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The role of the *Aedes aegypti* Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides

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ABSTRACT

The Epsilon glutathione transferase (GST) class in the dengue vector *Aedes aegypti* consists of eight sequentially arranged genes spanning 53,645 bp on super contig 1.291, which maps to chromosome 2. One Epsilon GST, GSTE2, has previously been implicated in conferring resistance to DDT. The amino acid sequence of GSTE2 in an insecticide susceptible and a DDT resistant strain differs at five residues two of which occur in the putative DDT binding site. Characterization of the respective recombinant enzymes revealed that both variants have comparable DDT dehydrochlorinase activity although the isoform from the resistant strain has higher affinity for the insecticide. GSTe2 and two additional Epsilon GST genes, GSTe5 and GSTe7, are expressed at elevated levels in the resistant population and the recombinant homodimer GSTE5-5 also exhibits low levels of DDT dehydrochlorinase activity. Partial silencing of either GSTe7 or GSTe2 by RNA interference resulted in an increased susceptibility to the pyrethroid, deltamethrin suggesting that these GST enzymes may also play a role in resistance to pyrethroid insecticides.

1. Introduction

Aedes aegypti is the major vector of several human pathogens including the arboviruses dengue, chikungunya and yellow fever. Vaccines are not available for most mosquito borne diseases and hence control programmes rely extensively on vector control, which generally involves the use of chemical insecticides. The number of insecticide classes available to control public health pests is very limited and many mosquito populations have developed resistance to one or more of these classes. *Aedes aegypti*, is no exception, and populations of this cosmotropical species has evolved very high levels of resistance to the two major classes of insecticide currently used in dengue control: organophosphates and pyrethroids (Ranson et al., 2010). Although the organochlorine, dichlorodiphenyltrichloroethane (DDT), is now rarely used to control Aedes mosquitoes, high levels of resistance to this insecticide are also common in field populations (Harris et al., 2010; Polson et al., 2011).

Understanding the molecular basis of insecticide resistance is an important step in developing strategies to mitigate the resistance problem. Several studies have implicated the glutathione transferase (GST) enzyme family in conferring resistance to multiple classes of insecticides (Ranson and Hemingway, 2005). Insect GSTs have been classified into at least six classes but it is the Epsilon class that is most commonly associated with resistance (Ranson et al., 2001). The Epsilon GST class in *Ae. aegypti* consists of a cluster of 8 sequentially arrange genes that have been genetically mapped to chromosome 2, supercontig 1.291 (Lumjuan et al., 2007; Saavedra-Rodriguez et al., 2008). Previously we have shown that homodimers of one *Ae. aegypti* Epsilon GST, GSTE2 are very efficient at metabolizing DDT and that expression of this enzyme is elevated in a DDT and pyrethroid resistant population from Thailand (Lumjuan et al., 2005). More recent microarray studies have identified several additional members of this gene cluster that are expressed at elevated levels in insecticide resistant populations of *Ae. aegypti* (Marcombe et al., 2009; Strode et al., 2008) but their ability to bind or metabolize insecticides has not been determined. We therefore characterized additional members of this class in *Ae. aegypti* and provide evidence for a role of two additional Epsilon GSTs in conferring resistance to insecticides.

Abbreviations: GST, glutathione transferase; GSH, glutathione; CDNB, 1-chloro-2, 4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; CHP, cumene hydroperoxide; DDT, 1,1,1-trichloro-2, 2-bis-(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis-(p-chlorophenyl)ethane; DDTase, DDT dehydrochlorinase.

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2. Materials and methods

2.1. Mosquito strains

The New Orleans strain is a laboratory, insecticide susceptible strain of *Ae. aegypti* originally colonized by the Center for Disease Control, Atlanta, USA.

The PMD and PMD-R strains of *Ae. aegypti* originated from Chiang Mai Province, Thailand (Lumjuan et al., 2005). The PMD strain has a resistance ratio of approximately 2.5-fold to both DDT and permethrin compared to New Orleans susceptible strain determined by LT_{50} (Lumjuan et al., 2005). The PMD-R strain is a highly resistant strain and 100% survival was observed after 7 h on both 4% DDT and 0.75% permethrin papers. The resistance ratio of PMD-R compared to the PMD strain has been estimated at 25-fold for permethrin (Yanola et al., 2010).

The Isla Mujeres (IM) strain originated from Mexico and was kindly provided by Professor William Black, Colorado State University. The strain is able to survive 6 h exposure to 4% DDT and is approximately 300-fold resistant to permethrin compared to the susceptible New Orleans strain (Morou et al., 2010; Saavedra-Rodriguez et al., 2008).

Two amino acid substitutions the target site of DDT and pyrethroids, are present in the sodium channel of the IM strain compared to the susceptible New Orleans strain a V1016I replacement (allele frequency 0.6) and a F1534C substitution (frequency = 1.0) (Saavedra-Rodriguez et al., 2007; Harris et al., 2010). The F1534C mutation is also present in the PMD-R strain (Yanola et al., 2010).

2.2. Quantitative PCR

Total RNA was extracted from 3 biological replicates (10 mosquitoes per replicate) of 4th instar larvae, pupae, one-day-old adult males or females from each of the three strains using the PicoPure™ RNA Isolation Kit (Molecular Devices, CA, USA). Gene expression profiling analysis was performed using the GenomeLab GeXP Analysis System Multiplex RT-PCR assay (Beckman Coulter, UK). Multiplex primers were designed using the eXpress designer module of the eXpress Profiler software to generate PCR product sizes of between 100–400 nucleotides. Reverse transcription reactions were conducted according to GeXP Start Kit protocol. One hundred nanograms of total RNA were reverse transcribed with 0.5 mM of each reverse primer in Custom RT Reverse Primer Plex in the presence of 1 unit of reverse transcriptase and kanamycin RNA as internal control. The RT reaction was carried out at 48 °C for 1 min, 37 °C for 5 min, 42°C for 60 min and 95 °C for 5 min. An aliquot of 9.3 μl of cDNA from the RT reactions were used as the template for PCR reactions containing 1 × PCR buffer, fluorescently-labeled universal forward primer, 5 mM $MgCl_2$, 0.2 mM of each of gene-specific reverse primer and 0.175 units of Thermo-Start DNA Polymerase (ABgene, UK). The PCR consisted of 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 1 min. One microliter of PCR products were added to 38.5 μl of Sample Loading Solution containing 0.5 μl of DNA Size Standard-400 in 96-Well Sample Microplate and resolved by capillary electrophoresis on the Beckman CEQ8000. Transcript levels of GSTe5 and GSTe6 were determined using QuantiFast SYBR Green PCR Kit (Qiagen, UK) as previously described (Lumjuan et al., 2005). The primers used are shown in Table S1.

2.3. Expression of recombinant Epsilon GSTs

Total RNA was extracted from whole mosquitoes using Trizol reagent (Sigma–Aldrich, UK). Complementary DNA was synthesized using SuperScript III reverse transcriptase (Invitrogen Life Technologies, USA) as described in the manufacturer's protocol. PCR products generated with ProofStart DNA polymerase (Qiagen) using gene-specific primers (Table S2) were cloned into the pET 100-D/TOPO vector using the Champion pET directional TOPO Expression kit according to the manufacturer's instruction (Invitrogen Life Technologies). The construct was verified by DNA sequencing. The plasmids containing the Epsilon GST genes were transformed into *Escherichia coli* BL21 Star (DE3). The recombinant protein was produced after induction with isopropy β–D-thiogalactoside at 37 °C or room temperature for 4 h.

2.4. Epsilon GST purification

The pET 100-D/TOPO vector encodes an N-terminal polyhistidine (6 × His) fused to the recombinant protein. Protein purification was performed using HisTrap Ni affinity columns (GE Healthcare). The soluble fraction was applied to a 1 ml HisTrap column equilibrated with 40 mM imidazole in 1 × phosphate buffer (20 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.4). The column was washed with 20 ml of 40 mM imidazole in 1 × phosphate buffer and then 20 ml of 80 mM imidazole in 1 × Phosphate buffer. The bound proteins were eluted with 5 column volumes of elute buffer (500 mM imidazole in 1 × phosphate buffer). The fractions containing recombinant protein were pooled and then loaded onto a PD-10 column to eliminate imidazole and change the sample buffer to 50 mM phosphate buffer pH 7.4 or 6.5. The purified protein was concentrated using Amicon-4 column (Millipore, MA, USA) and stored at −20 °C in the presence of 50% glycerol.

The protein purity was verified by 12.5% polyacrylamide gel electrophoresis and Coomassie staining. The protein concentration was determined by the Bradford method using the Bio-Rad protein-assay dye reagent and Bovine serum albumin as a standard (Bradford, 1976). MALDI-TOF mass spectrometry analysis was used to confirm the identity of five of the purified recombinant Epsilon GSTs (Table S3).

2.5. Immunoblotting

Peptide antibodies were designed to specifically recognize GSTE2 and GSTE7. Peptide synthesis, generation of polyclonal antibodies in rabbits and affinity purification was performed by Pacific Immunology Corporation, USA. The peptides matched the carboxy terminal of the proteins and had the following sequences: GSTE2: QFVLSQKEKNAQKA; GSTE7: KVFLKQKLDENNKK. Western blot analysis was performed using 50 μg of protein isolated from batches of 10 mosquitoes of each strain/developmental stage as previously described (Lumjuan et al., 2005). The membrane was probed with a 1:50,000 and 1:2500 dilutions of antibodies against GSTE2 and GSTE7 peptides, respectively. The bound antiserum was detected by incubation with a 1:50,000 dilution of Peroxidase-labeled Anti-Rabbit Antiserum followed by visualization using ECL Advance Blotting Detection Kit (GE Healthcare).

2.6. Characterization of Epsilon GSTs

GST activity against 1-choro-2, 4-dinitrobenzene (CDNB) and 1, 2-dichloro-4-nitrobenzene (DCNB) and glutathione peroxidase activity was determined as described previously (Habig et al., 1974; Simmons et al., 1989). The GST activity toward 4-hydroxy-2-nonenal (4-HNE) was determined by the rate of the consumption of 4-HNE at 224 nm (Alin et al., 1985). DDT dehydrochlorinase activity was determined by conversion of DDT to DDE detected by HPLC as described previously (Prapanthadara et al., 2000). Kinetic studies were performed as described (Lumjuan et al., 2007). The results were analyzed by non-linear regression analysis using GraphPad

Prism v4.0 software (GraphPad Software, Inc., San Diego, USA). Inhibition assays were performed using the standard assay conditions containing 1 mM GSH and 1 mM CDNB.

2.7. RNA interference

Gene-specific primers (Table S4) with the T7 RNA promoter sequence (TAATACGACTCACTATAGGGAGA) appended to the 5′ end were used to clone partial cDNAs of GSTe2 and GSTe7. A plasmid containing a 653 bp fragment of the enhanced green fluorescent protein (EGFP) with T7 linkers has been previously described (Lycett et al., 2004). Double stranded RNA (dsRNA) was synthesized using the MEGAscript RNAi Kit (Ambion Inc., Austin, Texas) and purified using MEGAclear columns (Ambion). The purified products were concentrated by ethanol precipitation and the dsRNA was resuspended to 2 μg/μl of dsRNA. Twenty four hour females from the IM strain were injected in the thorax with 69 nl of dsRNA or phosphate buffered saline (PBS) using a Nanoinjector II (Drummond Scientific Company) as described previously for *Anopheles gambiae* manipulations (Lycett et al., 2006).

Daily mortality was recorded and four days after injection the mosquitoes were exposed to either 4% DDT for 240 min or 0.05% deltamethrin for 180 min according to standard WHO protocols (WHO, 1998). Each experiment was repeated three times, using 20–26 mosquitoes for each group. The bioassay data for the control and treatment groups were compared using a two tailed paired *t*-test. The efficiency of the silencing was determined by Western blots. Twenty five micrograms of protein from a pool of 10 mosquitoes were resolved on 12% SDS-PAGE gel, 4 days after injection with dsRNA or PBS. Proteins were transferred to a nitrocellulose membrane and probed with a 1:1000 and 1:500 dilutions of antibodies against GSTE2 and GSTE7 peptides, respectively for 1 h. Peroxidase-labeled anti-rabbit antibody (1:10000; Amersham) was used as a second antibody. After incubation with ECL Western Blotting substrate (Pierce), proteins were visualized by exposing Hyperfilm to the membrane.

3. Results

3.1. Three Epsilon GSTs are over expressed in a DDT resistant strain of A. aegypti

The sequence similarity between the putative protein sequences of the eight members of the *Ae. aegypti* Epsilon GST class ranges from 26.2% to 66.2% with the highest similarity observed between GSTE2 and GSTE5 (Table 1).

The transcript profiles of the Epsilon GSTs in the three strains of *Ae. aegypti*, New Orleans, PMD and PMD-R, were first analyzed using a multiplex quantitative PCR assay using the predicted sequences from the *Ae. aegypti* genome database. In this first analysis *GSTe2*, *GSTe3*, *GSTe4* and *GSTe7* were successfully amplified in all three *Ae. aegypti* strains. Subsequent sequencing revealed that the initial primers used for *GSTe5* and *GSTe6* encompassed polymorphic residues. Hence new primer sets were designed and used in single plex quantitative PCR reactions using SYBR green. We were unable to quantify the transcript levels for *GSTe1* and *GSTe8* by either methodology. A previous study also found that *GSTe1* transcripts were not detectable by microarray (Strode et al., 2008) suggesting that the expression level of this gene is very low. Nevertheless we were able to successfully amplify the full length transcripts of both *GSTe1* and *GSTe8* using two rounds of PCR amplification during the preparation of constructs for recombinant protein expression indicating that these genes are not silent pseudogenes.

GSTe2 is expressed at significantly higher levels in the highly resistant PMD-R strain than in the PMD strain or the laboratory susceptible New Orleans strain in all life stages analyzed (Fig. 1) as reported earlier (Lumjuan et al., 2005). The fold change between NO and PMD-R ranges from 1.4 in pupae to 2.9 in adult males, two additional GSTs, *GSTe5* and *GSTe7*, were also over expressed in the PMD-R strain (maximum 16.9-fold increased expression of *GSTe5* in adult female stage, 7.8-fold in pupae stage for *GSTe7*). No significant difference in expression of *GSTe3*, *GSTe4* or *GSTe6* was observed between the strains (Fig. 1).

Table 1
Percentage similarity between *Aedes aegypti* GST proteins.

	Similarity (%)							
	GSTE1	GSTE2	GSTE3	GSTE4	GSTE5	GSTE6	GSTE7	GSTE8
GSTE1	***	40.7	37.8	40.7	38.8	35.4	34.4	27.3
GSTE2		***	49.3	54.3	66.2	43.4	41.3	30.8
GSTE3			***	51.6	45.5	38.5	57.8	27.1
GSTE4				***	50.9	46.2	40.2	25.8
GSTE5					***	38.9	38.3	30.8
GSTE6						***	33.9	29.0
GSTE7							***	26.2
GSTE8								***

Percentage similarity was determined using the DNASTAR software (DNAStar, Madison, WI, USA.) The highest pairwise identity, between GSTE2 and GSTE5, is underlined.

Affinity purified peptide antibodies against *Ae. aegypti* GSTE2 and GSTE7 were used to determine whether the transcript level also reflected the amount of protein in the different mosquito strains. The specificity of the antibody was confirmed using recombinant *Ae. aegypti* Epsilon GSTs. (Fig. S1). Western blots, where equal amounts of protein from each strain were loaded in respective wells, confirmed that both GSTE2 and GSTE7 were present at greatly elevated levels in all life stages of the PMD-R strain compared to the PMD or susceptible strain (Fig. 2).

3.2. Two Epsilon GSTs have DDT dehydrochlorinase activity

Full length transcripts of the eight Epsilon GSTs were amplified from the PMD-R strain and cloned into the pET 100-D/TOPO vector. Pilot expression experiments were performed to optimize the expression conditions. Recombinant GSTE1-1, GSTE3-3, GSTE5-5 and GSTE7-7 were expressed in the soluble forms at the culture temperature 37 °C. For GSTE2-2, GSTE4-4 and GSTE8-8 the majority of the protein was confined to the insoluble inclusion bodies at this temperature and therefore expression was conducted at room temperature (25 °C). This temperature reduction greatly increased the solubility. The recombinant proteins were affinity purified using the HisTrap FF column, and then checked for purity by SDS-PAGE (Fig. S2). For seven of the GSTs, a single band of approximately 28 kDa, including N-terminal His-tag was obtained. The identity was confirmed by peptide mass fingerprinting (Table S3). For GSTE6, a purified protein could not be obtained (Fig. S2).

All seven recombinant Epsilon GSTs showed catalytic activity towards the model substrates CDNB, 4-HNE and CHP (Table 2). All, except GSTE7, also cleaved the substrate DCNB. Note that the concentrations of substrates used in these assays were those suggested by Habig et al. (1974). These were selected to facilitate comparison with specific activities of GSTs from other species. However, kinetic analysis revealed that the K_m^{GSH} values for some of the Epsilon GSTs, notably GSTE1-1, were surprisingly high (Table 3) and well above the physiological concentration of GSH in most animal cells. Thus GSH may have been rate limiting in some reactions and specific activities may have been underestimated.

Only GSTE2-2 and GSTE5-5 were able to metabolize DDT to DDE. The DDT dehydrochlorinase activity of GSTE2-2 was similar to that reported earlier (Lumjuan et al., 2005). GSTE5-5 exhibited a much

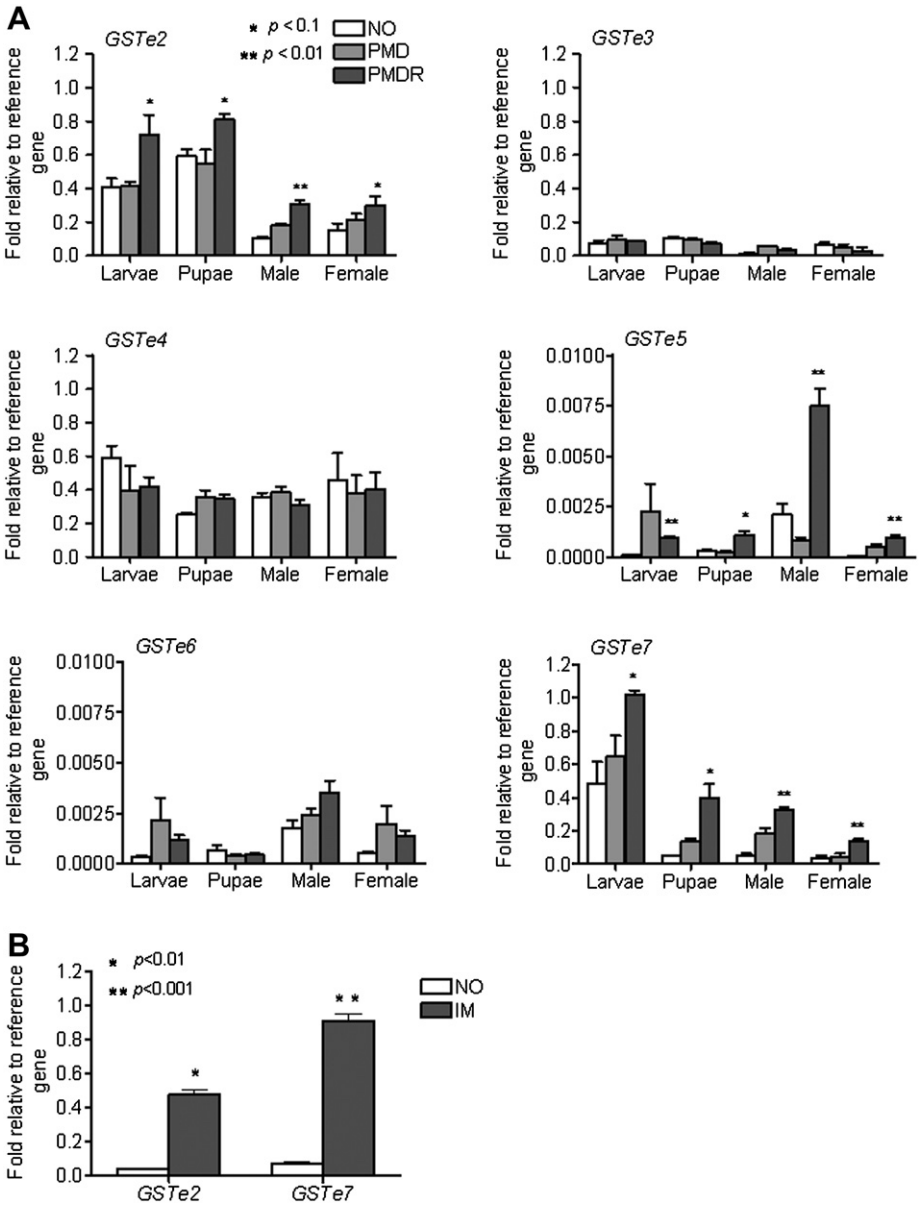

Fig. 1. Quantitative PCR analysis of Epsilon GSTs in *Aedes aegypti* from PMD-R strain (A). The mRNA copy numbers were determined by comparison with known concentrations of standard plasmids and normalized against the copy number of the ribosomal S7 transcript. Error bars indicate standard error of the mean. Statistically significant differences were evaluated with ANOVA test followed by pair-wise *t*-test ($p < 0.1$ indicated by * and $p < 0.01$ as **) relative to New Orleans strain. The expression profiles of GSTe2, GSTe3, GSTe4 and GSTe7 were analyzed using multiplex quantitative PCR, whereas GSTe5 and GSTe6 were determined using SYBR Green kit as described in the methods. Expression profiles of GSTe2 and GSTe7 in female of *Aedes aegypti* from IM strain (B). Three independent experiments were performed. Error bars indicate standard error of the mean. The differences in gene expression were statistically evaluated by paired-*t*-test.

lower level of DDT dehydrochlorinase activity but the activity was still over 5 -fold higher than reported for Delta GSTs (Ranson et al., 1997). The kinetic parameters of the recombinant *Ae. aegypti* Epsilon GSTs against CDNB are shown in Table 3. Of the seven Epsilon GSTs, GSTE3-3 has the highest maximum velocity of 61.5 ± 3.5 μmol/min/mg protein with highest turnover rates being achieved by GSTE3-3 and GSTE5-5.

3.3. Two alternative alleles of GSTE2 differ in their affinity for DDT

The putative amino acid sequence of GSTE2 was determined for both the insecticide susceptible New Orleans strain and the highly DDT resistant PMD-R strain. Five fixed substitutions were identified: S111L, F115C, V150I, A178E and E198A. Given that the New

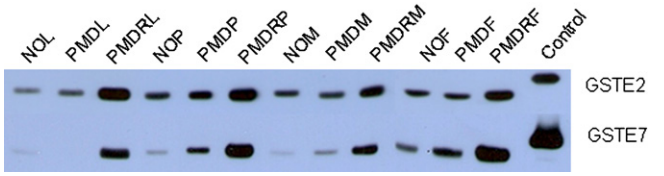

Fig. 2. Western blot analysis of GSTE2-2 and GSTE7-7. Equal amounts of protein (50 μg) from 10 mosquitoes from New Orleans (NO), PMD and PMD-R strains in four developmental stages were analyzed; larvae (L), pupae (P), adult male (M), and adult female (F) and purified His-tagged GSTE2-2 and GSTE7-7 (50 ng) were resolved on 12.5% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with a 1:50,000 and 1:2500 dilutions of antibodies against GSTE2 and GSTE7 peptides, respectively for 1 h. Peroxidase-labeled anti-rabbit antibody (1:50,000; Amersham) was used as a second antibody. Proteins were visualized by enhancing the chemiluminescence using ECL Advance Blotting Detection Kit (Amersham).

Table 2
Specific activities of recombinant *Aedes aegypti* Epsilon GSTs.

Enzyme	CDNB	DCNB	4-HNE	CHP	DDTase activity
	μmol/min/mg				nmol DDE/μg
GSTE1-1	1.2 ± 0.1	0.05 ± 0.01	0.01	0.11	ND
GSTE2-2NO	6.7 ± 0.1	2.21 ± 0.04	0.32 ± 0.04	0.19 ± 0.03	5.79 ± 0.14
GSTE2-2^{PMD-R}	6.9 ± 0.5	3.19 ± 0.12	0.48 ± 0.02	0.14 ± 0.01	6.95 ± 1.21
GSTE3-3	15.0 ± 0.5	0.73 ± 0.08	0.75 ± 0.03	0.84 ± 0.04	ND
GSTE4-4	21.6 ± 0.9	0.86 ± 0.07	0.56 ± 0.05	0.32 ± 0.03	ND
GSTE5-5	10.5 ± 1.0	3.02 ± 0.41	0.16	0.22 ± 0.01	0.05
GSTE7-7	4.7 ± 0.4	ND	0.26 ± 0.03	0.15 ± 0.04	ND
GSTE8-8	7.1 ± 0.6	1.62 ± 0.17	0.93 ± 0.08	0.99 ± 0.06	ND

GST activity against CDNB and DCNB were determined in the presence of 1 mM GSH and 5 mM GSH, respectively. Three independent assays were performed. Result shows mean ± SD. ND indicates to not detectable.

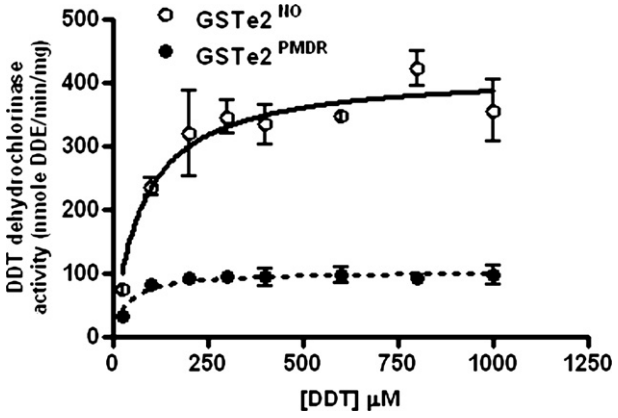

Fig. 3. Michaelis–Menten enzyme kinetics for GSTE2-2 variants. Recombinant GSTE2-2 variants were incubated with various concentration of DDT in the presence 2.5 mM of GSH. The reaction was incubated at 28 °C for 20 min. Dicofol was spiked as an internal control. The reaction was extracted and subjected for HPLC analysis as described in the text.

Orleans strain has been in colony for several decades, we also sequenced the coding sequence of GSTE2 from 10 individual *Ae. aegypti* mosquitoes collected from Chiang Mai city and Mae Sariang, Mae Hong Son Province in Northern Thailand in 2009. Both haplotypes were observed (8 identical to the PMD-R strain, 2 to the New Orleans strain) confirming that both alleles are found in wild populations of *Ae. aegypti*. Three of the amino acid substitutions are semi conservative and unlikely to affect the catalytic activity. However residues 111 and 115 form part of the putative DDT binding pocket (Wang et al., 2008) (Fig. S3) and hence the substitution of hydrophobic for polar residues at these sites may influence the ability of these enzymes to metabolize DDT. We therefore decided to characterize the New Orleans variant to see if this enzyme differed from the PMD-R variant in its catalytic properties and crucially in its ability to metabolize DDT.

The two GSTE2 variants had similar specific activities against the model substrates (Table 2) but the New Orleans variant had a lower maximum velocity and turnover rate for CDNB than the PMD-R variant (Table 3). The DDT dehydrochlorinase activity of the two variants was not significantly different (Table 2). However, the kinetics of the reaction did differ considerably (Fig. 3) with the PMD-R variant having a lower K_m for DDT (37.8 ± 12.5 μM versus 79.6 ± 23.6 μM) but also a lower V_{max} (104.3 ± 5.3 versus 418.5 ± 26.0 nmol DDE formation/min/mg).

3.4. Epsilon GSTs are inhibited by insecticides from all four major classes

The inhibition of GST activity against CDNB in the presence of 10 μM of 11 different insecticides from 4 classes (organochlorine, organophosphate, pyrethroid and carbamates) is shown in Table 4. The CDNB conjugation activity of GSTE2-2 is the least affected by insecticides with very little inhibition observed even with DDT, which is a known substrate for this enzyme. Many of the other enzymes were strongly inhibited but each showed a unique inhibition pattern. The kinetics of inhibition of six of the Epsilon GSTs was determined for permethrin. Five were inhibited in a non-competitive manner whereas inhibition of GSTE2-2 by permethrin was uncompetitive (Table S5). These results suggest that permethrin binds the enzymes in a different site to the CDNB active site. The binding of permethrin to non-catalytic site of the enzyme leading to sequestration of the insecticide has been proposed in the mechanism of resistance (Kostaropoulos et al., 2001).

3.5. Partial silencing of GSTe2 or GSTe7 increases susceptibility to deltamethrin

Two Epsilon GSTs, *GSTe2* and *GSTe7*, were targeted for RNAi experiments to determine whether suppression of these genes impacted on the resistance phenotype. RNAi was not attempted with *GSTe5*, as both the very low level of transcript of this gene in all strains (Fig. 1) and the lack of a specific peptide antibody, precluded analysis of the level of knockdown. Initially we had planned to conduct RNAi experiments on the PMD-R strain, in keeping with the rest of the study. Unfortunately live material from this strain was not available and hence an alternative strain, Isla Mujeres from Mexico, was used. The transcript levels of both *GSTe2* and *GSTe7* are highly elevated in this strain compared to the susceptible NO strain (>10-fold for *GSTe2* and >12-fold for *GSTe7* Fig. 1B) and hence this is an appropriate alternative to the PMD-R strain.

Targeting of *GSTe2* or *GSTe7* by injection of dsRNAs into adults resulted in partial silencing of the expression of the targets when

Table 3
Kinetic parameters of recombinant *Aedes aegypti* Epsilon GSTs.

	V_{max} (μmol/min/mg)	K_m^{GSH} (mM)	K_m^{CDNB} (mM)	k_{cat} (s-1)	k_{cat}/K_m^{GSH} (m M^{-1} s^{-1})	k_{cat}/K_m^{CDNB} (m M^{-1} s^{-1})
GSTE1-1	18.8 ± 1.7	26.1 ± 3.3	1.13 ± 0.21	10	0.4	9
GSTE2-2NO	13.4 ± 0.6	13.6 ± 3.4	0.05 ± 0.01	11	0.8	220
GSTE2-2^{PMD-R}	16.5 ± 0.6	7.0 ± 1.2	0.03 ± 0.01	23	3.3	763
GSTE3-3	61.5 ± 3.5	3.0 ± 0.7	0.41 ± 0.06	46	15.0	111
GSTE4-4	36.8 ± 2.2	3.8 ± 0.7	0.08 ± 0.02	23	5.9	282
GSTE5-5	44.5 ± 3.1	13.2 ± 4.4	0.19 ± 0.05	51	3.9	268
GSTE7-7	10.2 ± 1.1	2.2 ± 0.5	0.46 ± 0.13	7	3.2	15
GSTE8-8	8.3 ± 0.4	4.1 ± 1.2	0.35 ± 0.05	6	1.4	17

Three independent assays were performed. Results show mean ± SD. Kinetic studies were determined by varying the concentration of GSH (1.25–40 mM) at fixed saturating CDNB of 2 mM (except GSTE2-2 variants where 1 mM CDNB was used), or fixed saturating concentrations of GSH of 40 mM (20 mM for GSTE3-3 and GSTE7-7) and CDNB from 0.0125 to 2.0 mM.

Table 4
Inhibition of GST activity in the presence of various insecticides.

% Inhibition Insecticides (10 μM)	Isoenzyme						
	GSTE1-1	GSTE2-2	GSTE3-3	GSTE4-4	GSTE5-5	GSTE7-7	GSTE8-8
Organochlorines:							
DDT	0	3	42	4	2	34	24
DDE	5	3	61	0	6	34	18
Dieldrin	45	0	59	5	14	28	0
Lindane	0	0	1	0	0	0	0
Organophosphate:							
Chlorpyrifos	0	2	31	0	0	0	0
Temephos	2	4	100	35	77	26	17
Pyrethroids:							
Permethrin	0	1	75	27	54	7	6
Deltamethrin	6	9	93	11	29	90	40
Carbamate:							
Bendiocarb	0	0	0	0	0	0	0
Propoxur	0	0	99	0	58	0	0

The GST activity was assayed using 1 mM CDNB and 1 mM GSH as substrates in the absence and presence of 10 μM insecticides. Three independent assays were performed. Results show mean value.

assessed by western blotting (Fig. S4). Female mosquitoes, injected with dsRNA against either GST, were less able to survive exposure to the insecticide deltamethrin than cohorts that had been injected with either dsEGFP or PBS controls (Fig. 4). The effect was particularly pronounced for *GSTe2*. Partial silencing of this gene resulted in a greater than 8-fold increase in deltamethrin induced mortality in the GST injected group compared to the control EGFP injected group. Partial silencing of *GSTe7* increased the % mortality after deltamethrin exposure from 8% to 42%.

Surprisingly, partial silencing of *GSTe2* or *GSTe7* had no effect on mortality of the IM strain after DDT exposure (Fig. 4).

4. Discussion

Three GST genes are over expressed in the DDT/permethrin resistant PMD-R strain. Two of these genes, GSTe2 and GSTe7 were also found to be over expressed in microarray studies on the same strain (Strode et al., 2008). The third, GSTe5, is expressed at very low levels, which may be below the detection limit of microarray experiments. GSTE2-2 had previously been implicated in DDT resistance but here we show that GSTE5-5 is also able to detoxify this insecticide, albeit at a much lower rate (140-fold lower specific activity) that GSTE2-2.

Two variants of GSTE2, which differ at five sites, including two in the putative DDT binding pocket were characterized. In situ modeling predicted that these non- conservative substitutions would result in a wider more polar DDT binding pocket in the GSTE2 variant found in the PMD-R strain. Although the specific activities of these two enzyme variants for DDT were not significantly different, the variant found in the resistant colony had a higher affinity for the insecticide. Interestingly, both alleles were detected in a random sample of 10 individuals of *Ae. aegypti* collected in Northern Thailand but no mixed haplotypes were observed. The correlation between the allelic variants and the resistance phenotype has not been investigated in field populations but it is possible that both increased transcription and allelic variation in the *GSTe2* gene contribute to the very high level of DDT resistance of the PMD-R strain.

The role of GSTE7 in DDT resistance remains unclear. This gene is highly expressed in multiple DDT/pyrethroid resistant populations as compared to susceptible strains (Marcombe et al., 2009; Strode et al., 2008) but the recombinant GSTE7-7 has no DDT dehydrochlorinase activity. One possibility is that up regulation of *GSTe7*, *GSTe2* and *GSTe5* are controlled by a single *cis*-acting factor and the fact that these three genes are found sequentially on the chromosome may support a model of coordinated regulation. However this

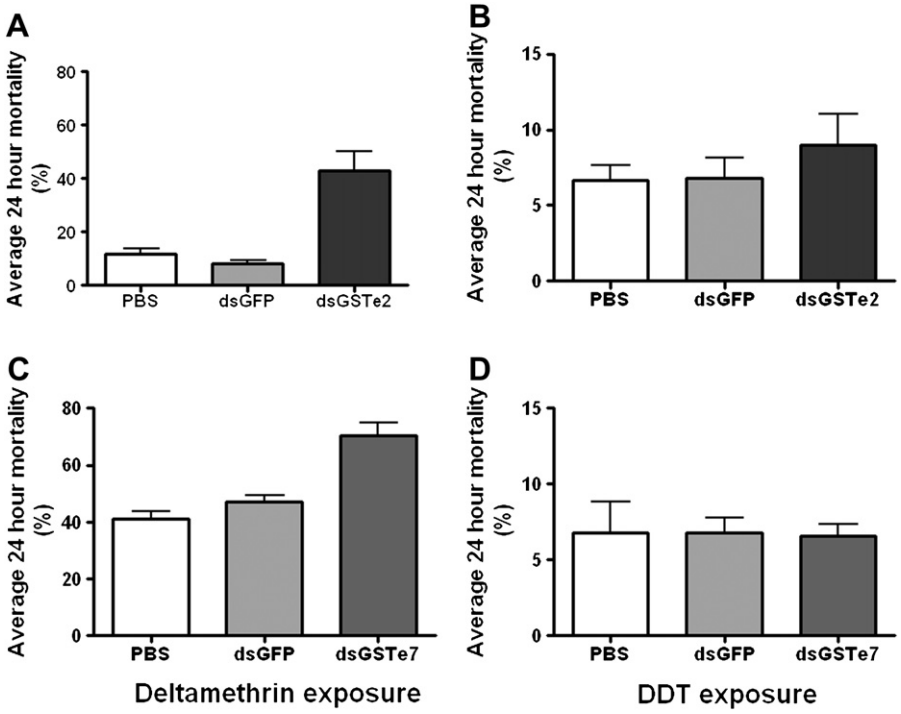

Fig. 4. Effect of partial silencing of GSTe2 or GSTe7 on ability to withstand insecticide exposure. One-day-old females from the Isla Mujeres strain of *Ae. aegypti* were injected with dsRNA against either GSTe2 GSTe7 or EGFP. A control group was injected with PBS. Three days post injection the mosquitoes were exposed to insecticide papers impregnated with 0.05% deltamethrin (Panel A and C) or 4% DDT (Panel B and D). Morality was recorded 24 h later. Results show the means from 3 separate experiments. The minimum number of mosquitoes injected in any group was 75.

factor would have to work bidirectionally since *GSTe7* is arranged in the opposite orientation to *GSTe2* and *GSTe5* (Lumjuan et al., 2007). Partial silencing of *GSTe2* or *GSTe7* had no apparent effect on the ability of the IM strain to survive DDT exposure. However, it should be noted that the level of DDT resistance is extremely high in this strain, conferred at least in part, by two amino acid substitutions in the voltage gated sodium channel, the target site of DDT. The presence of more than one resistance mechanism complicates analysis of the RNAi experiments. Unfortunately, target site resistance is now very widespread in *Ae. aegypti* (Garcia et al., 2009; Kawada et al., 2009; Martins et al., 2009) and it is very difficult to find a strain without this mechanism to examine experimentally.

The role of GSTs in pyrethroid resistance has been largely unproven to date. GSTs may help protect against the oxidative stress induced by pyrethroids (Vontas et al., 2001). Indeed all of the *Ae. aegypti* Epsilon GSTs do show peroxidase activity, but the level is not significantly higher in the two GSTs, GSTE7 and GSTE2, repeatedly found over expressed in pyrethroid resistant populations. Other studies have proposed that GSTs are protective against insecticides by sequestering the pyrethroid (Kostaropoulos et al., 2001). Here we provide evidence that the Epsilon GSTs, GSTE2 and GSTE7 are involved in conferring resistance to the pyrethroid deltamethrin in the IM strain but the mechanism by which this occur still remains to be resolved.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ibmb.2010.12.005.

References

Alin, P., Danielson, U.H., Mannervik, B., 1985. 4-Hydroxyalk-2-enals are substrates for glutathione transferase. FEBS Lett. 179, 267–270.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Garcia, G.P., Flores, A.E., Fernandez-Salas, I., Saavedra-Rodriguez, K., Reyes-Solis, G., Lozano-Fuentes, S., Guillermo Bond, J., Casas-Martinez, M., Ramsey, J.M., Garcia-Rejon, J., Dominguez-Galera, M., Ranson, H., Hemingway, J., Eisen, L., Black, I.W., 2009. Recent rapid rise of a permethrin knock down resistance allele in *Aedes aegypti* in Mexico. PLoS Negl. Trop. Dis. 3, e531.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Harris, A., Rajatileka, S., Ranson, H., 2010. Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. Am. J. Trop. Med. Hyg. 83, 277–284.

Kawada, H., Higa, Y., Komagata, O., Kasai, S., Tomita, T., Thi Yen, N., Loan, L.L., Sanchez, R.A., Takagi, M., 2009. Widespread distribution of a newly found point mutation in voltage-gated sodium channel in pyrethroid-resistant *Aedes aegypti* populations in Vietnam. PLoS Negl. Trop. Dis. 3, e527.

Kostaropoulos, I., Papadopoulos, A.I., Metaxakis, A., Boukouvala, E., Papadopoulou-Mourkidou, E., 2001. Glutathione S-transferase in the defence against pyrethroids in insects. Insect Biochem. Mol. Biol. 31, 313–319.

Lumjuan, N., McCarroll, L., Prapanthadara, L.A., Hemingway, J., Ranson, H., 2005. Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti*. Insect Biochem. Mol. Biol. 35, 861–871.

Lumjuan, N., Stevenson, B.J., Prapanthadara, L.A., Somboon, P., Brophy, P.M., Loftus, B.J., Severson, D.W., Ranson, H., 2007. The *Aedes aegypti* glutathione transferase family. Insect Biochem. Mol. Biol. 37, 1026–1035.

Lycett, G.J., Kafatos, F.C., Loukeris, T.G., 2004. Conditional expression in the malaria mosquito *Anopheles stephensi* with Tet-On and Tet-Off systems. Genetics 167, 1781–1790.

Lycett, G.J., McLaughlin, L.A., Ranson, H., Hemingway, J., Kafatos, F.C., Loukeris, T.G., Paine, M.J., 2006. *Anopheles gambiae* P450 reductase is highly expressed in oenocytes and in vivo knockdown increases permethrin susceptibility. Insect Mol. Biol. 15, 321–327.

Marcombe, S., Poupardin, R., Darriet, F., Reynaud, S., Bonnet, J., Strode, C., Brengues, C., Yebakima, A., Ranson, H., Corbel, V., David, J.P., 2009. Exploring the molecular basis of insecticide resistance in the dengue vector *Aedes aegypti*: a case study in Martinique Island (French West Indies). BMC Genom. 10, 494.

Martins, A.J., Lima, J.B., Peixoto, A.A., Valle, D., 2009. Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of *Aedes aegypti* Brazilian populations. Trop. Med. Int. Health 14, 1351–1355.

Morou, E., Dowd, A.J., Rajatileka, S., Steven, A., Hemingway, J., Ranson, H., Paine, M., Vontas, J., 2010. A simple colorimetric assay for specific detection of glutathione-S transferase activity associated with DDT resistance in mosquitoes. PLoS Negl. Trop. Dis. 4.

Polson, K.A., Brogdon, W.G., Rawlins, S.C., Chadee, D.D., 2011. Characterization of insecticide resistance in Trinidadian strains of *Aedes aegypti* mosquitoes. Acta Trop. 117, 31–38.

Prapanthadara, L., Koottathep, S., Promtet, N., Suwonkerd, W., Ketterman, A.J., Somboon, P., 2000. Correlation of glutathione S-transferase and DDT dehydrochlorinase activities with DDT susceptibility in *Anopheles* and *Culex* mosquitoes from northern Thailand. Southeast Asian J. Trop. Med. Publ. Health 1 (31 Suppl.), 111–118.

Ranson, H., Burhani, J., Lumjuan, N., Black, W., 2010. Insecticide resistance in dengue vectors. TropIKA.net J. 1 (1).

Ranson, H., Cornel, A.J., Fournier, D., Vaughan, A., Collins, F.H., Hemingway, J., 1997. Cloning and localization of a glutathione S-transferase class I gene from *Anopheles gambiae*. J. Biol. Chem. 272, 5464–5468.

Ranson, H., Hemingway, J., 2005. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Glutathione Transferases. Comprehensive Molecular Insect Science – Pharmacology, vol. 5. Elsevier, Oxford, pp. 383–402.

Ranson, H., Rossiter, L., Ortelli, F., Jensen, B., Wang, X., Roth, C.W., Collins, F.H., Hemingway, J., 2001. Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. Biochem. J. 359, 295–304.

Saavedra-RodriguezStrode, K.C., Strode, C., Flores Suarez, A., Fernandez Salas, I., Ranson, H., Hemingway, J., Black, W. C. t., 2008. Quantitative trait loci mapping of genome regions controlling permethrin resistance in the mosquito *Aedes aegypti*. Genetics 180, 1137–1152.

Saavedra-Rodriguez, K., Urdaneta-Marquez, L., Rajatileka, S., Moulton, M., Flores, A.E., Fernandez-Salas, I., Bisset, J., Rodriguez, M., McCall, P.J., Donnelly, M.J., Ranson, H., Hemingway, J., Black, W. C. t., 2007. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*. Insect Mol. Biol. 16, 785–798.

Simmons, T.W., Jamall, I.S., Lockshin, R.A., 1989. Selenium-independent glutathione peroxidase activity associated with glutathione S-transferase from the housefly, *Musca domestica*. Comp. Biochem. Physiol. 94, 323–327.

Strode, C., Wondji, C.S., David, J.P., Hawkes, N.J., Lumjuan, N., Nelson, D.R., Drane, D.R., Karunaratne, S.H., Hemingway, J., Black, W. C. t., Ranson, H., 2008. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. Insect Biochem. Mol. Biol. 38, 113–123.

Vontas, J.G., Small, G.J., Hemingway, J., 2001. Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. Biochem. J. 357, 65–72.

Wang, Y., Qiu, L., Ranson, H., Lumjuan, N., Hemingway, J., Setzer, W., Meehan, E., Chen, L., 2008. Structure of an insect epsilon class glutathione S-transferase from the malaria vector *Anopheles gambiae* provides an explanation for the high DDT-detoxifying activity. J. Struct. Biol. 164, 228–235.

WHO, 1998. Report of the WHO Informal Consultation. Test Procedures for Insecticide Resistance Monitoring in Malaria Vectors, Bio-efficacy and Persistence of Insecticides on Treated Surfaces WHO/CDS/CPC/MAL/98.12.

Yanola, J., Somboon, P., Walton, C., Nachaiwieng, W., Prapanthadara, L., 2010. A novel F1552/C1552 point mutation in the *Aedes aegypti* voltage-gated sodium channel gene associated with permethrin resistance. Pestic. Biochem. Physiol. 96, 127–131.