to the previous total. Cumulative percentage that provides an easier way to compare different sets of data was calculated within each class interval by dividing the cumulative frequency by the total number of observations (*n*), then multiplying it by 100 (the last value will always be equal to 100%), as follows: cumulative percentage = (cumulative frequency / *n*) × 100.

Distribution of mosquitoes by their GST activity in each category is shown graphically by taking upper endpoint of the class intervals of GST values on *x*-axis, cumulative frequency on primary *y*-axis, and cumulative percentage frequency on secondary *y*-axis. The corresponding class interval that was cut by a line drawn through the curve against the cumulative 50% mark was the midclass interval of GST activity, and this class interval is a range, having a lower and higher endpoint.

Comparison of mean GST activity between the DDT-resistant and susceptible *An. annularis* and also between the five categories of *An. culicifacies* was done using one-way analysis of variance, followed by post hoc test (least significant difference) for multiple comparisons.

**Results**

Results of the susceptibility tests confirmed that *An. culicifacies* and *An. annularis* are phenotypically re-
An. fluviatilis male mosquitoes of was field-collected phenotypically DDT-resistant feature of all the five categories was that their origin activity, is also indicated in the figures. The common tested mosquitoes were distributed by their GST active percentage of samples of the five categories of The cumulative frequency distribution and cumulative SE and the median GST activity are given in Table 2. The number of mosquito samples tested for I–V categories of An. culicifacies together with the mean ± SE and the median GST activity are given in Table 2. The cumulative frequency distribution and cumulative percentage of samples of the five categories of An. culicifacies by GST activity are shown in Fig. 1. The class interval of GST activity, above which 50% of the tested mosquitoes were distributed by their GST activity, is also indicated in the figures. The common feature of all the five categories was that their origin was field-collected phenotypically DDT-resistant female mosquitoes of An. culicifacies. In category III, because there was no significant difference in GST activity between the isofemale families (P > 0.05 by one-way analysis of variance), all were considered together under one category. As the analysis indicated, in category I, the median GST activity was 71.8 µmol/min/mg (the midclass interval of GST activity was 61–80 µmol/min/mg) (Fig. 1a). Compared with this, it was relatively higher in category II (106.6 µmol/min/mg) (Fig. 1b) and category III (123.5 µmol/min/mg) (Fig. 1c). When the enzyme assay was done directly with the field-collected mosquitoes (without raising F1 progeny), the median GST activity was 5.9 µmol/min/mg in category IV (not exposed to DDT paper) (Fig. 1d) and 31.2 µmol/min/mg in category V (exposed to 4% DDT paper) (Fig. 1e). The mean GST activity in these two categories was significantly lower than that in the other three (I–III) categories (P < 0.05 by post hoc multiple comparisons) (Table 2).

The GST activity was measured in F1 progeny raised from wild-caught phenotypically DDT-resistant (n = 333) and DDT-susceptible (n = 41) populations of An. annularis. Compared with the GST level in the susceptible population (mean ± SE = 33.3 ± 6.8 µmol/min/mg), there was a significant (F = 48.396, df = 1, P < 0.001), 3-fold increase in GST activity in the resistant population (103.0 ± 5.0 µmol/min/mg), indicating the involvement of GST in conferring resistance to DDT. Similar to An. culicifacies (category I), in the resistant population of An. annularis, the class interval of GST activity corresponding to cumulative 50% was 61–80 µmol/min/mg (Fig. 2a); the median GST activity was 74.6 µmol/min/mg. But, in the susceptible population, the midclass interval of GST activity was 1–20 µmol/min/mg (Fig. 2b); the median GST activity was 12.8 µmol/min/mg. When percentage frequency distribution of both DDT-resistant and DDT-susceptible females by GST activity was plotted, the maximum GST activity range the susceptible individuals had was 101–200 µmol/min/mg. Furthermore, it was observed that up to an activity range of 21–40 µmol/min/mg, proportion of susceptible individuals was relatively more, from 41 to 60–101-200 µmol/min/mg resistant mosquitoes were higher in proportion and the range above 101–200 µmol/min/mg did not include any susceptible individual (Fig. 3).

An. fluviatilis collected from Malkangiri and Koraput districts were phenotypically susceptible to DDT. The mean GST activity ± SE in An. fluviatilis was 61.6 ± 8.1 µmol/min/mg (n = 207). The range 1–20 µmol/min/mg was the midclass interval of GST activity with a median of 20.0 µmol/min/mg (Fig. 4).

### Discussion

Insect populations survive the effect of toxic insecticidal compounds by different physiological mechanisms, including reduced target site sensitivity and elevated detoxifying enzyme production (Martinez-Torres et al. 1998). Elevated level/up-regulation of GSTs is one such defense mechanism and has been

### Table 1. Number of fully fed female mosquitoes of the three vector species exposed to 4% DDT for 1 h and their corrected mortality recorded after 24 h of holding

<table>
<thead>
<tr>
<th>District</th>
<th>No. tests</th>
<th>No. replicates</th>
<th>No. fully fed female mosquitoes exposed</th>
<th>Corrected mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. annularis</td>
<td>Malkangiri</td>
<td>14</td>
<td>27</td>
<td>527</td>
</tr>
<tr>
<td></td>
<td>Koraput</td>
<td>18</td>
<td>61</td>
<td>753</td>
</tr>
<tr>
<td>An. culicifacies</td>
<td>Malkangiri</td>
<td>5</td>
<td>20</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>Koraput</td>
<td>7</td>
<td>28</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>Tinsukia (Assam State)</td>
<td>2</td>
<td>2</td>
<td>44</td>
</tr>
<tr>
<td>An. fluviatilis</td>
<td>Malkangiri</td>
<td>3</td>
<td>7</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Koraput</td>
<td>3</td>
<td>6</td>
<td>90</td>
</tr>
</tbody>
</table>

Mean GST values with same letters do not differ significantly by one-way analysis of variance, followed by multiple comparisons using post hoc test (least significant difference).

### Table 2. Number of mosquito samples tested under each category of An. culicifacies together with mean and median GST activity

<table>
<thead>
<tr>
<th>Category</th>
<th>Details</th>
<th>No. tested</th>
<th>Mean GST ± SE (µmol/min/mg)</th>
<th>Median GST (µmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>F1 progeny (unfed 1-d-old females) raised from wild-caught females</td>
<td>305</td>
<td>195.7 ± 23.1a</td>
<td>71.8</td>
</tr>
<tr>
<td>II</td>
<td>F1 progeny (unfed 1-d-old females) raised from wild-caught females that survived the exposure to DDT (4%)</td>
<td>256</td>
<td>242.2 ± 19.9a</td>
<td>108.6</td>
</tr>
<tr>
<td>III</td>
<td>F1 isofemales (unfed 1-d-old) raised from wild-caught females that survived the exposure to DDT (4%) impregnated paper</td>
<td>224</td>
<td>172.7 ± 10.3a</td>
<td>123.5</td>
</tr>
<tr>
<td>IV</td>
<td>Wild-caught fully fed females</td>
<td>134</td>
<td>20.6 ± 3.3b</td>
<td>5.9</td>
</tr>
<tr>
<td>V</td>
<td>Wild-caught fully fed females that survived the exposure to DDT (4%) impregnated paper</td>
<td>124</td>
<td>73.4 ± 11.8b</td>
<td>31.2</td>
</tr>
</tbody>
</table>
Fig. 1. Number of phenotypically DDT-resistant An. culicifacies by GST activity: (a) category I, (b) category II, (c) category III, (d) category IV, and (e) category V.
reported to be associated with DDT resistance in mosquitoes by detoxification. The elevation is attained by overexpression of target genes under selection pressure of mosquitoes (Ding et al. 2003) that results in increased production of enzyme facilitating insecticide detoxification, thereby development of resistance (Mouches et al. 1987). Enzyme assay to detect the GST-mediated DDT resistance mechanism has been used in many areas (Ranson et al. 1997). Increased GST activity in *Anopheles sacharovi* and *Anopheles subpictus* was implicated for their resistance development to DDT (Clark and Shamaan et al. 1984, Hemingway et al. 1992, Verhaeghen et al. 2009) and in DDT-resistant population of *An. gambiae*, the activity of GST was found at an elevated level (Hemingway et al. 1985, Prapanthadara et al. 1995).

The current study demonstrated a significant increase in GST activity in DDT-resistant *An. annularis* compared with the DDT-susceptible population of this species; the elevated level of the enzyme in the resistant population was the biochemical response by which DDT gets detoxified. In DDT-resistant *An. culicifacies*, although there was no DDT-susceptible strain for GST estimation and for direct comparison, the estimated median GST activity (71.8 µmol/min/mg) was almost the same as estimated in the DDT-resistant *An. annularis* (74.6 µmol/min/mg), suggesting that the GST activity estimated in *An. culicifacies*...
could be an elevated level for detoxification of DDT. Furthermore, GST activity in the DDT-resistant *An. culicifacies* and *An. annularis* was significantly higher than that in the DDT-susceptible *An. fluviatilis* (the major malaria vector in the study area), which had a median GST activity of 20.0 µmol/min/mg. Although the three vector species were prevalent in the same area and exposed to insecticides, particularly DDT, for a long period, the vector species differed in their response to DDT; within a genus, two species (*An. culicifacies* and *An. annularis*) acquired resistance and one species (*An. fluviatilis*) did not.

Involvement of GSTs in detoxifying DDT in *An. culicifacies* was also confirmed by comparing GST activity in the categories II–V with that in the category I. The categories II and III clearly showed a higher level of GST activity in F1 individuals when their mothers were exposed to DDT-impregnated paper than the F1 progeny of field-caught females without any such exposure in the laboratory. The increased GST activity could be the response to the DDT exposure for detoxification of this insecticide toward conferring resistance against it. Further verification on DDT detoxification by GSTs was done by comparing the GST activity in the two categories of wild-caught mosquitoes, not exposed to DDT paper (category IV) and that survived the exposure (category V), with that of the F1 progeny of either wild-caught or of wild-caught and DDT-exposed mothers (categories I–III). The mean and the median GST activity in the two wild-caught categories were found much lower. The difference in the median GST activity between the wild-caught females and F1 progeny could be the result of the exposure of mosquitoes to various xenobiotic compounds like DDT, Malathion, in the field. Therefore, when the wild-caught mosquitoes were directly subjected to the enzyme assay, the GST levels were significantly lower because a considerable volume of GST was already used for the detoxification process (Hemingway and Brogdon 1998). In insect biological system, DDT molecules are dechlorinated by GST into water-soluble dichloro diphenyl dichloro ethylene (DDE) molecules, which are excreted in urine.
Herath and Jayawardena (1988) have detected the presence of DDE molecules in DDT-resistant mosquitoes by high performance liquid chromatography analysis.

Because the degree of increase in GST activity in the DDT-resistant population of *An. culicifacies* could not be measured in the absence of its susceptible population, it was necessary to determine a resistance threshold of GST activity for this vector species so as to use in the field for screening resistance populations. The median or the midclass interval of GST activity was considered as the threshold for DDT resistance, because in 50% of the tested known DDT-resistant phenotypes, the GST activity was above this level. Although median value alone will do for this, the midclass interval was also considered, as it has a range that might be more relevant for field screening. Accordingly, the midclass interval of GST activity, 61–80 μmol/min/mg (with a median GST activity of 71.8 μmol/min/mg), found in the F1 progeny of wild-caught females, which were not exposed to any DDT-impregnated paper in the laboratory (category I), could be taken as the resistance threshold of GSTs for *An. culicifacies*. In the case of *An. annularis*, taking the maximum GST activity in the DDT-susceptible individuals into consideration, as reported for *An. gambiae* by Brogdon et al. 1997, the resistance threshold of GSTs to DDT could be >200 μmol/min/mg. Or else, as decided in the case of *An. culicifacies*, based on cumulative 50% and its corresponding class interval of GST activity, 61–80 μmol/min/mg or the median GST activity of 74.6 μmol/min/mg could be the resistance threshold.

The resistance threshold of GSTs estimated for the two vector species needs to be validated with field-resistant populations in areas having different spray history. The outcome of this prelude study is a lead to identify the specific class of GSTs involved in conferring resistance to DDT and to develop a molecular marker/tool to measure the expression status of the

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**Fig. 3.** Resistance threshold of GST activity to DDT in *An. annularis*.

**Fig. 4.** Number of phenotypically DDT-susceptible *An. fluviatilis* by GST activity.
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