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Endocrine-Disrupting Effects of Nonylphenol, Bisphenol A, and p,p′-DDE on *Rana nigromaculata* Tadpoles

F.-X. Yang,[1] Y. Xu,[1] S. Wen[1,2]

[1] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Donghu Nan Road 7, Wuhan 430072, People's Republic of China
[2] Graduate School of the Chinese Academy of Sciences, Yuquan Road 19A, Beijing 100039, People's Republic of China

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Amphibians are excellent bioindicators of the general health of the environment because of their highly permeable skin and their lifecycle which goes through both aquatic and terrestrial stages (Noriega and Hayes 2000). Sex steroids and thyroid hormones play vital roles in the development and growth of amphibians. Androgens, such as testosterone, are essential for normal spermatogenesis but also to maintain the normal integrity of the male secondary sex organs. Besides the physiological functions, androgens can be converted into estrogens which have a major influence on sexual characters and other activities in the female. It is well known that the metamorphosis of amphibians is an endocrine-mediated pathway (Gray et al. 2002). The developmental delay and changes in amphibians, especially the effects on metamorphosis, are believed to be influenced by disruptions of thyroid hormones by endocrine disrupting chemicals (EDCs) (Iwamuro et al. 2003).

It has been reported that many chemicals in the environment can mimic natural hormonal actions and disrupt endocrine function, thereby affecting development and reproduction in human beings and wildlife (Colborn et al. 1993). Nonylphenols (NPs) in the environment mainly originate from the breakdown of nonylphenol ethoxylates (NPEs) which are nonionic surfactants. Bisphenol A (BPA) is used in the manufacture of polycarbonate, epoxy resins, and other plastics. p,p'-DDE is a most persistent metabolite of DDT which had been widely used as a pesticide in agriculture. Their occurrences and levels in aquatic environment are well documented (Porter and Hayden 2002; Staples et al. 1998; Misumi et al. 2005). Some studies (Thibaut and Porte 2004; Pait and Nelson 2003; Mills et al. 2001) have identified endocrine effects of the three chemicals on fish, but there is still a lack of information on their potential effects on amphibians, especially on metamorphosis. Furthermore, the combined effects of these three chemicals on aquatic animals, especially on amphibians, are seldom reported. The aim of the present study is to investigate the combined effects of NP, BPA and p,p'-DDE on tadpoles of the black-spotted pond frog (*Rana nigromaculata*) as these chemicals are often found in the aquatic environment (Petrovic and Barcelo 2001; Sapozhnikova et al. 2004).

Correspondence to: Y. Xu

MATERIALS AND METHODS

BPA (≥95%) was obtained from Fluka. Technical NP, p,p'-DDE (99%) and 4-amino-3-hydroxy-1-naphthalenesulfonic acid (95%) were purchased from Aldrich. The other chemicals and solvents were analytical grade. The chemicals were dissolved in dimethylsulfoxide (DMSO). The testosterone (T) radioimmunoassy kit and total thyroxin (TT4) radioimmunoassy kit were both obtained from TianJing Jiuding Medical and Bioengineering Corp., China.

The embryos of the black-spotted pond frog (*Rana nigromaculata*) were collected from the field and hatched in the laboratory. Before imposing the treatments, the tadpoles were maintained in dechlorinated water for five days after they were hatched. A group of 30 tadpoles for a treatment were maintained in each tank that contained 6L of dechlorinated water. NP and BPA were applied at three different concentrations: 2μg/L(low), 20μg/L(intermediate), and 200μg/L(high). The concentrations of combined treatments of BPA+NP and BPA+NP+p,p'-DDE were 2+2μg/L(low), 20+20μg/L(intermediate), 200+200μg/L(high), and 2+2+0.5μg/L(low), 20+20+5μg/L(intermediate), 200+200+50μg/L(high) respectively. Each treatment was duplicated. The final concentrations of DMSO in dechlorinated water were no more than 0.01%. The tadpoles maintained in dechlorinated water and DMSO were used as the blank control (control) and solvent control (DMSO) respectively. Half the volume of water was renewed with fresh dechlorinated water and corresponding chemicals every three days. The tadpoles were fed with fish food every day. The tanks were kept at 25±2℃ and under a 12h light-12h dark cycle. Five tadpoles from each tank were sampled and weighed after exposure for 15, 30, 45 and 60 days (four tadpoles were sampled and weighed for the high concentration treatment of BPA+NP for 60 days). For analysis, all five tadpoles were pooled together to supply enough biomass for analysis and stored at -20℃.

The samples were homogenized with phosphate buffered saline (137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4 \cdot 7H_2O$, 1.4mM KH_2PO_4, pH 7.3) and centrifuged at 12,000×g and 4℃ for 15 min. The supernatant was transferred to a new tube for determination of T and TT4 with radioimmunoassay kits. The analysis was performed by a GC-1200 Gamma Radioimmunoassay Counter. Milli-Q water was used as the procedural blanks in both determinations. The standard deviations were below 6.2% and 5.8% for T and TT4 analysis, respectively. To assess the estrogenic effects of EDCs, the presence of plasma vitellogenin (VTG) in males or immature oviparous vertebrates has been broadly accepted as a biomarker in aquatic systems (Marin and Matozzo 2004). However, determination of VTG using immunoassay was not possible in the present study due to the lack of specific antibody. Therefore, alkaline-labile phosphate (ALP) was used to indicate the presence of VTG (Kramer et al. 1998) in the tadpoles. The samples were homogenized with Milli-Q water and centrifuged at 12,000×g and 4℃ for 15 min. The supernatant was transferred to a new tube for the analysis of ALP, which was determined by the method of Griswold et al. (1951). Each concentration of T, TT4

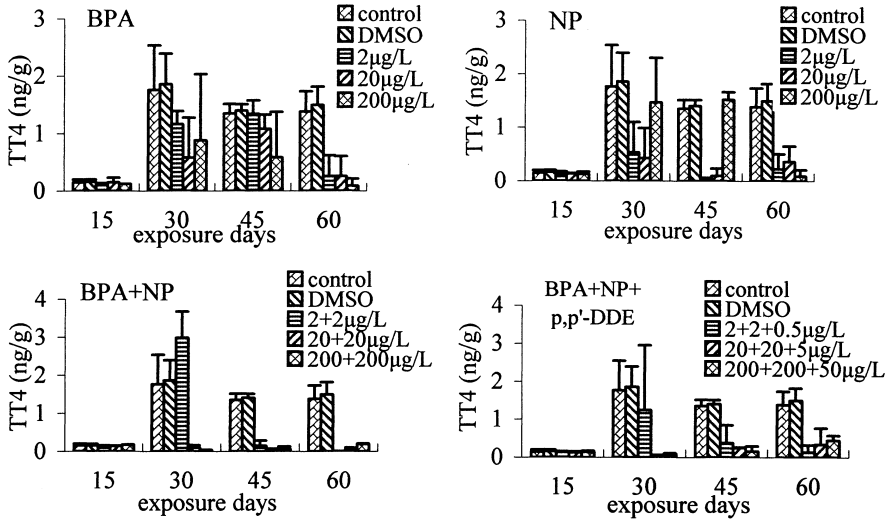

Figure 1. Effects of the tested chemicals on total thyroxine (TT4) of the tadpoles.

and ALP was shown as the average value for 5 tadpoles. Statistical analysis was performed using a one-way ANOVA to determine significance groups with SPSS 10.0 (SPSS Inc. USA). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Malformations of tail flexure in some tadpoles exposed to the high concentrations of the chemicals and mixtures were found during metamorphosis (after 45-day exposure). The percentages for malformations were 10.0% for 200μg/L BPA, 16.7% for 200μg/L NP, 13.3% for 200+200μg/L BPA+NP, and 13.3% for 200+200+50μg/L BPA+NP+p,p'-DDE at the end of exposure respectively. The tadpoles with tail flexure grew up into young frogs after the completion of metamorphosis. It is well known that metamorphosis of amphibians is related to the thyroid axis which is a potential target of EDCs action (Gray et al. 2002). The metamorphic abnormalities of the tadpoles in the study may result from the disruption of thyroid hormone.

Inhibition of TT4 was observed in all tadpoles after 60 days exposure when compared to the control (Figure 1), although the inhibition was not significant. BPA and NP were reported to weakly interact with amphibian transthyretin (TTR) which has been investigated thoroughly as a target site for EDCs (Yamauchi et al. 2003). The binding of EDCs to mammalian TTRs is believed to increase the plasma clearance rate of L-thyroxine (T4), resulting in decreased serum T4 concentrations (Morse et al. 1996). A recent study has reported BPA blocked the thyroid hormone-induced resorption of the tail segments of larval *Xenopus laevis* in vitro and in vivo (Iwamuro et al. 2003). But the mechanism of the chemical-induced tail flexure resulting from disruption of the thyroid system remains

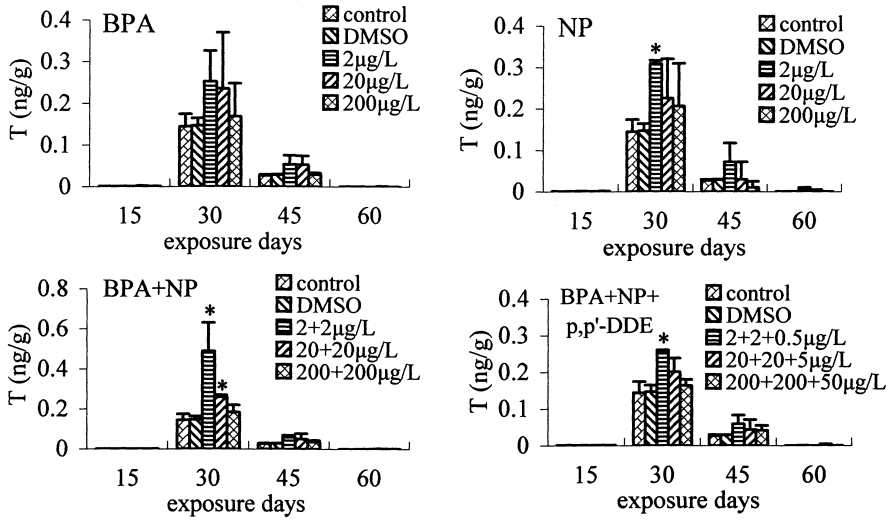

Figure 2. Effects of the tested chemicals on testosterone (T) in the tadpoles.
*: $P<0.05$ (compared to the control).

unclear. The failure of the tail to straighten may be explained as developmental retardation (Mann and Bidwell 2000) or effects on notochord formation as observed with dithiocarbamate pesticide (Haendel et al. 2004). Furthermore, no evidence indicates that the malformations resulted from changes of T and ALP in the study. So, further studies are needed to understand the relationship between this malformation and the disruption of the thyroid system, if any.

The effects of the chemicals on T are shown in Figure 2. The concentrations of T in the tadpoles exposed for 15 days were below the limit of detection (the LOD of analytical instrument was 0.1ng/ml). With continual exposure, the highest T concentrations were observed in all groups treated with the low dose of each chemical and mixture after 30 days of exposure. After that, the T concentrations declined. The time when T could be detected in the present study was similar to the results reported by Kang et al (1995). Moreover, Kang et al. also reported that tadpole gonads and interrenals were the only tissues capable of steroid biosynthesis and the interrenals were more active than the gonads in tadpoles but not in juveniles. That might be one of the causes that resulted in the decrease of T in the tadpoles after 45 and 60 days exposure. An increase in body weight of the tadpole can lead to decreased T level due to the expression of T concentration based on body weight.

Recent studies reported that NP disrupted the metabolic elimination of testosterone in daphnids (LeBlanc et al. 1999). This suggests the potential for both indirect androgenic and estrogenic effects, either through the actions of elevated T levels or through increases in E_2 levels due to increased levels of aromatizable substrate. On the other hand, it was reported that BPA significantly decreased

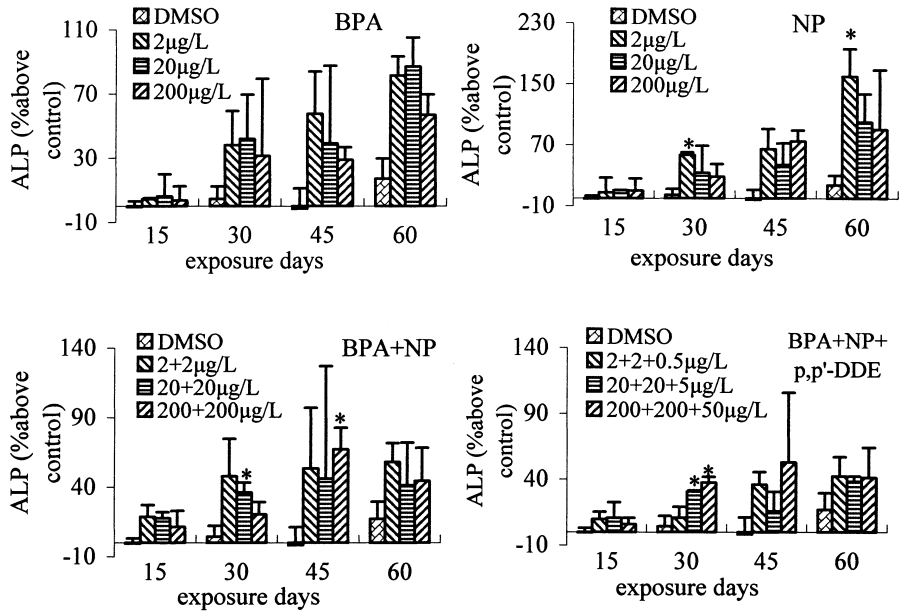

Figure 3. ALP inductions in the tadpoles exposed to the tested chemicals.
*: $P<0.05$ (compared to the control).

testosterone 2α-hydroxylase (T2AH) and testosterone 6β-hydroxylase (T6BH) activities in rat liver and resulted in an increase in T (Takeuchi and Tsutsumi 2002). Moreover, a trend of decreasing T levels was observed from low dose to high dose exposure in all groups after 30 and 45 days treatment. The result indicated the low dose exposure induced higher response. Murono et al. (1999) reported that exposure of cultured neonatal rat Leydig cells to increasing concentrations of octylphenol (1 to 2000 nM) and 10 mIU/mL human chorionic gonadotropin (hCG) for 24 h had a biphasic effect on testosterone formation, with lower concentrations (1 and 10 nM) actually increasing testosterone levels (10 to 70% above control), while higher octylphenol concentrations (100 to 2000 nM) progressively reduced testosterone from peak levels.

ALP concentrations in the tadpoles of each treatment generally increased along with the exposure time (Figure 3). Induction of ALP in the NP-exposed groups was higher than in the BPA-exposed groups. BPA and NP have been demonstrated to bind to amphibian estrogen receptors (Lutz and Kloas 1999) and induce feminization of male *Xenopus laevis* larvae (Kloas et al. 1999). In primary cultured amphibian hepatocytes, NP stimulated VTG-mRNA synthesis at a concentration of 10^{-8} M while BPA needed 10^{-7} M for a similar increase (Kloas et al. 1999). The estrogenic effect of BPA on stimulating VTG expression was lower than NP. The similar result was also obtained in the current experiment that the increase of ALP induced by NP was higher than that induced by BPA at the same concentration. Furthermore, elevated T levels induced by BPA and NP can also contribute to increased ALP concentrations by elevating E_2 transformed from

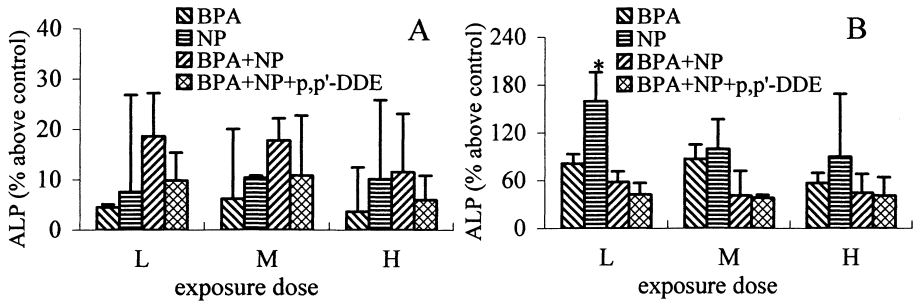

Figure 4. ALP inductions in the tadpoles after 15 (A) and 60 (B) days exposure. L: low, M: intermediate, H: high. *: $P<0.05$ (compared to the control).

increased T levels in the treated tadpoles. In the four groups, it was also observed that the low doses of exposure induced higher ALP levels than the high doses of exposure in most cases.

The combined effects on ALP in the initial (15 days) and final (60 days) stages of exposure were monitored (Figure 4). The increase in ALP induction in the tadpoles exposed to the three concentrations of BPA+NP was higher than the tadpoles exposed to BPA and NP individually after 15 days exposure. However, after 60 days of exposure, the ALP levels in the tadpoles exposed to BPA+NP were lower than in the tadpoles exposed to BPA and NP individually. Furthermore, p,p'-DDE prevented the increase of ALP induced by BPA+NP in the tadpoles treated with the mixture of the three chemicals after 15 and 60 days. Similarly, Tan et al. (2003) reported that the effects induced by the mixture of NP and BPA were markedly decreased compared with the effects induced by either NP or BPA which had significant effects in a study of pubertal development in juvenile male rats. It could be speculated that the estrogenic pathway of BPA was disrupted by NP (Matthews et al. 2001). The varied responses of ALP induced by BPA+NP at different exposure times also indicated the complexity and importance of the time-response relationship of chemical exposure. Previous studies based on in vitro competitive binding assays using mammalian androgen receptors indicated that p,p'-DDE was anti-androgenic (Kelce et al. 1995). These reports coincide with our results that p,p'-DDE inhibits the levels of T and ALP induced by BPA+NP.

Hormesis is a phenomenon with a stimulatory response under exposure to low dose of stress, such as chemicals and radiation, and an inhibitory response at high dose. In this study, we observed that these chemicals induced stimulatory effects on T and ALP at low-dose. More studies have confirmed the stimulatory effects at low dose, and a biphasic or inverted U-shaped dose-response relationship with some kind of hormesis (Calabrese 2001). It is believed that this kind of dose-response correlation plays a crucial role in EDCs studies. Nevertheless, further exploration of the relevance of hormesis to the long-term effects could be desirable for an overall ecological risk assessment of EDCs.

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