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Exposure to 17α-Ethinylestradiol Alters Brain Histology and Behavioural Response in Fish, *Channa punctatus* (Bloch. 1793)

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Abstract One of the key ingredients in oral contraceptives and the most extensively researched endocrine disruptor globally is 17α-ethynylestradiol (EE2), a synthetic estrogen. The primary way it gets into the environment is via wastewater discharges. In this work, *Channa punctatus*, a freshwater vertebrate model fish, was used to examine the potential effects of ecologically relevant concentrations of EE2 on behaviour and brain histology. Fish that had been laboratory acclimated and were in good condition were split into three groups and given medium treatment exposure to one treatment at each concentration (5, 10, and 20 ng/L of EE2) for 28 days along with the controls in order to study the potential biological pathways. When the concentration of EE2 was raised after 28 days of exposure, there was an increase in structural damage to the brain. The degradation of brain cells with cytoplasmic vacuolization, alterations in the quantity of grey and white matter, and neuronal necrosis were also noted. The Group 4 (20 ng/L) group showed the worst damage to the brain tissues of both males and females. Male brains have the highest levels of necrosis (22.23±0.54) and vacuolization (16.37±0.44), while female brains exhibit lower levels of necrosis (13.73±0.24) and vacuolization (6.3±0.24), respectively. When exposed to 17α-ethinylestradiol, the test fish displayed various unusual behaviours, including restlessness, asymmetrical swimming movements, loss of balance, motionlessness, and sluggish movement. When exposed to 17α-ethynylestradiol, *Channa punctatus* internal brain structure had severe histopathological abnormalities, and its behaviour responded differently from that of control fish, demonstrating the severity of synthetic hormones.

Keywords *Channa punctatus*, Brain Histology, 17α-Ethinylestradiol, Behaviour

1. Introduction

Diverse chemicals found in aquatic environments have the potential to disturb the endocrine system, which in turn may have an impact on organisms. The main sources of steroids in aquatic environments are sewage waste and animal rearing. In the influents and effluents of wastewater treatment facilities (WWTPs), swine farm wastewaters, surface water, and even groundwater, elevated concentrations of steroids have regularly been found [1,2]. According to Aris et al. and Laurenson et al. [3,4], 17α-ethinylestradiol (EE2) is the most powerful endocrine disrupting chemical (EDC) polluting the aquatic environment. Its widespread worldwide presence in effluents of sewage treatment plants (STPs) and surface water [3] and reclaimed water is a matter of great concern [5-8]; it is 10 times more active than estradiol itself and has

a predicted adverse effect level of 0.1 ng/L for water-living organisms. According to Ternes and Kolpin et al. [9,10], it is found in effluents in quantities ranging from 1 ng/L to as high as 2–300 ng/L (U.S. streams). According to Aris et al. [3], EE2 is persistent in the environment and exhibits evidence of biomagnification. Mammal fertility and behaviour have been demonstrated to be impacted by EDC effects at the level of tissue organization in the brain [11-12]. In addition to transcription factors, endogenous hormones like gonadal steroids also play a critical role in the regulation and direction of brain development [13,14].

Histopathology has been extensively used as a biomarker in the evaluation of various stressors (microbial pathogens, toxic compounds, nutritional deficiency, and adverse environmental conditions), both in the laboratory [15] and in field studies [16]. It is an effective tool for visualising the structural changes caused by stress in cells and tissues. As a consequence, an organism experiences negative physiological changes as a whole. Vacuolation of the brain parenchyma and mild enlargement of the cerebrum's pyramidal cells are two characteristics of histopathological alterations in the brain. Vacuolation could have resulted from micorsomal and mitochondrial malfunctions brought on by glycolysis. The chemical's neurotoxic character was shown by the loss of Nissl substances and glial cell responses, including the appearance of glial nodules in certain areas [17].

By regulating, integrating, and managing biological systems, hormones coordinate an individual's physiology and behaviour. As a result, hormones in the environment may have an impact on an animal's behaviour [18]. Although toxicants discharged into natural systems have been shown in several studies to have deadly consequences [19], it has only recently become clear that these chemicals may also have non-lethal behavioural impacts [20]. The behavioural effects of EDCs on freshwater fish are of special relevance since these species are in constant and direct contact with EDCs from a range of sources, such as treated sewage effluent and agricultural runoff [21]. Individual EDCs are often examined in isolation during laboratory studies that quantify EDC effects; however, EDC combinations may have behavioural effects that are distinct from those of a single exposure, such as synergistic or antagonistic effects [22]. There is evidence that EDCs have an impact on non-reproductive behaviour. Fish reproductive behaviour has been extensively examined; however, there is little information on non-reproductive behaviour brought on by EDC exposure. Adult EDC exposure has been linked to changes in bottom living, hazardous behaviour, and school performance [23-27]. Natural estrogens may have neuromodulatory effects on vertebrate locomotor activity by directly influencing neuron function, as shown in earlier investigations on vertebrates [28-30]. Numerous behavioural activities, including courtship displays, foraging, and escape from a dangerous region, may be affected by changes in locomotor activity [31]. Animals' aggressive behaviour facilitates the preservation of a territory or the formation of a dominance hierarchy, which facilitates access to resources including mates, the best places to forage, and shelter [32].

According to several studies, estrogenic EDCs reduce male dominance behaviour and plasma levels of endogenous male hormones (such as 11-ketotestosterone) or change courting behaviour to favour males that are unable to take care of their eggs [33,34]. However, since hormones control behavioural reactions driven by aggressiveness or fear, EDCs may potentially have an impact on an individual's fitness by altering crucial survival behaviours. Fish antipredator and risk-taking behaviours may potentially be affected by EDCs [21]. According to Sih et al. [35], neuroendocrine pathways may be in charge of behavioural syndromes, in which people exhibit linked behaviours in a variety of social or environmental circumstances.

According to Segner [36], an increasing number of chemicals, including those from wastewater treatment facilities, agricultural runoff, industrial effluents, and urban runoff, are polluting aquatic ecosystems and having a negative impact on fish populations. An increasing number of studies over the past few decades have focused on the impact of EDCs on the ability of various fish species to reproduce (Marlatt et al., current issue). But very little is known about the specific mechanism of action of EDCs, and very little research has focused on the impact of EDCs in fish before the commencement of pubertal development. However, research shows that exposure to EDCs causes the following in fish: 1) disruption of gonad development and sex differentiation, causing intersex; 2) abnormal gonad differentiation, affecting the number of germ cells and causing episodes of sterility; and 3) changes in the timing of puberty. The purpose of the current investigation is to look at histological changes in the brain and behavioural reactions of the fish *Channa punctatus* since earlier research has shown the effects of exposure to the synthetic hormone 17α-ethinylestradiol [37].

2. Material and Methods

2.1. Collection and Acclimatization of Test Animal

With the assistance of fishermen, 130 live *Channa punctatus* (15±2 cm and 30±5 g) were procured from adjacent ponds in the month of October 2022. The fish were transported to the lab in a large, well-ventilated bucket and submerged for 5 minutes in a 0.05% KMnO4 solution to eliminate any skin infections. According to protocol, fish were given two weeks to become used to the lab environment [38]. The physicochemical parameters, such as DO, hardness, TDS, alkalinity, temperature, and pH, were observed and confirmed to be within the established limits [39]. Fish were fed artificial fish meal at a regular rate of 2% of body weight twice a day [40].

2.2. Test Chemical

SIGMA-ALDRICH, Co., 3050 Spruce Street, St. Louis, MO 63103, USA; 314-771-5765, produced 17α-Ethynylestradiol (EE2) (>98%). In order to establish specified stock solutions at each test concentration of 0, 5, 10, and 20 ng/L, stock solutions of EE2 in ethanol (purity of 99.9%) were first created.

2.3. Experimental Setup

The experiment was conducted as static. Only healthy and well-acclimatised fish were divided into four groups of three triplicates, each with 12 fish. One control group and three exposure groups each received 5, 10, and 20 ng/L concentrations of EE2 for 28 days, with samples being taken every 7, 14, 21 and 28 days from each group. Throughout the 28-day study period, there was no fish mortality noted. The handling and laboratory-based experimentation procedures were carried out in accordance with the Institutional Animal Ethics Committee's (IAEC, Registration No. 1861/GO/Re/S/16/CPCSEA) published criteria.

2.4. Identification of Male and Female Fish

The gonads of the fish in the sample are paired; they are long, elongated structures that are located ventral to the swim bladder and dorsal to the alimentary canal. The mesenteries held them to the bodily cavity. The size of males was often greater than that of females. Males have an extended genital entrance, while females have a circular aperture. The gonads were taken out of each specimen after it was dissected. The gonads of each specimen were examined to determine their sex. The sex ratio was determined by dividing the proportion of the two sexes by two.

2.5. Histological Evaluation

For histological evaluation n=4 samples were taken throughout the 28-day study period. Clove oil was used as an anaesthetic before the treatment and control groups' fish were slain, and the brains were then removed and stored in Bouin's fixatives. Tissues were processed using the method outlined by Bernet et al. [41] in order to prepare them for histological analysis. To put it quickly, after a 24-hour fixation in Bouin's solution, tissues were dehydrated with alcohol. After being dehydrated, paraffin wax was used to create tissue blocks. Sagittal slices (5 m thick) of the paraffin blocks were cut for histological investigation, and they were then stained with hematoxylin and eosin stains. Photomicrographs of stained sections were taken using light microscopy (a Nikon or Eclipse camera fitted with a Spot Inside DS-Fi2 digital camera). The damage to brain tissue was examined using ImageJ software; version ImageJ comes with 64-bit Java 1.8.0_172 image software.

2.6. Behaviour of Fish

Video-based behavioural monitoring is now often used to collect quantified behavioural data for aquatic risk assessment. A popular method for gathering quantitative behavioural data for aquatic risk assessment is video-based behavioural monitoring. The test fish's behaviour after being exposed to 17α-ethynylestradiol in each tank was regularly observed by eye as well as captured on camera and recorded. Anhui Sharetonic Data Technology Co., Ltd., Anhui Province, China, BHR5003IN, produced the camera that was used to track the changes in the behaviour of the test fish. The fish were recorded on video, which was then thoroughly examined to count the number of lunges, chases, bites, and violent side-to-side and quivering displays they made towards the intruder.

3. Result and Discussion

Histopathology is the study of an illness's or damage's outward symptoms via microscopic inspection of tissue. For information on the alterations caused by chemicals at the tissue and cellular level, toxicological histopathology is helpful [42-44]. Any chemical compound's harmful effects might potentially target any of an animal's tissues or organs. A histological analysis gives insight on the kind of tissue change and the degree of damage, which in turn reveals the compound's toxicity. Different parts of the fish brain are focused on different kinds of significant responsibilities. These compulsive modifications to the brain's tissue might impair a certain function in fish by preventing it from performing its purpose. The fish's physiological and behavioural characteristics are changed as a result. This supports the fish's respiratory issues, loss of harmony, and erratic swimming patterns that were reported in the present inquiry. Due to the adverse effects of fenvalerate on the mind, tremors and convulsions were seen in rainbow trout [45]. Fish mind vascular expansion was seen after exposure to 2,4-D and endosulfan independently, according to Nordin et al. [46]. Pugazhvendan et al. [47] observed that *Ophiocephalus punctatus* exposed to malathion pesticide had dispersed brain cells, severe rot, and a lack of separation in the synapses.

All of the fish in Group 1, the control group, had normal brain tissue in both the male and female fish (Figs. 1 a and b), and there was no evidence of damage to the neural cells. While groups 2, 3, and 4, which were subjected to 5 ng/L, 10 ng/L, and 20 ng/L of EE2 for 28 days, respectively, showed degeneration of neuronal cells, including necrosis and vacuolization (Figs. 2 a & b and 3 a & b). The degree of necrosis in the brains of female fish increased by 9 folds, from 1.43 ±0.12 to 13.73 ±0.24, between the 7th day sample of group 1 (with exposure to 0 ng/L of EE2) and the 28th day sample of group 4 (with exposure to 20 ng/L of EE2). When analysing each group separately, it was observed that group 1 had a degree of necrosis that varied between

1.43 ±0.12 and 1.5 ±0.22, which was not as significant (P > 0.05) as the ranges for the other three groups: group 2's range was between 3.17 ± 0.23 and 6.13 ±0.24, group 3's range was between 7.4 ±0.24 and 9.67 ±0.24, and group 4's range was between 11.2 ±0.25 and 13.73 ±0.24. The amount of necrosis in the male fish's brain increased by 24 times, from 1.43 ±0.22 to 22.23 ±0.54, between the 7th-day sample of group 1 (with exposure to 0 ng/L of EE2) and the 28th (final-day) sample of group 4 (with exposure to 20 ng/L of EE2). When each group was examined separately, it was observed that group 1 had a degree of necrosis that varied between 1.43 ±0.22 and 1.47 ±0.22, which was not as significant (P > 0.05) as the ranges for the other three groups: group 2's range was 8.3 ±0.53 to 12.27 ±0.54, group 3's range was 13.3 ±0.54 to 16.57 ±0.54, and group 4's range was 18.63 ±0.45 to 22.23 ±0.54.

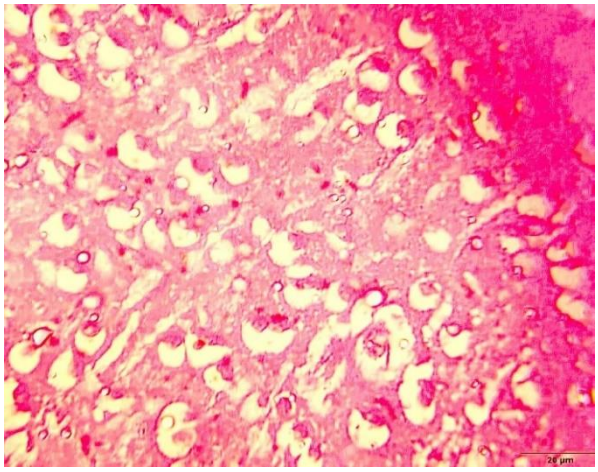

Figure 1a. Histology of brain of female fish Channa punctatus of control group showing normal condition on exposure to 17α-ethynylestradiol for 28 days

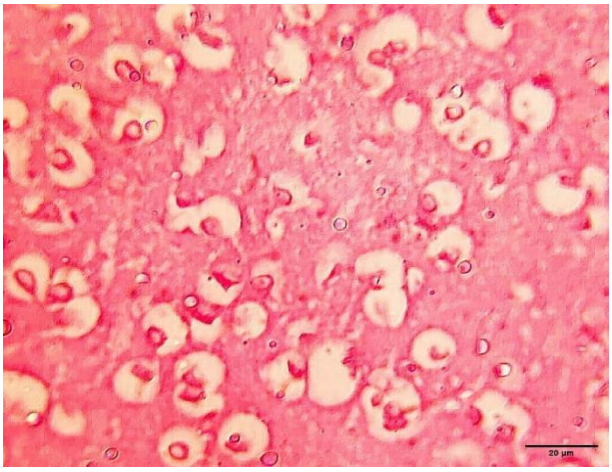

Figure 1b. Histology of brain of male fish Channa punctatus of control group showing normal condition on exposure to 17α-ethynylestradiol for 28 days

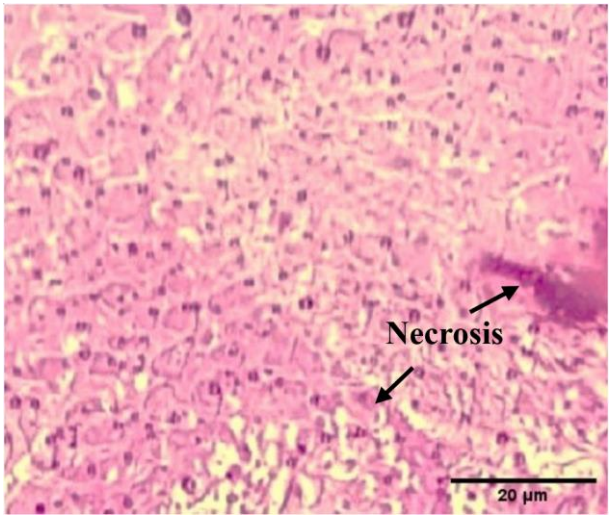

Figure 2a. Histology of brain of female fish Channa punctatus exposed to 20 ng/L showing necrosis on exposure to 1*7α-ethynylestradiol for 28 days*

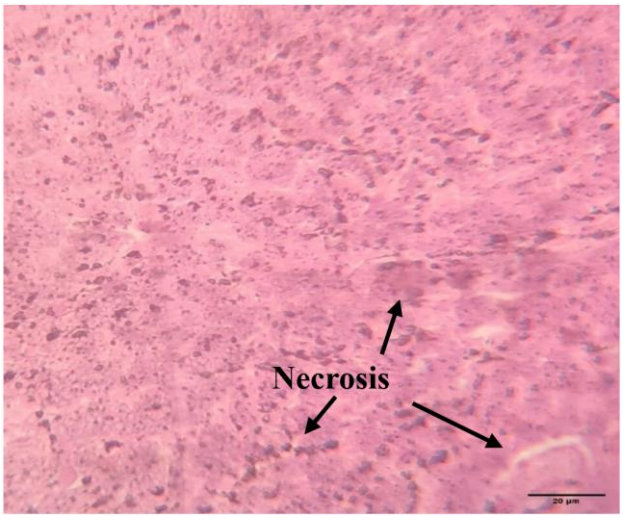

Figure 2b. Histology of brain of male fish Channa punctatus exposed to 20 ng/L showing necrosis on exposure to 17α-ethynylestradiol for 28 days

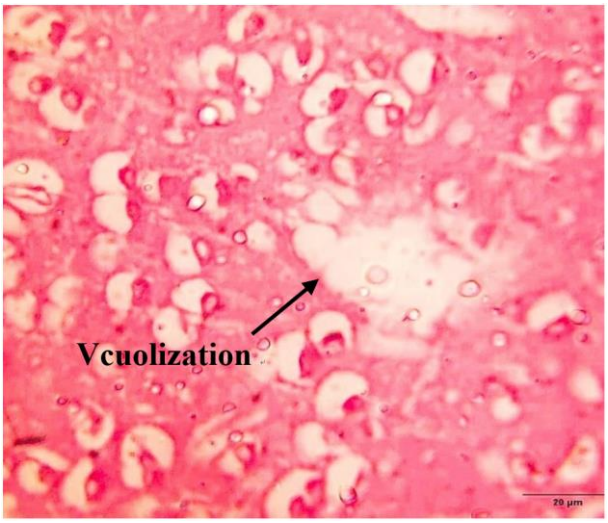

Figure 3a. Histology of brain of female fish Channa punctatus exposed to 20 ng/L showing vacuolization on exposure to 17α-ethynylestradiol for 28 days

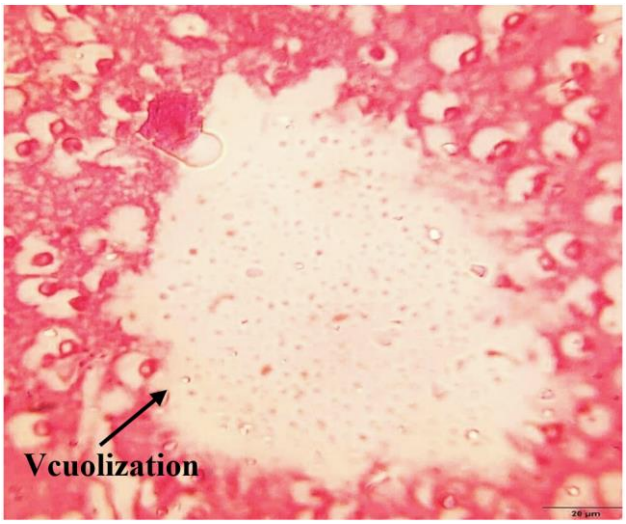

Figure 3b. Histology of brain of male fish Channa punctatus exposed to 20 ng/L showing vacuolization on exposure to 1*7α-ethynylestradiol for 28 days*

Neuronal vacuolation is a hallmark of hydropic degeneration, which may cause neuronal loss in the dorsal root ganglia and the growth of nodules in Nageotte to replace the lost neurons. It goes without saying that "apoptosis" is the only way that neurons might conceivably die in the eyes of certain neuropathologists. However, this is untrue. We have shown in Krinke's work that cytoplasmic vacuoles may be readily produced in an experimental setting and that they are signs of neuronal death due to the toxicity of extraordinarily high dosages of pyridoxine (vitamin B6) [48]. When comparing the level of vacuolization in the brains of female fish from group 1's 7th day sample to group 4's 8th day sample, the level of vacuolization rose similarly by nine times, from 0.67±0.13 to 6.3±0.24. (Table 1). When analysing each group separately, it was observed that group 1's degree of vacuolization ranged between 0.67±0.13 and 0.68±0.12, which was not as significant (P > 0.05) as the other three groups, whose ranges were as follows: group 2's range was between 1.73±0.15 and 3.17±0.21, group 3's range was between 2.17±0.22 and 4.3±0.22, and group 4's range was between 4.27±0.25 and 6.3±0.24. So, it can be inferred that as EE2 concentration grew from 5 ng/L to 20 ng/L and exposure time extended from 7 days to 28 days, the damage to brain tissue significantly increased (Tables 1 and 2).

Table 1. Mean value along with standard error of damage observed in the histology of brain of female fish *Channa punctatus* on exposure to 17α-ethynylestradiol for 28 days

Groups	Conc. (ng/L)	Exposure period	Vacuolization	Necrosis
Group 1	0 ng/L	7 days	0.67±0.13	1.43±0.12
		14 days	0.67±0.13	1.47±0.13
		21 days	0.68±0.12	1.43±0.13
		28 days	0.68±0.12	1.5±0.22
Group 2	5ng/L	7 days	1.73±0.15	3.17±0.23
		14 days	2.17±0.18	4.27±0.24
		21 days	2.57±0.21	5.5±0.25
		28 days	3.17±0.21	6.13±0.24
Group 3	10ng/L	7 days	2.17±0.22	7.4±0.24
		14 days	2.77±0.21	8.13±0.25
		21 days	3.63±0.21	8.83±0.26
		28 days	4.3±0.22	9.67±0.24
Group 4	20ng/L	7 days	4.27±0.25	11.27±0.25
		14 days	4.87±0.24	11.97±0.24
		21 days	5.7±0.23	12.83±0.23
		28 days	6.3±0.24	13.73±0.24

Table 2. Mean value along with standard error of damage observed in the histology of brain of male fish *Channa punctatus* on exposure to 17α-ethynylestradiol for 28 days

Groups	Conc. (ng/L)	Exposure period	Vacuolization	Necrosis
Group 1	0 ng/L	7 days	0.68 ±0.12	1.43 ±0.22
		14 days	0.67 ±0.13	1.5 ±0.23
		21 days	0.67 ±0.13	1.47 ±0.23
		28 days	0.68 ±0.12	1.47 ±0.22
Group 2	5ng/L	7 days	4.37 ±0.23	8.3 ±0.53
		14 days	5.13 ±0.24	9.67 ±0.54
		21 days	5.8 ±0.25	11.3 ±0.45
		28 days	6.23 ±0.24	12.27 ±0.54
Group 3	10ng/L	7 days	8.2 ±0.34	13.3 ±0.54
		14 days	8.83 ±0.35	14.77 ±0.45
		21 days	9.3 ±0.36	15.83 ±0.66
		28 days	9.73 ±0.34	16.57 ±0.54
Group 4	20ng/L	7 days	10.4 ±0.33	18.63 ±0.45
		14 days	12.63 ±0.54	19.67 ±0.54
		21 days	14.67 ±0.45	20.2 ±0.45
		28 days	16.37 ±0.44	22.23 ±0.54

In the brain of male fish, when the degree of vacuolization was compared between the 7th day sample of group 1 and the 28th day sample of group 4, it also increased by 24-fold, from 0.68 ±0.12 to 16.37 ±0.44 (Table 2). When examining each group separately, it was observed that group 1 had a degree of vacuolization that varied between 0.68 ±0.12 and 0.68 ±0.12, which was not as significant ($P > 0.05$) than the ranges of the other three groups: group 2's range was 4.37 ±0.23 to 6.23 ±0.24, group 3's range was 8.2 ±0.34 to 9.73 ±0.34, and group 4's range was 10.40 ±0.33 to 16.37 ±0.44. With rising EE2 concentrations from 0 ng/L to 20 ng/L and an increasing time period from 7 days to 28 days, brain tissue damage increased dramatically. In an experiment investigating histological indications of sublethal toxicity of copper ions in *Labeo catla*, [49] found similar results.

3.1. Changes in Behaviour of Test Fish

In fish and other aquatic species, animal behaviour offers a crucial integrated measurement of neuroendocrine function. According to Scott and Sloman [50] behaviour serves as a connection between ecological processes at the scale of populations and communities and physiological processes at the scale of the individual animal. Changes in social dominance and courting behaviours might indicate the possibility of adverse impacts on the lifetime reproductive success of exposed animals in the case of environmental pollutants that operate on the fish endocrine system [51].

Channa puctatus behaviour was significantly altered in the current study as a result of exposure to EE2. Unstable behaviour, uneven swimming movements, loss of stability, motionlessness, and sluggish movement were observed, as well as a decrease in swimming activity, a change in pigmentation, and bottom dwelling throughout the experiment.

They first stopped moving and gathered at the bottom, but after some time, they resumed their regular pace of swimming. During the second exposure week, the colour patterns in group 3 (10 ng/L) aquaria receiving EE2 were transformed from normal to brilliant, while the controls and the aquaria of groups 2 and 4 treated with 5 and 20 ng/L concentrations maintained their typical colouring. Zebrafish exposed to lead showed reduced learning and changed colour preferences [52,53].

Controlling the fish's hormone flow is one of the duties of the medulla oblongata, which is located underneath the cerebellum. Additionally, it regulates the rhythmic contraction of the gill muscles during breathing, the heart, and the smooth muscles of the internal organs. With the greatest concentration of EE2 dissolved in aquaria, the degree of necrosis and vacuolization in this region were observed, and as a result, the highest rate of air gulping was seen in fish from groups 3 (10ng/l) and 4 (20 ng/L). When there is not enough oxygen in the water, test animals will air gulp, coming to the surface to get oxygen from the air. Additionally, fish in group 4 (20 ng/L) exposed to EE2 swim erratically and exhibit circular movement. Fipronil generates notochord degeneration and locomotor abnormalities in zebrafish embryos and larvae, and it causes neuronal hyperexcitation by blocking GABAergic neurotransmission [54].

It is possible to hypothesize that the increased lethargy of EE2-exposed spotted snakeheads is due to estrogenic neurotoxicity because of the processes of EE2 disruption. In fact, estrogens have previously been proven to be neurotoxic in mammals, inducing variations in locomotor activity [29] and the motor-sensory endocrine axis of vertebrates tends to be well preserved [30].

According to the study's findings, behavioural endpoints may be very helpful in determining the danger of EDCs in wild fish populations. However, we must consider that field animals are exposed to chemical combinations, which may result in behavioural reactions that are different from those of single exposures. In fact, according to Sarria et al. [21] this seems to be the case for combinations of EE2 and the androgenic substance tributyltin.

This research assessed the association between hormone levels and levels of behaviour, drawing on behavioural endocrinological investigations that have shown that androgens influence the presence or absence of nest building and territorial activity [55,56].

In their F1 generation of zebrafish, Nash et al. [57] showed that low and environmentally relevant concentrations of EE2 (5 ng/L) significantly reduced reproductive success. Their findings have been confirmed in other species, including minnows [58] and medaka [59]. Fenske et al. [60] observed gonadal feminization in 100% of exposed zebrafish at a dosage of just 3 ng/L EE2

throughout development, which clearly has an impact on the survival of the species. A long-term experimental lake study by Kidd et al. [61] showed that fathead minnow populations completely collapsed after being exposed to 5–6 ng/L EE2.

4. Conclusions

To the best of our knowledge, this is the first investigation into how the EDC EE2 affects freshwater fish behaviour and brain histology. Growing attention is being paid to environmental issues, including how to safeguard them and what effects contaminants have on the health of living things. One class of contaminants that has lately caught the attention of scientists is sex hormones. According to the material that is currently available, estrogen-related hormones are the most significant of them in terms of their effects on the environment. They may have a variety of detrimental effects on health or the operation of organisms that are directly or indirectly connected to them when they occur in the environment. These effects may include feminization, dysregulation of biological processes involved in reproduction, degradation of organismal health generally, problems in the control of apoptotic processes, or even encouragement of processes involved in the development of cancer. Even with the use of current filtering techniques, it is difficult to completely remove estrogen from the aquatic environment.

Because several of its concentrations cause significant alterations in the histology of the brain and behaviour of the fish *Channa punctatus*, the use of EDCs, particularly 17α-ethynylestradiol, should be carefully monitored.

Contributions from Authors

All writers contributed to the completion of this work. Experimental planning, data analysis, graph production, and paper writing were all handled by author Zainab Khatoon. The experiment was carried out, and test animals were gathered by author Nooreen Fatima. The manuscript's final editing was carried out by author Vivek Kumar. Sunil P. Trivedi, the author, oversaw the experiment and provided advice on article development. The final text was reviewed and approved by all writers.

Compliance with Ethical Standards

According to the guidelines set out by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), the University of Lucknow in Lucknow established an Institutional Animal Ethics Committee (IAEC) with registration number 1861/GO/Re/S/16/CPCSEA. The authors carried out the experiment in accordance with the CPCSEA's specified procedures.

REFERENCES

[1] Huang, G.Y., Liang, Y.Q., Liu, Y.S., Shi, W.J., Liu, S.S., Hu, L.X., Xie, L.T., Ying, G.G., 2019a. Swine farm wastewater discharge causes masculinization of western mosquitofish (*Gambusia affinis*). Environ. Int. 123, 132–140. https://doi.org/ 10.1016/j.envint.2018.11.066.

[2] Zhang, J.N., Ying, G.G., Yang, Y.Y., Liu, W.R., Liu, S.S., Chen, J., Liu, Y.S., Zhao, J.L., Zhang, Q.Q., 2018. Occurrence, fate and risk assessment of androgens in ten wastewater treatment plants and receiving rivers of South China. Chemosphere 201, 644–654. https://doi.org/10.1016/j.chemosphere.2018.02.144.

[3] A. Z. Aris, A. S. Shamsuddin, and S. M. Praveena, "Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: A review," *Environment International*, vol. 69. Elsevier Ltd, pp. 104–119, 2014. doi: 10.1016/j.envint.2014.04.011.

[4] J. P. Laurenson, R. A. Bloom, S. Page, and N. Sadrieh, "Ethinyl estradiol and other human pharmaceutical estrogens in the aquatic environment: A review of recent risk assessment data," *AAPS Journal*, vol. 16, no. 2. Springer New York LLC, pp. 299–310, 2014. doi: 10.1208/s12248-014-9561-3.

[5] K. L. Thorpe *et al.*, "Relative potencies and combination effects of steroidal estrogens in fish," *Environ Sci Technol*, vol. 37, no. 6, pp. 1142–1149, Mar. 2003, doi: 10.1021/es0201348.

[6] J. S. Denny *et al.*, "comparison of relative binding affinities of endocrine active compounds to *Fathead minnow* and *Rainbow trout* estrogen receptors," 2005.

[7] D. J. Caldwell, F. Mastrocco, P. D. Anderson, R. Länge, and J. P. Sumpter, "Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol, and 17α-ethinylestradiol," *Environ Toxicol Chem*, vol. 31, no. 6, pp. 1396–1406, Jun. 2012, doi: 10.1002/etc.1825.

[8] S. Mompelat, B. Le Bot, and O. Thomas, "Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water," *Environ Int*, vol. 35, no. 5, pp. 803–814, 2009, doi: 10.1016/j.envint.2008.10.008.

[9] T. A. Ternes, "Occurrence of drugs in German sewage treatment plants and rivers," *Water Res*, vol. 32, no. 11, pp. 3245–3260, 1998.

[10] D. W. Kolpin*et al.*, "Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: A national reconnaissance," *Environ Sci Technol*, vol. 36, no. 6, pp. 1202–1211, 2002.

[11] D. Crews and J. A. McLachlan, "Epigenetics, evolution, endocrine disruption, health, and disease," *Endocrinology*, vol. 147, no. 6. Jun. 2006. doi: 10.1210/en.2005-1122.

[12] S. M. Dickerson and A. C. Gore, "Estrogenic environmental endocrine-disrupting chemical effects on reproductive neuroendocrine function and dysfunction across the life cycle," *Reviews in Endocrine and Metabolic Disorders*, vol.

8, no. 2. pp. 143–159, Jun. 2007. doi: 10.1007/s11154-007-9048-y.

[13] K. I. Matsuda, H. Mori, and M. Kawata, "Epigenetic mechanisms are involved in sexual differentiation of the brain," *Reviews in Endocrine and Metabolic Disorders*, vol. 13, no. 3. pp. 163–171, Sep. 2012. doi: 10.1007/s11154-012-9202-z.

[14] W. C. J. Chung and A. P. Auger, "Gender differences in neurodevelopment and epigenetics," *PflugersArchiv European Journal of Physiology*, vol. 465, no. 5. pp. 573–584, May 2013. doi: 10.1007/s00424-013-1258-4.

[15] P. W. Wester and J. H. Canton, "The usefulness of histopathology toxicity studies in aquatic," 1991.

[16] S. J. Teh, S. M. Adams, and D. E. Hinton, "Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress," *Aquatic toxicology*, vol. 37, no. 1, pp. 51–70, 1997.

[17] Azize Al sawafi, A. G., Wang, L., & Yan, Y. (2017). Cadmium Accumulation and Its Histological Effect on Brain and Skeletal Muscle of Zebrafish. *Journal of Heavy Metal Toxicity and Diseases*, *02*(01). https://doi.org/10.21767/2473-6457.100017

[18] O. H. Meléndez-Fernández, J. A. Liu, and R. J. Nelson, "Circadian Rhythms Disrupted by Light at Night and Mistimed Food Intake Alter Hormonal Rhythms and Metabolism," *Int J Mol Sci*, vol. 24, no. 4, p. 3392, 2023.

[19] G. E. Howe, R. Gillis, and R. C. Mowbray, "Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae," 1998.

[20] T. Brodin, J. Fick, M. Jonsson, and J. Klaminder, "Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations," *Science (1979)*, vol. 339, no. 6121, pp. 814–815, Feb. 2013, doi: 10.1126/science.1226850.

[21] M. P. Sária, J. Soares, M. N. Vieira, L. Filipe, M. M. Santos, and N. M. Monteiro, "Rapid-behaviour responses as a reliable indicator of estrogenic chemical toxicity in zebrafish juveniles," *Chemosphere*, vol. 85, no. 10, pp. 1543–1547, 2011, doi: 10.1016/j.chemosphere.2011.07.048.

[22] M. P. Sária, J. Soares, M. N. Vieira, L. Filipe, M. M. Santos, and N. M. Monteiro, "Rapid-behaviour responses as a reliable indicator of estrogenic chemical toxicity in zebrafish juveniles," *Chemosphere*, vol. 85, no. 10, pp. 1543–1547, 2011, doi: 10.1016/j.chemosphere.2011.07.048.

[23] A. M. Bell, "An endocrine disrupter increases growth and risky behavior in threespined stickleback (*Gasterosteus aculeatus*)," *HormBehav*, vol. 45, no. 2, pp. 108–114, Feb. 2004, doi: 10.1016/j.yhbeh.2003.09.009.

[24] T. L. Dzieweczynski, B. A. Campbell, J. M. Marks, and B. Logan, "Acute exposure to 17α-ethinylestradiol alters boldness behavioral syndrome in female Siamese fighting fish," *HormBehav*, vol. 66, no. 4, pp. 577–584, Aug. 2014, doi: 10.1016/j.yhbeh.2014.08.005.

[25] A. E. Wibe, G. Rosenqvist, and B. M. Jenssen, "Disruption of male reproductive behavior in threespine stickleback *Gasterosteus aculeatus* exposed to 17β-estradiol," *Environ Res*, vol. 90, no. 2, pp. 136–141, 2002.

[26] Å. E. Wibe, A. Billing, G. Rosenqvist, and B. M. Jenssen, "Butyl benzyl phthalate affects shoaling behavior and bottom-dwelling behavior in threespine stickleback," *Environ Res*, vol. 89, no. 2, pp. 180–187, 2002, doi: 10.1006/enrs.2002.4360.

[27] J. Xia, C. Niu, and X. Pei, "Effects of chronic exposure to nonylphenol on locomotor activity and social behavior in zebrafish (*Danio rerio*)," *Journal of Environmental Sciences*, vol. 22, no. 9, pp. 1435–1440, Sep. 2010, doi: 10.1016/S1001-0742(09)60272-2.

[28] P. G. Mermelstein, J. B. Becker,'%, and D. J. Surmeier3, "Estradiol Reduces Calcium Currents in Rat Neostriatal Neurons via a Membrane Receptor," 1996.

[29] M. J. Kelly, J. Qiu, E. J. Wagner, and O. K. Rønnekleiv, "Rapid effects of estrogen on G protein-coupled receptor activation of potassium channels in the central nervous system (CNS)," *J Steroid Biochem Mol Biol*, vol. 83, no. 1–5, pp. 187–193, 2002.

[30] P. S. Dickinson, "Neuromodulation of central pattern generators in invertebrates and vertebrates," *Current Opinion in Neurobiology*, vol. 16, no. 6. pp. 604–614, Dec. 2006. doi: 10.1016/j.conb.2006.10.007.

[31] E. E. Little, R. D. Archeski, B. A. Flerov, and V. I. Kozlovskaya, "E Behavioral Indicators of Sublethal Toxicity in Rainbow Trout," 1990.

[32] R. Gerlai, "Zebra Fish: An Uncharted Behavior Genetic Model," 2003.

[33] M. Saaristo, J. A. Craft, K. K. Lehtonen, and K. Lindström, "An endocrine disrupting chemical changes courtship and parental care in the sand goby," *Aquatic Toxicology*, vol. 97, no. 4, pp. 285–292, May 2010, doi: 10.1016/j.aquatox.2009.12.015.

[34] K. Shenoy, "Environmentally realistic exposure to the herbicide atrazine alters some sexually selected traits in male guppies," *PLoS One*, vol. 7, no. 2, Feb. 2012, doi: 10.1371/journal.pone.0030611.

[35] A. Sih, A. M. Bell, J. C. Johnson, and R. E. Ziemba, "Behavioral syndromes: An integrative overview," *Quarterly Review of Biology*, vol. 79, no. 3. pp. 241–277, Sep. 2004. doi: 10.1086/422893.

[36] Segner, H., 2011. Reproductive and Developmental Toxicity in Fishes, in: Reproductive and Developmental Toxicology. Elsevier Inc., pp. 1145–1166. https://doi.org/10.1016/B978-0-12-382032-7.10086-4.

[37] Gonzalez, J. A., Histed, A. R., Nowak, E., Lange, D., Craig, S. E., Parker, C. G., Kaur, A., Bhuvanagiri, S., Kroll, K. J., Martyniuk, C. J., Denslow, N. D., Rosenfeld, C. S., & Rhodes, J. S. (2021). Impact of bisphenol-A and synthetic estradiol on brain, behavior, gonads and sex hormones in a sexually labile coral reef fish. *Hormones and Behavior*, *136*. https://doi.org/10.1016/j.yhbeh.2021.105043

[38] R. M. Burress, *Development and evaluation of on-site toxicity test procedures for fishery investigations*, vol. 68. US Department of the Interior, Fish and Wildlife Service, 1976.

[39] APHA. (2017). Standard Methods for Examination of water

and wastewater. 23rd editi. Rodger B. Baird. Andrew D. Eaton EWR, editor. Washington, DC 20001-3710: American Public Health Association.

[40] D. Dogan, C. Can, A. Kocyigit, M. Dikilitas, A. Taskin, and H. Bilinc, "Dimethoate-induced oxidative stress and DNA damage in *Oncorhynchus mykiss*," *Chemosphere*, vol. 84, no. 1, pp. 39–46, 2011, doi: 10.1016/j.chemosphere.2011.02.087.

[41] D. Bernet, H. Schmidt, W. Meier, P. Burkhardt-Holm, and T. Wahli, "Histopathology in fish: proposal for a protocol to assess aquatic pollution."

[42] U. Sivaiah, "Azadirachtin And Monocrotophos Effect On Haematological Teratological Biochemical And CytoArchitectural Studies in Albino Mice," 2006.

[43] B. Velmurugan, M. Selvanayagam, E. I. Cengiz, and E. Unlu, "Histopathological changes in the gill and liver tissues of freshwater fish, *Cirrhinus mrigala* exposed to dichlorvos," *Brazilian Archives of Biology and Technology*, vol. 52, no. 5, pp. 1291–1296, Sep. 2009, doi: 10.1590/S1516-89132009000500029.

[44] Tripathi M, Mishra RP, and Girdoniya V, "Histopathological changes in liver of a teleost fish *Catla catla* treated with 1.2% lindane," vol. 2, no. 1, pp. 17–19, 2011, [Online]. Available: http://www.bioinfo.in/contents.php?id=68

[45] S. P. Bradburyjames M Mckim and A. R. Coats, "Physiological Response of Rainbow Trout (*Salmo gairdneri*) to Acute Fenvalerate Intoxication'," 1987.

[46] I. L. Nordin, N. Ibrahim, S. A. Ahmad, N. L. Hamidin, F. A. Dahalan, and M. Y. A. Shukor, "Endosulfan Toxicity to *Anabas testudineus* and Histopathological Changes on Vital Organs," in *E3S Web of Conferences*, EDP Sciences, Mar. 2018. doi: 10.1051/e3sconf/20183402055.

[47] S. R. Pugazhvendan, N. J. Narendiran, R. G. Kumaran, S. Kumaran, and K. M. Alagappan, "Effect of malathion toxicity in the freshwater fish *Ophiocephalus punctatus*-A histological and histochemical study," *World Journal of Fish and Marine Sciences*, vol. 1, no. 3, pp. 218–224, 2009.

[48] G. Krinke, H. H. Schaumburg, P. S. Spencer, J. Suter, P. Thomann, and R. Hess, "Pyridoxine megavitaminosis produces degeneration of peripheral sensory neurons (sensory neuronopathy) in the dog.," *Neurotoxicology*, vol. 2, no. 1, pp. 13–24, 1981.

[49] J. M. Patel and A. Bahadur, "Histopathological manifestations of sub lethal toxicity of copper ions in *Catla catla*.," *American-Eurasian Journal of Toxicological Sciences (AEJTS)*, vol. 3, no. 1, pp. 1–5, 2011.

[50] G. R. Scott and K. A. Sloman, "The effects of environmental pollutants on complex fish behaviour: Integrating behavioural and physiological indicators of toxicity," *Aquatic Toxicology*, vol. 68, no. 4. pp. 369–392, Jun. 30, 2004. doi: 10.1016/j.aquatox.2004.03.016.

[51] S. M. Zala and D. J. Penn, "Abnormal behaviours induced by chemical pollution: A review of the evidence and new challenges," *Animal Behaviour*, vol. 68, no. 4. pp. 649–664, Oct. 2004. doi: 10.1016/j.anbehav.2004.01.005.

[52] Bault, Z. A., Peterson, S. M., and Freeman, J. L. (2015). Directional and Color Preference in Adult Zebrafish: Implications in Behavioral and Learning Assays in Neurotoxicology Studies. J. Appl. Toxicol. 35, 1502–1510. doi:10.1002/jat. 3169

[53] Xu, X., Weber, D., Burge, R., and VanAmberg, K. (2016). Neurobehavioral Impairments Produced by Developmental Lead Exposure Persisted for Generations in Zebrafish (*Danio rerio*). Neurotoxicology 52, 176–185. doi:10.1016/j.neuro.2015.12.009.

[54] Tingle, C. C. D., Rother, J. A., Dewhurst, C. F., Lauer, S., and King, W. J. (2003). "Fipronil: Environmental Fate, Ecotoxicology, and Human Health Concerns," in Reviews of Environmental Contamination and Toxicology. 1–66. Editor G. W. Ware (New York, NYNew York: Springer). doi:10.1007/978-1-4899- 7283-5_1.

[55] B. Borg, "Stimulation of reproductive behaviour by aromatizable and non-aromatizable androgens in the male three-spined stickleback, *Gasterosteus aculeatus* L," in *Proc V Congreurop Ichthyol Stockholm*, 1987, pp. 269–271.

[56] C. Bornestaf, E. Antonopoulou, I. Mayer, and B. Borg, "Effects of aromatase inhibitors on reproduction in male three-spined sticklebacks, *Gasterosteus aculeatus*, exposed to long and short photoperiods," 1997.

[57] J. P. Nash *et al.*, "Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish," *Environ Health Perspect*, vol. 112, no. 17, pp. 1725–1733, Dec. 2004, doi: 10.1289/ehp.7209.

[58] R. Lange *et al.*, "Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*)," 2001. [Online]. Available: http://nbn-resolving.de/urn:nbn:de:bsz:352-opus-50153

[59] G. C. Balch, C. A. Mackenzie, and C. D. Metcalfe, "alterations to gonadal development and reproductive success in japanese medaka (*Oryzias latipes*) exposed to 17α-ethinylestradiol," 2004. [Online]. Available: http://www.fon.hum.uva.nl/Service/Statistics/Wilcoxon

[60] M. Fenske, G. Maack, C. Scha ̈fers, S. Scha ̈fers, and H. Segner, "an environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *Danio rerio*," 2005.

[61] K. A. Kidd *et al.*, "Collapse of a fish population after exposure to a synthetic estrogen." [Online]. Available: www.pnas.orgcgidoi10.1073pnas.0609568104.