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Responses of the zebrafish hypothalamic–pituitary–gonadal–liver axis PCR array to prochloraz are dependent on timing of sampling

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ABSTRACT

A PCR array, based on expression of genes along the hypothalamic–pituitary–gonadal–liver (HPGL) axis of fish, has been suggested as a useful method for screening of endocrine-disrupting chemicals (EDCs). However, effects of circadian rhythm on responses of the HPGL axis to exposure to chemicals were unknown. In this study, profiles of expression of genes along the HPGL axis and concentrations of 17β-estradiol (E2) in blood plasma of female zebrafish were compared at two sampling times of day (8:00 AM and 7:00 PM). Prochloraz (PCZ) was selected as a model chemical to evaluate differences in responses of the HPGL axis at these two times of day. Profiles of responses of concentrations of E2 in plasma and expressions of genes along the HPGL axis genes were different between the two times of sampling. Concentrations of E2 were less, and abundances of mRNA for several genes along the HPGL axis were significantly greater or lesser when samples were collected at 7:00 PM than they were when samples were collected at 8:00 AM. Exposure to three concentrations of PCZ (3, 30 or 300 μg/L) for 48 h resulted in significantly lesser concentrations of plasma E2 and caused compensatory up-regulation of genes included in hypothalamus, pituitary and ovary. Expressions of genes along the HPGL were more responsive to PCZ at 8:00 AM than they were when samples were collected at 7:00 PM. Correlations among parameters in samples collected at the two times indicated the effects might be due to different concentrations of E2 in plasma due to exposure to PCZ.

1. Introduction

Since endocrine-disrupting chemicals (EDCs) disrupt the endocrine system of wildlife and humans and thus cause possible long-term effects on health, during the past few decades, they have received worldwide attention (Hotchkiss et al., 2008; Kavlock et al., 1996; Lamb et al., 2014). Considering the complicated nature of endocrine systems of humans and animals, real-time PCR arrays especially for the hypothalamic–pituitary–gonadal–liver (HPGL) axis of fishes are effective when used to evaluate effects of EDCs on expression of genes or prioritize EDCs (Ankley et al., 2012; He et al., 2012; Liu et al., 2013; Villeneuve et al., 2009; Zhang et al., 2008a). Profiles of expressions of genes of the HPGL axis show considerable promise for evaluation of toxic potentials of EDCs (Ankley et al., 2006; Zhang et al., 2005). These genes are included in the hypothalamus, pituitary, gonad or liver, representing synthesis of enzymes that produce gonadotropins released in the hypothalamus and pituitary, and cholesterol transport and steroidogenesis in gonads, and production of yolk protein precursor vitellogenin (VTG) in liver of fish (Ankley and Johnson, 2004; Dufour et al., 2010). Systematic monitoring of genes along the HPGL axis of fish exposed to EDCs of concern provides further insights into adverse outcomes (Ankley et al., 2009a) and supports some aspects of regulatory decision-making. Compared to previous traditional approaches (e.g., measurement of enzyme activity), PCR of genes along the HPGL axis is more sensitive and flexible and allows for simultaneous screening of multiple genes in multiple tissues at any stage of development or reproduction (Zhang et al., 2008a).

Regulation of reproduction in fishes is primarily via coordinated interactions of expression of genes along the HPGL axis (Villeneuve et al., 2008). The dynamic nature of HPGL axis is closely related to regulation of the circadian rhythm (Zhao et al., 2015). For example,

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the endogenous circadian rhythm is able to produce timed signals to increase biosynthesis of gonadotropin-releasing hormone (GnRH) and stimulate pituitary gonadotrope cells to rapidly secrete luteinizing hormone (LH). Furthermore, the surge of luteinizing hormone (LH) at the end of the light phase clearly shows a circadian pattern of release in the presence of E2 in female mice (Christian et al., 2005; Legan and Karsch, 1975). In human breast carcinoma cells, expression of the estrogen receptor α (ER-α) is associated with the clock gene Per2 (Gery et al., 2007).

Although the fish HPGL axis PCR array has been suggested as an effective method for screening of EDCs, to the best of our knowledge, no studies have been performed to evaluate effects of circadian rhythm on responses of genes along the HPGL axis of fish exposed to EDCs. It was hypothesized that responses of the HPGL axis of zebrafish PCR array to an EDC by use of a PCR array, would be dependent on timing of sampling. To test the hypothesis, profiles of expression of genes along the HPGL axis and concentrations of E2 in blood plasma of zebrafish exposed to prochloraz (PCZ) collected at two times of sampling (8:00 AM and 7:00 PM) were compared. Prochloraz is an imidazole fungicide, and registered for various agricultural and horticultural uses throughout the world (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=73665). PCZ was selected because it is a potent inhibitor of cytochrome P450 c17α-hydroxylase/17,20-lyase (CYP17) and aromatase (CYP19) and is a model compound for studying changes in HPGL axis gene mRNA abundance using a PCR array (Ankley et al., 2009b, 2005). In fish, the gene product of cyp17 is responsible for synthesis of testosterone (T) and cyp19 catalyzes conversion of T to E2. Exposure to PCZ decreased concentration of plasma E2 and caused compensatory up-regulation of genes of the hypothalamic–pituitary–gonadal (HPG) of small fish (Ankley et al., 2009b; Liu et al., 2011). Zebrafish was selected because their reproduction is influenced by the HPGL axis which is closely regulated by circadian rhythm.

2. Materials and methods

2.1. Chemicals and reagents

Prochloraz (PCZ) and MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) were obtained from Sigma (St. Louis, MO, USA). TRIzol regent was obtained from Invitrogen (New Jersey, NJ, USA). Reverse transcription and SYBR Green kits were purchased from Takara (Dalian, Liaoning, China). 17β-estrogen (E2) enzyme immunoassay (EIA) kits were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). All the other chemicals used in this study were of analytical grade.

2.2. Fish and chemical exposure

Stock solutions of PCZ were prepared in dimethyl sulfoxide (DMSO). Adult zebrafish (*Danio rerio*, AB strain) maintenance was performed according to previously described methods (Dang et al., 2015). Briefly, 6-month old zebrafish were cultured in flow-through tanks at 28 ± 0.5 °C with a 12:12 light/dark cycle. Fish were fed twice daily with commercial brine shrimp (*Artemia nauplii*). Before use in experiments, males and females were acclimated for 2 weeks in 15-L tanks filled with 10 L of carbon-filtered water. Experiment included two parts: in the first part, after the acclimation period, sixteen females from two replicated tanks were sampled at 8:00 AM or 7:00 PM to compare physiological expression profiles of HPGL axis and concentrations of E2 in plasma at the two times of sampling. Tissues from brain and pituitary, ovary, and liver were preserved in TRIzol reagent for subsequent quantification of expressions of genes and blood was collected for quantification of E2 in plasma. In the second part, females and males were exposed to 0, 3, 30 or 300 μg PCZ/L (0, 8, 80 or 800 nM PCZ) for 48 h. Exposures included two subsets: one started at 8:00 AM and samples were collected at 8:00 AM; the other started at 7:00 PM and was sampled at 7:00 PM. Each treatment included two replicated tanks and each tank included 8 female and 8 male zebrafish. Half of the water in each tank was replaced by carbon-filtered water including appropriate concentrations of PCZ daily. Exposure concentrations were selected based on information from previous studies (Dang et al., 2015; Liu et al., 2011). Both experimental and control groups received 0.01% DMSO, which has been shown to not affect reproductive system of fishes (Han et al., 2013). After 48 h of exposure, only female fish were anesthetized in 0.03% MS-222 and sampled since previous studies demonstrated that based on PCR array, responses of the HPGL axis to PCZ were similar, but female fish were more sensitive (Dang et al., 2015; Ankley et al., 2009b). In zebrafish, females produce eggs every morning, and males must be present for ovulation and spawning to occur. Therefore, responses of the zebrafish HPGL axis PCR array to PCZ in this study might be different between the presence and absence of males. In previous studies, to evaluate responses of fish HPGL axis PCR array to chemicals, female and male fish were exposed together (Ankley et al., 2009b; Zhang et al., 2008b). Therefore, to compare our results to previous studies male zebrafish was also used in this study although they were not sampled. The two times of sampling, 8:00 AM and 7:00 PM were selected because aquarium lights were routinely turned on at 7:00 AM and turned off at 7:00 PM. Therefore, the circadian rhythm of fish at these two times of sampling represented the greatest difference. Duration of collections of samples was limited to 2 h. In this study, experimental procedures were carried out following the approved protocol by Institutional Animal Care and Use Committee (IACUC) of Huazhong Agricultural University.

2.3. Measurement of plasma E2 concentrations

Blood was collected from the caudal vein of each fish. Plasma was obtained through centrifugation (5000*g* for 5 min at 4 °C). Plasma from four individual females was pooled to form a composite sample. There were four replicates for each treatment. Plasma was stored at −80 °C until analysis. Concentrations of E2 in plasma were quantified by use of Cayman EIA kits as described previously (Liu et al., 2009). Briefly, plasma from each pooled sample was diluted with 500 μL ultrapure water and then extracted thrice with 2 mL of ethyl ether. The ether phase was collected and evaporated. After that, residues were dissolved in 50 μL EIA buffer provided in the kit and E2 was quantified following manufacturer's instructions. The intra- and inter-assay coefficients of variance (CV) were <10%.

2.4. RNA isolation and quantitative real-time PCR assay

Samples employed for RNA isolation were from individual zebrafish. Isolation of total RNA was performed using TRIzol regent following the manufacturer's instructions. Qualities of RNA were determined by use of a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.) and 1% agarose-formaldehyde gel electrophoresis with gelred staining. Purities of samples of RNA were between 1.8 and 2.0 (260 nm/280 nm ratio) and RNA integrities (RIN) were ≥8.0. Five hundred ng of total RNA was reverse transcribed to first-strand cDNA using PrimeScript™ RT reagent kit (Takara, Dalian, Liaoning, China).

Quantitative, real-time PCR was performed using Minimum Information for Publication of Quantitative Real-Time PCR Experiment (MIQE) Guidelines (Bustin et al., 2009). Applied Biosystems StepOne Plus Real-time PCR System (Foster City, California, USA) was used to perform quantitative real-time PCR. Primers were

designed according to previously described methods (described in Supporting information) and the housekeeping gene 18S small subunit rRNA (18S rRNA) was used as an internal control (Dang et al., 2015). Thermal cycling was set at 95 °C for 2 min, followed by 40–45 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve was used to check out the purity and specificity of PCR productions in each assay. Expressions of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method. Each treatment included 6–8 fish. For each gene, two replicates were performed in each plate.

2.5. Statistical analyses

Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Normality of data set was examined using the Kolmogorov-Smirnow test. If necessary, data were log-transformed to approximate normality. Homogeneity of variances was checked by Levene's test. A one-way analysis of variance (ANOVA) followed by Tukey's multiple range test was used to evaluate differences between the control and exposure groups. The independent variable was time of sampling in the comparison of parameters tested between 8:00 AM and 7:00 PM, and the independent variable was concentration of PCZ in comparisons of parameters tested between control and exposure groups. Nonparametric Spearman correlation analysis was used to examine the relationship between decreasing percentages of plasma E2 concentrations and gene number with statistically significant change in expression in HPGL axis. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Survival

No mortality of female or male zebrafish exposed to 0, 3, 30 or 300 μg PCZ/L for 48 h was observed.

3.2. Profiles of expression of genes of HPGL axis genes and concentrations of E2 in plasma at the two times of sampling

Mean concentrations of E2 in blood plasma of females were 750.41 ± 51.00 and 593.40 ± 19.45 pg/mL at 8:00 AM and 7:00 PM, respectively (Fig. 1). Concentrations of E2 in plasma were significantly difference in plasma E2 concentration between the two times of sampling.

Compared with 8:00 AM, a significant up-regulation in expression of gonadotropin-releasing hormone 3 (*gnrh3*) and *gnrh3r* (gonadotropin-releasing hormone 3 receptor) was observed at 7:00 PM, while expressions of other genes in brain, including follicle stimulating hormone beta (*fsh*-β), luteinizing hormone beta (*lh*-β), *gnrh2*, *gnrhr1*, *gnrhr2* and *gnrhr4* were not changed (Fig. 2A). In ovary, expression of steroidogenic acute regulatory protein (*star*) was significantly up-regulated at 7:00 PM compared with that at 8:00 AM (Fig. 2B). Expression of vitellogenin 1 (*vtg1*), vitellogenin-2 (*vtg2*) and estrogen receptor 1 (*er-1*) in liver was down-regulated at 7:00 PM compared with that at 8:00 AM (Fig. 2C).

3.3. Exposure to PCZ caused different responses in plasma E2 concentration at the two times of sampling

At 8:00 AM, exposure to 30 or 300 μg PCZ/L, resulted in 52.9% and 60.2% lesser concentrations of E2 in plasma, respectively, while the lesser concentration of exposure (3 μg/L) did not change concentrations of E2 in plasma (Fig. 3). At 7:00 PM, only exposure to 300 μg PCZ/L resulted in significantly (40.9%) lesser concentrations of E2 in plasma, while exposure to each of other concentrations (3 or 30 μg/L) did not change concentrations of E2 in plasma (Fig. 3).

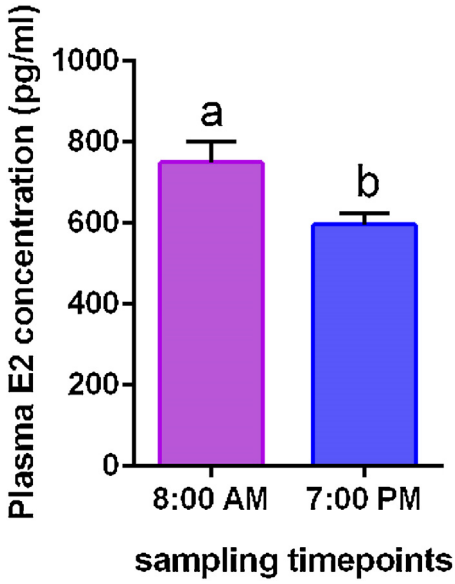

Fig. 1. Concentrations of E2 in plasma of female zebrafish at two times of sampling (8:00 AM and 7:00 PM). Values represent mean ± SEM. Each concentration contains 4 replicates, and each replicate was a composite of 4 fish. Letters indicate significant differences between the two times ($P = 0.045$).

3.4. Exposure to PCZ caused different responses in the expression of HPGL axis genes at the two times of sampling

Expressions of twenty genes involved in the HPGL pathway were investigated (Fig. 4). At 8:00 AM, treatment with 3 μg PCZ/L for 48 h significantly up-regulated the expressions of *gnrh3*, *gnrhr1* and *gnrhr4* in brain, while expressions of other genes of the HPGL axis were not changed. Exposure to 30 μg PCZ/L caused up-regulation of expression of some genes in brain (*gnrhr1*, *gnrhr2*, *gnrhr3*, *gnrhr4*) and in ovary (*star and er1*), while expressions of *vtg1*, *vtg2* and *er1* in liver were significantly down-regulated compared with the control. Expressions of some genes in brain and ovary were significantly changed after exposure to 300 μg PCZ/L. Genes significantly up-regulated in brain were *gnrh3*, *gnrhr3*, *gnrhr4* and in ovary were aromatase (*cyp19a*), *star* and *er-1*. In liver, significant down-regulation in expression of *er-1*, *vtg1* and *vtg2* was observed, while expression of estrogen receptor 2α (*er2α*) was significantly up-regulated compared with expression in the control (Fig. 4).

At 7:00 PM, only exposure to 300 μg PCZ/L caused significant changes in expressions of HPGL axis genes. Exposure to 300 μg PCZ/L for 48 h significantly changed expression some genes in brain, ovary and liver. In brain, expressions of *gnrhr2*, *gnrhr4* and *fsh*-β were up-regulated, compared with their expressions in control. In ovary, expression of follicle stimulating hormone receptor (*fshr*), *cyp19a* and *er-1* were significantly up-regulated. In liver, treatment with 300 μg PCZ/L significantly down-regulated expression of *vtg-1* and *er-1* and expression of *er-2α* was up-regulated compared with the control (Fig. 4). Detailed results of HPGL axis PCR array in brain, ovary and liver are presented in the Supporting information (Figs. S1, S2 and S3).

3.5. Correlations between decreasing percentages of concentrations of E2 in plasma and number of HPGL axis genes exhibiting significant responses in expression

Correlations between decreasing percentages of changes in concentrations of E2 in plasma and the number of HPGL axis genes with statistically significant changes in expression upon PCZ exposure were examined (Table 1). The decreasing percentages of

Table 1
Spearman rank correlation coefficients (number) and probabilities (*) between decreasing percentages of plasma E2 concentrations and the number of HPGL axis genes with significant changes in expression upon PCZ exposure in female zebrafish (n = 8).

Spearman rank correlation	The number of HPGL axis genes with significant changes in expression			
	Brain	Ovary	Liver	Total
Decreasing percentages of plasma E2 concentration	0.802 $P = 0.055$	0.833* $P = 0.039$	0.880* $P = 0.013$	0.928* $P = 0.008$

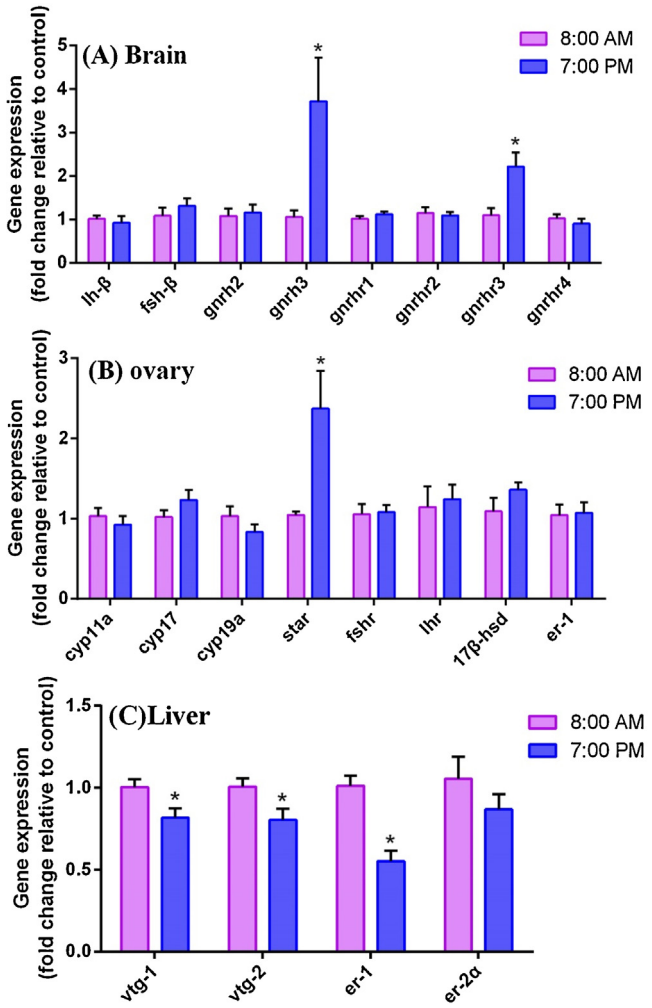

Fig. 2. Profiles of relative expression of genes of the HPGL axis in female zebrafish at 7:00 PM compared with those collected at 8:00 AM. Values represent mean ± SEM. Significant differences from the control are indicated by $^*P < 0.05$. Each group contains 6–8 biological replicates. Relative expressions of genes were calculated by the $2^{-\Delta\Delta Ct}$ method.

concentrations of E2 in plasma were significantly correlated with the number of significant responsive genes in ovary, liver and the whole HPGL axis of zebrafish exposed to various concentrations of PCZ. No significant correlation was observed between the decreasing percentages of changes in concentrations of E2 in plasma and the number of significant responsive genes in brain, although a relatively great correlation coefficient was obtained.

4. Discussion

The PCR array for genes on the HPGL axis of fish has been suggested for screening of EDCs, but to the best of our knowledge, no information is available for evaluating effects of the circadian rhythm on responses of HPGL axis to exposure to EDCs. The purpose of the present study was to determine the more appropriate

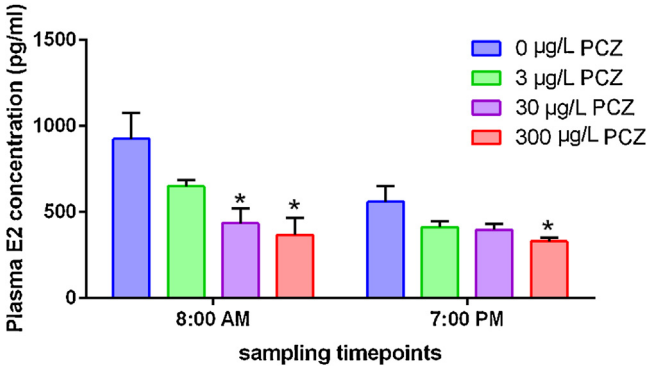

Fig. 3. Effects of various concentrations of PCZ on concentrations of E2 in blood plasma of female zebrafish at two times of sampling (8:00 AM and 7:00 PM). Values represent mean ± SEM. Significant differences from the control (0 μg PCZ/L) are indicated by $^*P < 0.05$. Each concentration contains 4 replicates, and each replicate contains 4 individual fish.

time of sampling and understand possible mechanisms involved in compensatory responses of the HPGL axis, which were triggered by decreases in concentrations of E2 in plasma after exposure to PCZ. In this study, for the first time, we report that time of sampling resulted in different responses of HPGL axis in female zebrafish exposed to various concentrations of PCZ for 48 h. Responses observed in samples collected at 8:00 AM were more sensitive than those collected at 7:00 PM, and thus 8:00 AM might be a more appropriate time of sampling than 7:00 PM. Furthermore, the decreasing percentages of changes in concentrations of E2 in plasma were significantly correlated with the number of significant responsive genes in ovary, liver and the whole HPGL axis.

Differences in profiles of expression of genes of the HPGL axis and concentrations of E2 in plasma between the two times of sampling were observed. Due to physiological regulation of the circadian internal clock, the HPGL axis of zebrafish undergoes dynamic alterations within a 24-h period (Tonsfeldt and Chappell, 2012; Vatine et al., 2011). In this study, significant differences in concentrations of E2 in plasma of female zebrafish at 8:00 AM and 7:00 PM were observed. In fish, vtg is the precursor for egg yolk which is normally synthesized in liver in response to endogenous estrogen via estrogen receptors (e.g., er-1), and expression of *vtg* and *er-1* in liver is positively correlated with concentrations of E2 in plasma (Hutchinson et al., 2006). This is consistent with our results. Compared with 8:00 AM, a significant down-regulation of *vtg* and *er-1* in female liver was observed. Also, a significant up-regulation of *gnrh 3* and *gnrhr 3* in brain and *star* in ovary was observed in liver collected at 7:00 PM compared with those collected at 8:00 AM In fish, gnrh can induce synthesis of fsh and lh through interaction with gnrhr, and then fsh and lh are secreted by the pituitary and bind to their receptors in the ovary to induce steroidogenesis (Zhang et al., 2008b). Therefore, up-regulation of these genes (*gnrh 3*, *gnrhr 3* and *star*) was considered to be a compensatory response triggered by the circadian clock.

Exposure to PCZ caused different responses in concentrations of E2 in plasma observed at the two times of sampling. Previous studies have suggested that cyp19a activity was a more sensitive target for PCZ than other steroidogenic cytochrome P450 (cyp) enzymes

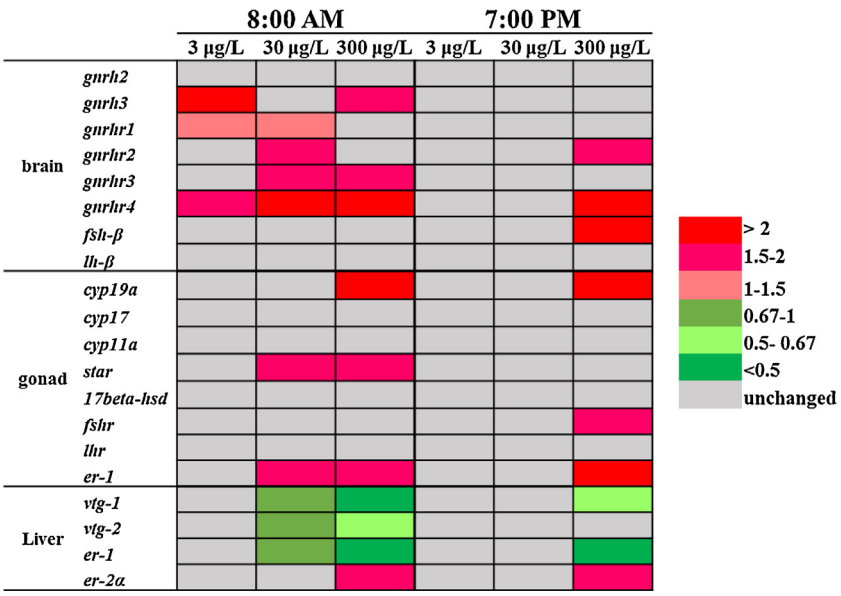

Fig. 4. Profiles of r'elative **e**xpressions of genes of the HPGL axis of female zebrafish exposed to various concentration of PCZ at 8:00 AM or 7:00 PM. Each concentration contains 6–8 replicates. Two controls were included, and relative expressions of genes at the two times were calculated by use of the $2^{-\Delta\Delta Ct}$ method using the corresponding control.

(e.g., cyp17), and exposure to PCZ more strongly decreased concentrations of E2 in plasma, compared with other steroid hormones, such as testosterone (Ankley et al., 2009b). Therefore, only E2 was quantified in plasma due to limited volumes of plasma (Ankley et al., 2009b; Dang et al., 2015). In this study, initial effects of PCZ on concentrations of E2 in plasma were surprisingly rapid (Skolness et al., 2011). After 48-h exposure to PCZ, significant decreases in concentrations of E2 in plasma were observed. These results are consistent with previous studies in small fish (e.g., fathead minnow and zebrafish) (Ankley et al., 2009b, 2005; Dang et al., 2015; Liu et al., 2011; Skolness et al., 2011). Furthermore, a difference in responses of concentrations of E2 to PCZ at the two times of sampling was observed. Concentrations of E2 in plasma were significantly less after exposure to 30 or 300 μg PCZ/L at 8:00 AM, while at 7:00 PM significantly lesser concentrations of E2 in plasma were observed in fish exposed to the greatest concentration (300 μg PCZ/L). Different effects on concentrations of E2 in plasma at the two times of sampling might be due in part, to different cyp19a activities that resulted in different concentrations of E2 in plasma at the two times of sampling (926.05 pg E2/mL at 8:00 AM and 560.84 pg E2/mL at 7:00 PM).

Compensatory responses of genes of the HPGL axis were different between the two times of sampling. Lesser concentrations of E2 in plasma, caused by exposure to PCZ might be responsible for responses of HPGL axis PCR array. Consistent with the postulated mode of action, PCZ effectively inhibited biosynthesis of E2. Thus, the significantly lesser concentration of E2 in plasma would cause compensatory responses of the HPGL axis, such as changes of expressions of genes. Furthermore, Ankley et al. (2009b) reported that the HPGL axis maintains dynamic homeostasis through various feedback mechanisms when organisms are exposed to certain EDCs. Up-regulation of genes along this axis lagged behind the decrease in concentrations of E2 in plasma of fishes exposed to EDCs, and thus was considered as a compensatory response or indirect response (Ankley and Villeneuve, 2015). Therefore, our results were consistent with those of previous studies, where exposure to PCZ significantly up-regulated expression of genes including brain and ovary. Recently, it was reported that exposure to PCZ caused a dose-dependent up-regulation of genes along HPGL axis in zebrafish (Dang et al., 2015). Furthermore, in this study, a difference in compensatory responses of genes of the HPGL axis was observed between the two times of sampling. In samples collected at 8:00 AM, compensatory up-regulation of genes were observed in all the treatment groups (3, 30 or 300 μg PCZ/L), while for 7:00 PM compensatory up-regulations of genes were only observed after exposure to the greatest concentration of PCZ. The difference might be due to different inhibitory effects of PCZ on concentrations of E2 in plasma at the two times.

Decreasing percentages of changes in concentrations of E2 in plasma were positively correlated with the number of HPGL axis genes with statistically significant changes in expression upon PCZ exposure. Concentration of E2 in blood plasma is usually used as an essential parameter in endocrine-disrupting study, and decrease of E2 is correlated with activation of compensation responses. Therefore, it was hypothesized that various compensatory responses of the HPGL axis genes might be caused by differences in decreasing percentages of changes in concentrations of E2 in plasma. The relatively large correlation coefficients between decreasing percentages of concentrations of E2 and the number of HPGL axis responsive genes confirmed our hypothesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.03.022.

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Supporting Information

Responses of the Zebrafish Hypothalamic-Pituitary-Gonadal-Liver Axis PCR Array to Prochloraz are Dependent on Timing of Sampling

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Table S1. Sequences of primers for the genes tested

Gene name	Sequences of primers (5'-3')	Accession number
er1	Forward: ggtccagtgtggtgtcctct	NM_152959.1
	Reverse: cacacgaccagactccgtaa	
er2α	Forward: agcttgtgcacatgatcagc	NM_180966.2
	Reverse: gctttcatccctgctgagac	
gnrh2	Forward: actggtctcacggctggtat	NM_181439.4
	Reverse: aaatcacgaatgagggcatc	
gnrh3	Forward: cagcactggtcatacggttg	NM_182887.2
	Reverse: ccttcagcatccacctcatt	
gnrhr1	Forward: ggtggagagccttgtctgag	NM_001144980.1
	Reverse: gggctgaaaccaataccaga	
gnrhr2	Forward: acagcgtgagcaaaacattg	NM_001144979.1
	Reverse: tgagcacaaactcagcatcc	
gnrhr3	Forward: aacagacatgatcccgaagg	NM_001177450.1
	Reverse: aggttcccgaacacaaacag	
gnrhr4	Forward: ttgcaatcattcgctctttg	NM_001098193.1
	Reverse: gattgcattgccctttgatt	
fshβ	Forward: acagcacacccagaaggtct	NM_205624.1
	Reverse: agctccccagtctgttgtgt	
lhβ	Forward: gagacggtatcggtggaaaa	NM_205622.2
	Reverse: aacagtcgggcaggttaatg	
fshr	Forward: cgtctcttttgtgcactgga	NM_001001812.1
	Reverse: gtggcaattccacacttcct	
lhr	Forward: aaaaggacgagtcgctgaaa	AY424302.1
	Reverse: aaaacaagaagcagggcaga	
hmgr	Forward: tcaactggattgagggaagg	NM_001079977.1
	Reverse: agcgttataaccaccgatgc	
star	Forward: aagggttggatcccaaaaac	NM_131663.1
	Reverse: tagctatgggtgggatgagg	
cyp11a	Forward: acagcctgctcagtgtcctt	NM_152953.2
	Reverse: agcaccgtcttcaggcttta	
3βhsd	Forward: agtgtcgcacatcgtctcag	AY279108.1
	Reverse: cagtcggaccagcttttctc	
cyp17	Forward: tggagctctttgcatgtttg	AY281362.1
	Reverse: agctcagcatctccacgttt	
17βhsd	Forward: catcatggacgtcaatctgc	AY306005.1
	Reverse: tctcacaagcgccctctatt	
cyp19a	Forward: ccgttcttatggcaggtgat	AF226620.1
	Reverse: ttgtgtggtcgatggtgtct	
vtg1	Forward: ctgcgtgaagttgtcatgct	NM_001044897.2
	Reverse: gaccagcattgcccataact	
vtg2	Forward: tactttgggcactgatgcaa	AY729644.1
	Reverse: agacttcgtgaagcccaaga	
18S rRNA	Forward: ttgttggtgttgttgctggt	NM_200713
	Reverse: ggatgctcaacaggggttcat	

Figure S1

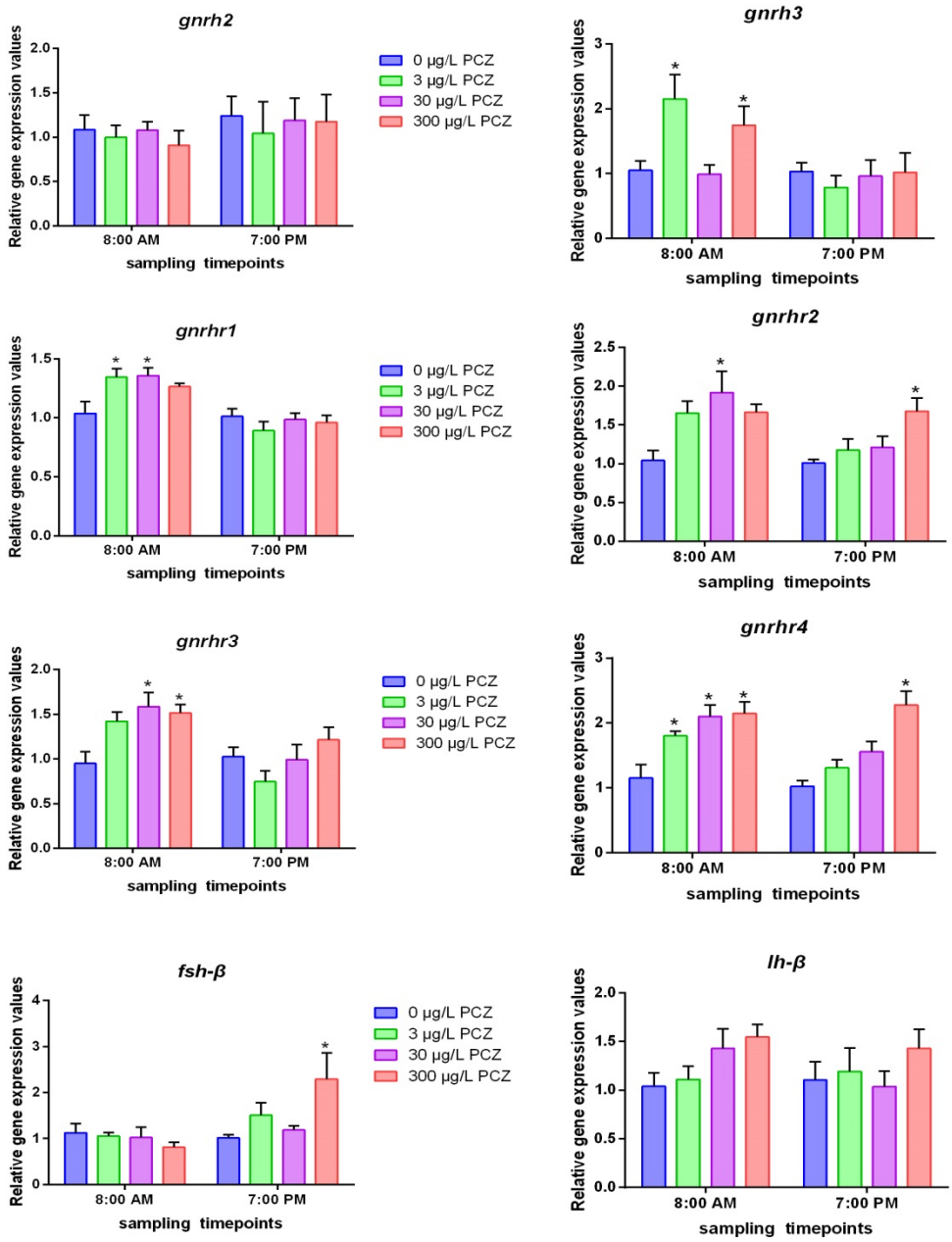

Figure S1: Effects of prochloraz on expressions of genes in brain of female zebrafish at 8:00 AM or 7:00 PM samplings. Values represent mean ± SEM. Significant differences from the control are indicated by *$P < 0.05$. Each concentration contains 6-8 replicates. Two controls were included, and relative expressions of genes at the two times of collection (8:00 AM and 7:00 PM) were calculated by the $2^{-\Delta\Delta Ct}$ method using the corresponding control.

Figure S2

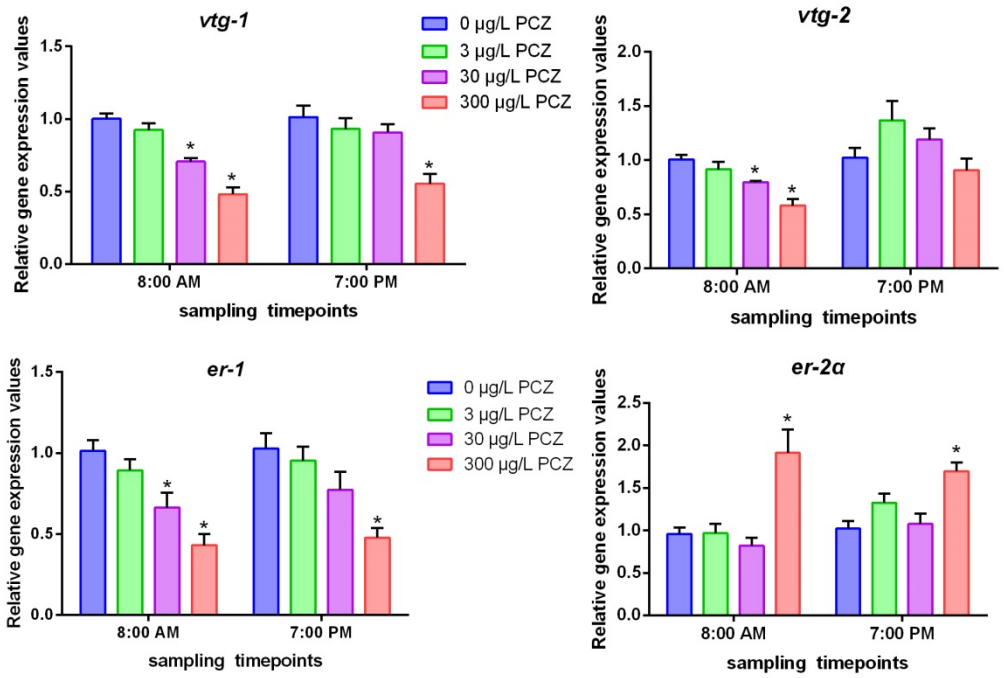

Figure S2: Effects of prochloraz on expressions of genes in liver of female zebrafish collected at 8:00 AM or 7:00 PM. Values represent mean ± SEM. Significant differences from the control are indicated by $*P < 0.05$. Each concentration contains 6-8 biological replicates. Two controls were included, and relative expressions of genes at the two times of collections (8:00 AM and 7:00 PM) were calculated by the $2^{-\Delta\Delta Ct}$ method using the corresponding control.

Figure S3

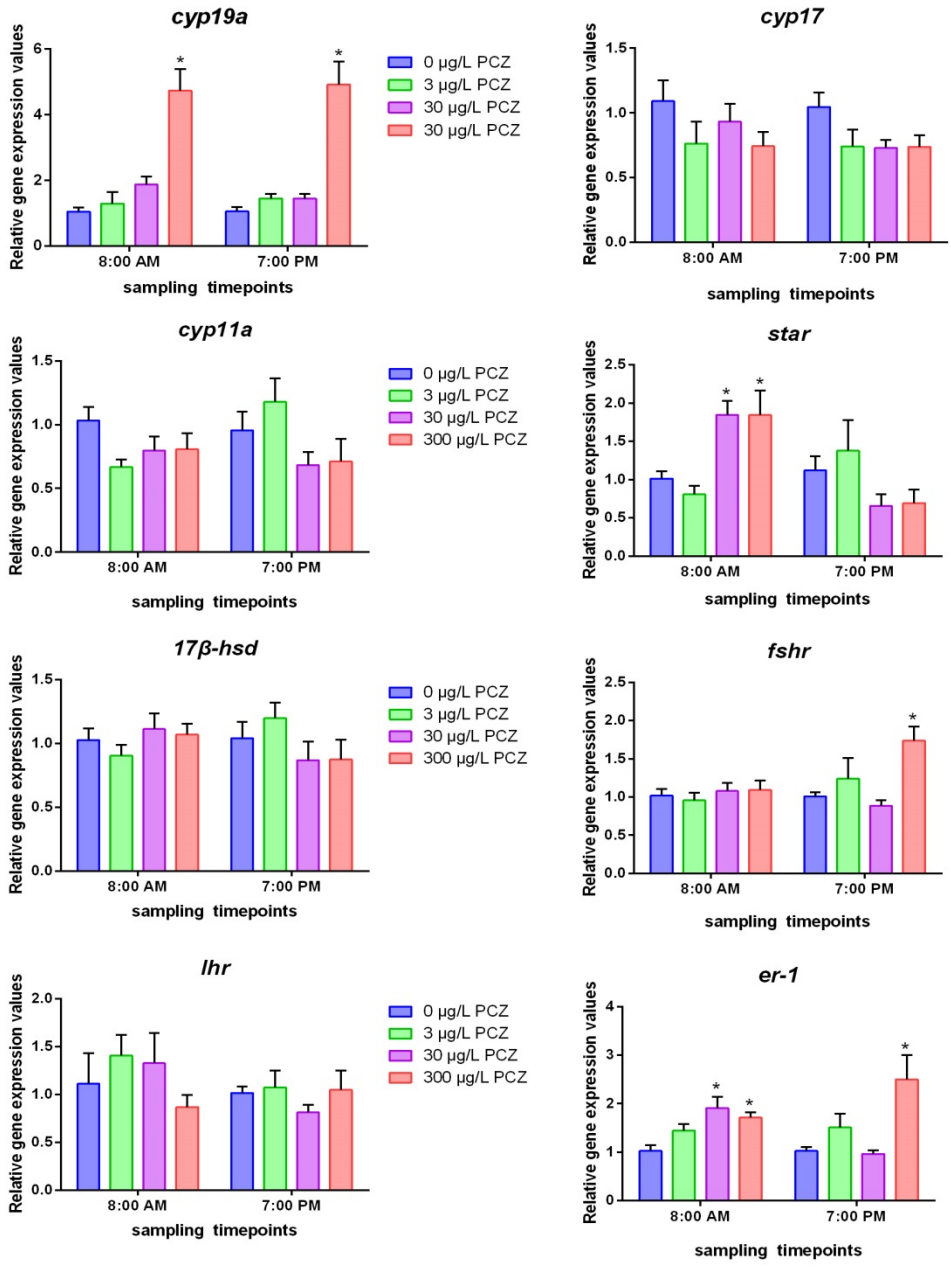

Figure S3: Effects of prochloraz on expressions of genes in ovary of female zebrafish collected at 8:00 AM or 7:00 PM. Values represent mean ± SEM. Significant differences from controls are indicated by *$P < 0.05$. Each concentration contains 6-8 biological replicates. Two controls were included, and relative expressions of genes at the two collections (8:00 AM or 7:00 PM) were calculated by the $2^{-\Delta\Delta Ct}$ method using the corresponding control.