Effect of Nonylphenol on Plasma Vitellogenin of Male Adult Guppies (*Poecilia reticulata*)

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ABSTRACT: Adult male guppies (*Poecilia reticulata*) were exposed to 4-nonylphenol (NP) at nominal concentrations of 10, 60, and 150 μg/L for 7, 14, or 21 days. Significant induction of vitellogenin (VTG) was found in male guppies exposed to 1 μg/L of 17β-estradiol and 60 or 150 μg/L of NP after 7, 14, or 21 days. Maximum induction was seen after 14 days of treatment at these concentrations. On the other hand, significant induction of VTG in male guppies exposed to 10 μg/L of NP was observed only after 21 days of treatment. A time-dependent tendency of reduction of the gonadosomatic index (GSI) in male guppies treated with 17β-estradiol or NP was observed, but no corresponding concentration-dependent tendency was detected. There were also no differences in the liver somatic index (LSI) of male fish treated with various concentrations of NP during the above-mentioned exposure periods. Preliminary data presented in this study strongly suggests that measurement of plasma VTG induction in male guppies is a suitable and rather sensitive indicator of exposure to estrogenic chemicals. Environ Toxicol 20: 53–59, 2005.

Keywords: guppy; *Poecilia reticulata*; nonylphenol; vitellogenin; endocrine disruption; biological indicator; gonadosomatic index

INTRODUCTION

There is increasing concern about the potential risk in wildlife and humans of exposure to man-made chemicals possessing endocrine-disrupting ability (Colborn et al., 1993). Among aquatic animals, fish are especially prone to exposure to endocrine disruptors in their ambient environment, and those in the wild can exhibit a variety of reproductive problems including masculinization of females (Cody et al., 1997), feminization of males (Jobling et al., 1998), altered sex hormone levels and gonadal abnormalities (Hashimoto et al., 2000), and reduced fertility (Jobling et al., 2002). Therefore, fish can be used as experimental models in studies of endocrine disruption effects and also can serve as an early-warning indicator of effects that may later become apparent in other wildlife species and in humans.

Before predicting the possible effects of endocrine disruptors in wildlife, it is important to assess the exposure status of endocrine disruptors in organisms in their environment. Induction of vitellogenin (VTG) in male fish has been widely used as an indicator of exposure to xenoestrogens both in field and in laboratory conditions. VTG is a yolk precursor protein produced in the liver under the stimulation of ovarian estradiol and is normally found in the blood of female fish, whereas plasma VTG levels in male fish are normally very low or not present (Kime et al., 1999). However, VTG synthesis and release can be induced in the

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livers of male fish if exposed to exogenous estrogen (Kime et al., 1999). The presence of plasma VTG in male fish therefore is considered a sensitive biomarker of exposure to both estrogens and xenoestrogens (Sumpter and Jobling, 1995).

The guppy, which is very well adapted to the streams of Southeast Asia (Wildianarko et al., 2000), is a viviparous tropical fish with a short life cycle. It is widely used as a test organism in investigations of aquatic toxicity because it is a readily available and easily handled species. This fish model offers several reproductive endpoints for assessing the cellular, behavioral, morphological, histological, and population-level effects of endocrine disruptors (Bayley et al., 1999, 2002; Haubruge et al., 2000; Baatrup and Junge, 2001; Toft and Baatrup, 2001, 2003; Larsson et al., 2002; Kinnberg and Toft, 2003; Kinnberg et al., 2003). However, only limited information is available on the VTG production of this species in response to exposure to xenoestrogens (Wester et al., 1985).

The objective of this study was to investigate the effects of 4-nonylphenol (NP) on VTG induction in male adult guppies. NP was selected as a testing chemical because of its industrial, commercial, and household applications all over the world (Bennie, 1999). This compound was detected in surface water and sediment in the rivers of Taiwan (Ding et al., 1999; Wang et al., 2001), and was found to be estrogenic both *in vivo* and *in vitro* (Nimrod and Benson, 1996). In this study we have demonstrated a dose–response relationship of plasma VTG induction in male guppies in response to NP during three different exposure periods.

MATERIALS AND METHODS

Chemicals and Reagents

4-Nonylphenol was obtained from Fluka (Switzerland). Trizma base, ethylenediamine tetraacetic acid (EDTA), and glycerol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetone, 17β-estradiol, MS222, aprotinin, heparin, and all other chemicals used in electrophoresis were obtained from Sigma Chemical Company (Saint Louis, MO, USA).

Animals and Experimental Design

Adult male guppies were obtained from a local supplier and acclimated in the laboratory to a temperature of 24°C $\pm$ 1°C and a 12-h photoperiod for at least 7 days prior to the experiments. The guppies were fed daily with commercial dry flake food (AZOO 9 in 1 guppy pellet, AZOO Company, Taiwan). To eliminate the risk of leached potential endocrine disruptors, plastic materials were avoided in all aquariums and plumbing. Dechlorinated tap water was stored in a stainless-steel tank before use. The fish were exposed to either NP at nominal concentrations of 10, 60, and 150 μg/L, 17β-estradiol at a nominal concentration of 1 μg/L as a positive control, or acetone alone at a nominal concentration of 30 ppm as a solvent control. The test chemicals were dissolved in acetone, and the test water was replaced three times a week in all beakers in order to maintain treatment concentrations. Each group of six male guppies was kept in 5 L of water in a glass beaker at the beginning of each treatment, and half of the guppies were removed from each beaker after different exposure periods (7 or 14 days; 14 or 21 days; 7 or 21 days). There were 21 guppies in each treatment group at the beginning of exposure. The fish were inspected daily for mortality during the entire experimental period.

Tissue Preparation

After 7, 14, or 21 days of exposure, all fish were exposed to water containing heparin (2000 U/L) for 30 min and then were anesthetized in MS222 (300 μg/L) for 15 min. Blood samples were collected by cardiac puncture into a microsyringe and transferred to plastic Eppendorf tubes containing aprotinin. Because the fish were small in size, blood from two or three guppies was contributed to make up one blood sample in this study. Blood samples were centrifuged at 10 000 $\times$ g for 10 min, and the resulting plasma volume of each sample was measured with a 10-μL Hamilton syringe. The resulting plasma was then stored at liquid nitrogen until analyzed. Whole-body, liver, and testis wet weights of each guppy also were measured. The gonadosomatic index (GSI) was calculated as the percentage of the testis weight to the whole-body weight. The liver somatic index (LSI) was calculated as the percentage of the liver weight to the whole-body weight.

Vitellogenin Determination

Semiquantification of plasma VTG concentration was carried out by gel electrophoresis followed by densitometric analysis of the VTG band as described by Janssen et al. (1997). In brief, plasma samples [0.05 M Tris (pH 6.8), 2.15 mM EDTA, 1% SDS, 1% β-mercaptoethanol] were combined with an equal volume of native sample buffer (8% glycerol, 0.025% bromophenol blue) and placed into nearly boiling water (95°C) for 10 min to denature the proteins. Then plasma samples were analyzed by reducing sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% stacking gel and a 7.5% running gel of polyacrylamide. The gels were run in a Hoefer Might Samll II SE250 Mini-Vertical Electrophoresis Unit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with a constant current of 40 V for 30 min and 120 V for another 60 min at room temperature. Gels were stained with 0.02% Coomassie blue R-250 for 15 min to indicate protein bands

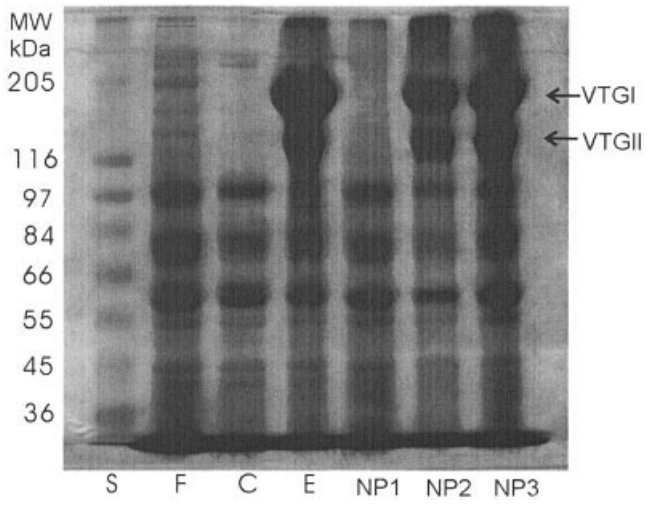

Fig. 1. SDS-PAGE gel from guppy plasma samples treated with NP or 17β-estradiol after 14 days of treatment. Lane 1 (S), molecular weight protein standards; lane 2 (F), reference female; lane 3 (C), control male; lane 4 (E), 1 μg/L 17β-estradiol–treated male; lane 5 (NP1), 10 μg/L NP–treated male; lane 6 (NP2), 60 μg/L NP–treated male; lane 7 (NP3), 150 μg/L NP–treated male.

and destained first with 25% methanol and 10% acetic acid, followed by 5% methanol and 7% acetic acid overnight. The resulting gels were digitized and then analyzed using Imagequant 5.0 (Molecular Dynamics, Sunnyvale, CA, USA). A reference sample from the aliquots of the pooled plasma collected from 36 adult female guppies was also analyzed in each gel. Therefore, the relative amount of VTG protein from different treatment groups could be calculated against this reference sample in each gel.

Statistical Analyses

All data were expressed as the mean $\pm$ standard deviation (SD). Differences among the treatment groups were analyzed with the nonparametric Kruskal–Wallis test using the Minitab statistical program (Version 13.2). If a significant result were found, the Mann–Whitney U test was used to determine which treatment groups were significantly different from the controls. Differences were considered significant at $P \leq 0.05$.

RESULTS

Identification of VTG

Two protein bands were detected in the plasma samples from adult female and 17-estradiol-treated male guppies but not from normal adult male guppies (Fig. 1).

These two bands (VTG I and VTG II) were tentatively identified as guppy vitellogenin in our SDS-PAGE gels. The molecular weights for VTG I and VTG II were estimated to be 191 and 156 kDa, respectively. Only VTG I protein appeared to be highly expressed and was used to quantify VTG induction in male guppies in the present study.

Dose- and Time-Dependence of NP-induced VTG

NP induced dose-dependent production of VTG in male guppies at different exposure periods. Densitometric analysis indicated that the level of VTG in male fish was about 82%–89% of that in female fish at 10 μg/L of NP during three different exposure periods (Fig. 2). There were about 2-, 4-, and 14-fold increases in VTG levels in male guppies exposed to 10 μg/L of NP compared to control male guppies for 7, 14, and 21 days of treatment, respectively; however, significant induction of VTG in male guppies was only observed after a 21-day exposure ($P = 0.0307$). On the other hand, significant induction of VTG was found in male guppies exposed to 1 μg/L of 17β-estradiol, 60 and 150 μg/L of NP after all three periods of treatment (Fig. 2). NP-induced (60 and 150 μg/L) and 17β-estradiol-induced (1 μg/L) VTG levels in male guppies were already at their maximum after 14 days of treatment and remained at a similar level of VTG induction after 21 days of treatment (Fig. 2).

Effect of NP on GSI and LSI in Male Guppies

There were no differences in body weight among the treatment groups (data not shown). Clear dose- or time-related mortality was not observed in our treatment groups. The

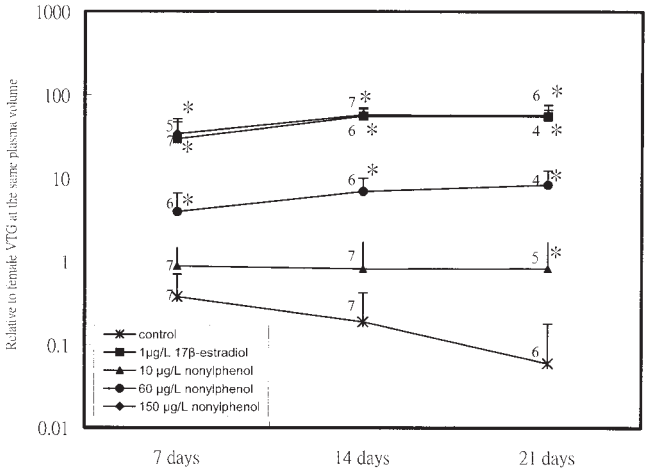

Fig. 2. Relative plasma vitellogenin (mean $\pm$ SD) in male guppies relative to that in reference female plasma samples after 7, 14, and 21 days of exposure to NP or 17β-estradiol, respectively. Numbers next to means indicate number of composite plasma samples per respective treatment. An asterisk denotes a significant difference from the respective controls at $P < 0.05$.

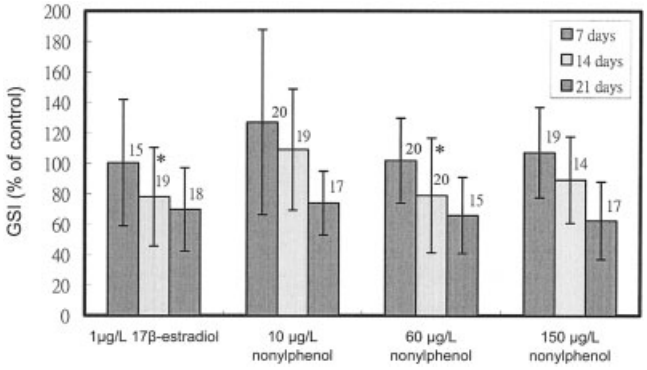

Fig. 3. Effects of 17β-estradiol and NP on the GSI (mean $\pm$ SD) in male guppies after 7, 14, or 21 days of treatment. Numbers next to means indicate number of fish per respective treatment. An asterisk denotes a significant difference from the controls with the same exposure time at $P < 0.05$.

percentage of survival in most treatment groups was above 80%, except for the fish exposed to 1 μg/L of 17β-estradiol for 7 days (71%), 150 μg/L of NP for 14 days (67%), and 60 μg/L of NP for 21 days (71%). Although a time-related tendency toward a reduction of the GSI in male guppies treated with 17β-estradiol or NP was shown, the GSI in guppies treated with 1 μg/L of 17β-estradiol or 60 μg/L of NP after a 14-day exposure decreased significantly (Fig. 3). A plot of the GSI as a function of plasma VTG showed a significant negative correlation ($r = -0.522$, $P = 0.046$; Fig. 4). In addition, there were no differences in the LSI of male fish treated with different concentrations of NP for different exposure times. The LSI of male guppies treated with 1 μg/L of 17β-estradiol was significantly higher than that of the control after 7 days of exposure but not significantly different than that of the control after 14 or 21 days of exposure (data not shown).

DISCUSSION

The purpose of this study was to examine the usefulness of plasma VTG production in male guppies as a biomarker to detect estrogenic exposure. Although Wester et al. (1985) reported that VTG production was found in juvenile male guppies exposed to 0.01 mg/L of 17β-estradiol or 1 mg/L of β-hexachlorocyclohexane after 2 weeks of treatment, a quantitative dose–response relationship of the VTG induction was lacking. In our study we measured plasma VTG induction in male guppies exposed to different concentrations of NP after 7, 14, or 21 days of treatment. Significant induction of VTG was found in male guppies exposed to 1 μg/L of 17β-estradiol, 60 and 150 μg/L of NP after 7, 14, or 21 days of treatment, and VTG induction was already at its maximum after 14 days of treatment. This result is in good agreement with reports from similar studies that mentioned that plasma VTG induction of male fish was a sensitive indicator of estrogenic exposure (Mills et al., 2001; Pait and Nelson, 2003).

In our study gel electrophoresis showed two protein bands related to estrogen-induced proteins, presumably VTG, with estimated molecular weights of 191 and 156 kDa. This result is in agreement with those of other studies. Wester et al. (1985) identified increased VTG production in juvenile male guppies exposed to 1 mg/L of β-hexachlorocyclohexane after 2 weeks of treatment, with estimated molecular weights of 200 and 140 kDa. Van den Belt et al. (2003) reported two protein bands with molecular weights of 193 and 138 kDa for zebrafish (*Danio rerio*) and 177 and 138 kDa for juvenile rainbow trout (*Oncorhynchus mykiss*), using gel electrophoresis. Fukada et al. (2003) also found two protein bands with molecular weights of 190 and 156 kDa after SDS-PAGE for carp (*Cyprinus carpio*). In their study further evidence was provided by the fact that these proteins were seen only in female fish and 17β-estradiol-treated males but not in normal male plasma. In the present study exposure of male guppies to NP resulted in the same protein bands as those exhibited in the plasma of female guppies and 17β-estradiol-treated males in a concentration-dependent manner.

Compared with the published data on the lowest-observed-effect concentration (LOEC) of plasma VTG induction in fish exposed to NP (Table I), the LOEC of NP (60 μg/L of NP in a 1-week exposure) found for male guppies in our study was at the lower end of the range (4–500 μg/L of NP) reported for male fish. This further suggests that the measurement of plasma VTG induction in male guppies is a suitable and rather sensitive indicator of NP exposure. In our experimental conditions no further induction of VTG in male guppies occurred between 14 and 21 days of treatment. On the other hand, the significant

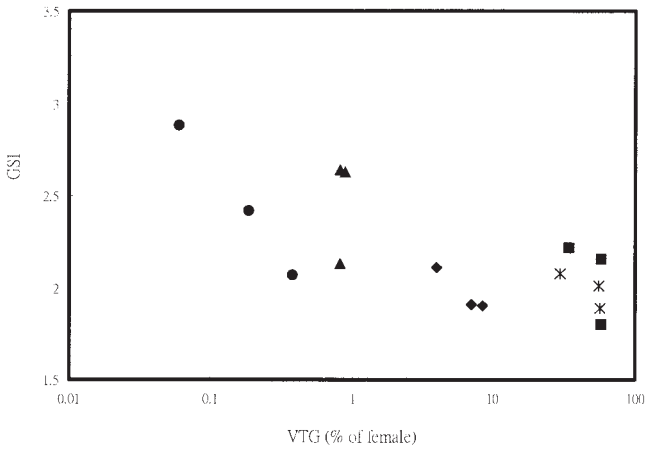

Fig. 4. Correlation ($r = -0.522$, $P = 0.046$) between plasma VTG induction and GSI in male guppies exposed to NP for 7, 14, or 21 days of treatment (●: control; *: 1 μg/L of 17β-estradiol; ▲: 10 μg/L of NP; ◆: 60 μg/L of NP; ■: 150 μg/L of NP).

TABLE I. Lowest observed effect concentration (LOEC) of plasma VTG induction in different fish exposed to waterborne NP

Species	Status	Exposure Type (nominal concentration used)	LOEC VTG Induction	Reference
Rainbow trout (*Oncorhynchus mykiss*)	male	3-week flow-through (0.5–65 μg/L)	24.5 μg/L (20.3 μg/L)[a]	Jobling et al., 1996
	juvenile male	3-week semistatic (20–500 μg/L)	100 μg/L	Van den Belt et al., 2003
	juvenile male	3-week semistatic (25–100 μg/L)	50 μg/L	Tremblay and Kraak, 1998
Sheephead minnow (*Cyrinodon variegates*)	male	3-week flow-through, (1–80 μg/L)	10 μg/L (5.4 μg/L)[a]	Hemmer et al., 2001
Carp (*Cyprinus carpio*)	male	28 to 31 day flow-through, (0.1–10 μg/L)	no induction observed	Villeneuve et al., 2002
	juvenile male	42 days (exposure condition unknown) (4–256 μg/L)	4 ug/L	Huang and Wang, 2001
Japanese medaka (*Oryzias latipes*)	male	1-day semistatic (20 μg/L)	20 μg/L	Foran et al., 2000
	male	4–day semistatic (20 μg/L)	20 μg/L	Foran et al., 2000
Zebrafish (*Danio rerio*)	male	3-week semistatic (20–500 μg/L)	500 μg/L	Van den Belt et al., 2003
	young male	from 2 to 60 days posthatch semistatic (10–100 μg/L)	30 μg/L	Hill and Janz, 2003
Goldfish (*Carassius auratus*)	male	5-day static (500 μg/L)	500 μg/L	Kitamura et al., 1999
Platyfish (*Xiphophorus maculates*)	male	4-week semistatic (80–1280 μg/L)	80 μg/L	Kinnberg et al., 2000
Guppy (*Poecilia reticulata*)	male	1-week semistatic (10–150 μg/L)	60 μg/L	This study
	male	3-week semistatic (10–150 μg/L)	10 μg/L	This study

[a] Measured actual NP water concentration.

induction of VTG in male guppies treated with 10 μg/L of NP compared to that in the controls after 21 days of exposure should be interpreted with caution. This might have occurred as a result of a reduced background level of VTG of the corresponding control fish, as the relative VTG levels in male guppies treated with 10 μg/L of NP remained almost the same (82%–89% of reference female VTG levels) across three exposure durations (Fig. 2). According to our results, a 7-day exposure is sufficient to detect 60 μg/L of NP-induced VTG production in male guppies.

Freshwater concentrations of NP have been reported in the range of <0.01–180 μg/L, and the average concentration of NP is usually less than 10 μg/L (Bennie, 1999). A study of 40 rivers in Taiwan revealed an average NP concentration of 4.87 μg/L, with a range of 0.89–50 μg/L (Wang et al., 2001). The actual NP concentrations in test water have been reported as approximately .33–.50 and .17–.25 of the nominal concentration after 48 and 72 h, respectively (Gray and Metcalfe, 1997; Kinnberg et al., 2000). The actual NP concentrations tested in the present study were roughly estimated, by changing the water 3 times a week, as .25–.33 of the nominal concentrations based on our experimental scheme. Therefore, male guppies exposed to 10, 60, and 150 μg/L of NP in the present study can be considered exposed to NP at environmentally relevant concentrations. It further suggests the usefulness of this fish model for detecting potential exposure to xenoestrogens in environmental samples.

A decreased GSI can be used as an indicator of gonadal dysfunction resulting from decreased hypothalamic, pituitary, or gonadal activity (Kime, 1999). In fact, decreased GSI was commonly observed, although less pronounced, after estrogenic exposure in fish (Jobling et al., 1996; Christiansen et al., 1998a; Kinnberg et al., 2000; Mills et al., 2001). In our study VTG induction was significantly correlated with a reduced GSI in male guppies. We observed an exposure-related decrease of the GSI in male guppies at each NP concentration, but a dose-related decrease of the GSI was less obvious for each exposure duration (Fig. 3). That there was not a strong concentration-related tendency of the GSI in guppies in the present study might be a result of the narrow NP concentration range used.

In general, an increased LSI is expected from VTG induction resulting from enhanced liver metabolism, leading to an enlarged liver. However, according to published data, an increased LSI is not a good indicator of estrogenic

exposure. For example, Christiansen et al. (1998a) found no significant difference in the LSI of male eelpout (*Zoarces viviparous*) treated with NP or 17β-estradiol after 25 days of injection. In a different study, these investigators (Christiansen et al., 1998b) reported a significant increase in the LSI of rainbow trout treated with diethylstilbestrol (DES), 17α-ethinyl estradiol, and bisphenol A, but not in fish treated with 17β-estradiol, NP, butylbenzylphthalate, and dibutylphthalate. Juvenile male summer flounder (*Paralichthys dentatus*) treated with octylphenol also showed no change in the LSI, whereas 17β-estradiol-treated fish (2 and 20 mg/kg) exhibited an increased LSI after 4 weeks of treatment (Mills et al., 2001). Interestingly, Verslycke et al. (2002) reported that no concentration-dependent changes in the LSI was observed in rainbow trout exposed to waterborne 17α-ethinyl estradiol after 14 days of treatment, but a significant dose-dependent increase of the LSI was found in fish injected with 17α-ethinyl estradiol. In our study we also observed no significant changes in the LSI of male guppies treated with NP or 17β-estradiol, except for a significant increase in the LSI of fish exposed to 1 μg/L of 17β-estradiol after 7 days of treatment.

CONCLUSIONS

The results of this study demonstrated that NP and 17β-estradiol exert similar effects on induction of the GSI and of VTG in male guppies and also confirmed that NP is estrogenic in fish. Indeed, guppies can be considered a suitable and sensitive species for the measurement of plasma VTG for estrogenic exposure at environmentally relevant concentrations by using the semiquantitative SDS-PAGE method. Furthermore, it will be useful to develop guppies as an integrated fish model suitable not only for assessing the effects of xenoestrogens but also for detecting exposure to xenoestrogens.

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