Diethylstilbestrol at environmental levels affects the development of early life stage and target gene expression in Japanese Medaka (*Oryzias latipes*)

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Abstract In this study, the biologic effects of DES on the early life and adult life stages of Japanese medaka (*Oryzias latipes*) were evaluated. At the early life stage, the fertilized eggs were exposed to 1–1000 ng/L diethylstilbestrol (DES) for 15 days and the hatched larvae were continually exposed to the same concentrations for an additional 25 days. Significant adverse effects on hatchability, time to hatching and mortality rate occurred at DES concentrations of 100 and 1000 ng/L, while the abnormality (scoliosis and abdominal swelling) rate was significantly increased at 10 ng/L and above. After exposure, the fish were maintained in charcoal-dechlorinated tap water for a further 30 days. Only the male gonadosomatic index (GSI) at 1000 ng/L was significantly increased. At concentrations greater than 1 ng/L, estrogen receptor α (ER α) mRNA in both sexes and vitellogenin-I (Vtg-I) mRNA in males were significantly down-regulated; while Vtg-I mRNA in females was significantly up-regulated. When sexually mature medaka were exposed to 10 and 1000 ng/L DES for 21 days, only the GSI in females was significantly decreased at 1000 ng/L. At 10 and 1000 ng/L, ER α mRNA in both sexes was significantly down-regulated, while Vtg-I mRNA in males was significantly up-regulated. These findings showed that DES at the environmental concentration of 10 ng/L can affect the early life stage development of medaka and alter liver ER α and Vtg-I gene

expression. Therefore, if we only focused on these sensitive toxicity endpoints such as ER α and Vtg-I mRNA expression, DES has a strong estrogenic effect on Japanese medaka.

Keywords Diethylstilbestrol · *Oryzias latipes* · Early life stage · Vitellogenin gene · Estrogen receptor gene

Introduction

Estrogens play an important role in the reproductive biology of vertebrates and have the ability to bind and activate estrogen receptors, thereby inducing endocrine responses that may cause reproductive impairment (Atteke et al. 2003). Estrogens such as estrone (E1), 17 α -ethinylestradiol (EE2) and β -estradiol 17-valerate (EV) at low concentrations (0.2–10 ng/L) have shown significant estrogenic effects on fish (Zha et al. 2008; Lei et al. 2013a, b). These estrogenic effects include decreasing reproductive success and the hatchability of embryos, changing sexual determination and sex behavior, and increasing the rate of intersex. Some representative marker genes such as vitellogenin (Vtg) and estrogen receptor (ER) genes have been used as early warning signals to evaluate the estrogenic effects of endocrine disrupting chemicals (EDCs) on aquatic organisms (Zhang and Hu 2008; Huang et al. 2010; Brieno-Enriquez et al. 2012). Medaka fish express at least three distinct types of ER (ER α , ER β 1 and ER β 2) (Hawkins et al. 2000; Chakraborty et al. 2011) and ER α appears to play a significant role in major reproductive physiological functions in females. Vtg is an egg yolk precursor protein that is synthesized primarily in the liver of females. In male and juvenile fish, Vtg levels are generally low and the Vtg gene is normally silent. However, it may be activated and regulated by xenoestrogens (Mitsui et al. 2007). Numerous studies

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have reported that potent EDCs such as β -estradiol (E2) and EE2 can alter Vtg and ER α gene expression in fish (Scholz and Gutzeit 2000; Andreassen et al. 2005; Huang et al. 2010). In our previous studies, we also found that the levels of ER α and Vtg-I mRNA in the liver of Japanese medaka exposed to EV, E1 and estriol (E3) at environmental concentrations of 1–10 ng/L can be altered (Lei et al. 2013a, b, 2014). However, few studies have reported on synthetic estrogens such as diethylstilbestrol (DES) which alters Vtg and ER α gene expression in fish (Adedeji et al. 2012).

DES was the first synthetic estrogen originally and was used in clinical therapy to prevent miscarriages. However, the use of DES as a human pharmaceutical was banned in 1972 (Adedeji et al. 2012) due to its adverse effects on the female reproductive tracts. DES is still used in terrestrial livestock as a growth promoter in some parts of the world such as Asia (Adedeji et al. 2012). DES may also enter the environment via aquaculture operations as it is used to promote growth and to produce monosex (female) populations of fish (Potts and Phelps 1995; Adedeji et al. 2012). Detectable concentrations of DES in surface water from different locations have been reported to range from slightly below 1 to approximately 20 ng/L (Pojana et al. 2007; Yang et al. 2008; Lei et al. 2009; Chen et al. 2010; Wang et al. 2011). Although DES has been demonstrated to be effective in inducing a female-specific pattern of development in fish species, only a few studies have reported the estrogenic effects of DES in fish (Panter et al. 2002; Zhong et al. 2005; Paul-Prasanth et al. 2011). However, these studies have not identified the no observed effect concentration (NOEC) of DES. Adedeji et al. (2012) showed that the NOEC of DES for hepatic transcripts of Vtg and ER α in sexually mature fathead minnows was 1 ng/L, which indicated that DES was quite potent in fish. This is consistent with in vitro work by Lange et al. (2012) with cloned ERs from a variety of fish species, suggesting that the potency of DES may be comparable to, or even exceed, that of EE2. However, in the study by Adedeji et al. (2012), the exposure period was only 4 days. With regard to the different life stages of fish exposed to DES at environmentally relevant levels, the influence of DES on a range of estrogen-responsive endpoints such as embryonic development, early sexual differentiation and responding target gene expression in fish have not yet been systemically studied.

Therefore, the aim of this study was to provide more information on the effects of DES at relatively low concentrations on a range of estrogen-responsive endpoints in Japanese medaka. In addition, a water recovery experiment was designed in order to see whether effects of DES in exposed fish were reversible or not after withdrawal of DES. The corresponding endpoints included time to hatching, hatchability, sex ratio, gross abnormality rate, the hepatosomatic index (HSI), and the gonadosomatic index

(GSI). In order to understand the molecular mechanisms associated with the action of DES, we analyzed the marker genes, Vtg-I and ER α , which express the estrogen effects in the liver of fish using real-time PCR.

Materials and methods

Chemicals and fish

DES was purchased from Sigma-Aldrich, USA. The purity of DES is higher than 99 %. The different concentrations of DES were prepared in high-performance liquid chromatography (HPLC)-grade dimethyl sulfoxide (DMSO). All the other chemicals are analytical or HPLC grade.

The source of Japanese medaka (d-rR strain) used for the experiment and the maintenance of the stock fish were described in our previous studies (Lei et al. 2013a, b). The early life stages of medaka are considered to be relatively sensitive to toxic chemicals (Ishibashi et al. 2004).

Exposure and experimental design

The exposure experiment of early life stage of fish

The embryo stage exposure experiments of medaka fish were described elsewhere (Lei et al. 2013a). Briefly, spontaneously spawned eggs were carefully collected from the ventral side of stock females (about 80 females) within a few hours of natural fertilization (less than 4 h) and then were checked for fertilization using a dissecting microscope. For each exposure level, 90 embryos were randomly separated into three equal groups (i.e. triplicate groups/exposure) and were exposed to DES nominal concentrations of 1, 10, 100, and 1000 ng/L in dilution water (charcoal-dechlorinated tap water) for 15 days. In addition, dilution water controls (DWC) and solvent controls (SC) were included in the experimental design. The embryos in each group were placed in a glass dish containing 100 mL of each test solution and eighty percent of each test solution was renewed every 48 h. Embryos were observed twice daily and dead embryos were removed. Hatchability, time to hatching and abnormalities (scoliosis and abdominal swelling identified by macroscopic observation) were recorded.

The hatched larvae were continuously maintained at the same dose range for an additional 25 days. The test solution was renewed every 48 h. The fish were observed daily and all dead fish were removed and recorded. After exposure, the genetic sex ratio was counted simply by the color of fish. In the d-rR strain of medaka, sex-linked genes determine orange-red coloration in males and white color in females (Edmunds et al. 2000; Scholz and Gutzeit 2000). Generally,

the male or female medaka can be distinguished by the color of fish about 15 days post-hatch (Sun et al. 2007).

Then treated and control fish were randomly assigned to 5-L glass tanks (5 females and 5 males/tank) and were maintained in charcoal-dechlorinated tap water. Duplicate aquaria were conducted for each concentration and the charcoal-dechlorinated tap water was renewed once every 48 h. Fish were kept for an additional 30 days.

The entire experiment duration was 70 days. The medaka fish were fed three times daily. Feeding consists of newly hatched brine shrimp (*Artemia nauplii*) for days 0–7, and a commercial granule food (TetraMin, Germany) and a diet supplement of newly hatched brine shrimp from day 7+.

Adult fish exposure experiment

To evaluate the effect of DES at environment concentrations and higher than environment concentrations on growth and Vtg and ER gene expression in adult fish, sexually mature fish (~3 months old) were exposed to DES nominal concentrations of 10 and 1000 ng/L for 21 days. The exposure solution was renewed once every 48 h. The experimental fish were the offspring from the same pair of brood stock. Since the stock of Japanese medaka was raised in flow-through system with dechlorinated tap water, 3 days of acclimatization to experiment conditions were carried out before exposure. At the same time, DWC and SC were included in the experimental design. The SC aquaria and all DES exposure aquaria received 0.1 ml/L DMSO whereas the DWC received dechlorinated tap water only. Treated and control fish (males: females = 1:1) were assigned to 5-L aquaria (10 fish/tank) and two duplicates were conducted for each exposure level and control. The medaka fish were fed three times daily, once with newly hatched brine shrimp (*Artemia nauplii*) and twice with commercial food (TetraMin®, Germany).

Analytical chemistry

To evaluate real exposure concentrations of DES in test solution and determine suitable time of renewing test solution, the actual concentrations of DES in test solutions were measured after exposure 0, 24, and 48 h. Water samples were extracted and analyzed for DES by the following methods described elsewhere (Lei et al. 2009). Briefly, filtered water samples (1 L) of DES were acidified and concentrated on the solid-phase extraction cartridges of Oasis HLB (Hydrophilic-Lipophilic Balanced Copolymer). Then the analytes were eluted and the eluate was evaporated to just dryness. The residues of water samples were redissolved in 0.4 mL of hexane that contained 50 µL of the derivatization mixtures of *N,O*-bis(trimethylsilyl)-trifluoroacetamide containing 1 % of chlorotrimethylsilane and *N*-trimethylsilylimidazole (BSTFA/

TCMS/TMSI, 99:1:0.5, v/v/v) and 50 ng/mL of internal standard pyrene-*d*₁₀. The derivatization was performed at 60 °C for 30 min. Recovery of DES for different concentrations of spiked water samples ranged from 70 to 115 %. The limit of quantification (LOQ) was 0.2 ng/L. The derivatized samples were analyzed by gas chromatography 6890/mass spectrometry 5975 B ([GC–MS], Agilent, Santa Clara, CA, USA).

Specimen preparation

After 70- and 21-days test duration, all the fish from two tanks for each treatment and control were sampled for determination of various biological endpoints. The males and females were sacrificed by keeping them on ice for 1–2 min and were weighted. Then fish were dissected, and gonads and livers were removed and weighted for determination of GSI and HSI. After weighing, livers were flash-frozen in liquid nitrogen for further gene expression analyses. The gonadosomatic index (GSI %) was calculated as follows: $GSI = (\text{weight of the gonads}) \times 100 / (\text{weight of the total body})$. The hepatosomatic index (HSI %) was calculated as follows: $HSI = (\text{weight of the liver}) \times 100 / (\text{weight of the total body})$.

RNA isolation and real-time PCR

Total RNA was isolated from the liver tissue and then was purified from lysates using a spin column method (RNeasy; Qiagen) according to the manufacturer's instructions. RNA samples were treated with DNase I (Ambion, UK) to remove contaminating genomic DNA and repurified by spin column (RNeasy; Qiagen). The RNA samples were dissolved in ribonuclease-free water and stored at –80 °C until the process of reverse-transcriptase.

The reverse-transcriptase polymerase chain reaction was carried out with total liver RNA isolated from male and female fish. The reverse transcription reaction mixtures containing 10 µL of total RNA, 2 µL of random primers and diethyl pyrocarbonate-treated water (a total volume of 12 µL), were heated to 70 °C for 5 min and quickly chilled on ice. After cooling, 50 mM Tris–HCl buffer (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂, 2 mM deoxynucleotide triphosphate (10 mM each), 40 units of RNAasin (RNAase inhibitor; Promega), and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) were added to a total volume of 25 µL and incubated for 1 h at 37 °C. Thereafter, the reaction mixture was heated to 70 °C for 10 min to inactivate the reverse transcription.

Real-time PCR was performed in MX3005P real-time quantitative polymerase chain reaction system (Stratagene, USA) in a total volume of 25 µL, consisting of the Brilliant II Sybr Green QPCR master mix, 300 nmol/L each of forward and reverse primers. The thermal cycle parameters

used were: 10 min at 95 °C, 1 min at 57 °C and 30 s at 72 °C. All samples were analyzed in triplicate and the mean values of these triplicate measurements were used for the calculation of mRNA expression. Results were analyzed according to delta–delta Ct method. The primers of ER α and Vtg-I genes were reported by Zhang and Hu (2008). The housekeeping gene β -actin was used as the internal standard (Zhang et al. 2008a). The ER α and Vtg-I mRNA expressions were normalized for β -actin mRNA expression. Dissociation curve analysis was performed for each gene to check the amplification of untargeted fragments. Only one peak was observed for each amplification, indicating the amplification of the target gene only. Gene expression data were presented as fold changed relative to the control fish within the same treatment period.

Data analysis

All statistical analyses were carried out using SPSS 13.0 (SPSS, Chicago, IL, USA) and Origin 8.0 (OriginLab, Northampton, MA, USA). The experimental data were checked for assumptions of normality and homogeneity of variance using Kolmogorov–Smirnov one-sample test and Levene's test, respectively. Upon confirmation of normality and homogeneity of variance, parametric data were analyzed using one-way analysis of variance (ANOVA) with Post Hoc multiple comparisons by Dunnett's test to reveal statistical differences in toxicity level between controls and samples. When data failed either test, the nonparametric Kruskal–Wallis test was used, or data were log transformed and evaluated using ANOVA. The critical values for statistical significance were $p < 0.05$ and $p < 0.01$.

Results

Measured concentrations of DES

Water samples were collected at exposure time of 0, 24 and 48 h and the mean concentrations of DES in test solutions were measured (Table 1). DES was not detected in the SC and DWC. The mean measured concentrations of DES for three time points were between 70.0 and 100.8 % of the nominal concentrations. In general, at three exposure time of 0, 24, and 48 h, the measured concentrations of DES had little difference indicating that DES is relative stability in experiment solution. Therefore, the solution was renewed every 48 h.

Effects on the early life stage of medaka

Less than 4-h post-fertilization embryos of Japanese medaka were continuously exposed to DES for 15 days

through the major embryonic stages (blastula, gastrula, neurula and organogenesis), hatched and into the larvae stage. Then the fry were exposed to the same concentrations for the additional 25 days.

Exposure to 100 and 1000 ng/L DES significantly decreased the hatchability of fertilized eggs and time to hatching was significantly increased at 1000 ng/L DES relative to the controls (Table 2). After the additional 25-days exposure, the gross abnormalities in larvae were significantly increased at DES concentrations higher than 1 ng/L compared to the controls. The two most common abnormal features in larvae were scoliosis and abdominal swelling. At the same time, at 100 and 1000 ng/L DES, the gross mortality rate in larvae was also significantly increased. In addition, the female: male ratios in all treatments were higher than those in the controls, and there were twice as many females as males at 1000 ng/L DES. However, statistically significant differences of sex ratios were not found between treatments and controls.

Tissue somatic indices of medaka

Early life stage exposed to DES

Table 3 shows the HSI and GSI in males and females developed from post-fertilization and hatched larvae which were exposed to DES at the concentrations of 1–1000 ng/L for 40 days, followed by maintenance of the fish in dechlorinated tap water for a further 30 days. No significant changes in tissue somatic indices were observed between the water control and solvent control. In addition, the HSI and GSI in both sexes showed no significant differences between the control groups and DES groups, except that a significant increase of the GSI in males was observed at the highest exposure concentration of 1000 ng/L DES.

Sex reversal in males was identified by autopsy. The males which had red color on the tail and also contained a mature ovary were identified as sex reversal fish. Four males at DES concentration of 1000 ng/L were observed to show sex reversal and one of them had abnormality with abdominal swelling. Moreover, some reversal males began to lay eggs. During the water recovery period, mortality was not found at any treatment and control group (Table 4).

Adult fish exposed to DES

In 21-days exposure experiment, no significant changes in tissue somatic indices were observed between the water control and solvent control. In addition, the HSI in both sexes showed no significant differences between the control groups and DES groups. The GSI in females was significantly decreased ($p < 0.05$) in the 1000 ng/L DES group (Table 3). The GSI in males presented a dose-dependent

Table 1 Measured and mean ($\pm$ standard deviation [SD]) concentrations of DES in the test solutions

Nominal concentration	Measured concentration (% nominal)			mean $\pm$ SD
	Hours after exposure			
	0 h	24 h	48 h	
DWC	0	0	0	0 $\pm$ 0
SC	0	0	0	0 $\pm$ 0
1 ng/L	1.1 (110.0 %)	0.8 (80.0 %)	0.7 (70.0 %)	0.9 $\pm$ 0.2 (86.7 $\pm$ 20.8 %)
10 ng/L	9.5 (95.0 %)	9.0 (90.0 %)	9.0 (90.0 %)	9.1 $\pm$ 0.3 (91.2 $\pm$ 2.9 %)
100 ng/L	97.6 (97.6 %)	94.1 (94.1 %)	90.8 (90.8 %)	94.2 $\pm$ 3.4 (94.2 $\pm$ 3.4 %)
1000 ng/L	950.1 (95.0 %)	1008.1 (100.8 %)	818.4 (81.8 %)	925.5 $\pm$ 97.2 (92.5 $\pm$ 9.7 %)

DWC dilution water (charcoal-dechlorinated tap water) control; SC solvent control

Table 2 Hatchability and time to hatching of fertilized eggs exposed to DES for 15 days and gross abnormality rate, gross mortality rate and sex ratio for early life stage of fish exposed to DES for 40 days

DES (ng/L)	Hatchability (%) (hatched fish number)	Time to hatching (days)	Gross abnormality rate (%) (abnormality number)	Gross mortality rate (%) (mortality number)	Sex ratio (female: male)
DWC	92.2 $\pm$ 1.9 (83)	11.7 $\pm$ 1.2	10.8 $\pm$ 6.1 (9)	8.5 $\pm$ 5.8 (7)	1.2 $\pm$ 0.2 (41:35)
SC	91.1 $\pm$ 1.9 (82)	11.3 $\pm$ 1.2	8.6 $\pm$ 2.2 (7)	7.4 $\pm$ 3.8 (6)	1.1 $\pm$ 0.3 (39:37)
1	86.7 $\pm$ 6.7 (78)	11.7 $\pm$ 0.6	11.8 $\pm$ 4.8 (9)	11.8 $\pm$ 8.5 (9)	1.2 $\pm$ 0.1 (37:32)
10	81.1 $\pm$ 13.9 (73)	12.3 $\pm$ 1.2	17.9 $\pm$ 1.0 (13)** p = 0.003	17.8 $\pm$ 12.5 (12)	1.2 $\pm$ 0.3 (34:27)
100	76.7 $\pm$ 8.8 (69)* p = 0.049	12.7 $\pm$ 1.5	38.7 $\pm$ 7.2 (27)** p = 0.002	24.8 $\pm$ 3.3 (17)** p = 0.004	1.6 $\pm$ 0.2 (31:20)
1000	67.8 $\pm$ 11.7 (61)* p = 0.027	14.7 $\pm$ 0.6* p = 0.011	30.9 $\pm$ 8.4* (19) p = 0.011	27.8 $\pm$ 2.5 (17)** p = 0.001	2.0 $\pm$ 1.0 (29:15)

The asterisks (*) and (**) denote statistically significant difference compared with the solvent control (SC) (p<0.05; p<0.01), respectively

DWC dilution water (charcoal-dechlorinated tap water) control; SC solvent control

Table 3 Hepatosomatic (HSI) and gonadosomatic (GSI) index of medaka fish for two life stage exposures

DES (ng/L)	Early life stage exposure		Adult stage exposure	
	HSI females/males	GSI females/males	HSI females/males	GSI females/males
DWC	4.65 $\pm$ 0.85/2.71 $\pm$ 0.85	5.20 $\pm$ 1.78/0.86 $\pm$ 0.40	3.32 $\pm$ 0.80/3.04 $\pm$ 0.65	5.16 $\pm$ 1.14/0.94 $\pm$ 0.25
SC	4.28 $\pm$ 0.66/2.83 $\pm$ 0.74	5.13 $\pm$ 1.54/0.74 $\pm$ 0.27	3.57 $\pm$ 0.50/3.12 $\pm$ 0.51	5.24 $\pm$ 1.08/0.97 $\pm$ 0.17
1	3.73 $\pm$ 0.67/3.35 $\pm$ 1.21	5.25 $\pm$ 1.74/0.70 $\pm$ 0.06	–	–
10	4.73 $\pm$ 1.00/2.88 $\pm$ 0.73	6.38 $\pm$ 1.29/0.89 $\pm$ 0.25	3.10 $\pm$ 0.82/3.02 $\pm$ 0.54	5.72 $\pm$ 1.02/1.20 $\pm$ 0.32
100	3.74 $\pm$ 0.54/2.62 $\pm$ 0.83	6.01 $\pm$ 0.69/0.85 $\pm$ 0.13	–	–
1000	4.15 $\pm$ 1.09/3.58 $\pm$ 0.38	6.70 $\pm$ 1.29/2.09 $\pm$ 1.27*p = 0.024	3.31 $\pm$ 0.26/3.14 $\pm$ 0.49	4.05 $\pm$ 0.99/1.49 $\pm$ 0.55

The asterisks (*) denote statistically significant difference compared with the solvent control (p < 0.05). Data are presented as the mean (n = 10 males and 10 females for each exposure level) and standard deviation (SD)

DWC dilution water (charcoal-dechlorinated tap water) control; SC solvent control; DWC dilution water (charcoal-dechlorinated tap water) control; SC solvent control

increase in the 10 and 1000 ng/L DES groups relative to the controls. However, statistically significant differences of the GSI in males were not found compared to the controls.

Sex reversal was not found at any DES treatment and control group by autopsy. In addition, mortality was also not observed at any treatment and control group. However,

Table 4 The survival, abnormality and sex reversal of medaka fish after 30 days water recovery

DES (ng/L)	Exposed females/males	Surviving females/males	Abnormality females/males	Females	Sex reversal males
DWC	10/10	10/10	0/0	10	0
SC	10/10	10/10	0/0	10	0
1	10/10	10/10	0/0	10	0
10	10/10	10/10	0/0	10	0
100	10/10	10/10	0/0	10	0
1000	10/10	10/10	0/1	10	4

DWC dilution water (charcoal-dechlorinated tap water) control; SC solvent control

two females presented abnormality with abdominal swelling in the 1000 ng/L DES group.

Quantification of ER α and Vtg-I mRNA by real-time PCR

Early life stage exposed to DES

The ER α mRNA was significantly ($p < 0.05$) down-regulated in female and male fish at DES concentrations greater than 1 ng/L (Fig. 1). Vtg-I mRNA levels in female and male fish showed different changing trends. At 10 ng/L DES and above, significant up-regulation of Vtg-I mRNA was found in females relative to the controls ($p < 0.05$ for 10 ng/L, $p < 0.001$ for 100 and 1000 ng/L). In contrast, male Vtg-I mRNA was significantly down-regulated at DES concentrations greater than 1 ng/L ($p < 0.05$).

Adult fish exposed to DES

In the 21-days exposure experiment in adult fish, the ER α mRNA levels in both sexes were significantly down-regulated at 10 and 1000 ng/L DES treatments relative to the controls (Fig. 2). Vtg-I mRNA levels in female and male fish showed opposing trends. In the 10 and 1000 ng/L DES exposure groups, significant up-regulation of Vtg-I mRNA expression was found in males ($p < 0.01$), while Vtg-I mRNA in females was markedly down-regulated only at 1000 ng/L DES ($p < 0.01$).

Discussion

Embryonic development and growth of fish

During the early life stage of fish exposed to DES, higher concentrations of DES caused adverse effects on the normal developmental process in embryos and the survival of larval fish, while a high gross abnormality rate was observed in fertilized eggs and hatched fry exposed to DES

concentrations of 10 ng/L and above. Other studies have also reported that estrogens can interfere with normal embryonic development. For example, Lee et al. (2012) found that the three xenoestrogens, nonylphenol (NP), E2 and bisphenol A (BPA) retarded the embryonic development and increased the time to hatching of the transgenic medaka embryos. EV at concentrations of 10 ng/L and above had adverse effects on hatchability and time to hatching, and a high gross abnormality rate was also observed (Lei et al. 2013a). DES with a lipophilicity $\log k_{ow}$ of 5.07 (Kuster et al. 2004) can easily partition into lipid compartments (e.g. membranes). Therefore, DES would be expected to transfer from water into the developing embryo during the exposure time and decrease hatchability, increase time to hatching and have a teratogenic effect on embryos. The female: male ratio in all DES treatments was higher than that in the control, although statistically significant differences were not found. Estrogens are active at sex steroid receptors and theoretically possess the potential to alter normal sexual differentiation in amphibians or fish to produce more females. Newly hatched tadpoles exposed to EE2 develop a female-biased phenotypic sex-ratio in both juvenile and adult frogs (Pettersson et al. 2006). Zha et al. (2008) reported that the phenotypic sex ratio of female: male in both the F0 and F1 generations of rare minnow after exposure to EE2 was significantly higher than that in controls. However, these female-biased phenotypic sex-ratios were obtained based on morphology or anatomy. Lei et al. (2013a, b, 2014) reported that exposure to EV, E1 and E3 resulted in an alteration of the genotypic sex ratio in Japanese medaka. However, these authors did not perform a statistical analysis of the sex ratio between treatments and controls. To our knowledge, only one paper has reported a significant alteration in genotypic sex ratio in fish exposed to antiestrogens such as tamoxifen, where exposure caused a significant increase in the proportion of F1 genetic male Japanese medaka relative to the controls (Sun et al. 2007). The observed change in sex ratio may be caused by differential sensitivity of the embryos or larvae to DES. In the present study, exposure to DES at 100 ng/L or greater resulted in the death of more than 40 % of the embryos and larvae. If

Fig. 1 Relative expression of ER α and Vtg-I mRNA levels in the liver of medaka fish by qPCR with regard to β -actin for early life stage exposed to DES. Data are given as mean $\pm$ standard deviation. $n = 10$ fish for each group and values are determined in triplicate.

DWC dilution water (charcoal-dechlorinated tap water) control; SC solvent control. The asterisks (*single asterisks*) and (*double asterisks*) denote statistically significant difference compared with the solvent control ($p < 0.05$; $p < 0.01$), respectively

Fig. 2 Relative expression of ER α and Vtg-I mRNA levels in the liver of medaka fish by qPCR with regard to β -actin for adult fish exposed to DES. Data are given as mean $\pm$ standard deviation. $n = 10$ fish for each group and values are determined in triplicate.

DWC dilution water (charcoal-dechlorinated tap water) control; SC solvent control. The asterisks (*single asterisks*) and (*double asterisks*) denote statistically significant difference compared with the solvent control ($p < 0.05$; $p < 0.01$), respectively

embryos or larvae with the XY chromosome are more sensitive to DES than embryos or larvae carrying the XX chromosome this may lead to a high death rate of embryos and

larvae with the XY chromosome, and the high female: male ratios may be presented. Specific reasons for the change in the sex ratio require further study.

GSI and HSI are general measures of the growth status of specific tissues in fish. When fish in the early life stage were exposed to DES, the GSI of genetic males significantly increased at the highest concentration of DES. Sex reversal of genetic males was also found at this concentration. The higher GSI of genetic male fish can be attributed to sex reversal. In these sex reversal male fish with red-colored tails, the testes were replaced by ovaries. Sex reversal in fish exposed to EDCs has been reported previously and may involve estrogenic chemicals eliciting female characteristics in males or androgenic chemicals producing male traits in females (Edmunds et al. 2000; Sun et al. 2007; Lei et al. 2013a). Sex reversal males are considered to be the consequence of the transformation from testes to ovaries during early gonadal development under the direct influence of environmental estrogens (Zha et al. 2008). The sex of genetic male medaka can be functionally reversed if estrogens such as E1, E2, EE2 and EV are applied during a sensitive period of embryonic or juvenile development (Kishi et al. 2006; Pettersson et al. 2006; Lei et al. 2013a, b). Even if a weakly estrogenic pesticide such as *o,p'*-DDT is present during the critical period of gonadal development, it can profoundly alter sex (Edmunds et al. 2000). Exposure to DES during the embryonic and larval stages of medaka fish leading to sex reversal in genetic males has been reported previously (Paul-Prasanth et al. 2011). In addition, aquaculturists have used the DES to produce more females in fish populations (Potts and Phelps 1995). The GSI in females and HSI in both sexes were not significantly altered in the DES groups relative to the controls. Taken together, these results showed that exposure to DES has long-lasting effects on gonadal development in genetic males (sex reversal) even when fish at an early life stage were exposed to DES.

In the 21-days exposure experiment in adult fish, the HSI and GSI in the males and females showed no significant differences relative to the controls, except for a significant decrease in the GSI in females at the greater exposure concentration of 1000 ng/L DES. The low GSI in females may be due to ovary degeneration (Scholz and Gutzeit 2000). Seki et al. (2002) reported the regression of ovaries in adult female medaka exposed to 488 ng/L EE2 for 3 weeks using tissue histopathology. Decreased GSI was also observed in female rare minnows exposed to EE2 (Scholz and Gutzeit 2000; Zha et al. 2008), 4-NP, as well as 2,4-dichlorophenol (2,4-DCP) (Zhang et al. 2008a, b). In the present study, two females showed abdominal swelling at 1000 ng/L DES. Therefore, these results show that DES exposure can cause ovary degeneration in adult females.

ER α and Vtg-I gene expression

As sensitive biomarkers of estrogenic action, ER and Vtg are involved in many important physiological processes

(Matozzo et al. 2008). ER α is auto-regulated by the presence of estrogen response elements (EREs) and expressed in many tissues in fish species (Petit et al. 1999; Zhang et al. 2008b). Vtg production in the liver is generally under the control of the ER pathway in fish (Ishibashi et al. 2008). Flouriot et al. (1997) consider that the alteration of Vtg mRNA levels depends directly on the number of ERs, which are also regulated at the mRNA level by estrogen treatment in fish. We therefore postulate that DES, a synthetic estrogen, can also alter ER and Vtg mRNA levels in both sexes. DES at concentrations greater than 1 ng/L significantly altered ER α and Vtg-I mRNA levels in males and females when exposed at two life stages. In addition, 1 ng/L may be the NOEC of DES for Vtg-I and ER α gene expression in medaka fish in the present study. This is consistent with the work by Adedeji et al. (2012) who also reported that the NOEC of DES was 1 ng/L for hepatic transcripts of Vtg and ER α in adult fathead minnows exposed to DES for 4 days.

When fish at an early life stage were exposed to DES, expression of the hepatic estrogen-responsive gene, ER α , was significantly down-regulated in females at DES concentrations greater than 1 ng/L and in males at DES concentrations greater than 10 ng/L. DES can bind ER α and it was found to be approximately 50-fold less potent than E2 affinity for ER α in an in vitro bioassay (Luo et al. 2011). E2 is an endogenous estrogen and its physiological concentration is generally in ng/ml levels in fish, and plays important roles in regulating gonad development, sex differentiation and related gene expression in fish (Bhandari et al. 2003; Nakamura et al. 2009). Therefore, the down-regulation of ER α mRNA expression suggests that a potential competitive effect may occur when endogenous E2 coexists with other exogenous ER α binding EDCs like DES. Similar results have also been reported where ER α gene expression in the liver was significantly reduced in both sexes of Japanese medaka exposed to different concentrations of E2 (Chakraborty et al. 2011). Vtg mRNA expression in females and males showed completely different changing trends. In general, the Vtg gene can be up-regulated in males by exposure to estrogenic substances (Adedeji et al. 2012; Lei et al. 2013a, b). However, in the present study, Vtg mRNA expression in females was significantly up-regulated at DES concentrations of 10 ng/L and above, and in males was markedly down-regulated at 100 and 1000 ng/L. Zhang et al. (2008b) also found that Vtg mRNA expression in females was significantly up-regulated, and was down-regulated in males (although statistically significant differences were not observed) when Chinese rare minnows (*Gobiocypris rarus*) were exposed to 2,4-DCP concentrations of 0.3 mg/L and above for 21 days. The mechanism by which DES up-regulates Vtg mRNA levels in females and down-regulates Vtg

mRNA levels in males is unknown, but deserves further study.

After adult stage exposure to DES for 21 days, ER α mRNA levels in both sexes were significantly decreased, which was similar to the change in ER α mRNA expression in fish exposed to DES during the early life stage. Vtg mRNA expression following adult stage exposure showed the different changing trends compared to early life stage exposure. Previous work showed that ER can act as a transcription repression factor (An et al. 1999), and can activate Vtg mRNA expression in male fish. Vtg gene expression can be significantly up-regulated in adult male fathead minnows by short-term exposure to DES for 4 days (Adedeji et al. 2012). Other studies have also reported a significant up-regulation of hepatic Vtg mRNA levels in male fish exposed to estrogens such as E2 and E1, with down-regulation (although no statistically significant differences were observed) of hepatic ER mRNA levels in females (Arukwe et al. 2001; Boyce-Derricott et al. 2009; Lei et al. 2013b). These results are similar to those in our study on adult life stage of fish exposure to DES for 21 days.

However, other studies have reported that liver Vtg and ER mRNA levels increase simultaneously in fish after estrogen treatment in fish (Atteke et al. 2003; Andreassen et al. 2005). In this study, ER and Vtg mRNA levels in males at the early life stage exposed to DES and in females at the adult life stage exposed to DES showed the same changing trends. In addition, ER mRNA expression in both sexes showed a downward trend following the two exposure conditions. However, Vtg mRNA levels were significantly increased in females treated with DES at the early life stage and in males treated with DES at the adult life stage. The mechanism by which DES results in different Vtg mRNA levels in both sexes at different life stages is not yet known, but may involve both competition and the synthesis of specific mRNAs. Presumably when the early life stages of fish were exposed to DES, followed by maintenance in charcoal-dechlorinated tap water, the remaining DES in female fish combines with endogenous E2 for ER up-regulating Vtg gene transcription in females. In the adult life stage, exposure to DES can directly activate ER receptor leading to transcriptional events and Vtg synthesis in male fish. However, it is important to bear in mind that the ER-mediated pathway is regulated by many other factors including co-activators and co-repressors and by phosphorylation and interactions with other signaling pathways (Andreassen et al. 2005). Therefore, the detailed mechanisms by which DES up-regulates Vtg mRNA levels in females at the early life stage exposure and in males at the adult life stage exposure require further study to find more reasonable explanation.

Conclusions

Under our experimental conditions, environmentally relevant concentrations (1 and 10 ng/L) of DES had no adverse effects on embryo hatching and the mortality of hatched larvae. However, DES at 10 ng/L had a teratogenic effect on embryos and hatched larvae indicating that DES affected the development of early life stages in Japanese medaka. In addition, exposure to DES at the environmental concentration of 10 ng/L markedly altered the ER and Vtg mRNA levels in both sexes at the two life stages examined. Therefore, when ER α and Vtg-I mRNA expression are used as toxicity endpoints to assess the estrogenic effects of DES, DES is potent in medaka fish.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Any studies in this paper using Japanese medaka fish were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

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