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Comparative responses in rare minnow exposed to 17β-estradiol during different life stages

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Abstract Present in the excrement of humans and animals, 17β-estradiol (E_2) has been detected in the aquatic environment in a range from several nanograms to several hundred nanograms per liter. In this study, the sensitivities of rare minnows during different life stages to E_2 at environmentally relevant (5, 25, and 100 ng l^{-1}) and high (1000 ng l^{-1}) concentrations were compared using vitellogenin (VTG) and gonad development as biomarkers under semistatic conditions. After 21 days of exposure, VTG concentrations in whole-body homogenates were analyzed; the results indicated that the lowest observed effective concentration for VTG induction was 25 ng l^{-1} E_2 in the adult stage, but 100 ng l^{-1} E_2 in the larval and juvenile stages. After exposure in the early life stage, the larval and juvenile fish were transferred to clean water until gonad maturation. No significant difference in VTG induction was found between the exposure and control groups in the adults. However, a markedly increased proportion of females and appearance of hermaphrodism were observed in the juvenile-stage group exposed to 25 ng l^{-1} E_2. These results showed that VTG induction in the adult stage is more sensitive than in larval and juvenile stages following exposure to E_2. The juvenile stage may be the critical period of gonad development. Sex ratio could be a sensitive biomarker indicating exposure to xenoestrogens in early-life-stage subchronic exposure tests. The results of this study provide useful information for selecting sensitive biomarkers properly in aquatic toxicology testing.

Keywords Biomarker · Estradiol · Hermaphrodism · Sex ratio · Vitellogenin

Abbreviations

dph	Days post hatch
LOEC	Lowest observed effect concentration
E_1	Estrone
VTG	Vitellogenin
E_2	17β-Estradiol
WBH	Whole-body homogenates
EDCs	Endocrine disrupter chemicals
ELISA	Enzyme-linked immunosorbent assay

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Introduction

Over the last decade, concern has been raised about the potential effects of endocrine-disrupting chemicals (EDCs) on the development and reproduction of humans and wildlife (Colborn et al. 1996). Effects of EDCs in fish include reduced fertility (decreased sperm number and quality, or egg number), induction of vitellogenin (VTG) in males and juveniles, and effects on the development of the gonads (Billard et al. 1981; Andersen et al. 2001; Aguayo et al. 2004). The Office of Research and Development of the United States Environmental Protection Agency (US EPA) has identified EDCs issues as one of six high-priority research areas (US EPA 1996; Kavlock et al. 1996). These chemicals include breakdown products of detergents, pesticides, plasticizers, and a variety of chlorinated compounds (Sumpter and Jobling 1995; Carballo et al. 2005). Considering the adverse physiological effects of EDCs on wildlife, many efforts have been made to develop and validate screening tests (Dizer et al. 2002; Ünal et al. 2007).

Now there is special concern about estrogenic chemicals among EDCs in the aquatic environment (Desbrow et al. 1998; Nath et al. 2007; Soto et al. 1995). Some estrogenic compounds such as alkylphenol polyethoxylates, their major metabolites, 4-nonylphenol (NP) or 4-tert-octylphenol (OP), bisphenol A, the natural steroid estrogens 17β-estradiol (E_2), estrone (E_1), and, to a lesser extent, the synthetic estrogen ethinlyestradiol (EE_2) have been measured in industrial and municipal sewage treatments works. These effluents represent the main source of estrogenic chemicals into the aquatic environment. However, many scientists have reported that low concentrations of natural steroid estrogens including E_2, E_1 and the manmade estrogen EE_2 can be detected simultaneously in the effluents from industrial and municipal sewage treatment plants (Desbrow et al. 1998; Snyder et al. 1999; Jin et al. 2005); in the environment these chemicals mainly originated from excrement of humans and animals, and their concentrations vary from several nanograms to several hundred nanograms per liter (Desbrow et al. 1998; Rodgers-Gray et al. 2001). In Israel, the concentrations of E_1 and E_2 in effluents varied between 48 and 141 ng l^{-1} (Shore et al. 1993). In America, the concentrations of E_2 in effluents from sewage treatment plants were below 3.7 ng l^{-1} (Snyder et al. 1999). In the recent literature most environmental reports mention higher levels of E_1 compared with E_2 (Servos et al. 2005; Johnson et al. 2005); moreover, it is known that excreted levels of E_1 are higher than those of E_2 (Jobling and Tyler 2006). Given these concentrations, those found in the aquatic environment, and its estrogenic potency, E_2 was considered as a central contributor to the estrogenicity of effluents from sewage treatment plants (Van den Belt et al. 2004; Desbrow et al. 1998; Folmar et al. 2002).

Zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*), and fathead minnow (*Pimephales promelas*) have been considered as fish models for screening EDCs by the Organisation for Economic Cooperation and Development (OECD) and the US EPA. Rare minnow (*Gobiocypris rarus*) is a Chinese freshwater cyprinid (Re and Fu 1983). It has many attractive features that make it a suitable organism for aquatic toxicity tests. These advantages include small size (adult 2–8 cm), wide temperature range (0–35°C), being easily cultured in the laboratory, large numbers of eggs (with an average of 266 eggs per hatch and being a continuous batch spawner), short duration of embryonic development (72 h at 26°C), and short life cycle (about 4 months) (Wang 1999). Meanwhile, it has been proved to be sensitive to heavy metals and xenoestrogens (Zhou et al. 2002; Lu and Shen 2002; Liao et al. 2006). Recently rare minnow has been recommended as a fish model for aquatic toxicity testing in China. Many scientists have reported endocrine-disrupting effects on rare minnow (Liao et al. 2006; Zha et al. 2007, 2008), but to date there are no reports in the literature on the process of sex development in rare minnow.

In this study, the sensitivities of Chinese rare minnow during different life stages to E_2 levels similar to the concentrations in the aquatic environment were studied using VTG and gonad development as biomarkers. The results from this study provide useful information for selecting sensitive biomarkers properly for aquatic toxicology testing.

Materials and methods

Experiment animals and hormone treatment

Rare minnows were cultured in our laboratory. During the test, the fish were maintained in a light/dark cycle of 14:10 h at 23–26°C and reared in 8-l glass aquaria under static conditions. Rare minnows in three different stages (larva, 0 days post hatch (dph); juvenile, 21 dph; adult, mature male) were exposed to E_2 (Sigma) at concentrations of 5, 25, 100, and 1000 ng l^{-1} for 21 days and fed with *Artemia nauplii* twice a day. Each group had a replicate group and the number of fish exposed in each group was 50. Only dimethyl sulfoxide (DMSO) was added in the control group. The concentrations of DMSO in all the aquaria were within 0.01%. The exposure solution was renewed once a day. Mortalities were recorded daily and dead fish were removed from the tanks daily. After 21 days exposure, ten fish from each treatment group were sampled at random and stored in a refrigerator at −80°C. After the early-life-stage exposure, the larval and juvenile fish were transferred to 100-l glass aquaria filled with clean water until gonad maturation under semistatic conditions. During this period, 80% of the volume was renewed once every 2 days. The test equipment and chambers were cleaned once a week. Then the blood was sampled in the adult fish, and the gonad was weighed and then fixed in Bouin's solution. Condition factor is expressed as weight (g)/length $(cm)^3 \times 100$.

Preparation of whole-body homogenates (WBH) and blood sampling

Fish were thawed on ice and individually homogenized with 0.5 ml (larval and juvenile fish) and 2.5 ml (adult fish) ice-cold phosphate-buffered saline (PBS; pH 7.5) in a glass homogenizer. The homogenate was then centrifuged at 13,000 × g for 15 min at 4°C, and the supernatant was withdrawn and immediately frozen at −80°C. The mature fish exposed to E_2 during the early life stages were netted from the exposure chambers and anaesthetized with MS-222 (0.5 g l^{-1}). After anaesthesia, the caudal peduncle was partially severed and blood was collected with a heparinized microhematocrit capillary tube. The plasma was quickly isolated by centrifugation for 3 min at 15,000 × g and stored in a centrifuge tube with 0.13 units of aprotinin (a protease inhibitor) at −80°C until the analysis of VTG (US EPA 2002).

Enzyme-linked immunosorbent assay (ELISA) procedure

The development of indirect enzyme-linked immunosorbent assay (ELISA) was followed by the method that has been shown to be suitable for determination of VTG concentration in the rare minnow (Zhong et al. 2004). Purified carp VTG was used as the VTG standard and polyclonal antibodies against carp VTG were produced in rabbits. There was a linear response in the range 10−350 ng ml^{-1} purified carp VTG standard. The sensitivity of the ELISA was 4.5 ng ml^{-1}; intra-assay variation was 3.8% (n = 12) and inter-assay variation was 11.4% (n = 12). Serial dilutions of WBH from female rare minnow showed parallelism with the carp VTG standard (Zhong et al. 2004). These characteristics made the method suitable for quantifying VTG concentrations in rare minnows exposed to xenoestrogens. Both the standard and samples were diluted with 0.025 M Tris–HCl (pH 7.5) in the range 10–500 ng ml^{-1}. The concentration of VTG in WBH was normalized to the body mass of the corresponding sample and expressed in μg g^{-1} fish. The concentration of VTG in plasma was normalized to the protein mass of the corresponding sample and expressed in μg g^{-1} protein. Total protein concentration was measured by the Bradford method (Bradford 1976) using bovine serum albumin as a standard.

Histological analysis

Fish sampled at 150 days post-hatch were killed by MS-222 anaesthetic. The gonads of the fish were removed and fixed in Bouin's solution for analyzing abnormalities. After washing with 50% ethanol, samples were dehydrated, embedded in paraffin, serially sectioned (7 μm) transversely, and stained with hematoxylin and eosin. All sections of gonads were examined by light microscopy.

Statistics

Values are expressed as mean ± standard deviation (SD). The data for the different treatments were

subjected to one-way analysis of variance (ANOVA) with a Tukey post-test. *P*-values below 0.05 were taken as significant. The data were statistically analyzed with the software Origin 6.0.

Results

Effects of growth in rare minnow exposure to E_2

The mortality of larval and juvenile rare minnow gradually increased with increasing E_2 concentration. Moreover, the lengths and weights of larval rare minnow exposed to 100 ng l^{-1} E_2 were significantly higher than those of the control group; the lengths, weights, and condition factors of larval rare minnow exposed to 1000 ng l^{-1} E_2 were significantly higher than those of the control group, but there were no significant differences between the exposure group and the control group in the growth of juvenile and adult rare minnows exposed to E_2 (Table 1). The results demonstrated that the growth of rare minnow during the larval stage was more easily affected by pollutants in the environment.

VTG induction in rare minnow exposure to E_2 during different stages

ELISA was employed to measure VTG concentration in the WBH of rare minnow exposed to E_2 at concentrations of 5, 25, 100, and 1000 ng l^{-1} during three different stages for 21 days. The results indicated that the lowest observed effect concentrations (LOECs) to induce VTG was 25 ng E_2 l^{-1} for adult rare minnow and 100 ng E_2 l^{-1} for larval and juvenile rare minnow (Fig. 1). After early-life-stages exposure, the larval and juvenile fish were transferred to clean water until gonad maturation. There was no significantly difference in VTG induction between the exposure group and the control group (Fig. 2).

Sex ratio in rare minnow after early-life-stages exposure to E_2

After exposure during the early life stages, larval and juvenile fish were transferred to clean water until gonad maturation. Sex ratios following the gonadal development are given in Fig. 3. The sex ratio in the control was 42:58 (male:female). In contrast, all groups exposed to

Table 1 Effects of length, weight, condition factor, and mortality determined in rare minnow following exposure to 17β-estradiol for 21 days during different stages

	E_2 concentration (ng l^{-1})	Weight (mg)	Length (cm)	Condition factor	Mortality (%)
Larva	Control	4.04 ± 1.30	0.762 ± 0.068	0.88 ± 0.13	0
	5	4.38 ± 1.14	0.796 ± 0.049	0.87 ± 0.20	6
	25	4.49 ± 0.93	0.808 ± 0.042	0.84 ± 0.09	7
	100	5.67 ± 1.07*	0.840 ± 0.040*	0.95 ± 0.11	26
	1000	6.68 ± 1.55*	0.831 ± 0.059*	1.17 ± 0.24*	58
Juvenile	Control	15.49 ± 4.80	1.025 ± 0.098	1.38 ± 0.15	0
	5	16.50 ± 2.99	1.034 ± 0.062	1.48 ± 0.16	3
	25	13.28 ± 5.44	0.983 ± 0.101	1.32 ± 0.20	2
	100	16.11 ± 5.11	1.024 ± 0.084	1.46 ± 0.21	6
	1000	15.89 ± 3.27	1.060 ± 0.045	1.32 ± 0.14	15
Adult	Control	1152.85 ± 367.49	3.65 ± 0.42	2.32 ± 0.19	0
	5	1034.61 ± 232.52	3.48 ± 0.31	2.44 ± 0.18	0
	25	1192.54 ± 244.44	3.60 ± 0.24	2.53 ± 0.20	0
	100	1104.28 ± 342.59	3.50 ± 0.32	2.53 ± 0.31	0
	1000	958.26 ± 161.29	3.42 ± 0.24	2.40 ± 0.37	2

Values are means ± SD ($n = 10$). Condition factor is expressed as weight (g)/length $(cm)^3 \times 100$. Asterisk denotes significant difference from control at $P < 0.05$

Larva: 0–21 days post hatch; juvenile: 21–42 days post hatch; adult: 150–171 days post hatch

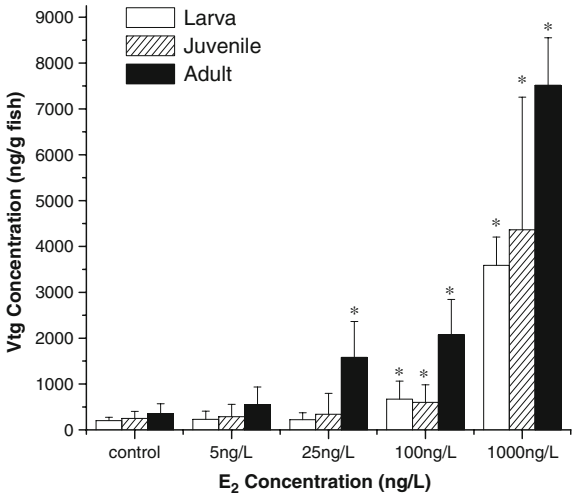

Fig. 1 Vitellogenin induction in rare minnow following exposure to 17β-estradiol for 21 days during different life stages. Values are means ± SD ($n = 10$). Asterisk denotes significant difference from the control at $P < 0.05$. Larva: 0–21 days post hatch; juvenile: 21–42 days post hatch; adult (mature male): 150–171 days post hatch

E_2 showed an elevated proportion of females. No males were observed in the 100 ng l^{-1} group. Moreover, in juvenile fish exposed to 25 ng l^{-1} E_2, testes-ova could be detected at an incidence of 9% (Figs. 3, 4). These results demonstrate that the juvenile stage in rare minnow may be the critical stage for sex development, and that the sex ratio in adult fish could be changed following exposure to E_2 during early life stages.

Sex development in rare minnow after early-life-stages exposure to E_2

Fish killed at 150 days post hatch were evaluated for gonadal development. The histological changes of ovaries of all observed female fish after early-life-stages exposure to 5 and 25 ng l^{-1} E_2 were similar to those in the control. There were many immature oocytes in the groups exposed to 100 ng l^{-1} E_2 during the early life stages (Fig. 5). Histological inspection of the ovaries revealed different stages of oocyte development in the control group (Fig. 5).

Discussion

Phenotype of sex differentiation in fish could be easily affected by pollutants in the aquatic environment (Devlin and Nagahama 2002), and sex development and reproductive capability in fish could also be changed by estrogenic compounds (Länge et al. 2001; Segner et al. 2003). It was considered that sex differentiation was controlled by sex steroids in many teleosts (Piferrer 2001; Devlin and Nagahama 2002). The undifferentiated gonad was easily affected by sex steroids, so this stage was called the sex-labile period (Piferrer 2001; Devlin and Nagahama 2002). The sex-labile period differed in different fish from hatching to the juvenile stage (Piferrer 2001). It is important to know the sex-labile period, and the gonad and reproductive capability could be permanently affected by short-term exposure during this period. The primary advantage of using larval or juvenile fish in an endocrine screen was the reduction in costs associated with maintenance in the assay. The use of larval or juvenile fish also allowed for a corresponding reduction in the quantity of water and test chemical required for the test. So it was helpful for us to know the labile period in screening and evaluating EDCs after exposure during the early life stage (Koger et al. 2000). In

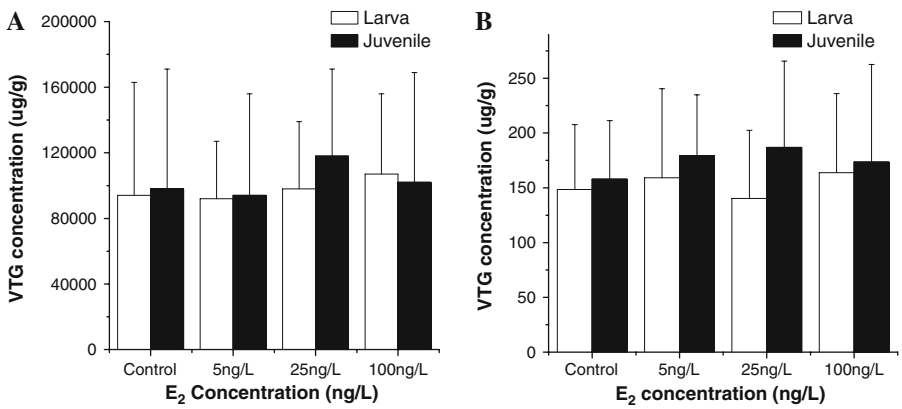

Fig. 2 Vitellogenin concentration in adult fish after exposure to 17β-estradiol during early life stages. Values are means ± SD ($n = 10$). (**a**) Female, (**b**) Male

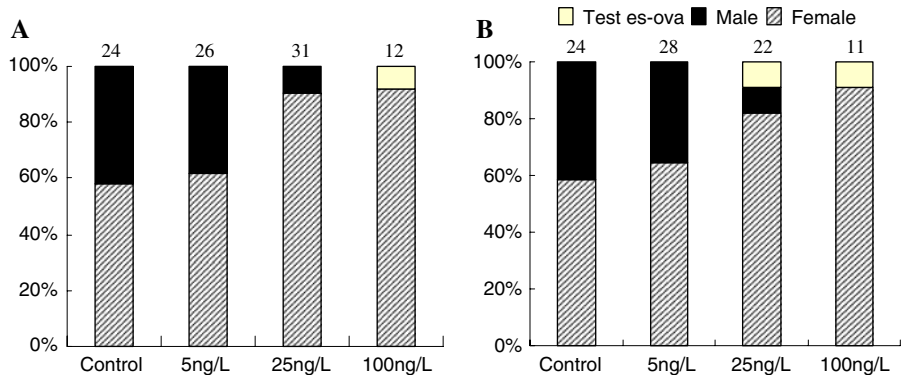

Fig. 3 Sex ratios of rare minnow in adult fish exposed to 17β-estradiol during early life stages. Sex ratio was determined by histological analysis of the gonad in 150 days post-hatch rare minnow. The *n* value is shown at the top of each column. (**a**) 0–21 days post hatch, (**b**) 21–42 days post hatch

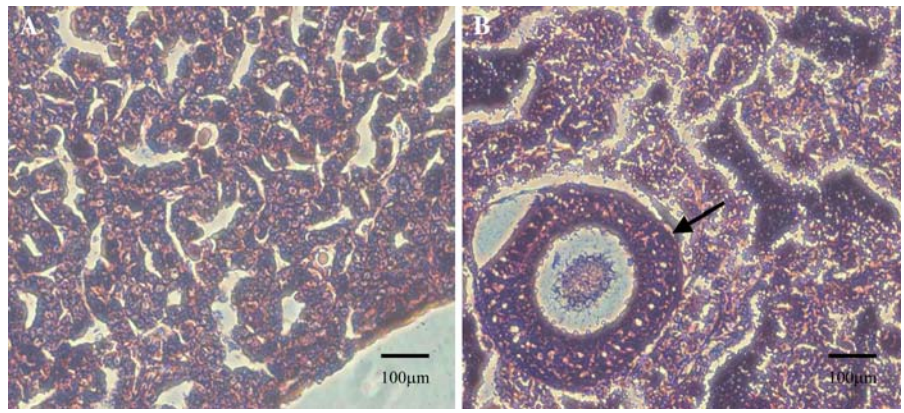

Fig. 4 Light micrograph of testis in adult fish exposed to 17β-estradiol during early life stages (stained with hematoxylin and eosin, 100×). Arrows indicate oocytes within the testicular tissue. (**a**) Control, (**b**) 25 ng l^{-1} 17β-estradiol group exposed during 21–42 days post hatch

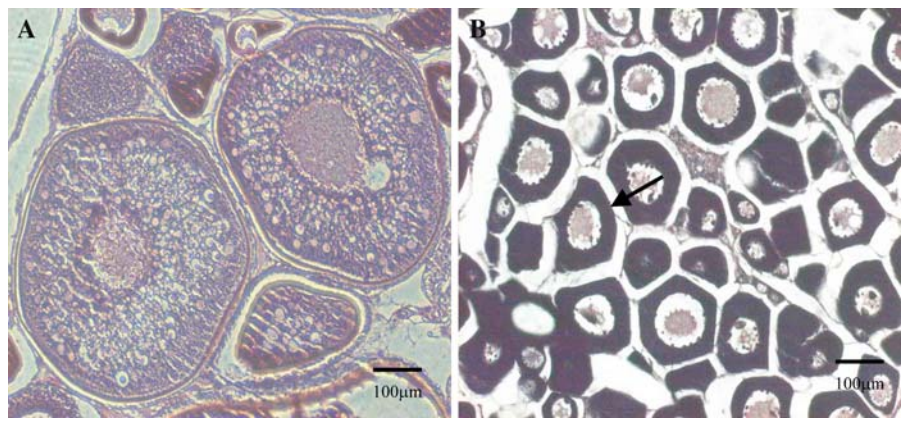

Fig. 5 Light micrograph of ovaries in adult fish exposed to 17β-estradiol during early life stages (stained with hematoxylin and eosin, 100×). Arrows indicate immature oocytes within the ovarian tissue. (**a**) Control, (**b**) 100 ng l^{-1} 17β-estradiol group exposed during 0–21 days post hatch

this study, the effects of E_2 exposure on rare minnow during different life stages were compared; the aim was to develop a test system during the early life stage and understand the fundamental process of sex development in rare minnow.

ELISA was employed to measure VTG concentration in the WBH of rare minnow exposed to E_2 at concentrations of 5, 25, 100, and 1000 ng l^{-1} during three different stages for 21 days. The results demonstrated that the LOECs to induce VTG were 25 ng E_2 l^{-1} for rare minnow during the adult stage and 100 ng E_2 l^{-1} for rare minnow during the larval and juvenile stages. These data indicate that early life stages in rare minnow are sensitive to estrogens, and exposure during these stages could result in abnormal vitellogenin induction. These results were similar to those of the study by Legler et al. (2000), who demonstrated that the estrogen receptor subtypes α and β were expressed very early in the development of zebrafish (from 1 dph) and that exposure to E_2 upregulated expression of estrogen receptor genes. Tyler et al. (1999) reported that VTG synthesis in fathead minnow was shown to occur in fish exposed to E_2 during early-life stages. The results also suggested that VTG induction following exposure to E_2 during the adult stage was more sensitive than during the larval and juvenile stages. One possible explanation for these results was that the expression of estrogen receptor in the adult stage was greater than in the larval and juvenile stages. Brion et al. (2004) utilized zebrafish to evaluate sensitivities of VTG induction following exposure to E_2 for 21 days during three different stages. They also observed that VTG induction in the adult stage was more sensitive than in the larval and juvenile stages. The LOEC for VTG induction was 10 ng l^{-1} when rainbow trout (*Oncorhynchus mykiss*) were exposed to E_2 for 14 days (Thorpe et al. 2001); but in other cyprinoids, the LOECs for VTG induction were between 27 and 100 ng l^{-1}, including fathead minnow (Panter et al. 1998) and roach (*Rutilus rutilus*; Routledge et al. 1998); and the LOEC of sheepshead minnow (*Cyprinodon variegates*) for VTG induction even reached 200 ng l^{-1} (Folmar et al. 2000). The variation seen in VTG induction highlights the importance of taking into account interspecies differences when evaluating the effects of xenoestrogen exposure in fishes.

After early-life-stage exposure, rare minnows in the larval and juvenile stages were transferred to clean water until maturation. There was no significant difference in VTG induction between the control group and the exposure group. The results showed that VTG induced by early-life-stage exposure was completely metabolized following a period of depuration. Many other studies have demonstrated similar results that VTG induced by xenoestrogen during early development was reversible after cessation of xenoestrogen exposure (Brion et al. 2004; Rodgers-Gray et al. 2001). So VTG concentration in adult fish is not suitable as a biomarker to indicate the effects of xenoestrogen exposure during early development.

The process of sex differentiation in fish could be effected by exposure to estrogens, and it could result in changes of sex ratio and presence of fish with testi-ova (Brion et al. 2004; Andersen et al. 2003). The beginning of sexual differentiation in other fish species differs in the different sexes. In carp (*Cyprinus carpio*), sex differentiation in females usually occurs between 50 and 60 dph, whereas male gonads remained undifferentiated until 90 dph (Komen et al. 1995). In Japanese medaka, sex differentiation of the male testis occurs around 13 dph, whereas sex differentiation of females takes place before hatching (Yamamoto 1975). In zebrafish, sex differentiation occurs between 22 and 34 dph simultaneously (or at least over a very brief period in time) in males and females (Hsiao and Tsai 2003). Sex differentiation of fish is a highly labile process and exposure to xenoestrogens during the labile period can lead to complete sex reversal (Piferrer 2001). In our study, rare minnows exposed during early life stages were transferred to the clean water until maturation. The sex ratio in the control was 42:58 (male:female). In contrast, all groups exposed to E_2 showed an elevated proportion of females. No males were observed in the 100 ng l^{-1} group. Moreover, in juvenile fish exposed to 25 ng l^{-1} E_2, testes-ova could be detected at an incidence of 9%. The development of gonads in female fish was delayed in the 100 ng l^{-1} group following early-life-stage exposure. These results demonstrate that in rare minnow the juvenile stage may be the critical stage for sex development, and the sex ratio in adult fish could be changed following exposure to E_2 during the early life stages. Brion et al. (2004) utilized zebrafish to evaluate the effects of sex ratios following exposure to E_2 for 21 days during early life stages. They observed that the sex ratio of zebrafish skewed toward females after exposure to E_2 during the larval

stage (0–21 dph), but no significant change in sex ratio was found in the treated groups compared with the controls during the juvenile stage (21–42 dph). This can be explained by the difference in timing of sexual differentiation between rare minnow and zebrafish.

In conclusion, the adult stage is more sensitive than the larval and juvenile stages in VTG induction following exposure to E_2, and the juvenile stage may be the critical stage for gonad development. Sex ratio could be a sensitive biomarker to indicate exposure to xenoestrogens in early-life-stage subchronic exposure testing. The results of this study provide useful information for selecting sensitive biomarkers properly in aquatic toxicology testing.

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