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AQUACULTURE STUDIES

In Vitro Effects of 4-Nonylphenol on Oocyte Maturation and Ovarian Steroid Hormone Dynamics in the Catfish *Heteropneustes fossilis*

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Abstract

Alkylphenols are known endocrine-disrupting chemicals present in water bodies as contaminants, affecting aquatic life adversely. In this study, post vitellogenic follicles of the catfish *Heteropneustes fossilis* were *in vitro* exposed to 4-nonylphenol (4-NP) at concentrations of 0.1, 0.5, 1, 10, 100 and 200 ng/mL for 12 and 24 h. Germinal vesicle breakdown (GVBD), an indicator of final oocyte maturation (FOM), and the mortality of ovarian follicles were monitored. Levels of estradiol-17β (E_2), testosterone (T), progestins; progesterone (P_4), 17α-hydroxyprogesterone (17-P), 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20β-DP), and cortisol (F) were measured by ELISA or HPLC. The exposure to lower concentrations of 4-NP (0.1, 0.5, 1 and 10 ng/mL) induced GVBD time dependently and the highest rate of induction was up to 25% in 0.5 ng/mL group. The higher concentrations decreased GVBD. 4-NP significantly increased the mortality rate of follicles in a dose-dependent manner. Concentrations ≥10 ng/mL produced more than 60% mortality. 4-NP elicited biphasic effects on all hormones measured, except in the level of 17-P. The levels of T, E_2, P_4, 17, 20β-DP and F increased at the lower concentrations and maximum increase was at 0.1 ng/mL dose for E_2, P_4 and 17, 20β-DP, at 0.5 ng/mL dose for F and at 1 ng/mL dose for T. Then, they were declined with increasing 4-NP concentration. In contrast, 17-P level exhibited a continuous decline in a dose-dependent manner. From the results, it is inferred that the low concentrations of 4-NP stimulated GVBD. Increase in E_2 level might have lowered the rate of GVBD, despite the high level of 17,20β-DP, maturation-inducing steroid, and F. The cytotoxicity infused by 4-NP at higher concentrations adversely affected GVBD and steroid secretion, causing follicle mortality.

Introduction

4-Nonylphenol (4-NP) is a bio-degradation product of nonylphenol ethoxylates (NPE), which are the major non-ionic surfactants used in the manufacture of plastics, pesticides, and industrial detergents (Servos, 1999; Ying, Williams & Kookana, 2002). It is also used in detergents, oil varnishes, rubber auxiliaries and vulcanization accelerators, as antioxidants and corrosion inhibitors for petroleum products and sludge generation inhibitors for petroleum (Khim *et al*., 2001; Kortner & Arukwe, 2007). In consequence, its presence as an environmental toxicant is widely reported in varying proportions in water bodies, sediments, air and food (Bennie, 1999). Aquatic organisms such as fishes that drink water frequently or burrowed in mud become soft targets for the toxicant. 4-NP is a known endocrine-disrupting chemical (EDC), as per the list compiled by Ying & Kookana (2002) and Ying, Williams & Kookana, 2002. The ovary is a known target organ for EDCs and it is well established that 4-NP modulates reproductive processes like zonagenesis and vitellogenesis (Arukwe, Knudsen & Goksoyr, 1997; Arukwe, Goksoyr, Thibaut & Cravedi, 2000; Denslow & Sepulveda, 2007).Many studies have reported that 4-NP has weak estrogenic potency in fish (White, Jobling, Hoare, Sumpter &

Parker, 1994; Nimrod & Benson, 1996; Hill & Janz, 2003). 4-NP elevates plasma concentrations of estradiol-17β (E_2), vitellogenin, and zona radiata proteins in both male and female fish and causes gonad abnormalities, such as the induction of ovotestes in males (Gray & Metcalf, 1997; Kinnberg, Korsgaard, Bjerregaard & Jespersen, 2000). As an estrogenic agonist, 4-NP binds to estrogen receptor (ER) (Petrovic, Sole, de Alda & Barcelo, 2002; Ishibashi *et al.*, 2006; Vetillard & Bailhache, 2006).

E_2 is the major ovarian steroid involved in vitellogenesis, causing follicular growth and maturation (Babin, Carnevali, Lubzens & Schneider, 2007; Nagahama & Yamashita, 2008). After the completion of vitellogenesis, follicles undergo final oocyte maturation (FOM), which is induced by an ovarian progesterone derivative called as maturation-inducing steroid (MIS). In a large number of teleosts, the major MIS is 17, 20β-dihydroxy-4-pregnen-3-one (17, 20β-DP) (Nagahama, 1997; Mishra & Joy, 2006). The MIS stimulates the induction of a maturation-promoting factor (MPF), which is identified as cdc2 kinase and cyclin B complex (Nagahama,Yoshikuni, Yamashita, Tokumoto & Katsu, 1995; Nagahama & Yamashita, 2008) that initiates the migration of the germinal vesicle (GV) and its breakdown (GVBD). These morphological events are useful indicators of FOM.

Estrogens are poor inducers of FOM and maintain the meiotic arrest in the catfish *H. fossilis* and other teleosts (Sundararaj & Goswami, 1977; Goetz, 1983; Jalabert, Fostier, Breton, &Weil, 1991). When oocyte maturation is induced, E_2 level decreases and MIS level increases, which lift the meiotic arrest (Mishra & Joy, 2006). In adult fish, exposure to xenoestrogens such as diethylstilbestrol (DES) and bisphenol A (BPA) has been reported to cause increased or decreased vitellogenin production in both male and female fish and inhibited FOM (Scholz & Gutzeit, 2000; Sohoni *et al.*, 2001; Jobling & Tyler, 2003). Hwang, Kima, Kima, Leeb and Baeka (2010) reported that 4-NP elicited estrogen-agonistic effects on the development and maturation of *Tridentiger obscures* oocytes *in vitro*. They reported that 4-NP increased endogenous E_2 production at the vitellogenic stage and inhibited GVBD at the fully vitellogenic stage. In fish, exposure to DES led to increase in vitellogenin level and inhibited GVBD, and the estrogen agonist effect of DES was more as compared to NP (Zhong *et al.*, 2005; Hwang Kima, Kima, Leeb & Baeka 2010). Tokumoto, Tokumoto, Horiguchi, Ishikawa and Nagahama (2004) demonstrated that DES induced oocyte maturation in a manner similar to MIS. DES induced FOM by binding to membrane progestin receptors, thus mimicking progestins. A similar type of inhibition of progestin-induced GVBD by xenoestrogen has been demonstrated in the oocytes of Atlantic croaker, *Micropagonias undulates* (Ghosh & Thomas, 1995; Tokumoto, Tokumoto & Thomas, 2007).

Objective of the present study was to investigate dose-dependent *in vitro* effects of NP on FOM and on the underlying steroid hormone dynamics of *H. fossilis*. Oocyte mortality under *in vitro* incubation was also monitored to assess the toxicity of 4-NP.

Material and Methods

Chemicals

4-Nonylphenol (99% pure) was purchased from ACROS ORGANICS (Geel, Belgium). Estradiol-17β, testosterone, 17-hydroxy progesterone (17-P), progesterone (P_4), 17,20β-dihydroxyprogesterone (17,20β-DP), cortisol and 21-deoxycortisol were purchased from Sigma Aldrich (St. Louis, M.O, USA). All other chemicals were of analytical grade and purchased locally. For estradiol-17β assay, ELISA kit was purchased from Labor Diagnostika Nord GmbH & Co. KG (Am Eichenhain 1, 48531 Nordhorn, Germany. REF: FR E-2000). Degassed and filtered nanopure water (Barnstead international, Dubuque and IO, USA) was used for ELISA and HPLC assay.

Animal Collection and Maintenance

The experiments were performed in accordance with the guidelines of Banaras Hindu University for experimentation in animals and all care was taken to prevent cruelty of any kind. Mature female catfish *Heteropneustes fossilis* (40-50 g) were purchased from local fish market in the pre-spawning phase (May-June) of annual reproductive cycle. Their mean gonadosomatic index (GSI) was 8.035 ± 0.56. They were maintained in our laboratory under normal photoperiod (13.0 h Light: 11.0 h Day) and temperature (25±2º C) until used for the experiments. During whole period, they were fed daily with goat liver. A few fish were sacrificed randomly to determine maturation phase of their ovaries. The fish containing ovaries filled with dark green post-vitellogenic follicles (with oocyte diameter around 1.0 mm) were used in the study.

Preparation of Incubation Medium and Test Compounds

The incubation medium consisted of (in grams) NaCl 3.74, KCl 0.32, $CaCl_2$ 0.16, $NaH_2PO_4 \cdot 2H_2O$ 0.10, $MgSO_4 \cdot 7H_2O$ 0.16 and glucose 0.40 in one L of triple distilled water (Goswami & Sundararaj, 1971). As phenol red (indicator of pH) is estrogenic, it was omitted in the preparation of the medium. The pH was adjusted to 7.5 with 1N sodium bicarbonate and autoclaved. Penicillin (200000 IU) and streptomycin sulphate (200 mg) were added and filtered. The medium was stored at 4°C and was prepared fresh every week. 4-Nonylphenol (4-NP) was dissolved in acetone in separate dark brown bottle to obtain a stock solution of 1mg/mL. Just before the incubation, it was diluted with the incubation medium to give different working concentrations (0.1, 0.5, 1, 10,

50, 100 and 200 ng/mL).

Effects of 4-NPon GVBD and Mortality

All instruments and glassware were sterilized. Gravid female *H. fossilis* were sacrificed by decapitation and ovaries were transferred to a Petri dish containing fresh cooled incubation medium. Round, dark green post-vitellogenic ovarian follicles (1mm in diameter) were separated with fine brush and watchmaker's forceps. Batches of about 30-40 follicles were incubated in embryo cups containing 3 mL incubation medium or medium containing test compound (0.1, 0.5, 1, 10, 50, 100 and 200 ng/mL) at 24±2°C. In each experiment, follicles from 5 fish ovaries were used and the incubations were done in triplicates from each ovary. The medium was changed at every 4 h and replenished with fresh medium containing the planned amount of 4-NP. In control group, the follicles were incubated in medium containing the vehicle. At the end of the incubation (12 or 24 h), the follicles were cleared in a clearing solution (ethanol/acetic acid/formalin, 6:1:3. Trant and Thomas, 1988) and observed under a stereo binocular microscope. Translucent follicles without germinal vesicle (GV) were scored. Percentage of GV breakdown (GVBD) was calculated from the total number of follicles incubated that underwent GVBD. After 12 or 24 h, the numbers of dead follicles (opaque and deformed) were scored and percentage mortality was calculated from the total number of follicles incubated for GVBD. The percentage values of GVBD and mortality were transformed to arcsin values (angular transformation) by using the following formula:

$$\theta = \text{Sin}^{-1} \sqrt{(x/100)}$$

where θ is arcsin value and x is the percentage value that is to be converted to arcsin value (archive.bio.ed.ac.uk/jdeacan/statistics/tress4.html/angular transformation)

Effects of Co-incubation of 4-NP with MIS (17,20β-DP) and E$_2$ on GVBD

About 50 follicles were incubated in the medium containing 4-NP (0.5 or 50 ng/mL), 17, 20β-DP (1μg/mL), E$_2$ (10 ng/mL). In another experimental set up, the follicles were incubated in 4-NP (low or high dose) containing17, 20β-DP and E$_2$ at doses mentioned above. Control groups were maintained concurrently. After 12 and 24 h, the follicles were cleared and percentage GVBD was scored, as described above and percentage GVBD values were transformed to arcsin values.

Effects of 4-NP on Ovarian Steroid Hormone Dynamics in Vitro

In parallel experiments, ovarian slices (300–400mg) containing mature follicles were incubated in different concentrations of 4-NP for 12 or 24 h, as described above. The tissues and the incubation medium were collected separately or group-wise and stored at -80° C for the steroid assay.

Steroid extraction The tissues were homogenized in 4 volumes of cold PBS (0.02M phosphate buffered saline, pH 7.4) at 0°C for 5-10 sec. The homogenate was centrifuged at 5000g for 20 min at 4°C. The supernatant was collected and steroid extracted in 3 volumes of diethyl ether, for 3 times. Organic fractions were pooled, evaporated and dried under N$_2$ gas and stored at -20°C until further estimation. The incubation medium was used directly for the steroid extraction. For hormone assay, both the tissue supernatant and corresponding incubation medium were pooled to make the sample.Steroid concentrations were measured with specific ELISA kit or HPLC, as described below.

Estradiol-17β assay - E$_2$ was assayed using an ELISA kit according to the manufacturer's instructions. Briefly, 50 μL each of standard (0, 20, 100, 300, 800 and 3200 pg/mL) and samples were pipetted into the anti-E$_2$ IgG-coated plate wells. The immunoreaction was started by adding 100 μL of E$_2$-HRP conjugate solution to each well, followed by incubation at room temperature for 1 h. The content from each plate was removed and washed with 300 μL of distilled water for 5–6 times. Water was completely drained out from each well. Next 150 μL of tetramethylbenzidine (TMB) substrate was dispensed into each well and incubated at room temperature for 15 min in dark. Color development was stopped by adding 50 μL of stop solution (1M sulphuric acid). Absorbance was taken at 450 nm using a Multiscan microplate reader (Thermo Electron Corporation, USA).

Validation of the Steroid ELISA Assay

Response linearity - The response-concentration curve for E$_2$ was linear over 20 – 3200 pg/mL. The sensitivity of the assay was 10 pg/mL.

Cross reactivity - Cross reactivity of the E$_2$ antibody was determined for E$_2$, estriol, estrone, progesterone and cortisol. The cross reactivity was 100% for E$_2$, 1.6% for estriol, 1.3% for estrone, 0.1% for progesterone and 0.1% for cortisol.

Recovery- Known concentrations of E$_2$ were processed in the same manner as the samples. Percentage recovery of E$_2$ was 93 ± 2 (n=5). In samples, known amount of E$_2$ (800 pg/mL) was added and the recovery in the sample was 96.075 ± 5 (n=5). No correction was made for the loss.

Inter- and Intra-assay variations - The coefficients of inter- and intra-assay variations for E$_2$ were 10.1% and 9.3%, respectively.

HPLC Assay of Testosterone, Progestins and Cortisol

Testosterone, progestins (P$_4$, 17P and 17,20β-DP) and cortisol were assayed by high performance liquid chromatography (HPLC) with UV detection (Chourasia & Joy, 2012). In brief, the HPLC system (Shimadzu, Kyoto,

Japan) consisted of two pumps (LC-10ATVP), system controller (SCL-10AVP) and an UV detector (SPD-10AVP) with a variable wavelength (190–370 nm). The system was operated with Shimadzu Class VP series software. The analysis was made with a reversed phase C18 column (150 4.5 mm, i.d., 5 μm, Luna, Phenomenex) connected to a guard column filled with the same material and absorbance was taken at 240 nm. For separation of steroids, a HPLC gradient elution system was used at a flow rate of 1 mL/min. The mobile phase was composed of solvent A – 71% water and 29% acetonitrile and solvent B – 100% acetonitrile. The run consisted of a 30 min linear gradient from 100% A to 60% B and further from 30–35 min linear gradient from 60% B to100% B.

Preparation of standards and determination of retention time - Steroid standards (P_4, 17-P, 17,20β-DP, testosterone and cortisol) were dissolved in methanol separately to prepare stock solutions. From the stock solutions, serial dilutions were made with acetonitrile. The diluted standards were injected into a 20 μL loop of the HPLC system with the help of a Hamilton microliter syringe. The standards were tested individually at different concentrations to record detection limit and retention time under gradient condition. This was repeated thrice with each standard to verify the retention time. The steroids in mixture were also injected to obtain the elution profile and retention time.

Validation of the HPLC Assay

Response linearity - Different concentrations of the standards were injected into the column, five times to set up concentration vs. peak area curve. The response was linear within the concentration ranges used (5–50,000 ng/mL).

Recovery - Percentage recovery was calculated from the concentrations of the standards injected directly and that measured after the extraction. The percentage recovery (n = 3) was 86% for cortisol, 85% for testosterone, 86% for 17,20β-DP, 83% for 17-P and 93% for P_4.The samples were also co-eluted with the known concentrations of standards and their chromatograms were compared with the respective chromatogram of standards alone at the same concentration for identification of the peak in compounds (Figure 1). No correction was made for the losses.

Sensitivity - The minimum detection limit of the standards in individual run was 10 ng/mL.

Inter- and Intra-assay variations - Inter-and intra-assay variations were determined from five chromatograms, each using the same set or different sets of diluted standards. Inter-and intra-assay variation was 6-10% for cortisol, 3-6% for testosterone, 3-10.3% for 17,20β-DP, 3-13% for 17-P and 3-10% for P_4.

Statistical Analysis

The data of GVBD, mortality and steroid hormone levels were expressed as means ± SEM and tested for homogeneity.The data followed a normal distribution and were subjected to two way analysis of variance (ANOVA; $P<0.001$), followed by Newman–Keuls' test ($P<0.05$) (Table 1 and 2) for multiple group comparisons.

Results

Effects of 4-NP on GVBD and Mortality

The incubation of the follicles with different concentrations of 4-NP produced significant effects on %
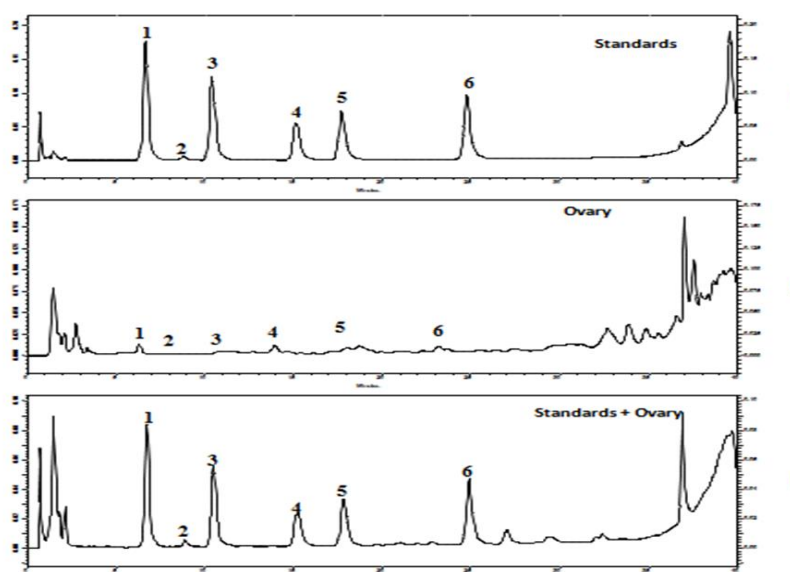
Figure 1. Chromatographic separation profile of (A) standard steroids: 1 – cortisol (Rt- 6.7), 2 – testosterone (Rt- 10.4), 3 - 17-P (Rt- 15.1), 4 - 17,20β-DP (Rt- 17.6), 5 - P4 (Rt- 24.8); (B) ovary sample; (C) standards + ovary sample.

GVBD at both time intervals (Figure 2 and Table 1, P<0.001). The GVBD response was higher at 24 h interval up to 10 ng/mL concentration and the duration effect was not significant at further higher concentrations. Low concentrations of 4-NP (0.1, 0.5, 1, 10 ng/mL) resulted in a significant increase in %GVBD compared to the control group, the highest response of about 25 % was obtained in the 0.5 ng/mL group at 24 h. At 50 ng/mL concentration, the stimulatory effect of 4-NP was significant only at 12 h. At 100 ng/mL, the stimulatory effect was attenuated further (12 h) and an inhibitory effect was noticed at 24 h. At 200 ng/mL, no GVBD was observed.

The incubation of the follicles with 4-NP at different concentrations (0.1, 0.5, 1, 10, 50, 100,200 ng/mL) for 12 and 24 h resulted in mortality of the follicles, which increased with the concentration (Figure 3 and Table 1, P<0.001). The mortality was less than 50% at low concentrations (0.1, 0.5 and 1ng/mL), but increased significantly (above 60%) at the higher concentrations (10, 50, 100 and 200 ng/mL) ($p<0.05$, Newman- Keuls' test).

Effects of Co-incubation with MIS and E_2

The incubation of the follicles with 17, 20β-DP which is the MIS in catfish stimulated GVBD significantly up to 70% at 12 h and above 80% at 24 h (Figure 4 and

Table 1. Statistical analysis using Two-way ANOVA followed by Newman–Keuls' test on the effects of 4-NP

	Two-way ANOVA(***P<0.001,** P<0.01, *P<0.1)		
	$F_{concentration}$	$F_{duration}$	$F_{concentration \times duration}$
Effects of 4-NP on GVBD	99.35***	120.61***	18.88***
Effects of 4-NP on Mortality	540.95***	43.49***	1.8*
Effects of co-incubation of 4-NP with MIS on GVBD	9660.08***	75.85***	68.18***
Effects of co-incubation of 4-NP with E_2 on GVBD	83.23***	11.79*	0.002

Table 2. Statistical analysis using Two-way ANOVA followed by Newman–Keuls' test on the effects of 4-NP on steroid hormone levels

	Two-way ANOVA(***P<0.001,** P<0.01, *P<0.1)		
Steroid hormones	$F_{concentration}$	$F_{duration}$	$F_{concentration \times duration}$
E_2	13.08***	2.47*	0.3
T	56.65***	0.17*	7.6**
P_4	79.71***	6.2**	3.8**
17-P	54.08***	1.17*	0.86*
17,20β-DP	117.3***	17.1***	6.32***
Cortisol	106.42***	79.34***	12.87***
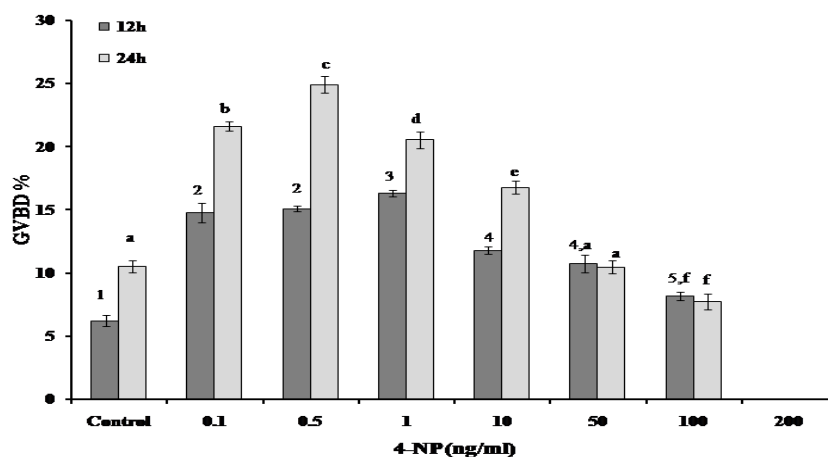
Figure 2. *In vitro* effects of different concentrations of 4-Nonylphenol (4-NP) on germinal vesicle breakdown (GVBD) in the catfish *Heteropneustes fossilis* after 12 and 24h incubations. Values are mean ± SEM. Statistical analysis was done using Two-way ANOVA followed by Newman–Keuls' test (P<0.05). Groups with the same letter and number are not significantly different from each other.

Table 1, P<0.001). The co-incubation with low or high dose of 4-NP (0.5 and 50 ng/mL, respectively) inhibited the MIS- induced GVBD; the inhibition was higher in the high dose group. In either case, the % GVBD remained significantly higher than that of the 4-NP groups.

The incubation with E_2 significantly inhibited the GVBD compared with the control group (Figure 5 and Table 1, P<0.001). But 4-NP stimulated GVBD in the E_2– co-incubated group with a higher effect in the low dose 4-NP combination group.

**Effects of 4-NP on Steroid Hormone Levels**

E_2 level: E_2 was assayed by the ELISA method. The incubation of the ovarian pieces with different concentrations of 4-NP showed an overall significant effect on E_2 at 12 and 24 h (Figure 6A and Table 2, P<0.001). The E_2 level changed in a biphasic manner, stimulated significantly in 0.1ng/mL 4-NP group at both 12 and 24 h, unchanged in 0.5 to 50 ng/mL groups and inhibited significantly at 100 and 200 ng/mL groups (P<0.05, Newman- Keuls' test).

T level: Testosterone was measured by HPLC method and it eluted at retention time of 10.4 min (Figure 6B). Testosterone showed an overall significant effect (Figure 6B and Table 2, P<0.001). Testosterone showed a duration-dependent biphasic effect (Figure 5-6B). At 24 h, its level increased in the low concentration groups i.e. 0.1, 0.5 and 1ng/mL and then unchanged or decreased at the higher concentration groups. At 12 h, T level increased significantly in 0.5, 1 and 10 ng/mL groups with a sharp rise at 1 ng/mL group, it remained
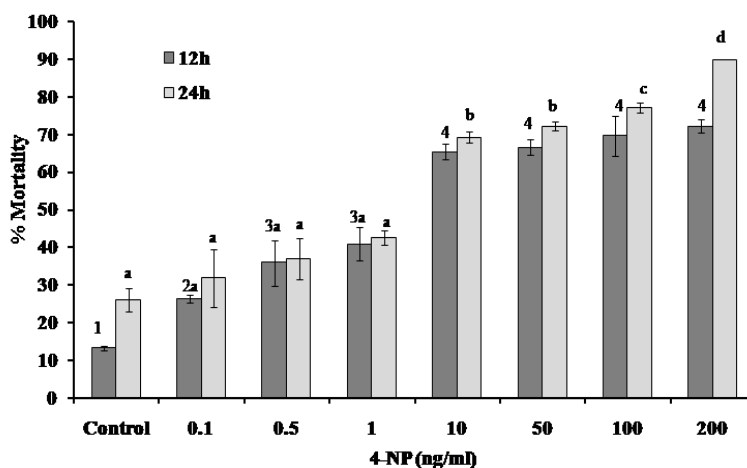
Figure 3. *In vitro* effects of different concentrations of 4-Nonylphenol (4-NP) on mortality rate of oocytes in the catfish *Heteropneustes fossilis* after 12 and 24 h incubations. Values are Mean ± SEM. Statistical analysis was done using Two-way ANOVA followed by Newman–Keuls' test (P<0.05). Groups with the same letter and number are not significantly different from each other.
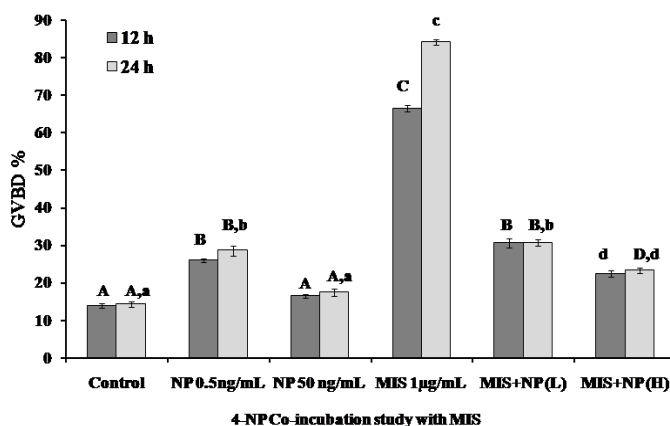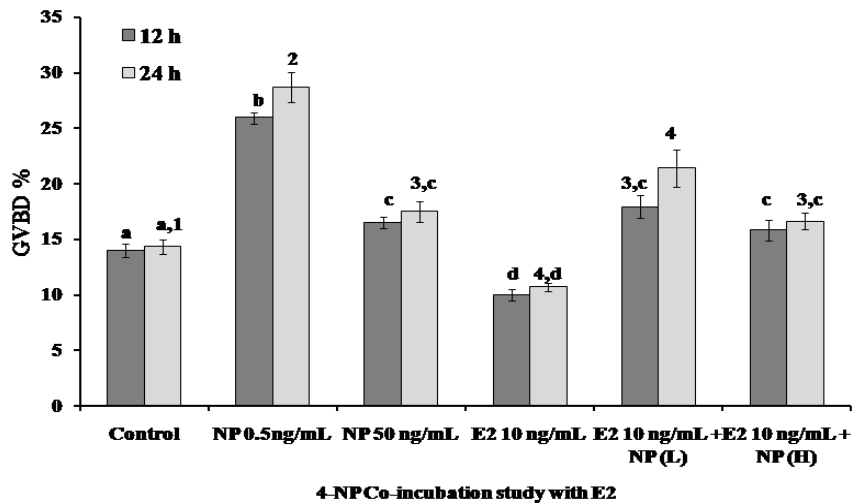

Figure 4. *In vitro* effects of co-incubation of post-vitellogenic follicles with 17, 20b-dihydroxyprogesterone and 4-NP for 12 and 24 h on GVBD in the catfish *Heteropneustes fossilis*. Values are Mean ± SEM. Statistical analysis was done using Two-way ANOVA followed by Newman–Keuls' test (P<0.05). Groups with the same letter are not significantly different.

unchanged in 50 and 100 ng/mL groups, and decreased significantly in the 200 ng/mL group.

E₂/T Ratio: The ratio of E₂/T has been used as a marker for endocrine disruption of steroid hormone levels. This ratio in catfish was calculated at different exposure time for each concentration (Figure 7). At 12 h exposure time, the ratio increased at 0.1 ng/mL concentration, but decreased subsequently in higher concentrations up to 1 ng/mL. The ratio E₂/T showed further increases at higher concentrations of 10 ng/mL and above. At 24 h exposure time, the E₂/T ratio decreased up to 1 ng/mL, and then increased consistently up to 100 ng/mL with a drastic increase at 200 ng/mL concentration.

Progestin level: Progestins were measured by the HPLC method and eluted at 15.1 min (17,20β-DP), 17.6

Figure 5. In vitro effects of co-incubation of post-vitellogenic follicles with 4-nonylphenol and E2 for 12 and 24 h on GVBD in the catfish Heteropneustes fossilis. Values are Mean ± SEM. Statistical analysis was done using Two-way ANOVA followed by Newman–Keuls' test (P<0.05). Groups with the same letter are not significantly different.
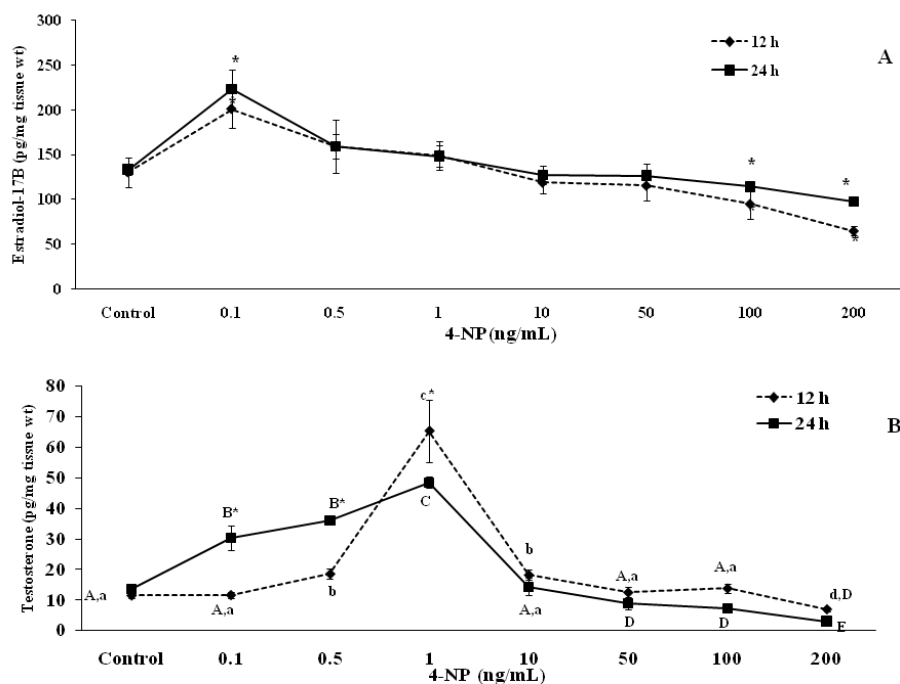
Figure 6. In vitro effects of incubation of post-vitellogenic follicles with 4-nonylphenol (4-NP) for 12 and 24 h on (A) estradiol-17β and (B) testosterone level in the catfish *Heteropneustes fossilis*. Values are Mean ± SEM. Statistical analysis was done using Two-way ANOVA followed by Newman–Keuls' test (P<0.05). Groups with the same letter are not significantly different.
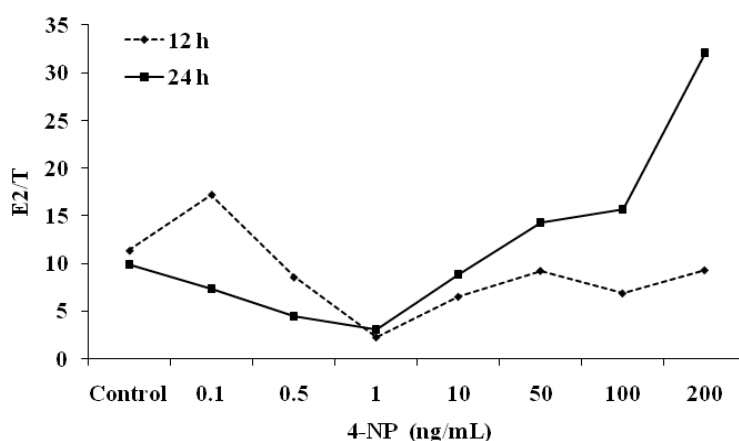
Figure 7. *In vitro* effects of 4-NP concentrations on estradiol-17β /testosterone (E₂/T) ratio in the catfish*Herteropneustes fossilis*. Values are Mean ± SEM. Statistical analysis was done using Two-way ANOVA followed by Newman–Keuls' test (P<0.05).

min (17-P) and 24.8 min (P₄) (Figure 1). Incubation of the ovarian tissues with different concentrations of 4-NP showed an overall significant effect on progestin (P₄, 17-P and 17,20β-DP) levels at both 12 and 24 h (Figure 8A, B, C and Table 2, P<0.001). 4-NP stimulated P₄ and 17,20β-DP at low doses (0.1, 0.5 and 1ng/mL) with a peak rise at the 0.1 ng/mL group. At higher concentrations (10, 50, 100 and 200 ng/mL), progestin levels decreased gradually. However, 17-P level was inhibited in all groups in a concentration-dependent manner at both intervals (P<0.05, Newman-Keuls' test).

Cortisol level: Cortisol was measured by HPLC method and eluted at 6.7 min (Figure6).The incubation of ovarian tissues with different concentrations of 4-NP showed an overall significant effects on corticosteroids at 12 and 24 h (Figure 9 and Table 2, P<0.001). The cortisol level showed a biphasic effect with a peak rise at 0.5 ng/mL concentration at 12 hand the level decreased progressively in the higher concentration groups. A similar pattern of change was also noticed at 24 h, but the increase in the 0.5 ng/mL group was low (P<0.05, Newman Kuel's test).

Discussion

Oocyte maturation is a critical prerequisite in gametogenesis upon which fertilization and embryonic development rely on. Many pesticides, including PCBs adversely affect this process in farm animals (Pocar, Brevini, Fischer, & Gandolfi, 2003). Studies in fishes with EDCs showed that these chemicals produced agonistic or antagonistic effects on oocyte maturation (Hwang Kima, Kima, Leeb & Baeka 2010; Tokumoto, Tokumoto & Horiguchi, 2005; Tokumoto, Tokumoto & Thomas, 2007; Hwang, Lee, Kim & Baek, 2008). We tested the effect of 4-NP on GVBD, an indicator of meiotic maturation. Our

data showed that the process of GVBD in catfish was affected by 4-NP exposures from low to high concentrations in an inverted U-shaped curve pattern. The U-shaped curves are commonly observed after exposure to EDCs and endogenous hormones (Denslow & Sepuldeva, 2008) and are reported as a result of different modes of action of 4-NP at low and higher concentrations. 4-NP elicited GVBD response between 0.1 and 10 ng/mL concentrations with a maximum response of about 25% in 0.5 ng/mL concentration at 24 h. At 12 h, the maximum response (about 16%) was noticed in the 1 ng/mL group. The GVBD response could be correlated with the increase in MIS level (see below). One reason for the low response to high 4-NP concentrations may be the mortality of follicle cells during the exposure. Even the low concentrations elicited mortality, albeit at a low rate. Another reason for a low turnout rate in GVBD response may be the estrogenic property of 4-NP. Hwang, Lee, Kim and Baek, 2008 and Hwang Kima, Kima, Leeb and Baeka 2010, demonstrated the estrogenic potency of 4-NP during *in vitro* oocyte maturation in dusky triple tooth goby *Tridentiger obscures* and greenling *Hexagrammos otakii* which showed increased E₂/T ratio. In the assay, these workers found that 4-NP inhibited GVBD in fully vitellogenic oocytes at concentrations of 0.45 and 4.54 nM, similar to DES at concentrations of 0.037, 3.73 and 37.26 nM (Hwang, Lee, Kim & Baek, 2008; Hwang Kima, Kima, Leeb & Baeka 2010). The GVBD inhibition was attributed to the estrogen agonistic action of 4-NP. The estrogenic property of 4-NP was also shown by Baek, Park, Lee & Kim (2003) in longchin goby *Chasmichthys dolichognathus* and by Giesy *et al*. (2000) in fathead minnow *Pimephales promelas*.In catfish, low concentrations of 4-NP up to 10 ng/mL stimulated GVBD and the higher concentrations inhibited it. The species
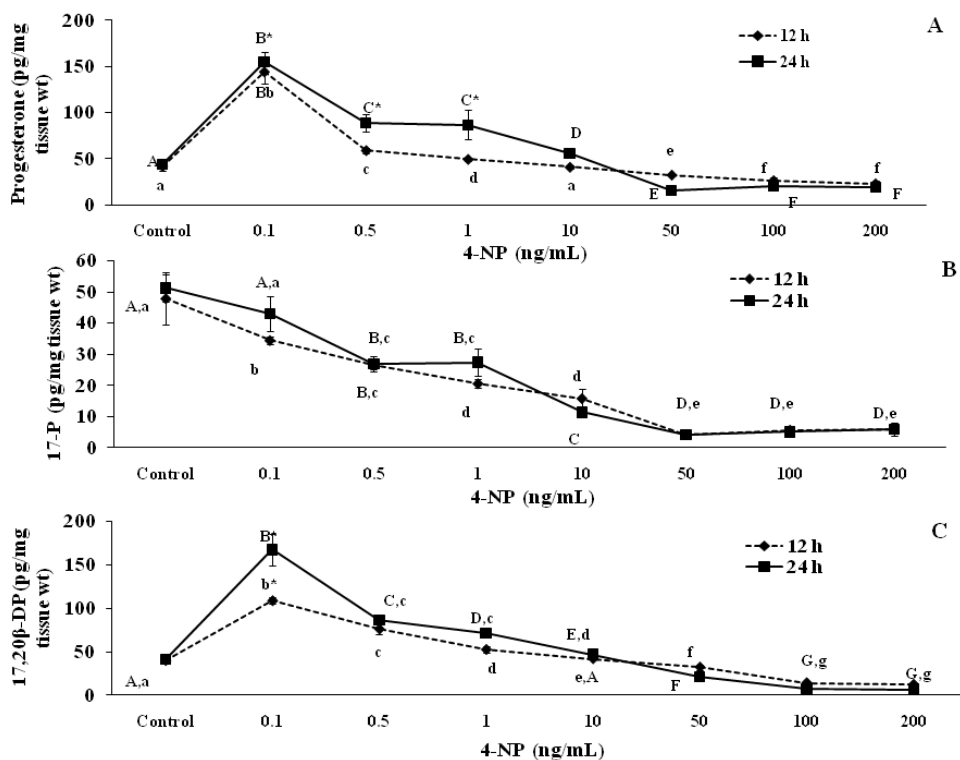
Figure 8. *In vitro* effects of incubation of post-vitellogenic follicles with 4-NP for 12 and 24 h on (A) progesterone, (B) 17-P and, (C) 17,20β-DP levels in the catfish *Heteropneustes fossilis*. Values are Mean ± SEM. Statistical analysis was done using Two-way ANOVA followed by Newman–Keuls' test (P<0.05). Groups with the same letter are not significantly different.
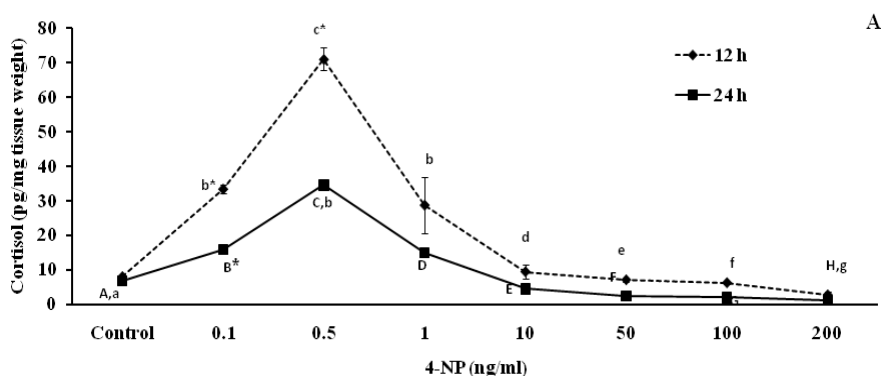
Figure 9. *In vitro* effects of incubation of post-vitellogenic follicles with 4-NP for 12 and 24 h on cortisol level in the catfish *Heteropneustes fossilis*. Values are Mean ± SEM. Statistical analysis was done using Two-way ANOVA followed by Newman–Keuls' test (P<0.05).Groups with the same letter are not significantly different.

variation may be one factor for different responses to 4-NP in the GVBD assay. In catfish, both GVBD and E_2 level increased concurrently in the 0.1 ng/mL group. Therefore, the increase in the E_2 level might have resulted in the low GVBD response despite the increase in MIS level. In the present study, the effects of 4-NP alone and in combination with E_2, MIS on the maturation of catfish oocytes were examined. We found

that treatment of oocytes with non-steroidal substance Tmx, and EDCs like 4-NP, E_2, MIS alone induced maturation as did DES. The estrogen inhibition of oocyte maturation is mediated via membrane GPR30 receptors by possible up-regulation of cAMP production (Pang & Thomas, 2009; Thomas, 1999). In the present study, E_2 had inhibitory effect on GVBD. However, in the presence of E_2, 4-NP stimulated GVBD response, the effect being

higher in the low dose exposure group (0.5 ng/mL). The mechanism of this action is not clear at present.

One of the objectives of the present study was to test the viability of catfish ovarian follicles on exposure to 4-NP. The follicles were exposed to different concentrations of 4-NP for 12 h and 24 h. The data show that all concentrations of 4-NP caused mortality in follicles, which increased linearly (a typical dose-response curve) with the exposure dose and time. Concentrations up to 1 ng/mL produced less than 50% mortality, while concentrations $\geq$ 10 ng/mL produced 60% or more mortality. From the above results, the lethal concentration-50 (LC_{50}) can be deduced to be 5ng/mL. Numerous studies have reported that eggs, embryos and larvae are the most sensitive stages affected by 4-NP exposure (Jobling, Sheahan, Osborne, Matthiessen,& Sumpter, 1996; Lahnsteiner, Berger, Grubinger & Weismann, 2005; Chaube, Gautam,& Joy, 2013). As fishes release gametes into water (spawning phase), and fertilization, hatching and larval development occur in it, water quality is an important determinant to support these processes. Chaube, Gautam,& Joy,(2013) described the deleterious effects of 4-NP on fertilization, early development and hatching. The mortality data further support the adverse effect of 4-NP on postvitellogenic follicles that impaired their ability to undergo FOM. As 4-NP is a persistent aquatic toxicant with concentrations varying from a few microgram to low milligram ranges (Servos, 2002b; Porter & Hayden, 2002; Soares, Guieysse, Jefferson, Cartmell,& Lester, 2008),its presence can lead to total dissipation of aquatic organisms, resulting in serious ecological imbalance and biodiversity loss.The dwindling populations of many freshwater fishes are in reality about to become endangered with the ever increasing aquatic pollution that destroys eggs, embryos and larvae (Chaube, Gautam,& Joy, 2013).

4-NP exposure affected the steroid hormone levels variously in a dose- and duration-dependent manner. Broadly, low concentrations stimulated steroid levels, while high concentrations decreased them. The inhibitions can be attributed to the cytotoxicity of 4-NP, as indicated by the mortality of follicles. The cytotoxic effects may lead to disturbances of the enzymes synthesizing/converting steroid hormones (Arkuwe, 2005; Kortner & Arkuwe, 2007). These authors have reported that 4-NP modulates StAR, P450scc, Cyp11β and cyclin B (a molecular marker of oocyte growth and maturation) expressions in the brain or ovary of Atlantic cod (*Gadus morhua*) or salmon (*Salmo salar*) in dose- and time-dependent manner. E_2 levels showed one fold increase after 7 days of 4-NP exposure in 1μM concentration and then a concentration-specific reduction in the higher concentrations (Kortner & Arukwe, 2007). At 14-day exposure, E_2 level showed 1 fold reduction in the 50 and 100 μM concentrations and no significant changes in the low concentration groups (Kortner & Arukwe, 2007).At 7-day exposure, 11-KT

levels increased 0.6 fold in 100μ M concentration, but decreased in lower concentrations. At 14-day exposure, 10 μM concentration produced a 2.5 fold increase in 11-KT level and reductions in other dose groups (Kortner & Arukwe, 2007). These results do not indicate a definite pattern of 4-NP action, either exposure-specific or concentration-specific, which may be due to multiple modes of action or targets. In catfish, the low concentrations (0.1, 0.5 ng/mL) stimulated E_2, but high concentrations (1.0, 10, 50, 100 and 200 ng/mL) unchanged or decreased E_2 levels at both 12 and 24 h. Testosterone levels were increased at 0.1, 0.5, 1.0 ng/mL concentrations, but decreased at 50, 100 and 200 ng/mL concentrations. The E_2/T ratio is considered a reliable index to assess endocrine disruption (Hwang Kima, Kima, Leeb & Baeka 2010; Folmar *et al.*, 2000; Labadie & Budzinski, 2006). Both BPA and *p*-NP lowered the ratio of androgens to estrogens in juvenile turbots(Labadie & Budzinski, 2006),but acted differently; *p*-NP decreased androstenedione and 11-KT levels, while BPA elevated estrone level. Both 4-NP and DES affected E_2/T ratio in dusky tripletooth goby, depending on the oocyte maturity stage (Hwang Kima, Kima, Leeb & Baeka 2010). In the vitellogenic and fully vitellogenic oocytes, high doses of 4-NP (45.38 and 453.82 nM) elevated the ratio. The low doses of 4-NP (0.045, 0.45, 4.54 nM) behaved differently, either it caused elevation or no change. In catfish, E_2/T ratio was influenced by both doses and exposure time. At 12 h exposure, the ratio increased at 0.1 ng/mL concentration, decreased to the lowest value in 1 ng/mL group, but increased further in10 ng/mL and above. At 24 h exposure, the E_2/T ratio decreased up to 1 ng/mL concentration, then increased up to 100 ng/mL consistently and with a drastic increase at 200 ng/mL concentration. From all this, it is evident that 4-NP exposure disturbed the pattern of E_2 and T secretion. Furthermore, the high dose of 4-NP appears to be more estrogenic.

The NP exposure not only affected the E_2/T ratio, but also the progestin metabolism. The post-vitellogenic follicles represent the threshold stage in the maturation process, during which E_2 decreases and progestin MIS such as 17α hydroxy, 20β- dihydroxy progesterone (17,20β-DP) or 17α,20β,21-trihidroxy-4-pregnen-3-one (17,20β-21-TP) increases to initiate maturational activity (Mishra & Joy, 2006; Rocha & Reis-Henriques, 1998; Pankhurst &Riple, 2000). The 0.1 ng/mL concentration of 4-NP increased both P_4 and 17,20β-DP (MIS) levels and the higher doses decreased the stimulatory effect or inhibited the levels below the control values. The 0.1 ng/mL dose interfered with the steroid metabolism, increasing simultaneously E_2 level on one hand and P_4 and MIS level on the other hand, quite akin to the control values. The GVBD response in the low concentrations (0.1, 0.5 and 1 ng/mL) can be attributed to the increase in MIS levels. At the same time, the low response can be attributed to the high E_2 level or mortality or both. 17-P, the precursor of 17,20β-DP

showed a decrease throughout all 4-NP exposure trials which could be due to its conversion to MIS.

Cortisol is the main glucocorticoid in fish and is secreted mainly by the inter-renal tissue (Jones *et al.*, 1980). In many fishes, cortisol is also secreted by the ovary and has been attributed MIS activity (Scott & Canario, 1987). The 4-NP exposure elevated the cortisol level in the low concentration groups up to 1 ng/mL (12 h) or 10 ng/mL at 24 h which might be due to stress. The toxicants are known to cause varying degrees of oxidative stress, depending on their dose, exposure time and chemical nature (Chitra, Latchoumycandane &Mathur, 2002; 2003; Ferreira, Moradas-Ferreira & Reis-Henriques, 2005). The increase in the cortisol level has coincided with the GVBD response, suggesting that it might be involved in the GVBD response as well. In catfish, cortisol and deoxycorticosterone induces GVBD (Sundararaj & Goswami, 1977; Goetz, 1983). The cortisol level decreased at higher concentrations, which might be due to cytotoxicity of 4-NP, resulting in high oocyte mortality.

Conclusion

In conclusion, the GVBD response of catfish to 4-NP can be compared to that of another xenoestrogens, DES. Like DES, 4-NP may be progestin-agonistic (Tokumoto Tokumoto, Tokumoto, Horiguchi, Ishikawa & Nagahama 2004; Tokumoto, Tokumoto & Horiguchi, 2005; Tokumoto, Tokumoto & Thomas, 2007). The low response may be due to mortality of follicles. Being a lipophilic macromolecule, 4-NP can pass through biomembranes and can cause cytotoxicity.

Acknowledgements

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