Dose-response to metaldehyde and cytochrome P450 levels in *Bradybaena similaris* populations which have and have not had previous pesticide exposure

S. Salmijah, P.L. Chan, K.K. Wong, A. Maimon and **B.S. Ismail,** School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM, Bangi, Selangor, Malaysia.

Summary

This study was designed to investigate the dose response of *Bradybaena similaris* to metaldehyde in populations which have had previous multiple exposure to the chemical at Cameron Highlands, Pahang and populations that never had exposure to any type of pesticides at Pos Piah, Perak. The base levels of cytochrome P450 (CYP) activities in the two populations were also investigated. The results showed that the 24 hour dose response profile of metaldehyde on *B. similaris* samples from Cameron Highlands differed from that observed on the *B. similaris* samples from Pos Piah. The snails from Cameron Highlands displayed the highest mortality of 85% at 4% metaldehyde and decreased thereafter. In contrast, the mortality rate of snails from Pos Piah was found to increase proportionally to 71.7% at the highest metaldehyde dose tested (7.0%). There was no significant difference (P >0.05) in the total cytochrome P450 hemoprotein measured from both the whole tissues and the hepatopancreas for the two populations. However, the CYP2B activity was significantly higher (P <0.05) in snails which have had unremitting exposure to metaldehyde, suggesting that CYP2B is selectively induced in these animals by metaldehyde.

Key words: *Bradybaena similaris*, cytochrome P450, metaldehyde, pesticide.

Introduction

In Malaysia, *Bradybaena similaris* (Fruticicolidae) is found predominantly in the upland, vegetable-growing areas particularly in Cameron Highlands, Pahang, where it infests soft tissued vegetables and fruits, such as cabbage, apple, carrot, tomato and asparagus (Salmijah *et al.* 2000). Farmers have been using molluscicides, particularly metaldehyde and methiocarb, intensively in these farms to reduce snail damage to the crops as well as to control their spread. The continuous applications of these pesticides may have resulted in the development of resistance in the *B. similaris* populations as indicated by the need to use increasingly higher dosages of metaldehyde to maintain satisfactory levels of control.

Resistance can develop in target organisms through two mechanisms: firstly, by enhancing the capacity of the organism to metabolically detoxify the pesticide, and, secondly, by altering the target sites of the organism to prevent binding of the pesticide (Brattsten *et al.* 1986). Previous investigations have shown evidence of the former mechanism occurring in the population under study, in the form of increased activities of CYP2B and glutathione-S-transferase (GST) in *B. similaris* exposed to metaldehyde (Salmijah *et al.* 2004).

The aim of this paper is to compare the dose response of *B. similaris* previously exposed to applications of molluscicides, particularly metaldehyde, for at least five years with a population that is believed never to have been exposed to any type of pesticide. The base levels of the total cytochrome P450 and one of the isoforms, the CYP2B were also measured, by assaying the pentoxyresorufin-O-depentylase (PROD) activities in the animals.

Materials and methods

Sample collection

Bradybaena similaris samples were collected from the Kea Farm, Brinchang, Cameron Highlands in Pahang, Malaysia, where molluscicides particularly metaldehyde had been used for at least five years and from Pos Piah, an aboriginal settlement where conventional farming is not practised and where there are no records of the use of any pesticides. In the laboratory, the snails were kept in polycarbonate containers (33.5 × 23.0 × 10.5 cm) layered with tissues drenched in distilled water to simulate their natural habitat. During the seven-day acclimatization period, prior to the experiment, the snails were given fresh cabbage, as well as chalk (as a source of calcium).

Metaldehyde baits were prepared by mixing 20% dried, crushed Chinese cabbage (*Brassica juncea*) leaves with the required amount of powdered metaldehyde (Fluka AG, Switzerland) to make the various dosages, and then made up to 100% (w/w) with bran. Some distilled water was added to the dry mixture to enable it to stick together and be extruded as pellets using a syringe. The pellets were then dried in the oven (35°C) and kept at 4°C before use. Control baits were prepared without metaldehyde.

Dose response experiment

A total of 420 snails having an average weight of 500–600 mg were collected from each study area, made to fast for 48 hours and divided into seven groups. Each 20 snail group was given metaldehyde baits at 0% (control), 0.5%, 1.0%, 2.0%, 3.0%, 4.0% and 5.0% a.i. for 24 hours. The snails from Pos Piah were initially given the same metaldehyde dosages but higher dosages of 6% and 7% were included for the repeat experiment as the mortality was found to be low even at the 5% metaldehyde rate tested. All treatments were done in triplicate. At the end of 24 hours, the mortality rate was scored when the snails showed no muscular response to tactile stimuli.

Total cytochrome P450 activity assay

The levels of cytochrome-P450 were measured in snails not subjected to metaldehyde treatment, collected from Cameron Highlands and Pos Piah, after the seven day acclimatization period in the laboratory.

1. Preparation of the microsomal fraction from *Bradybaena similaris*

Microsomal fractions were prepared according to the method of Liimaitainen and Hanninen (1982). The soft body tissues of the snails were separated from the shell and washed with distilled water. Samples of the hepatopancreas or whole tissues from ten randomly selected animals were homogenized in 0.1 mol phosphate buffer (pH 7.4) and centrifuged at 12 000*g* and 4°C temperature for 20 min (RC-5B Sorvall). The supernatant obtained was filtered through several layers of cheesecloth and re-centrifuged at 100 000*g* and 4°C temperature, for 1 hour (L3-50 Beckman). The microsomal pellet was then resuspended in the phosphate buffer (50 mmol, pH 7.5, 1:4 w/v), aliquoted into Eppendorf tubes and stored at -20°C until it was used.

2. Microsomal cytochrome P450 total haemoprotein assay

The total cytochrome P450 hemoprotein was determined according to Omura and Sato (1951), based on the binding of carbon monoxide with the reduced form of cytochrome P450. To 1.0 mL of the microsomal fraction in a tube was added 2.0 mL of 50 mmol phosphate buffer and 2.0 mg sodium dithionate, and the tube was inverted a few times to obtain an even mix. The sample was then saturated with carbon monoxide and the cytochrome

P450-carbon monoxide complex measured at 450 nm using a spectrophotometer (Shimadzu UV-160). The cytochrome P450 content (nmol mg^{-1} protein) was calculated using the absorbance constant of 91 $mmol^{-1}$ cm^{-1} for cytochrome P450.

3. Pentoxy-resorufin-O-depentylase (PROD) assay

The PROD activity was measured by the method described by Lubert (1985). The CYP2B-specific activity was determined by the rate of reduction of the substrate, pentoxyresorufin by NADPH to form resorufin which was used to determine the level of CYP2B in the samples.

Fifty µL of the microsomal fraction was added in triplicate to 2.0 mL 0.1 mol Tris-HCl (pH 7.8), 5.0 µL 1.0 mmol pentoxyresorufin in DMSO and 10 µL 50 mmol NADPH in 1% $NaHCO_3$. Both Tris-HCl and NADPH were pre-incubated to 37°C before use. The resorufin-forming rate was determined using a fluorometer (Turner Model 450) with the excitation wavelength fixed at 520 nm and emission wavelength at 585 nm. Calibration of each sample was done using 2.0 mL 0.1 mol Tris-HCl (pH 7.8), 50 µL microsomal fraction and 10.0 µL 0.1 mmol resorufin. The specific activity was expressed in nmol min^{-1} mg $protein^{-1}$.

Statistical analysis

The experiment was replicated thrice. The experimental data were subjected to analysis of variance and means were compared using the LSD test at the 5% level of significance.

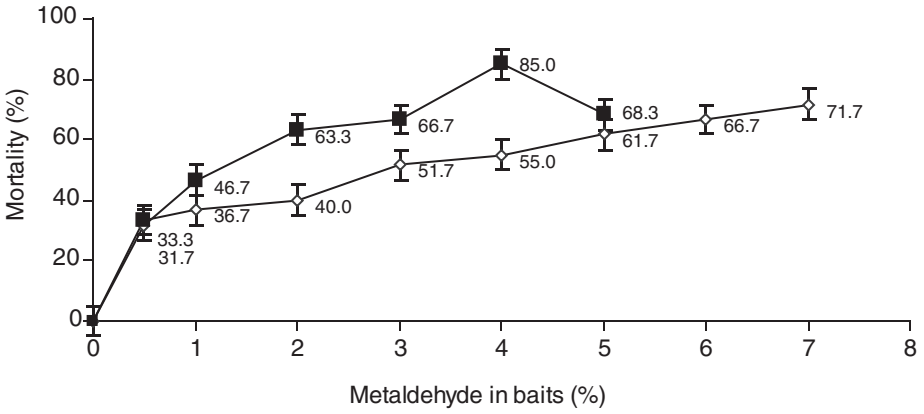

Figure 1. Dose response of metaldehyde to the mortality rate of the snail (*Bradybaena similaris*) collected from the Cameron Highlands (■) and Pos Piah (◊).

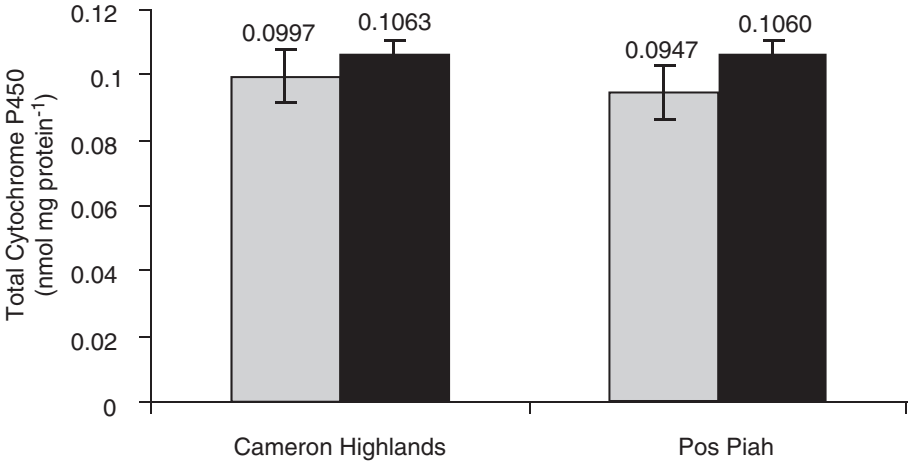

Figure 2. Total cytochrome P450 in whole tissues (grey) and hepatopancreas (black) of *Bradybaena similaris* collected from Cameron Highlands and Pos Piah. Values represent means ± standard deviation (n = 3).

Results and discussion

Figure 1 shows the percentage mortality rate of snails from Cameron Highlands and Pos Piah after being fed with metaldehyde bait for 24 hours. The mortality rate in the Cameron Highlands snails gradually increased with the metaldehyde dosage reaching the highest percentage (85%) at 4.0%. When the dosage was increased to 5% metaldehyde, the mortality rate dropped slightly. The highest mortality rate occurring at the dosages offered, is likely due to avoidance of the baits with higher concentration by snails, or a suppression of feeding by the animals. In our earlier work (Say, unpublished data) the amount of bait taken up by *B. similaris* diminished with increasing metaldehyde concentrations. Wedgewood and Bailey (1988) also reported a similar phenomenon on the effect of metaldehyde on terrestrial slugs.

The higher recorded mortality rate of 85% found in this study compared to 60% reported previously in animals from the same area (Salmijah *et al.* 2000) could be due to variation in animals among populations over a period of time (one year), or to the actual amount of bait they had ingested which is not recorded in this study.

For *B. similaris* from populations with no history of exposure to metaldehyde at Pos Piah, the mortality rate was found to increase linearly with increasing dosages of metaldehyde, reaching a maximum of 71.7% at 7.0% metaldehyde. This observation was totally unexpected. However, due to sample restriction, further studies to discover the dosage that would result in 100% mortality rate could not be pursued. Having never been exposed to any form of known pesticides, it was expected that the snails from Pos Piah would be susceptible to metaldehyde. The LD_{50} value for metaldehyde in the Pos Piah population (i.e. 2.30%) is twice that of the snails from Cameron Highlands (i.e. 1.26%). These results suggest that in the Pos Piah population, the avoidance mechanism is not in place and that they have an efficient detoxifying mechanism for xenobiotics, such as metaldehyde. It is likely that the snails from Pos Piah, living in the wild, actively induce high levels of xenobiotic metabolizing enzymes, as they feed on a variety of flavonoid-containing vegetation as opposed to those in the Cameron Highlands, which feed solely on cabbage. Gonzalez and Nebert (1990) reported that some plants produce metabolite suppression compounds called phytoalexins which render the leaves unpalatable. Feeding on leaves containing phytoalexins may induce the production of xenobiotic metabolizing enzymes such as cytochrome P450 to counter the opposing effects of this metabolite suppression compound. For both populations studied, there were no deaths recorded in the control, and a strong positive correlation (r^2 =0.893) between mortality rate and metaldehyde dosage.

All snails exposed to metaldehyde bait showed symptoms of sluggishness and excessive mucus secretion compared to the controls. These observations concurred with findings reported by Mills (1990) on

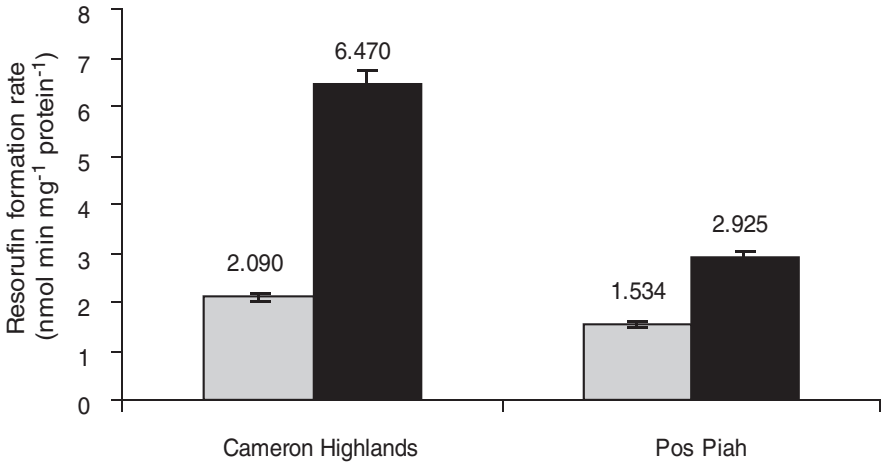

Figure 3. PROD activities in whole tissues (grey) and hepatopancreas (black) of *Bradybaena similaris* collected from Cameron Highlands and Pos Piah. Values represent means ± standard deviation (n = 3).

Lymnaea stagnalis exposed to metaldehyde. Mucus secretion is a reaction commonly displayed by snails to detoxify the metaldehyde (EXTOXNET 1998).

Total cytochrome P450 content in snails collected from the two locations were not significantly different (Figure 2). The levels in the whole tissues was slightly but not significantly lower than those of the hepatopancreas, ranging from 0.0947–0.0997 nmol mg protein^{-1} in whole tissue and 0.106 nmol mg protein^{-1} in the heptopancreas. These levels were in the same range as those of untreated *Lymnaea stagnalis* at 0.0301 nmole mg protein^{-1} (Willbrink *et al.* 1991). An earlier study using the *B. similaris* from the same area showed lower (0.001 nmol mg protein^{-1}) cytochrome P450 levels in the untreated animals. This difference is likely due to variation in the population under study over a period of time as discussed previously.

Figure 3 however shows that in both Cameron Highlands and Pos Piah populations, activities of CYP2B (PROD) were higher in the hepatopancreas than in whole tissues. This suggests that the hepatopancreas is likely to be the organ responsible for detoxification in the animal. These levels were generally higher in the Cameron Highlands population. Thus the data can also be interpreted as showing that CYP2B enhances the action of metaldehyde as the LD_{50} for Cameron Highlands is 1.26 and that for Pos Piah 2.30.

Acknowledgements

This work was supported by the Ministry of Science, Technology and the Environment of Malaysia (IRPA 08-02-02-0011EA185).

References

Brattsten, L.B., Holyoke, C.W., Leeper, J.R. and Raffa, K.F. (1986). Insecticide resistance: challenge to pest management and basic research. *Science* 231, 1255-60.

EXTOXNET. (1998). The Extension Toxicology Network. www. pmep.cce.cornell.edu/profiles/extonet/haloxyfopmethylparathion/metaldehyde.ext.html.

Gonzales, F.J. and Nerbert, D.W. (1990). Evolution of the P450 gene superfamily. *Trends in Genetics* 6, 182-6.

Liimaitainen, A. and Hanninen, O. (1982). Occurrence of cytochrome P450 in the earthworm *Lumbricous terrestris*. *In* 'Cytochrome P450: biochemistry biophysics and environment implication', eds E. Hietanan, M. Laitinen, and O. Hanninen. pp. 255-8. (Elsevier Biomedical Press, Amsterdam).

Lubert, R.A., Mayer, R.T., Cameron, J.W., Nims, R.W., Burke, M.D., Wolff, T. and Guengerich, F.P. (1985). Dealkylation of pentoxirexorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P450 by phenobarbital and other xenobiotics in rat. *Archives of Biochemistry Biophysics* 238 (1), 43-8.

Mills, J.D., McCrohan, C.R., Bailey, S.E.R. and Wedgwood, M.A. (1990). Effects of metaldehyde and acetaldehyde on feeding responses and neuronal activity in the snail, *Lymnaea stagnalis. Pesticide Science* 28, 89-99.

Omura, T. and Sato, R. (1951). The carbon monoxide-binding pigment of liver microsomes: solubilisation, purification and properties. *Journal of Biological Chemistry* 237 (7), 2379-85.

Salmijah, S., Chan, M.K., Kong, B.H., Maimon, A. and Ismail, B.S. (2000). Development of resistance in *Achatina fulica* Fer. and *Bradybaena similaris* Fer. towards metaldehyde. *Plant Protection Quarterly* 15 (1), 2-5.

Salmijah, S., Syed Zahirudin, S.Z. and Ismail, B.S. (2004). Glutathione S-transferase and cytochrome P450 monoxygenase activities of *Bradybaena similaris* treated with metaldehyde. *Plant Protection Quarterly* 19 (4), 151-4.

Wedgewood, M.A. and Bailey, S.E.R. (1988). The inhibitory effects of the molluscicide metaldehyde on feeding, locomotion and faecal elimination of three pest species of terrestrial slug. *Annals of Applied Biology* 112, 439-57.

Wilbrink, M., Groot, E.J., Jansen, R., Vriest, Y.D. and Vermeulen, N.P.E. (1991). Occurrence of a cytochrome P450 containing mixed function oxydase system in the pond snail, *Lymnaea stagnalis*. *Xenobiotica* 21 (2), 223-33.