Hazardous Effects of Effluent from the Chrome Plating Industry: 70 kDa Heat Shock Protein Expression as a Marker of Cellular Damage in Transgenic *Drosophila melanogaster* (*hsp70-lacZ*)

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Hazardous effects of an effluent from the chrome plating industry were examined by exposing transgenic *Drosophila melanogaster* (*hsp70-lacZ*) to various concentrations (0.05, 0.1, 1.0, 10.0, and 100.0 µL/mL) of the effluent through diet. The emergence pattern of adult flies was affected, along with impaired reproductive performance at the higher dietary concentrations of the effluent. Interestingly, the effect of the effluent was more pronounced in male than in female flies. The effect of the effluent on development of adult flies was concurrent with the expression pattern of the heat shock protein 70 gene (*hsp70*), both in larval tissues and in the reproductive organs of adult flies. We observed a dose- and time-dependent expression of *hsp70* in third instar larvae exposed for different time intervals. Absence of *hsp70* expression in larvae exposed to 0.1 µL/mL of the effluent indicated that this is the highest nontoxic concentration for *Drosophila*. The stress gene assay in the reproductive organs of adult flies revealed *hsp70* expression in the testis of male flies only. However, trypan blue dye exclusion tests in these tissues indicate tissue damage in the male accessory gland of adult flies, which was further confirmed by ultrastructural observations. In the present study we demonstrate the utility of transgenic *Drosophila* as an alternative animal model for evaluating hazardous effects of the effluent from the chrome plating industry and further reveal the cytoprotective role of *hsp70* and its expression as an early marker in environmental risk assessment. *Key words:* alternative animal model, biomarker, chrome plating industry, cytotoxicity, effluent, hsp70, reproduction, tissue damage, transgenic *Drosophila melanogaster*. *Environ Health Perspect* 111:1926–1932 (2003). doi:10.1289/ehp.6339 available via *http://dx.doi.org/* [Online 7 August 2003]

With rapid industrialization, many metallic wastes have become widely distributed over the earth's surface and are excessively concentrated in localized areas, particularly in regions of high population density. Chrome plating is one of the most actively operating industries worldwide. The problem becomes more pronounced with small-scale and unorganized growth of such industries in the vicinity of densely populated areas. These industries are more prevalent in underdeveloped and developing countries such as India. Effluent discharged from the chrome plating industry contains a large number of metals, including chromium, copper, nickel, zinc, manganese, and lead. The specific difficulty with industrial effluents is that they are very persistent and may be transported considerable distances by air, water, or the food chain, where they tend to accumulate, reaching concentrations in the upper trophic levels that are several orders of magnitude higher than those that originally existed, thus representing a continuous threat to the biota (Edmund et al. 1976). In complex effluents, toxic interactions among various toxicants can be synergistic (more than additive), antagonistic (less than additive), or simply additive. Only whole-effluent toxicity tests allow practical and effective assessment of combined effects of toxic substances (Gomez et al. 2001).

Over the last decade, biomarkers have been used in considerable research and have been valuable to the field of molecular epidemiology (Bennett and Waters 2000). In recent years, heat shock proteins have evolved as a very successful first-tier diagnostic tool to screen for adverse biologic effects induced by environmental chemicals. Because heat shock proteins are part of the machinery for defense, repair, and detoxification of cells, they become direct and specific markers of exposure and effect (Bierkens 2000). Homologues of heat shock proteins occur in every species of every kingdom of living things (Feder and Hofman 1999). Thus, the heat shock proteins represent a remarkable example of molecular "descent with modification" (De Maio 1999) at the levels of gene sequence, genomic organization, regulation of gene expression, and protein structure and function. Among the different heat shock proteins, the stress-inducible heat shock protein 70 (Hsp70) family is the most conserved and is also the most highly induced by cellular stresses (Feige and Polla 1995; Macario et al. 1999). Because of its responsiveness to diverse forms of stress, the heat shock response has undergone widespread application in biomonitoring and environmental toxicology (Ait-Aissa et al. 2003; de Pomerai 1996; Mukhopadhyay et al. 2002a, 2002b; Radlowska et al. 2002; Ryan and Hightower 1996).

In the present study we explored the toxic potential of an effluent from the chrome plating industry, using *hsp70* expression as a marker of cytotoxicity in transgenic *Drosophila melanogaster* (*hsp70-lacZ*) Bg^9 flies. We examined the emergence pattern of adult flies and their reproductive performance to determine whether the effluent affects their development. Finally, we attempted to determine the highest nontoxic concentration of the effluent in *D. melanogaster* by stress gene assay. *Drosophila* was used as a model organism because of its well-understood genetics and development. Moreover, the use of *Drosophila* falls within the recommendations of the European Centre for the Validation of Alternative Methods (Festing et al. 1998), whose goal is to promote the scientific and regulatory acceptance of alternative methods that are of importance in the field of bioscience and that reduce, refine, or replace the use of laboratory animals (Benford et al. 2000).

Materials and Methods

Fly strain. In the present study, we used a transgenic strain of *Drosophila melanogaster* (*hsp70-lacZ*) Bg^9 that expresses bacterial β-galactosidase as a response to stress (Lis et al. 1983). The flies and larvae were reared at 24 ± 1°C on standard *Drosophila* food (1 unit of *Drosophila* food contains 17 g maize powder, 15 g sugar, 6 g yeast, 1.5 g agar agar, 1 g nepagin (methyl *p*-hydroxybenzoate), 1 mL propionic acid, and 360 mL water).

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Concentrations of effluent. The effluent was collected from a chrome plating industry in the eastern part of India and mixed with *Drosophila* food medium. We used five different concentrations of the effluent, which was added to the food: 0.05, 0.1, 1.0, 10.0, and 100.0 µL/mL food. The control group received food with no effluent added.

Metal analysis of effluent. Effluent samples (50 mL) were digested in a mixture of nitric and perchloric acid (6:1 vol/vol) until a clear white residue remained at the bottom of the flask. This residue was dissolved in 0.1 *N* nitric acid made to a total volume of 20 mL in a volumetric flask. Metals were estimated by using an inductively coupled plasma atomic emission spectrophotometer (8440 Plasmalab; Labtam, Braeside, Victoria, Australia) (Cleseeri et al. 1998).

hsp70 ***expression in third instar larval tissues.*** To evaluate the cellular response to the effluent, third instar larvae of transgenic *D. melanogaster* were allowed to feed on normal food or food mixed with different concentrations of the effluent for 2, 6, 12, 24, and 48 hr (90–100 larvae per group). After feeding, the larvae were removed from food, washed thoroughly with Poels' salt solution (PSS) (Lakhotia and Mukherjee 1980) to remove adhering food particles, and then processed for *hsp70* expression studies as described below.

Soluble *o*-nitrophenyl-β-D-galactopyranoside assay. We followed a modified version of the method described by Stringham and Candido (1994). Briefly, after washing, the larvae were placed in a microcentrifuge tube (20 larvae/tube, five replicates 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. After incubation, the reaction was stopped by adding 300 µL 1 M Na_2CO_3. The extent of reaction was quantified by measuring the absorbance at 420 nm using a Cintra 20 ultraviolet spectrophotometer (GBC Scientific Equipment, Melbourne, Australia).

***In situ* histochemical β-galactosidase activity.** The larval tissues were explanted in PSS, and after a brief fixing in 2.5% glutaraldehyde and postwashing in 50 mM sodium phosphate buffer (pH 8.0), staining was performed with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) as described previously (Kar Chowdhuri et al. 1999; O'Kane and Gehring 1987).

Temperature shock treatment (positive control). Healthy third instar larvae were placed on a petri dish lined with moist filter paper and given temperature shock at 37 ± 1°C for 1 hr (Lakhotia and Singh 1989) followed by X-gal staining as described above.

In situ ***whole organ immunohistochemistry.*** We followed the method of Krebs and Feder (1997) with some modifications (Mukhopadhyay et al. 2002b). Briefly, internal tissues were dissected out in PSS, fixed in 2.5% glutaraldehyde, and permeabilized in phosphate-buffered saline (PBS) with Triton X-100. They were then incubated in 7Fb rat monoclonal antibody, which detects only the inducible form of Hsp70 in *D. melanogaster* (Velazquez and Lindquist 1984), challenged with horseradish peroxidase-conjugated rabbit anti-rat secondary antibody (Bangalore Genei Pvt. Ltd., Bangalore, India), and stained with the chromogenic substrate diaminobenzidine, as described previously (Sambrook et al. 1989).

Trypan blue staining in third instar larval tissues. The extent of tissue damage in larvae as a result of exposure to different concentrations of the effluent for 48 hr was assayed by dye exclusion test (Krebs and Feder 1997). Briefly, the internal tissues were explanted in PSS, washed once in PBS, immersed in 0.2 mg/mL trypan blue stain, and rotated for 30 min at room temperature. The tissues were then washed thoroughly in PBS and immediately scored for trypan blue staining.

Emergence pattern of adult flies. Freshly eclosed first instar larvae (± 0.5 hr) were transferred to normal food medium (control) and to food containing different concentrations of the effluent (50 larvae/vial, 10 vials/group). The number of flies emerging from different groups was recorded until all the flies emerged (Gayathri and Krishnamurthy 1981).

Pair mating and reproductive performance. We followed the method of Gayathri and Krishnamurthy (1981) with some modifications. Virgin male and female flies emerging from control and different concentrations of effluent-contaminated food were isolated and pair-mated in normal food vials. Pair mating was conducted in three different conditions per treatment group (30 pairs of flies per treatment group): *a*) treated male × treated female; *b*) treated male × normal female, and *c*) normal male × treated female. Flies were transferred to fresh vials every day for the next 10 days, and the number of eggs laid during this period was recorded. From the data obtained, we calculated total fecundity (number of eggs) and mean egg production by a female for 10 days. The total number of flies eclosed from the eggs laid during these 10 days of pair-mating was counted; the mean number of flies emerged per pair for 10 days gave a measure of reproductive performance.

hsp70 ***expression and trypan blue staining in the reproductive organs of adult fly.*** Reproductive organs of virgin male and female flies eclosing from control and treated groups were dissected out in PSS. *In situ* whole organ X-gal staining and immunohistochemistry was performed as described above.

Reproductive organs of virgin male and female flies eclosing from control and treated groups were dissected out in PSS. Trypan blue staining was performed as described above.

Ultrastructure of adult male accessory gland. Male accessory glands isolated from freshly eclosed adult flies in control and treated groups were fixed in a solution of 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2), postfixed in 2% osmium tetroxide, dehydrated, and embedded in Araldite 502 (Ted Pella, Inc., Redding, CA, USA). Ultrathin sections (70–90 nm thick) of the blocks were picked up on copper grids, stained with uranyl acetate and lead citrate, and analyzed under transmission electron microscopy at 80 kV (Tecnai 12; FEI/Philips Electron Optics, Eindhoven, the Netherlands).

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

Results

Metal analysis of effluent. Table 1 shows the concentration of the eight metals (cadmium, chromium, copper, iron, manganese, nickel, zinc, lead) analyzed for their presence in the effluent. Except cadmium, all the other metals were found to be present at a much higher

Table 1. Metal analysis of effluent from the chrome plating industry.

Metals present	Concentration (µg/mL)
Cadmium	ND
Chromium	230.3
Zinc	204.5
Iron	175.9
Nickel	120.8
Manganese	43.6
Lead	23.0
Copper	83.6

ND, not detected.

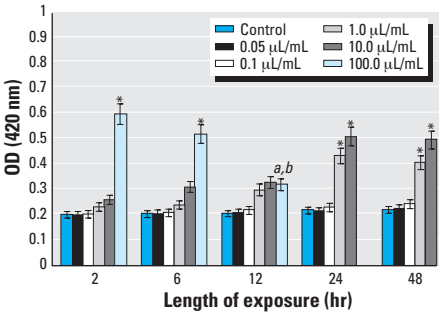

Figure 1. Assay of β-galactosidase activity in transgenic *D. melanogaster* (*hsp70-lacZ*) third instar larvae in controls and in larvae fed different concentrations of effluent from the chrome plating industry for different time periods. OD, optical density. Error bars indicate SE.
[a]$p < 0.05$ after 2 hr. [b]$p < 0.05$ after 6 hr. *$p < 0.05$ versus control.

concentrations than the permissible limit (Bureau of Indian Standards 1982).

hsp70 *expression in third instar larval tissues.* Soluble ONPG assay. Figure 1 shows quantitative *hsp70* expression by ONPG assay in third instar larvae of transgenic *D. melanogaster* exposed to different dietary concentrations of the chrome plating effluent for various time intervals. In the lowest concentration of the effluent (0.05 µL/mL), β-galactosidase activity was equivalent to that observed in the control. In the next higher concentration (0.1 µL/mL), we observed an increase in β-galactosidase activity with an increase in exposure time. However, such an increase in activity over time at this concentration was insignificant ($p > 0.05$) compared with the control. Larvae fed with 1.0 µL/mL and 10.0 µL/mL concentrations of the effluent-contaminated food showed significant increases in β-galactosidase activity after 24 hr of treatment (98% vs. 133% increase in activity, respectively, $p < 0.05$ compared with control) followed by an insignificant reduction in activity after 48 hr of treatment ($p > 0.05$ compared with β-galactosidase activity after 24 hr). At the highest concentration of the effluent (100.0 µL/mL), larvae exposed for 2 hr showed a maximum β-galactosidase activity (197% increase in activity, $p < 0.05$ compared with control), whereas a marked regression in the enzyme activity was seen after 12 hr of treatment (86% reduction, $p < 0.05$ compared with β-galactosidase activity after 2 hr). Because larvae in this group did not survive for 24 hr, no ONPG assay could be performed.

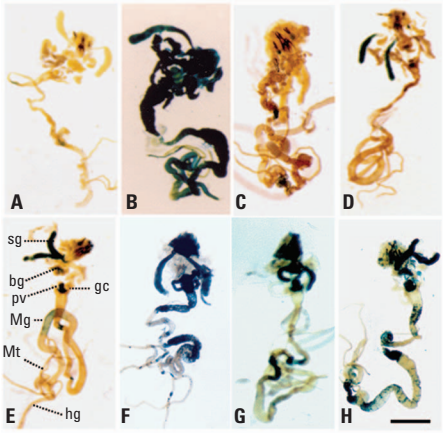

Figure 2. *In situ* β-galactosidase staining pattern in transgenic *D. melanogaster* (*hsp70-lacZ*) third instar larval tissues. (*A*) Control; (*B*) after temperature shock; (*C*) larvae exposed to 0.05 µL/mL effluent 48 hr; (*D*) larvae exposed to 0.1 µL/mL effluent 48 hr; (*E*) larvae exposed to 1.0 µL/mL effluent 2 hr; (*F*) larvae exposed to 1.0 µL/mL effluent 24 hr; (*G*) larvae exposed to 1.0 µL/mL effluent 48 hr; and (*H*) larvae exposed to 100.0 µL/mL effluent 2 hr. Abbreviations: bg, brain ganglia; gc, gastric ceca; hg, hindgut; Mg, midgut; Mt, Malpighian tubule; pv, proventriculus; sg, salivary gland. Bar = 200 µm.

***In situ* histochemical β-galactosidase activity.** *In situ* histochemical staining was performed to examine tissue-specific expression of *hsp70* after exposure to the effluent for different time intervals (Figure 2). In controls, no β-galactosidase activity was observed in 98% of larvae; in the remaining 2%, we observed a pale blue staining in the proventriculus (Figure 2A). Temperature-shocked larvae (positive controls) showed a maximum β-galactosidase activity in all the tissues except Malpighian tubules (Figure 2B). X-gal staining in the tissues of the larvae exposed to the lowest concentration of the effluent (0.05 µL/mL) was similar to that in control (97% of larvae showed no staining, and 3% showed a pale blue staining in the proventriculus; Figure 2C). At the next higher concentration of the effluent (0.1 µL/mL), no X-gal staining was evident in the exposed larvae until after 12 hr treatment, whereas a very weak staining was observed in the salivary gland and proventriculus of 95% of the larvae exposed at this dietary concentration for 24 hr (not shown). After 48 hr treatment in the effluent-contaminated food (0.1 µL/mL, a pale blue staining was evident in the salivary glands and proventriculus of the larvae, 93% of larvae showed a moderate blue staining, and 7% showed no staining (Figure 2D). However, the increase in X-gal staining at this concentration was not significant compared with the control, as determined with the ONPG data. Hence, we considered 0.1 µL/mL the highest nontoxic concentration of the effluent. In the next two higher concentrations of the effluent (1.0 and 10.0 µL/mL), maximum β-galactosidase activity was observed after 24 hr of treatment, followed by a regression in staining after 48 hr (Figure 2E–H). Salivary glands, brain ganglia, proventriculus, gastric ceca, and midgut tissues of third instar larvae exhibited a pale blue staining after 24 hr of feeding in food mixed with 1.0 µL/mL effluent (Figure 2F), whereas moderate staining was observed in the next higher group (10.0 µL/mL) involving the same tissues (data not shown). Third instar larvae grown on food contaminated with the highest concentration of the effluent (100 µL/mL) exhibited dark blue staining (in 97% larvae) in salivary gland, proventriculus, midgut, hindgut, gastric ceca, and brain ganglia after 2 hr of treatment (Figure 2H). Treatment of the larvae for 12 hr at this concentration resulted in reduction in β-galactosidase activity in the same tissues (data not shown).

***In situ* whole organ immunohistochemistry.** To examine whether the reporter gene assay reflects the native *hsp70* expression, we performed whole organ immunohistochemistry (Figure 3). Immunostaining of larvae with Hsp70-specific 7Fb antibody showing the original status of *hsp70* confirmed the transgenic observations.

Trypan blue staining in third instar larval tissues. Trypan blue is a vital dye that is not excluded in dead or dying cells and gives a reliable measure of tissue damage. Figure 4 shows trypan blue staining in third instar larval tissues of transgenic *Drosophila* in control and various concentrations of chrome plating effluent–treated groups after 48 hr exposure. Larvae fed the control diet (Figure 4A) and the lower two concentrations of the effluent-mixed food (0.05 and 0.1 µL/mL) did not exhibit any trypan blue staining (Figure 4B; data for 0.1 µL/mL concentration not shown). However, larvae exposed to 1.0 µL/mL effluent exhibited a pale blue staining in salivary gland, proventriculus, brain ganglia, and midgut tissues (data not shown). In the next higher concentration of the effluent (10.0 µL/mL), the exposed larvae exhibited a moderate blue staining in the salivary gland, proventriculus, brain ganglia, and midgut tissues (Figure 4C).

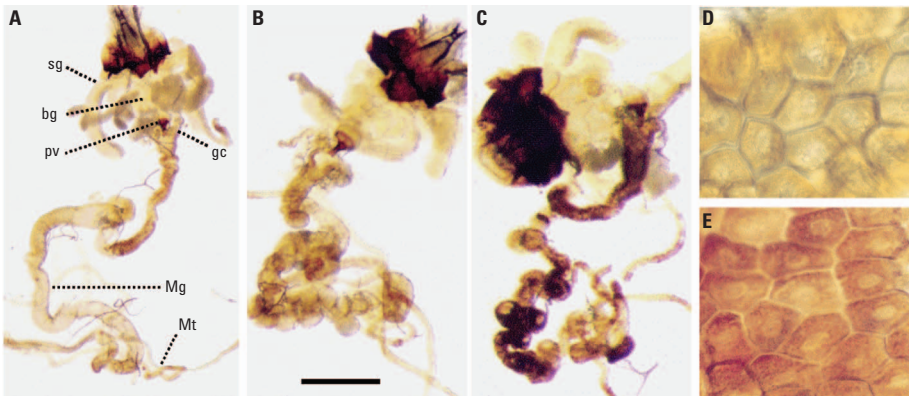

Figure 3. *In situ* immunohistochemical staining pattern in tissues of third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) using anti-Hsp70 antibody. (*A*) Control; (*B*) larvae exposed to 0.05 µL/mL effluent after 48 hr; (*C*) larvae exposed to 10.0 µL/mL effluent after 24 hr; and cells of the midgut at higher magnifications (250×) in (*D*) larvae fed 0.05 µL/mL effluent after 48 hr; and (*E*) larvae fed 10.0 µL/mL effluent after 24 hr. Abbreviations: bg, brain ganglia; gc, gastric ceca; Mg, midgut; Mt, Malpighian tubule; pv, proventriculus; sg, salivary gland. Bar = 200 µm.

Emergence pattern of adult flies. Figure 5 shows the pattern of emergence of flies after exposure of first instar larvae to different concentrations of effluent-contaminated food. Percentages of flies eclosed in the lower two concentrations of the effluent (0.05 and 0.1 µL/mL) were 94% and 96%, respectively; no delay in the emergence pattern was observed in these groups compared with the control. In the next two higher concentrations of the effluent (1.0 and 10.0 µL/mL), we observed a delay in the emergence of adult flies by 1 and 2 days, respectively, coupled with a lower percentage of adult flies eclosed (30% and 45% reduction, respectively). Adult flies did not emerge at the highest concentration of the effluent (100.0 µL/mL).

Pair mating and reproductive performance. Table 2 shows the effect of effluent from chrome plating industry on the reproduction of *Drosophila*. The mean daily egg production and the reproductive performance of flies fed food mixed with the lower three concentrations of chrome plating effluent (0.05, 0.1, and 1.0 µL/mL) since first instar larval stage were not significantly affected ($p > 0.05$) compared with the control. However, the flies fed food mixed with 10.0 µL/mL effluent showed a significant reduction ($p < 0.05$) in mean daily egg production and in reproductive performance. When we compared the effect of the effluent on individual sex, a more or less similar effect was observed in "treated male × treated female" and "treated male × normal female" categories. However, no significant reduction ($p > 0.05$) in reproduction was observed in the "normal male × treated female" category compared with the control.

hsp70 ***expression in the reproductive organs of adult fly.*** Testis from the control flies revealed β-galactosidase staining in a subset of late spermatogonial cells (Figure 6A). An overall increase in β-galactosidase activity was evident throughout the testis after heat shock (data not shown). Flies grown on food mixed with the lower three concentrations of the effluent (0.05, 0.1, and 1.0 µL/mL) exhibited β-galactosidase staining similar to that of control (data not shown). At the 10.0 µL/mL concentration, we observed an enhanced level of β-galactosidase activity in the somatic tip cells of spermatogonia and in the proximal part of the testis (Figure 6B). Interestingly, β-galactosidase staining was restricted to the male reproductive organs. Results obtained after immunostaining the adult reproductive organs with Hsp70-specific 7Fb antibody in control, positive control, and effluent-treated groups were comparable with those observed with β-galactosidase staining, thus confirming the native gene expression and nullifying any transgenic effect (Figure 6C).

Trypan blue staining in the reproductive organs of adult fly. No trypan blue staining was observed in the reproductive organs of adult control flies (Figure 7A) or in flies fed food mixed with the lower three concentrations of effluent (0.05, 0.1, and 1.0 µL/mL; data not shown). At the 10.0 µL/mL concentration, a moderate blue staining was evident in the accessory glands of male flies (Figure 7B). No trypan blue staining was observed in the female reproductive organs of adult flies in control or treated groups (data not shown).

Transmission electron microscopy. The ultrastructural morphology of the accessory glands in the control group (Figure 8A) was comparable with previous observations (Bairati 1968). The wall of the male accessory glands consists of a prismatic, single-layer epithelium, composed of two types of secretory cells: *a*) the more abundant "principal" or "main" cells, which are hexagonal, markedly electron opaque, and resting on the basal membrane; and *b*) the less abundant "secondary" cells, which are pear shaped and are usually surrounded by the main cells. The nucleus of the main cells presented both fine and dense granules plus a notable nucleolus typical of secretory cells. The cytoplasmic content was filled with numerous rough endoplasmic reticula. Golgi cisternae with large number of vesicles filled with osmiophilic contents were observed, which were finally secreted in the lumen of the gland. Numerous linearly arranged filamentous structures were also observed in the lumen of the gland.

A deviation from the normal ultrastructural morphology was evident in the male accessory gland of adult flies cultured on food mixed with the 10.0 µL/mL concentration of effluent. Both cell types showed signs of necrosis, as evident by necrotic nuclei (Figure 8B), comparatively higher vacuolization (Figure 8C), and disorganized endoplasmic reticula with fewer ribosomes (Figure 8D). Golgi cisternae were scarce, with very few vesicles. The lumen of the gland showed fewer filamentous structures.

Discussion

Effluent from the chrome plating industry was found to be highly toxic at higher concentrations, as revealed by *hsp70* expression, trypan blue staining, and overall effects on the development of the organism. This was further confirmed with the analysis of heavy metals present in the effluent. The Hsp70 synthesis increases in response to damaging stimuli, and it takes on new roles to provide defense for the cell to protect it from further damage (Hightower 1991). A number of studies in recent years have employed Hsp70

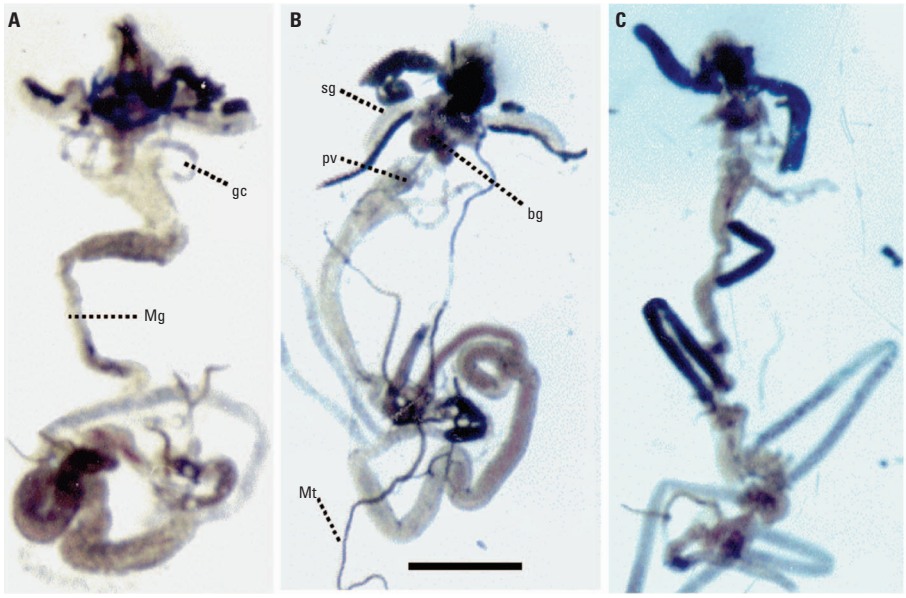

Figure 4. Trypan blue staining pattern in the third instar larval tissues of *D. melanogaster* (*hsp70-lacZ*). (*A*) Control; (*B*) larvae exposed to 0.05 µL/mL effluent 48 hr; and (*C*) larvae exposed to 10.0 µL/mL effluent 48 hr. Abbreviations: bg, brain ganglia; gc, gastric ceca; Mg, midgut; Mt, Malpighian tubule; pv, proventriculus; sg, salivary gland. Note the blue staining in the midgut tissues. Bar = 200 µm.

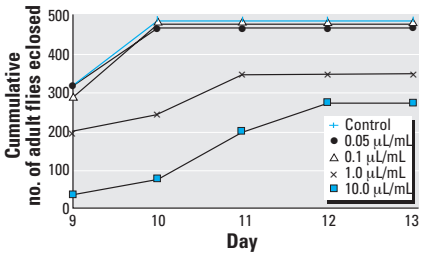

Figure 5. Emergence pattern of transgenic *D. melanogaster* (*hsp70-lacZ*) given control food and food mixed with different concentrations of effluent from the chrome plating industry.

in evaluating the toxic potential of such heavy metals as lead, chromium (Tully et al. 2000), nickel (Caroll and Wood 2000), cadmium (Blechinger et al. 2002), zinc (Ait-Aissa et al. 2000), or complex mixtures (Ait-Aissa et al. 2003; Radlowska et al. 2002).

An insignificant regression in *hsp70* expression was observed in the higher concentrations of the effluent once its maximum level was reached. That the regression was due to instability of the reporter gene was nullified by the native *hsp70* expression study, which mirrors the reporter gene activity pattern. Earlier reports showed that autorepression of *hsp70* takes place once its upper limit of cellular level is reached (Di Domenico et al. 1982; Nover and Scharf 1991; Stone and Craig 1990). Therefore, regression of *hsp70* under such condition may be due to attainment of the threshold limit in the cell. However, pale to moderate trypan blue staining observed in the exposed larvae at these concentrations indicates tissue damage. Hence, regression of *hsp70* due to availability of lesser numbers of viable cells also cannot be ruled out.

In the present study, we found *hsp70* expressed in salivary glands, brain ganglia, proventriculus, gastric ceca, and midgut of the exposed larvae. Because salivary glands and brain ganglia are nourished by the surrounding hemolymph, they are likely to be exposed when the toxicant reaches the hemolymph. Exposure of the larvae to different concentrations of the effluent occurs through feeding. Therefore, gut tissues of the larvae are likely to be exposed during this process, leading to induction of *hsp70*. Even *hsp70* was unable to protect the gut tissues from damage, as evidenced by trypan blue staining in this region, thus showing the severity of damage caused by the effluent. Malpighian tubules were not found to induce *hsp70* against the effluent used in the present study. This may be possibly explained by the time of assaying the β-galactosidase activity in the present study, as we reported previously (Mukhopadhyay et al. 2002b).

Development of the organism was affected in the present study, as evidenced by a delay in the emergence pattern of adult flies and a decrease in the number of flies that emerged at the higher effluent concentrations. Gayathri and Krishnamurthy (1981), using Agallol 3 [Bayer (India) Ltd., Mumbai, India], a mercurial fungicide, and Nazir et al. (2001), using chlorpyrifos, an organophosphate pesticide, observed similar effects in *D. melanogaster*. Such a change may be due to both genetic and environmental components (Bonnier 1960).

Results presented here from pair-mating of adult flies to examine gender sensitiveness to the test chemicals indicates that the male flies are more sensitive than female flies. Hsp70 synthesis in the male reproductive organs of freshly eclosed adult flies revealed a dose-dependent synthesis of the protein. Interestingly, *hsp70* expression was restricted only in the testis lobe. Dix and Hong (1998) showed that a wide range of environmental exposures trigger protective mechanisms in reproductive tissues that are mediated by heat shock proteins. The ability of Hsp70 to protect the testis lobes from damage was evident in our study by normal sperm morphology, motility (Mukherjee I, Kar Chowdhuri D. Unpublished data), and absence of trypan blue staining in this tissue. Interestingly, trypan blue stained positively in the male accessory glands of adult flies grown on food containing 10.0 µL/mL effluent, indicating tissue damage in this gland. This was further confirmed by the ultrastructural observations of the gland. Nonexpression of *hsp70* in this gland is intriguing, and at present we are unable to provide any plausible explanation for this.

The male accessory gland in *Drosophila* serves as the main organ of the male genital

Table 2. Effect of effluent from chrome plating industry on reproduction in *D. melanogaster* (*hsp70-lacZ*)*Bg*[9].

Group	Total fecundity (no. of eggs)	Eggs laid per female in 10 days (mean ± SE)	Fertility (%)	Reproductive performance
Control	2,350	235.0 ± 8.90	95.6	224.7 ± 7.6
0.05 µL/mL				
Treated male × treated female	2,330	233.0 ± 7.6	93.3	217.4 ± 6.9
Treated male × normal female	2,329	232.9 ± 8.1	91.1	212.2 ± 7.1
Normal male × treated female	2,345	234.5 ± 7.7	94.0	220.4 ± 7.0
0.1 µL/mL				
Treated male × treated female	2,328	232.8 ± 7.2	92.8	216.0 ± 7.2
Treated male × normal female	2,327	232.7 ± 7.3	92.0	214.1 ± 7.6
Normal male × treated female	2,331	233.1 ± 7.3	93.0	216.8 ± 7.3
1.0 µL/mL				
Treated male × treated female	2,319	231.9 ± 8.2	93.3	216.4 ± 6.8
Treated male × normal female	2,322	232.2 ± 7.9	93.6	217.3 ± 7.6
Normal male × treated female	2,329	232.9 ± 7.7	91.1	212.2 ± 7.3
10.0 µL/mL				
Treated male × treated female	1,990	199.0 ± 8.6*	86.6	172.3 ± 8.1*
Treated male × normal female	1,998	199.8 ± 8.3*	88.4	176.6 ± 7.8*
Normal male × treated female	2,318	231.8 ± 7.3	91.3	211.6 ± 7.8

*$p < 0.05$ versus control.

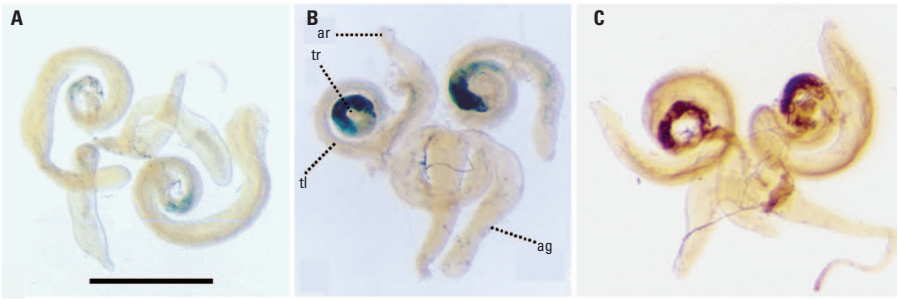

Figure 6. *In situ* β-galactosidase staining (*A* and *B*) and immunohistochemistry using anti-Hsp70 antibody (*C*) in reproductive organs of adult male *D. melanogaster* (*hsp70-lacZ*). (*A*) Control; (*B* and *C*) flies exposed to 10.0 µL/mL effluent. Abbreviations: ag, accessory gland; ar, apical region; tr, terminal region; tl, testis lobe. Bar = 200 µm.

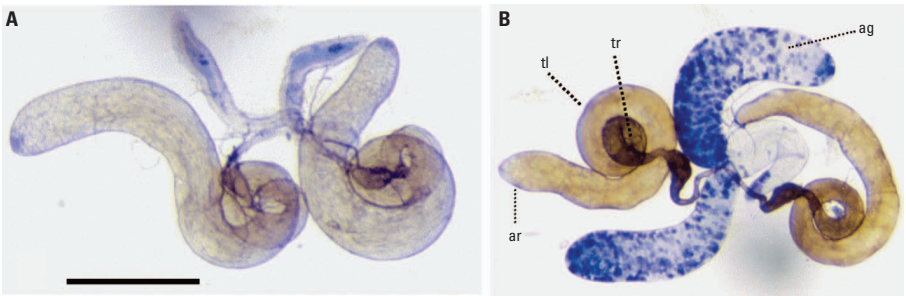

Figure 7. Trypan blue staining in reproductive organs of adult male *D. melanogaster* (*hsp70-lacZ*). (*A*) Control (note the absence of staining). (*B*) Fly exposed to 10.0 µL/mL effluent (note the absence of staining in testis and blue staining in accessory glands). Abbreviations: ag, accessory gland; ar, apical region; tl, testis lobe; tr, terminal region. Bar = 200 µm.

apparatus in charge of producing the noncellular portion of the sperm (Bairati 1968). The adult male accessory glands of *D. melanogaster* synthesize and secrete a few peptide molecules, which are transmitted to the female during mating (Chen et al. 1988; Wolfner 1997). The mated female has an elevated rate of egg laying, stimulation of oviposition, and sexual receptivity (Chapman et al. 1995; Wolfner 1997). The secondary cells, on the other hand, discharge granular filamentous bodies into the lumen of the gland, where they aggregate to form linearly arranged filamentous structures. The role of filamentous bodies has been described previously by Bairati (1968). A significant reduction in fecundity and reproductive performance of adult flies grown on food mixed with 10.0 µL/mL chrome plating effluent (flies did not eclose at the highest concentration of 100.0 µL/mL) in the present study may be attributed to the extensive damage in both the cell types in these groups, thereby affecting the secretion and formation of filamentous structures in the accessory gland lumen. Filamentous structures were very few in number in this group, unlike in controls. Previous studies have shown that increased number and volume of Golgi vesicles signifies acidification of the Golgi compartments (Zhang et al. 1993), which may be linked with aggregation of the subunits of filamentous structures. The reduction in the number of Golgi vesicles signifies a deviation from the original pH and hence may affect the aggregation and packaging process of the filamentous bodies in the treated groups.

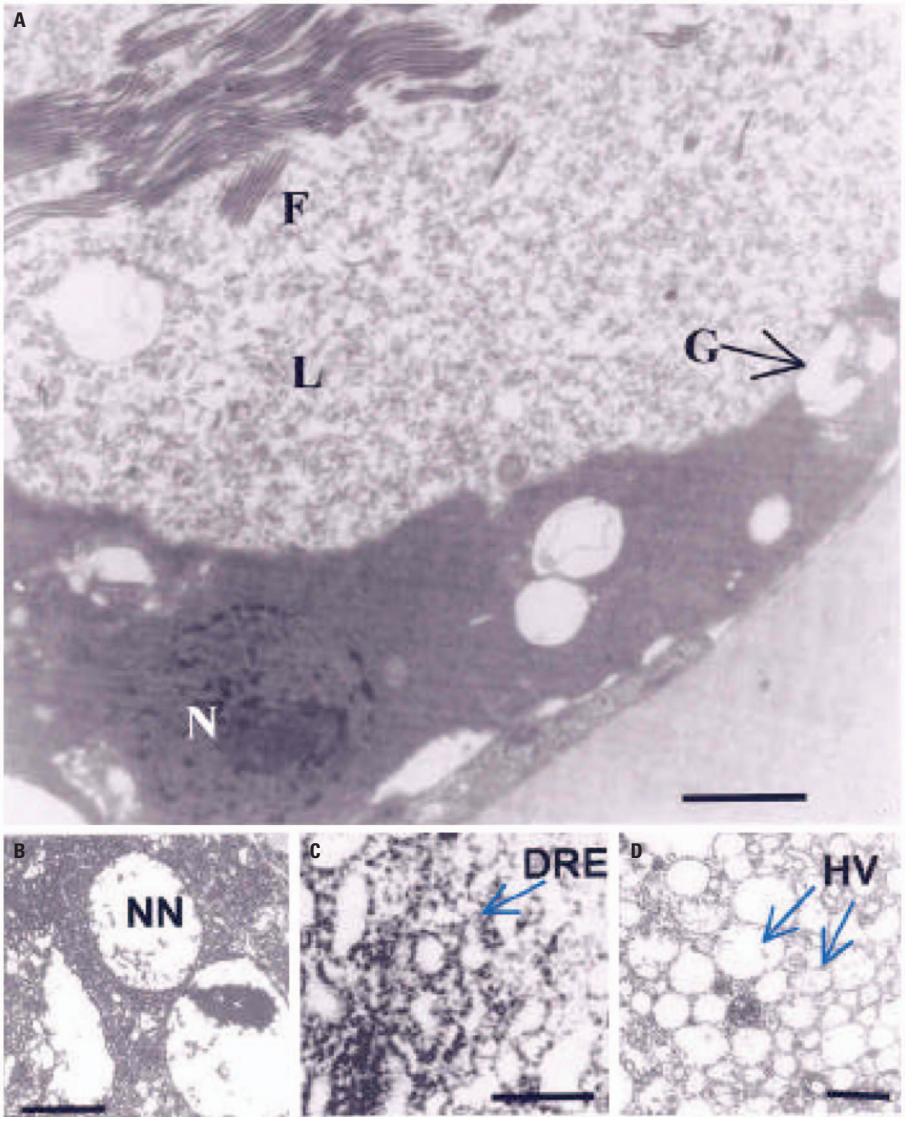

Figure 8. Electron photomicrograph showing ultrastructure of adult male accessory gland cells of transgenic *D. melanogaster* (*hsp70-lacZ*). (*A*) Control (bar = 1 µm); and (*B–D*) flies exposed to 10.0 µL/mL effluent from the chrome plating industry (bar = 100 nm). Note the intact nucleus (N), lumen (L) filled with granular bodies, Golgi vesicles (G), and organized filamentous bodies (F) in control cells (*A*). In the treated flies, a necrotic nucleus (NN; *B*), disorganized endoplasmic reticula with reduced number of ribosomes (DRE; *C*), and higher vacuolization (HV; *D*) can be seen.

During the past decade, issues of animal use and care in toxicology research and testing have become fundamental concerns for scientists, not only because of hue and cry from animal rights organizations but also because of difficulty in interpreting data due to intraspecies variation and exorbitant costs (Benford et al. 2000). This has led researchers to encourage the use of alternative animals in toxicologic studies.

Guven et al. (1994a, 1994b) and Guven and de Pomerai (1995) have successfully constructed a transgenic *Caenorhabditis elegans* strain (*hsp70-lacZ*) and used this in soil ecotoxicologic studies. Halloran et al. (2000) have cloned zebrafish promoter for the inducible *hsp70* gene and made stable transgenic lines of zebrafish that express the gene for the reporter green fluorescent protein under the control of an *hsp70* promoter. Recently, Blechinger et al. (2002) have used this transgenic zebrafish model to measure cadmium toxicity in early larval stage. They further demonstrated that enhanced green fluorescent protein expression in this strain of fish acts as an accurate and reproducible indicator of cell-specific induction of *hsp70* gene expression, which is sensitive enough to detect cadmium at doses below the median combined adverse effect concentration and the median lethal concentration.

The tiny fruit fly *Drosophila* is a well-established animal model for geneticists and developmental and molecular biologists. In recent years, our laboratory has made a significant contribution by successfully employing transgenic *D. melanogaster* as an alternative animal model for toxicologic research (Kar Chowdhuri et al. 1999, 2001; Mukhopadhyay et al. 2002a, 2002b; Nazir et al. 2001, 2003a, 2003b, 2003c). The study presented here is the first of its kind to demonstrate the toxicity of a whole effluent, using Hsp70 as a marker and transgenic *D. melanogaster* as a model animal. *Drosophila* proved to be a very sensitive model and inexpensive to employ, especially in a Third-World setting, because it would require little equipment. Technicians can be easily trained in the use of fly culture and analysis.

Within this context, a pertinent question is whether the engineered fly has a potentially enhanced capability to detect harmful substances compared with the wild-type model. Although the transgenic fly enables researchers to bypass the metabolic labeling to detect cytotoxicity against stressors by a quick, simple, inexpensive reporter gene assay, it does not possess any enhanced capacity of detecting chemicals compared with the normal fly. However, it will be interesting to explore if the engineered fly has better capacity to detect lower concentrations of harmful substances

compared with higher animal models. Although we have no such comparative data at present, recent studies by Hirsch et al. (2003) indicate that fly and human have similar dose–response relationship with lead.

The effect of the whole effluent on the reproduction of the fly was also explored in the present study. Male flies were found to be more susceptible to the effluent than females. Such observations tempt us to speculate that the fly model could be valuable in reproductive toxicology risk assessment. However, this warrants further investigation. Protection by *hsp70* against tissue damage in the testis, along with its nonexpression in the accessory glands leading to cellular damage, further confirms the cytoprotective role of *hsp70* and consolidates the evidence for the use of *hsp70* as a bioindicator in risk assessment. However, further studies in this direction with *hsp70* need to be done before reaching a definitive conclusion regarding the use of *hsp70* as a biomarker of environmental pollution.

References

Ait-Aissa S, Pandard P, Magaud H, Arrigo AP, Thyband E, Porcher JM. 2003. Evaluation of an in vitro *hsp70* induction test for toxicity assessment of complex mixtures: comparison with chemical analyses and ecotoxicity tests. Ecotoxicol Environ Safety 54:92–104.

Ait-Aissa S, Porcher J, Arrigo A, Lambre C. 2000. Activation of the *hsp70* promoter by environmental inorganic and organic chemicals: relationships with cytotoxicity and lipophilicity. Toxicology 145:147–157.

Bairati A. 1968. Structure and ultrastructure of the male reproductive system in *Drosophila melanogaster* Meig. Monit Zool (Ital) 2:105–182.

Benford DJ, Hanley BA, Bottrill K, Oehlschlager S, Balls M, Branca F, et al. 2000. Biomarkers as predictive tools in toxicity testing. Altern Lab Anim 28:119–131.

Bennett DA, Waters MD. 2000. Applying biomarker research. Environ Health Perspect 108:907–910.

Bierkens JGEA. 2000. Applications and pitfalls of stress proteins in biomonitoring. Toxicology 153:61–72.

Blechinger SR, Warren JT Jr, Kuwada JY, Krone PH. 2002. Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line. Environ Health Perspect 110:1041–1046.

Bonnier G. 1960. Experiments on hybrid superiority in *Drosophila melanogaster*. II. Rate of development from egg hatching to eclosion. Genetics 46:86–91.

Bureau of Indian Standards. 1982. ISI Standards for Surface Water Quality for Various Uses. IS: 2296. New Delhi:Government of India.

Caroll S, Wood EJ. 2000. Exposure of human keratinocytes and fibroblasts in vitro to nickel sulphate ions induces synthesis of stress proteins Hsp72 and Hsp90. Acta Derm Venereol 80:94–97.

Chapman T, Liddle LF, Kalb JM, Wolfner MF, Partridge L. 1995. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. Nature 373:241–244.

Chen PS, Stumm ZE, Aigaki T, Balmer J, Bienz M, Bohlen P. 1988. A male accessory gland peptide that regulates reproductive behaviour of female *D. melanogaster*. Cell 54:291–298.

Cleseeri LS, Greenberg AE, Eaton AD. 1998. Standard Methods for the Examination of Water and Wastewater. 20th ed. Baltimore, MD:United Book Press.

De Maio A. 1999. Heat shock proteins: facts, thoughts, and dreams. Shock 11:1–12.

de Pomerai D. 1996. Heat shock proteins as biomarkers of pollution. Hum Exp Toxicol 15:279–285.

Di Domenico BJ, Bugaisky GE, Lindquist S. 1982. The heat shock response is self regulated at both the transcriptional and post-transcriptional levels. Cell 31:593–603.

Dix DJ, Hong RL. 1998. Protective mechanisms in germ cells: stress proteins in spermatogenesis. Adv Exp Med Biol 444:137–143.

Edmund B, Besselievre PE, Schwartz M. 1976. The Treatment of Industrial Wastes. 2nd ed. New York:McGraw Hill.

Feder ME, Hofmann GE. 1999. Heat shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282.

Feige U, Polla S. 1995. Heat shock proteins: the *hsp70* family. Experientia 50:979–986.

Festing MFW, Baumans V, Combes DR, Halder M, Hendriksen FM, Howard BR, et al. 1998. Reducing the use of laboratory animals in biomedical research: problems and possible solutions. Altern Lab Anim 26:283–301.

Gayathri MV, Krishnamurthy NB. 1981. Studies on the toxicity of mercurial fungicide Agallol3 in *Drosophila melanogaster*. Environ Res 24:89–95.

Gomez CE, Contento L, Carsen AE. 2001. Toxicity tests to assess pollutants removal during wastewater treatment and the quality of receiving waters in Argentina. Environ Toxicol 16:217–224.

Guven K, de Pomerai DI. 1995. Differential expression of *hsp70* proteins in response to heat and cadmium in *Caenorhabditis elegans*. J Therm Biol 20:355–363.

Guven K, Duce JA, de Pomerai DI. 1994a. Evaluation of a stress-inducible transgenic nematode strain for rapid aquatic toxicity testing. Aquat Toxicol 29:119–137.

———. 1994b. Calcium moderation of cadmium stress explored using stress-induced transgenic strain *Caenorhabditis elegans*. Comp Biochem Physiol C 110:61–70.

Halloran MC, Sato-Maeda M, Warren JT, Su F, Lele Z, Krone PH, et al. 2000. Laser-induced gene expression in specific cells of transgenic zebrafish. Development 127:1953–1960.

Hightower LE. 1991. Heat shock, stress proteins, chaperones and proteotoxicity. Cell 66:191–194.

Hirsch HV, Mercer J, Sambaziotis H, Huber M, Stark DT, Torno-Morley T, et al. 2003. Behavioral effects of chronic exposure to low levels of lead in *Drosophila melanogaster*. Neurotoxicology 24:435–442.

Kar Chowdhuri D, Nazir A, Saxena DK. 2001. Effect of three chlorinated pesticides on *hsr*ω stress gene in transgenic *Drosophila melanogaster*. J Biochem Mol Toxicol 15:173–186.

Kar Chowdhuri D, Saxena DK, Viswanathan PN. 1999. Effect of hexachlorohexane (HCH), its isomers and metabolites on Hsp70 expression in transgenic *Drosophila melanogaster*. Pestic Biochem Physiol 63:15–25.

Krebs RA, Feder ME. 1997. Tissue specific variation in *hsp70* expression and thermal damage in *Drosophila melanogaster* larvae. J Exp Biol 200:2007–2015.

Lakhotia SC, Mukherjee T. 1980. Specific activation of puff 93D of *Drosophila melanogaster* by benzamide and the effect of benzamide on the heat shock induced puffing activity. Chromosoma 81:125–136.

Lakhotia SC, Singh AK. 1989. A novel set of heat shock polypeptides in Malpighian tubules of *Drosophila melanogaster*. J Genet 68:129–138.

Lis JT, Simon JA, Sutton CA. 1983. New heat shock puffs and β-galactosidase activity resulting from transformation of *Drosophila* with an *hsp70-lacZ* hybrid gene. Cell 35:403–413.

Macario AJL, Lange L, Ahring BK, De Macaraio EYC. 1999. Stress genes and proteins in the Archaea. Microbiol Mol Biol Rev 63:923–967.

Mukhopadhyay I, Nazir A, Mahmood K, Saxena DK, Das M, Khanna SK, et al. 2002a. Toxicity of argemone oil: effect on *hsp70* expression and tissue damage in transgenic *Drosophila melanogaster* (*hsp70-lacZ*)*Bg*^{9}. Cell Biol Toxicol 18:1–11.

Mukhopadhyay I, Saxena DK, Kar Chowdhuri D. 2002b. Toxicity of cypermethrin: *hsp70* as a biomarker of response in transgenic *Drosophila*. Biomarkers 7:501–510.

Nazir A, Mukhopadhyay I, Saxena DK, Kar Chowdhuri D. 2001. Chlorpyrifos induced *hsp70* expression and effect on reproductive performance in transgenic *Drosophila melanogaster* (*hsp70-lacZ*) *Bg*^{9}. Arch Environ Contam Toxicol 41:443–449.

———. 2003a. Evaluation of toxic potential of captan: induction of *hsp70* and tissue damage in transgenic *Drosophila melanogaster* (*hsp70-lacZ*)*Bg*^{9}. J Biochem Mol Toxicol 17:98–107.

———. 2003b. Evaluation of the no observed adverse effect level of solvent dimethyl sulfoxide in *Drosophila melanogaster*. Toxicol Mech Methods 13:147–152.

Nazir A, Saxena DK, Kar Chowdhuri D. 2003c. Induction of *hsp70* in transgenic *Drosophila*: biomarker of exposure against phthalimide group of chemicals. Biochem Biophys Acta 1621:218–225.

Nover L, Scharf KD. 1991. Heat shock proteins. In: Heat Shock Response (Nover L, ed). Boca Raton, FL:CRC Press, 41–128.

O'Kane CJ, Gehring WJ. 1987. Detection in situ of genomic regulatory elements in *Drosophila*. Proc Natl Acad Sci USA 84:9123–9127.

Radlowska M, Pemp K, Owiak J. 2002. Stress-70 as indicator of heavy metals accumulation in blue mussel *Mytilus edulis*. Environ Int 27:605–608.

Ryan JA, Hightower LE. 1996. Stress response as molecular biomarkers for environmental toxicology. In: Stress Inducible Cellular Responses (Feige U, Morimoto RI, Yahara I, Polla BS, eds). Boston, MA:Birkhauser-Verlag, 411–424.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor, NY:Cold Spring Harbor Laboratory Press, 18.47–18.75.

Stone DE, Craig EA. 1990. Self regulation of 70 kilodalton heat shock proteins in *Saccharomyces cerevesiae*. Mol Cell Biol 10:1622–1632.

Stringham G, Candido EPM. 1994. Transgenic *hsp16-lacZ* strains of the soil nematode *Caenorhabditis elegans* as biological monitors of environmental stress. Environ Toxicol Chem 13:1211–1220.

Tully DB, Collins BJ, Overstreet JD, Smith CS, Dinse GE, Mumtaz MM, et al. 2000. Effects of arsenic, cadmium, chromium and lead on gene expression regulated by a battery of 13 different promoters in recombinant HepG2 cells. Toxicol Appl Pharmacol 168:79–90.

Velazquez JM, Lindquist S. 1984. *Hsp70*: nuclear concentration during environmental stress and cytoplasmic storage during recovery. Cell 36:655–662.

Wolfner MF. 1997. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. Insect Biochem Mol Biol 27:179–192.

Zhang GF, Driuoich A, Staehelin LA. 1993. Effect of monensin on plant Golgi: re-examination of the monensin induced changes in cisternal architecture and functional activities of the Golgi apparatus of sycamore suspension—cultured cells. J Cell Sci 104:819–831.