

Research Article

Alteration in the histology of gonads and hormone levels of fish *Channa punctatus* on exposure to 17- α ethynylestradiol

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Abstract

It has been demonstrated that effluents discharged from sewage treatment facilities allow synthetic estrogens, widely employed in contraceptives and other pharmaceutical applications, to reach the aquatic environment. One of the most biologically active xenoestrogens in the aquatic environment is the synthetic hormone 17- α ethynylestradiol (EE2), raising reproductive issues in the fish population. The present study chose *Channa punctatus* fish to study the effects of EE2 on gonad histology and the calculation of estrogen and testosterone levels. The experimental setting was separated into four groups, with Group I as control with 0 ng/L of EE2. The other three groups viz., Group II to IV, had 5 ng/L, 10 ng/L, and 20 ng/L concentrations of EE2, respectively, and samples were obtained after the durations of 7, 14, 21, and 28 days. The findings showed that as 17- α ethynylestradiol concentrations increased, testosterone levels decreased from 10.88 \pm 0.24 ng/dL to 4.91 \pm 0.24 ng/dL, while estrogen levels increased from 42.7 \pm 2.22 pg/mL to 120.18 \pm 4.54 pg/mL. The number percentage of primary growth oocytes, previtellogenic oocytes and vitellogenic oocytes in the histology of ovary decreased from 28.45 \pm 1.42 to 10.43 \pm 0.47; 16.22 \pm 0.81 to 3.15 \pm 0.16 and 14.81 \pm 0.89 to 2.14 \pm 0.11 respectively while in testis, the number percentage of spermatogonia, spermatocytes decreased from 21.4 \pm 0.86 to 15.1 \pm 0.68 and 97.1 \pm 3.88 to 54.2 \pm 3.25. In contrast, the percentage of mature spermatids increased from 20.8 \pm 0.94 to 40.2 \pm 2.41. Since synthetic estrogens adversely affect aquatic animals, especially fish, they should be treated properly before releasing them into the aquatic environment.

Keywords: 17- α ethynylestradiol, *Channa punctatus* Estrogen, Histology, Testosterone

INTRODUCTION

Estrogens and androgens, which can be natural (found in nature) or synthetic, are the most significant sex hormones employed in fish aquaculture (synthesized or produced in a laboratory). Natural and synthetic estrogens' possible effects on aquatic environments have recently attracted a lot of attention. Concerns have been raised about the potential role of environmental estrogens as important factors in the reproduction of various European freshwater fish populations (Amenyogbe *et al.*, 2020). 17- α ethynylestradiol (EE2)

is of particular concern due to its longer half-life and proclivity to bioconcentrate in tissues among ECs (Estrogenic Chemicals), and studies have shown that it is 10–50 times more powerful in fish than naturally generated estrogen (De Wit M. *et al.*, 2010). A synthetic hormone called 17- α ethynylestradiol (EE2) is used in this study. It is a derivative of estradiol (E2). One of the main organisms at risk from endocrine disrupting chemicals (EDCs) is fish (Gong *et al.*, 2016). It has been demonstrated that in some fish species, the binding affinity of EE2 to the estrogen receptor is up to five times higher than that of E2 (Thorpe *et al.*, 2003).

Comparing EE2 to naturally occurring E2, the increased receptor affinity suggests that EE2 may be a more potent estrogenic chemical inducing an estrogenic response (Aris *et al.*, 2014). Multiple fish species have been reported to be adversely affected by EE2 under environmental and laboratory conditions. Due to their known negative effects on aquatic creatures (most notably fish) at very low concentrations, certain groups of environmental (exogenous) steroids constitute a significant water quality problem. (Gunnarsson *et al.*, 2019, Runnalls *et al.*, 2010)

Synthetic steroid hormones are thought to be far more resistant to environmental degradation than natural steroid hormones, which means they will likely be present in the environment for longer periods of time. *Channa punctatus* is the fish used to explore how 17- α ethynylestradiol affects hormones and the gonads. It is favoured as a food during recovery because of its deliciousness, high protein content, lack of intramuscular spines, high nutritional value, and therapeutic properties (Haniffs *et al.*, 2009). As a result of exposure to the synthetic hormone 17- α ethynylestradiol, the experiment's goal is to track changes in hormone levels and examine histological alterations in the gonads of the fish *Channa punctatus*. It has been demonstrated that exposing zebrafish to the synthetic oestrogen 17- α ethynylestradiol (EE2) has a number of negative effects, such as the feminization of male fish, decreased reproductive potential, and altered embryonic development (Foster and Brown, 2018).

In the fish ovary, EE2 influences the frequency of primary growth oocytes (PG), vitellogenic oocytes (VO), and previtellogenic oocytes (PV), and it also has a significant impact on the number of spermatogonia (SG), spermatocytes (SC), and mature spermatids (MS). Primary growth oocytes (PG) are small cells (Grier, 2012) (12–20 μ m in diameter in methacrylate slices) that are found in clusters or nests with bigger primary oocytes of a similar size. The following developmental stage, during which vitellogenic oocytes (VO) (425–1070 μ m in diameter) are formed, is marked by the buildup of eosinophilic yolk bodies in the ooplasm. The fish testis is made up of a variety of cells that are in various developmental stages, including the mitotic, or spermatogonial, phase, which results in the production of spermatogonia. The meiotic phase, has primary and secondary spermatocytes, and the spermiogenic phase, sees haploid spermatids emerge from meiosis and differentiate into motile, flagellated, haploid spermatozoa (Xie *et al.*, 2020). As a result, exposure to EE2 can impair fish reproduction (Jackson *et al.*, 2019). On exposure to 17-ethynylestradiol, the levels of male and female hormones are also known to fluctuate more than the normal amount. Thus, the present study aimed to analyse toxicity induced by 17 α -ethynylestradiol in both male and female fish *Channa punctatus* by assessing changes in the levels of their gonadal hor-

mones and by observing alterations in their gonadal histology.

MATERIALS AND METHODS

Chemicals

EE2 (17- α Ethynylestradiol, > 98% purity; Sigma Aldrich, St. Louis, MO) stock solutions were made using ethanol as the solvent (lab-grade; Thermo-Fisher Scientific, Hampton, MA). In 0.002% ethanol, the nominal EE2 exposure doses were 0 (solvent control), 5 ng EE2/L, 10 ng/L, and 20 ng/L.

Collection and acclimating of test animals

Channa punctatus (Bloch, 1973), also known as Girai, Spotted Murrel, and Snakehead Murrel, is chosen as the test animal in the current experiment. Livehealthy *C. punctatus* freshwater fish that were 16 \pm 2 cm in length and 35 \pm 5 g in weight were bought from a local fishmarket in Lucknow. Fish were immersed for 5 minutes in a 0.05% potassium permanganate (KMnO₄) solution to ensure they were clear of skin diseases. The fish were kept in glass aquariums that were filled with 100 litres of tap water two days before the start of the acclimatisation phase. Before the trial, the fish were acclimatised for roughly 14 days in properly maintained laboratory settings. Artificial fish meal of brand optimum was used to feed the fish. The faeces and other waste were regularly removed to prevent the aquariums from having a high ammonia concentration. Every effort was made to maintain optimal conditions throughout fish acclimation (APHA, 2017).

Layout and design of the experiment

Experimental setups were established at the Department of Zoology, University of Lucknow. Following a 14-day acclimatization period, non-infectious fish were chosen at random and exposed to 17- α ethynylestradiol for 28 days. Samples were taken on the 7th, 14th, 21st, and 28th days. Fish, each having 30 individuals, were divided into four groups. Group I was used as a benchmark (de-chlorinated tap water- control). Group II received 5 ng/L of the synthetic hormone 17- α ethynylestradiol, and Group III received 10 ng/L of this hormone, and 20 ng of 17- α ethynylestradiol was given to Group IV. This was completed based on the earlier work of Kidd *et al.* (2007). Three fish from each Group were anaesthetized with 0.1% diethyl ether and slaughtered for the experiment after the appropriate exposure times were completed (n = 3 fish from each Group). This was done before blood and tissue samples were taken for hormone analysis tests and histological studies of the gonads.

Compliance with Ethical standards

As per the requirements and for control and supervision of experiments on animals (CPCSEA), Govern-

ment of India, an institutional Animal Ethics Committee (IAEC) vide Registration No. 1861/GO/Re/S/16/CPCSEA, University of Lucknow, Lucknow, the authors followed the protocols as stated in the CPCSEA for executing the experiment.

Hormone analysis

The males and females were first distinguished; unlike males with an extended genital entrance, females have a circular genital aperture. The females are believed to have a bloated abdomen and can be identified by diffuse black blotches in the ventral region, while the males are supposed to have black spots on a yellow underbelly. The ventral fin in males should occasionally extend beyond the vent, whereas the ventral fin in females is small and never touches the vent (Farid *et al.*, 2020)

Blood sampling

In the morning, live males and females were given blood samples from the lateral caudal vein with heparinized 22-gauge needles within 10 to 20 minutes to measure progesterone, 17- β estradiol, and testosterone levels. Collected blood samples were centrifuged at 3000 rpm for 20 minutes to extract the serum, which was then used to calculate the levels of progesterone, testosterone, and 17- β estradiol (Lawrence *et al.*, 2020).

Measurement of steroid hormones

Radioimmunoassay was used to determine the plasma concentrations of sex steroids (testosterone, progesterone, and 17- β estradiol) (Abraham 1969, Rinchard *et al.*, 1993). By detecting the precipitation created by the interaction of the protein and the antiserum, a quantitative immunoassay approach can be utilised to determine the level of 17-methyl testosterone, progesterone, and 17- β estradiol in plasma (Shimizu *et al.*, 1985).

Histological analysis

Fish from all four groups had their gonads examined (Lange *et al.*, 2001). Fish were dissected for this purpose after being properly anaesthetized and killed with the anaesthetic clove oil; their gonads were then removed, dehydrated, and processed for paraffin embedding. On microscopic slides, 8–10 5 mm-thick sections of each gonad tissue were created. These slides were mounted on Canada balsam after being stained with hematoxylin and cleaned with xylene. Under a light microscope, the stained slices were examined, and photos of various observations were recorded and stored in JPEG format at 40x magnification.

Statistical analysis

A one-way ANOVA test was used to analyse mean differences, followed by Duncan multiple comparison tests, and a significance level of $P > 0.05$ was applied to each test. Using SPSS 27, all data were reported as

mean \pm standard deviation.

RESULTS AND DISCUSSION

Ovary

When fully developed, the ovaries, paired organs, take up a large portion of the abdominal cavity ventral to the swim bladder (Gupta and Mullins, 2010). They are held in place by a mesentery called the mesovarium. It is connected to the abdominal cavity, the dorsal portion of the swim bladder close to the kidneys, and the abdominal cavity's wall is positioned just lateral to them.

Oogonia/primary growth oocytes (PG)

Oogonia (and/or the smallest oocytes present (Selman *et al.*, 1993); are little cells with a diameter of 12 to 20 μ m in methacrylate slices) that occur in clusters or nests with bigger primary oocytes and oocytes of comparable size. After exposure for 7, 14, 21, and 28 days, the frequencies of PG in the ovary of the fish *C. punctatus* in Group I which served as the control- were nearly constant with negligible change for all test concentrations. After all exposure periods, there was a significant ($p > 0.05$) decrease in PG levels in Groups II, III, and IV. However, the range of frequencies of Groups II, III and IV showed significant changes by decreasing with increase in concentration of EE2 (Table 1). The percentage of PG was observed to have fallen by 2.7 times from 28.45 \pm 1.42 to 10.43 \pm 0.47 when 7 days of Group I (which included no EE2) when compared to 28 days of group IV (which contained 20ng/L of EE2).

Oocytes that are previtellogenic (PV)

At 7, 14, 21, and 28 days, the frequency of PV in Group I showed negligible shifts. (Table 1).. After all exposure periods, lower PV levels were seen in groups II, III and IV. When comparing day 7 of Group I with day 28 of group IV, it was discovered that the PV percentage had fallen by five times, from 16.22 \pm 0.81 to 3.15 \pm 0.16 (Table 1). After 7, 14, 21, and 28 days of exposure, the frequency in Group I for VO was nearly unchanged (Table 1).

Oocytes with vitellogenin (VO)

The next developmental stage is the beginning of vitellogenesis, which produces vitellogenic oocytes (425–1070 μ m in diameter), which are distinguished by an increase in eosinophilic yolk bodies in the ooplasm. The yolk bodies of fathead minnows are also just marginally sudanophilic (Ankley *et al.*, 2003). When comparing day 7 of Group I with day 28 of group IV, it was discovered that the proportion of VO had reduced seven times, from 1.72 \pm 0.10 to 12.43 \pm 0.62. Thus, as the levels of EE2 increased from the lowest concentration of 5ng/L to the greatest concentration of 20ng/L, they quickly reduced as well. This clearly illustrates how the degree of maturation decreased with increasing EE2 exposure

(Table 1). This showed shifting percentages of PG, VG, and VO at various amounts of 17-ethynylestradiol. Fig. 1 and 2 depict PG, PV, and VO in the histology of ovary of fish *C. punctatus*.

Similar observations were made in the control group, where females had a full spectrum of oocytes, including developing and mature stages (oogonia, previtellogenic oocytes, and vitellogenic oocytes) (Van Der Ven et al., 2003). In the present experiment, the control group displayed varied stages of oocytes. When compared with the control (0 concentration of EE2), with group IV having the highest concentration 20ng/l of EE2, histological evaluation revealed a decrease in the frequency of PG by 2.7 folds, VO by 7 folds, and PV by 5 folds, indicating a decrease in maturation. Zeilinger (2009) made an almost identical observation when he observed the histology of female ovary exposed to mid and high concentrations of drospirenone. When the comparison was made with the vehicle control, the histomorphometric evaluation revealed a decreasing amount of PG from 34.2 to 17.8 %, mature vitellogenic stage oocytes from 26.0 to 2.2 %, and previtellogenic oocytes from 26.0 to 2.2% .

Similarly, EE2 exposure in females lowered their level of maturation. When zebrafish (*Danio rerio*) were exposed to EE2, additional studies found reductions in the amount of vitellogenic oocytes, atresia, a lower ovary-somatic index, and significant ratios of fish with non-defined sex (Hill and Janz, 2002; Van et al., 2003; Maack and Segner, 2004). However, data showed that EDC exposure causes: 1) disruption of gonad development and sex differentiation, generating intersex; 2) aberrant gonad differentiation, affecting the quantity of germ cells, resulting in episodes of sterility in freshwater fish (Delbès et al., 2022). Therefore, sexual im-

maturity in adult females is caused by exposure to environmentally relevant amounts of EE2 during the time of sexual development (Stefan et al., 2016).

Testis

The testes are long organs that are spread out from the head to the tail of the body. The mesorchium connects the testes to the dorsal peritoneal wall. The testes are situated beneath the swim bladder and communicate with the outside world via the genital pore. (Hernández et al., 2022). Spermatogonia (SG): As a consequence of multiple mitotic divisions of a single spermatogonia, a cluster or cyst is composed of medium-sized cells with a spherical, light-basophilic nucleus called spermatocytes (Richard et al., 2005). In the present study, at 7, 14, 21, and 28 days, respectively, SG in Group I, which acted as the control, had negligible change. After all exposure times, there were significantly ($p > 0.05$) lower SG levels in groups II, III and IV (Table 2). When day 7 of Group I (0 ng/L of EE2) was compared to day 28 of Group IV (20 ng/L of EE2), it was found that the proportion of SG had dropped by 1.4 times, from 21.4 ± 0.86 to 15.1 ± 0.68 .

Spermatocytes (SC)

Spermatocytes are small cells that go through meiosis II to create spermatids (Richard et al., 2005). They have smaller, more basophilic nuclei than primary spermatocytes. The frequency of SC in Group I remained almost constant with negligible change. After all exposure times, lower SC levels were seen in groups II, III, and IV. The percentage of SC was observed to have fallen by 1.8 times, from 97.1 ± 3.88 on day 7 of Group I to 54.2 ± 3.25 on day 28 of group IV (Table 2).

Table 1. Number percentage (Mean ± SE) of primary growth oocyte, previtellogenic oocyte and vitellogenic oocyte in the histology of ovary histology in *Channa punctatus* on exposure to 17- α ethynylestradiol for 28 days

Groups	Conc. (ng/L)	Exposure period	Primary Growth Oocytes (PG) %	Pre vitellogenic oocyte (PV) %	Vitellogenic Oocytes (VO) %
Group I	0 ng/L of EE2	7 days	28.45 ± 1.42	16.22 ± 0.81	14.81 ± 0.89
		14 days	28.44 ± 1.70	16.21 ± 0.73	14.68 ± 0.59
		21 days	28.43 ± 1.28	16.20 ± 0.65	14.7 ± 0.81
		28 days	28.43 ± 1.42	16.23 ± 0.89	14.69 ± 0.80
Group II	5ng/L of EE2	7 days	25.21 ± 1.26	14.83 ± 0.74	12.43 ± 0.62
		14 days	23.44 ± 1.41	13.68 ± 0.55	11.76 ± 0.65
		21 days	21.84 ± 1.09	11.54 ± 0.58	10.66 ± 0.53
		28 days	20.61 ± 1.03	10.44 ± 0.57	9.36 ± 0.56
Group III	10ng/L of EE2	7 days	19.81 ± 1.19	9.26 ± 0.42	8.44 ± 0.38
		14 days	18.29 ± 0.91	8.41 ± 0.42	7.84 ± 0.39
		21 days	16.91 ± 0.85	7.41 ± 0.30	6.92 ± 0.35
		28 days	15.66 ± 0.78	6.32 ± 0.38	5.24 ± 0.26
Group IV	20ng/L of EE2	7 days	14.70 ± 0.88	5.47 ± 0.33	4.53 ± 0.27
		14 days	13.19 ± 0.53	4.48 ± 0.22	3.63 ± 0.20
		21 days	11.95 ± 0.66	3.83 ± 0.23	2.83 ± 0.16
		28 days	10.43 ± 0.47	3.15 ± 0.16	2.14 ± 0.11

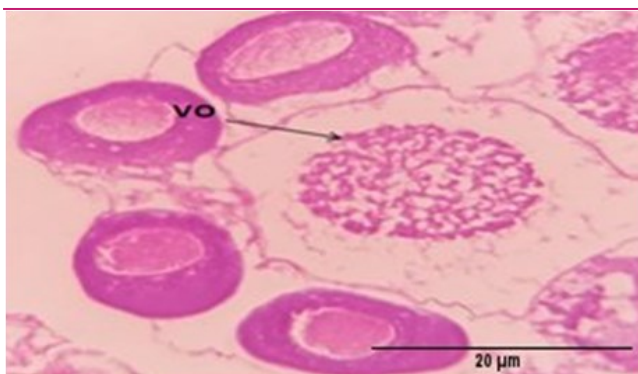


Fig. 1. Histology of ovary in *Channa punctatus* showing PV and VO

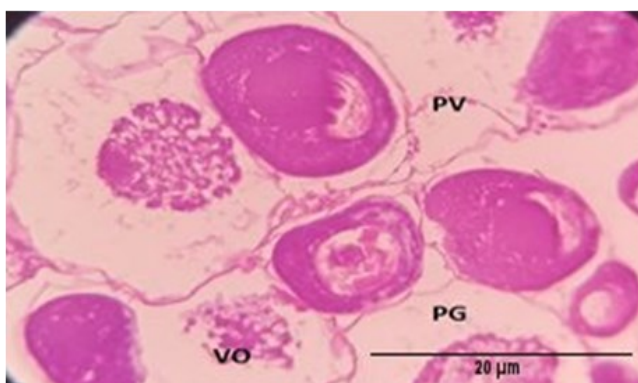


Fig. 2. Histology of ovary in *Channa punctatus* showing PG, PV and VO

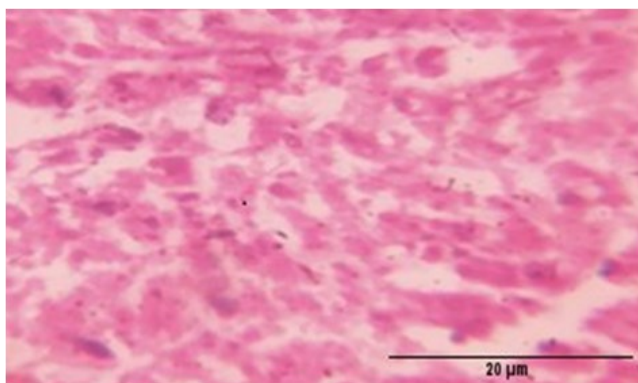


Fig. 3. Histology of testis of *Channa punctatus* showing SG and MS

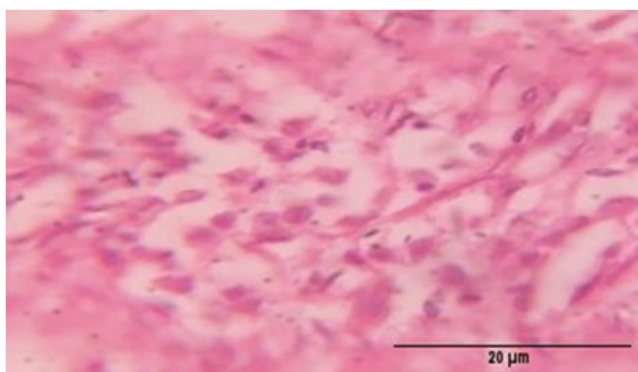


Fig. 4. Histology of testis of *Channa punctatus* showing SC

Mature spermatids (MS)

Spermatids develop into spermatozoa (Richard L. Leinoe *et al.*, 2005) and contain a tiny basophilic nucleus. The frequency of MS in Group I showed negligible change (Table 2). Following exposure periods of 7, 14, and 28 days, respectively, MS values increase as EE2 concentrations rise. Similar elevated MS levels were seen in groups II, III, and IV following all exposure periods. The percentage of MS was found to have doubled between day 7 of Group I and day 28 of group IV, from 20.8 ± 0.94 to 40.2 ± 2.41 . This clearly showed that as concentration increases from lowest to highest, the levels of EE2, count of SG and SC decrease while the frequency of MS rises. The percentages of SG, SC, and MS change following the concentrations of 17-ethynylestradiol (Table 2). Fig. 3 and 4 show SG, SC and MS in the histology of testis in *C. punctatus*.

The present experiment's control testicles revealed several germ cell phases comparable to the five stages of freshwater drum germ cell development viz. primary spermatogonia, secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa are the first five components of spermatozoa (Hernández *et al.*, 2022). In the present study, the frequency of SG and SC decreased by 1.4 and 1.8 folds in the testis histology when comparing the lowest concentration (control) with the highest concentration (Group IV); however, the frequency of MS increased by 2 folds. Similar observations were made by Jana Zeilinger (2009) who observed the histology of male fish testis exposed to mid and high concentrations of drosiprenone, the count of spermatogonia cysts decreased from the control to the highest concentration, from 20.8 to 16%, and the average number of counted spermatocytes per field of view decreased from 95.8 in control to 51 cysts in the group with high concentration. Similarly, at the mid and high concentration showed an increase in the mature spermatids from 22.5 to 39.1% (Zeilinger *et al.*, 2009). Thus, the sexual maturity of male fish increases with increase in mature spermatids.

Consequently, it can be inferred that testosterone and estradiol are the main hormones that regulate the sexual characteristics of male and female animals, including fish. This study uses a synthetic hormone called 17- α ethynylestradiol (EE2), which is an estradiol (E2) derivative. Table 3 shows the computed hormone levels for various periods, including mean values and standard errors for several groups, including the control group for the fish *C. punctatus* (Bloch).

Estradiol level in female fish was estimated to be around three times higher than testosterone levels in male fish, rising from 42.7 ± 2.22 to 120.18 ± 4.54 , indicating that after 28 days of exposure, estradiol values significantly increased from day 7 to day 28. Estradiol levels increased from Group II to Group IV as exposure concentration increased. However, in the control group (Group I), there was no discernible difference between

Table 2. Number percentage (Mean \pm SE) of spermatogonia, spermatocytes and mature spermatids in the histology of testis in fish *Channa punctatus* on exposure to 17- α ethynylestradiol for 28 days

Groups	Conc. (ng/L)	Exposure period	Spermatogonia (SG) %	Spermatocytes (SC) %	Mature Spermatids (MS) %
Group 1	0 ng/L of EE2	7 days	21.4 \pm 0.86	97.1 \pm 3.88	20.8 \pm 0.94
		14 days	1.31 \pm 21.8	96.9 \pm 5.8	21.4 \pm 0.86
		21 days	21.3 \pm 1.17	96.8 \pm 5.3	22.7 \pm 1.36
		28 days	21.2 \pm 1.27	97.1 \pm 4.86	23.8 \pm 1.31
Group 2	5ng/L of EE2	7 days	20.1 \pm 1.20	84.2 \pm 5.10	24.2 \pm 1.21
		14 days	19.9 \pm 0.80	80.8 \pm 4.04	25.6 \pm 1.02
		21 days	19.8 \pm 1.00	79.3 \pm 3.97	27.8 \pm 1.67
		28 days	19.8 \pm 1.18	77.2 \pm 3.17	29.6 \pm 1.48
Group 3	10ng/L of EE2	7 days	18.5 \pm 0.93	68.2 \pm 3.41	30.2 \pm 1.81
		14 days	18.1 \pm 0.80	66.5 \pm 3.33	31.8 \pm 1.59
		21 days	17.6 \pm 1.1	64.3 \pm 3.54	33.4 \pm 1.84
		28 days	17.1 \pm 0.85	62.1 \pm 3.73	35.2 \pm 1.41
Group 4	20ng/L of EE2	7 days	16.7 \pm 0.84	60.2 \pm 3.01	39.7 \pm 1.99
		14 days	16.2 \pm 0.97	58.8 \pm 2.94	39.8 \pm 1.79
		21 days	15.6 \pm 0.86	56.7 \pm 2.27	40.1 \pm 1.60
		28 days	15.1 \pm 0.68	54.2 \pm 3.25	40.2 \pm 2.41

Table 3. Concentration level (Mean \pm SE) of estradiol and testosterone in fish *Channa punctatus* on exposure to 17- α ethynylestradiol for 28 days

Groups	Conc. (ng/L)	Exposure period	Estradiol (pg/mL)	Testosterone (ng/dL)
Group 1	0 ng/L of EE2	7 days	42.7 \pm 2.22	10.88 \pm 0.24
		14 days	42.7 \pm 2.23	10.88 \pm 0.33
		21 days	42.7 \pm 2.23	10.88 \pm 0.33
		28 days	42.7 \pm 2.22	10.88 \pm 0.24
Group 2	5ng/L of EE2	7 days	49.25 \pm 2.33	9.32 \pm 0.23
		14 days	55.12 \pm 2.54	8.66 \pm 0.24
		21 days	65.9 \pm 2.55	8.18 \pm 0.22
		28 days	68.94 \pm 2.54	7.48 \pm 0.24
Group 3	10ng/L of EE2	7 days	68.94 \pm 3.54	7.2 \pm 0.24
		14 days	75.96 \pm 3.45	6.67 \pm 0.25
		21 days	82.24 \pm 3.56	6.18 \pm 0.26
		28 days	88.5 \pm 3.54	5.91 \pm 0.24
Group 4	20ng/L of EE2	7 days	96.71 \pm 4.05	6.45 \pm 0.25
		14 days	104.93 \pm 4.33	5.92 \pm 0.24
		21 days	113.2 \pm 4.45	5.42 \pm 0.23
		28 days	120.18 \pm 4.54	4.91 \pm 0.24

the samples taken on the 7th and 28th days ($P < 0.05$). This indicated an increase in estradiol level as the exposure duration rose from day 7 to day 28. A two-fold decrease in testosterone level, from 10.88 \pm 0.24 to 4.91 \pm 0.4, was seen when the exposure duration was extended from 7 to 28 days and when we moved from Group I to Group IV. However, when moved from day 7 to day 28, there was no discernible change in the tes-

tosterone level in the control group (group 1) ($P < 0.05$) (Table 3).

The estrogenic hormones in a river can cause male fish to become feminized (Sun *et al.*, 2019). Hence, it is possible to induce sex reversal by treating fish with estradiol or testosterone (Mousavi-Sabet, 2011). Feminization occurred in some teleost species when the hormonal balance shifted favouring 17 β -estradiol,

whereas masculinization occurred when the hormonal balance shifted in favour of 11-ketotestosterone. Therefore, when 17 α -ethynylestradiol, a synthetic female hormone, is administered, feminization will be facilitated, increasing the levels of estradiol (the female reproductive hormone) and decreasing the levels of testosterone (the male reproductive hormone) (Rougeot *et al.*, 2007). EDCs (Endocrine-disrupting chemicals) have produced an imbalance in organisms' reproductive hormones over the last two decades (Shirdel *et al.*, 2020). Thus, the present study shows that male reproductive hormone decreases and female reproductive hormone increases with increasing concentration of EE2, leading to sex reversal and feminization and might lead to changes in sex ratio.

Conclusion

The current study showed that endocrine active substances at levels commonly found in sewage treatment effluents and surface waters could change the endocrine physiology and gonadal histology of individual organisms and may have a negative impact on the gender structure of fish populations. Exposure to environmentally relevant quantities of EE2 particularly causes the feminization of fish, resulting in variations in sex ratios in populations and opening up new avenues for future research. Exposure to EE2 also promotes sexual immaturity in individual adult females, affecting the population's reproductive success. This study concludes that fish exposure to EE2 alone or in combination with other xenobiotics can endanger fish reproductive health and population patterns. As a result, it is also vital to monitor the impact of EE2 and make the necessary efforts to minimise its release into the environment to provide sustainable and better aquatic ecosystems. More in vivo exposure studies on the effects of environmentally important EDC combinations and other chemicals, such as medicines, are required.

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Conflict of interest

The authors declare that they have no conflict of interest.

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