Table 1. Classification of flies according to species and sex (Font Groga site, Barcelona).

Species	Number	Percentage
D. subobscura (♂)	5	0.91
D. subobscura (♀)	61	11.11
D. simulans (♂)	230	41.89
D. melanogaster (♂)	1	0.18
D. melano / simulans (♀)	240	43.72
D. suzukii (♀)	7	1.28
D. phalerata (♂)	1	0.18
D. phalerata (♀)	1	0.18
D. hydei (♂)	1	0.18
Scaptomyza sp.	2	0.36
Total	549	100

0.474 and 0.294, respectively. For these indexes, the trend to decrease detected in the last two years continues (Rosselló *et al.*, 2016; Esteve and Mestres, 2015).

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Flubendiamide inflicts tissue damage and alters detoxification status in non-target dipteran insect, *Drosophila melanogaster*.

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Abstract

This study aims to assess the safety of a lepidopteran insecticide, Flubendiamide, in a non-target dipteran model insect, *Drosophila melanogaster*, at tissue/cellular and enzyme/protein levels. Enhanced blue coloration through Trypan blue dye exclusion test suggests greater tissue damage. Furthermore, dose-dependent increase ($p < 0.05$) in the cytochrome P450 1A1 enzyme activity suggests activation of the Phase-I detoxifying mechanism. Thus, this study confirms Flubendiamide-induced toxic stress in *Drosophila* that might be replicated in other non-target organisms. Keywords: Cytochrome P450, *Drosophila*, Flubendiamide, Tissue damage.

Introduction

Flubendiamide ($C_{23}H_{22}F_7IN_2O_4S$, CAS No: 272451-65-7), a lepidopteran insecticide, is widely used in agriculture and has been suggested to be chemically safe for non-target insects like *Drosophila melanogaster* (Tonishi *et al*., 2005). Approximately 60 µg/mL Flubendiamide has been recommended for use in case of cotton by Fluoride Action Network Pesticide Project (2007), whereas proposals of US EPA (2010) for soya bean and grain are up to 60 and 103 µg/mL. The recommended Indian field doses in case of paddy and cotton are 50 µg/mL and 100 µg/mL (Government of India, Ministry of Agriculture, 2009). But recent studies revealed that Flubendiamide at very low concentration (far below the agricultural doses) may elicit severe effects on stress gene expression, neurophysiology, and external morphology of dipteran non-target *D. melanogaster* (Sarkar *et al*., 2015a, 2015b). Several workers recognized *Drosophila* as a remarkable model organism for pesticide-induced toxicity monitoring studies (Aurosman Pappus *et al*., 2017; Dutta *et al*., 2017; Rajak *et al*., 2017).

Cytochrome P450 belongs to a group of heme-containing, mono-oxygenase enzymes, which are present in eukaryotes in large numbers. There are 85 functional P450 genes present in *Drosophila melanogaster* (Tijet *et al*., 2001), whereas humans have 57 (Lewis, 2004). Several researchers suggested Cytochrome P450 enzymes to be involved in metabolism of several endogenous steroids as well as xenobiotic compounds (Chung *et al*., 2009). P450s are reported to metabolize numerous pharmaceutical drugs (Nebert and Russell, 2002), pesticides (Joussen *et al*., 2007), and plant toxins (Mao *et al*., 2006). Chung *et al*. (2009) suggested that CYP450 is found in brain, midgut, hindgut, Malpighian (renal) tubules, fat body, gonads, and so forth of *D. melanogaster* at the time of detoxification. Thus, the present study aims to observe the tissue damage through the dye exclusion test and to monitor changes in activity of cytochrome P450 mono-oxygenase enzymes in *D. melanogaster* larvae to identify the effect of the chemical on the organism as well as the response of the organism (detoxification ability) towards the chemical-induced stress at sub-lethal concentrations.

Materials and Methods

Drosophila *strain*

Drosophila melanogaster Oregon 'R' were reared at 25±1ºC and 65% relative humidity on Standard *Drosophila* Medium (SDM) containing agar-agar, corn meal, sucrose, and yeast in 360 mL distilled water (Dutta *et al*., 2014). As preservative and fungicide, 1 mL Propionic acid and 5 mg Nipagin were used.

Dye exclusion assay

The degree of tissue damage was observed in 3rd instar larvae through Trypan blue dye exclusion test following Krebs and Feder (1997) with some modifications. First instar larvae of *D. melanogaster* were subjected to chronic exposure to Flubendiamide (0.25, 0.5, 2, and 3 µg/mL) at different treatment concentrations. Treated (0.25 – 3 µg/mL) as well as control larvae were dissected cautiously to expose insect gut on a grooved slide using Poel's Salt Solution (PSS), and were carefully washed in phosphate buffer saline (PBS) for three times. 0.2 mg Trypan blue (Himedia, India) solution in 1ml PBS was used to stain tissues for 30 minutes with continuous shaking at room temperature (24±1°C) and again washed with phosphate buffer saline (PBS) three times. Immediate observation revealed tissue damage indicated by blue coloration under binocular microscope (Model: Magnus MS-24). The experiment was performed in triplicate sets.

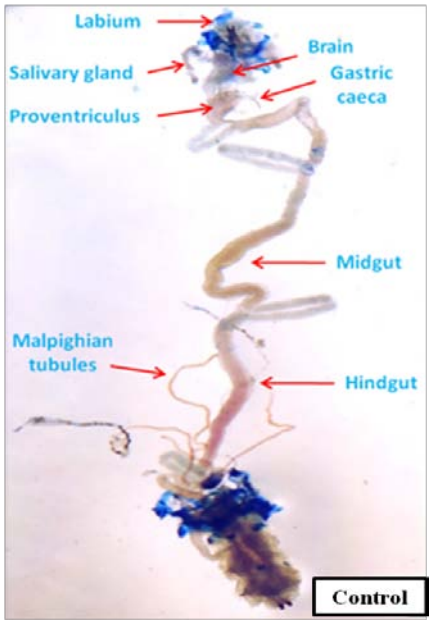
Activity of larval Cytochrome P450 (*CYP450*) *in* Drosophila (*EROD assay*)

EROD assay was carried out to observe the activity of Cytochrome P450 (CYP450 1A1) according to the method described by Klotz *et al*. (1984) with some modifications. Healthy third instar Flubendiamide-treated (0.25 – 3 µg/mL) larvae were collected from food and rinsed in Poel's salt solution (PSS). 10% homogenates were prepared with sodium pyrophosphate buffer (pH 7.4) and centrifuged at 14000 RCF for 20 min. Then, supernatants collected containing crude cytosolic (post-mitochondrial fraction) protein was used for the assay. The entire process was carried out at 4°C. Resorufin (RR) standard curve was prepared using Tris-Cl buffer for unknown samples referencing and protein was estimated following Lowry *et al*. (1951).

The typical reaction mixture contains 0.1 M Tris-Cl buffer (pH 7.8) including 0.1 M NaCl, 2 µM 7-ethoxyresorufin, and 20 µg microsomal protein to compose a total volume of 1 mL. 0.5 mM NADPH was added to initiate the reaction. Activity of 7-ethoxyresorufin o-deethylase (EROD) was recorded after 2 min at 25-30°C in UV-Vis Spectrophotometer (Model: Shimadzu UV-1800) at 572 nm. The experiment was done in triplicate sets with ten repetitions.

Statistical analysis

Non parametric Kruskal-Wallis test, followed by mean comparison Steel-Dwass-Critchlow-Fligner (*Post hoc test*) test according to the methods of Zar (1999) was performed using statistical software XLSTAT 2010, to calculate the exposure dependent differences in larval CYP450 level of different treatment concentrations in comparison to control counterparts. $p < 0.05$ was considered as the level of significance.



Results

Effect of Flubendiamide in larval tissue damage (*Trypan blue dye exclusion Assay*)

Figure 2 has revealed differential patterns of coloration in 3rd instar larval body of *D. melanogaster* exposed to different concentrations of Flubendiamide (0.25 – 3 µg/mL) after Trypan blue dye exclusion assay. Following Krebs and Feder (1997), scoring procedure was adopted based on an average composite index per larva with the help of the photographs (Table 1). From the scores, it appeared that all the treatment concentrations (0.25 – 3 µg/mL) manifested a significant ($p < 0.05$) increase in blue color which is reflected in greater Trypan blue score in comparison to control counterpart (Figure 1). Maximum tissue damage due to Flubendiamide exposure was observed after 3 µg/mL treatment (Figure 2). The uniform blue color noticed at the anterior and posterior skin regions of control as well as treated larvae might be due to the injury caused during the time of dissection. This color was ignored during scoring.

Figure 1. Trypan blue staining pattern in control 3rd instar larvae of *Drosophila melanogaster*. This figure is a representation of the different parts of larvae given special attention during observation.

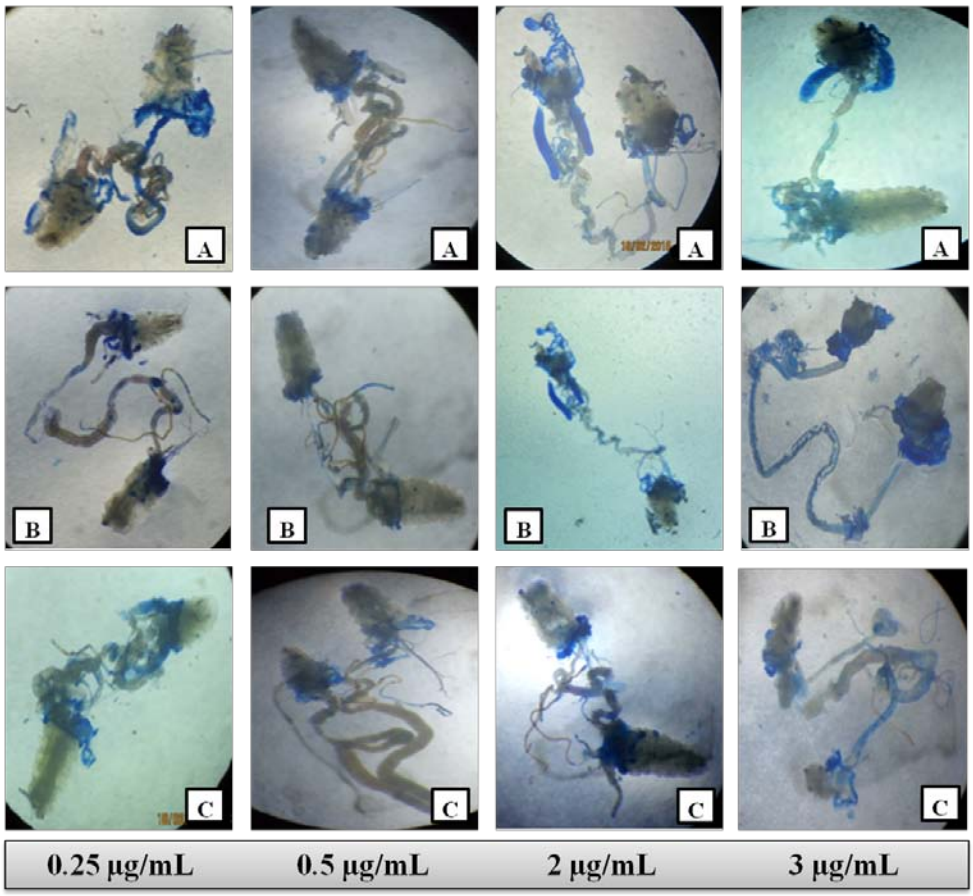


Figure 2. Trypan blue staining pattern in different tissues of third instar larvae of *Drosophila melanogaster* treated with 0.25 – 3 µg/mL Flubendiamide. Experiments were carried out in triplicate sets and scoring was done depending on development and intensity of blue color in different body parts.

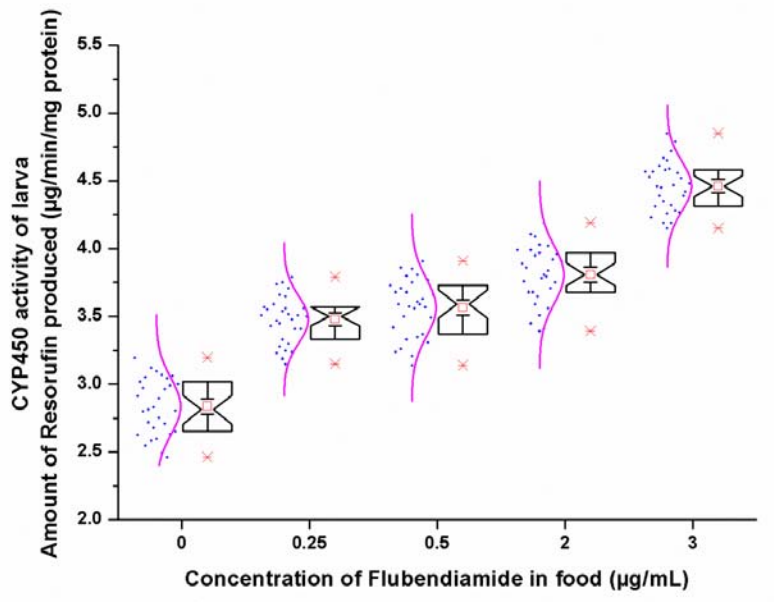


Figure 3. Variation in CYP450 activity calculated through amount of resorufin formed (µg/min/mg protein) in *D. melanogaster* larvae after treatment with Flubendiamide. The data are expressed in notch box plot, where blue dots stand for real data, pink curve denotes normal curve, and notch box represents middle 50% data (26% - 75%, *i.e.*, 2nd and 3rd quartile data). Moreover, small red boxes within the notch boxes indicate mean values, vertical lines within notch boxes symbolize standard errors (SE), notches of the notch boxes signify median values, whereas lower and upper cross of notch boxes are 1st (1% - 25%) and 4th (76% - 99%) quartile data, respectively.

Table 1. Quantification of Trypan Blue staining, an indicator of tissue damage, observed in 3rd instar larvae of *D. melanogaster* exposed in different concentrations (0.25 – 3 µg/mL) of Flubendiamide.

Conc. Of Flubendi-amide (µg/mL)	Group	B	SG	P	GC	FG	MG	HG	MT	PS	Score	Mean	S.E.
Control	A	0	0.5	0	0	1	0.5	0	0	0	2	2.33	0.3
	B	0	1	0	0	0.5	1	0	0	0	3		
	C	0	0.5	0.5	0	0	1	0	0	0	2		
0.25	A	1	1	0.5	1	0	3	3	0	0.5	10	10.5*	0.2
	B	1	2	2	1	0.5	1	1	1	1	10.5		
	C	2	2	1	1	1	1	1	2	0	11		
0.5	A	2	2	0.5	1	1	1	1	2	1	11.5	10.7*	0.3
	B	1	1	1	1	0.5	1	2	2	1	10		
	C	2	1	1	1	1	2	1	1	1	1		
2	A	1	3	1	1	1	2	1	1	1	14	12.8*	0.5
	B	2	3	1	1	1	2	1	1	1	13		
	C	1	2	1	1	1	2	0.5	2	1	11.5		
3	A	3	3	2	1	1	2	2	1	0.5	15.5	13.2*	0.4
	B	2	2	1	0.5	2	2	1	0.5	0	11		
	C	1	1	1	2	1	2	1	2	2	13		

B- brain; SG- salivary gland; P- proventriculus; GC- gastric caeca; FG- foregut; MG- midgut; HG- hindgut; MT- Mapighian tubule and PS- posterior skin.
The numerical '0' = no color, '0.5'= very pale blue color, '1'=pale blue color, '2'=moderate blue color, '3'= dark blue color. In the present study scoring pattern was adopted from Krebs and Feder (1997) with some modifications. The experiments were carried out in triplicate sets and each set consisted of 20 larvae. '*' represents significant difference from control group ($p<0.05$).

Effect of Flubendiamide in larval Cytochrome P450 assay (*EROD*)

Cytochrome P450 (CYP6 family) activity was measured by the formation of Resorufin (µg)/ min/ mg protein. This present study revealed that all the Flubendiamide-treated 3rd instar *D. melanogaster* larvae were significantly different ($p < 0.05$) in Cytochrome P450 activity from control counterpart (2.84±0.04). Treatment with (0.25 – 3 µg/mL) test chemical revealed a significant ($p < 0.05$) increase (22.63%, 25.79%, 34.28%, and 62.54%) in Resorufin production or enzyme activity as compared with control (Figure 3).

Discussion

Trypan blue is a vital dye which can easily infiltrate through the cell membrane of dead cells or tissues, whereas live cells with intact cell membrane resist the dye from penetration. When cells or tissues were exposed to Trypan blue, then dead or dying cells were unable to eliminate the dye and dead cells or tissues reveal distinct blue color. On the contrary, live cells do not absorb any dye and remain unchanged. In the present study, all the treated larvae (0.25 – 3 µg/mL) were observed with blue coloration in their brain, salivary gland, proventriculus, gastric caeca, foregut, midgut, hindgut, and Mapighian tubule as compared with control counterparts (Figure 1 and Figure 2). Furthermore, quantification of Trypan Blue staining (according to Krebs and Feder, 1997), an indicator of tissue damage, revealed a significant ($p < 0.05$) dose dependent increase in treated *Drosophila* larvae exposed in different concentrations (0.25 – 3 µg/mL) of test chemical (Table 1). Flubendiamide and/or metabolites of Flubendiamide might cause toxic stress on larvae that result in tissue or cellular damage. Thus larvae exposed to Flubendiamide reveal an increase in dead cell number as observed through trypan blue dye exclusion test, thereby confirming the Flubendiamide-induced stress. Dose-dependent greater tissue damage might be due to reduced number of viable cells as suggested by Gupta *et al*. (2005a) in *Drosophila* exposed to an organophosphate pesticide, Nuvan. Several workers reported tissue damage in response to chemical stress (Siddique *et al*., 2013; Dutta *et al*., 2017; Rajak *et al*., 2017). As the test chemical might cause cellular or tissue damage, initiation of detoxification was observed to overcome the stress.

As suggested by Mukhopadhyay and Chottopadhyay (2014), all organisms have their own detoxifying systems for overcoming toxic stress. Normally, detoxifying system is composed of Phase I, Phase II, and Phase III pathways (Rajak *et al*., 2017). In phase I pathway, toxic substances convert into polar and water soluble compounds facilitated following pathways (Phase II and Phase III) act upon and are excreted from body through bile or kidney (Benson and Di Giulio, 1992). Cytochrome P450 is very well known for its Phase I detoxifying mechanism against xenobiotic compound including pesticides (Joussen *et al*., 2007; Chung *et al*., 2009). Hence, insects exposed to chronic sub-lethal Flubendiamide (0.25 – 3 µg/mL) might be under stress that activates Cytochrome P450 detoxifying system (Phase I). In the present study, treated larvae of *D. melanogaster* show a concentration dependent significant ($p < 0.05$) increase of Cytochrome P450 enzyme activity in comparison to control, thereby confirming a greater Flubendiamide-induced stress (Figure 3). Similar increase in Cytochrome P450 activation was also found in *Drosophila melanogaster* in response to toxic stress of NaF (Dutta *et al*., 2017). As Flubendiamide is a fluoride containing chemical (Tonishi *et al*., 2005), thus, free fluoride ion released from test chemical might be responsible for toxic stress (oxidative stress) (Guo *et al*., 2003). Thus, this study confirms Flubendiamide-induced toxic stress in *Drosophila melanogaster* and might show similar hazardous effects to other related non-target organisms.

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Appl-GAL4 driven transcription in adult heads.

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Drosophila melanogaster is one of the most important invertebrate model organisms to study the aging process. The proper application of genetic tools that depend on the GAL4/UAS bipartite gene expression system to overexpress or downregulate genes of interest in aging research requires GAL4 drivers that provide well-characterized expression levels in specific adult tissues. It was previously reported that Appl-GAL4 driven expression of UAS-Atg8a lines increased maximal and average lifespan, while the widely used pan-neuronal elav-GAL4 driver did not provide similar effect (Simonsen, 2008), suggesting that Appl-GAL4 provides stable adult expression. To assess the expression level and expression pattern of Appl-GAL4 in the adult nervous system we decided to characterize UAS transcription driven by the P{w[+m*]=Appl-GAL4.G1a}1, y[1] w[*] line (BDSC stock no. 32040) that is advertised to express GAL4 in the nervous system.

In order to determine UAS transgene expression levels driven by Appl-GAL4 during aging we collected freshly eclosed *Appl-GAL4/w; UAS-GFP/+* progeny of *Appl-GAL4* females and *w; UAS-GFP* males and maintained them at 25°C by passing them to fresh vials every 2-3 days. We prepared RNA samples from heads of 1 day, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, and 6 weeks old female flies (10 heads per sample, three biological replicates per age group) using QIAzol Lysis Reagent (Qiagen). We generated first strand cDNA from 0.5 µg RNA per sample using TaqMan Reverse Transcription Reagents (Thermo Scientific) with random hexamer primers, then measured transgene expression levels in a PikoReal Real-Time PCR system (Thermo Scientific) using GoTaq qPCR Master Mix (Promega) with primers specific for pUAST (Fw: CTG TGG TGT GAC ATA ATT GGA CAA, Rev: TGC TCC CAT TCA TCA GTT CC A, designed for the SV40 polyA/terminator region in the pUAST vector) and with primers for the rp49 housekeeping gene that was used for normalization. qPCRs were performed in duplicates and transcript levels were determined by setting Ct values against cDNA template calibration curves. We found that the transcript level of UAS-GFP significantly decreased over time (Spearman's correlation coefficient $\rho = -0.60553$, $P = 0.00363$). A gradual reduction in Appl-GAL4 driven UAS-GFP expression was most pronounced after 2 weeks of age, with heads of 4 and 6 weeks old flies having transcript levels below 60% of that of freshly eclosed adults (Figure 1).

Next, we investigated the expression pattern of Appl-GAL4 in the adult nervous system by visualizing GFP expression in dissected brains of 1 week old *Appl-GAL4/w; UAS-GFP/+*females under a Nikon Eclipse