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17β estradiol induced ROS generation, DNA damage and enzymatic responses in the hepatic tissue of Japanese sea bass

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Abstract The importance of endocrine disrupting chemicals and their effects on fish has been documented in recent years. However, little is known about whether the estrogenic compound 17β estradiol (E2) causes oxidative stress in the hepatic tissue of fish. Therefore, this work tested the hypothesis that E2 might cause oxidative stress in the Japanese sea bass *Lateolabrax japonicus* liver. To test this hypothesis, its effects on reactive oxygen species (ROS) production, DNA damage, antioxidants and biotransformation enzyme were investigated in two different size groups (fingerling and juvenile groups) following 30 days exposure. Results showed that there was a good relationship between the E2 exposure concentration, plasma E2 level and ROS generation. In addition ROS production correlated negatively with 7-ethoxyresorufin-O-deethylase activity and positively with DNA damage and lipid peroxidation (LPO). Antioxidant enzymes such as superoxide dismutase and catalase did not show any significant relation with ROS, LPO and DNA damage. In contrast, glutathione mediated enzymes showed a good relationship with the above parameters suggesting that the glutathione system in fish might be responsible for protection against the impact of E2 and also indicating a possible adaptive response during exposure periods. In addition, it was observed that fingerling was more susceptible to E2 exposure than juvenile fish. The present study provided strong evidence that the ROS level increased significantly in the liver of E2 exposed fish, and that ROS might serve as a biomarker to indicate estrogen contamination.

Keywords *Lateolabrax japonicus* · Estradiol · ROS · DNA damage · LPO · EROD

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Introduction

Endocrine disrupting chemicals (EDCs) are found in aquatic environments, often in complex mixtures, and representing a potential hazard to aquatic species (Sumpter and Johnson 2005). Steroid compounds such as estrone, 17β estradiol (E2) and 17α ethynylestradiol (EE2) are more abundant estrogenic compounds in aquatic environments and are capable of exerting an impact on organisms (induction of vitellogenin and vitelline envelope proteins) even at very low concentrations (1 ng L^{-1}) (Thomas-Jones et al. 2003). Xenobiotics present in the environment can stimulate the production of reactive oxygen species (ROS), which results in oxidative damage to aquatic organisms (Livingstone 2001). ROS are formed in various metabolic steps in organisms and have been associated with the etiology of hepatic neoplasia in vertebrate groups. Environmental contaminants are known to induce ROS formation during biotransformation via redox reactions (Giulio et al. 1989) and there is increasing evidence that prolonged xenobiotic exposure causes ROS formation in marine organisms (Mather-Mihaich and Di Giulio 1986; Giulio et al. 1989). Although antioxidant enzymes in animals play a key role in preventing cellular damage caused by ROS (Mates et al. 1999), cellular ROS accumulation can lead to oxidative stress in an individual and result in various types of tissue damage and disease when the dysfunction of

antioxidation occurs with overproduction of reactive oxygen intermediate species (Janssen et al. 1993).

Elevated levels of estrogen in vivo leading to DNA damage are reported in both mammals and fish, and E2 causes oxidative DNA damage in fish (Maria et al. 2008; Rempel et al. 2008). Metabolism of E2 leads to the production of semiquinones and quinones, which produce free radicals through redox cycling (Cavalieri et al. 2000). Oxidation of DNA and lipid peroxidation (LPO) may be the important early markers of such damage in the tissues of marine organisms. Earlier studies report that free radicals are generated during redox cycling of estrogens and could cause to damage cellular macromolecules (Seacat et al. 1997). Cytochrome P450 plays a critical role in the oxidative metabolism of endogenous compounds such as steroids and other xenobiotics (Carrera et al. 2007). In fish, it is reported that the phase I biotransformation system responds in a very selective manner to estrogenic compounds (Arukwe et al. 1997). Cytochrome P450 1A (CYP1A) induction is commonly measured as 7-ethoxyresorufin-O-deethylase (EROD) activity, which has already been used as a biomarker when studying exposure to estrogenic compounds (Arukwe et al. 1997; Sole et al. 2000; Teles et al. 2004, 2005; Carrera et al. 2007). In addition it is also reported that lipid peroxides may play an important role in estrogen-induced carcinogenesis (Wang and Liehr 1995).

In recent years a variety of biomarkers have been used to monitor the effect of E2 on fish and most of the studies focus on biotransformation, osmoregulation, hepatic enzymes, genotoxicity, lipid peroxidative damage and antioxidant responses (Arukwe et al. 1997; Sole et al. 2000; Teles et al. 2004, 2005; Carrera et al. 2007; Ahmad et al. 2009), were restricted to short term aqueous exposure (1–10 days), or were as a result of intraperitoneal injection (Maria et al. 2008). To our knowledge, no long-term study on the generation of ROS due to E2, and its subsequent toxic effects on fish hepatic tissues has been carried out. This investigation is, therefore, the first attempt to evaluate ROS effects on the modulation of antioxidant parameters such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), reduced glutathione (GSH), and Glutathione S-transferase (GST) in different life stages of Japanese sea bass after long-term exposure to E2. Related parameters observed included DNA damage, LPO, EROD activity and other antioxidant enzymes. The purpose of comparing two different size groups of *L. japonicus* in this study was to understand the possible differences in their responses when exposed to the same concentrations of E2 for the same period of exposure. Two different concentrations (200 and 2000 ng L^{-1}) and three different exposure periods were employed in the present study and the concentration of E2 used in the present study was chosen based on previous work (Thilagam et al. 2009).

Materials and methods

Fish

Japanese sea bass (*L. japonicus*) of two different sizes were obtained from Zhang Pu fish culture farm in Fujian Province, China and were acclimatized for 10 days to laboratory conditions as mentioned in our previous study (temperature 24 ± 1°C; salinity of 30 ± 1‰; pH 7.8 ± 0.1) (Thilagam et al. 2009). Fish with a length of 7 ± 1 cm were considered as fingerlings and fish with a length of about 15 ± 2 cm were considered as juveniles. The fish were fed with fresh prawn flesh at the rate of 3% of their body weight, and water was removed daily along with the waste feed and fecal material.

Chemicals

Estrogen (E2, purity, 99%), Bis-benzimide, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), resorufin, 7-ethoxyresorufin, 1-chloro-2,4-dinitrobenzene (CDNB), GSH, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), malondialdehyde, pyrogallol, and thiobarbituric acid were purchased from Sigma (Sigma Chemicals, St. Louis, MO USA) and all other chemicals used were of analytical grade with 99% purity.

Toxicity test for biomarkers

Experimental exposure conditions were similar to those described in our previous paper (Thilagam et al. 2009). Briefly, E2 stock solution was prepared at 1 mg L^{-1} using ethanol (HPLC grade). To assess the changes in biomarkers, fingerling and juvenile fishes were divided into four groups: Group I was reared in normal seawater, group II was set for the solvent control (95% ethanol), group III and IV were exposed to seawater containing 200 and 2000 ng L^{-1} of E2. Fish were exposed in glass aquaria (90 × 60 × 60 inches) containing 100 L of seawater/test solution. The test solution was renewed daily. To avoid additional stress during renewal only 90% of test solution was renewed. The fish were fed with fresh prawn flesh during the 30 days experimental period. After 5, 15 and 30 days of exposure, 3 fish from each group were killed to assess the changes in biomarkers. Duplicate experimental chambers were maintained for all concentrations. Blood samples were collected as described previously (Thilagam et al. 2009). There was no significant difference between the solvent and blank

controls, and we therefore show only the results of the solvent control in the figures and tables.

Plasma E2 concentration

The concentration of E2 in the blood was measured in fingerling and juvenile fish plasma using a diagnostic ELISA direct immunoenzymatic kit (Nanjing Jiancheng Bioengineering Institute, Jiancheng, China) following the manufacturers instructions. Briefly, 25 μL of samples (including the standard and blank control) were loaded into the appropriate wells and 100 μL of estradiol-horseradish peroxidase conjugate reagent was added to each well. Next, 50 μL of rabbit anti-estradiol reagent was added to each well and mixed thoroughly for 30 s and then incubated at 22°C for 90 min. After incubation, micro-wells were rinsed and flicked 5 times with deionized water. To each well 100 μL of 3,3′,5,5′-tetramethylbenzidine was added and mixed for 20 s and incubated at 22°C for 20 min. The reaction was stopped by adding 50 μL of 0.16 M sulfuric acid (stop solution) to each well and the plate was then read (within 15 min) at 450 nm using a microplate reader.

Protein measurement

Total protein contents were determined according to Bradford (1976) using bovine serum albumin as a standard.

Evaluation of DNA strand breaks

The alkaline unwinding assay used in the present study was adapted from Shugart (1988). A liver sample was gently cut into fine pieces and incubated with 1 mL of TNE buffer (0.05 M Tris, 0.1 M NaCl, 0.1 M EDTA, 0.5% SDS, pH 8.0) at 37°C for 24 h. After incubation, 150 μL of saturated NaCl was added and the mixture centrifuged at 12,000×*g* for 20 min. The supernatant was added to an equal volume of buffered phenol/chloroform/isoamyl alcohol (PCI) (24:25:1, v/v/v, pH 8.0) and gently mixed. The sample was allowed to settle for 5 min before centrifuging at 12,000×*g* at 4°C for 5 min. The aqueous layer was transferred to a new centrifuge tube and the PCI extraction repeated. The aqueous layer was then digested using 5 μL of Ribonuclease A (10 mg mL^{-1}) for 30 min at 37°C and extracted successively using equal volumes of PCI. The DNA was precipitated from the resulting aqueous layer by adding 2 volumes of cold absolute ethanol and a 1/10 volume of 3 M sodium acetate buffered to pH 5.2. The sample was centrifuged at 12,000×*g* for 15 min and the supernatant decanted. Finally, the pellet was rinsed with 500 μL of 70% ethanol, air dried and then dissolved in 400 μL of TE buffer (10 mM Tris, 1 mM EDTA). The DNA sample was separated into two equal portions for fluorescence determination of double stranded DNA (dsDNA) and single-stranded DNA (ssDNA). The fluorescence of dsDNA and ssDNA was measured using a spectrofluorimeter with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Data concerning the DNA unwinding technique were expressed as F values, determined by dividing the double strand value by the double plus single strand value in the sample.

Preparation of samples for ROS measurement, EROD, LPO and antioxidant activity

The liver was dissected, rinsed with ice-cold normal saline (0.91% w/v of NaCl) and stored at −80°C until analysis, when it was homogenized in 4 volumes of ice-cold Tris buffered saline (10 mM Tris–HCl, 0.1 mM EDTA-2Na, 10 mM sucrose, 0.8% NaCl, pH 7.4) with a glass-homogenizer. The homogenate was centrifuged at 500×*g* for 10 min, the fat layer removed and the resulting supernatant centrifuged at 3,000×*g* for 30 min, followed by 10,000×*g* for 30 min at 4°C. The supernatant was further centrifuged at 100,000×*g* for 60 min in an HIMAC CS150GXL (Japan) micro-ultracentrifuge to obtain the cytosolic and microsomal fractions. All the antioxidant and associated enzyme assays were carried out using the cytosolic fraction and the microsomal pellets were used for the determination of EROD activity.

Liver EROD determination

Microsomal pellets were resuspended in 500 μL of Tris–HCl buffer. Cytosolic and microsomal protein contents were measured using the method of Bradford (1976), using bovine serum albumin (BSA) as the standard. In the microsomal fraction, EROD activity was determined as described in Burke and Mayer (1974) using micro plate reader (TECAN A-5082, Genios, Austria) at 535/585 nm excitation/emission wavelengths.

ROS measurement

ROS was measured based on the methods of Driver et al. (2000) with slight modifications. Homogenate (20 μL), 100 μL physiological saline and 5 μL of Dichlorodihydrofluorescein diacetate (DCFH-DA) were added to each well and the plates were incubated at 37°C for 30 min. The conversion of DCFH to the fluorescent product dichlorofluorescein (DCF) was measured using a TECAN spectrophotometer with excitation at 485 nm and emission at 530 nm. Background fluorescence (conversion of DCFH to DCF in the absence of sample) was corrected for by the inclusion of parallel blanks.

Antioxidant and lipid peroxidation measurements

CAT activity was determined according to Sinha (1972). SOD activity was measured as the degree of inhibition of auto-oxidation of pyrogallol at an alkaline pH using the method of Marklund and Marklund (1974). GPx was assayed by measuring the amount of GSH consumed in the reaction mixture according to the method of Rotruck et al. (1973). GSH was estimated using the method of Moron et al. (1979) and reading the optical density of the yellow substance formed when DTNB was reduced by glutathione at 412 nm. GST activity was measured using the CDNB substrate following conjugation of the acceptor substrate with glutathione as described in Habig et al. (1974). LPO was measured according to Devasagayam and Tarachand (1987). The color developed was measured at 532 nm and the MDA content of the sample was expressed as nmol of MDA formed/mg protein.

Statistical analyses

SPSS software version 11.0 for Windows was used for the statistical analysis. Results are reported as mean ± S.D. of six observations per group and the significance was tested. The data were processed using two-way analysis of variance followed by Tukey's multiple-comparison post hoc test to identify statistical differences among individual treatment groups. Principal component analysis (PCA) and a correlation matrix were used to assess the interrelationships among the parameters used. "Varimax Rotation" was used for extraction and deriving factors in the PCA and the Pearson correlation coefficient was used in the correlation matrix. Differences were statistically significant when $P < 0.05$ and 0.01, respectively.

Results

Both E2 concentrations resulted in significant increases in plasma E2 in fingerlings after 5 and 15 days (Fig. 1). However, after 30 days only the higher concentration of E2 significantly increased plasma E2. Plasma E2 concentrations responded in a dose- and time-dependant manner during exposure.

Sea bass exposed to E2 had higher ROS production and DNA damage in their liver (Figs 2, 3). The generation of ROS was induced in both fingerling and juvenile groups exposed to the higher concentration (after 15 and 30 days, respectively). Similarly the juvenile group exposed to 2000 ng L^{-1} showed an increase in ROS after 5 days of exposure (Fig. 2). DNA integrity in the fingerling liver sample decreased significantly when they were exposed to higher concentrations of E2 after both 15 and 30 days

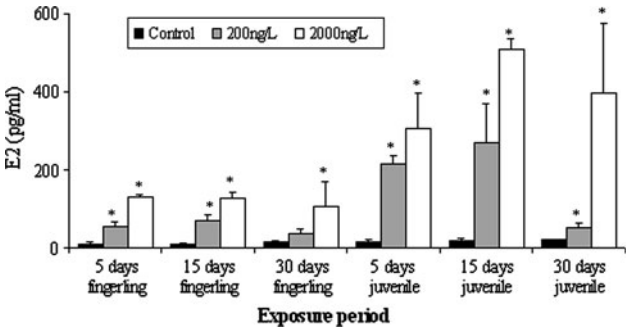

Fig. 1 Plasma E2 concentrations in fingerling and juvenile groups of *L. japonicus*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups were indicated with *asterisks* (* $P < 0.05$)

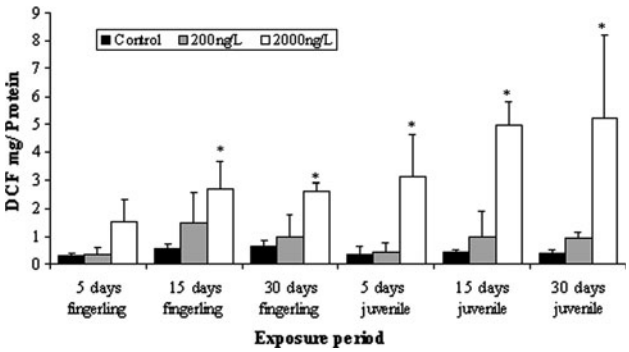

Fig. 2 Effect of E2 on ROS measurement in the liver of fingerling and juvenile groups of *L. japonicus*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups were indicated with *asterisks* (* $P < 0.05$)

(Fig. 3). Similarly, the DNA integrity decreased significantly in juvenile fish exposed to higher concentration in all the exposure periods. However, the DNA integrity in the juvenile group decreased for both concentrations only after 15 days (Fig. 3).

Modulations of the antioxidant enzymes, EROD activity and LPO content in the hepatic tissues of sea bass exposed to different concentration of E2 are shown in Tables 1 and 2. The SOD activities increased significantly after 5 days in both groups when they were exposed to higher E2 concentration (Tables 1, 2) and decreased significantly after 15 days in fingerlings exposed to lower concentration of E2 (Table 1). The CAT activity decreased significantly after 15 days when fingerlings were exposed to both E2 concentrations (Table 1), but the CAT activity in the juvenile group did not show any significant modulation compared with the control group. The GSH level decreased significantly in fingerlings exposed to both E2 concentrations

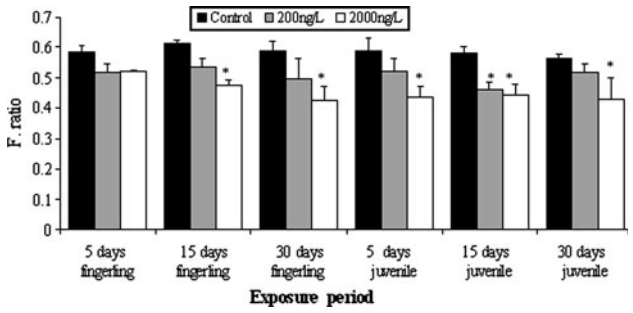

Fig. 3 Effect of E2 on DNA damage in the liver of fingerling and juvenile groups of *L. japonicus*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups were indicated with *asterisks* (* $P < 0.05$)

after 5 and 15 days, but decreased only at higher concentration after 30 days (Table 1). A similar decrease in GSH level in the juvenile group was observed after 5 and 15 days at higher E2 concentration (Table 2). The GPx activity was significantly reduced in both groups exposed to E2 concentrations after 15 days (Tables 1, 2), but decreased in the juvenile group after 5 days when the fish were exposed to the lower concentration of E2 (Table 2). The GST activity was significantly induced in fingerlings after 5 and 15 days of exposure to both E2 concentrations. A similar increase was also observed in the juvenile group after 15 and 30 days of exposure (Table 1, 2). The LPO content increased in both the fingerling and juvenile groups exposed to E2 concentration after 5 and 15 days (Tables 1, 2), but was reduced to the normal level after 30 days of exposure in fingerlings exposed to both E2 concentrations (Table 1), whilst the LPO level in the juvenile group exposed to E2 was still significantly increased in comparison to the control group (Table 2).

The EROD activity in liver normalized to microsomal protein content was significantly reduced in fingerlings after 5 days (higher concentration), and after 15 and 30 days (both concentrations) of exposure (Table 1). Similarly the EROD activity decreased in the juvenile group exposed to the higher concentration of E2 after 15 and 30 days (Table 2).

The Pearson correlation matrix calculated is given in Table 3a and b. The correlation between individual parameters produced similar results to those of PCA and showed significant ($P < 0.01$; $P < 0.05$) association between the parameters studied. There were good correlations between plasma E2 and other parameters (except for SOD, CAT and GPx) in both the fingerling and juvenile groups. The coefficients of correlation in most cases were greater than 0.645. ROS generated in the liver showed a significant relationship with DNA damage, LPO, EROD activity and glutathione mediated enzymes in the juvenile group. Similarly, it showed correlation with DNA damage, EROD activity and GSH in the fingerling group. Our study also showed a significant relation ($P < 0.05$) between ROS and GST activity for the juvenile group (Table 3b). Correlation between the LPO and glutathione mediated enzymes showed significant relationships, but the fingerlings showed high correlation only between LPO with GSH and GST enzymes (Table 3a).

The rotated component matrix, developed by PCA on the measured parameters for both groups, is given in Table 4. The dimensions of the parameters were reduced from 10 original variables to three principal factors for the fingerling and two principal factors for the juvenile group using PCA with an eigen value >1.0. For fingerlings, the first three factors accounted for 89.39% of the overall variance of the data. The percentage of variance explained by different principal components was as follows: 55.32, 19.74, 14.28, 5.95, 2.08, 1.79, 0.55 and 0.23%. Similarly for the juvenile group, the first two factors accounted for 85.83% of the overall variance of the data and the percentage of variance explained by different principal components was as follows: 69.39, 16.43, 6.46, 2.99, 1.77, 1.61, 0.72 and 0.59%.

Discussion

Elevated levels of plasma E2 concentration leading to DNA damage in fish are reported (Rempel-Hester et al. 2009). A similar result was observed in the present study with plasma E2 concentrations increasing in sea bass exposed to E2 and resulting in ROS production and corresponding antioxidative responses in the liver. This caused oxidative damages exemplified by LPO and DNA damage. Correlations between plasma E2 concentration and ROS, DNA damage, LPO, EROD activity and antioxidant enzymes demonstrated that E2 might induce oxidative stress in the hepatic tissue of sea bass.

Xenobiotic induced ROS can cause oxidative damage and the mechanisms are involved in the redox cycling catalysis by flavoprotein, reaction of O_2 and ROS with redox, autoxidation, enzyme induction and depletion of antioxidant defences (reviewed in Livingstone 2001). Earlier studies report that E2 induces ROS (Felty et al. 2005) and produces oxidative stress (Patel and Bhat 2004) in hamster kidney cells. In our study, ROS caused significant induction in fingerlings (sevenfold, fivefold and fourfold) that of their respective control after 5, 15 and 30 days of exposure and similarly 9-fold, 11-fold and 14-fold higher in juvenile groups after these exposure periods. It was also interesting to note that the induced ROS levels corresponded to the DNA damage produced in the liver of sea bass with a strong significant correlation between ROS

Table 1 Effect of E2 on antioxidants, lipid peroxidation and EROD activity in fingerlings of *Lateoiabrax japonicus*

	5 days			15 days			30 days		
	Control	200 ng L^{-1}	2000 ng L^{-1}	Control	200 ng L^{-1}	2000 ng L^{-1}	Control	200 ng L^{-1}	2000 ng L^{-1}
SOD	109.69 ± 30.8	147.45 ± 44.7	233.25 ± 76.0*	95.28 ± 7.80	39.38 ± 26.9*	135.54 ± 78.7	84.94 ± 32.2	92.97 ± 85.7	140.53 ± 65.5
CAT	1.3 ± 0.16	1.12 ± 0.34	1.0 ± 0.19	1.52 ± 0.17	0.98 ± 0.09*	0.42 ± 0.11*	1.09 ± 0.57	1.02 ± 0.64	2.45 ± 0.74
GSH	16.68 ± 1.57	9.12 ± 2.69*	8.42 ± 4.40*	19.56 ± 5.41	8.54 ± 2.04*	5.3 ± 2.04*	19.50 ± 5.32	10.86 ± 7.52	7.88 ± 3.09*
GPx	11.27 ± 3.77	9.13 ± 1.64	15.8 ± 2.80	9.70 ± 1.24	3.7 ± 1.17*	2.02 ± 0.78*	10.89 ± 6.0	4.6 ± 1.63	3.43 ± 1.27
GST	4.87 ± 0.43	26.79 ± 14.5*	34.42 ± 5.35*	10.13 ± 2.89	28.36 ± 9.38*	47.85 ± 1.3*	10.23 ± 2.08	20.02 ± 13.9	15.58 ± 10.2
LPO	0.04 ± 0.03	0.51 ± 0.21*	0.38 ± 0.19*	0.12 ± 0.03	0.32 ± 0.09*	0.4 ± 0.14*	0.08 ± 0.03	0.16 ± 0.04	0.18 ± 0.02*
EROD	0.28 ± 0.12	0.27 ± 0.21	0.08 ± 0.01*	0.2 ± 0.08	0.08 ± 0.02	0.08 ± 0.01*	0.22 ± 0.04	0.07 ± 0.02*	0.05 ± 0.01*

Values of SOD and GST were expressed as U/mg protein; CAT expressed as μmol of H_2O_2 consumed/min/mg protein; GSH expressed as μg/mg protein and GPx expressed as μmol of GSH oxidized/min/mg protein; LPO expressed as nmol of MDA released/mg protein; EROD activity expressed as pmol resorufin/mg protein/min. Data represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followedby Tukey's post hoc test was used. The significant difference between control and exposure groups were indicated with asterisks (* $P < 0.05$)

Table 2 Effect of E2 on antioxidants, lipid peroxidation and EROD activity in juveniles of *Lateolabrax japonicus*

	5 days			15 days			30 days		
	Control	200 ng L^{-1}	2000 ng L^{-1}	Control	200 ng L^{-1}	2000 ng L^{-1}	Control	200 ng L^{-1}	2000 ng L^{-1}
SOD	138.85 ± 52.1	160.78 ± 38.9	282.13 ± 40.0*	128.6 ± 51.9	77.58 ± 28.5	76.34 ± 60.9	113.88 ± 72.7	94.36 ± 53.1	64.23 ± 8.8
CAT	1.94 ± 0.62	2.16 ± 0.65	3.16 ± 0.75	1.90 ± 0.79	1.26 ± 0.49	0.96 ± 0.20	1.60 ± 0.71	2.16 ± 0.60	1.06 ± 0.24
GSH	19.03 ± 2.09	18.18 ± 5.97	11.92 ± 0.31*	16.24 ± 8.38	6.27 ± 1.87	5.28 ± 0.56*	16.05 ± 7.84	8.68 ± 2.45	8.0 ± .94
GPx	18.02 ± 1.31	11.12 ± 2,17*	11.74 ± 4.51	12.09 ± 1.56	4.71 ± 0.46*	3.30 ± 0.78*	6.97 ± 3.73	2.68 ± 0.36	2.10 ± 0.33
GST	9.29 ± 3.86	16.07 ± 5.62	21.40 ± 8.94	6.34 ± 2.41	47.47 ± 27.6*	46.62 ± 27.3*	4.74 ± .05	46.56 ± 9.91*	58.42 ± 32.53*
LPO	0.03 ± 0.01	0.39 ± 0.21*	0.15 ± 0.02*	0.02 ± 0.01	0.42 ± 0.17*	0.7 ± 0.11*	0.03 ± 0.01	0.29 ± 0.09*	0.57 ± 0.16*
EROD	0.26 ± 0.06	0.25 ± 0.12	0.20 ± 0.03	0.30 ± 0.15	0.17 ± 0.05	0.04 ± 0.01*	0.18 ± 0.06	0.1 ± 0.05	0.06 ± 0.002*

Values of SOD and GST were expressed as U/mg protein; CAT expressed as μmol of H_2O_2 consumed/min/mg protein; GSH expressed as μg/mg protein and GPx expressed as μmol of GSH oxidized/min/mg protein; LPO expressed as nmol of MDA released/mg protein; EROD activity expressed as pmol resorufin/mg protein/min. Data represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followedby Tukey's post hoc test was used. The significant difference between control and exposure groups were indicated with asterisks (* $P < 0.05$)

Table 3 Correlation matrix for measured parameters in (a) fingerlings and (b) juveniles of *Lateolabrax japonicus* exposed to different concentration of E2

	ROS	DNA	EROD	LPO	SOD	CAT	GSH	GPX	GST	E2
(a) Fingerlings										
ROS	1.000	−0.790*	−0.821**	0.292	0.231	0.088	−0.708*	−0.583	0.606	0.841**
DNA		1.000	0.712*	−0.428	−0.336	−0.205	0.859**	0.0.582	−0.526	−0.764*
EROD			1.000	−0.199	−0.134	−0.023	0.663	0.497	−0.531	−0.716*
LPO				1.000	0.417	−0.434	−0.752*	−0.121	0.839**	0.645*
SOD					1.000	0.024	−0.371	0.495	0.376	0.605
CAT						1.000	0.175	−0.026	−0.606	−0.107
GSH							1.000	0.483	−0.819**	0.869**
GPX								1.000	−0.303	−0.234
GST									1.000	0.813**
E2										1.000
(b) Juveniles										
ROS	1.000	−0.817**	−0.756*	0.732*	−0.139	−0.348	−0.673*	−0.521	0.672*	0.878**
DNA		1.000	0.706*	−0.773*	−0.006	0.156	0.797*	0.609	−0.778*	−0.908**
EROD			1.000	−0.771*	0.501	0.534	0.836**	0.870**	−0.834**	−0.664
LPO				1.000	−0.472	−0.581	−0.755*	−0.699*	0.832**	0.876**
SOD					1.000	0.912**	0.429	0.609	−0.517	−0.100
GAT						1.000	0.455	0.553	−0.483	−0.353
GSH							1.000	0.836**	−0.906**	−0.698**
GPX								1.000	−0.811**	−0.500
GST									1.000	0.680*
E2										1.000

* Correlation is significant at the 0.05 level (2-tailed), ** correlation is significant at the 0.01 level (2-tailed)

and DNA damage (Table 3a, b). In healthy animals, there is a balance between oxidative stress and antioxidant defenses but, due to environmental contamination, excess ROS is generated in tissues which ultimately results in oxidative damage to key molecules such as DNA, protein and lipid (Livingstone 2001) and this will affect the homeostasis of the fish.

Free-radical intermediates and ROS formed during biotransformation of xenobiotics can initiate macromolecular changes, namely DNA damage, necrosis and apoptosis (van der Oost et al. 2003). Estrogens cause DNA damage in mammals even at low concentration (10 nM) (Wellejus et al. 2004). Studies in mammals show that estrogens are linked with oxidative DNA damage and DNA adduct formation, probably through the production of catechol metabolites via hydroxylation at the 2 or 4 position (Cavalieri et al. 2002; Wellejus et al. 2004) as cited in Rempel-Hester et al. (2009). Moreover, during catechol metabolism hydrogen, peroxide and hydroxyl radicals produced through redox cycling can oxidize the DNA bases and the tissues/cells which have a low level of detoxification enzymes (namely catechol-O-methyltranferase, quinone reductase, or P450 reductase) and are more susceptible to DNA damage (Cavalieri et al. 2002). It has been reported that E2 can enhance DNA damage in fish (Maria et al. 2008; Rempel-Hester et al. 2009). However, the role of ROS as a possible cause of DNA damage has been left out.

CYP enzymes are inhibited due to ROS mainly at the transcriptional level by inhibition of mRNA synthesis (Risso-de Faverney et al. 2000), but sometimes due to increasing degradation of mRNA, at the post transcriptional level (Delaporte and Renton 1997) or at the protein level when the xenobiotics act directly as mechanism-based inhibitors (Watson et al. 1995). Earlier studies show that E2 affects hepatic CYP1A activity in fish (Elskus 2004; Vaccaro et al. 2005) and that the phase I biotransformation system in fish also responds to steroids in a very selective manner (Arukwe et al. 1997). Also, the hepatic EROD activities are significantly reduced in different fish species exposed to E2 (Arukwe et al. 1997), EE2 (Sole et al. 2000), E2 and 4-NP alone (Arukwe et al. 2001; Vaccaro et al. 2005) or to co-exposures E2 and 4-NP in *Sparus aurata* (Teles et al. 2004, 2005). In the present study, the EROD activity was found to be lower at all exposure periods when both groups were exposed to higher concentration of E2. This might be due to E2 interaction with CYP1A metabolism pathways in sea bass hepatocytes through an inhibition of the EROD activity. Such effects

are of environmental concern, since this inhibition of CYP1A activity affects xenobiotic metabolism and toxicity (Hawkins et al. 2002). Our data agreed with the earlier reports, since a reduction in EROD activity was observed in E2-treated fish, which was dose dependent. The decrease in EROD activity was observed in both concentrations in fingerlings. However, in the juvenile group only the higher concentration reduced EROD activity after 15 and 30 days exposure. The difference observed may be due to the differences in the group sizes. Conversely, Teles et al. (2006) report that no significant change in EROD activity is observed after 10 days of exposure to E2.

The ROS induction could enhance oxidation of polyunsaturated fatty acids leading to LPO. The interaction of ROS with cell membrane produced more LPO, which is a manifestation of oxidative stress. The observed results clearly indicated that the induction of LPO in sea bass liver under E2 stress might be due to the difference in the amounts of ROS generated and scavenged by antioxidants (CAT or SOD). The results clearly revealed that fish exposed to E2 for 5 and 15 days showed an enhanced LPO level and the increasing LPO level corresponded to the ROS formed and the DNA damage produced, and the correlation between these measured parameters was highly significant in the juvenile group (Table 3b). LPO content in fingerlings did not show significant correlation with ROS induction and DNA damage; however it showed a good relationship with antioxidant defense. A rise in LPO content in E2 exposed fish might be due to the microsomal metabolism of estrogen and microsome mediated redox cycling, which gives rise to oxyradicals capable of oxidizing membrane lipids. Maria et al. (2008) report that intraperitoneal injection of E2 increases the LPO content in juvenile sea bass (*Dicentrarchus labrax*). Conversely, the same authors report that fish exposed to water diluted E2 does not show any significant increase in LPO content. All these observations emphasize the possibility of quantitatively, as well as qualitatively, different routes of metabolism for either a natural steroid or a xenobiotic with estrogenic capacity, and further study is needed to understand the effects of estrogenic compounds on this mechanism.

Table 4 Rotated component matrix developed by principal component analysis for fingerling and juvenile group (only value >0.40 are listed here)

Parameters	Fingerlings			Juveniles	
	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2
DNA	0.936	–	–	−0.975	–
ROS	−0.931	–	–	0.889	–
E2	−0.819	–	0.401	0.929	–
GSH	0.820	−0.470	–	−0.775	0.481
EROD	0.858	–	–	−0.707	0.601
GST	−0.551	0.813	–	0.745	−0.552
LPO	–	0.776	–	0.776	−0.496
GPX	0.641	–	0.720	−0.563	0.685
SOD	–	–	0.941	–	0.974
CAT	–	−0.901	–	–	0.884
Eigen values	5.53	1.97	1.42	6.94	1.64
Cumulative percentage	55.32	75.11	89.39	69.4	85.83

SOD is the first enzyme to deal with oxyradicals by accelerating the dismutation of superoxide generated, and CAT is a peroxisomal haemoprotein which catalyses the removal of H_2O_2 formed during the reaction catalyzed by SOD. ROS thus generated may reduce the levels of SOD and this leads to reduction of CAT activity as a chain reaction, or reduced SOD and CAT activity might induce excess ROS production. In this sense, the antioxidant was modulated in E2-treated fish. However, we did not find any relationship between the modulation of SOD and CAT activity in liver along with an increase of ROS generation, DNA damage or LPO.

Teles et al. (2005) report that GST activity increases in fish exposed to E2. Similar results were observed in the present study and the increase in GST activity was significant, except after 30 days of exposure. The increase in GST activity observed in the current study was suggestive of an increased hepatic steroid catabolism in fish. It is well known that GSH plays a major role in cellular metabolism and free-radical scavenging. In general, GSH serves as a cofactor for GST, which facilitates the removal of certain xenobiotics and other reactive molecules from the cells, and it also interacts directly with ROS for their detoxification, as well as performing other critical activities in the cell. The depletion of GSH can result in cell degeneration due to oxidative stress caused by xenobiotics (Zhang et al. 2008). In the present study, the GSH contents in the liver were significantly reduced in fingerling exposed to both E2 concentrations up to 15 days, and also decreased in the juvenile group exposed to the higher concentration. These results imply that the depletion of GSH level in hepatic tissue may lead to a change of balance between the oxidative and antioxidant systems.

The present study revealed the important role of glutathione in responding to E2 induced stress rather than SOD and CAT. The correlation analysis showed glutathione associated enzymes had a significant relation with plasma E2 and similarly with the ROS generated and LPO in the liver. In the present study GPx activity was reduced when the fish were exposed to E2 and this reduction in GPx activity might have been attributed to the decline in glutathione concentration. Reduced glutathione may be

consumed by direct interaction with E2 molecules or due to increased consumption of glutathione as a ROS scavenger. The data revealed that the activity of these antioxidants, especially glutathione mediated antioxidant enzymes was influenced by E2 exposure, indicating the possibility of an increase of ROS in the tissue. Thus, the decrease in GSH concentrations, the reduction in GPx activity, and the increase in GST activity reflected antioxidant response against the deleterious effects caused by E2.

The resulting PCA model showed a clear trend with increasing E2 concentration and duration of exposure. For instance, E2 and ROS; LPO and GST; and GSH, GPx and EROD were grouped together in juveniles and, similarly, in fingerlings LPO and GST; E2 and SOD; and DNA and EROD were grouped together (figure not shown). In the loading plot we also observed that several variables were correlated. For example, with increasing E2 concentration, the variables plasma E2, ROS, LPO and GST increased. Based on this result we speculated that increasing E2 concentration in the exposure medium and the duration of exposure to some extent induced the plasma E2 concentration, which may have induced the ROS production leading to an increased LPO and induced GST activity. Moreover, from the results, it is very clear that glutathione mediated enzymes were more associated with a response to the increase in oxidative stress caused by E2.

Although many reports are available on estrogen effects on marine animals, the mechanisms involving toxicity of E2 exposure on antioxidant response in marine cultured fish has, until now, not been established. The results of the present study showed that Japanese sea bass were sensitive to E2 exposure. Sea bass showed some level of adaptation during long-term exposure (30 days) to E2 induced stress, possibly by the interaction (or balanced effects) between oxidative stress and their antioxidant system during long-term exposure. As we observed in our earlier study, the toxic effect of E2 on one system clearly led to subsequent effects on other systems, which resulted in disruption of general physiological functions. The effect of E2 on antioxidant reaction in both groups revealed that the effect was dose dependent. The higher concentration of E2 caused significant effects; however the low concentration chosen in the present study did not show any significant effects during long-term exposure, indicating the possibility of adaptation to low concentration by the fish. Although the rise in E2 concentration in the plasma of both groups led to the production of ROS, in fingerlings the ROS generated did not show any relationship with antioxidant response and LPO. This might have been due to the difference in scavenging activity in both size groups; however, the exact reason is unknown and needs to be further elucidated.

In conclusion, the present study demonstrated that a rise in plasma E2 concentration can cause stress responses in both fingerling and juvenile fish in terms of producing ROS, DNA damage, LPO, decrease in EROD and modulation of antioxidant enzymes. The responses observed varied with the duration of the stress and between groups, and the correlation analysis revealed that there was a direct link between plasma E2 and ROS The comparative study on both groups clearly showed the EDC-induced modulation of antioxidant and biotransformation enzymes that were observed in both fingerlings and juveniles of sea bass. These effects depended on toxicant concentrations and the duration of exposure. Decreased GSH levels and GPx activity and an induction in GST activity in the liver might suggest a critical role of glutathione mediated enzyme function against the deleterious effects of E2 in cell protection. The other antioxidant enzymes studied such as SOD and CAT were either unaffected or partially involved in responding to oxidative stress induced by E2. This comparative study on different stages after prolonged exposure should extend our knowledge and help us to understand more about oxidative stress induced by E2 in different life stages of marine cultured fish and the potential role of antioxidant systems in the prevention of damage.

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References

Ahmad I, Maria VL, Pacheco M, Santos MA (2009) Juvenile sea bass (*Dicentrarchus labrax* L.) enzymatic and non-enzymatic antioxidant responses following 17beta-estradiol exposure. Ecotoxicology 18:974–982

Arukwe A, Forlin L, Goksoyr A (1997) Xenobiotic and steroid biotransformation enzymes in Atlantic salmon (*Salmo salar)* liver treated with an estrogenic compound, 4-nonylphenol. Environ Toxicol Chem 16:2576–2583

Arukwe A, Kullman SW, Hinton DE (2001) Differential biomarker gene and protein expressions in nonylphenol and estradiol-17 beta treated juvenile rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol Part C 129:1–10

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Burke MD, Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab Dispos 2:583–588

Carrera EP, Garcia-Lopez A, del Rio MDM, Martinez-Rodriguez G, Sole M, Mancera JM (2007) Effects of 17 beta-estradiol and 4-nonylphenol on osmoregulation and hepatic enzymes in gilthead sea bream (*Sparus auratus*). Comp Biochem Physiol Part C 145:210–217

Cavalieri E, Frenkel K, Liehr JG, Rogan E, Roy D (2000) Estrogens as endogenous genotoxic agents—DNA adducts and mutations. J Natl Cancer Inst Monogr 27:75–93

Cavalieri EL, Devanesan P, Bosland MC, Badawi AF, Rogan EG (2002) Catechol estrogen metabolites and conjugates in different regions of the prostate of noble rats treated with 4-hydroxyestradiol: implications for estrogen-induced initiation of prostate cancer. Carcinogenesis 23:329–333

Delaporte E, Renton KW (1997) Cytochrome P4501A1 and cytochrome P4501A2 are down regulated at both transcriptional and post-transcriptional levels by conditions resulting in interferon-alpha/beta induction. Life Sci 60:787–796

Devasagayam TP, Tarachand U (1987) Decreased lipid peroxidation in the rat kidney during gestation. Biochem Biophys Res Commun 145:134–138

Driver AS, Kodavanti PR, Mundy WR (2000) Age-related changes in reactive oxygen species production in rat brain homogenates. Neurotoxicol Teratol 22:175–181

Elskus AA (2004) Estradiol and estriol suppress CYP1A expression in rainbow trout primary hepatocytes. Mar Environ Res 58:463–467

Felty Q, Xiong WC, Sun D, Sarkar S, Singh KP, Parkash J, Roy D (2005) Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers. Biochemistry 44:6900–6909

Giulio RTD, Washburn PC, Wenning RJ, Winston GW, Jewell CS (1989) Biochemical responses in aquatic animals: a review of determinants of oxidative stress. Environ Toxicol Chem 8:1103–1123

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

Hawkins SA, Billiard SM, Tabash SP, Brown RS, Hodson PV (2002) Altering cytochrome P4501A activity affects polycyclic aromatic hydrocarbon metabolism and toxicity in rainbow trout (*Oncorhynchus mykiss*). Environ Toxicol Chem 21:1845–1853

Janssen YM, Van Houten B, Borm PJ, Mossman BT (1993) Cell and tissue responses to oxidative damage. Lab Invest 69:261–274

Livingstone DR (2001) Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull 42:656–666

Maria VL, Ahmad I, Santos MA (2008) Juvenile sea bass (*Dicentrarchus labrax* L.) DNA strand breaks and lipid peroxidation response following 17beta-estradiol two mode of exposures. Environ Int 34:23–29

Marklund S, Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–474

Mates JM, Perez-Gomez C, Nunez de Castro I (1999) Antioxidant enzymes and human diseases. Clin Biochem 32:595–603

Mather-Mihaich E, Di Giulio RT (1986) Antioxidant enzyme activities and malondialdehyde, glutathione and methemoglobin concentrations in channel catfish exposed to DEF and n-butyl mercaptan. Comp Biochem Physiol C 85:427–432

Moron MS, Depierre JW, Mannervik B (1979) Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochem Biophys Acta 582:67–78

Patel MM, Bhat HK (2004) Differential oxidant potential of carcinogenic and weakly carcinogenic estrogens: Involvement of metabolic activation and cytochrome P450. J Biochem Mol Toxicol 18:37–42

Rempel MA, Wang Y, Armstrong J, Schlenk D (2008) Uptake of estradiol from sediment by hornyhead turbot (*Pleuronichthys verticalis*) and effects on oxidative DNA damage in male gonads. Mar Environ Res 66:111–112

Rempel-Hester MA, Hong H, Wang Y, Deng X, Armstrong J, Gully J, Schlenk D (2009) Site-specific effects of 17beta-estradiol in hornyhead turbot (*Pleuronichthys verticalis*) collected from a wastewater outfall and reference location. Environ Res 109:552–558

Risso-de Faverney C, Lafaurie M, Girard JP, Rahmani R (2000) The nitroxide stable radical tempo prevents metal-induced inhibition of CYP1A1 expression and induction. Toxicol Lett 111:219–227

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179:588–590

Seacat AM, Kuppusamy P, Zweier JL, Yager JD (1997) ESR identification of free radicals formed from the oxidation of catechol estrogens by Cu_2^+. Arch Biochem Biophys 347:45–52

Shugart LR (1988) Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. Aquat Toxicol 13:43–52

Sinha AK (1972) Colorimetric assay of catalase. Anal Biochem 47:389–394

Sole M, Porte C, Barcelo D (2000) Vitellogenin induction and other biochemical responses in carp, *Cyprinus carpio*, after experimental injection with 17 alpha-ethynylestradiol. Arch Environ Contam Toxicol 38:494–500

Sumpter JP, Johnson AC (2005) Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. Environ Sci Technol 39:4321–4332

Teles M, Gravato C, Pacheco M, Santos MA (2004) Juvenile sea bass biotransformation, genotoxic and endocrine responses to beta-naphthoflavone, 4-nonylphenol and 17 beta-estradiol individual and combined exposures. Chemosphere 57:147–158

Teles M, Pacheco M, Santos MA (2005) *Sparus aurata* L. liver EROD and GST activities, plasma cortisol, lactate, glucose and erythrocytic nuclear anomalies following short-term exposure either to 17beta-estradiol (E2) or E2 combined with 4-nonylphenol. Sci Total Environ 336:57–69

Teles M, Pacheco M, Santos MA (2006) Biotransformation, stress and genotoxic effects of 17beta-estradiol in juvenile sea bass (*Dicentrarchus labrax* L.). Environ Int 32:470–477

Thilagam H, Gopalakrishnan S, Bo J, Wang KJ (2009) Effect of 17beta-estradiol on the immunocompetence of Japanese sea bass (*Lateolabrax japonicus*). Environ Toxicol Chem 28:1722–1731

Thomas-Jones E, Thorpe K, Harrison N, Thomas G, Morris C, Hutchinson T, Woodhead S, Tyler C (2003) Dynamics of estrogen biomarker responses in rainbow trout exposed to 17beta-estradiol and 17alpha-ethinylestradiol. Environ Toxicol Chem 22:3001–3008

Vaccaro E, Meucci V, Intorre L, Soldani G, Di Bello D, Longo V, Gervasi PG, Pretti C (2005) Effects of 17beta-estradiol, 4-nonylphenol and PCB 126 on the estrogenic activity and phase 1 and 2 biotransformation enzymes in male sea bass (*Dicentrarchus labrax*). Aquat Toxicol 75:293–305

van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149

Wang MY, Liehr JG (1995) Lipid hydroperoxide-induced endogenous DNA adducts in hamsters: possible mechanism of lipid hydroperoxide-mediated carcinogenesis. Arch Biochem Biophys 316:38–46

Watson DE, Menard L, Stegeman JJ, Di Giulio RT (1995) Aminoanthracene is a mechanism-based inactivator of CYP1A in channel catfish hepatic tissue. Toxicol Appl Pharmacol 135:208–215

Wellejus A, Bornholdt J, Vogel UB, Risom L, Wiger R, Loft S (2004) Cell-specific oxidative DNA damage induced by estrogen in rat testicular cells in vitro. Toxicol Lett 150:317–323

Zhang X, Yang F, Xu Y, Liao T, Song S, Wang J (2008) Induction of hepatic enzymes and oxidative stress in Chinese rare minnow (*Gobiocypris rarus*) exposed to waterborne hexabromocyclododecane (HBCDD). Aquat Toxicol 86:4–11