Fish Aqua Sci 12(4), 293-298, 2009

KOSFAS FISHERIES AND AQUATIC SCIENCES
The Korean Society of Fisheries and Aquatic Science

# Effects of Nonylphenol and 2,2',4,6,6'-pentachlorobiphenyl on *in vitro* Sex Steroid Production in Maturing Oocytes of the Yellowfin Goby, *Acanthogobius flavimanus*

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Several studies have reported that nonylphenol (NP) and 2,2',4,6,6'-pentachlorobiphenyl (PCB104) exhibit estrogenic activity. To investigate the estrogenic potency of NP and PCB104 during oocyte maturation, fully vitellogenic oocytes (0.76 mm diameter in average) of yellowfin goby, *Acanthogobius flavimanus*, were exposed *in vitro* to these chemicals at different concentrations (0.1, 1, 10, 100, and 1,000 ng/mL) with the exogenous precursor 17α-hydroxyprogesterone (17αOHP) 50 ng/mL in the presence or absence of human chorionic gonadotropin (HCG). The production of testosterone (T), estradiol-17β (E2), and 17α,20β-dihydroxy-4-pregnen-3-one (17α20βOHP) in response to NP or PCB104 were measured by radioimmunoassay. Steroid levels were also expressed as E2/T and E2/17α20βOHP ratios. In the absence of HCG, no significant differences in either NP or PCB104 treatment groups were observed. In the presence of HCG, NP treatment did not show significant differences in the production of T, E2, and 17α20βOHP at any concentrations tested, but E2/T ratios were decreased at concentrations of 0.1, 1, 10, and 1,000 ng/mL compared with the control group. PCB104 decreased E2 production at concentrations of 0.1, 10, and 1000 ng/mL, but did not show significant differences in the production of T and 17α20βOHP at any concentration tested. While E2/T ratios were decreased at PCB104 concentrations of 0.1, 1, 10, and 1,000 ng/mL, E2/17α20βOHP ratios were also decreased at 0.1, 10, and 1,000 ng/mL compared with the control. Results indicate that both NP and PCB104 appeared to have antiestrogenic effects during this phase.

Key words: Nonylphenol, 2,2',4,6,6'-pentachlorobiphenyl, Steroid hormone, Estrogenic-antagonist, Yellowfin goby

## Introduction

Most studies of endocrine-disrupting chemicals (EDCs) have focused on the effects of estrogenic chemicals (Folmar et al., 1996, 2001; Nimrod and Benson, 1996). These chemicals, when introduced into aquatic environments, are able to mimic endogenous hormones, especially reproductive sex steroids such as estrogens and androgens, and may therefore have the potential to disrupt endocrine-mediated processes in exposed fish (Colborn and Clement, 1992).

In fish, as in most vertebrates, steroid hormones are critical to maintain hypothalamus-pituitary-gonad axis function, and feedback controls on the system are achieved largely through alterations in steroid production (Ankley et al., 2009). In female fish, estradiol-17β (E2), testosterone (T), and progestogens are the main gonadal steroids. Plasma E2 increases during the vitellogenic phase of the oocyte and then declines as oocytes complete vitellogenesis. A holding phase then occurs during which the aromatase enzyme is switched off and the follicle secretes testosterone. Progestogens are important during the final maturation of the oocyte leading to spawning. Two of these, 17α,20β-dihydroxy-4-pregnen-3-one (17α20βOHP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (17α20β21OHP), have clear roles in this res-

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pect, but other steroids may also be involved (Kime, 1993).

Nonylphenol (NP) and 2,2,4,6,6-pentachlorobiphenyl (PCB104) are estrogenic in various aquatic animals such as zebrafish and catfish (Sumpter and Jobling, 1995; Nimrod and Benson, 1996; Billsson et al., 1998). These compounds have been found to be widely dispersed in the environment, including in waters, aquatic sediments, and groundwater. NP is a degradation product of nonylphenol ethoxylates (NPE), which are the major non-ionic surfactants used in plastics, pesticides, and industrial detergents (Talmage, 1994; Maguire, 1999; Ying et al., 2002). Many experiments with fish have focused mainly on the estrogenic activity of NP in freshwater species using vitellogenin assays during the vitellogenic stage.

The induction of vitellogenin, which is related to E2 production, is a widely used end point for detecting the effects of estrogenic activity (Nichols et al., 2001). In amphibians, E2/progesterone (E/P) ratios have been used as indicators of the vitellogenic status of ovarian follicles *in vitro*: vitellogenic follicles synthesize primarily estrogens and express a high E/P ratio, whereas mature, preovulatory follicles synthesize principally progesterone and express low E/P ratios (Sretarugsa et al., 1997).

PCB104 is a member of a group of highly chlorine-substituted, non-coplanar PCB congeners, which may also exhibit estrogenic and antiestrogenic activities (Fielden et al., 1997; Bonefeld-Jørgensen et al., 2001). Increased estrogenic activity has been observed for PCB104 following hydroxylation on the para position (Fischer et al., 1998). The effects of PCB104 have been tested mostly on mammalian species, and limited information is available on its estrogenic effect on fish (Choi et al., 2003).

In this study, we investigated the potential estrogenic or other effects of NP and PCB104 on steroid production in a marine fish, *Acanthogobius flavimanus,* using a steroid hormone assay to measure E2, T and 17α20βOHP. Sex steroid levels were also expressed as E2/T and E2/17α20βOHP ratios. This species inhabits coastal waters of Korea, Japan, and China.

## Materials and Methods

### Chemicals

Both 4-nonylphenol and PCB104 (Aldrich Chemical, Milwaukee, WI, USA) were prepared as stock solutions (mg/mL) by dilution in ethanol. These were diluted further in ethanol. The ethanol concentration in the incubation medium was kept at less than 0.1%. Standard 17α-hydroxyprogesterone (17α OHP), T, E2, and 17α20βOHP were purchased from Sigma Chemical (St. Louis, Missouri, USA) or Steraloids, Inc. (Wilton, NH, USA). Antiserum for T was purchased from Sigma Chemical, and those for E2 and 17α20βOHP were a kind gift from Dr. Alexis Fostier (INRA, Rennes, France). Radioactive [2,4,6,7-$^3$H]-testosterone and [2,4,6,7-$^3$H]-17ß-estradiol were obtained from Amersham Life Science (London, England). Radioactive [1,2,6,7-$^3$H]-17α20βOHP was obtained by enzymatic conversion from [1,2,6,7-$^3$H]-17α-hydroxyprogesterone following Scott et al. (1982).

### Experimental fish and oocyte incubation *in vitro*

The *A. flavimanus* used in this study were captured in coastal waters of Busan, Korea during the spawning season (April-June). Oocytes were separated into groups using fine forceps. Oocytes with average diameters of 0.76 mm (fully vitellogenic stage; Park, 2004) were used for incubation. After separating the ovaries into small pieces in ice-cold balanced salt solution (132.96 mM NaCl, 3.09 mM KCl, 0.28 mM $MgSO_4 \cdot 7H_2O$, 0.98 mM $MgCl_2 \cdot 6H_2O$, 3.40 mM $CaCl_2 \cdot 6H_2O$, and 3.65 mM HEPES), approximately 20 follicle-enclosed oocytes were incubated in the presence of 17α-hydroxyprogesterone (17αOHP) as a precursor in each well of 24-well culture plates containing 1 mL of Leibovitz L15 medium (Gibco, USA) with or without 50 IU/mL of human chorionic gonadotropin (HCG; Sigma). The pH and osmolarity of the media were adjusted to 7.9 and 300 mOsm, respectively. At the start of incubation, NP and PCB104 were added to the media at concentrations of 0.1-1,000 ng/mL. The plates were incubated for 24 h at 12°C with constant gentle shaking.

### Radioimmunoassay

After incubation, steroids in aliquots of medium were extracted twice using five volumes of ethylacetate:cyclohexane (1:1). Then, the T, E2, and 17α20βOHP levels were measured by RIA following Kobayashi et al. (1988). The intra-assay coefficients of variance were 2.3% (n=3), 3.4% (n=3), and 3.2 % (n=4) for the T, E2, and 17α20βOHP assays, respectively, and the respective inter-assay coefficients of variance were 12.5% (n=5), 11.5% (n=5), and 9.5% (n=8). The minimum detectable limits were 10, 12.5, and 10 pg/mL for T, E2 and 17α20βOHP, respectively.

### Statistics

All data were expressed as means with the standard

error of the means (SEM). SPSS 11.0 for Windows was used for the Kruskal–Wallis test followed by the Bonferroni adjustment. A value of $P<0.05$ was considered statistically significant.

## Results

### Effects of NP on steroid production

In NP with or without HCG treatment (Fig. 1), no significant differences were found in the production of each steroid compared with 17αOHP controls. No significant differences were observed in the ratios of E2 to T (E2/T) and E2 to 17α20βOHP (E2/17α20β OHP) without HCG treatment (Fig. 2). In the presence of HCG, 0.1, 1, 10, and 1,000 ng/mL of NP decreased the E2/T ratio (55.29±5.14 vs. 85.05± 14.99, 41.22±3.12 vs. 85.05±14.99, 60.51±1.04 vs. 85.05±14.99, 43.66±6.21 vs. 85.05±14.99, respectively) compared with 17αOHP controls ($P<0.05$), although no significant differences in E2/17α20βOHP ratio were observed.

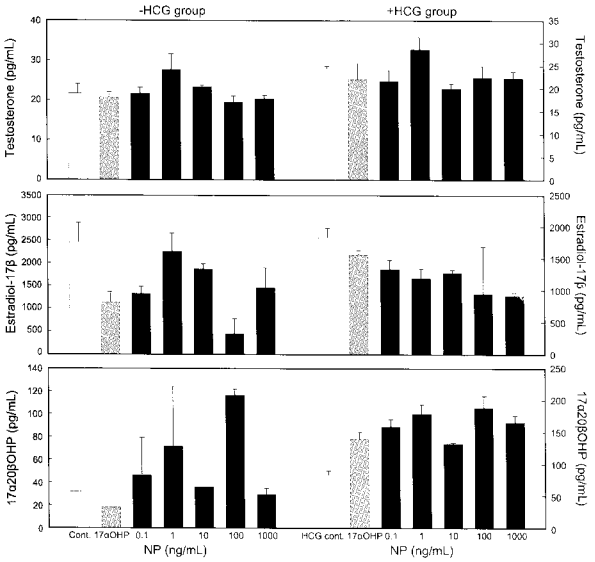


Fig. 1. Effects of NP on *in vitro* steroid production in yellowfin goby oocytes (oocyte diameter=0.76 mm) in the presence or absence of 50 IU hCG after a 24 h incubation. Values are mean±SE of three replicate wells with 20 oocytes/well.

### Effects of PCB104 on steroid production

In PCB104 without HCG treatment (Fig. 3), no significant differences were found in the production of each steroid compared with 17αOHP controls. In the presence of HCG, exposure to 0.1, 10, and 1,000 ng/mL PCB104 with HCG resulted in a significant decrease in the production of E2 compared with 17αOHP controls (795.67±125.26 vs. 2,034.33± 828.74, 848.33±183.39 vs. 2,034.33±828.74, 1,312.33± 533.08 vs. 2,034.33±828.74 pg/mL, respectively, $P<0.05$), although no significant differences were observed in either T or 17α20βOHP.

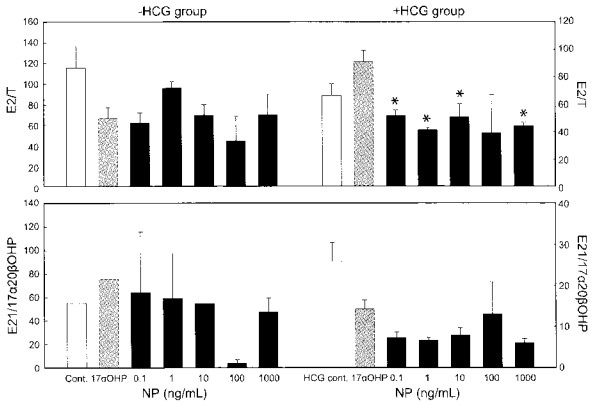


Fig. 2. Effects of NP on the E2/T and E2/17α20βOHP ratio in yellowfin goby oocytes (oocyte diameter= 0.76 mm) after a 24 h incubation. Values are the mean±SE of the ratio of each steroid in three replicate wells with 20 oocytes/well. Data were analyzed using the Kruskal-Wallis test followed by the Bonferroni adjustment. Asterisks show significant differences from controls ($P<0.05$).

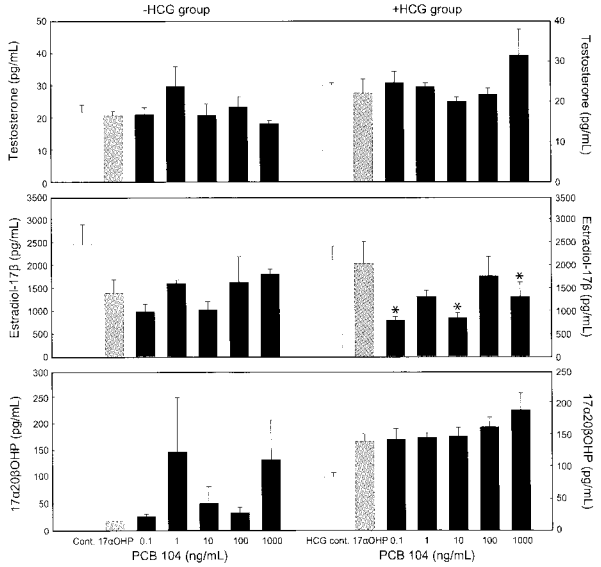


Fig. 3. Effects of PCB104 on *in vitro* steroid production in yellowfin goby oocytes (oocyte diameter= 0.76 mm) in the presence or absence of 50 IU hCG after a 24 h incubation. Values are mean±SE of three replicate wells with 20 oocytes/well. Data were analyzed using the Kruskal-Wallis test followed by the Bonferroni adjustment. Asterisks show significant differences from controls ($P<0.05$).

The E2/T and E2/17α20βOHP ratios (Fig. 4),

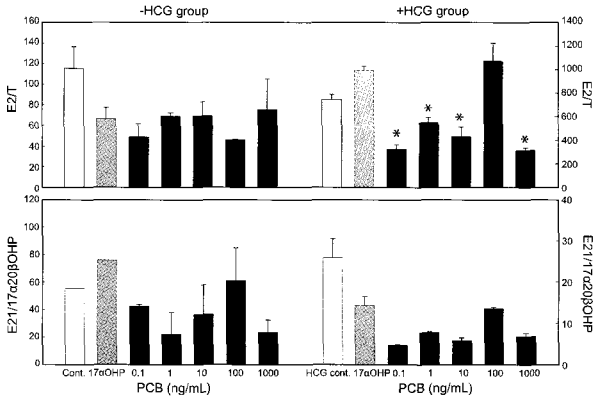


Fig. 4. Effects of PCB104 on the E2/T and E2/17α 20βOHP ratio in yellowfin goby oocytes (oocyte diameter=0.76 mm) after a 24 h incubation. Values are the mean±SE of the ratio of each steroid in three replicate wells with 20 oocytes/well. Data were analyzed using the Kruskal-Wallis test followed by the Bonferroni adjustment. Asterisks show significant differences from controls ($P<0.05$).

showed no significant differences compared with 17αOHP controls without HCG treatment. With HCG treatment, exposure to 0.1, 1, 10, and 1,000 ng/mL PCB104 decreased E2/T (32.72±6.07 vs. 91.04± 14.84, 55.12±7.01 vs. 91.04±14.84, 43.20±13.59 vs. 91.04±14.84, 31.48±3.57 vs. 91.04±14.84, respectively) and 0.1, 10, and 1,000 ng/mL PCB104 decreased E2/17α20βOHP (5.88±2.04 vs. 14.33±3.67, 5.84±1.09 vs. 14.33±3.67, 6.87±1.28 vs. 14.33± 3.67, respectively, $P<0.05$) compared with 17αOHP controls ($P<0.05$).

## Discussion

Exposure to synthetic or natural xenoestrogens appears to affect vitellogenin production in both male and female fish and to inhibit oocyte maturation (Scholz et al., 2000; Sohoni et al., 2001; Jobling et al., 2003). NP is estrogenic in various aquatic animals (Sumpter and Jobling, 1995; Nimrod and Benson, 1996; Billsson et al., 1998). Experiments with fish have shown that NP causes elevated plasma vitellogenin and zona radiata protein concentrations in both males and females, inhibition of spermatogenesis and induction of ovotestis in males, and altered gonadosomatic indices (Jobling et al., 1996; Arukwe et al., 1998, 2000).

In the present investigation, we compared the estrogenic potential of NP and PCB104 during oocyte maturation in the fully vitellogenic oocytes of yellowfin goby, *Acanthogobius flavimanus*. In the presence of HCG, we found that NP and PCB104 at concentrations of 0.1, 1, 10, and 1,000 ng/mL decreased E2/T ratios, and E2/17α20βOHP ratios were also decreased at PCB104 concentrations of 0.1, 10, and 1,000 ng/mL. These results suggest that NP and PCB104 have estrogen-antagonistic effects on the maturing oocytes.

Tollefsen (2002) reported that estrogenic effects refer to compounds mimicking the action of estrogens by binding to estrogen receptors and activating responsive genes. Estrogenic effects may also be mediated by the elevation of endogenous free plasma estrogen levels caused by competitive binding of xenobiotics to sex steroid binding globulins (Tollefsen et al., 2002). Anti-estrogenic effects refer to compounds with weak or no estrogenic activity but the ability to competitively bind to estrogen receptors. Furthermore, anti-estrogenic action can be mediated by affecting the expression and activity of the aromatase, which catalyses the final step in the synthesis of estrogens. Inhibition of this step leads to a reduction in E2 plasma concentrations, resulting in reduced vitellogenin levels in female fish (Scholz and Mayer, 2008).

In our previous studies, NP stimulated *in vitro* estrogen synthesis in fully vitellogenic oocytes of the longchin goby, *Chasmichthys dolichognathus* (Baek et al., 2003). NP has also been shown to have *in vitro* estrogenic potency on fully mature and vitellogenic oocytes by increasing E2/T and E2/17α20βOHP in the greenling, *Hexagrammos otakii* (Hwang et al., 2008). These different effects of NP on steroid production might be species-specific or depend on ovarian developmental stage.

PCBs have deleterious effects on the endocrine system because of their structural similarity to steroid hormones, especially estrogen. PCBs are considered to be potential endocrine disruptors due to their ability to act as estrogens, antiestrogens, and goitrogens (De et al., 2004). Several studies have reported that commercial PCB mixtures or individual congeners and/or their hydroxylated metabolites exhibit estrogenic or anti-estrogenic activity (Fielden et al., 1997; Bonefeld-Jørgensen et al., 2001). In a recent study, Westerlund et al. (2000) reported that PCB104 was highly toxic to zebrafish embryos following maternal exposure among 10 PCB congeners; the authors also found that PCB104 induced ER mRNA expression in the injected females similar to that found in exogenous E2 injected females (Westerlund et al., 2000). Moreover, PCB104 treatment was found to mimic the effects observed with E2. Following hydroxylation, PCB104 has the potential to become an E2 agonist. Determination of ER mRNA con-

firmed that PCB104 was capable of inducing ER in female zebrafish (Billsson et al., 1998). These results indicate that PCB104 has the ability to interact with estrogenic systems. Although it is difficult to compare data on estrogenic effects that has been evaluated by different methods and has used different species, our investigation showed that PCB104 showed estrogen-antagonistic effects by decreasing E2 and increasing 17α20βOHP levels in the fully vitellogenic stage, which is the threshold stage in the maturation process.

## Acknowledgements

This work was supported by a grant (PS-2006-028) from Pukyong National University.

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(Received 16 November 2009; Revised 4 December 2009; Accepted 26 December 2009)