Research reports

Development of resistance in *Achatina fulica* Fer. and *Bradybaena similaris* Fer. towards metaldehyde

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Summary

The possible role of glutathione S-transferase (GST) in resistance to metaldehyde in two snails, *Achatina fulica* Fer. and *Bradybaena similaris* Fer. was investigated. Dose-response experiments (range 0–5%) of baited metaldehyde, showed 100% mortality of *A. fulica* to 5% metaldehyde within 24 hours. Dose response for *B. similaris* over the same range (0–5%) showed highest mortality of 60% at 2.85% metaldehyde, thereafter mortality decreased significantly. When 3% and 0.475% baited metaldehyde were given repeatedly to *A. fulica* and *B. similaris* respectively, mortality rates decreased significantly; concomitant with increases in GST activities in both the snails. In *B. similaris,* ELISA assays showed progressive increases in GST concentrations ($P<0.05$) for each consecutive metaldehyde treatment. There was also positive correlation ($r=0.97$) between specific activities of GST to the intensities of signals for the GST protein as determined by Western and densitometric analyses. These latter observations suggest the synthesis of new GST in *B. similaris* to cope with higher metaldehyde concentrations in its tissues.

Introduction

Achatina fulica (Achatinidae) and *Bradybaena similaris* (Fruticicolidae) are two of the most important land snail pests in Malaysia. *A. fulica* is known to thrive on fresh and rotting plants (Purchon 1977) and infests more than 500 plant species including cocoa, papaya, rubber trees, legumes and plants from the leguminosae and cucurbitaceae families (Watson 1985). *B. similaris* is emerging as an important pest and infests cabbages, carrots, Chinese cabbage and asparagus in Malaysia (Say 1995).

The molluscicidal activity of metaldehyde has been recognized since the 1930s (Ware 1978) and it has since been used most effectively against molluscs pests worldwide (Handerson and Martin 1990). Metaldehyde has been variously described as a contact poison (Jary and Austin 1937) and a stomach poison (Thomas 1948, Cragg and Vincent 1952). More recently, Say (1995), showed metaldehyde to be more potent as a contact poison (LC_{50} =74.42 mg g^{-1} snail) than a stomach poison (LD_{50} oral = 93.8 mg g^{-1}) in *B. similaris*. Metaldehyde has been reported to cause changes in feeding patterns and behaviour (Bailey and Wedgewood 1991), and the breakdown of the nervous functions (Mills *et al.* 1992). Histological studies in *Deroceras reticulatum* showed changes in the epithelium cells of the digestive system (Bourne *et al*. 1988) which adversely affect mucosal secretions (Triebskorn and Ebert 1989) and resulted in the death of these animals. Metaldehyde has been widely used to control snail pests including *B. similaris* and *A. fulica* in Malaysia. However, emerging resistance of snails to metaldehyde due to over use has reduced its effectiveness.

Organisms may cope with the presence of xenobiotics by increasing activities of xenobiotic metabolizing enzymes such as glutathione S-transferase (GST), nonspecific esterases, acetylcholine esterase, mixed function oxidases and hydrolases (Brown and Brogdon 1987). The glutathione S-transferases are multifunctional proteins that are important in initiating the detoxication of xenobiotics in organisms. GSTs act on a variety of aromatic and aliphatic xenobiotics including organophosphorus insecticides and epoxide compounds (Chasseaud 1979) as well as catalysing the conjugation of glutathione with a wide variety of electrophiles to form less toxic and water soluble substances which are then more easily excreted from the body (Habig *et al*. 1974). GSTs thus protect the cell against cytotoxic and genotoxic compounds, including secondary products of lipid peroxidation.

GST activity has been measured in a large variety of species including mammals, insects, plants and invertebrates (Kalinyak and Taylor 1982). The induction of GST in rats, insects and fish exposed to certain organic compounds has been studied to a great extent (Hayaoka and Dauterman 1982, Pearson *et al.* 1983). It has been shown that GST activity is influenced by these compounds which suggests that it plays a significant role in the development of resistance towards these compounds.

In the present study, we report the dose-response of *B. similaris* and *A. fulica* to baited metaldehyde. The possible role of glutathione S-transferase in the development of tolerance in animals repeatedly dosed with metaldehyde was also investigated.

Materials and methods

Achatina fulica were collected from housing estates near Bandar Bangi Baru, Selangor, Malaysia and *B. similaris* were obtained from a major cabbage plantation in Cameron Highlands, Pahang, Malaysia. Snails were starved for 48 hours prior to treatment to facilitate their food intake. Animals were adapted to the laboratory conditions in plastic containers (33.5 × 23.0 × 10.5 cm) on a diet of cabbages for at least one week before starting the experiments. The containers were lined with tissue papers wetted with distilled water to simulate their natural habitats. Powdered metaldehyde (95%) was obtained from Fluka AG, Switzerland.

For dose-response studies, snails were placed in plastic containers and given diets containing 1.0, 2.0, 3.0, 4.0, and 5.0% metaldehyde, for *A. fulica,* and 0.25, 0.475, 0.95, 1.90, 2.85, 3.8, and 5.0% for *B. similaris*. For each treatment, there were three replicates with 5 and 10 snails of each replicate for *A. fulica* and *B. similaris*, respectively. To aid bait and molluscicide intake, snails were starved for 48 hours prior treatment. Diets comprised of 20% powdered whole Chinese cabbage leaves, the required amounts of metaldehyde to make the various doses, and then made up to 100% with oat. Control animals received diets with no metaldehyde added. Mortality was scored when animals showed no muscular response to a sharp needle-prick.

To study the role of GSTs in tolerance development, animals were repeatedly given diets containing sub-lethal concentrations of metaldehyde i.e. 3.0% for *A. fulica* and 0.475% for *B. similaris*. For *B. similaris*, a total of 600 animals weighing approximately 300 mg each were divided into three replicates of 150 animals each and a control group. For *A. fulica,* each triplicate of treated and control group consists of 30 animals. Mortality scorings were made 24 hours after metaldehyde containing diets were given; the survivors were then placed in another container and given a diet of fresh cabbages to recover.

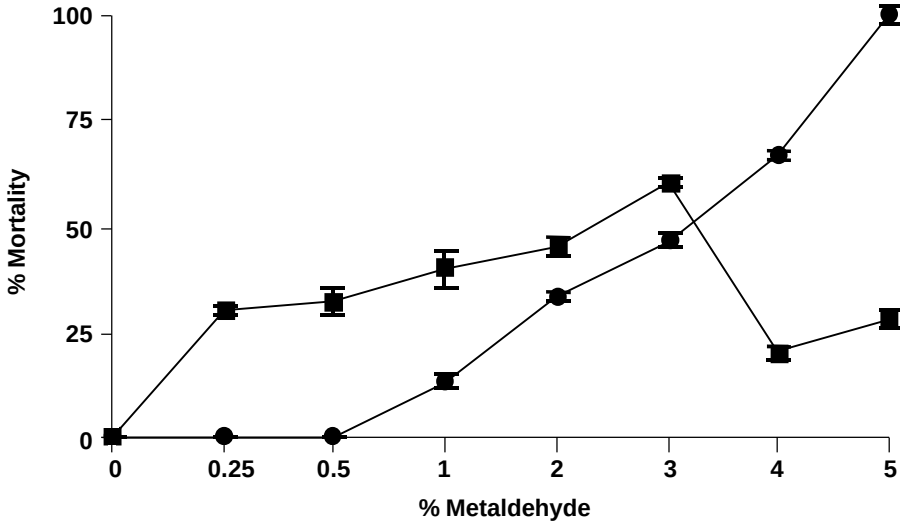

Figure 1. Dose response (24 hours) of *Achatina fulica* (● —— ●) and *Bradybaena similaris* (■ —— ■) towards metaldehyde. Error bars are ± SEM.

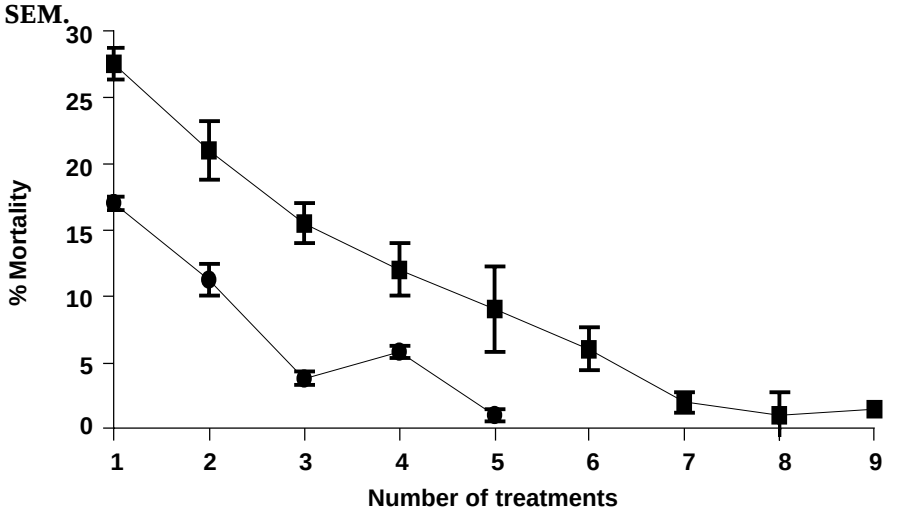

Figure 2. Mortality rates of *A. fulica* (● —— ●) repeatedly treated (5X) with 3.0% and *B. similaris* (■ —— ■) with 0.475% metaldehyde (9X) compared to their respective control groups. Each value is the mean of three replicates and percentage mortality at each consecutive treatment is calculated based on the number of animals at the beginning of the experiment. Error bars are ± SEM.

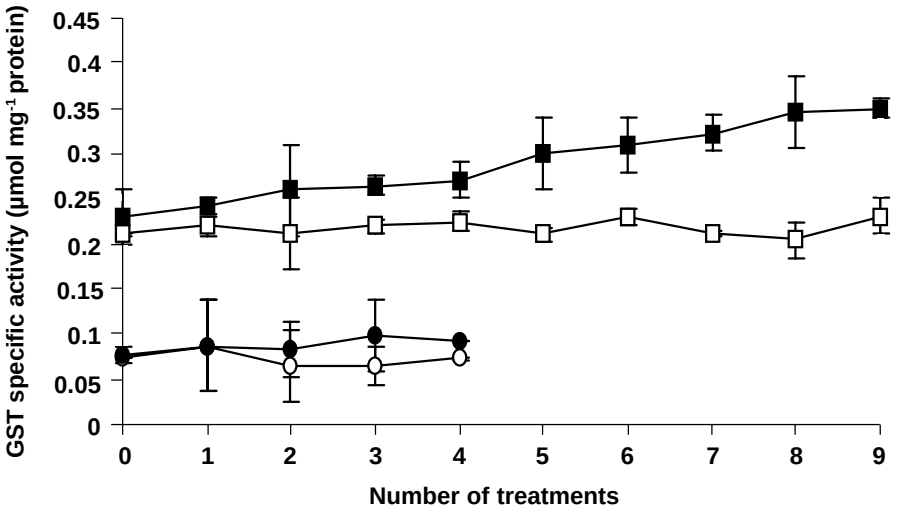

Figure 3. GST activity in *B. similaris* treated with 0.475% metaldehyde (■ —— ■) compared to control (□ —— □) and in *A. fulica* treated with 3.0% metaldehyde (● —— ●) compared to controls (○ —— ○). Error bars are ± SEM.

After 24 hours, the animals were again starved for 48 hours before the start of another treatment. The surviving snails (four animals for *A. fulica* and five animals for *B. similaris*) from each replicate were taken and whole body GST activity assayed.

The soft body tissue was separated from its shell and whole body tissues were pooled and homogenized in 0.1mM phosphate buffer, pH 7.3 (1:3; w/v) and then centrifuged at 10 000 g for 20 minutes at 4°C. The supernatant was kept at -30°C until assayed for GST activity and subsequently, protein analyses by ELISA and Western Blotting.

The GST activity was assayed according to the method of Balabaskaran *et al.* (1986) as modified by Surif *et al.* (1992). Assays were performed at 25°C using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate at a concentration of 1.2mM in 4% ethanol, in 0.2M arginine-HCl buffer, pH 8.3 and at GSH concentration of 0.015M. The activities were followed spectrophotometrically at 340 nm, using molar extinction coefficient of the thioether product of $9.6mM^{-1}cm^{-1}$. Protein concentrations were measured by the method of Lowry *et al.* (1951).

Polyclonal antibody anti-GST was raised in 'Local White' rabbits by the method of Nieschlag *et al.* (1975), using commercially obtained equine GST (Sigma Chemicals, USA) as the antigen. Quantification of the GST protein in each group of animals repeatedly treated with metaldehyde were done by indirect ELISA (Voller and Bidwell 1986).

For Western analyses, proteins were fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and transferred to nitrocellulose membrane according to the method of Towbin *et al.* (1979). The GST proteins were probed with the polyclonal antibody anti GST and the GST protein concentrations were estimated semi-quantitatively by densitometric analyses using Imaging Densitometer GS-670.

Results

The 24 hour dose-response of *A. fulica* and *B. similaris* towards metaldehyde in the range of 0–5% is shown in Figure 1. On the first day of each treatment, the snails secreted copious amounts of mucus; most probably due to injuries to their digestive system. Similar observations were reported by Wedgewood and Bailey (1988) on three terrestrial slug pests. Two days after metaldehyde treatment, the snails started to feed on the cabbage given to them, and no longer secreted any mucus. In *A. fulica*, maximum mortality rate (100%) was recorded at 5% metaldehyde. In contrast, for *B. similaris*, the highest mortality rate observed was 60% at 2.85% metaldehyde. In the latter, increasing the dosing rate resulted in further decreases

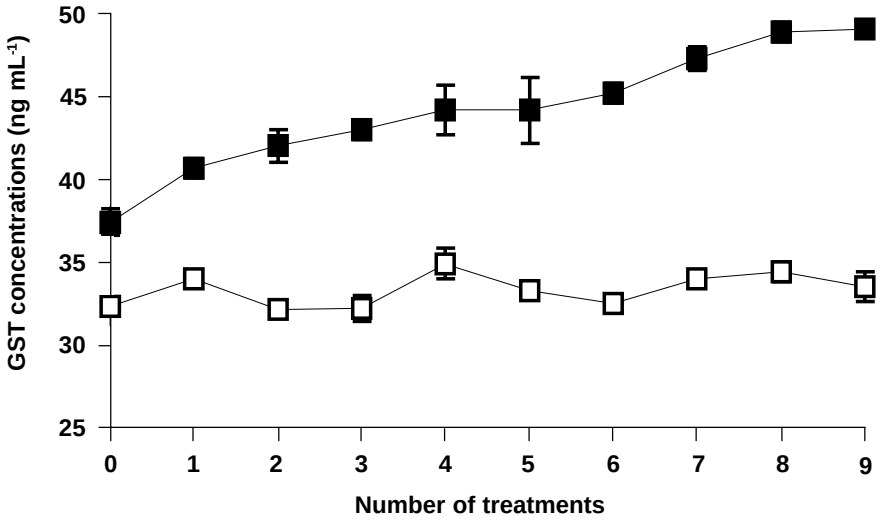

Figure 4. Concentrations of GST proteins in *B. similaris* treated with 0.475% metaldehyde repeatedly, as determined by indirect ELISA (■ —— ■) compared to controls (□ —— □). Error bars are ± SEM.

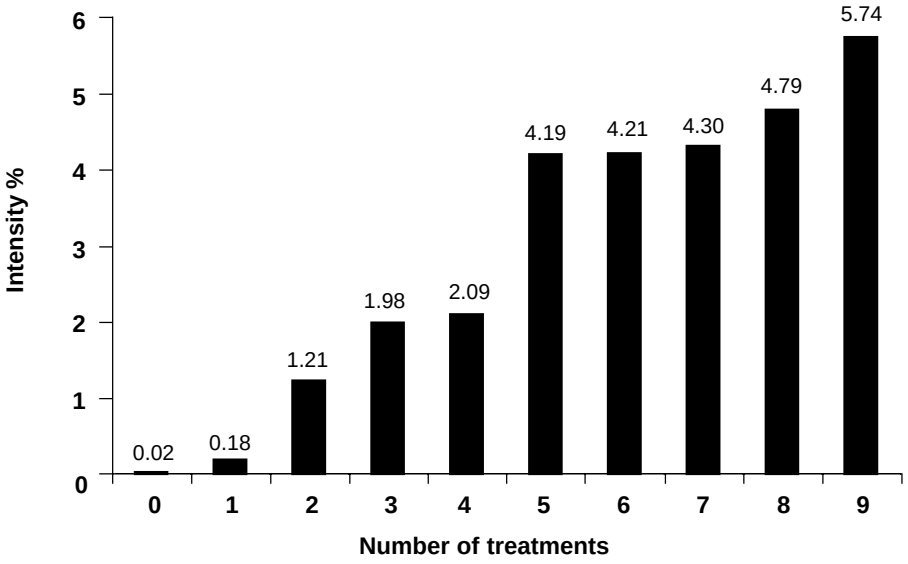

Figure 5. Densitometric analyses of Western blotting : percentage increase of GST protein in *B. similaris* repeatedly given 0.475 % metaldehyde compared to control animals.

in mortality rates. Closer inspection of the mortality curves for *A. fulica* and *B. similaris* showed some difference in the effect of metaldehyde in these two animals. In *A. fulica*, metaldehyde was not effective until the dose was increased to 1.0%; mortality then increased linearly until it reached 100% at 5% metaldehyde. In *B. similaris*, the animals were killed at much lower dose rates, i.e. 0.25%; the mortality rate then increased slowly to reach a peak at 2.85%, and thereafter, decreased significantly.

When *A. fulica* were treated with 3% metaldehyde repeatedly for five times, there were marked decreases in mortality rates; by the fifth treatment, the mortality rates were similar to those of the control (Figure 2). Similar phenomenon was also observed in *B. similaris* given repeated 0.475% metaldehyde treatment; whence the mortality rates showed a steady drop; reaching that of the control by the seventh treatment (Figure 2). The specific activities of GSTs in *B. similaris* showed marked increases compared to the control group during the course of the consecutive treatments, which were not so evident in *A. fulica* (Figure 3). Further quantification of the GST protein in these *B. similaris* by Indirect ELISA assay (Figure 4) showed significant differences in the level of treated animals compared to the control ($P<0.05$). Similarly, densitometric analyses of Western Blotting of treated animals' supernatant using anti-GST as the probe showed positive correlation ($r=0.97$) between GST specific activity to concentrations of GST proteins during the course of the repeated metaldehyde treatments (Figure 5).

Discussion

The difference in response of *A. fulica* and *B. similaris* towards metaldehyde is rather interesting. The higher doses needed by *A. fulica* to cause death is most likely due to their bigger mass; an average *A. fulica* weighs about 20–25 g while *B. similaris* weighs an average 200–500 mg. In *B. similaris*, the highest mortality (60%) was recorded at 2.85% metaldehyde, the mortality rates then declined at higher doses. At low doses, metaldehyde is thought to have an attractive feeding effect, but at a certain critical concentration it can depress the intake (Wedgewood and Bailey 1988). However, mortality rates of *A. fulica* was shown to be dose-dependent, reaching its highest (100%) at 5.0% metaldehyde. The *A. fulica* samples were collected from housing estates where metaldehyde has not been used to control them. *B. similaris* however were collected from a cabbage farm whose proprietor uses metaldehyde all year round to control the infestation of the cabbage leaves. It is thus probable that the survivors of the high metaldehyde dose consists of *B. similaris* population which have been selected to metaldehyde and therefore resistant to higher concentrations of metaldehyde.

When the animals were repeatedly treated with low, sub-lethal doses of metaldehyde, there were marked decrease in mortality with each consecutive dose in both animals; equalling those of control animals by the fifth treatment for *A. fulica* and the seventh for *B. similaris*.

The increases in GST specific activity which are concomitant with elevated protein levels during the course of repeated treatments suggest that new proteins were being synthesized to cope with the increased metaldehyde levels in their system. Hayaoka and Dauterman (1982) showed that GST activity was induced in various housefly strains exposed to phenobarbital and selected pesticides. In another study, the GST mRNA and protein levels were reported to be induced in mice in response to exposure to 2(3)-tertbutyl-4-hydroxyanisole (Pearson *et al.* 1983).

It can therefore be concluded that, exposure to prolonged periods of metaldehyde can induce the synthesis of glutathione S-transferase, one of the most important conjugation enzymes in the metabolism of xenobiotics in the snail, *B. similaris*. The role of other xenobiotic metabolizing enzyme in metaldehyde metabolism, especially cytochrome P450 which catalysed the first phase reactions is presently being investigated.

Acknowledgement

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