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Expression patterns of epsilon glutathione S – transferases genes in developmental stages of susceptible and DDT resistant lines of *Anopheles arabiensis* strains

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Abstract
Expression patterns of three epsilon GST genes were studied in developmental stages of two strains of *Anopheles arabiensis* (*An. arabiensis*) which differed in susceptibility to DDT. The mosquito strains reared to several generations were successively exposed to and selected for DDT resistance. Quantitative real-time PCR was used to compare the transcript levels of these genes in ontogenic stages of the mosquitoes. The expression patterns of *GSTe1* and *GSTe2* varied relatively in proportion to resistance status and sequential life stages while *GSTe4* was expressed invariably. Further investigation is needed to understand the factors regulating the expression of the individual genes in each life stage of *An. arabiensis*.

Keywords: *Anopheles arabiensis*, developmental stages, DDT, expression, GSTs, resistance

1. Introduction
Glutathione S – transferases (GSTs) are large family of enzymes involved in the metabolism of xenobiotic compounds and in mosquitoes, the Epsilon GSTs particularly have been implicated in resistance to DDT [1, 2, 3]. During development, mosquitoes live in diverse habitats and feed on various diets encountering harmful substances that must be rapidly detoxified. The adults feed on plant nectar and mammalian blood which is rich in potentially toxic substances such as heme, while larval diet often contains allelochemicals. The Anopheline mosquitoes typically breed in temporary rain pool, irrigation rice fields, hoof prints and other anthropogenic environments which are often contaminated with pesticides residues [4, 5]. Adults of Anopheline species such as *An. gambiae* and *An. arabiensis* that are endophilic may encounter insecticide treated surfaces when searching for human host or resting indoors after taking blood-meal [6, 7]. The broad spectrum of xenobiotic compounds which mosquitoes encounter has necessitated the development of extensive families of detoxification enzymes including the GSTs [8, 9]. Expression patterns of the different GSTs which have been studied in mosquitoes varied markedly between developmental stages and in addition also, that of Epsilon GSTs was influenced by previous exposure to insecticides. In investigating the life-stages profile of *GSTe2* in three strains of *Aedes aegypti*, [10] reported the highest expression level in larvae in the DDT-resistant PMD-R strain. Recent studies on expression profile using whole organism transcriptome approach have provided supporting evidence for developmental regulation of hundreds of genes including GSTs [8, 11, 12]. Have shown overexpression of GST gene cluster in the larval stages of *An. colluzzi* which was found breeding in organically polluted urban environment. Five out of eight epsilon class GSTs were shown to be constitutively expressed at high levels in one – day old adults and fourth stage larvae in *An. gambiae* [12, 13, 14]. Constructed *An. gambiae* specific microarray chips containing unique fragments of detox genes including 35 GSTs to compare expression profile of the detoxification genes between DDT-resistant and susceptible strains. They showed GSTe2 and four other genes to be strongly up-regulated in the DDT-resistant Zan/U compared to the susceptible Kisumu strain. Using the detox chips in life-stage gene expression analysis, [15] reported 2 – fold higher level of *GSTe1* and *GSTe2* in larvae than adults of *An. gambiae*. Until recently, attention has been focused on studies on *An. gambiae* ss GSTs and relatively little is known about *An. arabiensis* GSTs. *An. arabiensis*, a genetically close species to *An. gambiae* ss, is a major malaria vector in Africa [16, 17]. Overexpression of *GSTe4* has been reported in adults both from the field and laboratory populations of *An. arabiensis* resistant to DDT [18, 19].

We have isolated and identified eight epsilon GSTe genes from *An. arabiensis* MAT strain. (Yayo *et al.*, in press). In this work, the basal expression of three of the GSTs, *GSTe1*, *GSTe2* and *GST4* was investigated in the larval, pupal and adult stages of MAT-P and KGB-P strains which differed in their susceptibility to DDT. To further assess the effect of exposure to insecticides on expression of each of the three *An. arabiensis* GST genes, the experiments were repeated with mosquitoes from the KGB selected line. This strain, KGB-R

has been maintained on selection pressure with 4% DDT for over 20 generations. Quantitative real-time PCR was used to quantify the transcripts of the three *An. arabiensis* GST genes in life-stages of the susceptible and resistant strains. The initial copy number of each of the GST transcripts in the cDNA was quantified by comparison with standard curves, which were generated using serially diluted plasmids containing full-length cDNA from each of the Epsilon genes. Variation in concentration of the templates was normalized using the ribosomal protein gene sp7.We reported differential expressions of the genes between the ontogenic stages and between the strains such that *GSTe1* and *GSTe2* were significantly overexpressed in the KGB-P and KGB-R compared to MAT-P strains. The role of these genes in adaptation to environmental xenobiotics and potential functions in the other biosynthetic pathways during development of this malaria vector is discussed.

2. Methods

2.1 Mosquito Strains

Three *An. arabiensis* strains were used in this study. *An. arabiensis* MAT originated from Mozambique and was susceptible to DDT. KGB originates from Zimbabwe and showed low level resistance to DDT. Adult mosquitoes from the KGB strain were kept under selection pressure with 4% DDT according to the standard [21] to obtain the selected line, the KGB-R strain. Colonies of the three strains were reared under standard rearing conditions of 25^{oc} with 80% RH and 12 h of day/night cycle and maintained in insectary at Liverpool School of Tropical Medicine. Larvae were fed on diet of premeasured quantity of fish food TeraMin, Tetra Germany.

2.2 Testing mosquitos for susceptibility to DDT

Adult mosquitoes from the colonies of the parental lines of *An. arabiensis* MAT and *An. arabiensis* KGB strains were tested for susceptibility to DDT. Bioassays were performed according to WHO protocols using standard WHO susceptibility test kits and DDT impregnated papers. Batches of 20 to 25 non-blood fed adult mosquitoes were exposed to 4% DDT for I hour and mortality recorded after 24 hours [21]. Each test was replicated at least four times with different mosquito batches, to take into account inter-batch variability. The number of knocked-down mosquitoes was recorded at ten minutes intervals during the one hour exposure period. The KDT50 and KDT90 knock-down times were calculated by probit analysis using Minitab 14 and LDP line software programmes.

2.2.1 Selection for resistance to DDT

Mosquitoes from the *An. arabiensis* MAT and KGB strains, which survived previous exposures to DDT, were reared and their progeny subjected to selection using 4% DDT. Cohorts of 10 to 25 adult mosquitoes of both sexes were placed in WHO exposure tubes, with many replicates per generation (depending on the availability of mosquitoes), and exposed to DDT for 30 minutes. Exposure times were increased to 40, 45, 50 and 60 minutes gradually as the mortality decreased through selected generations. Final mortality for each cohort was recorded 24-hour post-exposure. Survivors from each test were put back into a new cage, blood fed and reared for further selection. Mosquitoes from both *An. arabiensis* MAT and KGB strains were selected up to F 20 generations each. Batches of mosquitoes from *An. arabiensis* MAT and KGB selected lines were tested for resistance to DDT following the WHO Susceptibility protocol [21].

2.3 cDNA synthesis

Total RNA was extracted from triplicate batches, each of 4th instar larvae, pupae or one-day old adults (5 male, 5 female) sampled from each of the three mosquito strains. The samples were homogenized in 400 µl of TRI reagent (SIGMA) and centrifuged at 12,000 g for 10 min at 4$^{o.}$ The supernatant was transferred to a DEPC- treated centrifuge tube, homogenized for 15 sec with chloroform and was incubated at 4^{oc} after which the upper aqueous phase containing RNA was transferred to a fresh tube. To precipitate the RNA, 200 µl of molecular grade isopropanol was added. The sample was mixed and incubated at room temperature for 10 min. After centrifugation at 12,000 g for 10 min at 4^{oc}, the pellet was air-dried and re-suspended in 26 µl of nuclease-free water. The RNA was treated with RQI DNase (Promega) to remove contaminating genomic DNA.

cDNA was synthesized from total RNA using superscript T^{M111}RNase H^{-} reverse transcriptase (Invitrogen) and an oligo (dT) adapter primer (5´-GACTCGAGTCGACATCGA (dT) $_{17}$ – 3´). One microgram of RNA was mixed with 1µg of the primer and heated to 65ºC for five minutes to dissociate the secondary conformation. The reaction mixture was first chilled on ice for 1 minute and then pre-warmed to 50ºC for 2 minutes after addition of 8µl of 5X first strand buffer (250mM Tris-HC1 pH 8.3, 375mM KCL, 15mM $MgCl_2$), 2µl of 10mM of each dNTP and 2µl of 0.1M DTT. One half microlitre of Superscript T^{M111}Rnase H^{-} reverse transcriptase was added and the reaction was incubated at 50ºC for 90 minutes. The reaction was then heated to 70ºC for 15 minutes to inactivate the enzyme. First strand cDNA samples were stored at -20ºC.

Primer pair sequences designed for the amplification of individual members of the *An. gambiae* Epsilon class GST genes [13] were used to amplify the full-length *GSTe1, GSTe2* and *GSTe4* genes using *An. arabiensis* cDNAs as templates. The PCR reactions contained 1.5mM $MgCl_2$, 0.2mM of each dNTPs, and 0.5 µm of each primer, 1 X reaction buffer (Qiagen) and 1.25 units of Taq DNA polymerase (Hotstar) in a final volume of 25µl. Annealing temperatures ranging from 50ºC – 60ºC were determined empirically for each gene using a gradient PCR machine PTC-200 (MJ Research). The cycling parameters were 95ºC for 15 minutes, followed by 30 cycles of 95ºC for 30 seconds, 50ºC – 60ºC for 30 seconds and 72ºC for 30 seconds and a final extension of 72ºC for 10 minutes. The PCR products were separated on a 2% (w/v) agarose gel in the presence of ethidium bromide (0.5µg/ml) and then visualized using the Gene Genius Bio Imaging system (Syngene). The PCR products of expected size were sub cloned into the P^{GEM}-T Easy Vector (Promega) and used as templates for sequencing. For each GST, three independent clones were sequenced using Beckman procedure. The resultant products were analysed on a Beckman CEQ800 capillary sequencer.

2.4 Plasmid Construction

The *GSTe1*, *GSTe2* and *GSTe4* standard plasmids were constructed by insertion of full-length cDNA from the MAT and KGB strains into the P^{GEM}-T Easy Vector. The ribosomal protein gene SP7 (accession no. AY 380336) [22] used as an internal control plasmid, was amplified using primers SPC and SPD (Table 1) and ligated into the P^{GEM}-T Easy Vector. The concentrations of the plasmids were determined using an ND-1000 spectrophotometer (Nanodrop Technologies).

2.5 Quantitative PCR

The *GSTe1*, *GSTe2*, *GSTe4* and *SP7* standard plasmids were diluted serially to concentrations ranging from 1 fg/µl to 1ng/µl. Quantitative PCR was used to quantify and compare the abundance of the transcripts from each of the 3 GST genes in the different life-stages of the MAT and KGB strains. In all cases the primers were designed to span an intron as a control against genomic DNA contamination, and for each of the GST genes, the primers were selected to avoid regions of allelic variation within the gene sequences (Table 1). Strain-specific primers were used for amplifying *GSTe1* and *GSTe4* genes from the MAT and KGB because of the nucleotide differences at the primer binding sites between the variant gene sequences from the strains. The plasmid and cDNA templates were amplified using the Quantitative SYBR Green PCR Kit (Qiagen). SYBR Green 1 is a double-strand DNA specific dye, which binds to the minor groove of the DNA helix during amplification cycles. Quantitative PCR was performed by amplifying 1µl of cDNA with 0.5µM of each primer and 1 x SYBR Green Master Mix in a final volume of 15µl. The conditions used for the amplification of each of the three Epsilon GSTs and the *SP7* genes are specified in Table 1. The PCR reaction was conducted for 35 cycles. Plasmid DNA standards and negative controls were included in the same plate, for each experiment. Three biological replicate samples from each strain and stage in the life-cycle were used as templates. A sample was analysed in duplicate in each experiment and results were averaged from three separate experiments. The incorporation of the SYBR Green 1 into the PCR products was quantified using the DNA Engine Opticon (MJ Research). The fluorescence was plotted in relation to the number of cycles and the crossing line was produced to obtain the standard concentration related to the cycle number. A straight line standard curve was obtained by plotting the cycle number against the logarithmic value of the standard concentration.

2.6 Gene copy number calculation

The initial copy number for each of the Epsilon GSTs was calculated automatically by measuring the fluorescence produced due to incorporation of the SYBR Green 1 dye into the double stranded PCR product. The value was then compared to the standard curve produced from the PCR amplification of the fragment of the particular gene from plasmids of known concentrations. From the sequences of the P^{GEM}- T Easy Vector and cDNA fragments that were inserted into the vector, the molecular weights (MW) of the plasmids used to produce the standards were calculated. The mRNA copy number of each transcript was computed by multiplying the value of plasmid molecular weight by Avogadro's constant.

3. Results

3.1 WHO susceptibility assays

The susceptibility levels to DDT of adult mosquitoes of the original parental populations of the *An. arabiensis* MAT and KGB strains were determined. There has been no previous record of exposure of either of the strains to any class of insecticide and the KGB has been used as a laboratory reference insecticide susceptible strain (Brooke, personal communication). The WHO test kits were used and tests were carried out using the WHO recommended discriminating dosage. About 338 two to three day old adult mosquitoes from the parental *An. arabiensis* MAT strains were exposed to 4% DDT for different time periods. The percentage mortality recorded 24 hours after exposure is shown (Table 2). The 87% mortality after exposure to DDT for 1 hour is indicating the presence of low level of resistant genotypes in the MAT parental colony.

In the KGB strain, 389 adult mosquitoes in batches of 20 – 25 were exposed at the four different time points and mortality was recorded 24 hours post exposure as shown (Table3). The 81.6% mortality after exposure to DDT for 1 hour suggests higher level of resistance genotypes in KGB than in the MAT colony.

After selection, the susceptibility tests with the diagnostic dose of DDT (4%) were repeated at different time points for the parental and selected populations of both *An. arabiensis* MAT and KGB strains. The percentage mortality as recorded and plotted against the exposure time is shown (Figure 1).

The LT50 values for DDT were 23.4 min and 33.2 min (resistant ratio 1.4) in the parental and selected colonies of *An. arabiensis* MAT strain. The slopes of the regression lines are 2.96 in the parental and 2.4 in the selected lines respectively (Table 4). In the KGB strain, the LT50 values were 33.5 min and 50.8 min and the corresponding slopes of the regression lines were 3.26 and 1.7 in the parental and selected populations respectively (Table 4). The change in slope of regression lines between KGB selected and parental indicates increased resistance in the selected population.

3.2 Initial copy number of GST transcript

The copy number of mRNA computed from the plasmids containing *GSTe1*, *GSTe2*, *GSTe4* and SP7 genes is shown in Table 5. The length of cDNA of each gene from *An. arabiensis* MAT and KGB strains and molecular weight of the plasmids is indicated. From the sequences of PGEM- T Easy Vector and cDNA fragments that were inserted into the vector, the molecular weights (MW) of the plasmids used to produce the standards were calculated. The length of each Epsilon GST and SP7 cDNAs, the AT and CG contents, the molecular weights of the plasmids and the initial copy number per fentogram (fg) of each Epsilon plasmid are given in the (Table 5).

3.3 Standard Curves

The standard curves produced from amplification of plasmids serially diluted to concentrations ranging from 1fg to ng are illustrated in Figure 2A. To assess the developmental expression pattern of Epsilon GSTs in *An. arabiensis* and the

effect of DDT resistance on the expression, the messenger RNA copy number of the Epsilon GSTs genes in different life-stages of MAT, KGB-P and KBG-R strains was determined. These three lines of *An. arabiensis* differed significantly in their response to DDT. The resistance ratio between MAT and KGB-P is 1.5 and the KGB-R has been selected for DDT resistance up to 20 generations. Complementary DNA from 3 biological replicate samples each taken from a batch of ten individuals were used as templates in quantitative real – time PCR. Three developmental stages: larvae (L), pupae (P), and adult (A) were assessed. A sample was analysed in duplicate in three independent experiments. From the standard curves shown in Figure 2 (A), the fluorescence values obtained with the cDNA samples in each life stage was extrapolated as illustrated in Figure 2 (B).

Differences were evaluated for each gene separately. One-way analyses of variances were applied initially to test for differences across all three life stages in all three lines simultaneously; significant results were then investigated in detail using the Turkey "honestly significant difference" multiple comparison test (a less conservative variant of the Bonferroni procedure) to protect against spurious significance due to multiple testing [23]. Statistical significance was set at the conventional 5% level for all tests. The expression of individual genes was compared between developmental stages within and between strains as follows:

3.3.1 GSTe1 expression levels

The observed levels of *GSTe1* expressed at each life stage, the geometric means and 95% confidence intervals for these observations are shown for each strain separately in Figure 3.

In the MAT strain the mean levels differed significantly in larvae compared to adult and pupae ($p < 0.001$ for all comparisons). The lowest average level was found at the pupal stage. The mean level at the adult stage was approximately 50% higher, while the mean level at the larval stage was 5.5 times greater than at the pupal stage. The expression of *GSTe1* in the KGB-P strain showed mean levels at the larvae and adult stages to be both significantly greater than that at the pupae stage ($p < 0.001$), but the larval and adult stages did not differ significantly ($p > 0.999$). The mean levels at the larvae and adult stages were approximately 4.5 times greater than at the pupal stage. In the KGB-R line, again, mean levels of *GSTe1* at the larvae and adult stages were both significantly greater than that at the pupae stage ($p < 0.001$), but the larvae and adult stages did not differ significantly ($p = 0.997$). The mean levels at the larvae and adult stages were approximately 20 times greater than at the pupal stage.

3.3.2 GSTe2 expression levels

For *GSTe2* the observed levels of expression, the geometric means and their 95% confidence intervals for these observations are shown in Figure 4. In the MAT –P strain the mean levels (≤ 0.19), at the larval and adult stages were both significantly greater than that at the pupal stage ($p < 0.001$), but the larval and adult stages did not differ significantly ($p = 0.977$). The mean levels at the larvae and adult stages were around 60% greater than at the pupal stage. For the KGB strain, in the KGB –P line, the mean level of *GSTe2* at the larvae stage was 2 and 2.5 times greater than the means at the pupae and adult stages respectively – both differences were highly significant ($p < 0.001$). However, the difference between the pupae and adult stages was not statistically significant ($p = 0.849$). In KGB-R line the mean level at the larvae stage was 3.8 and 2.6 times greater than at the pupae and adult stages respectively – both differences were highly significant ($p < 0.001$). The mean level at the adult stage was approximately 50% higher than that at the pupae stage – this difference was significant, but at a lower level ($p = 0.010$).

3.3.3 GSTe4 expression levels

The geometric means and 95% confidence intervals for the observed levels of *GSTe4* expressions at each life stage are shown for each strain in Figure 5. The mean level at the larval stage was 75% greater than that at the pupal stage – this difference was statistically significant ($p = 0.021$) in the MAT -P. However, no significant difference was found between the larvae and adult stages ($p = 0.525$), or between the pupal and adult stages ($p = 0.763$) in this strain. In KGB strain, the mean levels at the larval and pupal stages were 4.2 and 8.6 times greater than the mean at the adult stage respectively – both differences were highly significant ($p < 0.001$) in the KBP - P. And the mean level at the pupal stage was approximately double the mean level at the larvae stage ($p = 0.001$). In KGB-R line, the mean levels at the larvae and adult stages were 3.5 and 5.1 times greater than at the pupal stage respectively – both differences were highly significant ($p < 0.001$). The mean level at the adult stage was approximately 50% higher than that at the larval stage – but this difference was not statistically significant ($p = 0.176$).

3.4 Comparative expressions of GSTs in *An. arabiensis* strains

After testing for significant differences in levels of the expressions of the individual genes across life stages within strains, significant results were analysed further using the Turkey "honestly significant difference". This is a multiple comparison test which protects against false significance due to multiple testing. The relative expressions of each of the three genes in all the life stage in the strains are shown (Table 7).

4. Discussion

The expression patterns of *An. arabiensis* Epsilon *GSTe1*, *GSTe2* and *GSTe4* genes were investigated in developmental stages of the MAT – P and the two lines (KGB- P and KGB-R) of the KGB strain. These laboratory raised mosquito strains differed significantly in susceptibility to DDT. The expression levels of the three GST genes were measured at mRNA level. Results showed that these GST genes were significantly expressed more in the resistant compared to the susceptible strains. Several recent studies on mosquitoes have implicated Epsilon GSTs in resistance to various insecticides including DDT [8, 24, and 25]. The results demonstrated further the differential expression patterns of the individual GSTs in various developmental stages of the two *An. arabiensis* strains. Similar observations were also reported in DDT-resistant strains of the malaria vector *An. stephensi* in India [25]

and the African malaria vector *An. gambiae* [11, 12, 15]. *GSTe1* was expressed at higher levels in larval and adult stages than in pupae in all the strains [26]. Have reported over transcription of this gene in resistant DOB-R strain of *An. stephensi*. The consistent high expression of the gene in the larvae and adults may indicate its involvement in protecting the Anopheles mosquito against stressful effect of dietary and environmental chemicals to which it may be exposed at these stages of the life cycle [8, 27]. The ortholog *AgGSTe1* in *An. gambiae* possesses peroxidase activity and can protect cells against oxidative stress [28].

GSTe1 was over expressed 4.5 fold in larvae and 16 fold in adult in the KGB – P and KGB – R compared with the MAT – P, suggesting a positive correlation with resistance status of the strains. Recombinant *GSTE1* does not metabolize DDT, but it is possible the enzyme may be conferring some resistance to the secondary effects of insecticide exposure. GSTs with peroxidase activity have been implicated in pyrethroid resistance in a resistant strain of plant hopper [29] and Drosophila larvae exposed to oxidative stress [30]. However, whether *GSTe1* in *An. arabiensis* possess peroxidase activity remains to be investigated. Also in this study, the positive correlation of the expression of *GSTe1* with resistance status is not reflected in the KGB – P and KGB – R lines, in both of which, the mean levels of the expression in larvae and adults did not differ significantly ($P > 0.999$) [15]. Have reported higher expression level of *GSTe1* in larvae than in pupae and adult in *An. gambiae*.

GSTe2 was significantly over expressed in larvae and adult ($P < 0.001$) than in pupae in all strains. The orthologs of *GSTe2* in the mosquitoes *Aedes aegypti* and *An. gambiae* were also expressed at higher levels in larvae than in later developmental stages [10, 15]. Relative to the MAT –P strain, mean expression levels of *GSTe2* were 6.6 and 12.8 times greater in KGB – P and KGB – R ($p < 0.001$ for both comparisons). A positive correlation of high GSTe2 transcript with resistance status is indicated by the higher expression in KGB – R compared to KGB – P line ($p < 0.001$).

However, in this study, only the adult stage was selected with DDT and it is not known if the resistance observed in this life stage was conferred at the larval stage also. Nevertheless, the mechanism responsible for up regulation of *GSTe2* in larvae of *An. arabiensis* KGB – R line is unknown. Out of the three *An. arabiensis* GST genes that were investigated in this study, *GSTe2* was over expressed at consistently higher levels relative to *GSTe1* and *GSTe4* in all the life stages as has been reported [11, 15]. The high expression of this gene suggests that it may be ubiquitously expressed in mosquito tissues and may indicate a general housekeeping or biosynthetic role in addition to its involvement in specialized detoxification pathways [25].

In contrast, the expression of *GSTe4* fluctuated in all the strains in different life stages and without definite correlation with insecticide resistance status. For example, in the KGB – P, the gene was expressed at higher levels in the larvae and pupae than in adult but the expression was 3.5 and 5.1 times greater in larvae and adult stages than in pupae in the KGB R line. Functional studies on allelic variants of *An. arabiensis GSTE4* do not support direct role in detoxification of insecticide [31]. Similar observation has been made in *Aedes aegypti* ortholog, AeGSTe4 which was also expressed at significantly high level in pupae and adult in parental PMD but not in PMD – R strain [10]. However, in this study, the mean expression level of *GSTe4* in adult stage was significantly higher in the KGB – R strain than in MAT – P and KGB – P ($p < 0.001$), but the MAT – P and KGB – P strains did not differ significantly ($P = 0.394$). Regulation of GST expression is subject to a complex set of sex, tissue-specific and developmental factors as well as dietary and environmental parameters [8, 32]. The mechanisms responsible for the regulation of these genes at developmental stages of *An. arabiensis* need to be investigated. The knowledge on the susceptibility or detoxification capacities of different developmental stages is required to determine the stage at which resistance monitoring test should be directed.

5. Tables and Figures

Table 1: Primers used in quantitative real-time PCR

Gene	Primer Name	Primer Sequences (5′ to 3′)	cDNA size	Annealing	Extension	Reading
GSTe1	*GSTelqAF*	GTC AAT GAG GCA CTG CAC TTC	180	60ºC for 30 seconds	72ºC for 30 seconds	82ºC
	GSTelqAR	GTG ATC CGG CTA CGT AAT TG				
	GSTelAKR	AGC TCA GGT CAG CGA TCG TC				
GSTe2	*GSTe2*Qaf	ATC ACC GAG AGC CAC GCG ATC A	280	60ºC for 30 seconds		
	150HGSTR	GCC ACG GTT CGC TTC CTC GTA GTA				
GSTe4	15BGST	CGC CAT TCA AAC GAC CAT GCC	229	60ºC for 30 seconds		
	160HR	GAT GGC GTG GCT GTC CCA CAC G				
	*GSTe4*qAKR	CAT TGA TGG CGT GGC TGT CC				
SP7	SPC	GCA CGT CGT GTT CAT TGC CG	291	56ºC for 30 seconds		85ºC
	SPD	GAA CAT TAA CGT CAC GGC CAG TCA				

GSTe1AKR and *GSTe4qAKR* are the reverse primers used for amplifying *GSTe1* and *GSTe4* from the KGB strain

Table 2: Susceptibility of adult mosquitoes in parental MAT colony to DDT

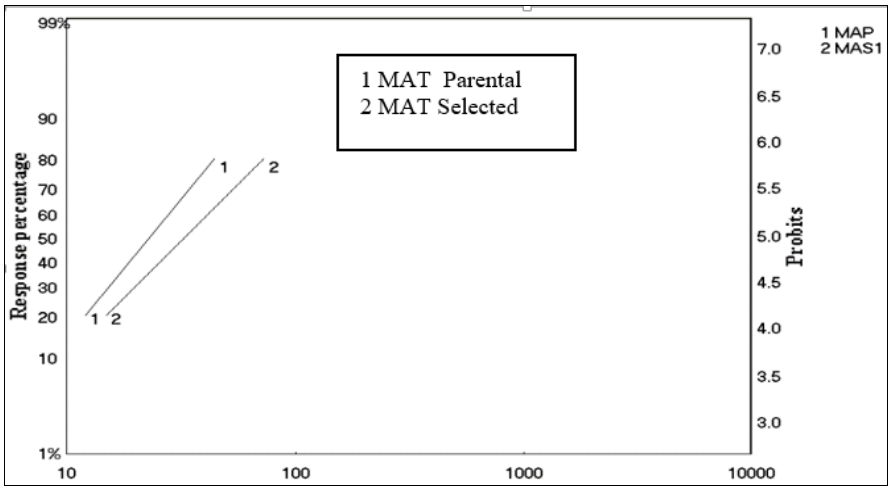
Time (min) Exposure	Number Tested	Number Dead	Number Alive	% Mortality 24 post-exposure
15	58	16	42	27.5
30	80	53	27	66.3
45	135	116	19	85.9
60	65	57	8	87.6

Initial scores for mortality from WHO diagnostic test kit for 4% DDT tested against adult mosquitoes (n=338) sampled from the F1 generation of parental line

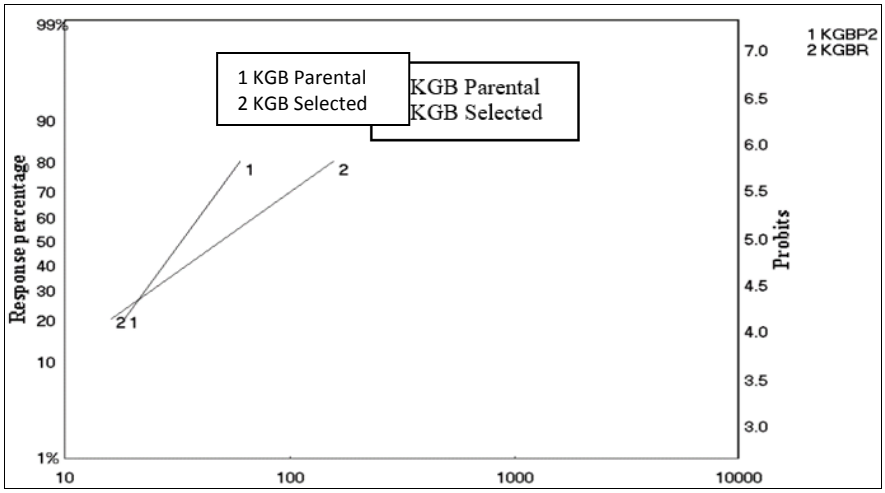
Table 3: Susceptibility of adult mosquitoes in parental KGB colony to DDT

Time (min) Exposure	Number Tested	Number Dead	Number Alive	% Mortality 24 post-exposure
15	67	13	54	18.6
30	85	38	47	44.7
45	98	65	33	66.3
60	148	121	27	81.6

Initial scores for mortality from WHO diagnostic test kit for 4% DDT tested against adult mosquitoes (n=398) sampled from the F1 generation of parental line



1A



1B

Fig 1A: Time – response curves for the parental (MAP), and selected lines (MAS) and 1B the parental (KGBP), and selected lines (KGBR) of the MAT and KGB strains exposed to exposed to 4% DDT

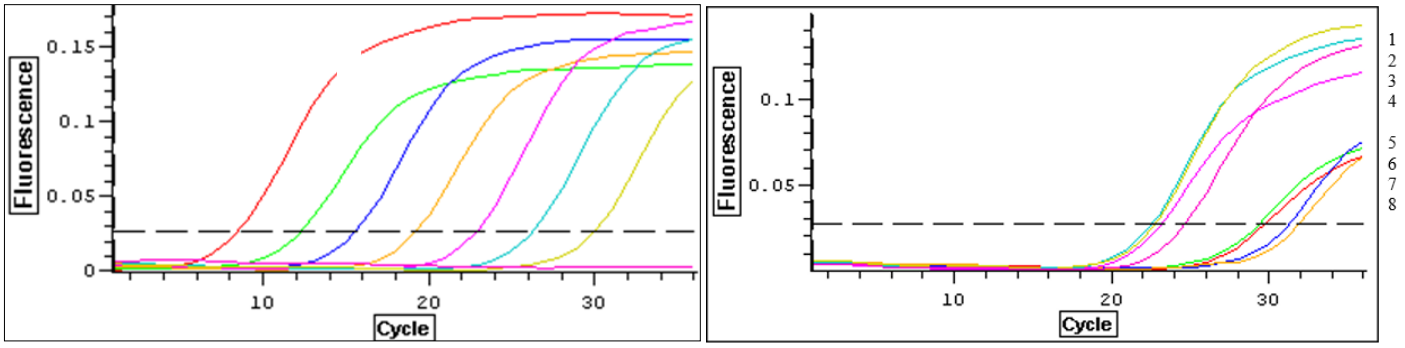
Table 4: Relative susceptibility of DDT (4%) based on time mortality relationships tested against parental and selected lines of *An. arabiensis* MAT and KGB strains

Strain	Line	Sample	No tested	LT50 (min)	CI	LT90	Slope	RR	X^2
MAT	Parental	F20	355	23.4	(20.3 – 26.1)	63.40	2.9 ± 0.3	1	0.703
	Selected	F20	290	33.2	(29.2 – 37.7)	88..96	2.40 ± 0.3	1.4	1.301
KGB	Parental	F20	360	35.1	(28.2 – 37 3)	95.06	3.26 ± 0.5	1.5	0.821

Table 5: Calculation of the initial copy number of epsilon GST cDNA plasmids

GenBank name	Length of cDNA (bp)	AT content	GC content	Molecular weight of full-length Cdna	Molecular weight of Plasmid	Initial copy No/fentogram
GSTe2	624	278	346	440118	22571373	234.2
GSTe1	675	313	362	475905	2607160	231
GSTe1K	676	294	382	475478	2606733	231
GSTe4	693	294	399	488309	2619564	236.4
GSTe4K	697	291	406	491899	2623154	236
SP7	489	232	257	344688	2475943	241.3

GSTe1K and GSTe4K are plasmids containing the genes amplified from KGB strain



2A **2B**

Fig 2: Quantification of *GSTe2* mRNA expression levels in Larvae and Adults *An. arabiensis* KGB strain. **(A)** SYBR Green 1 fluorescence acquisition by PCR products from serially diluted (1 ng to 1fg) standard plasmids and **(B)** biological replicates cDNA samples each extracted from 10 randomly selected individual larvae and adults from KGB selected line. 1 – 4 represent replicates larval samples and 5 – 8 adult samples. The X – axis represent the cycle number and the Y – axis indicates the value of the fluorescence acquisition. A to G in (A) indicate the concentrations ranging from 1 ng/µl to 1 fg/µl in the serially diluted plasmids containing the *GSTe2* insert.

Table 6: Quantitative PCR result of *An. arabiensis* GSTs Normalized cDNA copy number

Gene	Life stage	MAT	(95%CI)	KGB-P	(95%CI)	KGB-R	(95%CI)
GSTe1	Larvae	0.137±0.02	(0.123– 0.152)	0.614±0.06	(0.575 – 0.655)	0.618±0.12	0.536 – 0.701
	Pupae	0.023±0.023	0.019 – 0.28	0.123±0.02	(0.120 – 0.146)	0.03±0.01	(0.024 – 0.058
	Adult	0.036±0.01	(0.031 –0.041)	0.586±0.11	(0.521- 0.658)	0.566±0.10	(0.485– 0.660)
GSTe2	Larvae	0190±0.06	(0,152– 0.237)	1.249±0.23	(1.125 – 1.387)	2.426±0.55	(1.988– 2.962)
	Pupae	0.115±0.01	(0.110 –0.119)	0.507±0.10	(0.451 – 0.569)	0.635±0.0.09	(0.575– 0.701)
	Adult	0.172±0.04	(0.148 –0.199)	0.584±0.11	(0.516 - 0.661)	0.938±0.42	(0.672– 0.764)
GSTe4	Larvae	0.066±0.03	(0.045– 0.096)	0.151±0.02	(0134 – 0.171)	0.091±0.02	(0.075– 0.109)
	Pupae	0.038±0.01	(0.03 –0.046)	0.306±0.07	(0.265 – 0.355)	0.026±0.07	(0.021– 0.052)
	Adult	0.049±0.01	(0.042 –0.058)	0.036±0.01	(0.030 - 0.042)	0.135±0.07	(0.098– 0.018)

The GST transcript copy number was determined by normalizing with transcript copy number of ribosomal *Sp7* transcript.

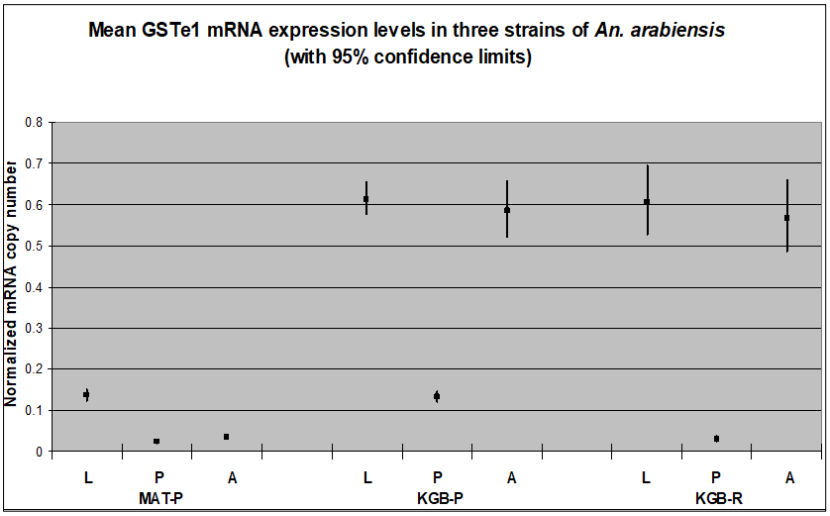


Fig 3: Geometric mean *GSTe1* mRNA expression levels in three strains of *An. arabiensis* (with 95% confidence limits)

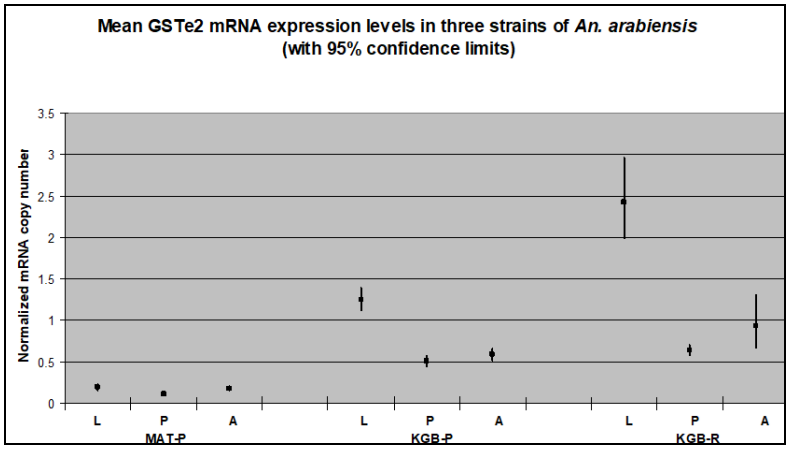


Fig 4: Geometric mean *GSTe2* mRNA expression levels in three strains of *An. arabiensis* (with 95% confidence limits)

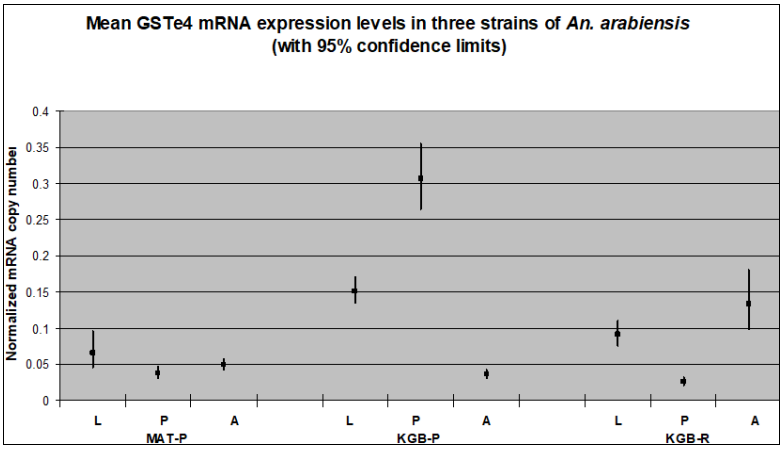


Fig 5: Geometric mean *GSTe4* mRNA expression levels in three strains of *An. arabiensis* (with 95% confidence limits)

Table 7: Comparative expressions of GSTs in developmental stages between strains

Gene	Life Stages	Ratio of Kgb-p/mat	Copy Kgb-r/mat	Number Kgb-r/kgb-p
GSTe1	Larvae	4.5***	4.5***	1
	Pupae	5.7***	1.3	0.2
	Adult	16.2***	15.7***	0.96
GSTe2	Larvae	6.5***	12.8***	1.9***
	Pupae	4.4***	5.5***	1.2
	Adult	3.4***	5.5***	1.6**
GSTe4	Larvae	2.2***	1.4*	0.6
	Pupae	8.8***	0.7	0.8
	Adult	0.7	2.8***	3.8***

The transcript copy number of each GST was determined by normalizing with transcript number of ribosomal SP7 gene. The ratio of the average copy is obtained by comparing the resistant with the susceptible in each pair. Statistical differences were evaluated using the Turkey "honestly significant difference" multiple comparison test (a less conservative variant of the Bonferroni procedure). Statistical significance was set at the conventional 5% level for all tests ($p < 0.001$ indicates by ***).

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