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_{7}IN_{2}O_{4}S, 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, F1, F2 and F3) consecutive generations, similar to the findings of NaF exposure in *Drosophila* (Dutta *et al.*, 2014b; Yiamouyiannis, 1983).

**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.

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 F₁, F₂ and F₃ 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, F₁, F₂ and F₃) 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, F₁, F₂ and F₃) 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 (F₁, F₂ and F₃)
Flies exposed to Flubendiamide (50 μg/mL and 100 μg/mL) in P generation had structural changes in the compound...
Flubendiamide induced alteration in eye morphology of *Drosophila*

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Eyes, which was not restricted to P generation but to the following 3 generations (F₁, F₂ and F₃) (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 F₂ (P<F₁<F₂) and then slightly decreased in case of F₃ (Figures 2 and 3).

**Effects of Flubendiamide on proportion/percentage of altered compound eye morphology (P, P₁, P₂ and P₃)**

*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 F₁ generation, 17.33±0.88% and 21.33±0.67% flies were found with altered compound eyes respectively. 25.33±0.88% and 23.33±0.33% flies were reported to have modification in their compound eye structure in F₂ generation, whereas a declining 13.33±0.33% and 18.67±0.67% flies in F₃ generation revealed altered eye phenotype (Figure 4).
Discussion

The compound eye of *D. melanogaster* consists of nearly 800 regular ommatidia with evenly distributed mechano-sensory 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-flubendiamide* treated flies, as reported by Yiamouyiannis (1983), Huang *et al.* (1995) at different treatment occasions, or 2) it may be autosomal / extra-chromosomal / 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’Avila *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 (Yiamouyiannis, 1983) is expected to cross the biological membranes either via non-ionic passive diffusion (Whittard, 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

| 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 | Significance |
|------------|--------------|
| F1-Generation | 0.00028     |
| F2-Generation | 0.00016     |
| F3-Generation | 0.0045     |
among P and F1 (Tukey test (Table 2) revealed significant (p<0.05) variation among P and F1 (p=0.00028), P and F2 (p=0.00016), F2 and F3 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 F2 generations and decreased in case of F1 and F3. 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 (F1, F2 and F3, 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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