Effects of 4-Nonlyphenol Exposure on *P. olivaceus* and *S. schlegeli* Vitellogenesis

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ABSTRACT : The effects of the estrogenic compound 4-nonylphenol (4-NP) on vitellogenesis in juvenile olive flounder (*Paralichthys olivaceus*) and rockfish (*Sebastes schlegeli*) exposed continuously at 10, 50 and 100 $\mu g\ \ell^{-1}$ levels for 7 days were compared. The expression of VTG mRNA level and protein using specific probes were examined. The levels of plasma estradiol-17 β (E_2) and testosterone (T) were assessed by radioimmunoassay (RIA). Plasma E_2 concentrations increased significantly in two female fish species exposed to 100 $\mu g\ \ell^{-1}$ of 4-NP over concentrations in control fish. Plasma T concentrations increased in *P. olivaceus*. Four days after exposure, the level of VTG mRNA expression increased in *P. olivaceus* and *S. schlegeli* exposed to 20 $\mu g\ \ell^{-1}$ of 4-NP. In addition, plasma VTG protein expression was seen in *P. olivaceus* and *S. schlegeli*. In *S. schlegeli* and *P. olivaceus* exposed to 4-NP, the changes were noticed mainly in hepatocytic vaculation after 7 days of exposure. Thus, 4-NP may disrupt vitellogenesis in immature fish both directly and indirectly via disrupted steroidogenesis and liver pathology. Immature *S. schlegeli* were the most sensitive to 4-NP exposure in vitellogenesis.

Key words : Vitellogenin, *Paralichthys olivaceus*, *Sebastes schlegeli*, 4-Nonylphenol, Steroidogenesis, Endocrine disrupter

INTRODUCTION

Nonylphenol (NP) is widely suspected as an endocrine disrupting compound (Nimrod and Benson, 1997). In the aquatic environment, nonylphenol (NP) originates mainly from the decomposition of nonylphenol ethoxylates in sewage treatment plants (Kime, 1998). Concentrations of nonylphenol in water ranged from 23.2 to 1,533.1 ng ℓ^{-1} in suspended particulate material and 6.8 to 932.0 ng g^{-1} dry wt. in sediment near industrial cities in Korea (Li et al., 2004a, b; Li et al., 2004). Nonylphenol was found even at a depth of 1,000 m at Siribesi trough in the East Sea, albeit at low levels (Kannan et al., 1998).

Alkylphenols have always shown estrogenic activity in various *in vitro* and *in vivo* assays (Gray and Metcalfe, 1997; Christensen et al., 1998; Giesy et al., 2000; Olsen et al., 2005; Tollefsen and Nilsen, 2007; Hwang and Baek, 2010). It has been reported that in male and immature fish, several alkylphenols can impair reproductive process by inducing plasma vitellogenin (VTG) (Korsgaard and Petersen, 1998; Parks et al., 1999). Cardinali et al. (2004) reported that nonylphenol treatment of male guppies induced VTG transcription and reduced the gonadosomatic index (GSI). However, females exposed to nonylphenol showed no alteration in reproduction. Other studies have reported a decrease in testis development and steroid levels in male rainbow trout (Lech et al., 1996; Jobling et al., 1996). These results show a clear need for the study of the mechanism and mode of action of 4-NP on the fish reproductive system. In addition, most of the studies on these compounds have focused so far on the stimulation of VTG in freshwater male fish as an indicator of possible "endocrine disruption" (Lomax et al., 1998).

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VTG, an estrogen inducible phospho-protein and complex precursor protein of egg yolk, is synthesized in the liver after stimulation of ovarian estrogens, transported to the ovary through the bloodstream and incorporated into the oocytes of teleost fish. VTG is observed in sexually mature females, whereas VTG levels in males and sexually immature fish are normally very low or undetectable. A number of environmental estrogens including alkylphenolic compounds also induce VTG synthesis in both males and females (Sumpter and Jobling, 1995). However, it is unclear how NP exerts its estrogenic effects *in vivo* and produces its physiological effects on fish during the life cycle and sexual differentiation. There are only a few studies that have investigated inter-species differences in 4-nonlyphenol effects on vitellogenesis in marine fish. Minor inter-species differences in the binding activity of fish ERs have been shown (Denny et al., 2005; Tollefsen et al., 2002; Tollefsen and Nilsen, 2007), but larger differences have also been shown among fish species of distant evolutionary relatives (Matthews et al., 2000). Beresford et al. (2000) have suggested that estrogenicity may dependent on ligand-binding affinity, and transcriptional and post-transcriptional regulation of ER-dependency. Thus, it is important to compare interspecies difference in estrogenic response and sensitivity in Korean coastal species.

In the present study, the effects of 4-NP on the estrogenic activity of two juvenile marine species (one-year old), namely, olive flounder (*Paralichthys olivaceus*) and rockfish (*Sebastes schlegeli*) were investigated. They are commercially and ecologically important fish species in Korean coast. The inter-species differential sensitivity of 4-nonylphenol on reproductive system was also examined. The study aims to clarify the estrogenic disruption from 4-NP exposure in Korean coastal species and to support the database of seawater quality management.

MATERIALS AND METHODS

1. Chemicals

4-nonylphenol was purchased from Fluka Chemical Biochemika (Buchs, Switzerland). Standard hormones, testosterone (T: 17β-hydroxy-3-oxo-androstene) and estradiol-17β (E_2; 1,3,5-estratriene-3,17-β-diol) were purchased from Sigma Chemical Co (Dorset, UK). Anti-testosterone and anti-estradiol-17β rabbit antibodies were purchased from CosmoBio Co. (Tokyo, Japan). Radioactive steroids, testosterone (T; 1,2,6,7-^{3}H) and estradiol (17β; 2,6,7-^{3}H), were purchased from NEN Life Science (Boston, MA, USA). Dextran-coated charcoal was purchased from Sigma-Aldrich (St. Louis, MO, USA) and Optiphase was purchased from Wallac Company (Turku, Finland). ABC Elite Kits and biotinylated anti-rabbit IgGs were purchased from Vector Laboratories (Burlingame, CA, USA) and Sigma-Aldrich, respectively.

2. Experimental Animals and Exposure to 4-Nonylphenol in a Continuously Flow-through System

Juvenile olive flounder (*Paralichthys olivaceus*) and rockfish (*Sebastes schlegeli*) were transferred from a fish farm in Geoje Island to the Korea Ocean Research and Development Research Institute. Before starting the experiment the fish were ensured to be free of any disease and then they were acclimated in the aquarium for two weeks. The exposure was carried out in 100 ℓ water tank using a flow-through water exposure system at 5 ℓ h^{-1}. Chemical stock solutions were infused at 50 $\mu\ell$ per cycle in a mixing chamber using a VersaPump 6 syringe dispenser module (Kloehn Ltd, Las Vegas, NV, USA). Photoperiod was maintained at 12/12 h light/dark. Water conditions were maintained at 18 ± 0.58℃, pH 7.7-8.0 and 87% oxygen saturation. Fish were exposed continuously to 4-NP concentrations of 20, 50 or 100 μg ℓ^{-1}. Seven fish were sampled randomly on each day on 1, 4 and 7 days after exposure. They were anesthetized with 0.3 $m\ell$ 2-phenoxyethanol (Sigma-Aldrich) per liter of water. Immediately after movement ceased, blood was drawn from the caudal vessels using a heparinized syringe and placed in a tube containing phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich)

at a final concentration of 1 mM. Blood was centrifuged at 2,200 g for 15 min at 4℃. Plasma was collected and stored at −80℃. The fish were killed by a blow on the head and the body length, weight, sex and gonadal weights (in mature individuals) were recorded. Samples of liver were taken, snap-frozen in liquid nitrogen and stored at −80℃ until analysis.

The hepatosomatic index (HSI = liver weight × 100/total body weight in g) and gonadosomatic index (GSI = gonad weight × 100/total body weight in g) were calculated for all samples.

3. Northern Blot Analysis

Total RNA of the individual liver tissues was extracted with ISOGEN (Wako Pure Chemical Industries, Ltd, Osaka, Japan). VTG-specific cDNA probes were generated by transcription with 5.0 μℓ of [α-^{32}P] dATP (New England Nuclear, Boston, MA, USA) using Random Primer labeling kits (Takara, Japan). Expression levels of VTG mRNA were estimated by northern blot analysis as described by Jung et al. (2006) and normalized by the expression level of β-actin. RNA was separated on 1% formaldehyde agarose gels, transferred to Hybond N^+ membranes (Amersham, Buckinghamshire, UK). RNA was prehybridized for 3 h in 50% formamide, 5 × Sodium Sodium Citrate buffer (SSC), 5 × Denhard's solution, 0.1% sodium dodecyl sulfate (SDS) and 10% blocking reagent (Roche, USA). Hybridization proceeded in a solution containing 50% formamide, 5 × SSC, 5 × Denhard's solution. 0.1% SDS and 10% blocking reagent, plus radiolabeled probes at 42℃ for 16 h. Following hybridization, blots were washed for 15 min each with low (2 × SSC, 0.5% SDS) and high (0.5 × SSC and 0.5% SDS), and washed again at 55℃. The membranes were analyzed with a BAS 2000 Bio-Image Analyzer (Fujix, Tokyo, Japan).

4. Radioimmunoassay

To extract steroids, 200 μℓ of plasma was mixed with 2 mℓ of diethyl ether. The ether phase was dried under nitrogen and reconstituted in 0.1% gelatin-phosphate buffer saline (pH 7.5). Plasma levels of E_2, T and 17-OHP were measured by RIA according to the method of Aida et al. (1984). The assay system had a working range between 30 and 3,840 pg $mℓ^{-1}$ for all of these steroids.

5. VTG Levels in Plasma

Plasma VTG concentration was measured Enzyme-linked Immunosorbent Assay (ELISA) method using poly and monoclonal antibodies against rockfish (Jung et al., 2006) and olive flounder VTG (Kim et al., 2006) in seven fish selected randomly from each treatment group.

6. Tissue Preparations for Light Microscopic Examination

Small pieces of liver were immediately extirpated and fixed in aqueous Bouin's solution. The tissues thus fixed were routinely dehydrated in graded series of alcohols, cleared in xylene and embedded in paraffin. Serial sections were cut at 6 μm and stained with hematoxylin-eosin.

7. Statistical Analysis

All data are expressed as the mean ± standard error of mean (SEM). Data were compared by analysis of variance (ANOVA) and significant differences between groups were determined by Duncan's multiple range test.

RESULTS

1. Liver and Gonadosomatic Index during the Experiment

There were few differences in the water quality among treatment tanks during 4-NP exposure studies. Within species, there were no significant differences in mean fish length and mass among treatment groups 4 and 7 days after exposure. There was no significant difference in GSI in *P. olivaceus* and *S. schlegeli* during experiment periods (Table 1). From 4 days after exposure, the level of HSI increased significantly to 2.033 ± 0.171%, 2.310 ± 0.349% and 2.330 ± 0.158% in *P. olivaceus*. Clear increases in HSI were

Table 1. Gonadosomatic index (GSI) in female *P. olivaceus*, female *S. schlegeli*, male *P. olivaceus*, and male *S. schlegeli* exposed to 4-nonylphenol

Species	Biomass index	NP concentration (㎍/ℓ)	Sex	4 days after exposure (mean±S.D.)	7 days after exposure (mean±S.D.)
P. olivaceous	GSI	0	Male	0.068±0.002	0.053
			Female	0.639±0.002	0.636±0.073
		20	Male	0.061	0.045±0.011
			Female	0.604±0.016	0.649
		50	Male	0.068	0.049
			Female	0.758±0.155	0.536±0.074
		100	Male	0.052±0.004	0.049±0.013
			Female	0.733	0.542
S. schlegeli	GSI	0	Male	0.030±0.008	0.036±0.025
			Female	0.179±0.002	0.143
		20	Male	0.031±0.007	0.040±0.011
			Female	0.166±0.008	0.121±0.006
		50	Male	0.037	0.046
			Female	0.169±0.019	0.136±0.013
		100	Male	0.034±0.052	0.044
			Female	0.18	0.143±0.024

Data are presented as means and standard errors.

Table 2. Hepatosomatic index (HSI) in *P. olivaceus*, and *S. schlegeli* exposed to 4-nonylphenol

Species	Biomass index	NP concentration (㎍/ℓ)	4 days after exposure (mean±S.D.)	7 days after exposure (mean±S.D.)
P. olivaceous	HSI	0	1.242±0.228	1.402±0.082
		20	2.033±0.1717*	2.421±0.271**
		50	2.310±0.349**	2.675±0.137**
		100	2.330±0.158**	2.298±0.087**
S. schlegeli	HSI	0	1.640±0.130	1.590±0.177
		20	2.079±0.076*	2.904±0.397**
		50	1.896±0.245*	2.670±0.136**
		100	1.816±0.565*	2.642±0.370**

Data are presented as means and standard errors; n = 4~7. Significantly different from control levels: *P<0.05; **P<0.01.

seen in *S. schlegeli* 4 days and 7 days after exposure, which increased significantly to 2.904 ± 0.397%, 2.670 ± 0.136% and 2.642 ± 0.370% (Table 2).

2. Effects of 4-NP on Steroid Hormone Production

The levels of plasma E_2 increased significantly to 133 ± 33.35 pg $ℓ^{-1}$ in female *P. olivaceus* exposed to 100 ㎍ $ℓ^{-1}$ and T levels increased 4 days after exposure in both sexes (Fig. 1). As shown in Figure 2, the plasma E_2 level increased to 73.40 ± 13.20 pmol $mℓ^{-1}$ at 4 day after exposure, and similarly it increased to 165 ± 38.80 pmol $mℓ^{-1}$ in female *S. schlegeli* treated with 100 ㎍ $ℓ^{-1}$ of 4-NP at

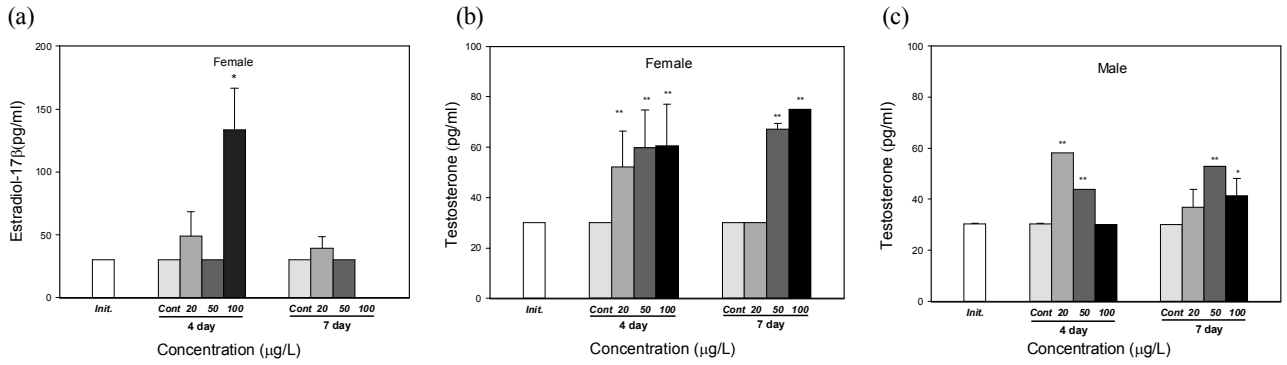

Fig. 1. Plasma steroid hormone levels in *P. olivaceus*. (a) level of estradiol-17β in females (b) level of testosterone in females and (c) level of testosterone in males exposed to 4-nonylphenol. Data are presented as means and standard errors; n = 7. Significantly different from control levels: **P*<0.05; ***P*<0.01.

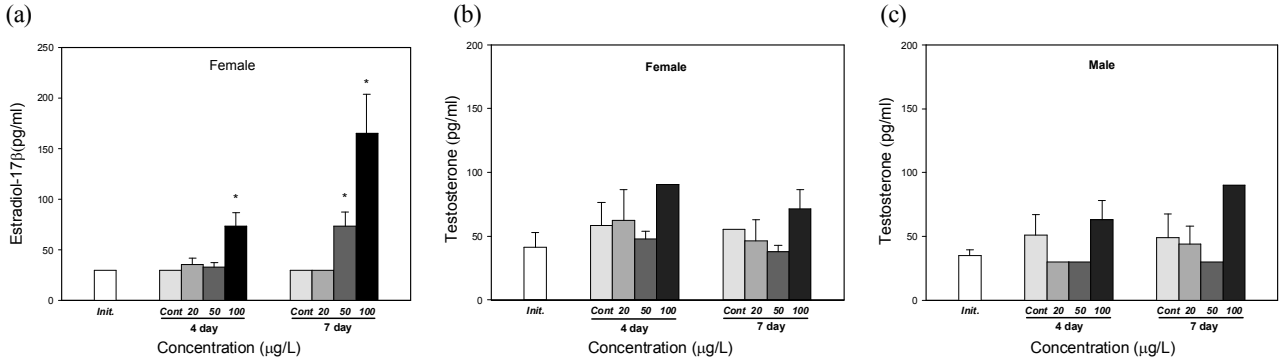

Fig. 2. Plasma steroid hormone levels in *S. schlegli*. (a) level of estradiol-17β in females (b) level of testosterone in females and (c) level of testosterone in males exposed to 4-nonylphenol. Data are presented as means and standard errors; n = 7. Significantly different from control levels: **P*<0.05.

7 days after exposure.

3. Expression of Vitellogenin mRNA and Protein Levels

VTG mRNA was significantly elevated showing a four- to five-fold increase in *P. olivaceus* in comparison to controls in all treatment at 4 days after exposure (Fig. 3a); a similar induction was seen in *S. schlegeli* from all treatment groups at 4 days after exposure (Fig. 3b). The VTG mRNA level of VTG *P. olivaceus* was three fold higher than that of *S. schlegeli*.

Plasma VTG protein increased significantly in *P. olivaceus* exposed to 100 $\mu g\ \ell^{-1}$ 4 days after exposure and in all treatment groups at 7 days of exposure (Fig. 4a). In *S. schlegeli*, significantly induced VTG protein was seen in all treatment groups during the experiment period (Fig. 4b). The level of plasma VTG of *S. schlegeli* increased more than that of *P. olivaceus*.

4. Effects on Liver Histopathology

There were no significant pathological abnormalities in liver of unexposed fish, but histopathological changes in liver caused by exposure to NP in fish were observed. In *S. schlegeli* and *P. olivaceus* exposed to 4-NP, the changes were mainly in hepatocytic vaculation after 7 days of exposure (Fig. 5). However, histopathological changes were

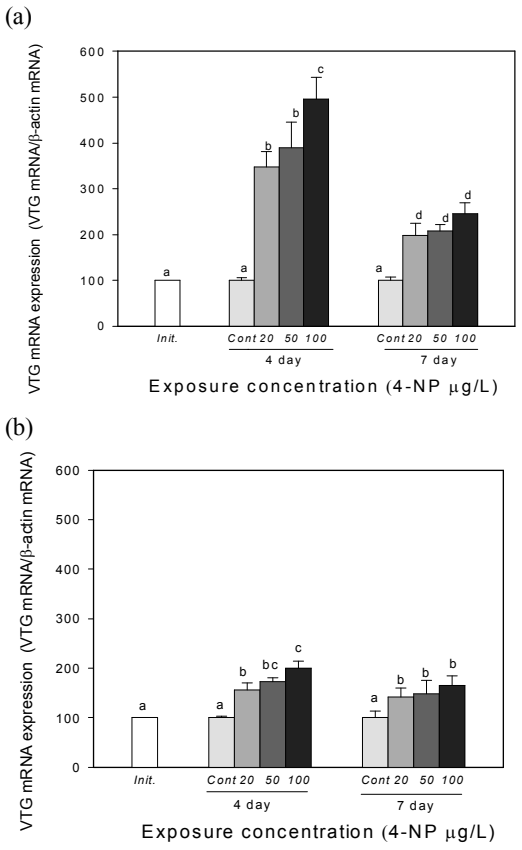

Fig. 3. Vitellogenin mRNA expression normalized to beta actin in (a) *P. olivaceus* and (b) *S. schlegeli* exposed to 4-nonylphenol. Data are presented as means and standard errors; n = 7. Significantly different from control levels: $^{**}P<0.01$

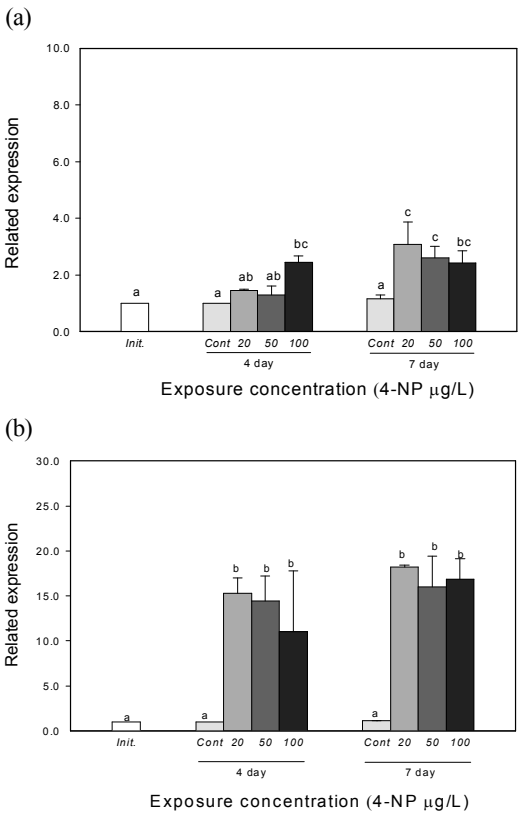

Fig. 4. Plasma VTG level in (a) *P. olivaceus* and (b) *S. schlegeli* exposed to 4-nonylphenol. Data are presented as means and standard errors; n = 7. Significantly different from control levels: $^{**}P<0.01$. Data were subjected to one- way ANOVA followed by Duncan's multiple-range test.

not seen in gonads in any treatment groups (data not shown).

DISCUSSION

NP-exposure can impair the gonadal development in immature and mature fish and several studies have indicated that exposure to 4-NP can lead to reduction in GSI (Jobling et al., 1996; Van den Belt et al., 2003; Kang et al., 2003; Brion et al., 2004). In the present study, no obvious difference in GSI was noticed in *P. olivaceus* and *S. schlegeli* during the experimental period. Scholz and Gutzeit (2000) have indicated that this decreased GSI might be from degeneration of the ovaries. Yang et al. (2006)

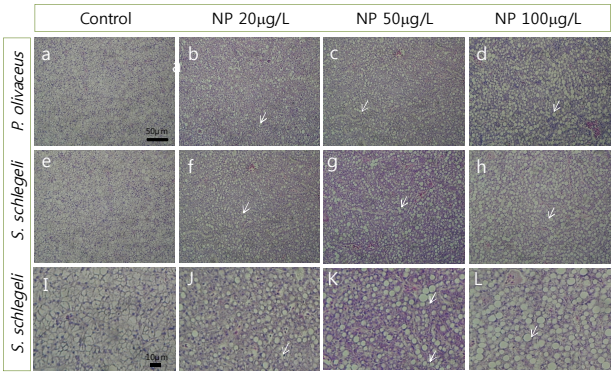

Fig. 5. Histological observation of liver in (a~d) : *P. olivaceus* and (e~h) : *S. schlegeli* exposed to 4-nonylphenol. Arrows indicate hepatocytic vacuoles. Magnification range: × 400 (a~h) and ×1,000 (I~L).

suggested that the difference in inhibitory effects of GSI might involve impaired gonadal development i.e., alterations in germ cell syncytia. In this study, all the fish species were immature and had similar gonadal development. Therefore, this difference in GSI might be due to species-specific difference for short-term exposure.

A significantly higher HSI was observed only in *P. olivaceus* and *S. schlegeli* after 7 days of exposure in comparison to controls. The increased HSI observed in NP-treated juvenile guppies might be due to higher VTG production in both sexes (Gimeno et al., 1998).

Exposure to 4-NP has been shown to increase the levels of plasma E_2 significantly in several studies (Schwaiger et al., 2002; Mosconi et al., 1998; Cavaco et al., 2003). The same result was observed in the present study. 4-NP exposure in two fish species increased the E_2 plasma values through induction. However, an opposite effect was found in Atlantic salmon, where exposure to 4-NP caused a 24-43% decrease in the plasma E_2 level (Arukwe et al., 1997); no effect at all was found in our previous study (Jung et al., 2006). Plasma T levels were not reduced in three male fish in all treatment groups through 4-NP exposure. There was significant increase in concentration of plasma T in *P. olivaceus*.

These data showed that two fish species treated with 4-NP showed difference in vitellogenesis. A significant increase in VTG was observed in *P. olivaceus* and *S. schlegeli*. It is interesting to note that the VTG mRNA level was higher in *P. olivaceus* than *S. schlegeli* but the VTG protein was expressed highest in *S. schlegeli*. The present data show clearly that sensitivity and half-life time of vitellogenesis differs and depends on fish species. Although 4-NP-exposure generally mimics VTG induction in the reproductive system, specific processes may differ within species.

The modulation of vitellogenesis caused by some estrogenic compounds, such as 4-NP, is mediated via binding to the ER or at the pituitary level (Jobling et al., 1996; Tollefsen et al., 2002). Because both male and female juvenile fish possess ER receptors in the liver (Soverchia et al., 2005; Inui et al., 2003), these data suggest that the ER might vary in its response between species. In addition, Luo et al. (2005) found that the differential effects of 4-NP treatment on the expression of the ERα gene in sockeye salmon could explain the different physiological effects observed between treatments. 4-NP has been shown to bind to the ER and to elicit a number of estrogenic responses *in vivo*. Although the ERs from closely related species exhibit similar binding affinities for endogenous and exogenous estrogens (Tollefesen et al., 2002), large differences in estrogen binding capacity have been demonstrated for the ERs from divergent species (Matthews et al., 2000). Increased ER concentrations could be explained through direct effects of 4-NP on ER gene expression (White et al., 1994; Nimrod and Benson, 1997; Arukwe et al., 1997). However, in our results, 4-NP induced significantly in plasma steroids level of fish and this difference might be due to a different mode of action. Our results suggest that 4-NP may disrupt the reproductive system by acting directly or indirectly on vitellogenesis (Giesy et al., 2000; Villeneuve et al., 2002). Clearly, more studies needed to understand this problem.

The present study demonstrated histopathological changes in the liver due to 4-NP-exposure in fish. Highly vacuolated cytoplasm was observed in 4-NP exposed fish. This is in agreement with similar finding in summer flounder exposed to 17β estradiol (Folmar et al., 2001) and rat exposed to nonylphenol (Hern´andez-Rodr´ıguez et al., 2007). Mollendorf (1973) suggested that vascular formation is a cellular defense mechanism against injurious substances to cells, these substances being segregated in vacuoles and thus were prevented from interfering with cellular metabolism. It has also been suggested that cytoplasmic vacuolation is mainly a consequence of disturbances in lipid inclusions and fat metabolism occurring during pathological disturbances (Zhang and Wang, 1984).

Thus, 4-NP might disrupt vitellogenesis and liver pathological disruption in immature fish both directly and indirectly

via disrupted steroidogenesis. Immature *S. schlegeli* was more sensitive to 4-NP exposure during vitellogenesis. There are obviously large interspecies differences in the response of fish to 4-NP.

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