Volume 65, Issue 5, 2021
Journal of Scientific Research
of
The Banaras Hindu University

4-Nonylphenol affects the structure and function of testis in catfish *H. fossilis* and *C. batrachus*

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Abstract: **Nonylphenol (4-NP) is a persistent organic water pollutant that disturbs many physiological processes in aquatic species. This experiment was taken up to compare the effects of 4-NP on the two indigenous male catfish *Heteropneustes fossilis* and *Clarias batrachus*. Effects of 4-NP (low dose: 64µg.L^{-1} and high dose: 160 µg.L^{-1}) on the behaviour, gonadosomatic index (GSI), structure, and function of testis of both fish were analyzed. Fishes were exposed during the preparatory to pre-spawning phase for 30, 45, and 60 days. There was a significant dose- and duration-dependent decrease in the GSI. Histopathological alterations were observed in the testes of both along with altered steroid biosynthesis. There was a dose and duration-dependent decrease in the level of testosterone. Estradiol 17β(E_2) level was significantly increased at a low dose for 30 days but significantly decreased at 45 and 60 days. E_2 level was significantly decreased at a high dose for all durations in testes of *H. fossilis* and *C. batrachus.* Even though the catfishes investigated were physiologically different their response toward the 4-NP toxicity was not significantly different. Thus, we can conclude that 4-NP affects the structure and function of testes as well as steroid hormone levels in both catfish species.**

Index terms- **E_2, GSI, LC_{50}, 4-NP, Testosterone.**

I . INTRODUCTION

Xenoestrogens or environmental estrogens are capable of perturbing spermatogenesis and hampers the fertility of fishes. Nonylphenol is one of the potent xenoestrogens (Vielela et al 2018, Desbrow et al 1998, Rodger-Gray et al 2000, Synder et al 2001). Nonylphenol is extensively used as industrial organic compounds and as a biodegradation byproduct of alkylphenol ethoxylates which are a form of industrial effluents and released in water bodies. Due to lipophilicity, it accumulates in tissues of aquatic species, and via biomagnification, it reaches the human population. Nonylphenol is used as non-ionic surfactants in industrial, agriculture, and domestic applications. Much research has been conducted and elucidated that the xenoestrogen attributes to the alteration of the development of the reproductive system in mammalian models (eg. rats, mice) and aquatic organisms (eg. fish, crustaceans) (Roig et al., 2014; Saravanan et al., 2019). Recently our laboratory studies, have reported that 4-NP has a severe effect on the viability of eggs and embryonic development of catfish *H. fossilis* (Chaube et al, 2013), and its bioaccumulation in various tissues and toxicity (Gautam et al., 2015), oocyte maturation and germinal vesicle breakdown (Gautam et al. 2018).

Among all living organisms, fishes are a good biological biomarker for indicating the toxicological risk of pollutants in aquatic ecosystems. Catfish *H. fossilis* and *C. batrachus* are some of the most popular high protein-rich and low-fat content edible native fish of the Asian subcontinent. It is also termed as popular poor fish and has a good potential for rural aquaculture (Chaube et al., 2019). In this study, we have investigated the comparative effects of 4-NP on histology and physiology of testes of catfish *H. fossilis* and *C.batrachus*. As, per the study report given by Paul et al, 2016 both catfishes have different content of essential, non-essential amino acid, and fatty acid content. The Vitamin A and D contents were significantly higher in magur (*C.batrachus*) when compared to singhi (*H. fossilis*). The Vitamin K content was significantly higher in singhi compared to magur. Monounsaturated fatty acid (MUFA) content was reported to be more in *H. fossilis* while polyunsaturated fatty acid content was more in *C. batrachus* (Paul et al 2016). This variation in their lipid content could reflect in their response towards lipophilic pollutants like 4-NP. They dwell in the bottom of the water bodies so, they are prone

to be largely exposed to various pollutants that tend to concentrate more in the sediments such as 4-NP. No comparative study has been conducted on these particular species of fish i.e.*H. fossilis* and *C. batrachus*. Hence in this present study, we decided to compare the effect of the same dose of 4-NP on both the physiologically different male catfishes concerning their reproductive function.

II. MATERIAL AND METHODS

A. Chemicals

4-Nonylphenol(liquid),99%, a mixture of isomers (CAS: 84852-15-3) was purchased from Acros Organics (Geel, Belgium). Hormone assays were performed by specific ELISA kits from Dia Metra (Giustozzi, Foligno, PG Italy for E_2 (REF-DKO003, LOT-4511A) and testosterone (REF-DKO002, LOT-4510A). All chemicals were of analytical grade and purchased locally.

B. Collection and acclimatization of animals.

To study the effect of 4-nonylphenol on male *H. fossilis* and *C. batrachus* 275 sexually mature male catfishes (30-40g; length=15±3.5cm) were bought from the local fish market of Varanasi, Uttar Pradesh during preparatory (March-April; 11.5L: 12.5D, 22±2˚c; GSI: 1.15 ± 0.04 percentage) phases. Fishes were maintained in the 20-L flow-through aquarium and they were disinfected with 0.1% $KMNO_4$ They were kept for one week in laboratory conditions under ambient temperature and photoperiod to overcome stressed conditions due to transportation. During the acclimatization and course of the experiment, they were fed on goat liver *ad libitum*. All experiments and methodologies were performed as per the guidelines and regulations of the Animal Ethics Committee of Banaras Hindu University, Varanasi (F.Sc./IAEC/2016-17/113S), and national guidelines for experimentation in animals. Intensive care was given to prevent cruelty of any kind.

C. Exposure to 4-NP

4-Nonylphenol (4-NP) was dissolved in ethanol and diluted with triple distilled water to obtain the desired concentrations (64 $\mu g.L^{-1}$ and 160 $\mu g.L^{-1}$). Based on the lethal concentration dose (LC_{50}) value these concentrations were selected as mentioned in Gautam et al. (2015).

From the preparatory phase, fishes that were acclimatized were maintained in three different tanks of 10-L capacity. Group 1, group 2, group 3 were control, low dose (64$\mu g.L^{-1}$ $1/25^{th}$ of LC_{50}), and high dose (160 $\mu g.\ L^{-1}$ $1/10^{th}$ of LC_{50}) respectively. Treatment was done for 30 days,45 days, and 60 days in semi-static conditions. After completion of the experiment, fishes were weighed and sacrificed by decapitation. Testes were dissected out, weighed fixed in Bouin's fluid, and stored in 70% alcohol. The GSI was calculated as GSI (%) = weight of gonad/ weight of fish x 100.

D. Histological analysis

Tissues were further processed for histology. Five µm paraffin sections were stained with hematoxylin and eosin routinely. Photomicrographs were taken by using a Leica DM LS microscope equipped with a Leica DFC310 FX camera (Leica DM 2000 LED, Germany). Histological demarcations were described by using the HE staining method.

E. Determination of 17β- estradiol and T concentrations

Steroid extraction from tissues was described earlier (Gautam, et al., 2018). E_2 and T levels were measured by enzyme-linked immunosorbent assays (ELISA), followed by the manufacturer's instructions. Optical density was read at 450nm in Market Microplate Absorbance Reader (BioRad, USA), and the concentration was expressed as ng/ml or ng/g. The intra-assay and inter-assay coefficients of variance for E_2 and T were found to be≤ 9% and ≤ 10% for E_2 and for T the intra-assay and inter-assay coefficients of variance were found to be ≤ 7.0% and ≤8.3% respectively.

F. Sperm Collection and analysis

Testis was dissected out from *H. fossilis* and *C. batrachus*, cut into small pieces, and macerated in mortar pestle with 0.9% NaCl solution (1:3.5, w/v). The sperm suspension was collected and centrifuged at 700xg for 2 min to segregate debris and 200µl of supernatant from the middle column was collected from each testis suspension (Lal et al 2013) and was immediately under the Laborlux microscope (Leica Microsystems, CMS, Gmbh, Germany) at 20x to observe the percentage of motile sperm at 24±2˚C. The sperm suspensions exhibiting more than 70-80% motility were pooled to avoid the variation of sperm activity of individual fish. The sperm concentration of pooled milt was determined by counting 200x diluted sperm using a Neubauer cell-counting chamber of hemocytometer (Marienfeld Superior, Lauda-Konigshofen, Germany). Samples were loaded and counted at four corners for each sample using a microscope (20x magnification) (Leica Microsystems, CMS, Gmbh, Germany) and results were analyzed as the number of spermatozoa per µl of milt.

The sperm viability test was analyzed by performing an eosin nigrosin sperm viability test (Moskovtsev and Librach, 2013). The staining solution was prepared with 1% eosin Y, 10%nigrosin, and 0.9% sodium chloride (Sigma-Aldrich Co, MO, USA) in distilled water.1ml of each sample was mixed with 1ml of staining solution1:1 ratio. The suspension was incubated for the 30s and then spread on the labeled microscope slide. The smear was air-dried and examined directly with light microscopy.

G. Statistical Analysis

Appraisal of data through a one-way analysis of variance (ANOVA) followed by post hoc test, Tukey's multiple range test (P<0.05). Data were expressed as mean± standard error mean (SEM) (N=5). In SPSS16 software all the statistical data were interpreted (SPSSInc, Chicago, IL, USA).

III. RESULTS AND DISCUSSION

A. Behaviour and physiological changes

Exposure to 4-NP affected the behaviour of both the catfishes, they showed erratic behaviour such as decreased movements, high pigmentation and increased mucus secretion. There was gradual decrese in the reflexes, feeding, and increase in pigmentation as well as mucous secretions with respect to time and dose. Similar such observation was reported by Ward et al, (2005) and Sharma et al, (2015), who reported erratic behaviour in catfishes due to 4-NP toxicity at acute as well as chronic exposures.

B. Gonadosomatic Index (GSI)

The 4NP exposure decreased GSI index from preparatory to spawning significantly in a concentration and season-dependent manner (Table. I and II). The 30, 45, and 60 days of exposure to low dose and high dose i.e.,64 µg.L-1 and 160 µg.L-1 respectively caused a gradual declined in GSI values in both the catfishes i.e., *H. fossilis* and *C. batrachus.* Others study also reported the decreased GSI value in nonmammalian aquatic vertebrates (Milnes et al 2006); in African sharptooth catfish, *Clarias gariepinus* (Sayed et al 2012); male Nile tilapia (Amer et al 2019), and rainbow trout, *Oncorhynchus mykiss* (Harris et al 2001) due to exposure to estrogenic contaminants. The reason behind the declined GSI value might be the reduction of gonadal mass of *H. fossilis* and *C. batrachus*. NP toxicity decreases the activity of hypothalamic – pituitary axis which may also be responsible for the decline of GSI value due do morphological and physiological changes in the gonads (Kime et al 1999, Cardinali et al 2004, Louiz et al 2009, Sayed et al 2012, Saravanan et al 2019).

Table .I: Effect of 4NP on the gonadosomatic index of *H. fossilis*

	Preparatory (P<0.001)	Pre-spawning (P<0.001)	Spawning (P<0.001)
Control	3.06 ± 0.01	5.56 ± 0.01	7.05 ± 0.01
64 µg.L^{-1} (Low dose 4NP)	2.3 ± 0.1	4.3 ± 0.1	5.13 ± 0.1
160 µg.L^{-1} (High dose 4NP)	2.01 ± 0.01	4.01 ± 0.01	4.54 ± 0.01

Table .II: Effect of 4NP on the gonadosomatic index of *C .batrachus*

	Preparatory (P<0.001)	Pre-spawning (P<0.001)	Spawning (P<0.001)
Control	3.54 ± 0.01	6.60± 0.01	8.03± 0.01
64 µg.L^{-1} (Low dose 4NP)	2.65± 0.1	5.02± 0.1	6.80± 0.1
160 µg.L^{-1} (High dose 4NP)	2.03± 0.01	4.52± 0.01	5.66± 0.01

C. Histological alterations in testes of H. fossilis and C. batrachus:

The cellular structure of control untreated testes of *H. fossilis* and *C. batrachus* shows the demarcation of a well-arranged cellular structure (Fig. 1A, & 2A). The lobule of the testis displayed a germinal cyst of different stages of spermatozoa. The seminiferous tubule was surrounded by interlobular connective tissue with well-marked Leydig cells. Each compartment was well organized into primary spermatogonia, secondary spermatogonia, Sertoli cells, lobule walls and at the central space called the lobular lumen, there was a preponderance of spermatozoa. Normal histological features of testes of *H. fossilis* and *C. batrachus* were studied from Okoye et al (2016); Saha, Ali, and Rashid (2014), and Madhu S. Singh and K.P. Joy (2000) respectively

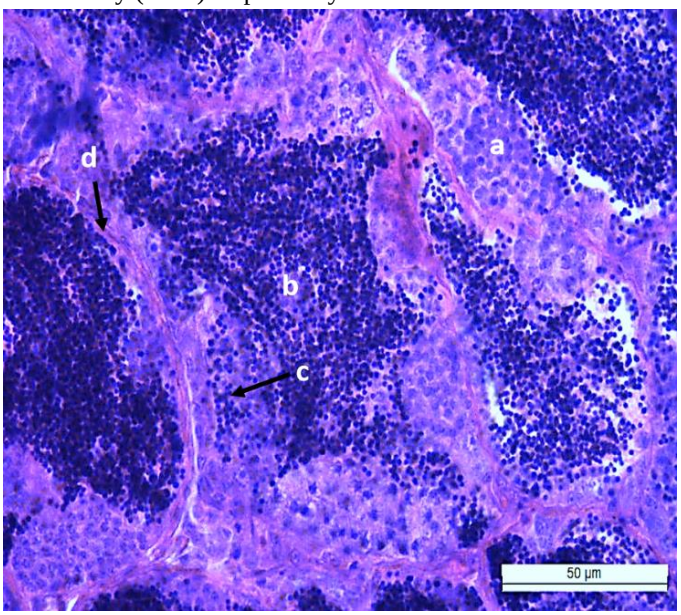

Fig. 1(A). Histological examination of *H. fossilis* (60 days parallel control) a- Spermatogonia; b-spermatozoa; c- Spermatid; d- interlobular connective tissue; Seminiferous tubule filled with spermatozoa, attain maximum size, Leydig cells are not prominent, lobules are distended. H and E stain, objective 40 x, scale bar-50µm.

After exposure to 4NP at low dose and high dose i.e., 64 $\mu g.L^{-1}$ and 160 $\mu g.L^{-1}$ respectively for 30, 45 and 60 days, significant differences in the cellular structure of testes were seen. In a low dose (64 $\mu g.L^{-1}$) group (Fig. 1B, & 2B) it causes the abnormal distribution of spermatozoa, discontinuous of interlobular connective tissue, degenerative spermatogonia, hyperplasia of interstitial tissue, clumping of cells, and necrosis of cells. In high dose (160 $\mu g.L^{-1}$;Fig 1C, & 2C) changes were greater like- fibrosis of interstitial tissue, vacuolization, the disintegration of germ cells, and cyst. Both the catfishes show almost the same results but Catfish *C. batrachus* shows more deformity as compare to *H. fosssilis*.

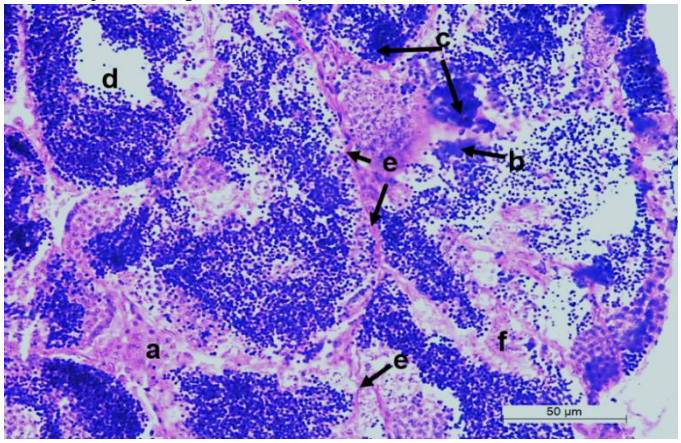

Fig. 1(b). Histological examination of *H. fossilis* **(**exposed to 4-NP Low dose, **60 days) a- necrotic s**permatogonia; b-necrotic spermatozoa; c- clumping of spermatozoa; d- vacuolization; e-Distorted interlobular connective tissue; f- disintegration of cyst;. H and E stain, objective 40x, scale bar-50µm.

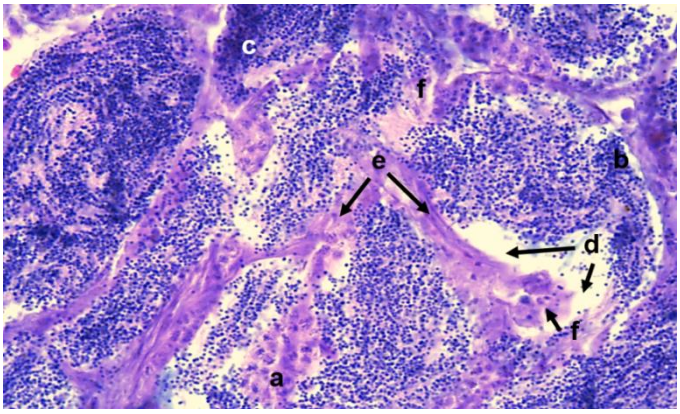

Fig. 1(C). Histological examination of *H. fossilis* **(**exposed to 4-NP High dose, 60 days)a- necrotic spermatogonia; b-necrotic spermatozoa; c- clumping of spermatozoa; d- vacuolization; e-Distorted interlobular connective tissue; f- disintegration of cyst;. H and E stain, objective 40x, scale bar-50µm.

Thickening of testicular lobule walls, indicative of fibrosis or fibroblastic proliferation has been reported in cod *Gadus morhua* fed Aroclor, a polychlorinated biphenyl (Sangalang et al. 1981), in guppies *Poecilia reticulata* exposed experimentally to methyl mercury (Wester 1991). This has been suggested to be a chronic tissue response to injury.

The present study showed ruptured and clumped germ cells, vacuolization in *H. fossilis*, (Fig. 1C) and fibrosis of interlobular connective tissues were seen prominently in *C. batrachus* (Fig. 2C). Similar observations were reported by Zade et al, (2018) in the histopathology of testes of African catfish *Clarias gariepinus* due to toxicity of 4-NP. More hyperplasia in interstitial tissue of testis of Nile tilapia exposed to 4-NP was reported by Ali et al (2014; and Shalaby and Migeed, (2012) who studied the impact of environmental contaminants on the testis of *Oreochromis niloticus*. Vergillo et al (2012) reported disorganization of the cyst's arrangement, vacuolization of germ cells, and sperm aggregation in the testis of *Gymnotus carapo* followed by 24 hours of Hg exposure.

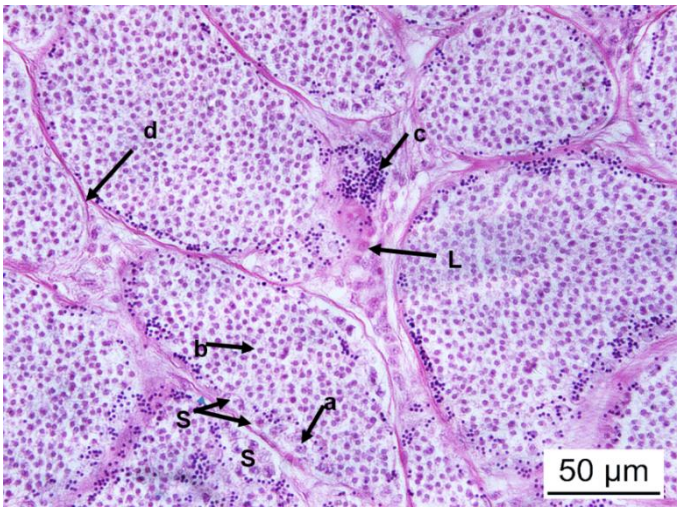

Fig. 2(A). Histological examination of testes of *Clarias batrachus* (60 days parallel control) :-a- primary spermatogonia; b – spermatid; c – spermatozoa; d- interlobular connective tissue; S- sertoli cell; L- leydig cells. H and E stain, objective 40x, scale bar-50µm.

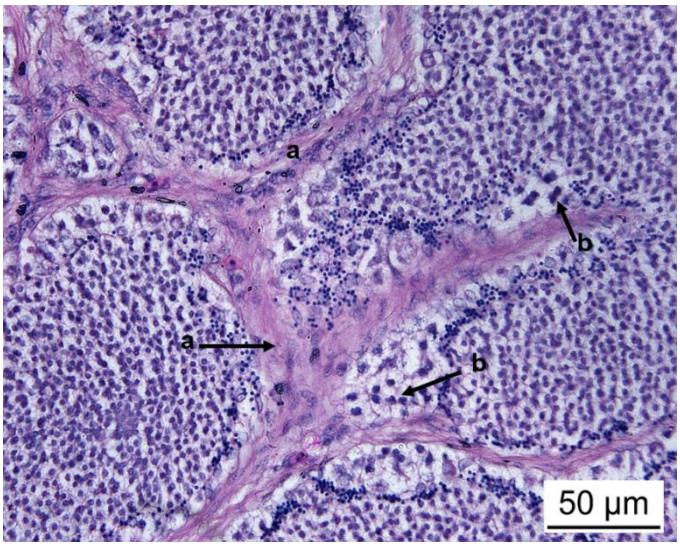

Fig. 2(B). Histological examination of testes of *Clarias batrachus* (exposed to 4-NP Low dose, 60 days) :- a- hyperplasia and distortion of

interstitial connective tissue, b- clumping of cells. H and E stain, objective 40x, scale bar-50µm.

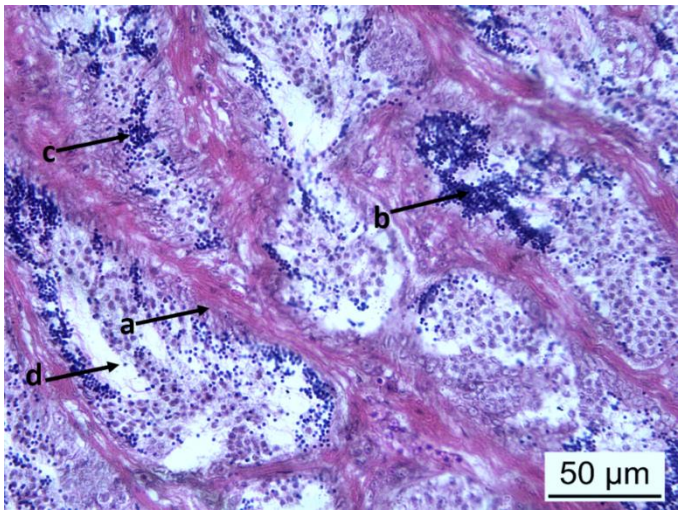

Fig. 2(C). Histological examination of testes of *Clarias batrachus* (exposed to 4-NP High dose, 60 days): a- fibrosis of interstitial tissue, b- clumping of cells, c- abnormal distribution of spermatozoa, d- vacuolization. H and E stain, objective 40x, scale bar-50µm.

Kumar et al (2007) also reported condensation of spermatogenic cells, the formation of intra and intertubular vacuoles in the testis of *Heteropneustes fossilis* after exposure of linear alkylbenzene sulphonate. The reason behind the testicular damage might be that the 4-NP can act indirectly via the hypothalamus-pituitary axis to alter gonadotropin synthesis and secretion or it may also act directly on the testis. Altered gonadotropin secretion resulting in disruption of sex steroid production, can have secondary effects on the testicular cells e.g. Sertoli cells, which are dependent upon the correct hormone level for normal functioning (Zade et al, 2018). Oestrogenic chemicals may also exert their effects directly on the testis via inhibition of androgen synthesis (Tradeau et al,1993.

C. ELISA Results

Estradiol-17β(E_2) and testosterone level in testes of *H. fossilis* and *C. batrachus* with exposure to 4-NP showed significant changes from preparatory season to spawning season. The present study depicts that 4NP downregulates the level of testosterone (T) hormone whether at a low dose(64 µg.L^{-1}) as well as a high dose(160 µg.L^{-1}) and induce a surge of Estradiol 17-β at a low dose(64 µg.L^{-1}) 30 days and significantly decrease at 45 and 60 days of treatment, but at high dose(160µg.L^{-1}) there was significant decline. (Sayed et al2017; Saravanan et al,2019)

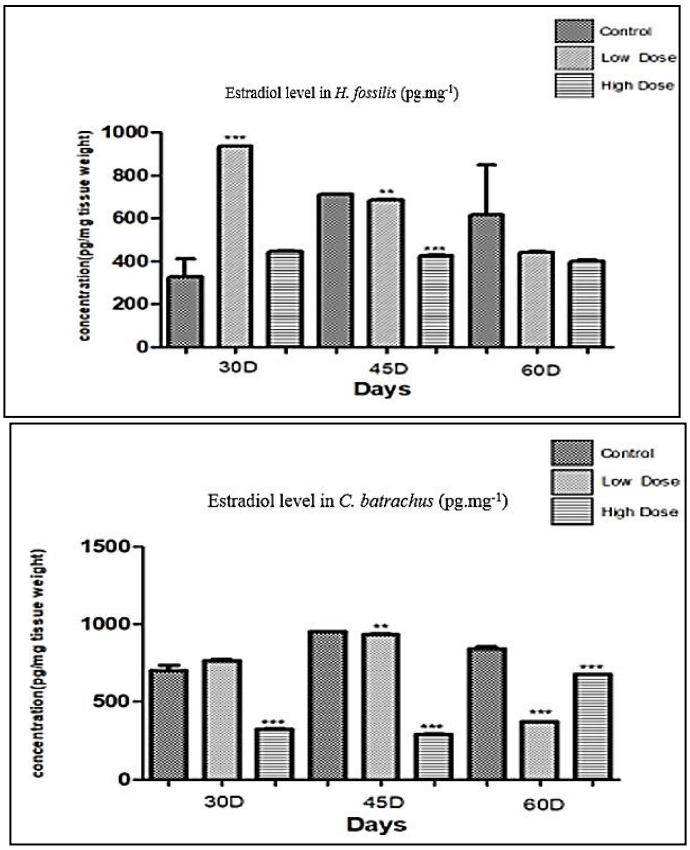

Fig. 3. Changes in estradiol 17-β in the testis of *H. fossilis* and *C. batrachus after* exposure to 4-NP in preparatory, pre-spawning to spawning phases. Data were expressed ad mean ± SEM (n=5). Assessment of data by one -way ANOVA (*P<0.1; **P<0.01; ***P<0.001), followed by Duncan's test (P<0.05). Stars denotes significant changes from the control.

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In this present study, we evaluated that level of 17β estradiol significantly increases in both the catfishes on 30 days exposure at low concentration (64µg.L^{-1}) but relatively decreases on long term exposure for 45 and 60 days at low(64µg.L^{-1}) as well as high concentration(160µg.L^{-1} Fig.3). Many studies have reported the modulation of estradiol level according to the exposure time and concentrations upon exposure to NP in different male animal models (Arukwe et al, 1997; Schwaiger et al, 2001 Saravanan et al, 2019). Ishibashi et al (2004) reported that there was no significant effect of NP on plasma E2 concentration in male goldfish. Aforesaid results suggest that NP may show different EDC potential on varied developmental and sexual stages of species. Altogether NP could alter the steroid biosynthesis pathway in fishes

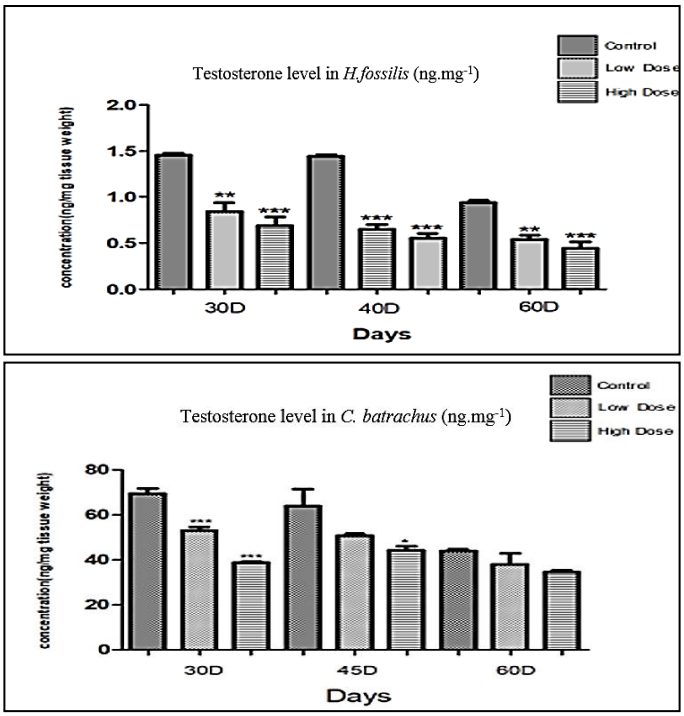

Fig. 4. Changes in testosterone in the testis of *H. fossilis* and *C. batrachus after* exposure to 4-NP in preparatory, pre-spawning to spawning phases. Data were expressed as mean ± SEM (n=5). Assessment of data by one -way ANOVA (*P<0.1; **P<0.01; ***P<0.001), followed by Duncan's test (P<0.05). Stars denotes significant changes from the control.

In this present study, the level of testosterone in both the catfishes *H. fossilis* and *C. batrachus* decreased significantly in respect to duration and dose (Fig.4). Many studies also showed similar results in support of our outcome. Saravanan et al (2019) reported that 11-KT concentrations were significantly decreased in both red seabream and black rockfish when NP concentrations were relatively high. Miles et al (2006) reported that depressed plasma androgens are a well-known characteristic of males exposed to anti-androgenic xenobiotics. There are several reports on decrease of plasma T levels after exposure to NP (Nichols et al (2001; Ishibashi et al, 2004; Zheng et al, 2019).

However comparative account of effect of NP on steroid hormone levels in testis of different catfishes in similar laboratory conditions and same dose were not found. We have here tried to report this effect on two physiologically different male catfishes and have found that both showed similar response to this estrogen mimicking pollutant. The imbalance between 17β estradiol and testosterone concentrations observed in this study may affect the phenotypic ratio and the development of reproductive organs (Hunter and Donaldson 1983). NP toxicity may directly induce damage on testis cells or Sertoli cells resulting in fluctuations of endocrine homeostasis and can also act indirectly by modulating gonadotropin synthesis and secretion through the hypothalamic and pituitary axis which results in an imbalance of sex steroid regulation and gonadal function (Kime,1999)

D. Effect of 4-NP on Sperm cells concentration and sperm Viability test

This present study showed that 4-NP caused a gradual decrease in a dose-dependent manner of sperm concentration and also there was a dose-dependent decrease in sperm viability rate in *H. fossilis* and *C. batrachus* during late preparatory and spawning season compared to the control groups respectively (Fig5, 6, &7). Other studies have also presented similar results showing decrease in sperm count and viability after exposure to xenoestrogens including 4-NP in various male fishes (Barley et al, 2002; Toft et al, 2003; Hara et al, 2007; Wang et al, 2018; Chen et al, 2020). Our results suggest that since 4-NP directly or indirectly imbalances the steroid hormone biosynthesis which regulate the normal gonadal function in male, hence there is decrease in the sperm viability causing reproductive dysfunction.Fig.6,7, and 8.

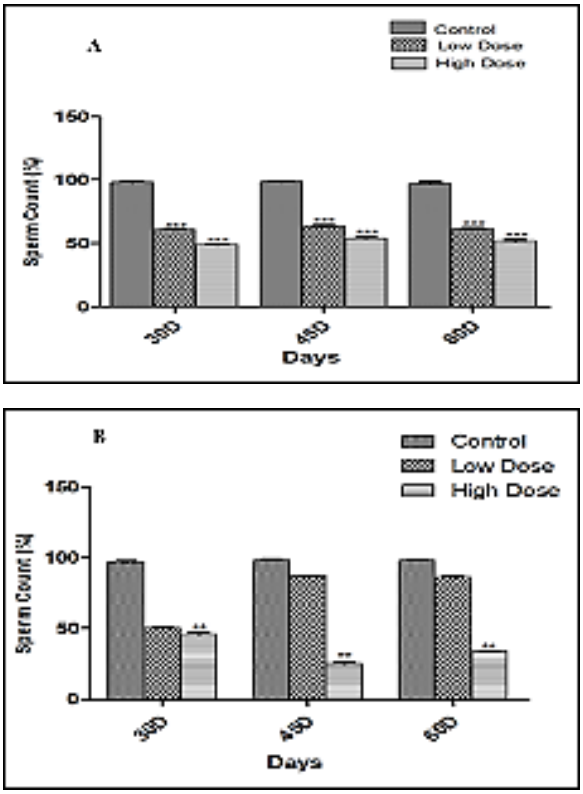

Fig. 5. Effects of 4-NP concentration of sperm cells in percentage of catfish (A) *H. fossilis* and (B) *C. batrachus* respectively during early pre spawning or late preparatory and spawning phase of reproductive cycle. Data were expressed as mean ± SEM (n=5). Assessment of data by one -way ANOVA (*P<0.1; **P<0.01; ***P<0.001), followed by Duncan's test (P<0.05). Stars denotes significant changes from the control.

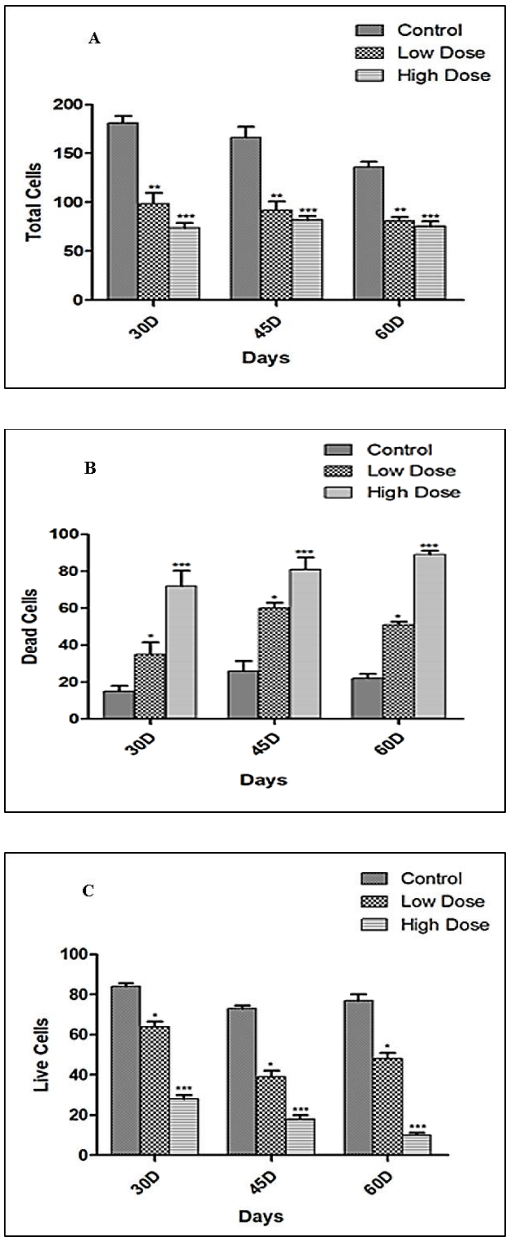

Fig. 6. Effects of 4-NP on viability of sperm cells of catfish *H. fossilis* during early pre spawning or late preparatory and spawning phase of reproductive cycle, (A) total no. of sperm cells, (B) total no. of dead sperm cells in percentage and (C) total no. of viable sperm cells in percentage. Data were expressed as mean ± SEM (n=5). Assessment of data by one -way ANOVA (*P<0.1; **P<0.01; ***P<0.001), followed by Duncan's test (P<0.05). Stars denotes significant changes from the control.

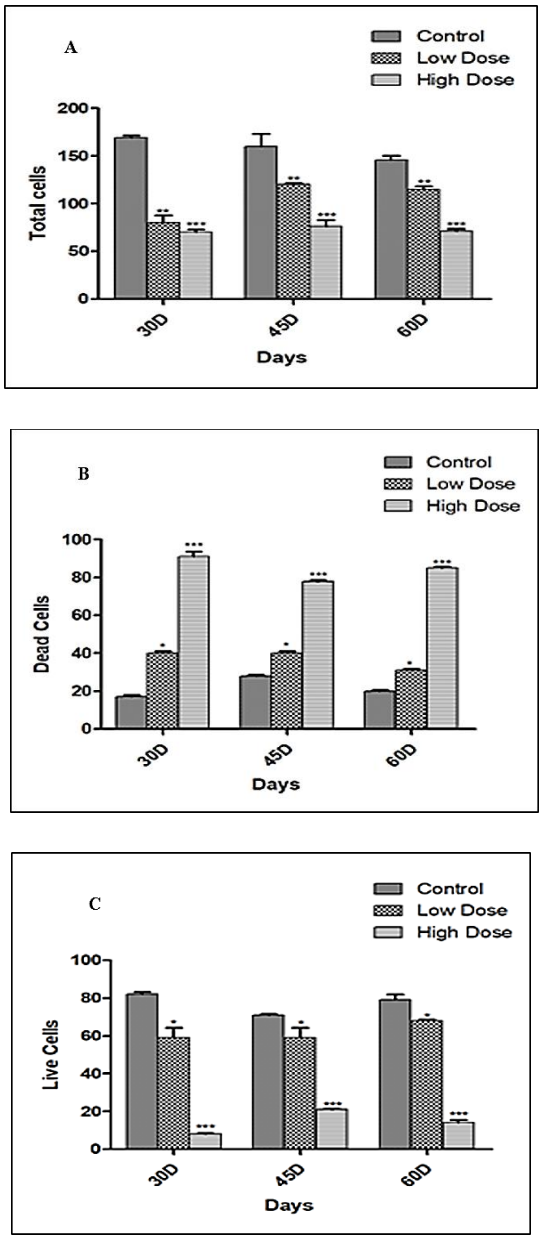

Fig. 7. Effects of 4-NP on the viability of sperm cells of catfish *C. batrachus* during early pre spawning or late preparatory and spawning phase of the reproductive cycle, (A) total no. of sperm cells, (B) total no. of dead sperm cells in percentage and (C) total no. of viable sperm cells in percentage. Data were expressed as mean ± SEM (n=5). Assessment of data by one -way ANOVA (*P<0.1; **P<0.01; ***P<0.001), followed by Duncan's test (P<0.05). Stars denotes significant changes from the control.

CONCLUSION

Results elucidated from the present study are that short term and long exposure of 4-NP can alter the reproductive physiology, endocrine parameters and disrupt spermatogenesis of male catfishes *H. fossilis* and *C. batrachus* which are having great economical and medicinal values. 4-NP has the potential to alter the endocrine homeostasis and increase estrogenic toxicity in male testis for short-term exposure at a low dose. It

significantly decreased the testosterone hormone level in the testis of catfishes. NP can also disrupt the cellular structure of the testis. Although the two fishes used for the study were physiologically different the effects of 4-NP on both the fishes were almost similar. It is therefore inferred that pollution by these waterborne xenoestrogens may result in significant economic losses to fisheries and aquaculture. So, there is an intense need for awareness and concern about such pollutants to prevent the threat it poses to the aquatic species and maintain balance in the aquatic ecosystem.

ACKNOWLEDGEMENT

The authors are thankful to CAS, Department of Zoology, Banaras Hindu University for providing the laboratory facilities.

CONFLICT OF INTEREST

The authors declare no conflicts of interest

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