



## Endocrine Disrupting Effect of Fenvalerate 20% EC and Mancozeb 80 % WP in adult Zebra fish (*Danio rerio*) using Vitellogenin as a Biomarker

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### ABSTRACT

An attempt was made to evaluate endocrine disrupting effect of pyrethroid (Fenvalerate 20% EC) and fungicide (Mancozeb 80 % WP) on adult Zebra fish (*Danio rerio*). The study was conducted as per OECD 229 guideline. Fish were exposed to Fenvalerate 20% EC (1, 3 and 10 µg/L) and Mancozeb 80 % WP (0.08, 0.2 and 0.8 mg/L) along with control and positive control (Prochloraz at 300 µg/L) for 21 days. Daily egg observation was done at all concentrations including control and positive control. At the end of 21 days, fish were euthanized by MS 222 and head and tail portions were collected from all fish. The tissue samples were homogenized and samples were analyzed for vitellogenin content using a Zebra fish vitellogenin ELISA kit. Spawning efficiency was affected in the treated groups. The average spawns were 12.74, 2.62 and 1.87 in fish treated with Fenvalerate and 15.82, 10.81 and 5.05 in Mancozeb-treated groups. In Prochloraz-treated group, the average spawn was 2.35. The control recorded an average spawn of 15.94. Significant differences ( $p < 0.05$ ) in vitellogenin were observed in male zebra fish exposed to Mancozeb 80% WP at the highest concentration tested (0.8 mg/L). In Fenvalerate 20% EC- treated groups, there was a statistically significant ( $p < 0.05$ ) difference (decrease in VTG concentration in females) was observed at all the concentrations tested (1, 3 and 10 µg/L). Hence it is pertinent to say that, both the chemicals have the potential to cause endocrine disruption, as evidenced from the vitellogenin analysis. Although a decrease in vitellogenin may not be originating solely from endocrine disruption, any positive result in vitellogenin assay indicates an evidence of *in vivo* endocrine activity and generally requires further clarification.

**Keywords:** *Danio rerio*, endocrine disruption, vitellogenin, ELISA.

### INTRODUCTION

The World Health Organization (WHO) defines an Endocrine Disruptor Chemical (EDC) as any exogenous substance that alters the endocrine function leading to health effects in an intact organism or its progeny. From a regulatory perspective, it is stated that EDCs are agents that alter the reproduction / development by directly affecting the hypothalamic-pituitary-thyroidal or hypothalamic-pituitary-gonadal (HPG) axes [1]. Many chemical classes covering natural and synthetic hormones, plant contents, plant protection chemicals, plasticizers, consumer products, industrial effluents and other pollutants [2]. These chemicals have the capability to initiate and aggravate diseases in all vertebrates including humans. This is explained by the fact that the differences in the basic chemical structures of hormones of various species is subtle, albeit the difference in functions [3].

Endocrine disruption has been investigated in fish worldwide. Recently, it has been shown that EDCs cause adverse health effects including developmental, neurological, endocrine [4] and reproductive alterations in different groups of vertebrates [5, 6]. The World Wildlife Fund has underlined the fact that many industrial and agricultural chemicals have the ability to disrupt the endocrine system and its hormones of many animals including fish. These chemicals are expected to affect the larval / developmental stages of fish, thereby interfering with sexual development, fertility and behavior (World Wildlife Fund). Fish are exposed to a variety of chemicals from various sources that can disrupt their endocrine system. Chemicals from industrial effluents, pesticides in agricultural run-offs, sewage effluents are all expected to be EDCs. In the natural environment, fish produce numerous eggs and sperms, giving an advantage to study the EDCs. In addition, the effect of potential EDCs can be studied in both male and female sexes.

For studying EDCs many markers are used in general practice – measurement of Steroid hormones, Organosomatic indices, hepatosomatic index and gonadosomatic index. Some EDCs alter the serum steroid levels, cause feminization of males, cause depression of gonads in various fish species. Recently, the Vitellogenin (vtg), a characteristic protein in females, associated with egg production is used as a biomarker for monitoring xenobiotics that mimic estrogen i.e., EDCs. Numerous studies have employed vtg to demonstrate the presence of estrogenic chemicals in the aquatic system [7]. The international scientific and regulatory community has considered the establishment of robust ecotoxicity test methods as a subject of high priority. New guideline [8] incorporating priority endpoints such as vitellogenin and gonad histology are now in place which can be useful for environmental monitoring was followed in the current study.

Fish are very closely associated with the environment they live in and act as useful model animals for assessing early damages caused by environmental pollutants. The endocrine system of fish has sharp differences in structure and hormones when compared to mammals, yet the reproductive endocrine system is very similar in fish and mammals. Thus, those chemicals that have an effect on the endocrine system of fish can be considered as potential disruptors in mammals, including humans [9]. Zebra fish (*Danio rerio*) is a small tropical fish that is found in India and various parts of South Asia and is considered as a very popular model organism in the field of developmental genetics and ecotoxicology. The advantages of this organism are its rapid development, the ease of maintenance and the enormity of off springs that it produces. The richness of data available on the genetic characterization of this fish makes it an ideal model for endocrine disruption studies and hence was employed in the current study.

The current investigation was undertaken to understand the endocrine disrupting potential of formulations of Fenvalerate (Fenvalerate 20% EC), Mancozeb (Mancozeb 80 % WP) at sub-lethal doses, by measuring the Vitellogenin content in the head and tail portions of the exposed fish. These two chemicals have been listed as suspected EDCs by the EPA and hence investigated.

## **MATERIALS AND METHODS**

### ***Test chemicals***

The test items (Fenvalerate 20% EC and Mancozeb 80% WP) were procured commercially and stored as per the directions of the manufacturer.

### ***Experimental Animals***

*Danio rerio* (Zebra fish) was obtained from commercial fish farm in Padappai, India. The weights of the fish were  $0.65 \pm 20\%$  for females and  $0.4 \pm 20\%$  for males. Fish were acclimatized to the laboratory conditions for 10 days and Fish were fed *ad libitum* throughout the holding period and during the exposure phase. The exposure phase is started with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals and actively spawning. The age of zebra fish was in the range of 16 ( $\pm 2$ ) weeks.

### ***Adult Zebra Fish Reproduction Test - 21 days***

The 21 day short term reproduction study to the fresh water fish, *Danio rerio* was conducted as per OECD 229 testing guidelines with some modifications (A static renewal system was used in place of a continuous flow-through system) (OECD, 2009). The test room was maintained with 12 hours light and 12 hours darkness controlled by an automatic timer and test temperature was maintained between 24 - 28°C. Glass aquaria (25 L capacity) were used as test Chamber. Exposure medium (blended water) was well water and reverse osmosis water in the mixture of 1:1 ratio.

The fish were placed in spawning cages within each aquarium and each replicate consisted of ten fish (five males and five females). The duration of the exposure of the fish to the test chemical was 21 days. The endpoints monitored on a daily basis were spawning and fecundity. During the test, physico-chemical parameters such as dissolved oxygen, temperature, and pH were measured in all test vessels once per week. Total hardness was measured in the controls and one vessel at the highest concentration once per week.

### ***Pilot Study***

A pilot study was conducted to determine the concentrations for the 21 day reproduction assay. Based on the pilot study, the LC<sub>50</sub> was determined as 0.054 mg/L for Fenvalerate 20% EC and 3.90 mg/L for Mancozeb 80 % WP. Based on these LC<sub>50</sub> values, three concentrations *viz.*, 1, 3 and 10 µg/L were fixed for Fenvalerate 20% EC and concentrations of 0.08, 0.2 and 0.8 mg/L were fixed for

Mancozeb 80 % WP. The highest concentration was the one fifth value of the corresponding LC<sub>50</sub>. The low and intermediate concentrations were fixed using geometric factor 3.2.

Three concentrations for test items of Fenvalerate 20% EC (1, 3 and 10 µg/L) and Mancozeb 80 % WP (0.08, 0.2 and 0.8 mg/L) with one control (blended water) along with positive control (Prochloraz at 300 µg/L) were used for the study. The study was conducted for 21 days. In this study, male and female zebra fish in a reproductive status were exposed together in test vessels with 5 male and 5 female for each test concentration.

#### **Preparation of Test Concentrations**

Test solutions of the chosen concentrations are prepared by dilution of a stock solution for each test item and also for positive control (Prochloraz). The exposure medium was renewed daily until 21 days of study period (Static renewal).

Egg production were recorded as the number of eggs/surviving female/day on a replicate basis. Eggs were removed daily from the test chambers. At day 21, i.e. at termination of the exposure, the fish were euthanized with appropriate amounts of MS-222. Tissue (head/tail) is then sampled for vitellogenin determination, as explained in the vitellogenin section.

#### **Vitellogenin Analysis**

At the end of the 21-day exposure period, the heads and tails of all the fish were cut, weighed and placed in 1.5 ml eppendorf tubes for determination of vitellogenin content. The samples were homogenized individually in buffer (Tris-HCl, pH 7.4 + 1% Protease inhibitor cocktail, Sigma) using a pistol. The homogenate was centrifuged at 13000 g at 4 °C for 30 minutes and the supernatant below the fat layer was collected to determine the Vtg concentration of each fish. Vitellogenin was measured using a commercially available pre-coated Vtg ELISA kit (Biosense Laboratories ®, Norway). Purified Vtg from zebra fish was used as a standard. The procedure was conducted according to the manufacturer's instructions. The absorbance was measured using a micro titer plate reader (Stat Fax - 2100) and the concentration of Vtg in each fish was calculated.

#### **Statistics**

The VTG data were analyzed by Modified Leven's test for homogeneity and subjected to one-way analysis of variance (ANOVA) using NCSS software (NCSS, version 2007; Number Cruncher Statistical Systems, Kaysville, Utah). A comparison of treated with control groups were done using Dunnett's test (parametric) on multiple pair-wise comparisons for post hoc comparison. If data was heterogeneous it was subjected to non-parametric test Mann-Whitney with Bonferroni adjustment (non-parametric) test. The alpha level at which all tests were conducted is 0.05.

## **RESULTS AND DISCUSSION**

### **Toxicity / Mortality**

No mortality / any other toxicity signs were recorded in any of the treated groups.

### **Physico-chemical parameters**

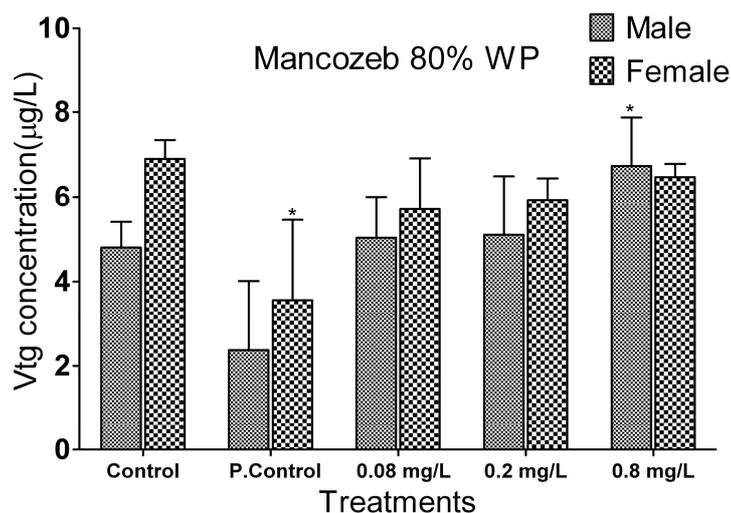
During the test, the physico-chemical parameters such as Dissolved oxygen, Temperature, pH and Total hardness were found to be within the ranges in control, positive control and test item concentrations as per OECD 229 (Table 1).

### **Spawning**

The spawning ability was calculated as the mean of the number of eggs hatched per day/female. According to OECD 229, average spawns of >10 eggs/female/day for each species is considered as normal. In the present study, it was observed that, in the treated groups the average spawns were 12.74, 2.62 and 1.87 in fish treated with Fenvalerate and 15.82, 10.81 and 5.05 in Mancozeb-treated groups. The control recorded an average spawn of 15.94. In Prochloraz-treated group, the average spawn was 2.35.

### **Vitellogenin analysis**

Prochloraz, the positive chemical used, induced a sharp decrease in VTG concentration in females that was statistically significant. Significant differences ( $p < 0.05$ ) in vitellogenin were observed in male zebra fish exposed to Mancozeb 80% WP (increased VTG concentration in males) at the highest concentration tested (0.8 mg/L) (Figure 1). No effect was observed in the females.



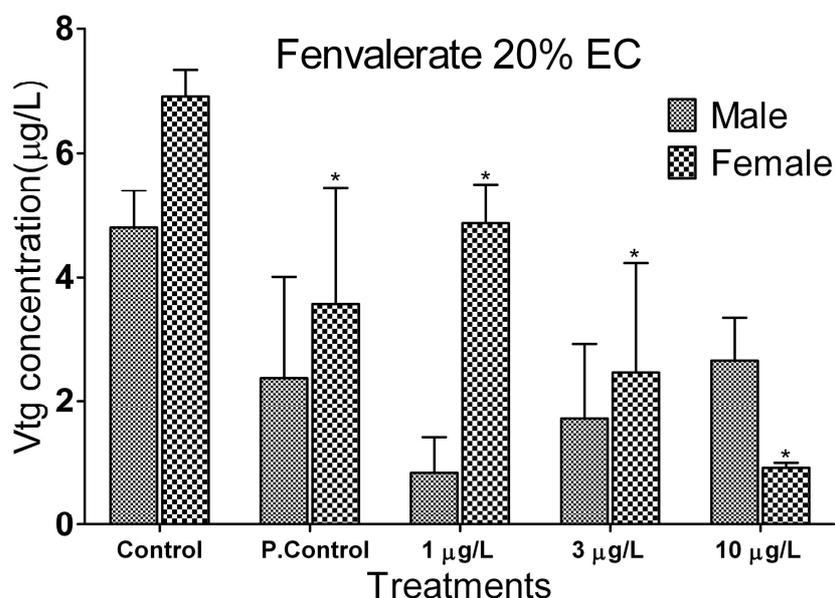
**Figure 1** indicates the effect of Mancozeb 80% WP in male and female zebra fish. Significant differences ( $p < 0.05$ ) in vitellogenin were observed in male zebra fish exposed to Mancozeb 80% WP (increased VTG concentration in males) at the highest concentration tested (0.8 mg/L). No effect was observed in the females.

Control - Represents Water control

P. Control - Represents Positive Control - Prochloraz at 300 µg/L

\* - Statistically significant values ( $p < 0.05$ )

In Fenvalerate 20% EC- treated groups, there was a statistically significant ( $p < 0.05$ ) difference (decrease in VTG concentration in females) was observed at all the concentrations tested (1, 3 and 10 µg/L) in females (**Figure 2**) and there was apparently no effect on males. Hence it is pertinent to say that, both the chemicals have the potential to cause endocrine disruption, as evidenced from the vitellogenin analysis.



**Figure 2** indicates the effect of Fenvalerate 20% EC in male and female zebra fish. There was a statistically significant ( $p < 0.05$ ) difference (decrease in VTG concentration in females) observed at all the concentrations tested (1, 3 and 10 µg/L) in females and there was apparently no effect on males.

Control - Represents Water control

P. Control - Represents Positive Control - Prochloraz at 300 µg/L

\* - Statistically significant values ( $p < 0.05$ )

**Table 1:** denotes the physico chemical parameters of the test solutions – Prochloraz, Fenvalerate 20 %EC and Mancozeb 80% WP

Parameters	Control (Blended water)	Positive control (Prochloraz)	Fenvalerate 20 %EC	Mancozeb 80% WP
Total hardness as CaCO <sub>3</sub> (mg/L)	208 - 216	198 - 207	210 - 218	202 - 216
Dissolved oxygen (%)	85.9 - 93.5	83.6 - 95.5	88.2 - 91.6	81.8 - 90.3
Temperature (°C)	24.2 - 25.7	24.5 - 25.3	24.7 - 25.2	24.1 - 25.0
pH	7.75 - 8.03	7.69 - 7.93	7.72 - 8.02	7.65 - 7.89

Endocrine disruption is a phenomenon that has been explored recently and hence there is a dearth in understanding of the risk that it imposes on human health and other organisms. Many pesticides are suspected to be causing endocrine effects at doses much lower than that is expected from conventional toxicological investigations and that are not covered by the current regulations of risk assessment. Exposure to pesticides and endocrine disruption has been studied in men involved in aerial application of DDT [10]. Recently, such exposures to pesticides has been implicated in various cancers [11, 12], miscarriage and many other reproductive disorders [12, 13], deformed genitals [14], birth defects [15], behavioural abnormalities [16] and distorted offspring sex ratios [12, 17]. Vitellogenesis is the process by which the estradiol, which is produced by the ovary, stimulates the liver to produce vtg, which is then incorporated into the yolk of the developing oocytes. Xenobiotics that mimic estrogen also tend to act on the receptors of the liver to induce the synthesis of vtg. Although both male and female fish have estrogen receptors, the livers of female fish are normally exposed to estrogens [18].

According to OECD 229, vitellogenin measurements are considered positive if there is a statistically significant increase in VTG in males ( $p < 0.05$ ), or a statistically significant decrease in females ( $p < 0.05$ ) at least at the highest dose tested compared to the control group, and in the absence of signs of general toxicity. From the results, it is pertinent to say that, both the chemicals – Fenvalerate 20% EC and Mancozeb 80% WP - have the potential to cause endocrine disruption, as evidenced from the vitellogenin analysis. Although a decrease in vitellogenin may not be originating solely from endocrine disruption, any positive result in vitellogenin assay indicates an evidence of in vivo endocrine activity and generally requires further clarification. Fenvalerate 20% EC is an Insecticide and acaricide that inhibits the proliferation of oestrogen-sensitive cells, antagonizes the action of progesterone [19, 20], which can be clearly observed from the results, all the concentrations of this chemical caused a statistically significant reduction in the vitellogenin content of female fish and Mancozeb 80% WP is a fungicide that inhibits the production of thyroid hormones [21], but the action of this chemical is not clear, however, owing to the effect that it has produced in the highest concentration in male fish, its potential to be an endocrine disruptor cannot be ignored. Further investigations into the detailed mechanisms of actions of these chemicals are suggested.

The endocrine system is a powerful chemical communication system that regulates homeostasis, stress, reproduction and fertility. The EDCs have received much attention owing to the fact that this system is extremely sensitive to pollutants [22]. It is a known fact that xenobiotics such as pesticides are released to the environment chiefly by anthropogenic activity. Endocrine disruption in fish, subsequent to pesticide exposure, is of prime importance since it can result in reproductive abnormalities. The structural similarities between the hormones and hormone receptors of different species, despite the taxonomical distance, are valuable indicators of human health risks. For example, Prolactin, that is responsible for osmoregulation in fish [23, 24], promotes mammary gland development and milk production in mammals [25].

From the point of pathology, the understanding goes that, in both fish and mammals, EDCs act in a similar fashion. However, the anatomical, physiological and pathological pathways may differ. This supports fish to be used as an alternative test species to study EDCs, suitable for hazard

identification. Exposure to carbamate insecticides has been related to increased mortality in salmon fish, reducing their survival when transiting from fresh to salt water [26 - 30].

Hence, from the results of the current investigation, it can be concluded that both Fenvelarete 20% EC and Mancozeb 80% WP could be potential endocrine disrupting chemicals. The exact mode of action needs to be delineated by further investigations. The results obtained can be considered as a preliminary hint about these chemicals and encourages in-depth analysis of the mechanisms involved therein.

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