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ORIGINAL ARTICLE

Flubendiamide induces trans-generational compound eye alterations in *Drosophila melanogaster*

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ABSTRACT

Pesticides are one of the major sources of environmental toxicity and contamination. This study reports potential of lepidopteran insecticide formulation, named Flubendiamide, in altering compound eye architecture and bristle pattern orientation for four consecutive generations (P, F1, F2 and F3) in a non-target diptera, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). The concentrations of the insecticide formulation selected for treatment of *Drosophila* (50 and 100 µg/mL) were in accordance with practiced Indian field doses (50 µg/mL for rice and 100 µg/mL for cotton). This study showed trans-generational insecticide-induced changes in the morphology of the compound eyes of the non-target insect *D. melanogaster*.

KEY WORDS: *Drosophila*; compound eye; Flubendiamide; ommatidia

Introduction

Indiscriminate use of pesticides leads to significant environmental contamination (KarChowdhuri *et al.*, 2001). Flubendiamide ($C_{23}H_{22}F_7IN_2O_4S$, CAS No: 272451-65-7), a contemporary lepidopteran insecticide formulation is responsible for calcium ion influx from muscle cytosol to lumen in target insects, which results in their muscle paralysis (Ebbinghaus-Kintscher *et al.*, 2006) leading to death. Doses of this benzene-dicarboxamide insecticide (Flubendiamide 20% WDG) used for pest control in India are 50 and 100 µg/mL for rice and cotton, respectively (Government of India, Ministry of Agriculture, Department of Agriculture and Cooperation 2009). The maximum residual levels (MRL) of Flubendiamide in rice and cotton crops are 0.2 and 1.0 mg/kg, respectively. The proposed average daily intake (ADI) value of Flubendiamide is 0.017 mg/kg bw/day (Sarkar & Roy 2017b). Since the formulation is targeted against lepidopteran insects, cross-reactivity leading to hazardous impact in non-target Dipterans, like *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) is quite unanticipated. In our previous studies, the neurotoxic potential of this chemical against *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) was discussed (Sarkar *et al.*, 2015a). In the present work we assessed the effects of Flubendiamide at Indian field doses on the compound eye morphology of *D. melanogaster,* a very accessible model organism (Rand, 2010; Sarkar *et al.*, 2017a). More specifically, the compound eye of *D. melanogaster* is yet again an established model used as an index for toxicity in environmental monitoring studies, where any possible alteration in its architecture would indicate the risk of exposure. Several studies on chemically induced alteration in compound eye have reported such variation in eye morphology (Podder *et al.*, 2012; Dutta *et al.*, 2014a; Sarkar *et al.*, 2015a). The study further explores the possibilities of trans-generational transfer of Flubendiamide induced alterations in the compound eye architecture for four (P, F_1, F_2 and F_3) consecutive generations, similar to the findings of NaF exposure in *Drosophila* (Dutta *et al.*, 2014b; Yiamouyiannis, 1983).

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Materials and Methods

Fly strain

Drosophila melanogaster Oregon R strain was maintained in Standard *Drosophila* Medium (SDM) containing 3 gm

agar-agar (Fisher Scientific, Mumbai, India), 17 g corn meal (Victoria Foods Private Limited, Delhi, India), 15 g sucrose (Fisher Scientific) and 9 g yeast (Merck Specialities Private Limited) in 360 mL distilled water at 22±1 °C (Dutta *et al.,* 2014a). 1 mL Propionic acid and 5 mg Nipagin were added as preservative and fungicide. Untreated larvae were maintained in a standard food medium as control.

Insecticide exposure

The formulation of Flubendiamide (TATA TAKUMI®) was used to prepare different concentrations of the test chemical in distilled water and mixed with SDM at a final concentration of 50 and 100 µg/mL. Thirty first instar larvae of *D. melanogaster* were introduced in each petri-plate (diameter – 9cm) containing SDM with or without insecticide and reared until adulthood (chronic exposure). Each experimental set up was maintained in triplicate sets (30 insects in triplicate sets per treatment concentration; 30×3=90; thus, N=90). One control set, free from additional chemical, was maintained for comparison with other treatment concentrations.

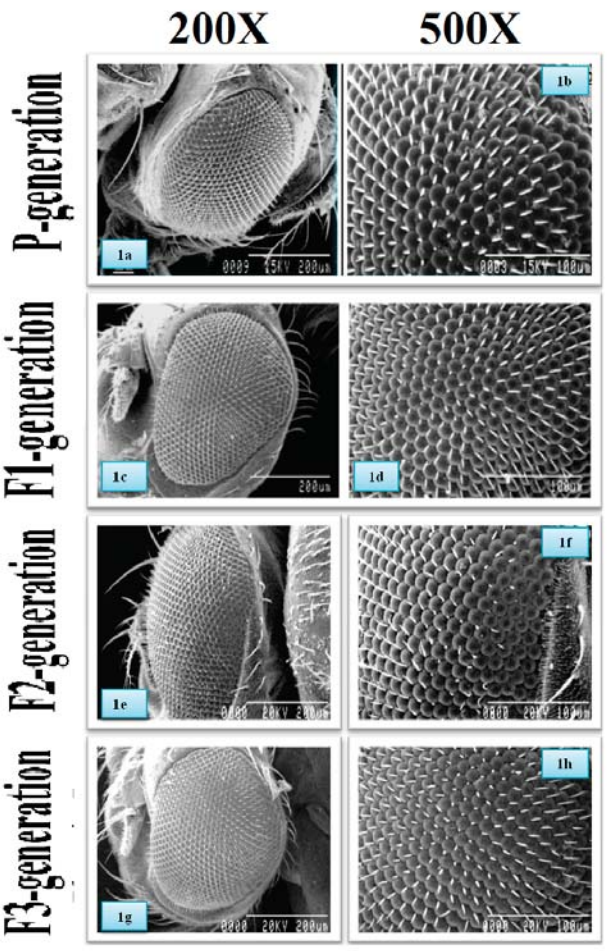

Figure 1(a–h). Electron-micrograph of compound eye of normal/wild type *Drosophila melanogaster* (P, F_1, F_2 and F_3 generations) at low (200×) and high magnification (500×). The insects were reared in a chemical free environment.

Study of consecutive generations

Fruit flies from parental generation were exposed to Flubendiamide from their first instar larval stage until they emerged as adults (chronic exposure). These P-generation adult flies were transferred to new petri-plates that were free from chemical and were maintained for successive generations. Thus the F_1, F_2 and F_3 generation flies were produced and maintained in additional chemical-free milieu.

Scanning electron microscopy

Randomly selected five (5) adult *D. melanogaster* (Oregon R strain) out of each group of thirty (30) experimental insects were used from each treatment category of all generations (P, F_1, F_2 and F_3) for scanning electron microscopy. As part of the preparation, they were taken for fixation in 2.5% gluteraldehyde for 2 hours and were then dehydrated with graded alcohols (Sarkar *et al.,* 2015a). Following fixation, the samples were processed using CPD Machine (HCP-2 HITACHI) for critical point drying. Finally, gold coating was performed using IB-2 Ion Coater (EIKO ENGINEERING) for better observation of the external morphology of the compound eye of *D. melanogaster* under scanning electron microscope (S-530 HITACHI).

Percentage of eye alterations

Twenty-five adult flies from each treatment category of all generations (P, F_1, F_2 and F_3) were carefully scrutinized under compound microscope (10X). At places, the distinct formation of ridge-groove structure and disorganized pattern of bristle orientation of the eyes after Flubendiamide treatment was considered as the "alteration". These significant percentages of compound eye abnormality were observed and recorded.

Statistical analysis

Two-way analysis of variance (ANOVA) was performed to find the significant variations in the occurrence of percentage alterations among the different generations followed by Tukey test according to Zar (1999) using the Statistical Package for Social Sciences (SPSS) version 16.

Results

Effects of Flubendiamide on compound eye morphology in P-generation

Flies receiving treatment in P generation (50 and 100 µg/mL) manifested distinct alterations in eye morphology (Figures and 3) when compared with control counterparts (Figure 1a–b). The total symmetry of eye morphology was changed with distinct grooves and ridges. The pattern of bristle orientation also revealed modification (Figures 2 and 3).

Effects of Flubendiamide on compound eye morphology in subsequent generations (F_1, F_2 and F_3)

Flies exposed to Flubendiamide (50 µg/mL and 100 µg/mL) in P generation had structural changes in the compound

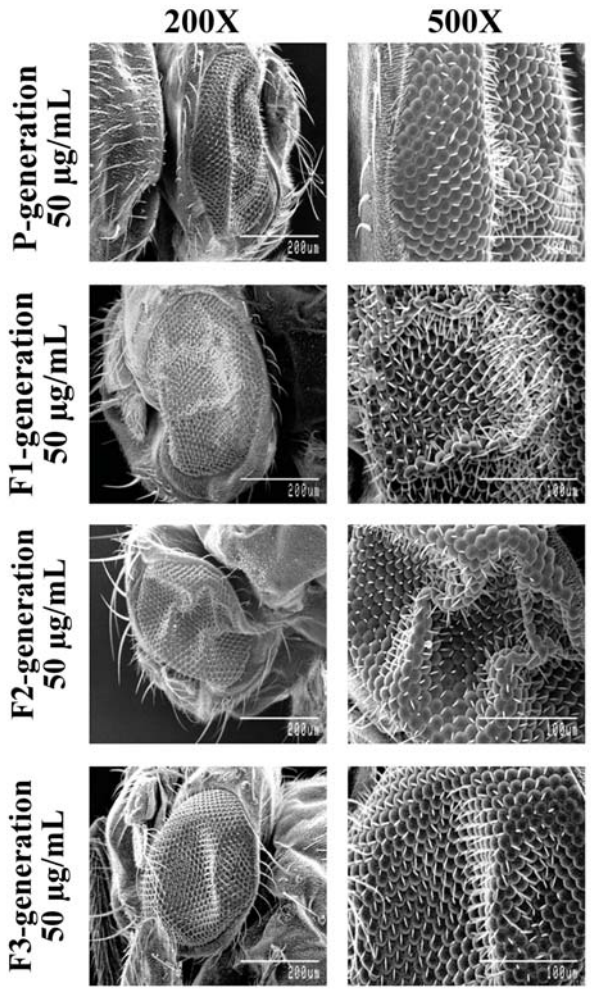

Figure 2. Scanning Electron micrographs of adult compound eye from *Drosophila melanogaster* of P, F_1, F_2 and F_3 generations where the insects received treatment with 50 µg/mL Flubendiamide in their P generation only.

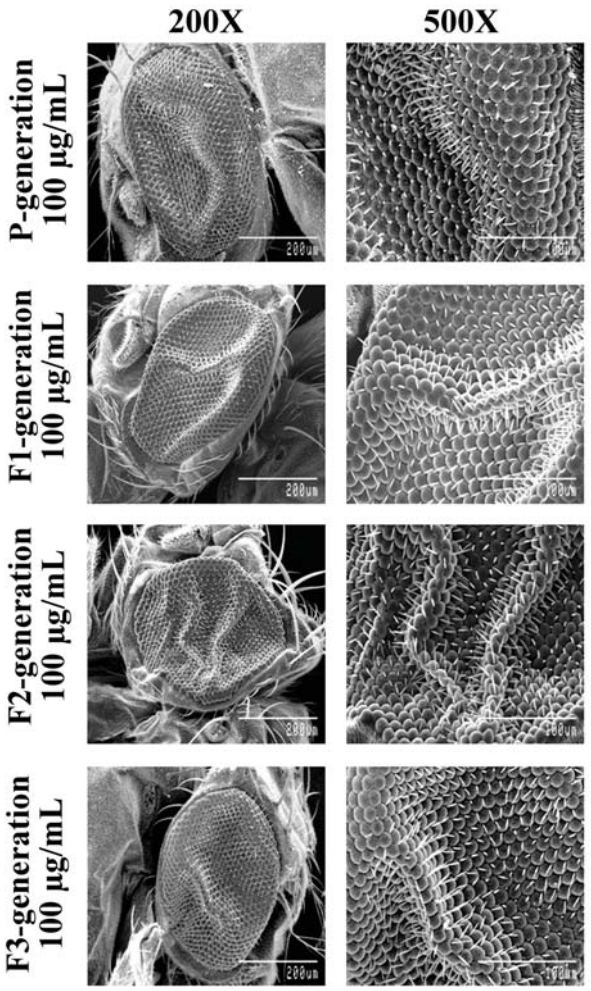

Figure 3. Scanning Electron micrographs of adult compound eye from *Drosophila melanogaster* of P, F_1, F_2 and F_3 generations. The insects were treated with 100 µg/mL Flubendiamide in the P generation only.

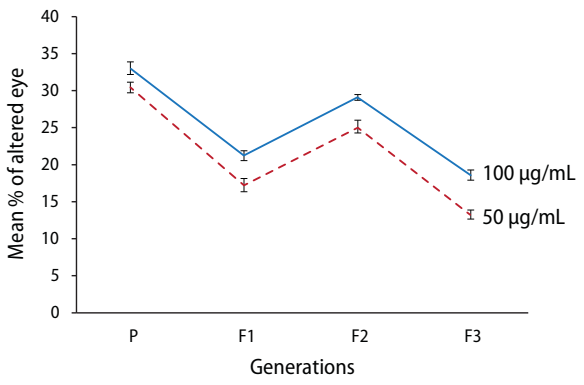

Figure 4. Graphical representation of percentage ± SE of alterations in compound eye structure for four generations (P, F_1, F_2, and F_3), when exposed to 50 (denoted by red line) and 100 µg/mL (denoted by blue line). Interestingly the control flies from all four generations were observed carefully but they did not manifest any structural alterations. Hence, percentage alteration could not be calculated for this group.

eyes, which was not restricted to P generation but to the following 3 generations (F_1, F_2 and F_3) (Figures 2 and 3) when compared with control counterparts (Figure 1c–h). The number of grooves and ridges as well as disoriented pattern of mechano-sensory bristles were increased in the subsequent generations up to F_2 ($P<F_1<F_2$) and then slightly decreased in case of F_3 (Figures 2 and 3).

Effects of Flubendiamide on proportion/percentage of altered compound eye morphology (P, F_1, F_2 and F_3)

D. melanogaster exposed to 50 and 100 µg/mL Flubendiamide in P generation revealed 30.67±0.67% and 33.33±0.88% alteration in compound eye morphology. In F_1 generation, 17.33±0.88% and 21.33±0.67% flies were found with altered compound eye respectively. 25.33±0.88% and 23.33±0.33% flies were reported to have modification in their compound eye structure in F_2 generation, whereas a declining 13.33±0.33% and 18.67±0.67% flies in F_3 generation revealed altered eye phenotype (Figure 4).

Discussion

The compound eye of *D. melanogaster* consists of nearly 800 regular ommatidia with evenly distributed mechanosensory bristles between them (Podder *et al.,* 2012) along with 8 photoreceptor neurons, accessory cells, 4 cone cells, 2 primary pigment cells, shared secondary and tertiary pigment cells, and bristle cells (Dutta *et al.,* 2014a). Any change in external eye morphology may interfere with signaling cascades regulating developmental processes (Dutta *et al.,* 2014a). Moreover, considering reflection symmetry with one axis of symmetry (Klingenberg, 2015), eyes from the treated flies revealed distinct alterations in structure when compared with control counterparts in terms of ommatidial arrangement and bristle pattern orientation.

Three hypotheses might be forwarded for trans-generational transmission of the altered phenotype in Flubendiamide treated fruit flies, 1) it might be due to the effect of the chemical on reproductive organs of P-generation flies, as reported by Yiamouyiannis (1983), Huang *et al.* (1995) at different treatment occasions, or 2) it may be autosomal / extra-chromosomal / mitochondrial / cellular inheritance as discussed by Xing *et al.* (2007) in case of trans-generational transfer of tumor factors in *D. melanogaster,* or 3) epigenetic factors like microRNAs (miRNAs), DNA methylation, and histone modification might play a distinct role in genetic inheritance and evolution, as suggested by Sharma (2015).

Several environmental factors like physical or chemical stress may elicit morphological alterations in *D. melanogaster* as discussed by D'Ávila *et al.* (2008). Waddington (1942) showed that alterations in the phenotype of fruit flies induced by (unusual) environmental conditions (high temperatures) could be fixed in a population by selective breeding. One of the probable reasons for the alterations to be transmitted to the following generations as suggested by Capy *et al.* (1998), might be due to change in location of Transposable Elements (TEs) in the host genome or due to alterations resulting from inbreeding in a small size population (Loeschcke *et al.,* 1997).

Interestingly, our previous studies on *D. melanogaster* also successfully demonstrated Flubendiamide-induced overexpression of a chaperon, Heat Shock Protein-70 (Hsp70) (Sarkar *et al.,* 2015b) along with Acetylcholinesterase (AChE) inhibition in neurons, as well as disoriented compound eye architecture (Sarkar *et al.,* 2015a). Since all HSPs belong to the same gene family, it could be assumed that they would manifest similar outcomes. One small α-crystalline-related heat shock protein (sHsp), Hsp27 is known to be synthesized during fly development by the induction of molting hormone ecdysone (Ireland *et al.,* 1982). This sHsp has its expression in adult brain, gonads (Arrigo & Tanguay, 1991), and ommatidial cells (cone, pigment and photoreceptor cells) (Marin *et al.,* 1996). Other sHsps are known to act together with α-crystalline in plasma membrane and cytoskeleton (Miron *et al.,* 1991; Lavoie *et al.,* 1993). A study on mammalian Hsp27 revealed its crucial role in signalling pathway between mitogens and actin polymerization at the membrane (Miron *et al.,* 1991; Lavoie *et al.,* 1993). Heat shock proteins of *Drosophila* (Hsps) have close proximity with vertebrate eye lens protein α-crystalline (Ingolia & Craig, 1982), which might indicate a common ancestry for both proteins. The second half of α-crystalline domain was found to have very conserved 83 residues in case of *D. melanogaster* and mammals (Southgate *et al.,* 1983). Any chemical stress may lead sHsps to form super aggregated structures (in lens cells), which is very well defined in case of mammalian Hsp27 (Mehlen & Arrigo, 1994) and α-crystalline (Klemenz *et al.,* 1991) proteins. So the effects seen in *D. melanogaster* compound eye might similarly be manifested in related organisms like mammals, including humans.

Several authors have established different genes responsible for normal eye development and vision, like Rab 11 (Tiwari & Roy, 2009) and Rab 6 (Alone *et al.,* 2005), fat facets (faf) (Huang *et al.,* 1995), nemo (nmo) (Choi & Benzer, 1994) gene, *etc.* Fluoride containing Flubendiamide (Sarkar *et al.,* 2014) is expected to cross the biological membranes either via non-ionic passive diffusion (Whitford, 1994) or in ionic form (Gutknecht & Walter, 1981). Cellular metabolism and physiology depend on the cell type, concentration and time of fluoride ion exposure (Barbier *et al.,* 2010). Fluoride has been demonstrated to be a potent activator of G-protein in virtually all cell types studied (Sternweis & Gilman, 1982). Increased amount of fluoride can cause chromosomal damage in the sperm cell and can lead to birth defects which can be transmitted through generations (Yiamouyiannis, 1983). In the present study, *D. melanogaster* was exposed to the fluoride containing chemical (Flubendiamide) from the

Table 1. Two-way analysis of variance (ANOVA) using SPSS software (version 10).

Source	Sum of squares	df	Mean Square	F	Significance
Generation	58.13	3	19.38	13.29	0.000131
Dose	7.04	1	7.04	4.83	0.043063
Gen*Dose	0.46	3	0.15	0.1	0.956089
Error	23.33	16	1.46		
Total	941	24			
Corrected Total	88.96	23			

The data show that at the 0.05 level the generation means are significantly different. At the 0.05 level, the dose means are also significantly different. Significance was calculated at $p<0.05$.

Table 2. Multiple comparisons between different generations using Tukey test based on observed means. Tukey test performed using SPSS software (version 10).

Generation	Generation	Significance
P-Generation	F_1-Generation	0.00028
	F_3-Generation	0.00016
F_2-Generation	F_3-Generation	0.0045

first instar larval stage until adulthood (chronic exposure) and revealed trans-generational transmission of altered compound eye phenotype similar to the finding of Dutta *et al.,* (2014b).

Percentage alterations in adult compound eye morphology following differential treatments in P generation and their variable expressions in subsequent generations revealed a gradual reduction of altered eye phenotype (F_3<F_1<P), except F_2 generation (Figure 4). This reduction might be due to withdrawn chemical exposure in the following generations. The ANOVA (Table 1) followed by Tukey test (Table 2) revealed significant (p<0.05) variation among P and F_1 (p=0.00028), P and F_3 (p=0.00016), F_2 and F_3 categories (p=0.0045). Thus the inter-generational variations of altered compound eye phenotype are evident.

Both treatment categories (50 and 100 µg/mL) exhibited significant enhanced number of affected phenotype in case of P and F_2 generations and decreased in case of F_1 and F_3. Since 75% human disease genes have their fly homolog (Pandey & Nichols, 2011) and some common mammalian genes are also known to have fly homolog like Rab gene (Bock *et al.,* 2001), pax6 (insect homolog of eyeless) *etc.*, the results of the present study are rather relevant in the light of the findings reported by Yiamouyiannis (1983), where a chemical like fluoride is seen to cause multiple genetic damage in insects, as well as animals including humans. Hence, the present work reports that non-target insect morphology is also an equal vulnerable target for pesticide hazard as that of its physiology.

Conclusion

In the present study, Flubendiamide, a lepidopteran insecticide, is found to alter compound eye structure of a non-target dipteran model insect, *Drosophila melanogaster.* The alterations were not confined to the exposed insects (P generation) only, rather insects from three subsequent generations (F_1, F_2 and F_3, who were never exposed to the chemical) also revealed alterations in their compound eye architecture. Thus irrational use of Flubendiamide in agricultural fields might pose serious health hazards for similar non-target insects and insect dependent organisms, including human beings.

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Conflict of interest

There is no conflict of interest regarding this paper.

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