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Toxicity of cypermethrin: hsp70 as a biomarker of response in transgenic *Drosophila*

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Heat shock protein induction is often associated with a cellular response to a harmful environment or to adverse life conditions. The main aims of our study were (1) to evaluate the cytotoxic potential of cypermethrin; and (2) to investigate the suitability of stress-induced heat shock protein Hsp70 as a biomarker for environmental pollutants in transgenic *Drosophila melanogaster* (*Hsp70-lacZ*)*Bg*^{9}. Different concentrations of cypermethrin (0.002, 0.2, 0.5 and 50.0 p.p.m.) were mixed with food. Third instar larvae of transgenic *Drosophila melanogaster* were allowed to feed on these mixtures for different time intervals (2, 4, 6, 12, 24 and 48 h). Following feeding, hsp70 induction and tissue damage were evaluated. In the highest concentration treatment group (50 p.p.m.), 100% larval mortality was recorded after 12 h exposure. Hsp70 was found to be induced even at the lowest concentration (0.002 p.p.m.) of the insecticide, while tissue damage was observed in the larvae exposed for 48 h. While an insignificant decline in hsp70 expression was observed in the larvae exposed to cypermethrin at a dietary concentration of 0.002 p.p.m. after 48 h compared with those exposed for 24 h, in the next two higher concentrations of the toxicant, a similar but significant decline in hsp70 expression was evident in the exposed larvae after 48 h. The present study reveals the cytotoxic potential of cypermethrin and further proposes that hsp70 induction in transgenic *Drosophila melanogaster* could be used as a sensitive biomarker in risk assessment.

Keywords: transgenic *Drosophila melanogaster*, cypermethrin, biomarker, hsp70, cytotoxicity, tissue damage

Introduction

Biomarkers have the potential either to be used as indicators of events at various stages in the manifestation of a toxic lesion, or to provide tools to test the relevance of proposed toxic mechanisms (Benford *et al*. 2000). Recent studies have provided compelling evidence that cells respond to damaging stimuli from a variety of environmental assaults, including exposure to xenobiotics, toxic metals, free radicals, metabolic poisons and heat, by increasing the synthesis of heat shock proteins (Welch 1993). Heat shock proteins are believed to buffer cells from damage under adverse conditions by maintaining the proteins in the proper conformation, by stabilizing unfolded precursor proteins prior to their assembly into multi-molecular complexes in the cytosol, and by guiding the newly formed as well as denatured proteins to attain their proper conformation, thereby protecting the cells from further damage (Craig *et al*. 1994, Morimoto *et al*. 1994, Barnes *et al*. 2001, King *et al*. 2002). Increased expression of heat shock proteins as a response

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to a variety of stimuli provides the potential for their use as biomarkers of chemical toxicity (Dilworth and Timbrell 1998). Among the stress genes, the 70 kDa heat shock protein (hsp70) is one of the most highly conserved and prominently expressed (Lindquist and Craig 1988). Several reports in recent years have shown that hsp70 is one of the best candidate genes for forecasting the cytotoxic effects of environmental chemicals (Kohler *et al*. 1998, Nazir *et al*. 2001, Mukhopadhyay *et al*. 2002).

Transgenic technology, using foreign reporter genes under the control of stress-inducible heat shock protein gene promoters affords a novel method for assessing heat shock protein induction (De Pomerai 1996). *Drosophila* was chosen as a model because its genome can be easily manipulated by P-element-mediated germ-line transformation. Moreover, the use of *Drosophila* falls within the recommendations of the European Centre for the Validation of Alternative Methods (ECVAM), whose goal is to promote the scientific and regulatory acceptance of alternative methods that are of importance to the biosciences and that reduce, refine or replace the use of laboratory animals (Festing *et al*. 1998, Benford *et al*. 2000).

Synthetic pyrethroids have gained importance world-wide due to their low mammalian toxicity and high insecticidal activity (Elliott *et al*. 1978). Consequently, the environmental occurrence of synthetic pyrethroids has become widespread (Casida 1980). In the present study, we explore the potential of hsp70 as a marker of toxicity against the very widely used pyrethroid insecticide cypermethrin. In addition, our study further focuses on the suitability of transgenic *Drosophila* for use as a model for evaluating toxicity.

Materials and methods

Fly strain

The flies and larvae of stock Bg^9, as described elsewhere (Lis *et al*. 1983), were cultured on standard food containing agar, corn meal, brown sugar and yeast at $24 \pm 1^{\circ}C$. Additional suspension of yeast was provided in Petri dishes for healthy growth.

Pesticide concentrations

Technical grade cypermethrin (purity 98.5%, Aimco Pesticides Ltd, India) dissolved in dimethyl sulphoxide (DMSO) (0.3% final concentration in food) was added to the food at four different concentrations: 50 p.p.m. (the recommended agricultural dose), 0.5 p.p.m. (1/100 of the agricultural dose), 0.2 p.p.m. (the maximum residue level [MRL] of cypermethrin in fruits; Chaddha 1992), and 0.002 p.p.m. (1/100 of the MRL).

Experimental design

Third instar larvae were allowed to feed on insecticide-treated food for six different time intervals (2, 4, 6, 12, 24 and 48 h). Following feeding, the larvae were taken out and washed thoroughly with Poels' salt solution (PSS) (NaCl 15 mM, NaH_2PO_4 6.4 mM, KCl 42 mM, $KHCO_3$ 1.8 mM, $CaCl_2$ 7.9 mM, $MgSO_4$ 20.8 mM; pH 6.95–7.0) (Lakhotia and Mukherjee 1980) to remove the adhering food. Control larvae received normal food, while DMSO controls received food mixed with 0.3% DMSO.

Soluble O-nitrophenyl-β-D-galactopyranoside assay

The method of Stringham and Candido (1994) modified by Nazir *et al*. (2001) was followed. Briefly, the larvae were transferred to microcentrifuge tubes after washing (20 larvae per tube, five tubes per group), permeabilized in acetone for 10 min and incubated overnight at 37°C in 600 µl *O*-nitrophenyl-β-D-galactopyranoside (ONPG) staining buffer. The reaction was stopped by adding 300 µl of 1 M Na_2CO_3. β-Galactosidase activity was quantified by measuring the absorbance at 420 nm using a Cintra20 GBCUV spectrophotometer.

Western blotting and hybridization

Protein samples prepared from the tissues of control and treated third instar larvae of the Bg^9 strain were separated using 12.5% linear sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 15 mA for 4 h (Singh and Lakhotia 1995). Western blotting and hybridization was performed according to the method of Sambrook *et al*. (1989) using an anti-Hsp70 rat monoclonal antibody, 7Fb, specific for the *Drosophila melanogaster* heat shock inducible Hsp70 family member (Velazquez and Lindquist 1984)(1:750). Immunodetection of Hsp70 was carried out following incubation of the membrane with the secondary antibody, rabbit anti-rat IgG-horse radish peroxidase (HRP) conjugate (1:1000) (M/S Bangalore Genei Pvt. Ltd), with diaminobenzidine tetrahydrochloride (DAB) as the substrate. The peak area of the immunopositive bands was calculated by densitometric analysis using the gel pro/gel blot software of GDS 7500 (UVP, England).

In situ *X-gal staining*

Approximately 90–100 larvae per group were dissected out in PSS, and internal tissues were fixed in 2.5% glutaraldehyde, washed in phosphate buffered saline (PBS) and stained in X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) as described elsewhere (O'Kane and Gehring 1987, Kar Chowdhuri *et al*. 1999).

In situ *immunohistochemistry*

The method of Singh and Lakhotia (1995) was followed with some modifications. In brief, larvae were dissected in PSS as above and fixed in 2.5% glutaraldehyde for 10 min. The tissues were then incubated with the anti-heat shock protein rat monoclonal antibody 7Fb (1:200), which is specific for *Drosophila melanogaster* Hsp70, challenged with HRP-tagged rabbit anti-rat secondary antibody (1:300) for 3 h, and stained with the chromogenic substrate (DAB).

Temperature shock treatment

Healthy third-instar larvae were placed on a petri dish lined with moist filter paper and given temperature shock at $37 \pm 1^{\circ}$C for 1 h (Kar Chowdhuri *et al*., 1999).

Trypan blue dye exclusion test

Briefly, 90–100 larvae per group were explanted in PSS to remove the internal tissues, washed once in PBS, immersed in 0.2 mg/ml trypan blue in PBS and rotated for 30 min at room temperature. The larvae were then rinsed thrice and washed for 30 min in PBS and immediately scored for trypan blue staining (Krebs and Feder 1997).

During the course of treatment, the larvae were observed for any overt signs of toxicity.

Statistical analysis

Statistical analysis was carried out by Student's *t*-test after ascertaining homogeneity of variance. Significance was ascribed at $p < 0.05$.

Results

During the course of treatment, the larvae did not exhibit any overt signs of toxicity, as evidenced by active crawling and boring habits, except at the highest dietary concentration (50 p.p.m.), when sluggish larval movement was observed.

ONPG assay

Figure 1 shows the quantitative β-galactosidase activity determined by ONPG assay as a measure of hsp70 induction in third instar larvae exposed to different dietary concentrations of cypermethrin for various time intervals. At the lowest concentration of the insecticide (0.002 p.p.m.), a significant increase ($p < 0.05$) in β-galactosidase activity was recorded in larvae following 24 h treatment (32% increase in β-galactosidase activity compared with control) followed by an insignificant decline (15%) in β-galactosidase activity after 48 h. Larvae exposed to 0.2 p.p.m. cypermethrin exhibited a significant ($p < 0.05$) elevation in

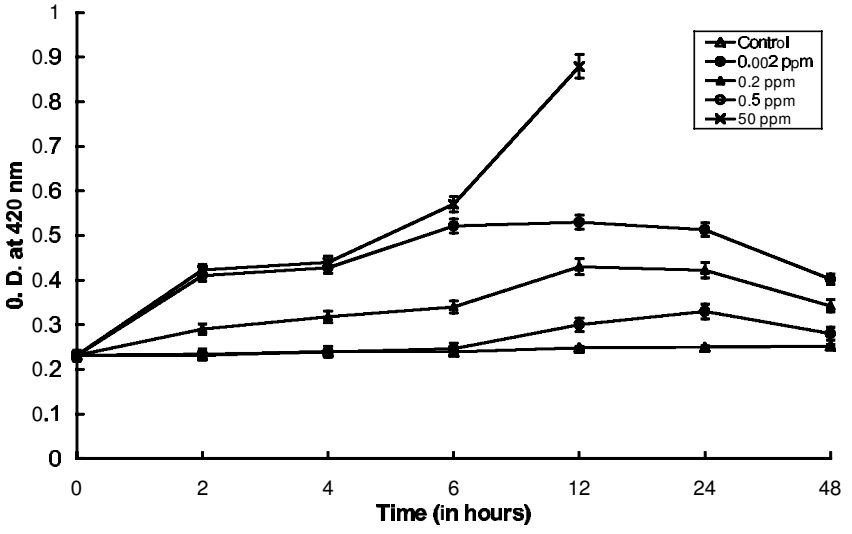

Figure 1. Soluble assay of β-galactosidase activity in transgenic *Drosophila melanogaster* (*hsp70-lacZ*)*Bg*^{9} third instar larvae in control and cypermethrin-treated conditions for different time periods. O.D., optical density.

β-galactosidase activity (26%) even after 2 h, which continued to increase significantly till 12 h (73%). However, a plateau in β-galactosidase activity in the exposed larvae was observed between 12 and 24 h treatment, followed by a significant decline ($p < 0.05$) after 48 h exposure (19% decline after 48 h). At the next highest dietary concentration of the toxicant (0.5 p.p.m), a similar but more pronounced effect was observed in the exposed larvae, with the plateau in β-galactosidase activity reached as early as 6 h and continuing until 24 h (78, 118 and 105% increase in β-galactosidase activity after 2, 6 and 24 h, respectively). This was followed by a significant decline in activity (22%) after 48 h exposure. At 50 p.p.m. cypermethrin, exposed larvae showed a maximum increase in β-galactosidase activity (254%) after 12 h. However, due to 100% larval mortality after 12 h exposure group, β-galactosidase activity could not be measured in the 50 p.p.m. cypermethrin dosed group after this time.

Western blotting

To investigate whether the decline in hsp70 expression in exposed larvae following 48 h treatment with insecticide is due to the instability of the reporter gene product or due to the decreased availability of cells, Western hybridization was performed. Figure 2 shows Hsp70 expression in control (lane 1), heat-shocked (lane 2) and cypermethrin-treated larvae (lanes 3–7). A 3.5 ± 0.4-fold increase in Hsp70 expression was observed in heat-shocked larvae (positive control; lane 2) compared with control larvae (lane 1). Similarly a 2.7 ± 0.2-fold increase in Hsp70 expression was observed in larvae treated with 50 p.p.m. cypermethrin for 12 h (data not shown). While a 1.6 ± 0.1-fold increase in Hsp70 expression was observed in larvae exposed to 0.002 p.p.m. cypermethrin for 24 h, a 0.2-fold reduction in expression of Hsp70 was evident after 48 h (lanes 3–4). Similarly, at the penultimate concentration of the toxicant (0.5 p.p.m.), a time-dependent

Figure 2. Western blot showing hsp70 in larval tissues of transgenic *Drosophila melanogaster* (*hsp70-lacZ*)Bg^9: lane 1, control; lane 2, heat-shocked larvae; lanes 3–4, 0.002 p.p.m. cypermethrin-treated larvae (lane 3, 24 h; lane 4, 48 h); lanes 5–7, 0.5 p.p.m. cypermethrin-treated larvae (lane 5, 2 h; lane 6, 12 h; lane 7, 48 h).

increase in Hsp70 expression was observed until 12 h exposure (2.0 ± 0.2-fold after 2 h to 2.5 ± 0.4-fold after 12 h), followed by a 0.2 ± 0.05-fold decrease in Hsp70 expression after 48 h exposure (lanes 5, 6 and 7).

In situ *X-gal staining*

To examine Hsp70 expression in the tissues of the larvae after feeding on cypermethrin-contaminated food, *in situ* X-gal staining was performed in control

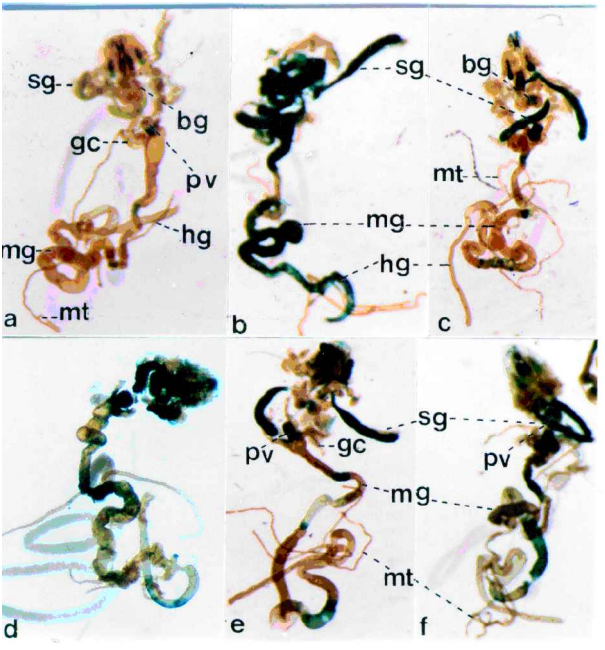

Figure 3. *In situ* β-galactosidase staining pattern in transgenic *Drosophila melanogaster* (*hsp70-lacZ*)Bg^9 third instar larval tissues: control (a); heat-shocked larvae (b); 0.2 p.p.m. cypermethrin-treated larvae after 4 h (c), 12 h (d) and 48 h (e); and 50 p.p.m. cypermethrin-treated larvae after 12 h (f). bg, brain ganglia; sg, salivary gland; gc, gastric caeca; pv, proventriculus; mg, midgut; hg, hind-gut; mt, Malpighian tubule. The bar represents 200 μm.

and treated larvae (figure 3). In the controls, 99% of the larvae did not show any β-galactosidase activity (a pale blue staining was observed in the remaining 1% larvae in the proventriculus region only) (figure 3a). Larvae given heat shock (positive control) reflected strong β-galactosidase activity in all the larval tissues except the Malpighian tubules and fat bodies in 96% of the larvae (figure 3b). At 0.002 p.p.m. cypermethrin, maximum β-galactosidase staining was evident after 24 h treatment; 96% of the exposed larvae showed moderate β-galactosidase staining in the salivary gland, brain ganglia, proventriculus and mid-gut region, while the remaining 4% did not stain at all. In the next higher dosed group (0.2 p.p.m.) a gradual increase in β-galactosidase staining was observed after 2, 4 and 6 h treatment (figure 3c, data for 4 and 6 h not shown), with maximum β-galactosidase activity being seen after 12 h exposure (figure 3d). The salivary gland, brain ganglia, gastric caeca, proventriculus and mid-gut tissues of 95% of the larvae showed high β-galactosidase staining, while in the remaining 5% a pale staining in the above mentioned tissues was observed. A regression in staining was evident in the larvae of the same treatment group after 24 and 48 h (figure 3e); the salivary gland, gastric caeca, brain ganglia and mid-gut tissues of 92% of the exposed larvae exhibited a moderate staining, while no staining was observed in the remaining 8%. Larvae exposed to 0.5 p.p.m. cypermethrin showed a similar β-galactosidase staining pattern in their tissues, but with higher intensity compared with those of the preceding group (data not shown). Strong β-galactosidase activity was observed in the highest dosage group (50 p.p.m.) following 6 h exposure. The salivary gland, brain ganglia, proventriculus, gastric caeca, imaginal discs and mid-gut tissues of 98% of the exposed larvae exhibited high β-galactosidase staining, while moderate staining was observed in the remaining 2% (data not shown). The intensity of β-galactosidase staining reached a maximum following 12 h exposure in this treatment group (figure 3f), with 97% of the larvae showing a very strong β-galactosidase staining involving the same tissues. Due to 100% larval mortality in after 12 h exposure to 50 p.p.m. cypermethrin, no data for β-galactosidase staining could be shown after this time.

In situ *immunohistochemistry*

To check whether the result obtained through the reporter gene assay (*in situ* X-gal staining) matches with the real status of Hsp70 after treatment with different concentrations of the insecticide, *in situ* immunohistochemical staining was performed (figure 4). The patterns of Hsp70 expression in the tissues of cypermethrin-treated larvae were comparable to those observed in the reporter gene assay (*in situ* X-gal staining).

Trypan blue staining

To investigate whether the insecticide can induce tissue damage in the larval tissues, trypan blue staining was performed (figure 5). A uniform staining in the head region of larvae from both the control and the treated groups was observed during the study. In the control group, 98% of the larvae stained negatively to trypan blue, even after 48 h exposure (figure 5a), while in the remaining 2% a faint blue staining was observed in the gastric caeca. Larvae exposed to 0.002 p.p.m. cypermethrin exhibited pale blue staining in the mid-gut in 91% of the larvae after

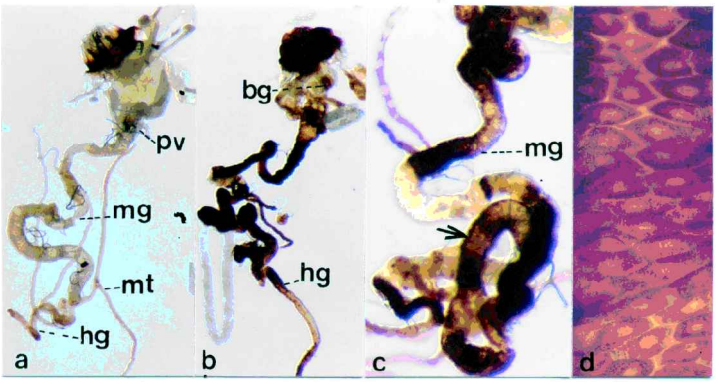

Figure 4. *In situ* immunohistochemical staining pattern in tissues of third instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*)Bg^9 using anti-Hsp70 antibody: control (a); heat-shocked larvae (b); 0.5 p.p.m. cypermethrin-treated larvae after 12 h (c); and cells of mid-gut (magnification ×250) of 0.5 p.p.m. cypermethrin-treated larvae as marked with arrow in c (d). bg, brain ganglia; pv, proventriculus; mg, mid-gut; hg, hind-gut; mt, Malpighian tubule. The bar represents 200 µm.

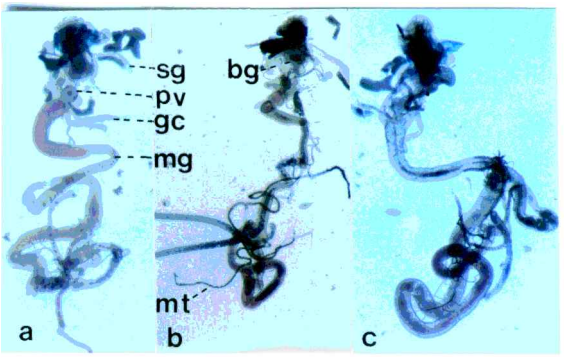

Figure 5. Trypan blue staining pattern in the third instar larval tissues of *Drosophila melanogaster* (*hsp70-lacZ*)Bg^9: control (a); 0.002 p.p.m. cypermethrin-treated larvae after 48 h (b); and 0.2 p.p.m. cypermethrin-treated larvae after 48 h (c). Note the blue staining in the mid-gut tissues. bg, brain ganglia; sg, salivary gland; gc, gastric caeca; pv, proventriculus; mg, mid-gut; mt, Malpighian tubule. The bar represents 200 µm.

48 h exposure (figure 5b). In the next two higher dosage groups (0.2 and 0.5 p.p.m.), the intensity of staining was found to be increased, with 94% and 92% of the larvae, respectively, exhibiting moderate blue staining in mid-gut, gastric caeca and brain ganglia tissues (figure 5c). Trypan blue staining in the highest dosage treatment group (50 p.p.m.) at these time points could not be accessed due to 100% larval mortality shortly after 12 h exposure.

Discussion

In the present study, Hsp70 expression was observed in the third instar larval tissues of Bg^9 even at the lowest concentration of the insecticide (0.002 p.p.m.). The sensitivity of a parameter is one of the important prerequisites of a suitable biomarker, and the sensitivity of the Hsp70 response was up to several orders of

magnitude higher than common measures of toxicity such as LC_{50} and EC_{20} (Werner and Nagel 1997).

A dose-dependent upregulation in Hsp70 expression was observed. Expression of Hsp70 in the different larval tissues was found to be delayed at the lowest concentration of the insecticide, while at the remaining three concentrations (0.2, 0.5 and 50 p.p.m.) Hsp70 expression was triggered more quickly. This may possibly be due to the severity of the action of the toxicant at higher concentrations compared with the lowest one (Kar Chowdhuri *et al.* 1999). Interestingly, a decline in Hsp70 expression was observed in all the treatment groups after 48 h, except for the highest concentration of the toxicant where 100% larval mortality occurred after 12 h. In this context, the question of whether such a decline is due to instability of the reporter gene product after 48 h or due to a reduction in the number of cells available for Hsp70 assay from 0.002, 0.2 and 0.5 p.p.m. cypermethrin-treated larvae following 48 h exposure is also addressed in the present study. Our results with Western hybridization and immunohistochemistry showing Hsp70 expression levels comparable to those observed in the reporter gene assay appear to negate the first possibility. However, the moderate trypan blue staining in the larval tissues observed under such conditions concurrent with a decline in Hsp70 expression could well be due to a reduction in the number of viable cells. The uniform trypan blue staining observed in the head region of the larvae in all the groups may be due to tissue damage during dissection.

It is important to remember that the effects of test chemicals on expression of stress proteins are tissue specific (Stringham and Candido 1994, Chapple *et al.* 1997). In the present study, higher dietary concentrations of the toxicant induced strong Hsp70 expression in brain ganglia rapidly as compared with the delayed expression seen at the lower concentrations. Pyrethroids are known to act as neurotoxicants, affecting the central nervous system. Cypermethrin has been shown to increase the cGMP content in mammalian brain (Abassy *et al.* 1983, Ruight 1985). The level of intoxication in rat brain following pyrethroid treatment was shown to be correlated with a critical concentration of the compound present in brain and is independent of the route of administration (Ruight 1985). One of the possibilities for rapid Hsp70 expression in brain ganglia at higher concentrations of the toxicant may be due to the presence of a critical amount of the compound. In addition, the possibility that the toxicant might affect nerve membranes by interfering with sodium channels as well as cGMP levels, thereby stimulating Hsp70 expression, cannot be ruled out. Salivary glands and imaginal discs were also found in the present study to induce Hsp70. Like brain ganglia, these two tissues are nourished by the surrounding haemolymph and therefore are likely to be exposed when the toxicant reaches the haemolymph. The mid-gut tissues of insects have the highest concentrations of cytochrome species and high microsomal oxidase activity (Wilkinson and Brattesten 1972). Hsp70 expression observed in the gut tissues may possibly be due to the metabolites generated by cytochrome species. Non-expression of Hsp70 in the Malpighian tubules is intriguing. Malpighian tubules comprise two distinct cell types, of which the more abundant broad cells show peak expression of Hsp70 well after most other tissues (Singh and Lakhotia 1995, Krebs and Feder 1997), which may be a possible reason for its non-expression over the time course tested. Recent studies have shown that vulnerable target organs for toxicants can be identified by stress gene expression (Hightower 1991), and regulation of stress proteins is achieved in a

stressor-specific manner. Therefore, it is likely that larval tissues showing Hsp70 expression are vulnerable sites for the toxicant.

The use of Hsp70 as a biomarker has been advocated by many (Ryan and Hightower 1996, Werner and Hinton 1999, Nadeau *et al*. 2001, Varo *et al*. 2002). Biomarker responses are need to be sublethal, quantifiable and reliable for interpretation (Pyza *et al*. 1997). Hence the detection of Hsp70 in *Drosophila* may serve as an early warning for the presence of potentially toxic agents in the environment.

In view of the above results, it is suggested that cypermethrin-induced Hsp70 expression may reflect the cytotoxic potential of the insecticide. The induction, quantification and differential expression of Hsp70 in various tissues further strengthens the evidence for the use of *Drosophila* as a useful bioindicator and of Hsp70 as a potential biomarker of environmental pollution.

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