Flubendiamide-induced HSP70 expression in transgenic *Drosophila melanogaster* (hsp70-lacZ)

Saurabh Sarkar, Sayanti Podder and Sumedha Roy*

Cytogenetics Laboratory, Department of Zoology, The University of Burdwan, Rajbati, Burdwan 713 104, India

Flubendiamide is a novel class of insecticides against lepidopteran insects. With a view to explore if this chemical is safe for non-targeted organisms, its effect was studied through assessment of heat shock protein (HSP70) expression in third instar larvae of transgenic *Drosophila melanogaster*, Bg9 (hsp70-lacZ). Dietary concentrations covering a range (5, 10, 20 and 40 µg/ml) of 20% flubendiamide were used for treatment of larvae for different durations (1, 3, 6 and 24 h). Reporter gene assay confirmed that HSP70 expression varied in tissues depending upon treatment concentration and exposure duration. The 5 µg/ml treatment stimulated higher stress response during the initial hours, which declined later (6 and 24 h). Nearly all tissues (humerus, brain, proventriculus, etc.) responded initially with the 20 µg/ml treatment, which declined with increasing exposure. Hence low concentration and short-term exposure of flubendiamide to non-target organisms seems to be highly effective as stressor and thus demands awareness in decreasing irrational use of the chemical.

Keywords: Beta-galactosidase, *Drosophila melanogaster*, flubendiamide, heat shock proteins.

ORGANISMS respond to adversities in their environment by a protective mechanism called stress response or heat shock response[1]. Heat shock response, one of the best known among the conserved responses, was first observed in *Drosophila melanogaster*[2]. Organisms facing any stress try to survive attack by expressing specific genes[3,4]. Such response mediated by the increased expression of genes encoding a group of proteins referred to as heat shock proteins (HSPs) or stress proteins[5] was initially reported to be heat-regulated[2]. Presently, the HSP70 family known to be induced by not only extreme temperatures but also by toxic chemicals as well as heavy metals[6], better qualifies for the broad term 'stressor proteins' coined by Boreham and Mitchell in 1994 (ref. 7). Among the different groups of HSPs (HSP22, HSP23, HSP27, HSP60, HSP70 and HSP83), HSP70 is an important part of the cellular machinery for protein folding, thereby having an active role in cellular defence[8]. Stress proteins act to cushion cells by maintaining the proteins prior to their congregation into multi-molecular complexes in the cytosol. These proteins act to direct the nascent as well as denatured proteins to achieve proper shape, thereby escaping additional deterioration[8]. Among the stress proteins, HSP70 is not only the largest and most extensively studied[9], but is also vastly maintained among prokaryotes and eukaryotes[1]. HSP70 synthesis increases under chemical stress when organisms are exposed to various pesticides[10], fungicides[11], food adulterants[12], solvents[13], etc. as HSP70 is responsible for a new but allied role to defend the cells from proteotoxicity[14]. Hence HSP70 is being used as a biomarker in monitoring the impact of several environmentally related chemicals on various invertebrates[3,10,13]. The test chemical, flubendiamide (CAS no. 272451-65-7) is the first commercial representative of benzene dicarboxamides or phthalic acid diamide, a novel class of insecticides highly active against lepidopteran insects[15]. Flubendiamide disrupts muscle function in insect cells by activating ryanodine receptors (ryanodine-sensitive calcium release channels; RyR)[16]. This insecticide is used in leafy green brassica, fruiting vegetables, pome and stone fruit, corn, cotton, grape, okra and tobacco[17]. *Drosophila* is recommended by the European Centre for the Validation of Alternative Methods for its use in environmental toxicity monitoring studies[18]. Hence *D. melanogaster* has been selected as a model for the present study which explores the status of stress response, if any, due to flubendiamide exposure. Though insects are the obvious target for insecticides, but being targeted against lepidopterans, the test chemical is expected to be ineffective for the non-target dipterans. Transgenic *D. melanogaster,* Bg9 (hsp70-lacZ), which expresses bacterial β-galactosidase in response to stress[19] has been used for the study. Thus, the study is targeted to explore the stress-inducing potential of the test chemical, if any, at concentrations lower than the suggested-field application range (rice 50 µg/ml, cotton 100 µg/ml, major uses of pesticides, Ministry of Agriculture, Government of India, 2009). This would stop the irrational and unscientific use of pesticides and thus help save non-target organisms from unintended hazards.

*For correspondence. (e-mail: roysumedha@gmail.com)

Materials and methods

Transgenic *D. melanogaster* strain which expresses bacterial β-galactosidase as a response to stress was used during the study[19]. The experimental flies and larvae were cultured on standard *Drosophila* food containing agar, corn meal, brown sugar and yeast (SDM) at 24 ± 1°C. Healthy third instar larvae were used as positive control. They were placed on a petri dish containing moist filter paper, following which they were given temperature shock at 37 ± 1°C for 1 h, as described previously[20].

Assay of *in situ* histochemical β-galactosidase activity (qualitative): Food containing various concentrations (5, 10, 20 and 40 µg/ml) of 20% flubendiamide WG (Takumi, TATA), was prepared and the third instar larvae were allowed to feed for various time intervals. Following Lakhotia and Mukherjee[21], the larvae thus feeding on food with different concentrations of the test chemical for different time intervals (1, 3, 6 and 24 h) were collected and washed thoroughly with Poel's salt solution (PSS). Following the dissection of the larvae, brief fixation in 2.5% glutaraldehyde was carried out followed by washing in 50 mM phosphate buffered saline (pH 8.0). Next, β-galactosidase histochemical staining was performed following the methods described in the literature[4,10,22].

Results and discussion

The differential treatments of third instar larvae with the test chemical for varied time durations (1, 3, 6 and 24 h) elicited heat shock protein (HSP70) expression in several body parts, namely labium, brain ganglion, salivary glands, humerus disc, proventriculus, hepatic caeca, midgut, hind gut, tracheoblast, gonadal disc, genital disc, posterior skin, Malpighian tubules and mouth parts. All treatment concentrations (5, 10, 20 and 40 µg/ml) caused differential HSP70 expression in different body parts in comparison to the control counterparts, where no colour developed indicating very low or undetectable HSP70. The colour demonstrates the stress due to chemical treatment. Table 1 and Figure 1 show that among all the organs/body parts of third instar larvae, some are responsive and have been specifically considered in this study. Among all the responsive tissues, maximum response is observed in the humerus disc (Figures 1 *a*, *d*, *g*, *h*, *k*, *l*, *n*, 2 *j*, *l*, 3 *g*, *j* and 4 *m*) followed by the midgut (Figures 1 *a–c*, *e*, *f*, *m*, 2 *e*, *h*, *k* and 3 *h*, *k*), when all the treatment concentrations and duration of exposure are taken into consideration. It is seen that all four concentrations (5, 10, 20 and 40 µg/ml) act as stress inducers. Interestingly, the 5 µg/ml-treated larvae (Figure 1 *a–g*) manifested higher response during the initial hours (1 and 3 h), followed by a lowering tendency later (6 and 24 h). More strikingly, with increased treatment concentration (10 µg/ml) the expression is seen to increase with increase in the duration of exposure (Figure 1 *a–l*). A concentration of 20 µg/ml showed maximum blue colour development, thereby marking maximum HSP70 expression on its sixth hour of exposure (Figure 3 *g–j*) followed by a drastic decline at the 24th hour (Figure 3 *g–j*), whereas least expression of HSP70 is noted in the 40 µg/ml treated larvae (Figure 1 *h–n*). With 40 µg/ml treatment (Figure 4 *a–m*), the HSP70 expression decreased as maximum tissues/body parts (labium, brain, proventriculus, hepatic caeca, hind gut, tracheoblasts, genitalia) were found to be nonresponsive to the stress. To analyse differential expression of HSP70 in different tissues, a two-way ANOVA was performed (Table 2) which clearly demonstrates that the stress protein expression varies significantly with respect to both concentration and duration of treatment. Difference in tissue responsiveness is also clear. These findings match the report of Krebs and Feder[23]. HSPs have been exploited as efficient biosensors to predict the toxic potential of several chemicals[24]. The present study demonstrating treatment and time-dependant variation in HSP70 expression in third instar larvae of *D. melanogaster* exposed to flubendiamide (20% WG) shows that all treatments increase HSP70 expression, which suggests the potentiality of the chemical to alter vital cellular functions affecting cellular integrity, thereby inducing HSP70 for its protective role[4]. Negative control larvae express undetectably low HSP70 (Figure 5 *b*) with respect to positive control (Figure 5 *a*), where dark blue colouration is seen and similarly varied expressions are noted with different treatment schedules, maximum with 20 µg/ml treatment (Figure 3) followed by 5 µg/ml (Figure 1) and a minimum with 40 µg/ml treatment (Figure 4). This reduced response at higher treatment concentration may be due to reduction in the number of viable cells, as reported by Kumar *et al.*[6]. The stress increases with initial increase in treatment concentration and duration. Hence larvae exposed to low concentrations (5, 10 and 20 µg/ml) till 24 h initiate a response that decreases with higher concentration (40 µg/ml), probably due to increased tissue damages (Figures 1–4). Similar to Das *et al.*[4], negligible expression is seen after 24 h continuous exposure in most of the responsive tissues. As suggested by Stringham and Candido[25], the effects of the chemical are tissue-specific as the humerus disc seems to be the most responsive to all treatments at varied exposure time (Figures 1 *a*, *d*, *g*, *h*, *k*, *l*, *n*, 2 *j*, *l*, 3 *g*, *j* and 4 *m*), followed by midgut (Figures 1 *a–c*, *e*, *f*, *m*, 2 *e*, *h*, *k* and 3 *h*, *k*), brain (Figures 1 *a*, *d*, *f–h*, *k*, *l*, 2 *h*, *i*, *j*, *l*, 3 *a*, *c*, *d*, *f*, *g*, *i*, *j* and 4 *c*, *f*). Larvae subjected to dietary exposure are expected to manifest HSP70 in body parts at close proximity to treated food more prominently than others. But expression in brain and humerus suggests that the chemical might inhibit certain enzyme activities and induce HSP70 expression[26]. Conversely, supranormal HSP70 expression can reduce specific enzyme (alcohol dehydrogenase and lactate

Table 1. Summary of *β*-galactosidase staining in the tissues/body parts of third instar larvae of transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg9 exposed to different concentrations (5, 10, 20 and 40 µg/ml) of flubendiamide for different time intervals (1, 3, 6 and 24 h). The experiments were carried out in triplicate sets and each set consisted of 20 larvae. Along with treated larvae, positive control (temperature shock, Figure 5 *a*) and a negative control (without any shock, Figure 5 *b*) have been maintained for clear demarcation from the ones expressing chemical-induced stress (scoring of the controls not shown in the table). 0, No colour, 1/2+, Very pale blue colour, +, Pale blue colour; ++, Moderate blue colour and +++, Dark blue colour. In the present study scoring pattern followed was similar to that of Kar Chowdhuri *et al.*[10]

Concentration (ppm)	Time (h)	Labium	Brain ganglion	Salivary gland	Humerus disc	Proventriculus	Hepatic caecum	Midgut	Hindgut	Tracheoblast	Gonad	Genitalia	Posterior skin
5	1	++	½+	½+	++	+	0	+++	0	0	0	0	++
5	3	++	+	++	++	++	0	++	0	0	0	0	+
5	6	+	½+	½+	+	½+	0	0	+	+	0	0	+
5	24	0	½+	++	+	++	0	++	0	0	0	½+	0
10	1	0	0	0	+	0	0	½+	0	0	0	0	0
10	3	0	0	0	0	0	0	++	0	½+	0	0	0
10	6	½+	+	0	½+	++	0	+	0	++	0	0	++
10	24	0	+	++	+	++	0	+	0	+	0	0	+
20	1	++	+	++	++	+	0	0	0	++	++	++	++
20	3	½+	½+	½+	0	+	0	0	+	+	0	0	+
20	6	+++	+++	+++	+++	+++	++	++	0	0	++	++	+
20	24	0	0	0	½+	0	0	++	0	0	0	0	0
40	1	0	0	0	+	0	0	½+	0	0	0	0	0
40	3	0	+	++	0	0	0	0	0	0	+	0	+
40	6	0	0	0	+	0	0	++	0	0	0	0	0
40	24	0	0	0	0	+	½+	0	0	0	½+	0	0

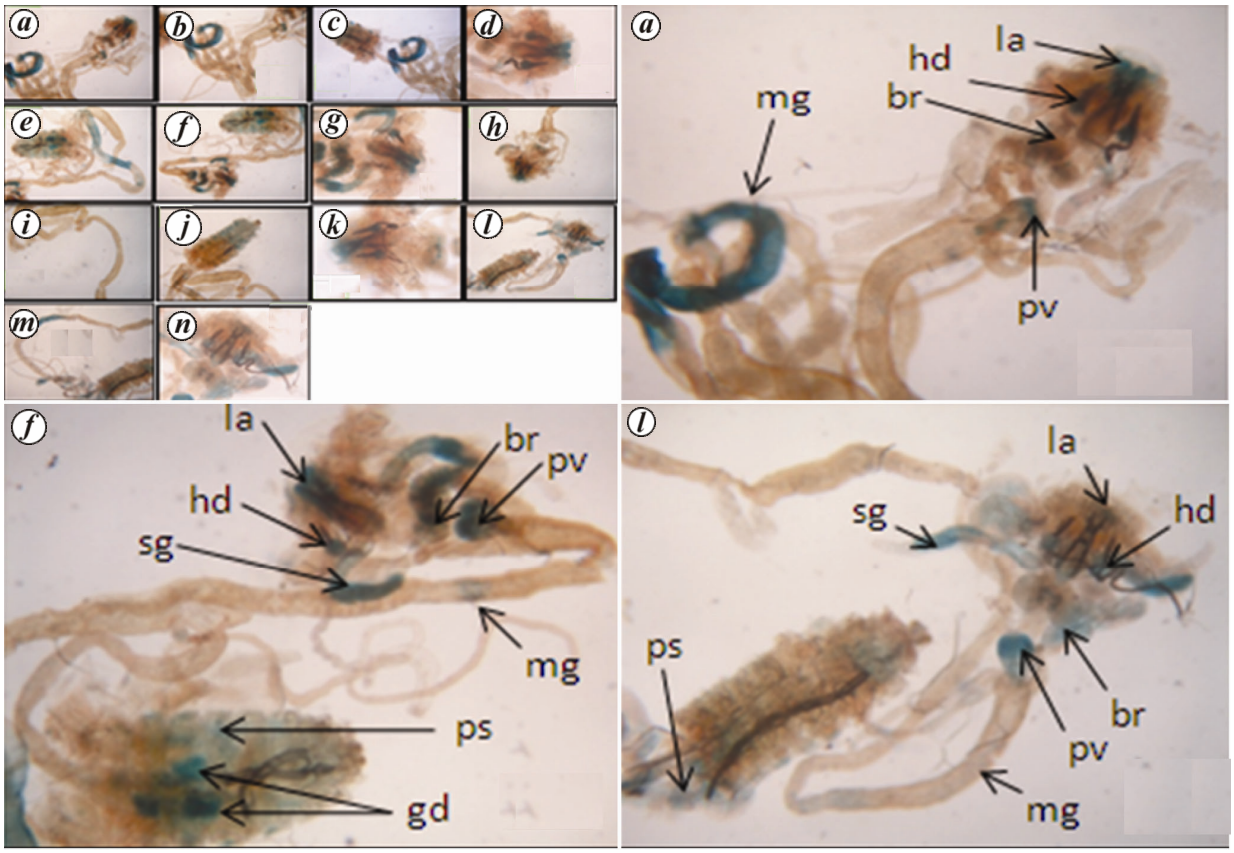

Figure 1 ***a–n.*** *β*-Galactosidase staining pattern in different tissues of third instar larvae of transgenic *Drosophila melanogaster* (hsp70-lacZ) after treatment with 5 µg/ml concentration of flubendiamide for different exposure time durations. ***a–d***, Staining pattern after 1 h; ***e–g***, After 3 h exposure; ***h–k***, After 6 h exposure, and ***l–n***, After 24 h exposure to the test chemical. ***a–c***, ***e***, ***f***, ***h***, ***i***, ***j***, ***l***, ***m*** show differential staining pattern under low magnification and ***d***, ***g***, ***k***, ***n*** show results under high magnification. ***a***, ***f***, ***l*** are the representative magnified figures which show variable staining pattern on different tissues. mg, midgut; br, brain; hd, humerus disc; la, labium; pv, proventriculus; sg, salivary gland; gd, gonadal disc; ps, posterior skin; hc, hepatic caeca; Mt, Malpighian tubule. Some of the plates are selected as representatives from Figures 1 to 4 where the larval body is viewed more prominently showing the differential staining pattern. Individual plates in composing each figure 1–4 are numbered from left to right.

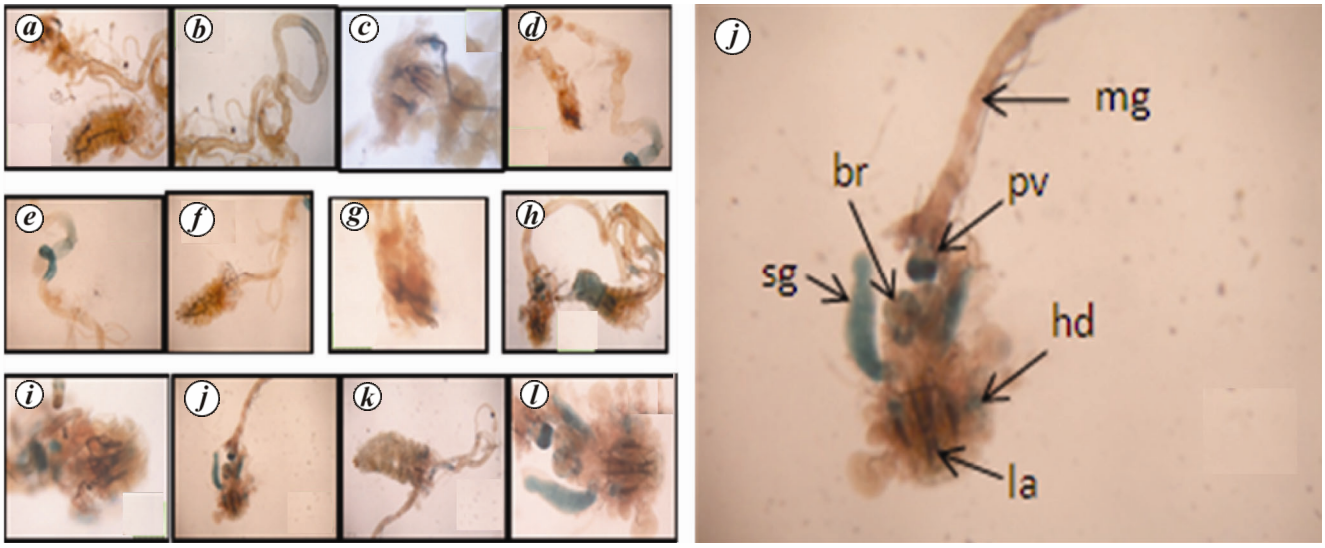

Figure 2 ***a–l.*** *β*-Galactosidase staining pattern in different tissues of third instar larvae of transgenic *D. melanogaster* (hsp70-lacZ) after treatment with 10 µg/ml of 20% flubendiamide for different exposure time durations (1, 3, 6, 24 h). ***a–c***, Staining pattern after 1 h; ***d–g***, After 3 h exposure; ***h***, ***i***, after 6 h exposure, and ***j–l***, after 24 h exposure to the test chemical. ***j*** is magnified for better viewing.

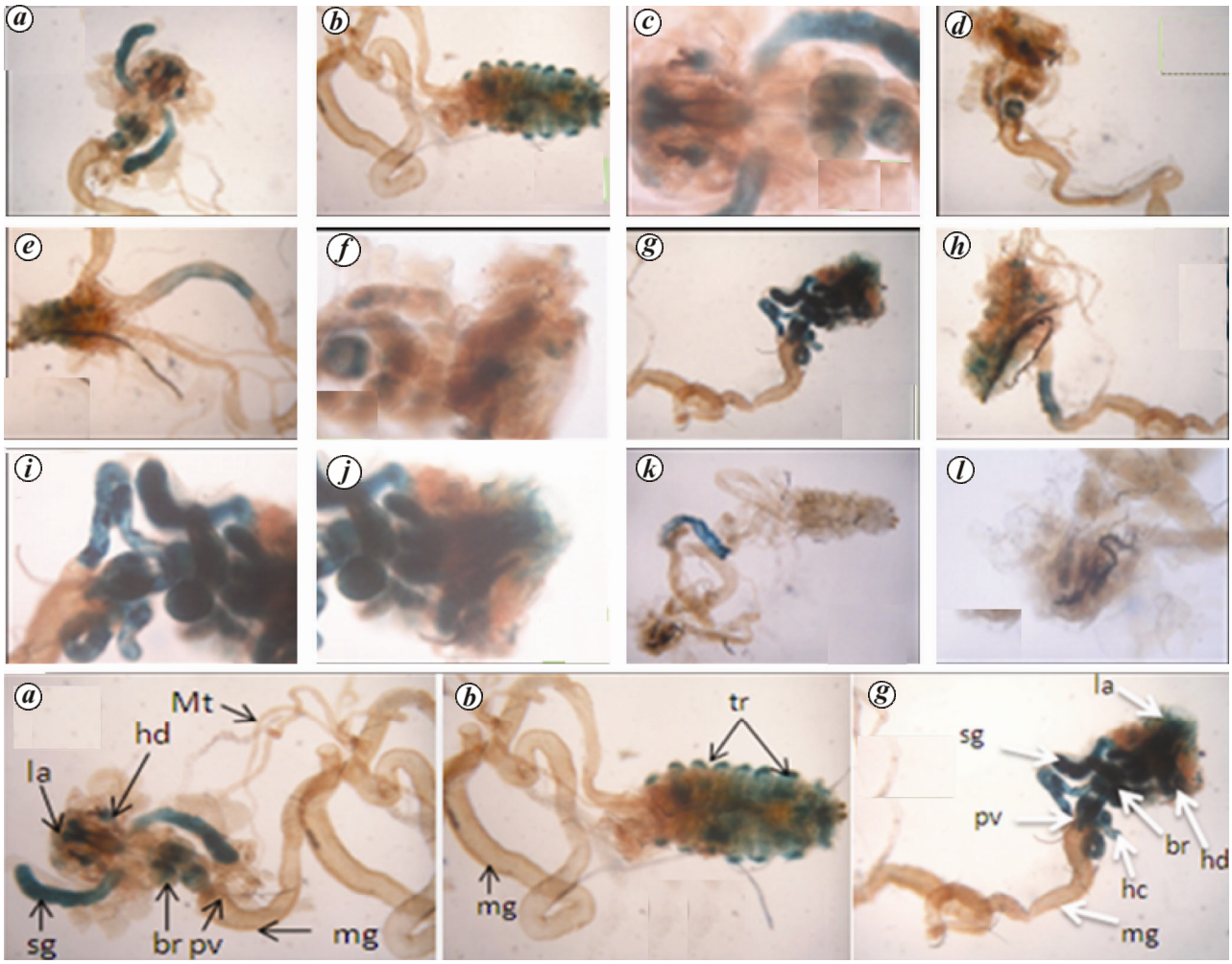

Figure 3 ***a–l.*** *β*-Galactosidase staining pattern in different tissues of third instar larvae of transgenic *D. melanogaster* (hsp70-lacZ) after treatment with 20 µg/ml concentration of flubendiamide for different exposure time durations. ***a–c***, Staining pattern after 1 h; ***d–f***, After 3 h exposure; ***g–j***, After 6 h exposure, and ***k–l***, after 24 h exposure to the test chemical. ***a***, ***b*** and ***g*** are magnified for better viewing of differential staining pattern in response to various treatments.

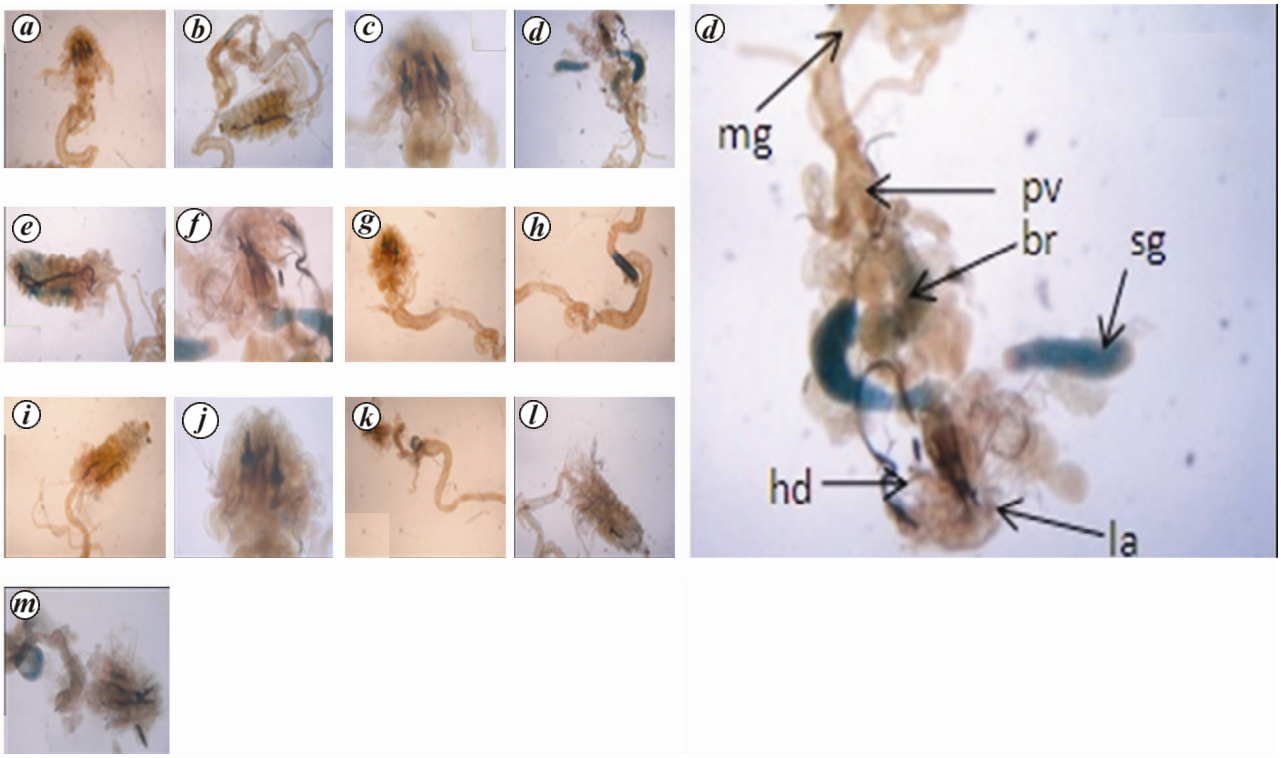

Figure 4 ***a–m***. *β*-Galactosidase staining pattern in different tissues of third instar larvae of transgenic *D. melanogaster* (hsp70-lacZ) after treatment with 40 µg/ml flubendiamide for different exposure time durations. ***a–c***, Staining pattern after 1 h; ***d–f***, After 3 h exposure; ***g–j***, After 6 h exposure, and ***k–m***, After 24 h exposure to the test chemical. ***d*** is magnified for better viewing.

Table 2. Analysis of variance (ANOVA)*

	df	Sum of squares	Mean square	*F* value	*P* value
A	3	709.64063	236.54688	946.1875	0
B	3	196.26562	65.42187	261.6875	0
Interaction	9	993.42188	110.38021	441.52083	0
Model	15	1899.32813	126.62188	506.4875	0
Error	32	8	0.25	–	–
Corrected total	47	1907.32813	–	–	–

At the 0.05 level, the population means of *A* are significantly different. At the 0.05 level, the population means of *B* are significantly different. At the 0.05 level, the interaction between *A* and *B* is significant.
*Statistical analysis was carried out by two-way ANOVA using Origin 8.5. Significance was calculated at $P < 0.05$.

dehydrogenase) activity in *D. melanogaster*[7]. Enzymes being the key regulators of metabolic pathways, when inhibited, might trigger cellular damage. Recent studies use stress gene assay to identify vulnerable target organs for toxicants[14] and their regulation being stress-specific, larval tissues expressing HSP70 are confirmed as vulnerable to the chemical. It has been reported that during abundance, cellular HSP70 has a tendency to bind nascent peptides. Existence of multiple copies of *hsp* genes in the *Drosophila* genome has greater relevance than simply supplying HSP proteins in short notice. The cost/benefit ratios of production of definite amount of hsp70 under stress vary among different cell types and physiological conditions, which suggests their developmental stage-specific regulation[5]. In this light, the present findings showing variation in HSP70 expression in different tissues in response to different concentrations and duration of treatment with the test chemical as can be seen in the Table 1, appear to be justified. The initial stress at the onset of treatment might have activated some drug metabolizing enzymes which help to combat the stress in *Drosophila*, just as in mammals[27]. It is also postulated that the increase in detoxifying enzymes helps to develop resistance, thereby decreasing sensitivity to the applied insecticides[28]. But increase in treatment concentration (20 µg/ml) and exposure duration (6 h) induces greater HSP70 activity, which might be due to shift in the balance between the stressor concentration and efficiency of

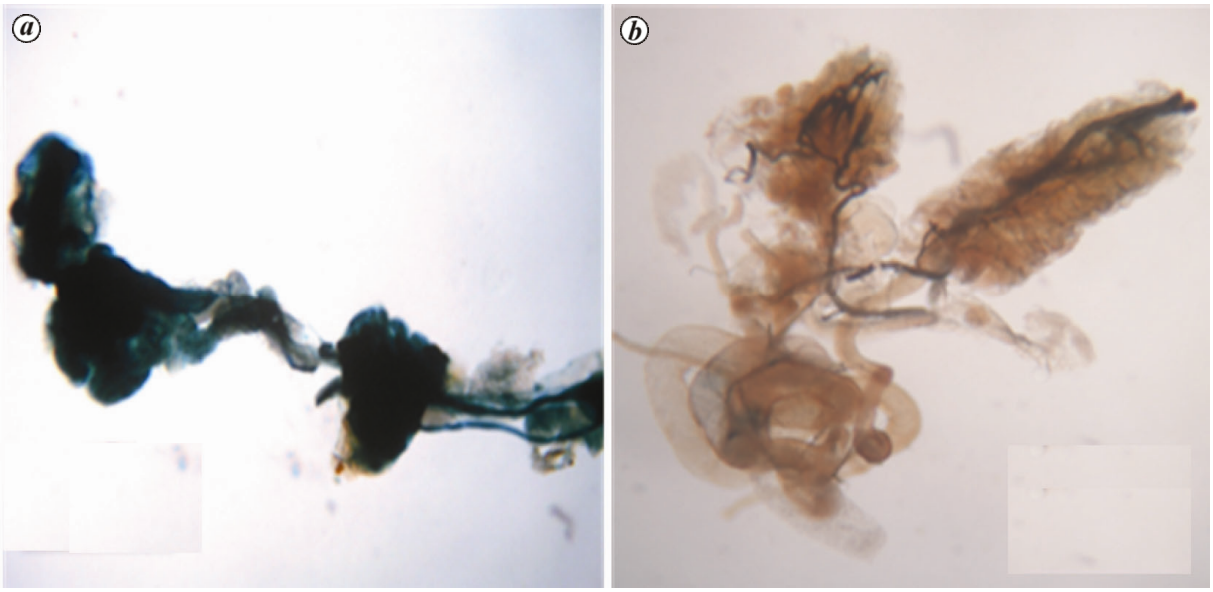

Figure 5. ***a***, *β*-Galactosidase staining pattern in positive control third instar larvae of transgenic *D. melanogaster* (hsp70-lacZ) maintained at a high temperature (37°C) facing a heat shock. ***b***, *β*-Galactosidase staining pattern in negative control, with third instar larvae of transgenic *D. melanogaster* (hsp–lacz) maintained at optimum temperature without any stress. No particular colour development is observed.

the detoxifying systems. Such studies on non-target organisms like *Drosophila* would facilitate evaluating the risk of unregulated use of chemicals in the case of other non-target organisms[29]. Very recently, flubendiamide has been seen to act as a neurotoxic chemical that inhibit acetylcholinesterase activity and subsequently produces compound eye deformities in the adult *D. melanogaster*[30].

Conclusion

Overall, our results demonstrate the stressor potential of flubendiamide (20% WG) in *D. melanogaster*. Regularly reported to be used as a pesticide at much higher concentrations in rice and cotton fields, this chemical can induce over-expression of HSP70 in *Drosophila.* This demonstrates the undesired stress on the non-target organisms following irrational exposure to insecticides.

1. Sanders, B. M., Stress proteins in aquatic organisms: an environmental perspective. *Crit. Rev. Toxicol.*, 1993, **23**, 49–75.
2. Ritossa, F., New puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia*, 1962, **18**, 571–573.
3. Lee, S. M., Lee, S. B., Park, C. H. and Choi, J., Expression of heat shock protein and hemoglobin genes in *Chironomus tentas* (Diptera, Chironomidae) larvae exposed to various environmental pollutants: a potential biomarker of freshwater monitoring. *Chemosphere*, 2006, **65**, 1074–1081.
4. Das, S. K., Podder; S., Akbari, S. and Roy, S., Impact of Thiovit®Jet treatment on HSP-70 expression, as a stress indicator in transgenic *Drosophila melanogaster*. *Proc. Zool. Soc. (Kolkata)*, 2011, **64**, 17–22.
5. Lakhotia, S. C. and Prasanth, K. V., Tissue and development specific induction and turnover of HSP70 transcripts from loci 87A and 87C after heat shock and during recovery in *Drosophila melanogaster*. *J. Exp. Biol.*, 2002, **205**, 345–358.
6. Kumar, V., Ara, G., Afzal, M. and Siddique, Y. H., Effects of methyl sulfonate on HSP70 expression and tissue damage in the third instar larvae of transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg9. *Interdisc. Toxicol.*, 2011, **4**, 159–165.
7. Boreham, D. R. and Mitchell, R. E., Regulation of heat and radiation stress responses in yeast by hsp104. *Radiat. Res.*, 1994, **137**, 190–195.
8. Craig, E. A., Weissman, J. S. and Horwich, A. L., Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. *Cell*, 1994, **78**, 365–372.
9. Morano, K. A., New tricks for an old dog: the evolving world of hsp 70. *Ann. NY Acad. Sci.*, 2007, **1113**, 1–14.
10. Kar Chowdhuri, D., Saxena, D. K. and Viswanathan, P. N., Effect of hexachlorocyclohexane (HCH), its isomers, and metabolites on HSP70 expression in transgenic *Drosophila melanogaster*. *Pestic. Biochem. Physiol.*, 1999, **63**, 15–25.
11. Das, S. K., Podder, S. and Roy, S., Effect of fungicide, Thiovit®Jet on several life history traits of *Drosophila melanogaster* (Diptera : Drosophilidae). *J. Appl. Biol. Sci.*, 2010, **4**, 31–36.
12. Mukhopadhyay, I., Nazir, A., Mahmood, K., Saxena, D. K., Das, M., Khanna, S. K. and Kar Choudhuri, D., Toxicity of argemone oil: effect on HSP70 expression and tissue damage in transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg9. *Cell. Biol. Toxicol.*, 2002, **18**, 1–11.
13. Nazir, A., Mukhopadhyay, I., Saxena, D. K. and Kar Choudhuri, D., Evaluation of the no-observed adverse effect level of solvent dimethyl sulphoxide in *Drosophila melanogaster*. *Toxicol. Mech. Methods*, 2003, **3**, 147–152.
14. Hightower, L. E., Heat shock, stress proteins, chaperones and proteotoxicity. *Cell*, 1991, **66**, 191–194.
15. Tohnishi, M. *et al.*, Flubendiamide, a novel insecticide highly active against lepidopterous insect pests. *J. Pestic. Sci.*, 2005, **30**, 354–360.
16. Ebbinghaus-Kintscher, U. *et al.*, Phthalic acid diamides activate ryanodine sensitive Ca^{2+} release channels in insect. *Cell Calcium*, 2006, **39**, 21–33.
17. Environmental Protection Agency, 2010; http://www.gpo.gov/fdsys/pkg/FR-2010-11-05/html/2010-27998.htm; last accessed on 28 May 2012.

18. Benford, D. J. *et al.*, Biomarkers as predictive tools in toxicity testing. The Report and Recommendations of ECVAM Workshop 40. *ATLA*, 2000, **28**, 119–131.
19. Lis, J. T., Simon, J. A. and Sutton, C. A., New heat shock puffs and β-galactosidase activity resulting from transformation of *Drosophila* with an hsp70-lacZ hybrid gene. *Cell*, 1993, **35**, 403–413.
20. Lakhotia, S. C. and Singh, A. K., A novel set of heat shock polypeptides in Malpighian tubules of *Drosophila melanogaster*. *J. Genet.*, 1989, **68**, 129–137.
21. Lakhotia, S. C. and Mukherjee, T., Specific activation of puff 93D of *Drosophila melanogaster* by benzamide and the effect of benzamide on the heat shock induced puffing activity. *Chromosoma* (*Berl*)., 1980, **81**, 125–136.
22. O'Kane, C. J. and Gehring, W. J., Detection *in situ* of genomic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 9123–9127.
23. Krebs, R. A. and Feder, M. E., Tissue specific variation in HSP70 expression and thermal damage in *Drosophila melanogaster* larvae. *J. Exp. Biol.*, 1997, **200**, 2007–2015.
24. Ait-Aissa, S., Pandard, P., Magaud, H., Arrigo, A. P., Thyband, E. and Porcher, J. M., Evaluation of an *in vitro* HSP70 induction test for toxicity assessment of complex mixture: comparison with chemical analysis and ecotoxicity tests. *Ecotoxicol. Environ. Saf.*, 2003, **54**, 92–104.
25. Stringham, E. G. and Candido, P. M., Transgenic hsp16-lacZ strains of the soil nematode *Caenorhabditis elegans* as biological monitors of environmental stress. *Environ. Toxicol. Chem.*, 1994, **8**, 1211–1220.
26. Baillie, A. C. and Wright, K., Biochemical pharmacology. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (eds Kerkut, G. A. and Gilbert, L. I.), Pergamon Press, New York, 1985, pp. 332–337.
27. Pai, S. B., Evaluation of mutagenic effect of the fungicide fenaminosulf in *Drosophila melanogaster*. *Mutat. Res.*, 1983, **119**, 289–291.
28. Devonshire, A. L. and Field, L. M., Gene amplification and insecticide resistence. *Annu. Rev. Entomol.*, 1991, **36**, 1–23.
29. Karatas, A. and Bahceci, Z., Toxic effects of diazinon on adult individuals of *Drosophila melanogaster*. *JABS*, 2009, **3**, 102–108.
30. Sarkar, S., Dutta, M. and Roy, S., Potential toxicity of flubendiamide in *Drosophila melanogaster* and associated structural alterations of its compound eye. *Toxicol. Environ. Chem.*, 2014, **96**, 1075–1087; doi: 10.1080/02772248.2014.997986

ACKNOWLEDGEMENTS. We thank the Head, Department of Zoology, The University of Burdwan; Prof. Jagat K. Roy, Banaras Hindu University, Varanasi; Prof. R. N. Chatterjee, Calcutta University, Kolkata; Dr G. Aditya, The University of Burdwan and Prof. A. R. Ghosh and Aaloke Mukherjee for help.

Received 10 February 2014; revised accepted 4 March 2015