Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*

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Selection of a laboratory colony of the brown planthopper *Nilaparvata lugens* with the pyrethroids permethrin and λ-cyhalothrin increased its resistance to both insecticides. Biochemical analysis and synergistic studies with metabolic inhibitors indicated that elevated glutathione S-transferases (GSTs) with a predominant peroxidase activity conferred resistance to both pyrethroids, whereas esterases conferred part of the resistance to permethrin. Purified esterases hydrolysed permethrin at a slow rate, but incubation of either pyrethroid or their primary metabolites with partially purified GSTs had no effect on the metabolic profile. Although GSTs were sensitive to inhibition by both pyrethroids, they did not serve as binding proteins, as previously hypothesized [Grant and Matsumura (1988) Insect Biochem. **18**, 615–622]. We demonstrate that pyrethroids, in addition to their neurotoxic effect, induce oxidative stress and lipid peroxidation in insects. Pyrethroid exposure induced lipid peroxides, protein oxidation and depleted reduced glutathione. Elevated GSTs in the resistant strains attenuated the pyrethroid-induced lipid peroxidation and reduced mortality, whereas their *in vivo* inhibition eliminated their protective role. We therefore hypothesize that the main role of elevated GSTs in conferring resistance in *N. lugens* is through protecting tissues from oxidative damage. Our study extends the GSTs' range of efficacy to pyrethroid insecticides and possibly explains the role of elevated GSTs in other pyrethroid-resistant insects.

Key words: insecticide, λ-cyhalothrin, permethrin, planthopper.

INTRODUCTION

Glutathione S-transferases (GSTs) are a major family of detoxification enzymes. They catalyse the conjugation of the tripeptide glutathione to electrophilic centres of lipophilic compounds, thereby increasing their solubility and aiding excretion from the cell. They possess a wide range of substrate specificities, including endogenous substrates, such as reactive unsaturated carbonyls, reactive DNA bases, epoxides and organic hydroperoxides produced *in vivo* as the breakdown products of macromolecules during periods of oxidative stress [1]. Thus GSTs play a vital role in protecting tissues against oxidative damage and oxidative stress. The lack of Se-dependent glutathione peroxidases (GPOXs) in insects increases the potential importance of the putative Se-independent peroxidase function of GSTs in antioxidant defence, with the principle function of reducing organic hydroperoxides within membranes and lipoproteins [2,3].

The GSTs in insects are primarily of interest because of their role in insecticide resistance. They are involved in the O-dealkylation or O-dearylation of organophosphorus insecticides [4], as a secondary mechanism in the detoxification of organophosphate metabolites [5] and in the dehydrochlorination of organochlorines [6].

Although GSTs have not been implicated directly in pyrethroid resistance, there are reports of elevated GSTs in pyrethroid-resistant wild-caught [7–9] or laboratory-selected insects [10]. The involvement of GSTs in pyrethroid resistance is further indicated by synergistic studies with specific GST inhibitors, such as Cibacron Blue, in *Blattella germanica* [8] and *Musca domestica* [9]. However, the mechanism of GST-based resistance remains unknown. Grant and Matsumura [10] suggested that GSTs could act as pyrethroid-binding proteins, thus serving in a non-catalytic capacity similar to their role in higher vertebrates binding haem, bilirubin and hormones [11].

The majority of reports of pyrethroid resistance implicate either enzymic oxidation and hydrolysis by microsomal monooxygenases and carboxylesterases, or non-metabolic changes in target-site sensitivity [12,13]. Resistance to pyrethroids has been reported previously in the brown planthopper (BPH) *Nilaparvata lugens* Stål, one of the most devastating insect pests of rice [14]. The underlying mechanisms of resistance were reported as enhanced oxidative metabolism and possibly elevation of carboxylesterases [14]. Esterase-based resistance to organophosphorous insecticides in *N. lugens* is due to a single differentially glycosylated elevated enzyme, via gene amplification [15,16]. The role of this esterase in permethrin resistance is unclear, as Chen and Sun [17] reported esterase-mediated hydrolysis of permethrin, but Karunaratne et al. [18] could not detect any enzyme–permethrin interaction.

Alteration of the sodium-channel kinetics is the principal mode of action of pyrethroids, resulting in the delay of sodium-channel closure and membrane repolarization, which leads to spontaneous repetitive nerve firing and convulsions [19]. Besides this neurotoxic effect, a cytotoxic and genotoxic potential has been indicated in higher vertebrates [20,21], which is partially due to the induction of oxidative stress and free-radical-mediated lipid peroxidation [22,23]. The oxidative stress, apart from its direct cytotoxicity, has an additive or accelerating effect on the neurotoxicity of Na^+ channel blockers [24].

Abbreviations used: GST, glutathione S-transferase; GPOX, glutathione peroxidase; BPH, brown planthopper; AU strain, Australian strain of BPH; SR strain, Sri Lankan strain of BPH; SRC, λ-cyhalothrin-selected SR strain; SRP, permethrin-selected SR strain; DFP, di-isopropyl fluorophosphonate; EA, ethacrynic acid; PB, piperonyl butoxide; DEF, *S*,*S*,*S*-tributyl phosphorothioate; ECOD, ethoxycoumarin O-de-ethylase; CDNB, 1-chloro-2,4-dinitrobenzene; DNPH, 2,4-dinitrophenylhydrazine; DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)-ethane.

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EXPERIMENTAL

Reagents

Permethrin [3-phenoxybenzyl-(1*RS*,3*RS*;1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclo-propane carboxylate] as a 60:40 *trans*/*cis* mixture and as 98% pure *cis*, λ-cyhalothrin [*RS*-α-cyano-3-phenoxybenzyl(*Z*)-(1*R*,3*R*)-3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethyl-cyclopropanecarboxylate] and the primary pyrethroid metabolites 3-phenoxy-benzoic acid (99% pure) and *m*-phenoxylbenzyl alcohol (99% pure) were purchased from British Greyhound (Birkenhead, Merseyside, U.K.). Di-isopropyl fluorophosphonate (DFP) was purchased from Boots Pure Drug (Nottingham, U.K.). Q-Sepharose Fast Flow, phenyl-Sepharose Fast Flow and PD-10 columns were purchased from Pharmacia (St. Albans, Herts., U.K.). Amicon Centriprep 10 and Centricon 10 units were purchased from Amicon (Stonehouse, Gloucestershire, U.K.). The Chiradex column (250 mm × 4 mm, inner diameter 5 μm, LiChroCart) was purchased from Merck (Lutterworth, Leics., U.K.). Piperonyl butoxide (PB; technical grade, 90%), ethacrynic acid (EA), biochemicals and all other reagents were purchased from Sigma (Poole, Dorset, U.K.) except where stated.

Insects

Two BPH populations were sampled from rice plants in Batalagoda, Sri Lanka (SR strain), and Ingham, Australia (AU strain), and maintained in culture in Cardiff on rice [25].

Bioassays

Bioassays were performed by topical application. Insecticide was delivered in 0.2 μl of acetone to the abdominal sternum of adult females using a Hamilton microsyringe. Controls were exposed to acetone alone. Insects were then transferred into plastic tubes containing rice seedlings and left at 28 °C for 48 h before mortality was scored. Survivors of exposure to different doses were snap-frozen and used for measurement of oxidative damage. For selection experiments the LD_{80} values (the log concentrations killing 80% of the insects) for permethrin and λ-cyhalothrin was determined for each generation. A mass selection was then undertaken at this dosage and survivors were used to establish the next generation.

Metabolic inhibitors were applied via a tarsal contact method. Inner surfaces of 30 ml glass scintillation vials (2.4 cm × 7.4 cm) were coated with different amounts of metabolic inhibitors in acetone and allowed to dry by rolling. Batches of insects were pre-exposed to inhibitors for 15 min, at the maximum sublethal dose of inhibitor (after a 24 h recovery period; 10 μg of PB, 1 μg of DFP and 12 mg of EA), 1 h prior to the insecticide application. The EA exposure was repeated 12 h after insecticide application.

Data from bioassays were subjected to log-time probit mortality data regression analysis using a program written by Dr C. Schofield (World Health Organisation, Geneva, Switzerland).

Biochemical assays

Esterase and GST activities of individual BPH adults were determined spectrophotometrically on a UV_{max} microtitre plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) as detailed by Hemingway et al. [25]. A unit corresponds to the hydrolysis of 1 μmol of ρ-nitrophenol acetate/min for the esterase assay and to the formation of 1 μmol of 1-chloro-2,4-dinitrobenzene-glutathione (CDNB-GSH) conjugated product/min for the GST assay. An estimate of total cytochrome P450 content was obtained as detailed by Brogdon et al. [26]. Ethoxycoumarin O-de-ethylase (ECOD) activity of *N. lugens* abdomens was measured on a F2 fluorescence absorbence reader (Labtech, Ringmer, East Sussex, U.K.), as described by Stumpf and Nauen [27]. For the ECOD assay a unit corresponds to the formation of 1 nmol of 7-hydroxycoumarin/min. Protein was assayed by using the Bio-Rad protein assay kit (Bio-Rad, Hemel Hempstead, Herts., UK) with BSA as the standard protein [28].

Se-independent GPOX activity was measured by aliquoting crude insect homogenate containing approx. 0.1 mg of protein in 50 mM potassium phosphate buffer (pH 7.0) into a reaction mixture that consisted of 1 mM EDTA, 0.2 mM NADPH, 1 unit/ml glutathione reductase and 1 mM GSH in a total volume of 300 μl. The mixture was incubated for 5 min at 25 °C before initiation of the reaction by the addition of *t*-butyl hydroperoxide (1.5 mM). Oxidation of NADPH at 25 °C was measured kinetically as the decrease in absorbance at 340 nm for 4 min [29]. Units are given as nmol of NADPH oxidized/min per mg of protein ($\epsilon = 6.22\ mM^{-1} \cdot cm^{-1}$). Activity values were subtracted from blanks for the non-enzymic oxidation of NADPH by the peroxide substrate and non-specific oxidation of NADPH by the crude homogenate.

Oxidized (GSSG) and total (GSH + GSSG) glutathione levels were measured using the method of Griffith [30]. GSH content was estimated by substracting GSSG from the total.

Measurement of lipid peroxides followed the Xylenol Orange assay of Hermes-Lima et al. [31]. Briefly, 0.02 g of insects were homogenized in 100 μl of ice-cold methanol. Homogenates were centrifuged at 10000 ***g*** for 5 min and two replicates of 30 μl of supernatant were transferred into 3 ml glass vials, to which were added 250 μl of $FeSO_4$ (1 mM), 100 μl of H_2SO_4 (0.25 M), 100 μl of Xylenol Orange (1 mM) and 520 μl of water. Samples were mixed and incubated for 15 min at 30 °C and absorbance was read at 580 nm. Units, in μmol of cumene hydroperoxide equivalents/g of wet weight, were obtained by comparison with a cumene hydroperoxide standard curve.

Protein carbonyl content was measured using the 2,4-dinitrophenylhydrazine (DNPH) method of Reznick and Packer [32]. Briefly, 0.15 g of insects were homogenized in 1 ml of 50 mM sodium phosphate buffer (pH 7.5) containing the protease inhibitors leupeptin (0.5 μg/ml), aprotenin (0.5 μg/ml) and pepstatin A (0.5 μg/ml), and 0.1% Triton X-100. Homogenates were centrifuged at 1000 ***g*** for 5 min and 300 μl aliquots of supernatant containing approx. 2 mg of protein were added to either 300 μl of 10 mM DNPH dissolved in 2 M HCl or 300 μl of 2 M HCl alone. Samples were incubated at room temperature in the dark for 1 h, with vortexing every 10 min, precipitated with 10% trichloroacetic acid and centrifuged for 3 min. The pellet was washed three times with 1 ml of ethanol/ethyl acetate, 1:1 (v/v), and redissolved in 1 ml of 6 M guanidine in 10 mM phosphate buffer adjusted to pH 2.3 with concentrated HCl. The difference in absorbance between the DNPH-treated and the HCl-treated samples was determined at 366 nm ($\epsilon = 22.0\ mM^{-1} \cdot cm^{-1}$). Results are expressed as nmol of carbonyl groups/mg of protein.

Addition of the maximum amount of pyrethroid possibly present in the treated insects (estimated from the amount of pyrethroid applied to the insects) to the control vials did not affect the assays measuring oxidative damage.

All biochemical data were subjected to ANOVA. Sceffe's or Dunnet's methods were used as *post hoc* tests. The differences described in the Results section and shown in the Figures were statistically significant at $P < 0.01$, unless otherwise stated.

Table 1 Response of *N. lugens* parental and selected strains to the pyrethroids permethrin and λ-cyhalothrin alone or after synergist exposure

Fiducial limits are shown in parentheses.

	LD_{50} (ng/insect)							
Treatment	AU	Slope	Parental SR	Slope	SRP	Slope	SRC	Slope
Permethrin	5.0 (1.2–10.9)	1.7	14.1 (9.0–18.4)	2.2	84.2 (62.6–107.0)	0.8	60.4 (46.0–79.2)	1.1
Permethrin + PB	3.4 (1.2–5.1)	2.1	7.4 (3.9–11.7)	1.9	73.0 (61.4–87.2)	1.3	51.2 (31.2–72.7)	1.2
Permethrin + DFP	3.2 (2.9–4.4)	1.7	7.0 (4.0–9.5)	1.7	55.7 (39.7–69.2)	0.6	60.0 (47.3–78.7)	1.0
Permethrin + EA	4.3 (2.1–6.9)	1.7	10.1 (7.1–12.8)	1.8	41.4 (30.8–53.6)	0.9	17.9 (14.1–24.8)	0.8
λ-Cyhalothrin	0.06 (0.02–0.09)	0.9	0.12 (0.08–0.15)	3.1	0.57 (0.39–0.80)	1.7	0.61 (0.46–0.75)	1.4
λ-Cyhalothrin + PB	0.01 (0.05–0.18)	1.4	0.04 (0.02–0.07)	2.6	0.46 (0.29–0.68)	1.9	0.49 (0.34–0.65)	1.8
λ-Cyhalothrin + DFP	0.06 (0.03–0.09)	0.8	0.12 (0.09–0.15)	3.0	0.54 (0.39–0.76)	1.6	0.60 (0.44–0.73)	1.5
λ-Cyhalothrin + EA	0.05 (0.03–0.08)	0.8	0.09 (0.06–0.13)	2.4	0.14 (0.10–0.17)	1.2	0.15 (0.11–0.18)	1.1

Purification of elevated esterases and GSTs

The BPH elevated esterases were purified from crude homogenates of the permethrin-selected strain by sequential Q-Sepharose, phenyl-Sepharose and hydroxylapatite column chromatography as outlined by Small and Hemingway [15]. The BPH elevated GSTs were partially partly purified from crude homogenates of both pyrethroid-selected strains by Q-Sepharose and *S*-hexylglutathione-agarose affinity column chromatography as described by Prapanthadara et al. [33]. Proteins bound to the *S*-hexylglutathione-agarose were eluted with 5 mM *S*-hexylglutathione. Fractions with esterase or GST activities (bound and unbound GSTs) were pooled and concentrated in Amicon Centriprep 10 units and buffers were exchanged using a PD-10 column, according to the manufacturer's instructions.

Insecticide interaction studies with partially purified esterases and GSTs

For simple inhibition studies, enzymes were preincubated for 15 min with a series of concentrations of permethrin and λ-cyhalothrin (0.01–0.03 mM) in the presence of acetonitrile (acetonitrile concentration of the medium, 1–5 %, v/v) as a carrier solvent. The remaining activity was determined as in the standard assays, except that ρ-nitrophenol caprate was used as a substrate in the esterase assay, and substrate concentrations for the GST assay were slightly modified to 0.3 mM CDNB and 2 mM GSH. The activities were divided by those measured in the control.

For metabolic studies, purified esterases (2 units/ml of reaction mixture; 0.1 M sodium phosphate buffer, pH 7.4) and partially purified GSTs (5–10 units/ml of reaction mixture; 0.1 M sodium phosphate buffer, 10 mM GSH, pH 6.5) were incubated with various concentrations of pyrethroids and/or primary pyrethroid metabolites (0.01–0.3 mM, acetonitrile 1–5 %, v/v) at 30 °C for 36 h. Combinations of both enzymes and/or crude homogenates were also tested. Aliquots (3 ml) of the reaction mixtures were taken every 2 h and pyrethroids and metabolites were extracted as described by Prapanthadara et al. [33].

To test the non-catalytic capacity of partially purified GSTs to bind pyrethroids or their primary metabolites, samples were taken from the incubation mixture of GSTs and pyrethroids at several time intervals (10 min–10 h) and passed through Amicon Centriprep 10 membranes. The eluted insecticides were extracted as above and quantified by HPLC, and the GST-bound insecticide fractions were calculated.

HPLC analysis for the detection and quantification of pyrethroids and their metabolites was carried out on a Chiradex column with a temperature-controlled water jacket. Peaks were integrated with the Beckman System Gold (166 UV/visible detector, 126 solvent module pump) and System Gold software. Optimal conditions for detection of permethrin and primary pyrethroid metabolites were slightly modified from those described by Sevcik et al. [34]. A mobile phase of 150 mmol of triethylamine with H_2SO_4 in water (pH 3.5)/acetonitrile (3:2, v/v) was used at a flow rate of 0.6 ml/min. Peaks were detected at 210 nm. Permethrin was analysed optimally at 10 °C and λ-cyhalothrin at 4 °C. Samples (50 μl) were injected manually using a Hamilton microsyringe. Quantities of λ-cyhalothrin, permethrin (*cis* and *trans*) and metabolites were calculated from standard curves established by HPLC analysis of known concentrations of authenticated standards.

Chemical synthesis of GSH-pyrethroid conjugates to produce standards of possible metabolites was not practical. However, aliquots of the GST-GSH-pyrethroid or primary metabolite reaction mixtures were analysed by MS for detection of possible pyrethroid-GSH conjugates. Mass spectral analysis was performed on a Fisons VG Platform spectrometer. Mass spectra were recorded between 0 and 1000 atomic mass units and only positive ions were studied.

RESULTS

Permethrin, a primary alcohol ester pyrethroid, was approx. 120-fold less toxic to BPHs than was λ-cyhalothrin, a secondary alcohol ester pyrethroid with an α-cyano group (Table 1). The SR strain was more resistant than the AU strain to both pyrethroids, and both strains had a heterogeneous response, as indicated by the slope values of the log-dosage probit mortality regression lines (results not shown). After 10 generations of continuous selection pressure of the SR strain at the 80 % mortality level with permethrin, the resultant strain (SRP) was 17-fold more resistant to permethrin than the AU strain. A similar λ-cyhalothrin-selected SR strain (SRC) was 10-fold more resistant to λ-cyhalothrin than the AU strain. Both selected strains were cross-resistant to the non-selecting pyrethroid (Table 1).

There was no evidence of increases in cytochrome P450 content or ECOD activity in either selected strain (Table 2). GST activity with the substrate CDNB increased from 0.25 to 0.41 and 0.43 units (64 and 70 % increases) between the parental (SR) and the SRP and SRC strains, respectively (Table 2). The GST-based peroxidase activity with the substrate *t*-butyl hydroperoxide was elevated from 2.9 to 4.0 and 4.1 units (38 and 41 %) and the total GSH from 74.0 to 86.4 and 87.8 μg/g of wet weight, respectively (Table 2). GST-based peroxidase activity of crude homogenates was GSH-dependent and totally inhibited by EA *in vitro*.

Table 2 Effect of pyrethroid selection on resistance ratios, enzyme activities and mean GSH content in SR strain

Values are the means ± S.E.M. from $n = 40$–60 samples. Resistance factors (RR) were calculated as the ratio of the resistant LD_{50}/susceptible LD_{50}. Specific activities are given in units/mg of protein. Total GSH content is given as μg/g of wet weight. In the same column, values with different superscript letters are significantly different ($P < 0.01$).

	Permethrin		λ-Cyhalothrin		Mean specific activity				
Strain	LD_{50}	RR	LD_{50}	RR	Esterase	GSTs	GPOX	ECOD	Mean GSH content
AU	5.0	1	0.06	1	0.35 ± 0.05^{a}	0.26 ± 0.03^{a}	2.7 ± 0.4^{a}	0.14 ± 0.02^{a}	71.9 ± 6.2^{a}
Parental SR	14.1	3	0.12	2	0.56 ± 0.05^{b}	0.25 ± 0.02^{a}	2.9 ± 0.3^{a}	0.13 ± 0.02^{a}	74.0 ± 4.1^{a}
SRP	84.2	17	0.57	9.5	0.82 ± 0.03^{c}	0.41 ± 0.03^{b}	4.0 ± 0.3^{b}	0.15 ± 0.01^{a}	86.4 ± 3.9^{b}
SRC	60.4	12	0.61	10	0.58 ± 0.06^{b}	0.43 ± 0.04^{b}	4.1 ± 0.5^{b}	0.14 ± 0.02^{a}	87.8 ± 4.7^{b}

Table 3 Inhibition of partially purified esterases and GSTs by permethrin and λ-cyhalothrin

The substrate ρ-nitrophenol caprate was used for the esterase assay. The results are the means ± S.E.M. from five separate experiments.

		Percentage remaining activity	
Insecticide	Concentration (μM)	Esterases	GSTs
Permethrin	25	84 ± 2.3	80 ± 3.0
	50	76 ± 3.4	77 ± 4.4
λ-Cyhalothrin	25	100	86 ± 2.8
	50	100	81 ± 4.1

PB, a synergist of the mono-oxygenase oxidative metabolic system increased pyrethroid toxicity to an equal extent in both the selected and parental strains. This suggests that although mono-oxygenases were present and interacted with both pyrethroids they did not have a major role in resistance to either pyrethroid. The specific esterase inhibitor DFP partially synergized permethrin resistance (synergistic ratio, 0.66) but was ineffective with λ-cyhalothrin resistance (Table 1).

In vivo, EA specifically inhibited 40% of the CDNB conjugating activity of GSTs and 85% of their peroxidase activity, whereas *S*,*S*,*S*-tributyl phosphorothioate (DEF) and Cibacron Blue were less effective. Both λ-cyhalothrin and permethrin resistance were largely eliminated by EA pre-exposure (synergistic ratios were 0.24 and 0.49, respectively), suggesting that the elevation of GSTs with glutathione peroxidase activity was the major underlying mechanism of resistance (Table 1). Pretreatment with DEF, an inhibitor of GSTs and esterases, also alleviated resistance (results not shown). The elimination of resistance by GST and esterase inhibitors and the lack of cross-resistance to DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)-ethane] in these strains excluded the possibility of nerve insensitivity playing a major role in this resistance.

Table 3 shows the interaction (percentage inhibition) of permethrin and λ-cyhalothrin with purified esterases and partially purified GSTs. Both insecticides only marginally inhibited CDNB-conjugating activity of GSTs. At concentrations of 50 μM the maximum inhibition achieved was 20% for permethrin and 18% for λ-cyhalothrin. Although permethrin did not inhibit the hydrolysis of ρ-nitrophenol acetate by purified esterases, as

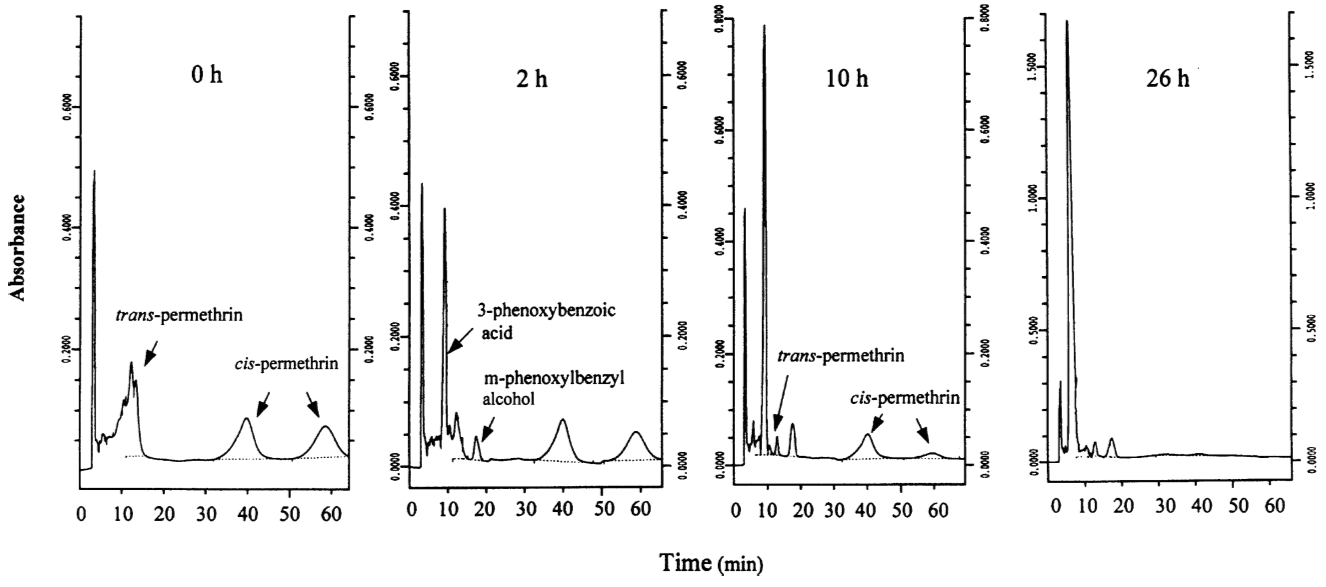

Figure 1 HPLC permethrin hydrolysis profile by purified BPH esterases

Hydrolysis of 95% of the *trans* and *cis* stereoisomers was completed after 10 and 26 h of incubation respectively.

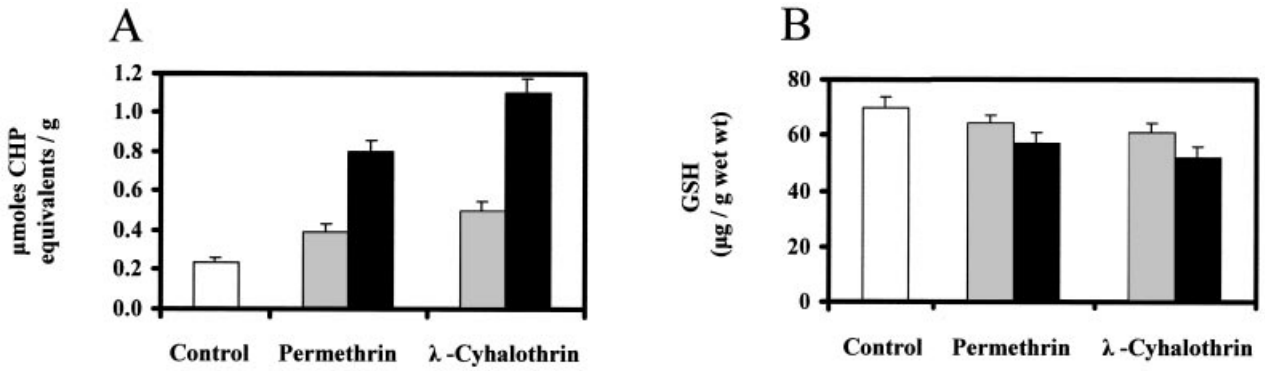

Figure 2 Effect of pyrethroids on lipid peroxides (A) and GSH content (B)

Adult SR females of the same age were treated with acetone (control, white bars), or pyrethroids at their LD_{10} (grey bars) or LD_{50} (black bars). Both pyrethroids at the LD_{10} and LD_{50} caused a significant increase in lipid peroxides and a respective decrease in the GSH content ($P < 0.01$). Values are means from 15–20 determinations for each bar. CHP, cumene hydroperoxide.

previously reported [18], it inhibited up to 24% of esterase activity with ρ-nitrophenol caprate, a substrate with lower affinity for BPH esterases (Table 3). λ-Cyhalothrin (at concentrations up to its solubility limits) did not inhibit the esterases.

HPLC analysis showed that permethrin was slightly hydrolysed by purified esterases. *Trans*-permethrin stereoisomers were hydrolysed at considerably higher rates than *cis* stereoisomers. Hydrolysis of 95% of both *trans* stereoisomers was achieved after 10 h of incubation, whereas the hydrolysis of the *cis* stereoisomers took 26 h to complete (Figure 1). An increase in the amounts of the esterases and/or addition of partially purified GSTs to the incubation mixture did not change the rates of permethrin hydrolysis, or the quantities of metabolites detected. Esterases did not metabolize λ-cyhalothrin. Partially purified GSTs incubated for up to 36 h with both pyrethroids and/or their primary metabolites did not have any effect on the metabolic profile, suggesting that no alteration occurred in pyrethroid molecules or their metabolites after incubation with GSTs and GSH. Incubation of crude homogenates (microsomal fraction) of the parental SR and selected SRC strains with λ-cyhalothrin indicated only a low rate of oxidative metabolism (results not shown). MS analysis showed that both control and GST-pyrethroid or primary metabolite reaction mixtures had identical mass spectra. No additional fragment ions, indicating possible formation of GSH-conjugates of either pyrethroid, were observed.

To test the non-catalytic capacity of partially purified GSTs to bind pyrethroids or their primary metabolites, samples from the GST-pyrethroid reaction mixtures were passed through an Amicon Centriprep 10 membrane. HPLC analysis of the eluate extracts showed no differences between incubation mixtures with or without GSTs, suggesting that although BPH GSTs were sensitive to inhibition by both pyrethroids, they did not act as binding proteins.

Having excluded the involvement of the elevated GSTs in pyrethroid metabolism or as non-catalytic binding proteins, we investigated their possible role in antioxidant defence, suggested by the elevated peroxidase activity. The potential of both pyrethroids for inducing oxidative stress to insects challenged with a minimally acute dose (LD_{10}) or the LD_{50} of permethrin or λ-cyhalothrin was assessed. Figure 2 shows that both pyrethroids induced marked changes in characteristic oxidative-stress- and lipid-peroxidation-related parameters. A significant dose-dependent enhancement in lipid peroxides occurred 1 day after

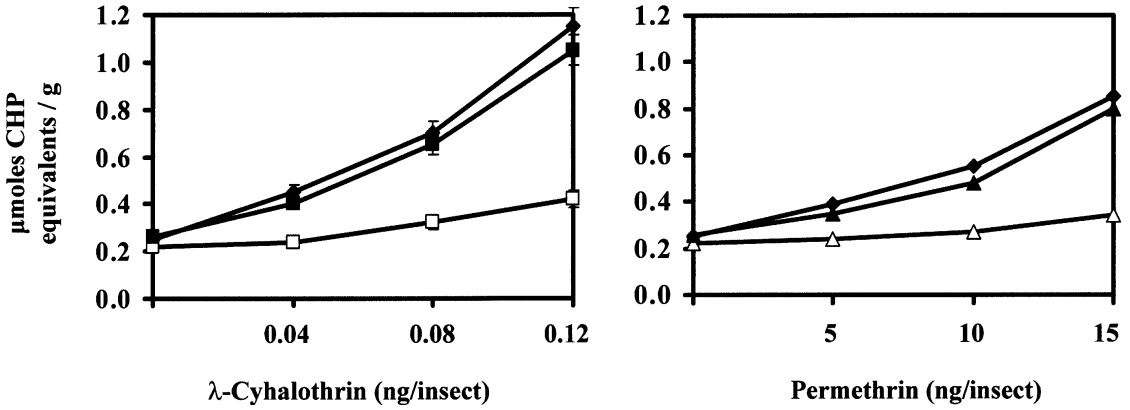

Figure 3 Effect of the *in vivo* inhibition of GSTs on lipid-peroxide levels induced by pyrethroids

Resistant strains SRP and SRC are indicated as □ and Δ, respectively, when treated with pyrethroids, and as ■ and ▲ when co-treated with EA. The susceptible parental SR strain treated with pyrethroids is indicated by ◆. Each value represents the mean ± S.E.M. from 10 determinations. CHP, cumene hydroperoxide.

treatment in insects exposed to either pyrethroid. The effect of λ-cyhalothrin was more pronounced than that of permethrin, as its application increased lipid peroxides by 2- and 5-fold for the LD_{10} and LD_{50} exposures, respectively, while the permethrin treatment increased them by 1.5- and 2.5-fold. Dose-dependent depletion of the reduced GSH content occurred 1 day after application of either pyrethroid (Figure 2). The GSSG content increased after exposure to either pyrethroid (results not shown). Both permethrin and λ-cyhalothrin increased carbonyl content 2 days after treatment, from 1.4 nmol/mg of protein to 1.8 and 1.9 nmol/mg of protein, respectively, for the LD_{50} treatment ($P < 0.01$). Carbonyl content peaked 2–3 days after pyrethroid treatment.

Figure 3 shows that elevated GSTs in the resistant strains, with a predominant peroxidase function, attenuated the pyrethroid-induced lipid peroxides while their *in vivo* inhibition eliminated their protective role. The effect of EA on the pyrethroid-induced lipid peroxides in the resistant strains virtually paralleled the mortality differences observed.

DISCUSSION

The role of GSTs in glutathione-dependent metabolism of organochlorine and organophosphorus insecticides and their metabolites is well documented [4–6]. We now demonstrate a further role for GSTs in insecticide resistance, through antioxidant defence, which extends their range of efficacy to pyrethroids.

Parkes et al. [3] showed that GSTs and GSH play a vital role in prevention (by conjugating reactive species and activated compounds) and/or repair (by detoxifying lipid peroxidation products and oxidized DNA bases) of oxidative damage in *Drosophila melanogaster*, and suggested their possible involvement in insecticide resistance. The co-ordination of GSH biosynthesis, GST expression and glutathione S-conjugate efflux-pump functions has a key role in antioxidant defence of higher vertebrates [35].

The level of GSH depletion and protein oxidation as a result of pyrethroid exposure, though probably not the main causal factor in lethality, indicates that pyrethroids induce strong oxidative damage in BPHs. However, the dramatic induction of lipid peroxides is directly associated with lethality. The effect of the α-cyano pyrethroid λ-cyhalothrin was more pronounced, supporting the hypothesis that the α-cyano group and its possible derivitives might inhibit the respiratory chain in mitochondria [20,35]. Our findings are consistent with studies in higher vertebrates that have shown a cytotoxic and genotoxic potency of pyrethroids, in addition to their neurotoxic function. The former is at least partially related to free-radical-mediated lipid peroxidation [21–23]. The oxidative stress, apart from its direct cytotoxicity, has an additive or accelerating effect on the neurotoxicity of pyrethroids. Mitochondria with impaired respiratory capacity during oxidative stress are unable to respond to increasing demand for ATP when ATP-driven Na^+ channels are inhibited, resulting in a dramatic enhancement of the initial toxicity caused by Na^+-channel blockers [24]. The mechanism involved in the induction of oxidative stress and lipid peroxidation by pyrethroids is unknown. The involvement of free radicals in the mono-oxygenase-based detoxification system, where oxidation reactions of xenobiotics require attack by reduced dioxygen-derived free radicals [36], suggests that O_2 may be liberated from cytochrome P450-mediated oxidative metabolism of pyrethroids to yield reactive species as stimulators of lipid peroxidation [37]. An alternative or additional explanation might be a more direct effect due to Ca^{2+} accumulation in the cell which leads to free-radical-mediated cell damage. Ca^{2+} accumulation after pyrethroid exposure may occur from the direct effect of pyrethroids on the Ca^{2+} channels [38], as exhibited by *Paramecium tetraurelia* [39], or due to energy deficits resulting in the inability to remove cytosolic Ca^{2+}. The development of an energy deficit might result from an increased demand for ATP to fuel the ATP-driven Na^+ channels during pyrethroid exposure, or the direct inhibitory effect of pyrethroid on ATPases [40].

We have shown that elevated GSH and GSTs in the resistant insects, with a predominant peroxidase function, attenuated the pyrethroid-induced lipid peroxidation and reduced mortality. GSH is a key component of the cellular defence against injury and lipid peroxidation damage and a co-factor for the activity of GSTs. These have a crucial role in the termination of free-radical cascades and the lipid-peroxidation chain reaction [41,42]. Inhibition of GSTs with EA, which has high affinity for GSTs with GPOX activity [33], alleviated their protective role and resistance. The number of GSTs elevated in resistant insects is not easily determined as the number of insect GST genes (> 50 in *Drosophila*) and the presence of many allelic variants at some loci make direct biochemical determination difficult [43].

The increase in the CDNB activity of GSTs between the parental and the insecticide-selected populations was not fully explained by the elevation in peroxidase activity, indicating that further GSTs without a peroxidase function were elevated. Multiple GSTs probably share a similar pro-oxidant mechanism of response, possibly controlled by the antioxidant-response element. Elevated GST isoenzymes might be responsible for the conjugation of GSH to secondary metabolic products of pyrethroids or epoxides (highly chemically reactive molecules that add to free amino groups of peptides) introduced during the oxidative metabolism of the pyrethroid molecules, or after cell injury [44].

Previous studies showed elevated GSTs and GSH in pyrethroid-resistant insects and demonstrated that specific GST inhibitors synergized pyrethroid resistance [7–9,45]. We found that BPH GSTs do not serve as binding proteins, as previously hypothesized [10]. Therefore it is possible that elevated GSTs in other pyrethroid-resistant insects play a similar role to that which we have demonstrated in the BPH. Furthermore, the elevation of many GST isoenzymes in insects resistant to organophosphorous and organochlorine insecticides may not be explained only by their role in GSH-dependent metabolism. The GSTs of *Anopheles gambiae* have been studied extensively because of their involvement in DDT resistance [33,46,47]. GSTs from a DDT-resistant strain of *A. gambiae* had an altered GST profile compared with susceptible insects [33]. Increases in the GSTs that possessed dehydrochlorination activity was recognized as the predominant resistance mechanism. However, the chromatographically fractionated peak of GST activity that had the highest increase (8-fold) in resistant insects had negligible DDT dehydrochlorinase activity. These GSTs possessed the predominant peroxidase activity [33], indicating that their function may be similar to that reported here for *N. lugens*.

Oxidative stress may be involved in the toxicity of organochlorine and organophosphorus insecticides [48,49]. Chlorinated cyclodiene insecticides, such as dieldrin and aldrin, induce lipid peroxidation and nuclear DNA single-strand breaks in higher vertebrates, and the use of antioxidants *in vivo* attenuates these damaging effects [50]. Surprisingly, little attention has been given to the role of antioxidant defence in insecticide-resistant insects. GSTs may contribute to the antioxidant defence in insecticide resistance, supplementing other primary resistance mechanisms. The complexity of the GST families in insects does not facilitate

simple detection of individual subunits or isozymes, such as those that possess peroxidase functions, when total GST activity is measured with CDNB, as in most insecticide-resistant studies. Common inhibitors that are used for the synergist studies, such as the esterase inhibitor DEF, are also equally good inhibitors of many GSTs. Hence, synergist studies alone may result in the classification of resistance as esterase-based, when GSTs may also be involved. Finally, most insecticide-resistance studies in which elevated GSTs have been detected are subsequently analysed by insecticide metabolism studies. Our results suggest that this type of interaction should not be assumed in all cases.

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REFERENCES

1 Hayes, J. D. and Pulford, D. J. (1995) The glutathione S-transferase supergene family. Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit. Rev. Biochem. Mol. Biol. **30**, 445–600

2 Fournier, D., Bride, J. M., Poire, M., Berge, J. B. and Plapp, F. W. (1992) Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. J. Biol. Chem. **267**, 1840–1845

3 Parkes, T. L., Hilliker, A. J. and Phillips, J. P. (1993) Genetic and biochemical analysis of glutathione S-transferases in the oxygen defence system of *Drosophila melanogaster*. Genome **36**, 1007–1014

4 Hayes, J. D. and Wolf, C. R. (1988) Role of glutathione transferase in drug resistance. In Glutathione Conjugation: Mechanisms and Biological Significance (Sies, H. and Ketterer, B., eds.), pp. 315–355, Academic Press, London

5 Hemingway, J., Miyamoto, J. and Herath, P. R. J. (1991) A possible novel link between organophosphorus and DDT insecticide resistance genes in *Anopheles*: supporting evidence from fenitrothion metabolism studies. Pest. Biochem. Physiol. **39**, 49–56

6 Clark, A. G. and Shamaan, N. A. (1984) Evidence that DDT-dehydrochlorinase from the house fly is a glutathione S-transferase. Pest. Biochem. Physiol. **22**, 249–261

7 Immaraju, J. A., Morse, J. G. and Hobza, R. F. (1990) Field evaluation of insecticide rotation and mixtures as strategies for Citrus Thrips (Thysanoptera: Thripidae) resistance management in California. J. Econ. Entomol. **83**, 306–314

8 Wu, D. X., Scharf, M. E., Neal, J. J., Suiter, D. R. and Bennett, G. W. (1998) Mechanisms of fenvalerate resistance in the german cockroach, *Blattella germanica* (L.). Pest. Biochem. Physiol. **61**, 53–62

9 Pospischil, R., Szomm, K., Londershausen, M., Schroder, I., Turberg, A. and Fuchs, R. (1996) Multiple resistance in the larger house fly *Musca domestica* in Germany. Pest. Biochem. Physiol. **48**, 333–341

10 Grant, D. F. and Matsumura, F. (1988) Glutathione S-transferase-1 in *Aedes aegypti* larvae. Purification and properties. Insect Biochem. **18**, 615–622

11 Clark, A. G. (1989) The comparative enzymology of the glutathione S-transferases from non-vertabrate organisms. Comp. Biochem. Physiol. **92**, 419–446

12 Shono, T., Ohsawa, K. and Casida, J. E. (1979) Metabolism of trans- and cis-permethrin, trans and cis-cypemethrin and decamethrin by microsomal enzymes. J. Agric. Food Chem. **27**, 316–325

13 Shono, T. (1985) Pyrethroid resistance: importance of the kdr type mechanism. J. Pest. Sci. **10**, 141–146

14 Dai, S. M. and Sun, C. N. (1984) Pyrethroid resistance and synergism in *Nilaparvata lugens* Stål (Homoptera: Delphacidae) in Taiwan. J. Econ. Entomol. **77**, 891–897

15 Small, G. J. and Hemingway, J. (2000) Differential glycosylation produces heterogeneity in elevated esterases associated with insecticide resistance in the brown planthopper *Nilaparvata lugens* (Stål). Insect Biochem. Mol. Biol. **30**, 443–453

16 Vontas, J. G., Small, G. J. and Hemingway, J. (2000) Comparison of insecticide resistance gene copy number, expression levels and esterase activity in insecticide susceptible and resistant *Nilaparvata lugens* (Stål). Insect Mol. Biol. **9**, 655–660

17 Chen, W. L. and Sun, C. N. (1994) Purification and characterization of carboxylesterases of a rice brown planthopper, *Nilaparvata lugens* (Stål). Insect Biochem. Mol. Biol. **24**, 347–355

18 Karunaratne, S. H. P. P., Small, G. J. and Hemingway, J. (1999) Characterization of the elevated esterase-associated insecticide resistance mechanism in *Nilaparvata lugens* (Stål) and other planthopper species. Int. J. Pest Manage. **45**, 225–230

19 Zlotkin, E. (1999) The insect voltage-gated sodium channel as target of insecticides. Annu. Rev. Entomol. **44**, 429–455

20 Gassner, B., Wuthrich, A., Scholtysik, G. and Solioz, M. (1997) The pyrethroids permethrin and cyhalothrin are potent inhibitors of the mitochondrial complex I. J. Pharmacol. Exp. Ther. **281**, 855–860

21 Hadnagy, W., Seemayer, N. H., Kuhn, K. H. and Leng, G. (1999) Induction of mitotic cell division disturbances and mitotic arrest by pyrethroids in V79 cell cultures. Toxicol. Lett. **107**, 81–87

22 Maiti, P. K., Kar, A., Gupta, P. and Chaurasia, S. S. (1995) Loss of membrane integrity and inhibition of type-I iodothyronine-monodeiodinase activity by fenvalerate in female mouse. Biochem. Biophys. Res. Commun. **214**, 905–909

23 Kale, M., Rathore, N., John, S. and Bhatnagar, D. (1999) Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species. Toxicol. Lett. **105**, 197–205

24 Chinopoulos, C., Tretter, L., Rozsa, A. and Adam-Vizi, V. (2000) Exacerbated responses to oxidative stress by an Na^+ load in isolated nerve terminals: the role of ATP depletion and rise of $[Ca^{2+}]_i$. J. Neurosci. **20**, 2094–2103

25 Hemingway, J., Karunaratne, S. H. P. P. and Claridge, M. F. (1999) Insecticide resistance spectrum and underlying resistance mechanisms in tropical populations of the brown planthopper (*Nilaparvata lugens*) collected from rice and the wild grass *Leersia hexandra*. Int. J. Pest Manage. **45**, 215–223

26 Brogdon, W. G., McAllister, J. C. and Vulule, J. (1997) Heme peroxidase activity measured in single mosquitoes identifies individuals expressing the elevated oxidase mechanism for insecticide resistance. J. Am. Mosq. Control Assoc. **13**, 233–237

27 Stumpf, N. and Nauen, R. (2001) Cross-resistance, inheritance and biochemistry of METI-acaricide resistance in *Tetranychus urticae* (Acari: Tetranychidae). J. Econ. Entomol., in the press

28 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

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

30 Griffith, O. W. (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. **106**, 207–212

31 Hermes-Lima, M., Willmore, W. G. and Storey, K. B. (1995) Quantification of lipid peroxidation in tissue extracts based on Fe(III)-xylenol orange complex formation. Free Radicals Biol. Med. **19**, 271–280

32 Reznick, A. Z. and Packer, L. (1994) Oxidative damage to proteins – spectrophotometric method for carbonyl assay. Methods Enzymol. **233**, 357–363

33 Prapanthadara, L., Hemingway, J. and Ketterman, A. J. (1993) Partial purification and characterization of glutathione S-transferase involved in DDT resistance from the mosquito *Anopheles gambiae*. Pest. Biochem. Physiol. **47**, 119–133

34 Sevcik, J., Lemr, K., Stransky, Z., Vecera, T. and Hlavac, J. (1997) Possible uses of micellar electrokinetic capillary chromatography and high-performance liquid chromatography for the chiral discrimination of some pyrethroids. Chirality **9**, 162–166

35 Hayes, J. D. and McLellan, L. I. (1999) Glutathione and glutathione-depedent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radicals Res. **31**, 273–300

36 White, R. E. (1991) The involvement of free radicals in the mechanism of monooxygenases. J. Pharmacol. Exp. Ther. **49**, 21–42

37 Klimek, J. (1990) Cytochrome P-450 involvement in the NADPH-depedent lipid peroxidation in human placental mitochondria. Biochim. Biophys. Acta **1044**, 158–164

38 Kadous, A., Matsumura, F. and Enan, E. (1994) High affinity binding of 3-verapamil to rat brain synaptic membrane is antagonized by pyrethroid insecticides. J. Environ. Sci. Health **29**, 855–871

39 Symington, S. B., Zhang, A. G., Karstens, W., Van Houten, J. and Clark, J. M. (1999) Characterization of pyrethroid action on ciliary calcium channels in *Paramecium tetraurelia*. Pest. Biochem. Physiol. **65**, 181–193

40 Reddy, P. M., Philip, G. H. and Bashamohideen, M. (1991) Inhibition of Mg^{2+} and Na^+-K^+ ATPases in selected tissues of fish, *Cyprinous caprio* under fenvalerate toxicity. Biochem. Int. **23**, 715–721

41 Klimek, J., Wozniak, M., Szymanska, G. and Zelewski, L. (1998) Inhibitory effect of free radicals derived from organic hydroperoxide on progesterone synthesis in human term placental mitochondria. Free Radicals Biol. Med. **24**, 1168–1175

42 Mark, R. J., Lovell, M. A., Markesbery, W. R., Uchida, K. and Mattson, M. P. (1997) A role for 4-hydroxynonenal, an aldehyde product of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid b-peptide. J. Neurochem. **68**, 255–264

43 Ranson, H., Prapanthadara, L. and Hemingway, J. (1997) Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain of *Anopheles gambiae*. Biochem. J. **324**, 97–102
44 Spiteller, G. (1996) Enzymic lipid peroxidation – a consequence of cell injury? Free Radicals Biol. Med. **21**, 1003–1009
45 Naumann, K. (1990) Synthetic pyrethroid insecticides, structures and properties. In Chemistry of Plant Protection (Bowers, W. S., Ebing, W., Martin, D. and Wegler, R., eds.), pp. 107–108, Springler-Verlag, Berlin
46 Ranson, H., Cornel, A. J., Fournier, D., Vaughan, A., Collins, F. H. and Hemingway, J. (1997) Cloning and localization of a glutathione S-transferase class I gene from *Anopheles gambiae*. J. Biol. Chem. **272**, 5464–5468
47 Ranson, H., Collins, F. H. and Hemingway, J. (1998) The role of alternative mRNA splicing in generating heterogeneity within the *Anopheles gambiae* class I glutathione S-transferase family. Proc. Natl. Acad. Sci. U.S.A. **95**, 14284–14289
48 Bachowski, S., Xu, Y., Stevenson, D. E., Walborg, E. F. and Klauning, J. E. (1998) Role of oxidative stress in the selective toxicity of dieldrin in the mouse liver. Toxicol. Appl. Pharmacol. **150**, 301–309
49 Hai, D. Q., Varga, S. I. and Matkovics, B. (1997) Organophosphate effects on antioxidant system of carp (*Cyprinus caprio*) and catfish (*Ictalurus nebulosus*). Comp. Biochem. Phys. C **117**, 83–88
50 Bagchi, D., Hassoun, E. A., Bagchi, M. and Stohs, S. J. (1993) Protective effects of antioxidants against endrin induced hepatic lipid peroxidation, DNA damage, and excretion of urinary lipid metabolites. Free Radicals Biol. Med. **15**, 217–222

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