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# Exposure to Fluralaner induces behavioral and reproductive alterations in Zebrafish (*Danio rerio*)

**Pradnya Patil, SS Sole, Purva Joshi, Vishal P Mote, RV Gaikwad, SU Gulavane and PV Meshram**

**Abstract**
The newly introduced insecticide fluralaner was evaluated for its possible endocrine-disrupting ability in the zebra fish model. Dependent on pilot study results, four concentrations were selected viz., 5 mg/l, 10 mg/L., 20 mg/l, and 100 mg/1 for a 21-days exposure period. Exposed males were mated with female zebra fish. Baseline data was also generated before exposure.
Changes in the behavior were observed in dose-dependent manner, confused behavior, and colliding with the walls may be due to the antagonist effect of fluralaner on GABA receptor. A significant decrease in spawn count was observed within the groups, which might be due to deceased oviposition and possible sperm toxicity. Comparison of fecundity within the respective groups to their pre-exposure fecundity values indicated statistically significant differences due to decrease implantations. The significant rise in the mortality percent of embryos is indicative of the possibility of adverse effects of fluralaner on sperm quality. Abnormal embryo count showed a significant difference within the groups to their respective pre-exposure values. The increased level of vitellogenin biomarker in the fluralaner exposed group is indicative of disruption in the endocrine system in male zebra fish. Hence it is concluded that fluralaner shows the potential for embryo toxicity due to endocrine disruption in male zebra fish.

**Keywords:** Endocrine disruption, Fluralaner, Vitellogenin, Zebrafish

## 1. Introduction
Fluralaner is a novel ectoparasitic belonging to the isoxazoline group (fig.1), used in veterinary medicine, particularly for dogs and cats, and marketed as a BRAVECTO® (Jia, *et al*., 2018[6]. Casida, *et al*., 2015 [2], Ozoe, *et al*., 2010 [10], Zhao, *et al*., 2015) Fluralaner has activity against γ- aminobutyric acid-(GABA) and glutamate-gated chloride channels (Gassel M, *et al*. 2014 [3], Kilp S. *et al*. 2016 [7]. Williams H. *et al*. 2015, Walther F. *et al*., 2014 [13], Rohdich N. *et al*., 2014 [11]). These chewable tablets are light brown to dark brown, with a smooth or slightly rough surface, and a practically round shape.
India is the world's third largest manufacturer of pharmaceuticals, with exports to over 65 countries. By 2020, the country would be ranked in the world's top 10 largest pharmaceutical markets. The large-scale production and extensive use of these compounds as well as their disposal from the medical center and discharge of domestic wastewater has resulted in environmental contamination (Satapathy *et al*., 2014) [12].
According to CVMP assessment report of Bravecto, unchanged parent fluralaner is one such compound, found primarily in feces (approx. 90% of the dose) after its oral administration to the dogs. This data demonstrated that veterinary personnel, and pet owner could be exposed to a significant amount of fluralaner from contact with fluralaner-treated dogs and cats. This compound through feces and urine may go to the water bodies and get accumulated in fishes and by consuming these fishes, human may get exposed to fluralaner. Fluralaner can also be potentially introduced into water systems, from the run off-site receiving waste product. It may also leach into the soil and water systems near the fluralaner manufacturing facilities which can affect the aquatic life and also to the human life. As there is very scarce research carried out on the effect of fluralaner on aquatic organisms, our study focuses to assess the effect of fluralaner on the reproduction of aquatic organisms. The present study was carried out according to OECD guidelines 229 for Fish Short Term Reproductive Assay (FSTRA).
Zebrafish species was chosen for the study as with other several compelling experimental advantages such as short time required for analyses, low cost, availability of different strains, knowledge of genome sequence, transparency of embryo, short life cycle, high fertility, smaller amount of compound required and it is also been approved by U.S.

Food and Drug Administration (FDA) and European Medical Agency (EMEA) for toxicity and safety assessments for Investigative New Drug (IND) approval. This small tropical freshwater teleost fish also has crucial genetic (85% with humans), anatomical and physiological homology with mammals. By keeping above facts in mind fluralaner assessed in *Danio rerio* for endocrine disruption (Gopi *et al*., 2012) [4].

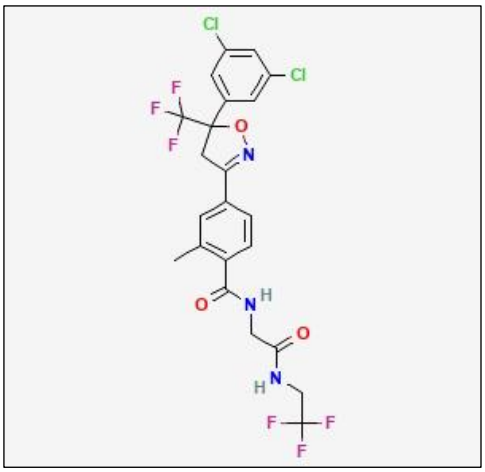


**Fig 1:** Structure of Fluralaner

## 2. Materials and Methods

### 2.1 Zebrafish

The zebrafish were maintained in the Department of Pharmacology and Toxicology, Mumbai Veterinary College, Parel as per the method described by Westerfield, (2000) [14] under optimum temperature 25-31 $^0$C. The circadian cycle of 14 hours of light and 10 hours of darkness was strictly maintained. They were housed in breeder tanks and separation tanks according to the need of experiment. The water was oxygenated with commercially available aerators. Regular cleaning of tanks and hygienic conditions are maintained. The feeding of fishes was as follows (Table 1).

They were acclimatized to laboratory conditions before the commencement of experiment. Permission from the institutional animal ethics committee was also obtained.

**Table 1:** Feeding schedule for fish

| Age (In days) | Morning (9.00am) | Afternoon (1.00pm) | Evening (6.00pm) |
|---|---|---|---|
| 0-6days | No feed | No feed | No feed |
| 6-60days | Larval Diet | Brine shrimp | Larval Diet |
| 60days onward | Brine shrimp | ------ | Dry trout Pellet |

### 2.2 Chemicals used in this study

**Test Drug:** Fluralaner: The International Union of Pure and Applied Chemistry (IUPAC) name for fluralaner is 4-[5-(3, 5-dichlorophenyl)-5-(trifluoromethyl)-4H-1,2-oxazol-3-yl]-2-methyl-N-[2-oxo-2-(2, 2, 2-trifluoroethylamino)ethyl] benzamide.

Chemicals used for embryo medium (given below) were procured from Fisher scientific and were stored at room temperature (27-28 $^o$C).

Sodium phosphate ($Na_2HPO_4$ $7H_2O$), CAS Number 10039-32-4, Potassium dihydrogen ortho phosphate ($KH_2PO_4$), CAS Number 7778-77-0. Calcium chloride (Dihydrate) ($CaCl_2$ $2H_2O$), Product Number 12135. Magnesium sulphate ($MgSO_4$ $7H_2O$), CAS Number 10034-99-8. Sodium hydrogen carbonate ($NaHCO_3$), CAS Number 144-55-8. Sodium hydroxide solution 1.0N (NaOH), 145057, MB/26/616/M-552-05/6/16. Sodium chloride (NaCl), CAS Number 7647-14-5. Potassium chloride (KCl), CAS Number 7447.

### 2.3 Experimental Design

Only male zebrafish were exposed to selected three concentrations of ractopamine. For each concentration, two replicates of eight male zebrafish were included. Thus, a total of 64 male zebrafish were exposed to fluralaner. Besides, some additional male zebrafish were kept with female zebrafish considering the physiological need for maintaining their reproduction. During mating, they were transferred to breeding tanks free of ractopamine in the ratio of 1 male: 2 females.

Male zebrafish were exposed to different concentrations of the drug through water and DMSO, as the drug was soluble in the water (National Institute of health). The male zebrafish separated in the separation tanks were exposed continuously for 21 days except for their weekly mating period of three hours (Boaru A *et al*., 2013) [1]. Based on the behavioral changes in zebra fish, male zebrafish were exposed to three different concentrations of fluralaner viz. 5 mg/L, 10 mg/L, 20 mg/L and 100 mg/L for 21 days (Table 2). They were mated with their female partners on every 8th day and the eggs deposited by the females after mating were collected, cleaned, and evaluated for spawn, fecundity, mortality, and abnormality percent (As per OECD guidelines 229).

**Table 2:** Group Distribution

| Drug | Group | | Number of fish (n) | Dose (mg/L) |
|---|---|---|---|---|
| Fluralaner | Group A | A1 | 8 | 5 mg/L |
| | | A2 | 8 | 5 mg/L |
| | Group B | B1 | 8 | 10 mg/L |
| | | B2 | 8 | 10 mg/L |
| | Group C | C1 | 8 | 20 mg/L |
| | | C2 | 8 | 20 mg/L |
| | Group D | D1 | 8 | 100 mg/L |
| | | D2 | 8 | 100 mg/L |
| | Control Male | | 64 | -- |
| | Control Female | | 128 | -- |

#### 2.3.1 Percent Reduction in Spawn Count

The reduction in spawn count at the weekly intervals of 8, 15, and 22 days over the control values for each of the duplicates was calculated. The values thus obtained were utilized for calculating the percent reduction in spawn count as follows:

$$\text{Percent}\frac{\text{Reduction in spawn count}}{\text{Spawn count (day 01) in spawn count}}\text{X100} = \text{reduction}$$

#### 2.3.2 Fecundity Percent (%)

On gross and microscopic examination, the live and dead embryos were distinguished. Live embryos appeared transparent while dead were milky crescent-shaped bodies within the egg. Under the inverted microscope (5X), the dead eggs appeared to have a coagulated mass while the live embryos exhibited the presence of a perfectly circular mass of yolk around which growth was detected during the observation period. The live count for the first day was considered as the initial live count and based on this, the fecundity percent was calculated as follows:

$$\text{Fecundity Percent} = \frac{\text{Live count (day 01)}}{\text{\% Spawn count (day 01)}} \text{X } 100$$

### 2.3.3 Mortality Percent (%)

Once transferred to 96 well plates, the embryos were further evaluated for 72 HPF for any further mortalities. The live and dead embryos observed during this period and the summation of dead embryos or larvae in the 72 HPF gave the total mortality and based on this, the mortality percent was calculated as follows:

$$\text{Mortality percent} = \frac{\text{Total mortality (0-72 HPF)}}{\text{Spawn count (day 01)}} \text{X100}$$

### 2.3.4 Abnormality Percent (%)

During the period of 72 HPF, observations were carried out for abnormalities in the growth and development of the embryo such as delayed hatching of larvae, abnormal yolk formation, heart oedema, and body bent. All abnormalities observed were enumerated and recorded as the abnormal count and the abnormality percent was calculated as follows:

$$\text{Abnormality \%} = \frac{\text{Total abnormality (0-72 HPF)}}{\text{Live count (day 01)}} \text{X } 100$$

### 2.3.5 Statistical Analysis

The percent reduction in spawn count, percent fecundity, percent mortality, and percent abnormality were calculated for group A, group B, group C, and group D, respectively, and plotted on a graph for comparison. One-way ANOVA was applied for the comparison of pre-exposure and post-exposure values at weekly intervals within each group with the help of the software sigma stat 4.0.

## 3. Result and discussion

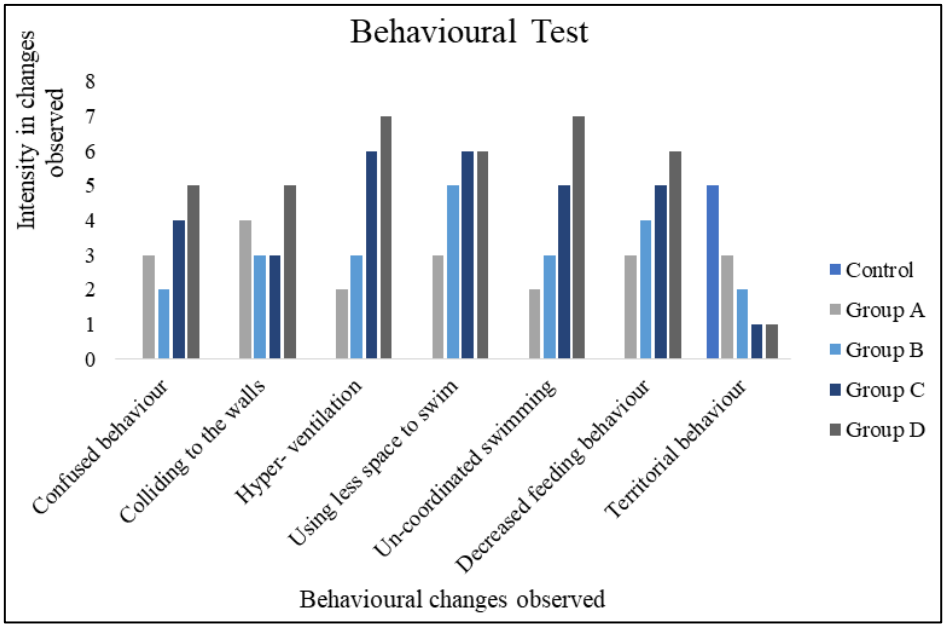
### 3.1 Behavioral test

Group A, B, C, and D treated with fluralaner showed significant changes in the behavior in the increasing concentration of the drug as compared to the control (Table 3.), (Figure 2).

**Table 3:** Behavioral changes observed after exposure of male zebrafish to fluralaner for 21 days

| | Control | Group A | Group B | Group C | Group D |
|---|---|---|---|---|---|
| Confused behavior | 0 | 3 | 2 | 4 | 5 |
| Colliding to the walls | 0 | 4 | 3 | 3 | 5 |
| Hyper- ventilation | 0 | 2 | 3 | 6 | 7 |
| Using less space to swim | 0 | 3 | 5 | 6 | 6 |
| Un-coordinated swimming | 0 | 2 | 3 | 5 | 7 |
| Decreased feeding behavior | 0 | 3 | 4 | 5 | 6 |
| Territorial behavior | 5 | 3 | 2 | 1 | 1 |

#Control-Unexposed males
#Group A-Fluralaner 5 mg/L
#Group B-Fluralaner 10 mg/L
#Group C-Fluralaner 20 mg/L# Group D-Fluralaner 100 mg/L




#Control-Unexposed males
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#Group B-Fluralaner 10 mg/L
#Group C-Fluralaner 20 mg/L
#Group D-Fluralaner 100 mg/L

**Fig 2:** Effect of fluralaner on the behavioral parameters

### 3.2 Assessment of Reproduction

The potential of fluralaner to affect the endocrine system and its effect on the male reproductive system was assessed by examination of embryos at 0 to 6, 24, 48, and 72 HPF using the following endpoints:

- Spawn count (total count of eggs).
- Fecundity percent.
- Mortality percent.
- Abnormal growth count.

### 3.3 Spawn Count

The enumeration of all the eggs collected in the embryo medium post-mating of adult zebrafish gave the total spawn count. Spawn count consisted of enumeration of both live and dead embryos in the zero to six hours post fertilization (HPF) time period. Percent reduction in spawn count over the exposure period was calculated and the results depicted significant ($p \leq 0.05$ and 0.001) reduction at all three recording periods in respect to their pre-exposure spawn count (Table 4 & 5), (Figure 3).

**Table 4:** Total spawn count in zebrafish after exposure of male zebrafish to fluralaner for 21 days

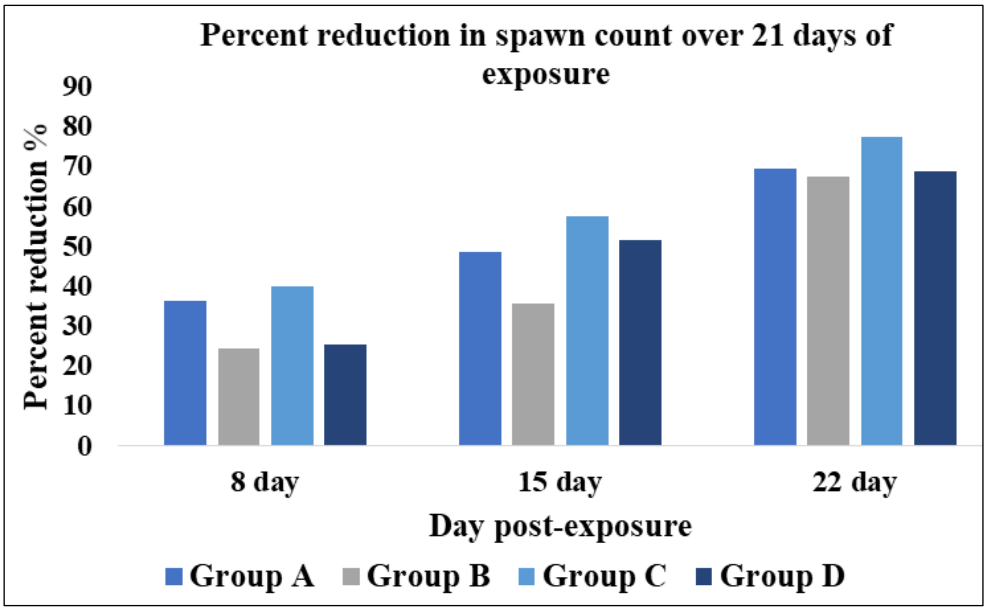
| | Group A | | | Group B | | | Group C | | | Group D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **A1** | **A2** | **Mean±S.E.** | **B1** | **B2** | **Mean±S.E.** | **C1** | **C2** | **Mean±S.E.** | **D1** | **D2** | **Mean±S.E.** |
| Pre-exposure | 1274 | 1255 | 1264.5±9.50* | 1317 | 1308 | 1312.5±4.50* | 1224 | 1247 | 1235.5±11.50* | 1284 | 1245 | 1264.5±11.50* |
| Post-exposure Day 8 | 828 | 817 | 822.5±5.50* | 915 | 934 | 924.5±9.50* | 852 | 848 | 850.0±2.00* | 803 | 812 | 807.5±4.50* |
| Post-exposure Day 15 | 627 | 651 | 639.0±12.00* | 723 | 733 | 728.0±50* | 672 | 665 | 668.5±3.50* | 608 | 612 | 610.0±2.00* |
| Post-exposure Day 22 | 398 | 381 | 389.5±8.50* | 387 | 394 | 390.5±3.50* | 378 | 380 | 379.0±1.00* | 304 | 324 | 314.0±10.00* |

#Control- Unexposed males
#Group A- Fluralaner 5 mg/L #Group B- Fluralaner 10 mg/L
#Group C- Fluralaner 20 mg/L#Group D- Fluralaner 100 mg/L

**Table 5:** Percent reduction in spawn count of exposed male zebrafish over the control values during the 21-day exposure period

| | Group A | | | Group B | | | Group C | | | Group D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **A1** | **A2** | **Mean±S.E.** | **B1** | **B2** | **Mean±S.E.** | **C1** | **C2** | **Mean±S.E.** | **D1** | **D2** | **Mean±S.E.** |
| **Post- exposure day 8** | 35.00 | 34.9 | 34.95±0.05* | 30.52 | 28.59 | 29.55±0.96* | 30.39 | 31.99 | 31.19±0.80* | 37.46 | 34.85 | 36.15±1.30* |
| **Post exposure Day 15** | 50.78 | 48.12 | 49.45±1.33* | 45.10 | 43.96 | 44.53±0.57* | 45.09 | 46.67 | 45.88±0.79* | 52.64 | 50.84 | 51.74±0.90* |
| **Post exposure day 22** | 68.75 | 69.64 | 69.19±0.44* | 70.61 | 69.87 | 70.24±0.37* | 69.11 | 69.52 | 69.31±0.20* | 76.32 | 73.97 | 75.14±1.17* |

Values are Mean ± SEM, One way ANOVA. Means with different alphabets differ significantly ($P \leq 0.05$); *$P < 0.05$ Vs Pre-exposure group SE- Standard Error of Mean. #Group A- Fluralaner 5 mg/L #Group B- Fluralaner 10 mg/L #Group C- Fluralaner 20 mg/L #Group D- Fluralaner 100 mg/L




#Control- Unexposed males
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#Group C- Fluralaner 20 mg/L
#Group D- Fluralaner 100 mg/L

**Fig 3:** Percent reduction in spawn count of exposed male zebrafish over the control values during the 21-day exposure period

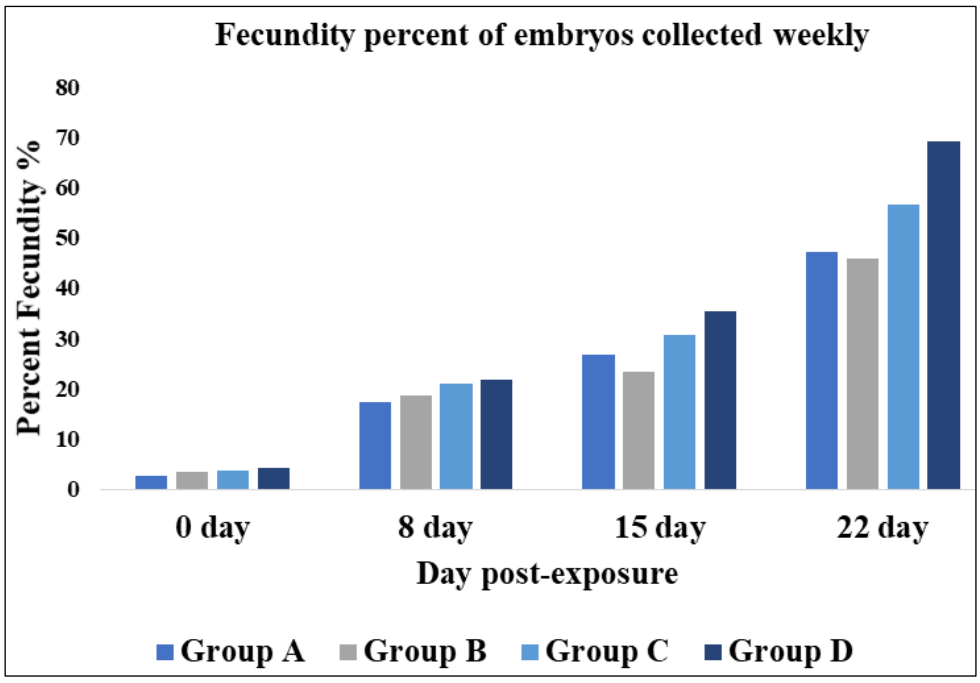
### 3.4 Fecundity percent (%)

The enumeration of all the live eggs collected in the embryo medium post mating of adult zebrafish gave the initial live count. Live count enumeration was carried out in the pre-exposure as well as the exposure groups of the concentrations 5 mg/L (Group A), 10 mg/L (Group B), 20 mg/L (Group C), 100 mg/L (Group D) at weekly intervals in 21 days. The fecundity percent was then calculated from the live count and spawn count obtained. Comparisons of the pre-exposure values to their exposed values indicated statistically significant ($p \leq 0.05$ and 0.001) reduction at all three recording periods (Table 6.), (Figure 4).

**Table 6:** Dead count and percent fecundity of embryos collected weekly

| | Group A | | Group B | | Group C | | Group D | |
|---|---|---|---|---|---|---|---|---|
| | **Pre-exposure observations** | | | | | | | |
| | **A1** | **A2** | **B1** | **B2** | **C1** | **C2** | **D1** | **D2** |
| Total spawn count | 1274 | 1255 | 1317 | 1208 | 1224 | 1247 | 1284 | 1245 |
| Dead count | 42 | 49 | 41 | 44 | 39 | 36 | 52 | 49 |
| Live count | 1232 | 1206 | 1276 | 1164 | 1185 | 1211 | 1232 | 1196 |
| % fecundity | 96.70 | 96.09 | 96.88 | 96.35 | 96.81 | 97.11 | 95.95 | 96.06 |
| Mean (±SE) | 96.40±0.30* | | 96.62±0.26* | | 96.96±0.15* | | 96.01±0.05* | |
| **Post-exposure observations** | | | | | | | | |
| **8 days post-exposure** | | | | | | | | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| Total spawn count | 828 | 817 | 915 | 934 | 852 | 848 | 803 | 811 |
| Dead count | 280 | 262 | 210 | 230 | 270 | 260 | 300 | 310 |
| Live count | 548 | 555 | 705 | 704 | 582 | 588 | 503 | 501 |
| % fecundity | 66.18 | 67.93 | 77.04 | 75.37 | 68.30 | 69.33 | 62.64 | 61.77 |
| Mean (±SE) | 67.05±0.87* | | 76.20±0.83* | | 68.81±0.51* | | 62.20±0.43* | |
| **15 days post-exposure** | | | | | | | | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| Total spawn count | 627 | 651 | 723 | 733 | 672 | 665 | 608 | 612 |
| Dead count | 270 | 240 | 250 | 260 | 240 | 270 | 290 | 320 |
| Live count | 357 | 411 | 473 | 473 | 432 | 395 | 318 | 292 |
| % fecundity | 56.93 | 63.13 | 65.42 | 64.52 | 64.28 | 59.39 | 52.30 | 47.71 |
| Mean (±SE) | 60.03±3.1* | | 64.97±0.45* | | 61.83±2.44* | | 50.00±2.29* | |
| **22 days post-exposure** | | | | | | | | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| Total spawn count | 398 | 381 | 387 | 394 | 378 | 380 | 304 | 324 |
| Dead count | 184 | 210 | 190 | 180 | 220 | 198 | 240 | 210 |
| Live count | 214 | 171 | 197 | 214 | 158 | 182 | 64 | 114 |
| % fecundity | 53.76 | 44.88 | 50.90 | 54.31 | 41.79 | 47.89 | 21.05 | 35.18 |
| Mean (±SE) | 49.32±4.44* | | 52.60±1.70* | | 44.84±3.05* | | 28.11±7.06* | |

Values are Mean ± SEM, One way ANOVA. Means with different alphabets differ significantly (P≤0.05); *P < 0.05 Vs Pre-exposure group SE- Standard Error of Mean. #Group A- Fluralaner 5 mg/L #Group B- Fluralaner 10 mg/L #Group C- Fluralaner 20 mg/L #Group D- Fluralaner 100 mg/L

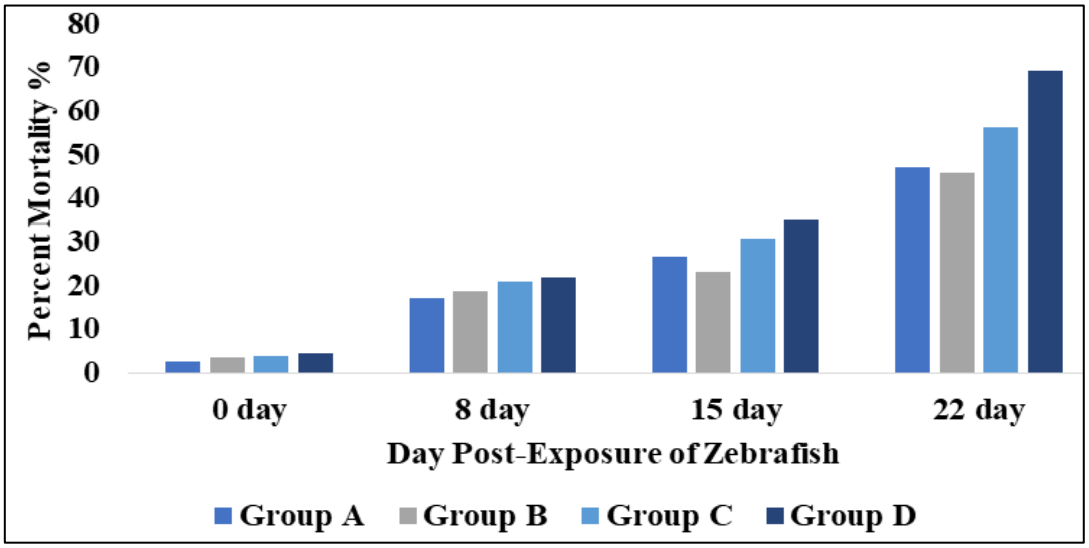



#Control-Unexposed males
#Group A-Fluralaner 5 mg/L
#Group B-Fluralaner 10 mg/L
#Group C-Fluralaner 20 mg/L
#Group D-Fluralaner 100 mg/L

**Fig 4:** Fecundity percent of embryos collected weekly over the period of 21 days of exposure of male zebrafish to test drugs

### 3.5 Mortality Percent (%)

The dead embryos observed during 0-72 HPF, were enumerated and recorded as dead count. The summation of dead embryos in the 72 HPF gave the total mortality and based on this, the mortality percent was calculated. Comparison of the pre-exposure values to their exposed values indicated significant ($p \leq 0.05$) reduction at all three recording periods (Table 7), (Figure 5).




#Control-Unexposed males
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#Group B-Fluralaner 10 mg/L
#Group C-Fluralaner 20 mg/L
#Group D-Fluralaner 100 mg/L

**Fig 5:** Percent Mortality in embryos recorded during post-spawning observation period of 0-72 HPF of embryos collected weekly

**Table 7:** Total and percent mortality recorded during post-spawning observation period of embryos collected weekly

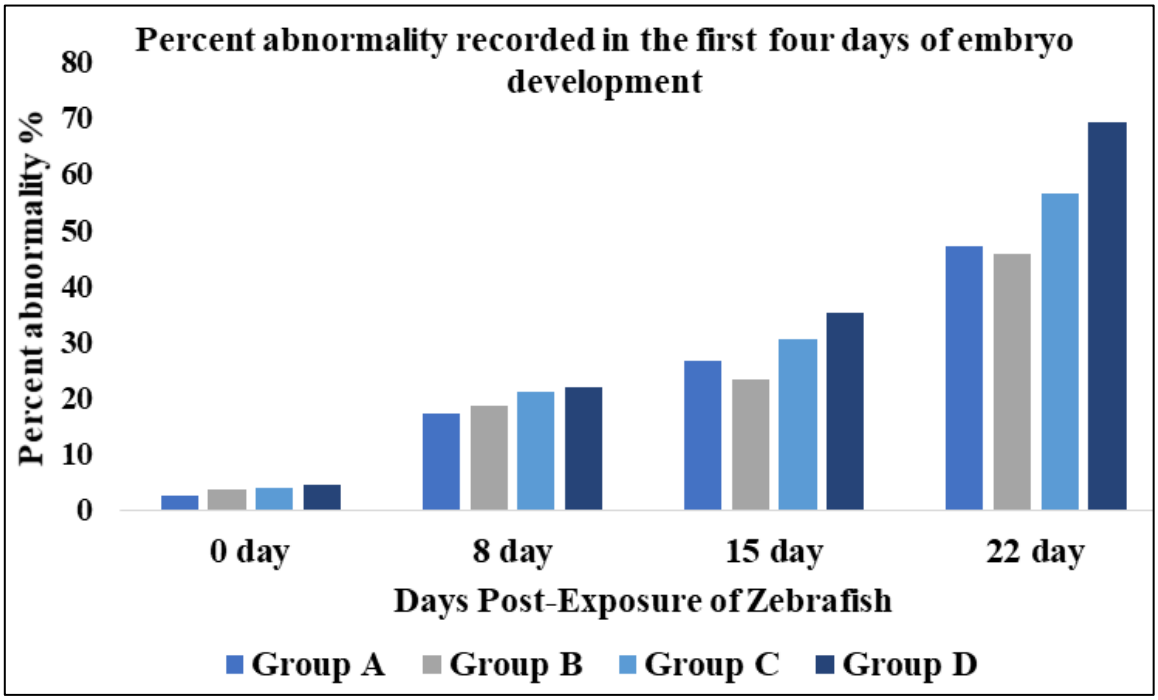
| | **Group A** | | **Group B** | | **Group C** | | **Group D** | |
|---|---|---|---|---|---|---|---|---|
| Control | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| 0-6 hours | 15 | 9 | 9 | 12 | 8 | 13 | 12 | 10 |
| 24 hours | 22 | 12 | 18 | 17 | 14 | 15 | 25 | 21 |
| 48 hours | 27 | 17 | 24 | 21 | 22 | 23 | 29 | 27 |
| 72 hours | 30 | 31 | 29 | 30 | 31 | 33 | 34 | 32 |
| Total | 94 | 69 | 80 | 80 | 75 | 84 | 100 | 90 |
| % Mortality | 7.37 | 5.49 | 6.07 | 6.11 | 6.12 | 6.73 | 7.78 | 7.22 |
| Mean (±SE) | 6.43±0.94* | | 6.09±0.02* | | 6.42±0.30* | | 7.5±0.28* | |
| | **8 days post-exposure** | | | | | | | |
| | **Group A** | | **Group B** | | **Group C** | | **Group D** | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| 0-6 hours | 22 | 35 | 24 | 28 | 67 | 72 | 67 | 71 |
| 24 hours | 34 | 48 | 38 | 32 | 89 | 85 | 75 | 78 |
| 48 hours | 52 | 69 | 42 | 38 | 96 | 92 | 87 | 86 |
| 72 hours | 68 | 78 | 48 | 43 | 102 | 98 | 98 | 107 |
| Total | 176 | 230 | 152 | 141 | 354 | 347 | 327 | 342 |
| % Mortality | 21.5 | 28.15 | 16.61 | 15.09 | 41.54 | 40.91 | 46.32 | 42.17 |
| Mean (±SE) | 24.82±3.32* | | 15.85±0.76* | | 41.22±0.31* | | 44.24±2.07* | |
| **15 days post-exposure** | | | | | | | | |
| | **Group A** | | **Group B** | | **Group C** | | **Group D** | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| 0-6 hours | 54 | 61 | 38 | 42 | 39 | 41 | 65 | 68 |
| 24 hours | 58 | 67 | 43 | 47 | 42 | 45 | 69 | 71 |
| 48 hours | 60 | 69 | 45 | 49 | 45 | 48 | 68 | 73 |
| 72 hours | 78 | 72 | 51 | 53 | 87 | 89 | 75 | 96 |
| Total | 250 | 269 | 177 | 191 | 213 | 223 | 277 | 308 |
| % Mortality | 39.87 | 41.32 | 24.48 | 26.05 | 31.69 | 33.53 | 45.55 | 50.32 |
| Mean (±SE) | 40.59±0.72* | | 25.26±0.78* | | 32.61±0.92* | | 47.39±2.38* | |
| **22 days post exposure** | | | | | | | | |
| | **Group A** | | **Group B** | | **Group C** | | **Group D** | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| 0-6 hours | 48 | 68 | 45 | 53 | 36 | 39 | 34 | 36 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 24 hours | 51 | 69 | 65 | 68 | 42 | 49 | 39 | 41 |
| 48 hours | 62 | 73 | 72 | 78 | 56 | 59 | 43 | 49 |
| 72 hours | 76 | 82 | 79 | 81 | 69 | 78 | 68 | 82 |
| Total | 237 | 292 | 261 | 280 | 203 | 225 | 184 | 208 |
| % Mortality | 59.64 | 76.64 | 67.44 | 71.06 | 53.70 | 59.21 | 60.52 | 64.19 |
| Mean (±SE) | 68.14±8.5* | | 69.25±1.81* | | 56.45±2.75* | | 62.35±1.83* | |

Values are Mean ± SEM, One way ANOVA. Means with different alphabets differ significantly (P≤0.05); *P < 0.05 Vs Pre-exposure group SE- Standard Error of Mean. #Group A- Fluralaner 5 mg/L #Group B- Fluralaner 10 mg/L #Group C- Fluralaner 20 mg/L #Group D- Fluralaner 100 mg/L

### 3.6 Abnormality percent

The enumeration of all abnormalities observed in the embryo and early-larval stage maintained in the embryo medium and observed for 72 HPF under the microscope which gave the abnormal count. The abnormalities observed in the embryo stage and early larval stage included abnormal yolk formation, heart edema, granule formation, and body bent defect in early larval stage, as shown in Fig. 8 which can be compared with normal embryo development (Fig.7). The percent abnormal count was calculated from the initial live count and abnormal count readings. Abnormality count and percent abnormality count enumeration was carried out in the control as well as the treatment groups of the concentration 5 mg/L (Group A), 10 mg/L (Group B), 20 mg/L (Group C), 100 mg/L (Group D), at weekly intervals in 21 days. The results for the same are presented in table 8 and data is graphically presented in figure 6.




#Control-Unexposed males
#Group A-Fluralaner 5 mg/L
#Group B-Fluralaner 10 mg/L
#Group C-Fluralaner 20 mg/L
#Group D-Fluralaner 100 mg/L

**Fig 6:** Weekly records of percent abnormality in embryos after exposing male zebrafish to Fluralaner

**Table 8:** Total and percent embryo defects recorded during post-spawning observation of embryos collected weekly from fluralaner exposed male zebrafish

| | **Pre-exposure** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Group A** | | **Group B** | | **Group C** | | **Group D** | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| Abnormal count | 31 | 36 | 46 | 51 | 43 | 51 | 52 | 58 |
| Percent abnormality | 2.43 | 2.86 | 3.87 | 3.28 | 3.51 | 4.08 | 4.04 | 4.65 |
| Mean (±S.E.) | 2.64±0.21* | | 3.57±0.29* | | 3.97±0.28* | | 4.34±0.30* | |
| **8 days post-exposure** | | | | | | | | |
| | **Group A** | | **Group B** | | **Group C** | | **Group D** | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| Abnormal count | 142 | 141 | 168 | 178 | 176 | 181 | 169 | 183 |
| Percent abnormality | 17.14 | 17.25 | 18.36 | 19.05 | 20.65 | 21.34 | 21.04 | 22.56 |
| Mean (±S.E.) | 17.19±0.05* | | 18.70±0.34* | | 20.99±0.34* | | 21.8±0.76* | |

| 15 days post-exposure | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Group A | | Group B | | Group C | | Group D | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| Abnormal count | 180 | 160 | 168 | 170 | 201 | 208 | 220 | 210 |
| Percent abnormality | 28.70 | 24.57 | 23.23 | 23.19 | 29.91 | 31.27 | 36.18 | 34.31 |
| Mean (±S.E.) | 26.63±2.06* | | 23.21±0.02* | | 30.59±0.68* | | 35.24±0.93* | |
| **22 days post-exposure** | | | | | | | | |
| | Group A | | Group B | | Group C | | Group D | |
| | A1 | A2 | B1 | B2 | C1 | C2 | D1 | D2 |
| Abnormal count | 189 | 178 | 170 | 188 | 208 | 220 | 205 | 230 |
| Percent abnormality | 47.48 | 46.71 | 43.92 | 47.71 | 55.02 | 57.89 | 67.43 | 70.98 |
| Mean (±S.E.) | 47.09±0.38* | | 45.81±1.89* | | 56.45±1.43* | | 69.20±1.77* | |

Values are Mean ± SEM, One way ANOVA. Means with different alphabets differ significantly (P≤0.05); *P < 0.05 Vs Pre-exposure group SE- Standard Error of Mean. #Group A- Fluralaner 5 mg/L #Group B- Fluralaner 10 mg/L #Group C- Fluralaner 20 mg/L #Group D- Fluralaner 100 mg/L

Normal development

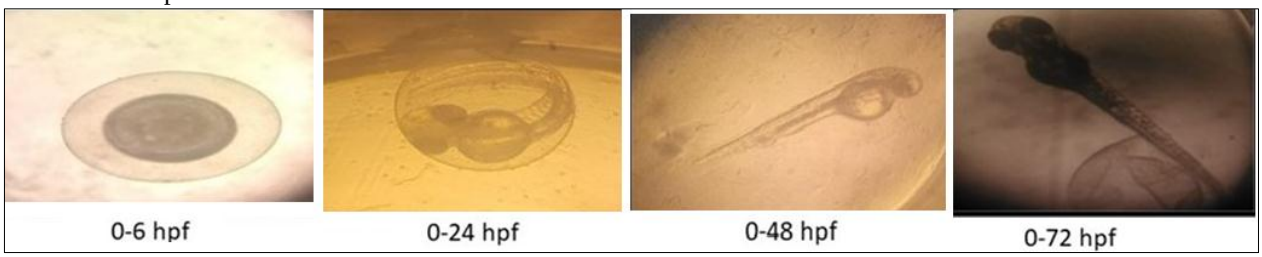



**Fig 7:** Embryo Normal Development

Abnormal embryos

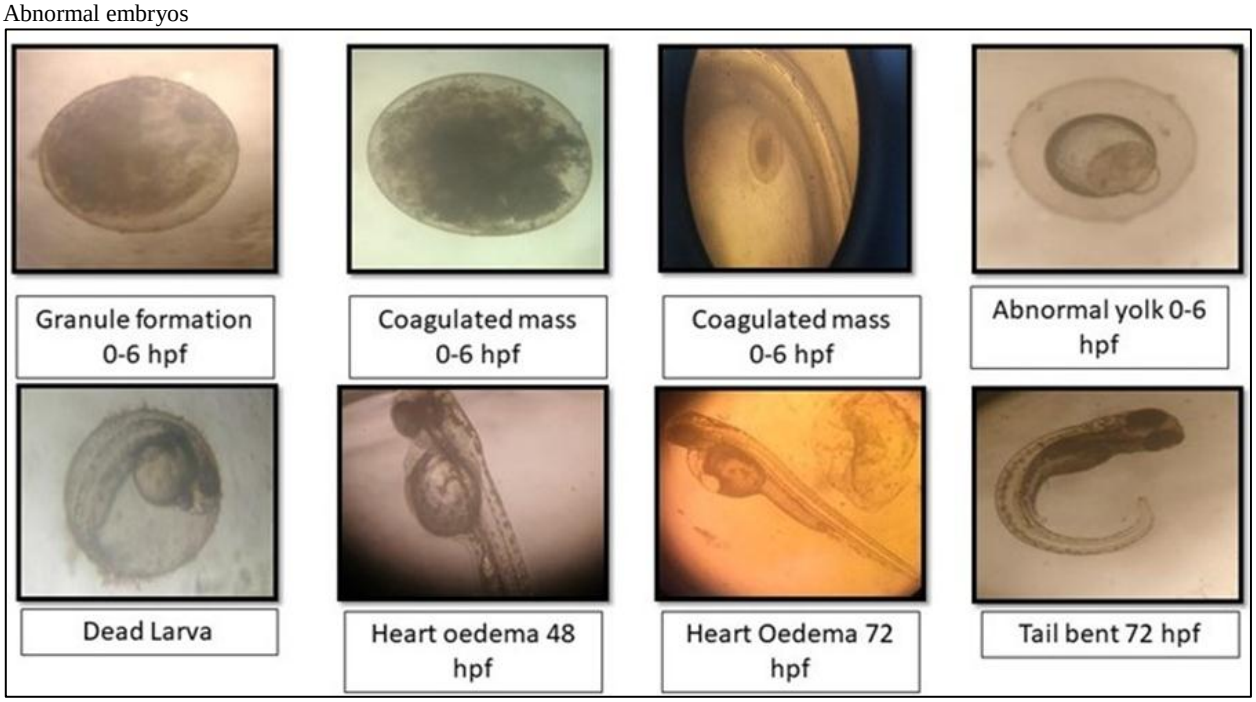



**Fig 8:** Abnormal embryos

### 3.7 Assessment of Vitellogenin (VTG) levels by ELISA

The quantitative assessment of fish vitellogenin in whole body homogenates of zebrafish using fish vitellogenin direct non-competitive sandwich ELISA kit gave an account for the ability of the tested drug to stimulate production of female egg yolk protein VTG in the exposed male fish. Estimation of vitellogenin was done in both control group (male and female) and the treatment groups of the concentrations 5 mg/L (group A), 10 mg/L (group B), 20 mg/L (group C) and 100 mg/L (group D), respectively. Percent increase in vitellogenin level in exposure groups was calculated and the results for the same are depicted in table 9 and 10, the graphical presentation for standard dilutions is depicted in figure 9, and the graphical presentation for an increase in vitellogenin concentrations in different exposure groups is depicted in figure 10.

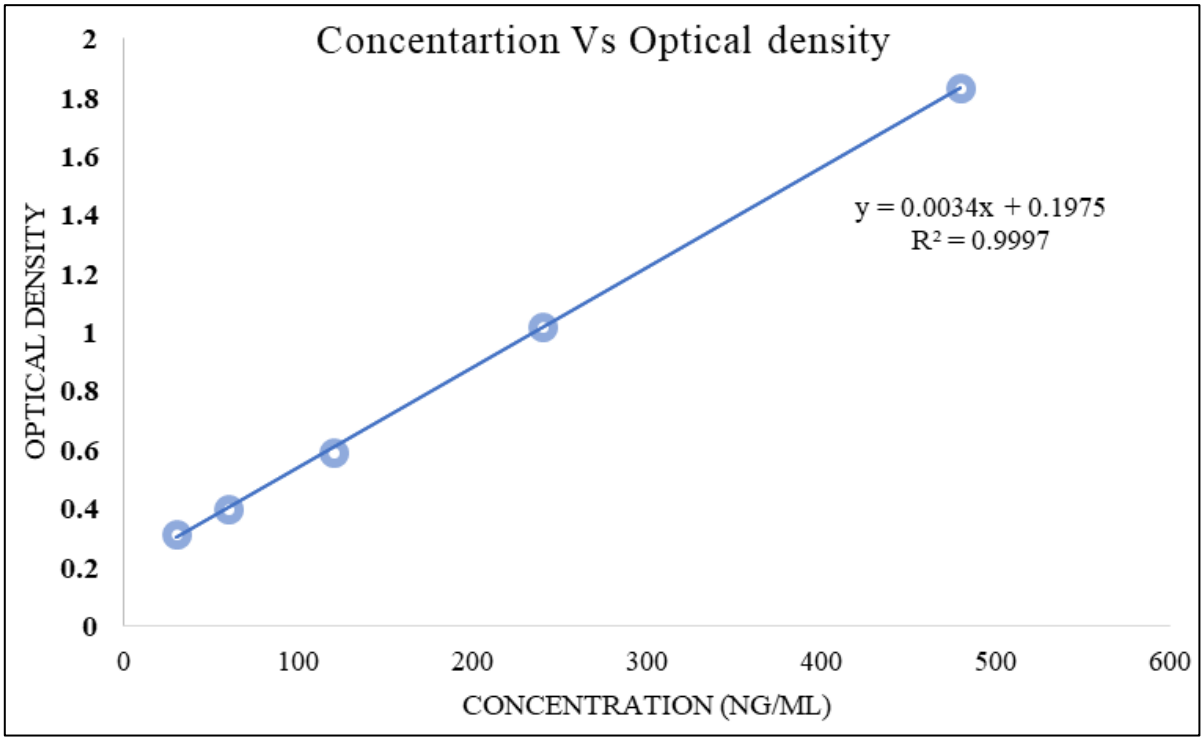



**Fig 9:** Standard curve of standard dilutions in vitellogenin ELISA

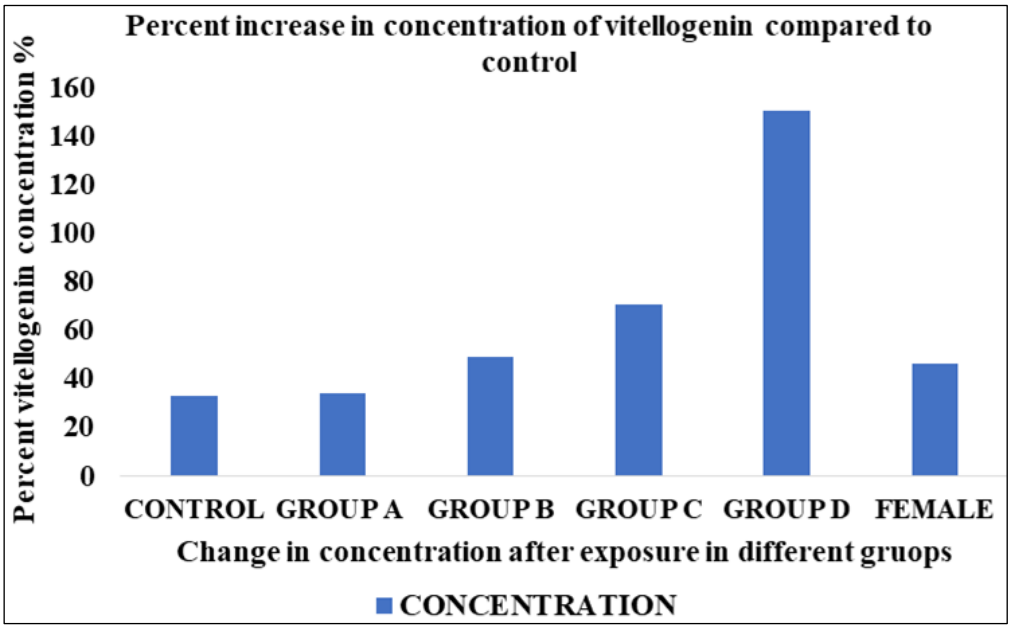
**Table 9:** Absorbance and concentration of vitellogenin in the control group and the treatment group respectively

| Sample No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Mean ± S.E. |
|---|---|---|---|---|---|---|---|---|---|
| Control (OD) | 0.28 | 0.29 | 0.34 | 0.35 | 0.27 | 0.28 | 0.38 | 0.29 | 0.31±0.01* |
| Conc. | 24.26 | 27.20 | 41.91 | 44.85 | 21.32 | 24.26 | 53.67 | 27.20 | 33.08±4.23* |
| Group A1 (OD) | 0.31 | 0.28 | 0.31 | 0.32 | 0.28 | 0.31 | 0.40 | 0.32 | 0.31±0.013* |
| Conc. | 33.08 | 24.26 | 33.08 | 36.02 | 24.26 | 33.08 | 59.55 | 36.02 | 34.9±3.88* |
| Group A2 (OD) | 0.32 | 0.30 | 0.33 | 0.34 | 0.26 | 0.32 | 0.35 | 0.28 | 0.31±0.01* |
| Conc. | 36.02 | 30.14 | 38.97 | 41.91 | 18.38 | 36.02 | 44.85 | 24.26 | 33.81±3.18* |
| Group B1 (OD) | 0.35 | 0.36 | 0.35 | 0.38 | 0.40 | 0.37 | 0.41 | 0.35 | 0.37±0.00* |
| Conc. | 44.85 | 47.79 | 44.85 | 53.67 | 59.55 | 50.73 | 62.5 | 44.85 | 51.09±2.45* |
| Group B2 (OD) | 0.38 | 0.35 | 0.36 | 0.36 | 0.37 | 0.36 | 0.35 | 0.34 | 0.35±0.00* |
| Conc. | 53.67 | 44.85 | 47.79 | 47.79 | 50.73 | 47.79 | 44.85 | 41.91 | 47.42±1.29* |
| Group C1 (OD) | 0.41 | 0.45 | 0.42 | 0.43 | 0.39 | 0.48 | 0.42 | 0.46 | 0.43±0.01* |
| Conc. | 62.5 | 74.26 | 65.44 | 68.38 | 56.61 | 83.08 | 65.44 | 77.20 | 69.11±3.03* |
| Group C2 (OD) | 0.43 | 0.48 | 0.41 | 0.45 | 0.39 | 0.46 | 0.45 | 0.48 | 0.44±0.01* |
| Conc. | 68.38 | 83.08 | 62.5 | 74.26 | 56.61 | 77.20 | 74.26 | 83.08 | 72.42±3.33* |
| Group D1 (OD) | 0.65 | 0.72 | 0.71 | 0.68 | 0.74 | 0.70 | 0.75 | 0.70 | 0.70±0.01* |
| Conc. | 133.0 | 153.6 | 150.7 | 141.9 | 159.5 | 147.7 | 162.5 | 147.7 | 149.5±3.33* |
| Group D2 (OD) | 0.71 | 0.69 | 0.72 | 0.75 | 0.71 | 0.68 | 0.72 | 0.73 | 0.71±0.00* |
| Conc. | 150.7 | 144.8 | 153.6 | 162.5 | 150.7 | 141.9 | 153.6 | 156.6 | 151.8±2.2* |
| Female (OD) | 0.32 | 0.36 | 0.41 | 0.38 | 0.35 | 0.36 | 0.34 | 0.32 | 0.35±0.01* |
| Conc. | 36.02 | 47.79 | 62.5 | 53.67 | 44.85 | 47.79 | 41.91 | 36.02 | 46.31±3.14* |

*OD = optical density, conc. = Concentration Values are Mean ± SEM, One way ANOVA. Means with different alphabets differ significantly (P≤0.05); *P < 0.05 Vs Pre-exposure group SE- Standard Error of Mean. #Group A- Fluralaner 5 mg/L #Group B- Fluralaner 10 mg/L #Group C- Fluralaner 20 mg/L #Group D- Fluralaner 100 mg/L

**Table 10:** Concentration of vitellogenin in the control group and the treatment group respectively

| Group | Mean concentration of vitellogenin | Mean ± S.E. |
|---|---|---|
| Control | 33.08 | 33.08±4.24* |
| Group A1 | 34.91 | 34.36±0.55* |
| Group A2 | 33.81 | |
| Group B1 | 51.09 | 49.26±1.84* |
| Group B2 | 47.42 | |
| Group C1 | 69.11 | 70.77±1.66* |
| Group C2 | 72.42 | |
| Group D1 | 149.57 | 150.68±1.12* |
| Group D2 | 151.8 | |
| Female | 46.31 | 46.31±3.14* |




#Control-Unexposed males
#Group A-Fluralaner 5 mg/L
#Group B-Fluralaner 10 mg/L
#Group C-Fluralaner 20 mg/L
#Group D-Fluralaner 100 mg/L
#Control-Unexposed males
#Group A-Fluralaner 5 mg/L
#Group B-Fluralaner 10 mg/L
#Group C-Fluralaner 20 mg/L
#Group D- Fluralaner 100 mg/L

**Fig 10:** Percent increase in vitellogenin levels in exposed male zebrafish over the control values after the 21 days exposure period

## 4. Conclusion

The emergence of new pharmaceutical compounds and their possible hazardous effects on the environment has been raised around the world. Among these compounds, fluralaner is a newly introduced insecticide which highly effective against ticks and fleas on dogs and cats (Jia, *et al*., 2018 [6], Casida, *et al*., 2015 [2], Ozoe, *et al*., 2010 [10], Zhao, *et al*., 2015). The unchanged fluralaner was found in feces which can be introduced into the environment through runoff water and also it could be rapidly accumulated in the water bodies. Among different targets of this agent, the endocrine system is an important target we have considered in this investigation to evaluate fluralaner for endocrine disrupting potential and its effect on reproduction in male zebrafish.

In the present study, exposure to different concentrations of fluralaner caused a significant reduction in the spawn count as compared to the pre-exposure count, which indicates that exposure to fluralaner had affected male reproduction in male zebrafish and as a result of that eggs spawned by the female has reduced, due to possible sperm toxicity.

In the present study, it was observed that there was a significant reduction in fecundity in all the exposed groups at all three recording periods as compared to its pre-exposure values. Reduced fecundity may be the outcome of increased oxidative stress, and hampered communication behavior.

A significant increase in the mortality count at all three recording periods in comparison to pre-exposure values indicated the effect of sperm released by exposed male zebrafish on the livability of the embryo. Accordingly, the results got in the present investigation are as per the discoveries of Kopeika *et al*., where a consistent rise in the mortality percent of embryos in their initial developmental stage was identified which is demonstrative of the chance of adverse effect on sperm quality.

In the present study, it was observed that there was a significant rise in the percent abnormalities in the embryo. Since embryo movement is essential for breaking the chorion and later shell for hatching, hence delayed and reduced hatching with inhibition of the embryo's movement in the eggs can be linked to the lethargic effects of drugs.

In conclusion, the present study suggests that fluralaner exposure at 5 mg/L, 10mg/L, 20mg/L, and 100mg/L in water to zebrafish, significantly affects behavior and the male reproductive system in zebrafish. As vitellogenin level, a biomarker for endocrine disruptions was also increased in male zebrafish with an increase in the dose of a test compound.

The present study is an introduction to the importance of Environment Risk Assessment (ERA) in relation to the entry of insecticide fluralaner into the environment and their effects on the endocrine system of non-target organisms including humans. It was observed that exposure to these concentrations can be dangerous to the development of the offspring and this is an exemplar of what can be anticipated in the coming years if relevant measures are not undertaken to check the entry of chemicals and their accumulation in the environment. Future investigation may be planned by conducting histopathology of male zebrafish, proteomic analysis, sperm examination, and hormonal assays of zebrafish. The screening of potential EDCs is fundamental for understanding the ecological and eco-toxicological consequences for the non-target organisms and the eventual fate of animals as well as mankind.

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