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Original Article

# Modulation of the reproductive hormone aromatase and vitellogenin in fumaronitrile-exposed *Oreochromis mossambicus*

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## ABSTRACT

Environmental chemical fumaronitrile that disrupts endocrine function has been linked to adverse effects, particularly on the reproductive system in *Oreochromis mossambicus*. Although there are several mechanisms through which fumaronitrile might alter the endocrine system, chemicals that mimic steroid hormones through an interaction with the estrogen receptor continues to receive considerable attention. The sex steroid hormones in *Oreochromis mossambicus* exposed to fumaronitrile were analysed by enzyme linked immmunosorbent assay (ELISA) to find the hormonal changes. The experiment was carried out using commercially available ELISA kit procured from Dimetra, Italy. The fumaronitrile-exposed *Oreochromis mossambicus* showed a significant ($P < 0.05$) reduction in testosterone (T) concentration when compared to control fish whereas the estradiol (E2) level in female fishes was not reduced by the fumaronitrile exposure both in plasma and ovary. Aromatase activity was significantly increased ($P < 0.05$) on the 100$^{th}$ day in testis (11%) and ovary (16%) by the fumaronitrile in the gonads of treated fishes as compared to the control fish. Hepatic vitellogenin (VTG) production was significantly ($P < 0.05$) increased in fumaronitrile-treated fish. Gonadal VTG was significantly higher in male and female fish exposed to fumaronitrile. This study demonstrates that fumaronitrile has estrogenic effects in *Oreochromis mossambicus* and can induce VTG protein concentrations, indicating disruption of endocrine homeostasis.

**KEYWORDS:** fumaronitrile, *Oreochromis mossambicus*, sex steroid hormones, aromatase, VTG.

## INTRODUCTION

Environmental chemicals that disrupt endocrine function have been associated with adverse effects, particularly on the reproductive system in wildlife and humans. Although there are several mechanisms through which environmental chemicals might alter the endocrine system, chemicals that mimic steroid hormones through an interaction with the estrogen receptor continues to receive considerable attention [1, 2]. Endocrinology hormones (endocrine substances) regulate growth, reproduction, metabolism, development, behaviour, immune function and stress, among other functions critical for life. Hormones are also important in many disease states including diabetes and cancer. Endocrine disruptors, such as atrazine, which mimic and interfere with hormone production and/or activity [3], can affect any of these processes. Natural estrogens play a major role in controlling reproduction in females and, to a lesser extent, in males. Physiological concentrations of estrogen are also essential for the maintenance of cell growth and several other biological activities. In addition to estrogen-responsive organs such as the uterus, breasts, and pituitary, estrogens also exert an effect at a number of other sites like

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kidney, liver, skeletal tissues, etc. Imbalance of the steady-state concentrations of estrogens is known to produce adverse effects [4]. In recent years, research have focussed their attention on animals associated with wetland or aquatic habitats receiving sewage or industrial effluents and agricultural runoff. Fish have received the greatest attention in most of the studies in aquatic toxicology. In fish, estrogenic responses have been associated with exposure to pesticides, pulp mill effluent/phyto estrogens, industrial waste and sewage effluent [5]. The aim of the present study is to investigate the aromatase activities, vitellogenin induction and the response of reproductive hormones in *Oreochromis mossambicus* exposed to fumaronitrile. VTG levels were measured as biomarkers for possible effects of fumaronitrile on their reproductive biochemistry.

## MATERIALS AND METHODS

### Experimental procedure

Mature male and female *Oreochromis mossambicus* fishes were obtained from the Veterinary University Training and Research Centre, Dharmapuri District, Tamil Nadu and maintained in the well-aerated aquarium separately for breeding. The laboratory temperature was maintained at 27 ± 05 ºC, the illumination cycle consisted of 12 h light and 12 h dark and the fishes were fed with food pellets. Physicochemical characteristics include temperature 27 ± 0.5 °C, pH 6.3 ± 0.4, Dissolved oxygen 6.5 ± 0.4 mg/L, salinity 0.6 ± 0.03 ppt, nitrite 0.04 ± 0.008 mg/L, and hardness (as CaCO3), 19.2 ± 0.05 mg/L. These parameters were measured according to the experimental procedures described in Standard Methods for the Examination of Water (APHA).

The newly hatched juveniles/fries were separated from their respective mother and maintained in 300 L fiber-reinforced plastic (FRP) tanks and were maintained at an ambient, controlled temperature of 28 ± 2 ºC under natural photoperiod. A total of 200 fry (0 day old fry) were separated and included in four equal treatment groups. Fifty individual fries in each group were exposed to three different concentrations (1/10 th of LC 50, 6 ppb, 1/20 th of LC 50, 3 ppb and 1/30 th of LC 50, 2 ppb) of fumaronitrile; the control was also maintained simultaneously. The frys were used for the experiment on the same day of hatching. The young ones were fed with commercial granular feed throughout the experimental period.

### Measurement of plasma and gonad steroids

The sex steroid hormones were analyzed using enzyme linked immunosorbent assay (ELISA). The experiment was carried out by using commercially available ELISA kit procured from Dimetra, Italy. The sex steroids were determined following the assay kit procedures and methods described by Cuisset *et al*. [6] and Nash *et al*. [7].

### Sample preparation for sex steroid hormones

Fishes administered with fumaronitrile for 60, 80 and 100 days were used for the experimental analysis. In each group (2 ppb, 3 ppb and 6 ppb concentration) the blood was collected from 4 fishes and analysed for the sex steroids testosterone and estradiol. Blood samples were taken from the caudal vessel into heparinised syringes.

### Plasma

Testosterone and estradiol were extracted from the plasma using diethyl ether, ethyl acetate/hexane (50:50) and methylene chloride. The organic extracts were evaporated to dryness using nitrogen stream gas. The resulting pellets were dissolved in 0.5 mL ELISA buffer. Testosterone, 11-ketotestosterone and 17β-estradiol assay standards were prepared. The plate was set-up; samples and standard absorbance were read at 412 nm wavelength using a micro plate reader (Synergy HT Multi-Mode Micro plate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA).

### Gonads

The procedure for steroid extraction in gonads was modified from D'Cotta *et al*., (2001) [8]. Briefly, 1 g sections of frozen gonads were homogenized twice in 4 ml of cold 95% ethanol and centrifuged for 20 min at 4500 rpm at 10 °C. The steroids in the aqueous phase were extracted by dichloromethane and after removing the aqueous phase, the extract in the dichloromethane was evaporated to dryness. Steroid concentrations were determined by ELISA as described in the case of plasma analysis.

### Aromatase activity (ARO)

After 60, 80 and 100 days of fumaronitrile exposure, the gonads were extracted following the protocol of Lephart (1991) and Simpson (1994). 500 mg of gonadal tissue was homogenized in 600 µL of ice-cold gonad buffer (50 mM $KPO_4$, 1 mM EDTA, 10 mM glucose-6-phosphate, (pH 7.4) and then centrifuged at 10,000 rpm at 4 ºC for 20 min. Supernatants of the extracts were analyzed for aromatase activity. Aromatase activity was expressed as f mol of androstenedione converted per hour per Milli gram protein.

### Measurement of vitellogenin (VTG)

After 60, 80 and 100 days of fumaronitrile exposure, blood samples were collected and centrifuged at 3,000 rpm for 20 min. The resulting plasma was collected and frozen at -20 °C until further analysis. Each plasma sample was analyzed for vitellogenin content. Liver and gonads were homogenized with a threefold volume of 0.02 M Tris-HCl buffer, pH 8.0 containing 2% NaCl, using a glass tissue homogenizer and then centrifuged at 12,000 rpm at 4 °C for 20 min. Supernatants of the extracts were analyzed for vitellogenin content. VTG was measured by ELISA [9].

### Statistical analysis

The values are expressed as mean ± SE. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) software package for windows (version 16.0). Post-hoc testing was performed for inter group comparisons using the least significant difference (LSD) test $p < 0.05$ was considered statistically significant.

## RESULTS

### Plasma and gonad concentrations of testosterone (T)

Both male and female *Oreochromis mossambicus* had detectable concentrations of T and E2 in their plasma and gonads. The fumaronitrile-treated male fish showed a significant ($p < 0.05$) reduction in plasma T concentrations (Figure 1a) and a significant ($p < 0.05$) increase in plasma E2 concentrations compared to control male fish (Figure 2a). Plasma concentrations of T in control males ranged between 19.053 ng/ml and 17.563 ng/ml whereas gonad concentrations of T in control males ranged between 5.321 ng/ml and 4.354 ng/ml over the study period (Figure 1b). At the highest

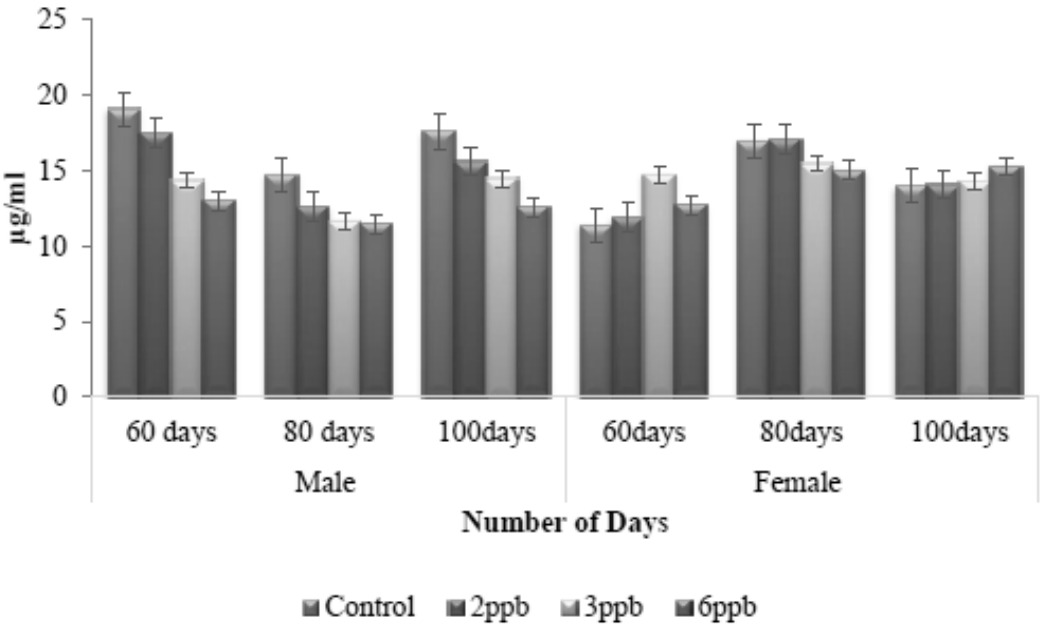


**Figure 1a.** Testosterone level in plasma of *Oreochromis mossambicus* at different concentration of fumaronitrile.

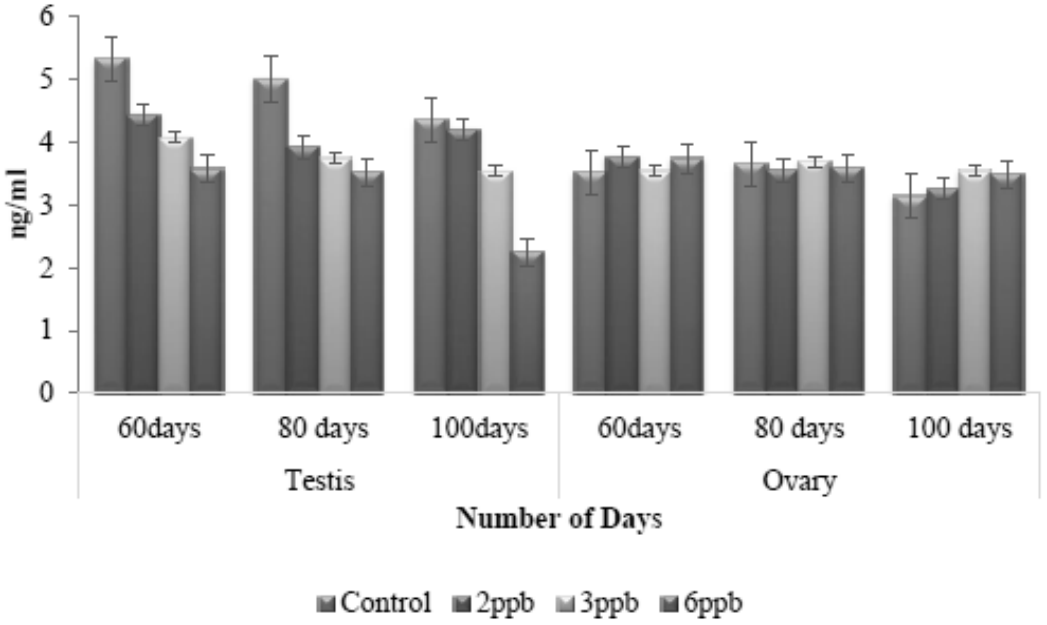


**Figure 1b.** Testosterone level in gonads of *Oreochromis mossambicus* at different concentration of fumaronitrile.

concentrations of fumaronitrile exposure to fish, there was a reduced plasma T (28.44%) and gonad T (48.43%) content at all sampling points of time ($p < 0.05$). In control female fish, mean concentrations of testosterone in the ovary was lower than in the plasma. In control females, plasma T concentrations and gonad T concentrations were lower than those of males (11.35 *vs*. 13.96 ng/ml & 3.52 *vs*. 3.13 pg/ml), respectively. In male there was a gradual decrease in plasma T concentration at 6 ppb fumaronitrile on the 100$^{th}$ day (28.44%) compared to control fish. The fumaronitrile-exposed *Oreochromis mossambicus* showed a significant ($p < 0.05$) reduction in T concentration when compared to control fish.

**Plasma and gonad concentrations of estradiol (E2)**

Estradiol concentrations in testis were slightly affected by all the three doses of fumaronitrile when compared to those of control plasma. The plasma E2 concentration of fumaronitrile-treated *O. mossambicus* was higher when compared to control. Plasma E2 concentration increased significantly ($p < 0.05$) on the 100$^{th}$ day from 25.376 to 43.402 pg/ml (71%) at the highest fumaronitrile concentration (Figure 2a). E2 in testis ranged between 25.5 and 44.74 pg/mg (75%) at the highest fumaronitrile concentration on the 100$^{th}$ day (Figure 2b) whereas E2 level in female fishes was not affected by fumaronitrile exposure, either in plasma or in the ovary. E2 concentration in the ovary on the 100$^{th}$ day at 6-ppb concentration was in the range between 32.643 and 45.129 pg/mg (33%) while, in plasma, it was higher in females than in males. No dose or time-related effects of fumaronitrile occurred in female.

**Gonadal aromatase activity**

Both male and female *O. mossambicus* treated with fumaronitrile showed aromatase activity. Gonadal aromatase activity was increased in fish treated with all concentrations of fumaronitrile, but it was not uniform; there was a lower elevation at 2 ppb than 6 ppb in the ovary with higher duration exposure. Gonadal aromatase activity increased and a low level variation among the three groups was observed (Figure 3). Aromatase activity was significantly increased ($P < 0.05$) on the 100$^{th}$ day in testis (11%) and ovary (16%) by the fumaronitrile in the gonads of treated fishes as compared to the control fish.

**Vitellogenin determination**

The plasma VTG concentrations in the treated male *O. mossambicus* on the 100$^{th}$ day at 6-ppb

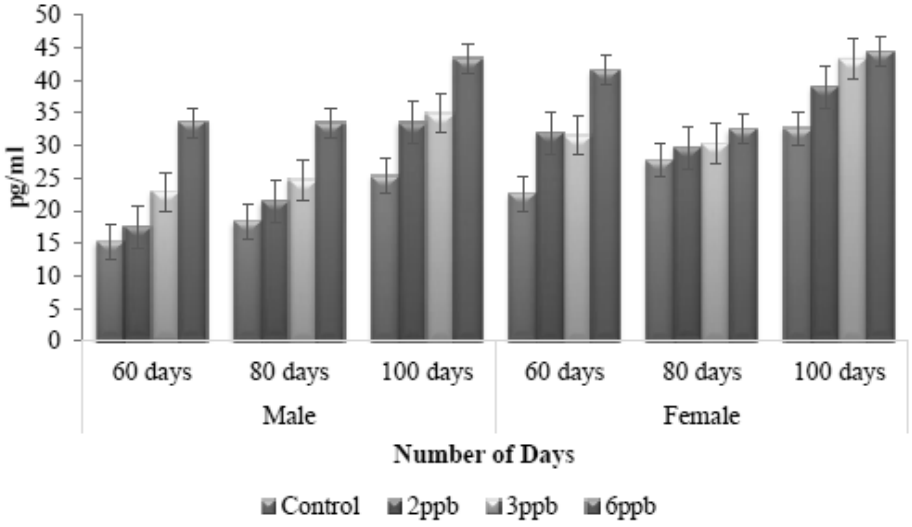


**Figure 2a.** Estradiol level in plasma of *Oreochromis mossambicus* at different concentration of fumaronitrile.

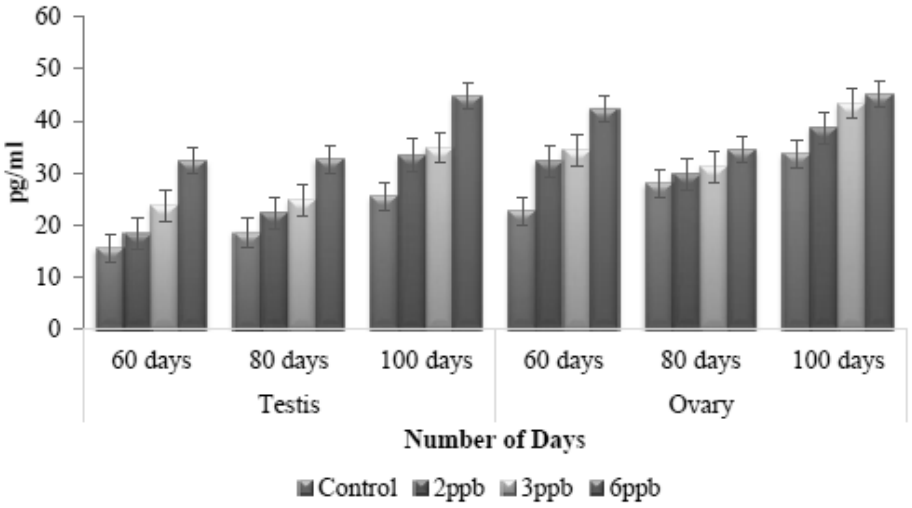


**Figure 2b.** Estradiol level in gonads of *Oreochromis mossambicus* at different concentration of fumaronitrile.

fumaronitrile increased in male by 39% (300.673 ng/ml) and in female by 7.4% (452.042 ng/ml) when compared to control (Figure 4a). The plasma VTG level was higher when compared to that of hepatic tissue. The plasma concentrations of VTG increased by twofold in the fumaronitrile-treated male fish when compared to liver. Plasma VTG concentrations in fumaronitrile-exposed male was significantly higher than control males ($P < 0.05$). Hepatic VTG production was significantly ($P < 0.05$) increased after fumaronitrile treatment in a dose dependent manner in male (79.95%) and female (32.35%) when compared to control (Figure 4b). Levels of VTG were higher in fumaronitrile-treated fishes. The female *Oreochromis mossambicus* had plasma VTG concentrations that were approximately four folds higher (452.042 ng/ml) than those in the control males (144.856 ng/ml). Gonadal VTG was significantly higher in male and female fish exposed to fumaronitrile treatments (Figure 4c). Low level induction of gonadal VTG was found in male fish exposed to fumaronitrile treatment. The exposure to fumaronitrile in female fish resulted in a slight

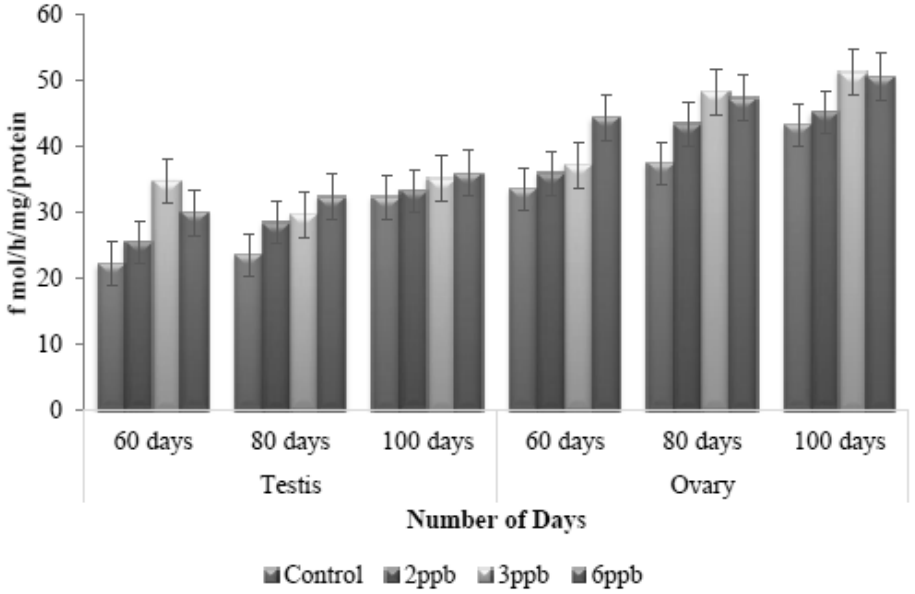


**Figure 3.** Gonadal aromatase activity of *Oreochromis mossambicus* at different concentration of fumaronitrile.

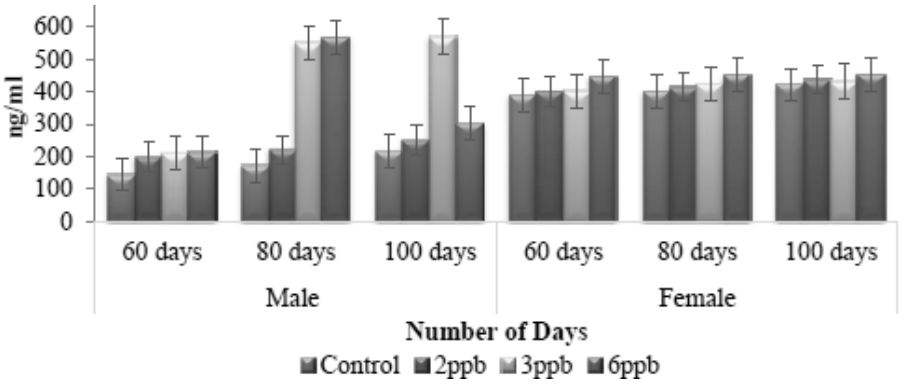


**Figure 4a.** Plasma vitellogenin content in *Oreochromis mossambicus* at different concentration of fumaronitrile.

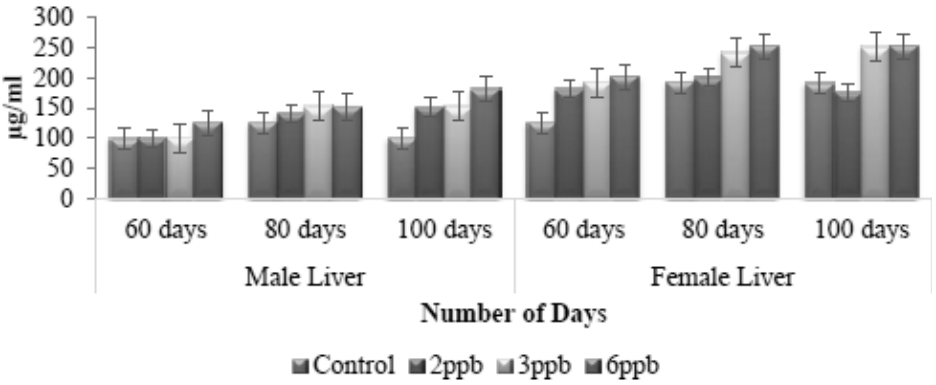


**Figure 4b.** Hepatic vitellogenin content in *Oreochromis mossambicus* at different concentration of fumaronitrile.

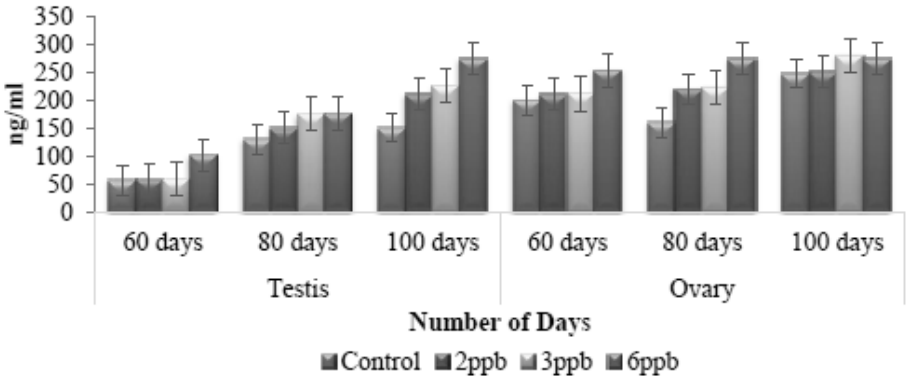


**Figure 4c.** Gonadal vitellogenin content in *Oreochromis mossambicus* at different concentration of fumaronitrile.

increase in concentrations of plasma, hepatic and gonadal VTG compared to control females. In this experiment however there was a significant increase in E2 concentration in plasma and gonad of male and female *O. mossambicus* and this could be in response to low level induction of VTG in male fish.

## DISCUSSION

In the present study altered steroid levels were observed in *Oreochromis mossambicus* exposed to fumaronitrile. The testosterone level was decreased in plasma and in testis of male fish whereas estradiol level was increased in the gonads and plasma of both male and female fishes. The testosterone level showed no effects in female fish that were exposed to fumaronitrile. Spano [10] reported that altered steroid hormone concentrations were observed in goldfish (*Carassius auratus*). The present findings evidenced that fumaronitrile doses can cause significant changes in the estradiol level at different duration in male fish. The effects on the sex steroids T and E2 can be attributed to the stimulatory effect of aromatase activity in male *O. mossambicus*. Further the effect was confirmed by the ELISA-based method that showed increased level of aromatase activity. In another study the effect of atrazine (ATZ) on steroid alteration was reported in mature male Atlantic salmon (*Salmo salar* L.) [3].

Friedmann [11] demonstrated a decreased level of T concentration in the goldfish gonads after 21 days of exposure to chemicals. The various doses of fumaronitrile experimented in *O. mossambicus* showed decreased level of testosterone in the plasma of male fish. In the present study it was revealed that the various doses (2 ppb, 3 ppb, and 6 ppb) of fumaronitrile could decreases testosterone concentration and increase concentration of estradial in plasma and testis of *O. mossambicus*. Our findings were consistent with the reports for other chemicals; however no reports were found for fumaronitrile. Hence the fumaronitrile doses focused in this study (2 ppb, 3 ppb, and 6 ppb) could cause decreased level of testosterone and this could affect the metabolism. The aromatase activity and vitellogenin in *Oreochromis mossambicus* exposed to different fumaronitrile doses were analysed. The interactions between fumaronitrile and the endocrine system were also analysed in the study. The aromatase level increased when treated with three different concentrations of fumaronitrile.

Our results support the current hypothesis that the mechanism of action of fumaronitrile induces the aromatase activity. Moreover aromatase activities in the gonads of *O. mossambicus* were increased by fumaronitrile doses. However, our results were similar to the previous study which was carried out in female zebra fish which revealed over-expression of aromatase activity [12]. Induction of aromatase activity has been reported in male and juvenile alligators when exposed to certain chemicals [13]. Fumaronitrile treatment at various doses can increase and enhance estradiol level in male *O. mossambicus*. These studies indicate that the estrogen levels increase in the developing gonad and plasma, which play a critical role in sex differentiation. Smith *et al*. [14] demonstrated

that the aromatase activity enhances in fishes and several reptiles during ovarian development. Different concentrations of fumaronitrile showed altered aromatase activity in *O. mossambicus*. It also showed considerable variations of aromatase activity in male and female fishes. However, activity of aromatase was increased in the gonads of *O. mossambicus*. A statistically significant increase in the ($p < 0.05$) aromatase activity was found in the gonads of *O. mossambicus* exposed to all the concentration of fumaronitrile. The effect of fumaronitrile on aromatase may explain the increase in estrogen and reduction in testosterone levels. The results supported the findings of Stoker *et al*. [15] and Spano [10] who stated that the cytochrome P450 is significantly needed for the vital activity of aromatase enzyme. Previous studies suggest that several chemicals can induce aromatase activity *in-vitro*. *O. mossambicus* tissues were bisected into plasma, liver and gonads for the VTG analysis by ELISA. The VTG content was predominantly high in the plasma and hepatic tissues. The VTG concentration was high in plasma and liver tissues when compared with the gonads. This is the first report on fumaronitrile chemical exposure in male/female *O. mossambicus* and the study confirmed a high level of VTG in plasma when compared to other organs. This result correlates with a previous study on other chemicals that can induce the level of VTG in male fish [16]. However no effects were described on VTG protein concentrations in adult goldfish (*Carassius auratus*) and male carp hepatocytes [10, 17, 18]. An earlier study also reported that increased level of VTG was observed in the Medaka fish (*Oryzias latipes*) [19]. However, in the case of *O. mossambicus*, the gonad VTG concentration was low when compared to plasma and liver. The breakdown products of alkyl phenol polyethoxylate surfactants are able to induce vitellogenesis in male rainbow trout (*Oncorhynchus mykiss*) [20]. However, in our study the various doses of fumaronitrile showed significant role in the increased level of estrogen and decreased level of testosterone. These results support the findings of Korsgaard *et al*. [21] who proposed that the liver was the main site of estradiol-induced *de-novo* synthesis of vitellogenin in fish [22]. Estradiol-induced VTG was also observed in the liver of cichlid fish (*Cichlasoma dimerus*). Seki Masanori *et al*. [23] also observed that hepatic VTG level was increased, especially in males, in ethinylestradiol-treated groups in a concentration-dependent manner. A very low level of VTG in gonads was observed throughout the study periods. A similar report on adult male medaka exposed for 4 weeks to 10 and 100 ng/l ethynylestradiol (EE2) in whole body homogenate showed a more than 2000-fold increase in VTG protein with respect to control levels [24].

## CONCLUSION

The results of this study showed that exposure to fumaronitrile affected the hormone concentrations in *Oreochromis mossambicus*. Fumaronitrile affect the endocrine system of *O. mossambicus via* an estrogen-like mechanism of action resulting in a reduction of plasma T, and an increase in plasma E2 due to up-regulation of aromatase. This study demonstrated that fumaronitrile chemical induces the expression of aromatase enzyme in *O. mossambicus*. VTG and aromatase activity was found to increase in the *O. mossambicus* treated with fumaronitrile. This study demonstrates that fumaronitrile has estrogenic effects in *O. mossambicus*. It further demonstrates that fumaronitrile can induce VTG protein concentrations, indicating disruption of endocrine homeostasis.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## ABBREVIATIONS

| | | |
|---|---|---|
| ELISA | : | Enzyme Linked Immunosorbent Assay |
| T | : | Testosterone |
| E | : | Estradiol |
| VTG | : | Vitellogenin |
| mL | : | Milliliter |
| nm | : | Nano meter |
| °C | : | degree Celsius |
| ng/L | : | Nanogram per liter |
| µg/L | : | Microgram per liter |
| pg/g | : | Pico gram per gram |
| ATZ | : | Atrazine |

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