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The effects of progesterone on transcriptional expression profiles of genes associated with hypothalamic–pituitary–gonadal and hypothalamic–pituitary–adrenal axes during the early development of zebrafish (*Danio rerio*)

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HIGHLIGHTS

- Zebrafish embryos were exposed to progesterone for 144 hpf.
- The transcriptional effects of progesterone on the HPG and HPA axes were evaluated.
- Progesterone could alter mRNA expression of target genes along HPG and HPA axes.
- The molecular effects of progesterone are time- and concentration-dependent manners.

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ABSTRACT

Progesterone (P4) has been reported in surface water, and it may have adverse effects on aquatic organisms. This study provided the transcriptional effects of P4 during the early development of zebrafish. Zebrafish embryos were exposed for 144 h post fertilization (hpf) to 0, 6, 45 and 90 ng L^{-1} P4, and transcriptional expression profiles of the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes were assessed every day. For the receptor signaling pathways, P4 significantly induced the transcript of *Pgr* gene above 45 ng L^{-1} at 72 and 144 hpf, but inhibited its transcript above 6 ng L^{-1} at 96 and 120 hpf. A significant up-regulation of *Vtg1* mRNA was observed at 6 ng L^{-1} P4 or higher at 24, 96 and 144 hpf. For the steroidogenic pathways, the transcriptional expression of *Cyp11a1* and *Hsd17b3* mRNAs was mediated by 6 ng L^{-1} P4 or higher according to different exposure time points. In addition, P4 resulted in a significant induction of *Cyp19a1a* and *Cyp11b* mRNA expression while it caused a significant inhibition of *Hsd11b2* mRNA expression above 6 ng L^{-1}. For the other target genes related to hypothalamic and pituitary hormones, P4 mainly modulated the transcripts of *Gnrh2*, *Fshb* and *Lhb* genes at 6 ng L^{-1} or higher. The overall results from the present study indicate that P4 at environmentally relevant concentrations could cause the potential effects on zebrafish reproductive and adrenal endocrine systems by interfering with the HPG and HPA axes.

Abbreviations: *P4*, progesterone; *Pgr*, progesterone receptor; *Esr1*, estrogen receptor 1; *Vtg1*, vitellogenin 1; *Ar*, androgen receptor; *Mr*, mineralocorticoid receptor; *Gr*, glucocorticoid receptor; *Star*, steroidogenic acute regulatory protein; *Cyp11a1*, cytochrome P450-mediated side-chain cleavage enzyme; *Cyp17*, 17-alpha-hydroxylase/17-20 lyase cytochrome P450; *Cyp19a1a*, ovarian cytochrome P450 aromatase; *Cyp11b*, 11-beta-hydroxylase; *Hsd3b*, 3-beta-hydroxysteroid dehydrogenase; *Hsd20b*, 20-beta-hydroxysteroid dehydrogenase; *Hsd17b3*, 17-beta hydroxysteroid dehydrogenase type 3; *Hsd11b2*, hydroxysteroid 11-beta dehydrogenase 2; *Gnrh2*, gonadotropin-releasing hormone 2; *Gnrh3*, gonadotropin-releasing hormone 3; *Fshb*, follicle stimulating hormone, beta polypeptide; *Lhb*, luteinizing hormone, beta polypeptide; *Crh*, corticotropin releasing hormone; *Pomc*, proopiomelanocortin.

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1. Introduction

Endocrine disruption by natural and synthetic steroids in aquatic ecosystems has drawn a global attention in recent years (Kloas et al., 2009; Christen et al., 2010). These steroids can enter aquatic environments via excretion (feces and urine) of human and animals, and result in potential adverse effects on the sexual differentiation and reproduction of aquatic vertebrates, such as fish and amphibians (Kloas et al., 2009). Most studies focused on the effects of estrogens and androgens, for example, vitellogenin development in male fish and a high incidence of intersexuality in UK wild roach by estrogens (Jobling et al., 1998), and spiggin induction in female three-spined stickleback by methyltestosterone (Hogan et al., 2008). However, there is limited research concerning progestins (progestogens), especially for natural progesterone (P4) and its effects on fish.

In humans, P4 is an important mediator during the menstrual cycle and pregnancy of females. It is involved in the regulation of oocyte growth and maturation as well as development of the endometrium and implantation in uterine (Spencer and Bazer, 2002). In addition, P4 is also known to have functions in spermatogenesis and sperm motility (Thomas, 2008). In teleost fish, two maturation-inducing hormones (MIHs), 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) represent the active progestins (Pinter and Thomas, 1999). These progestins can stimulate oocyte maturation in female fish, and spermatogenesis and sperm motility in male fish, which is similar to the function of P4 in humans (Pinter and Thomas, 1999; Thomas, 2008). P4 and 17α-hydroxyprogesterone also activate progesterone receptors (*Pgr*) to elicit their specific physiological responses in humans and fish.

P4 has been reported to be present in surface water at concentrations up to 199 ng L^{-1} (Kolpin et al., 2002). More importantly, P4 was also detected in the receiving surface waters and flush waters of a typical swine farm with concentrations up to 30.5 and 2470 ng L^{-1}, respectively (Liu et al., 2012). Given the critical functions of endogenous progestins in fish, exogenous P4 may interfere with natural endocrine system in fish. Recent studies have been conducted to assess the reproductive inhibition of synthetic progestins in fish (Zeilinger et al., 2009; Paulos et al., 2010; Runnalls et al., 2013; Han et al., 2014). Furthermore, Svensson et al. (2013, 2014) demonstrated that levonorgestrel had strong androgenic effects in three-spined stickleback, including induction of the male-specific glue protein spiggin and inhibition of vitellogenesis in females, and disruption of the androgen-dependent seasonal breeding cycle in males, which contribute to its reproductive impairment in fish. Additionally, recent studies also showed that exposure to natural P4 caused a significant decrease in sperm motility, fecundity and fertility of fathead minnow (Murack et al., 2011; DeQuattro et al., 2012). P4 treatment altered transcriptional expression of genes involved in steroid hormone receptor activity, circadian rhythm, cell cycle, cardiac hypertrophy and reproduction (Zucchi et al., 2012, 2013). However, so far, the toxicological effects of P4 on fish are largely unknown, especially for the effects on the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes during the early development of zebrafish.

Small freshwater fish such as zebrafish (*Danio rerio*) holds many similar cellular and physiological characteristics with higher vertebrates, such as the endocrine systems including steroid hormones and steroid hormone receptors (Reimers et al., 2004; Marguerie et al., 2006; Tokarz et al., 2013), and thus zebrafish is a perfect model to estimate the effects and mechanisms of action of endocrine-disrupting chemicals (EDCs). In adult zebrafish, the reproductive and adrenal endocrine systems are controlled primarily by HPG and HPA axes, respectively (Fig. 1). Therefore, disrupting any point in the HPG and HPA axes may impair the reproductive and adrenal endocrine systems.

The aim of the present study was to evaluate the transcriptional effects of P4 on the HPG and HPA axes during the early development stage of zebrafish. Zebrafish embryos were exposed to P4 for 144 h post fertilization (hpf) and the transcriptional profiles of target genes associated with the receptor signaling pathways (*Pgr*, *Esr1*, *Vtg1*, *Ar*, *Mr* and *Gr*), the steroidogenic pathways (*Star*, *Cyp11a1*, *Cyp17*, *Cyp19a1a*, *Cyp11b*, *Hsd3b*, *Hsd20b*, *Hsd17b3* and *Hsd11b2*) and other target genes (*Gnrh2*, *Gnrh3*, *Fshb*, *Lhb*, *Crh* and *Pomc*) were examined every day. The results from this study assist understanding the modulation of P4 on the transcriptional expression of target genes along with HPG and HPA axes in fish.

2. Materials and methods

2.1. Chemicals and reagents

Progesterone (abbreviated as P4, CAS: 57-83-0) was purchased from Tokyo Chemical Industry (Shanghai, China), with a purity of 98%. Ethanol was used as a carrier solvent for P4. Stock solution of P4 was made up in ethanol at a concentration of 1 mg mL^{-1} and then stored at −20 °C.

2.2. Maintenance of zebrafish and P4 exposure

Adult zebrafish (*Danio rerio*) were raised in the laboratory at 26 ± 1 °C under a natural photoperiod. Embryos were collected from healthy and spawning adults with the female–male ratio of 1:2 and examined under an inverted biological microscope. Approximately 160 embryos of blastula period (at 2–4 h post fertilization (hpf)) were transferred into 1000 mL glass beaker containing 500 mL exposure solution. Embryos were exposed to different doses of P4 at the nominal concentrations of 0, 5, 50 and 100 ng L^{-1} for 144 hpf. The experiment was performed in a semi-static system and the water in each glass breaker was replaced with fresh solutions every day. Both the solvent control and exposure groups received 0.001% ethanol only. Four replicate glass breakers were available for all treatment groups. The glass breakers were placed in an illuminated incubator at 26 ± 1 °C with a 14 h:10 h light:dark photoperiod. Dead embryos were recorded and removed daily. At time points of 24, 48, 72, 96, 120 and 144 hpf, 15 embryos/larvae from each glass breaker were randomly sampled and stored in RNAlater (Ambion) for the analysis of gene expression.

2.3. Measurement of P4 concentrations in exposure solutions

Since the exposure solutions were renewed every day, the actual exposure concentrations of P4 were determined at the beginning of exposure (0 h) and before water renewal (24 h) in the first day from all water samples (500 mL). The detailed analytical methodology for measurement of exposure concentrations of P4 in each grass breaker is available in our previous method (Liu et al., 2011). Briefly, 500 mL water sample was collected from each glass breaker for solid phase extraction. Extraction was performed by passing the water samples through CNWBOND LC-C18 SPE cartridges (200 mg, 3 mL) (Germany). The target compound was eluted from each cartridge with 10 mL ethyl acetate. The eluted solutions were dried with N_2 and re-dissolved in 1 mL methanol. Each final extract was stored at −20 °C for subsequent analysis by rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry (RRLC–MS/MS) (Agilent 1200 LC-Agilent 6460 QQQ, USA). The limit of detection (LOD) and the limit of quantitation (LOQ) of P4 were 0.05 ng L^{-1} and 0.17 ng L^{-1},

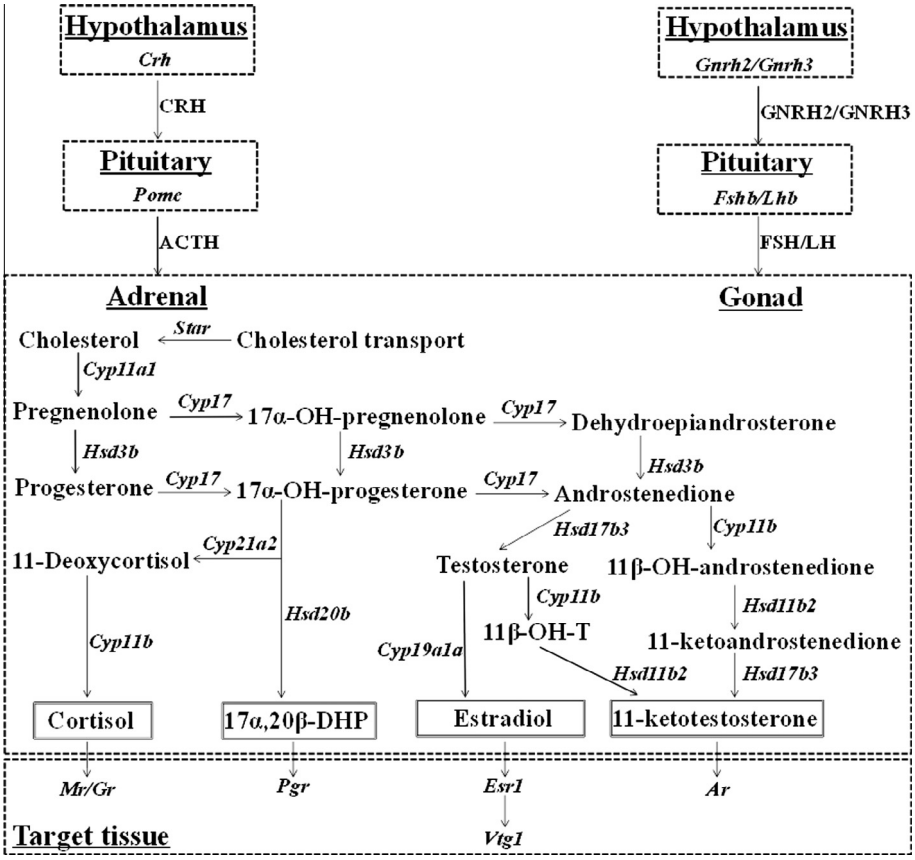

Fig. 1. A brief overview of hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes in zebrafish. Steroidogenic enzymes have been identified to date except for *Cyp21a2*.

respectively (Liu et al., 2011). The recoveries determined by spiking surface waters with known amounts of P4 (5 and 100 ng L^{-1}), were 92.4% and 102%, respectively (Liu et al., 2011).

2.4. Transcriptional expression analysis

Transcriptional expression analysis was carried out following our previous methods (Liang et al., 2015b). Briefly, prior to RNA isolation, RNAlater was removed. 15 embryos/larvae per sample were homogenized and used for extraction of total RNA using Trizol reagent (Invitrogen) following the manufacturer's instructions. The quality of total RNA was assessed using the electrophoresis on agarose gel stained with gelred (28S/18S rRNA intensity ratio about 2:1). Total RNA content was measured at 260 nm by using a SmartSpec™ Plus Spectrophotometer (Bio-Rad, USA) and the purity was determined by measuring 260/280 nm ratio (range, 1.8–2.0). The first strand complementary DNA (cDNA) was synthesized from 500 ng of total RNA by ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo) as described by the manufacture. The products from reverse transcription were diluted 4 times and 2.5 μL of each diluted sample was used for subsequent quantitative real-time PCR. The qRT-PCR was performed using the THUNDERBIRD SYBR® qPCR Mix (Toyobo) and analyzed on the Applied Biosystems ViiA™ 7 Dx (ABI). The specific primers for the target genes along with HPG and HPA axes (Table 1) were obtained from our previous published zebrafish primers sequences (Liang et al., 2015c). The thermal denaturation was 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melt curve was analyzed after each qRT-PCR reaction in order to ensure the amplification of single PCR product. The housekeeping genes, *β-actin*, ribosomal protein L13α (*RpL13α*) and elongation factor 1 alpha (*EF1-α*), were used as internal controls because of their relative stability in the early stage of zebrafish (Rongying et al., 2007). The expression levels of target genes were normalized to the average expression levels of *β-actin*, *RpL13α* and *EF1-α* mRNAs using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data are presented as relative mRNA expression ($\log_2$) at all time points.

2.5. Statistical analysis

Data on relative gene expression were statistically analyzed using SPSS (version 13.0). One-way analysis of variance (ANOVA) was used to calculate statistical significance between the exposure group and the solvent control group, followed by Tukey's multiple comparison tests. A probability of $P \leqslant 0.05$ was considered statistically significant differences. All quantitative data are expressed as mean ± standard error of mean (SEM).

3. Results

3.1. Measured concentrations of P4

The measured concentrations of P4 during the exposure period were found very close to the nominal concentrations, which suggest that P4 is constant during the exposure experiment. The average concentrations of P4 measured by RRLC–MS/MS were 6, 45 and 90 ng L^{-1} in the three exposure solutions, respectively. In addition, no P4 was detected in the control water sample. All exposure concentration data are presented in their average measured concentrations hereafter.

Table 1
Primers used for quantitative real-time PCR analysis and their sources.[a]

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)	GenBank number
Pgr	AGGCTTCTGGTTGTCATTATGG	TCCGGCGCACAGGTAGTTA	NM_001166335.1
Esr1	ACTCTCACCCATGTACCCTAAGG	CGGGTAGTATCCCACTGAAGC	NM_152959.1
Vtg1	CCTTGGAGAAAATTGAGGCTATC	CTGAATGAACTCGGGAGTGGTA	NM_001044897.2
Ar	CCACGAACCCCCGTTTATCT	TCCATCCATTCGCCCATCT	NM_001083123.1
Mr	ATCGGCTTGGATGCTATGG	TGGCTCGGTCTTAATGTGAGTTA	EF567113.1
Gr	TCACCACTTCAAGCGGACAG	GCTCCTGCTCCAACCATCTC	EF567112.1
Star	GCCTGAGCAGAAGGGATTTG	CCACCTGGGTTTGTGAAAGTAC	NM_131663.1
Cyp11a1	GAGGGGTGGACTCGGTTACTT	GCAATACGAGCGGCTGAGAT	AF527755.1
Cyp17	CTGCTCTGTTTAAGCCTGTTCTC	GCTGGCACAAATCCATTCATC	AY281362.1
Cyp19a1a	CGGGACTGCCAGCAACTACT	TGAAGCCCTGGACCTGTGAG	NM_131154.2
Cyp11b	CTGGGCCACACATCGAGAG	AGCGAACGGCAGAAATCC	DQ650710.1
Hsd3b	AGCCCATTCTGCCCATCTT	TGCCTCCTCCCAGTCATACC	AY279108.1
Hsd20b	TGGAGAACAGGCTGAGGTGAC	CGTAGTATCGGCAGAAGAGCAT	AF298898.1
Hsd17b3	ACATTCACGGCTGAGGAGTTT	ATGCTGCCATACGTTTGGTC	AY551081.1
Hsd11b2	CAACCCCAGGTGCGATACTAC	GCACGAGGCATCACTTTCTTCT	NM_212720.2
Gnrh2	GGTCTCACGGCTGGTATCCT	TGCCTCGCAGAGCTTCACT	NM_181439.4
Gnrh3	TGGTCCAGTTGTTGCTGTTAGTT	CCTGAATGTTGCCTCCATTTC	NM_182887.2
Fshb	GCAGGACTATGCTGGACAATG	CCACGGGGTACACGAAGACT	NM_205624.1
Lhb	GGCTGGAAATGGTGTCTTCTT	GGAAAACGGGCTCTTGTAAAC	NM_205622.2
Crh	GTCTGTTGGAGGGGAAAGTTG	CATTTTGCGGTTGCTGTGAG	BC085458.1
Pomc	GAGGGGAGTGAGGATGTTGTGT	TCGGAGGGAGGCTGTAGATG	AY158003.1
β-actin	TCTGGCATCACACCTTCTACAAT	TGTTGGCTTTGGGATTCAGG	AF057040.1
RpL13α	CCCTTCCCGTGGATCATATC	TTTGCGTGTGGGTTTCAGAC	NM_212784.1
EF1-α	GAGGAAATCACCAAGGAAGTCA	AATCTTCCATCCCTTGAACCAG	BC064291.1

[a] Liang et al. (2015c).

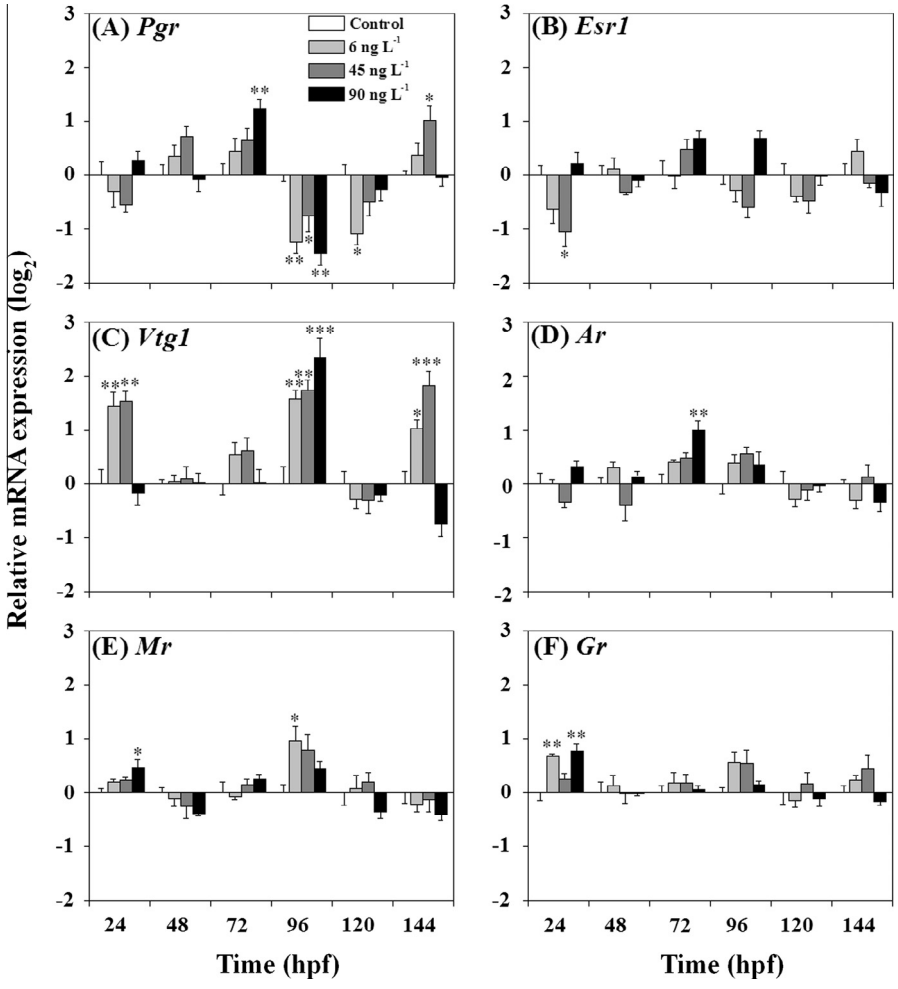

Fig. 2. Relative mRNA expression of *Pgr* (A), *Esr1* (B), *Vtg1* (C), *Ar* (D), *Mr.* (E) and *Gr* (F) in zebrafish embryos–larvae after exposure to 6, 45 and 90 ng L^{-1} P4. The results are expressed by mean ± SEM ($n = 4$ samples; each sample includes 15 embryos/larvae). $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$ and $^{***}p \leqslant 0.001$ indicate significant differences between the exposure group and the solvent control.

3.2. Transcriptional profiles of target genes related to the receptor signaling pathways after exposure to P4

P4 exposure did not cause significant changes in the survival rates in comparison with the control. The survival rates of control and exposure replicates were ⩾ 91.67%. There were significant alterations in mRNA expression of target genes related to the receptor signaling pathways following exposure to P4 (Fig. 2). For P4 exposure, a significant up-regulation of *Pgr* mRNA occurred at 90 ng L^{-1} at 72 hpf and at 45 ng L^{-1} at 144 hpf, whereas it significantly down-regulated the transcript of *Pgr* gene at all selected concentrations at 96 hpf and at 6 ng L^{-1} at 120 hpf (Fig. 2A). The expression of *Esr1* mRNA was inhibited at 24 hpf following exposure to 45 ng L^{-1} P4 (Fig. 2B). However, P4 resulted in significant induction of *Vtg1* mRNA expression at 6 and 45 ng L^{-1} for 24 and 144 hpf, and at all concentrations at 96 hpf (Fig. 2C). In addition, the transcripts of *Ar*, *Mr* and *Gr* were also up-regulated by P4 treatment at different time points, with significant up-regulation at 90 ng L^{-1} at 72 hpf for *Ar*, at 90 ng L^{-1} at 24 hpf and at 6 ng L^{-1} at 96 hpf for *Mr*, and at 6 and 90 ng L^{-1} at 24 hpf for *Gr* (Fig. 2D–F).

3.3. Transcriptional profiles of target genes related to the steroidogenic pathways after exposure to P4

P4 resulted in a significant up-regulation of *Cyp11a1* mRNA expression at all selected concentrations for 48, 72 and 120 hpf, and at 90 ng L^{-1} for 96 hpf, while a significant down-regulation was observed at 6 and 90 ng L^{-1} P4 at 144 hpf (Fig. 3B). In addition, P4 treatment resulted in the significant up-regulation of *Cyp19a1a* (at 6 and 45 ng L^{-1} for 96 hpf, and at 45 ng L^{-1} for 144 hpf), *Cyp11b* (at all treatment groups at 24 hpf) and *Hsd3b* (at 90 ng L^{-1} for 48 hpf) mRNA expression (Fig. 3D–F). P4 at all exposure groups displayed contrary effects on the transcript of *Hsd17b3*, with significant down-regulation at 24 hpf but up-regulation at 96 hpf (Fig. 3H). Additionally, at 120 hpf, P4 at 90 ng L^{-1} caused a significant decrease of *Hsd17b3* transcript, but the significant up-regulation was observed following exposure to 6 and 45 ng L^{-1} P4 at 144 hpf (Fig. 3H). A significant inhibition in the expression of *Hsd11b2* mRNA was observed at 45 ng L^{-1} at 96 hpf and at 90 ng L^{-1} at 144 hpf after exposure to P4 (Fig. 3I).

3.4. Transcriptional profiles of other target genes related to hypothalamic and pituitary hormones after exposure to P4

P4 at all exposure groups significantly induced the expression levels of *Gnrh2* mRNA at 24 hpf in comparison with the control, but it significantly suppressed the transcript of *Gnrh2* at 90 ng L^{-1} at 144 hpf (Fig. 4A). Similarly, the transcriptional levels of *Gnrh3* gene were significantly altered by 90 ng L^{-1} P4, with down-regulation at 24 hpf but up-regulation at 120 hpf (Fig. 4B). For the expression of *Fshb* mRNA, it was significantly up-regulated at 72 hpf but down-regulated at 144 hpf following exposure to 6 and 45 ng L^{-1} P4 (Fig. 4C). In addition, P4 exposure resulted in the significant induction of *Fshb* transcript at 120 hpf with the concentration of 90 ng L^{-1} (Fig. 4C). Also, a significant up-regulation of *Lhb* transcript was observed following exposure to P4 at different time points (at all treatment groups for 24 hpf, at 45 and 90 ng L^{-1} for 48 and 120 hpf, at 6 and 90 ng L^{-1} for 96 hpf, and at 45 ng L^{-1} for 144 hpf) (Fig. 4D). At 96 hpf, a significant down-regulation in the expression of *Crh* mRNA occurred at 90 ng L^{-1} P4 (Fig. 4E). Additionally, P4 led to significant induction of *Pomc* transcript at 90 ng L^{-1} at 24 hpf, while it significantly inhibited the transcript of *Pomc* gene at 45 ng L^{-1} at 48 hpf (Fig. 4F).

4. Discussion

The results from the present study showed that P4 could regulate the transcriptional profiles of some target genes associated with HPG and HPA axes at environmentally relevant concentrations during the early development of zebrafish, and that the

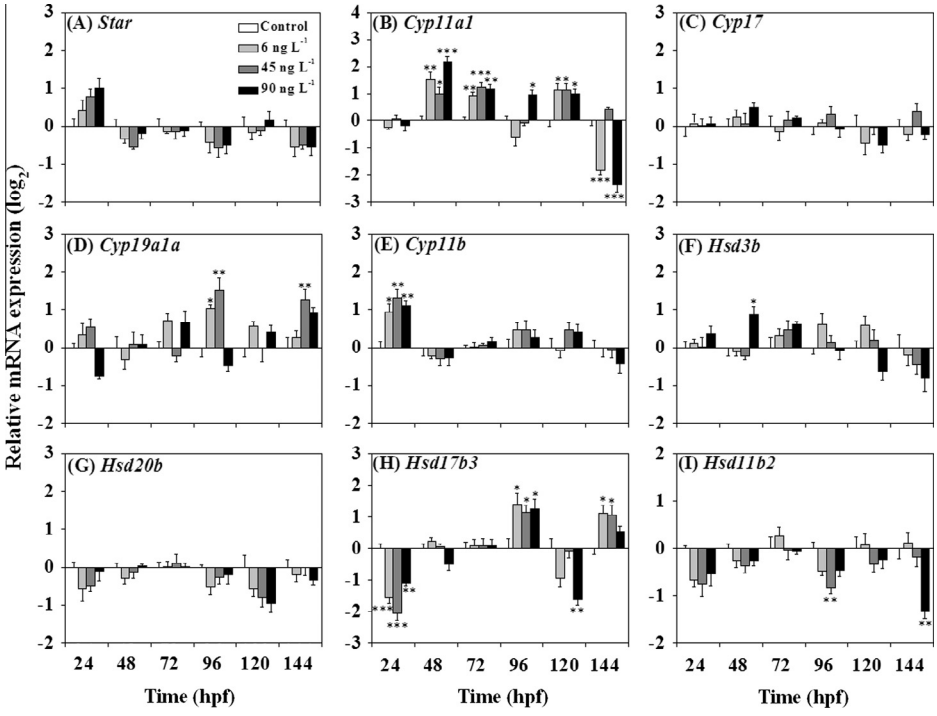

Fig. 3. Relative mRNA expression of *Star* (A), *Cyp11a1* (B), *Cyp17* (C), *Cyp19a1a* (D), *Cyp11b* (E), *Hsd3b* (F), *Hsd20b* (G), *Hsd17b3* (H) and *Hsd11b2* (I) in zebrafish embryos–larvae after exposure to 6, 45 and 90 ng L^{-1} P4. The results are expressed by mean ± SEM ($n = 4$ samples; each sample includes 15 embryos/larvae). $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$ and $^{***}p \leqslant 0.001$ indicate significant differences between the exposure group and the solvent control.

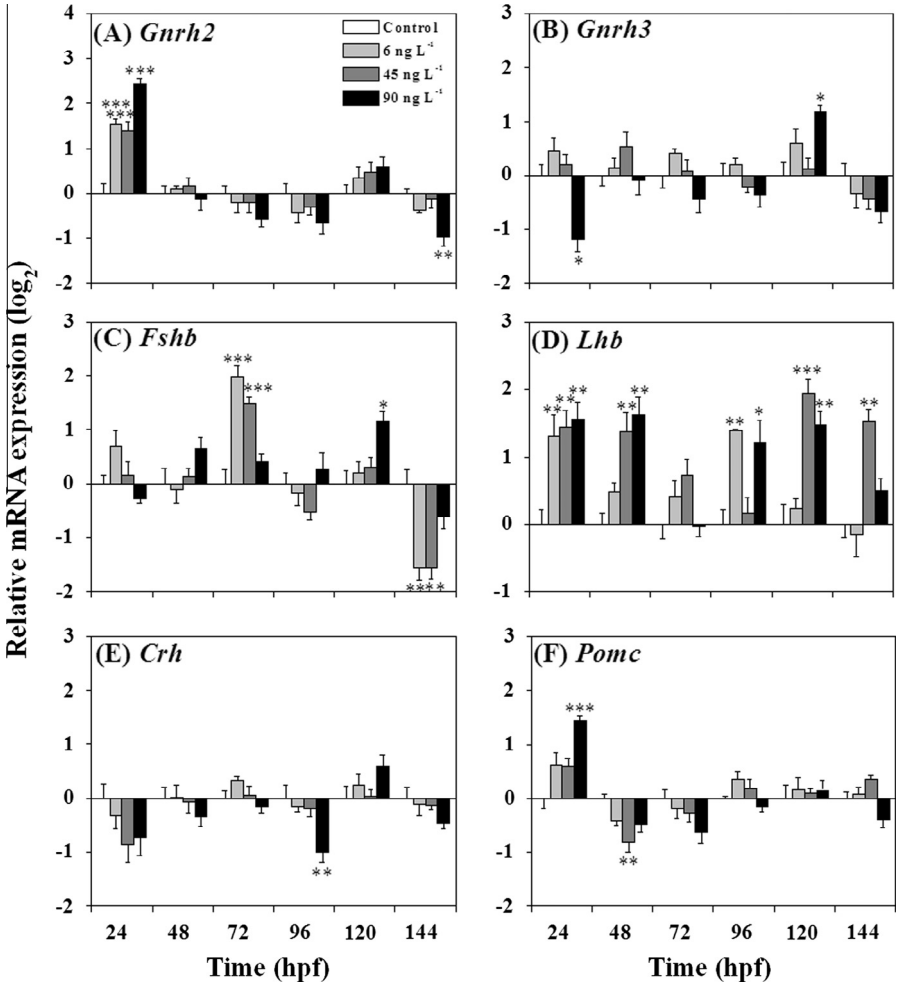

Fig. 4. Relative mRNA expression of *Gnrh2* (A), *Gnrh3* (B), *Fshb* (C), *Lhb* (D), *Crh* (E) and *Pomc* (F) in zebrafish embryos–larvae after exposure to 6, 45 and 90 ng L^{-1} P4. The results are expressed by mean ± SEM (n = 4 samples; each sample includes 15 embryos/larvae). $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$ and $^{***}p \leqslant 0.001$ indicate significant differences between the exposure group and the solvent control.

transcriptional patterns were variable at different exposure time points and exposure concentrations. Zucchi et al. (2012) investigated transcriptional expression levels of the receptor genes at different stages of zebrafish, and also found that transcriptional profiles were related to exposure time and concentration. In addition, we also observed some transcripts following the patterns of nonmonotonic dose–response (NMDRs). NMDRs are remarkably common in studies of EDCs, unlike the typical dose–responses observed in traditional toxicological studies (Vandenberg et al., 2012). When NMDRs occur, the effects of low doses cannot be predicted by the effects observed at high doses (Vandenberg et al., 2012). As with data in the present study, a safe dose determined from the highest concentration of P4 does not guarantee safety at the lowest and middle doses in the changes of mRNA expression (e.g., *Fshb* gene at 72 hpf). Therefore, NMDRs should be considered in risk assessment of these chemicals.

The present study showed that P4 had the stimulatory effects on the mRNA expression of *Pgr* at 72 and 144 hpf, but significantly inhibited its transcript at 96 and 120 hpf. In addition, *Pgr* was significantly up-regulated by P4 in juvenile zebrafish (at 60 d post fertilization) in our previous study (Liang et al., 2015a). However, Blüthgen et al. (2013) reported that P4 significantly inhibited the mRNA expression of *Pgr* in adult zebrafish. Different responses in embryos, juveniles and adults would indicate that transcriptional alterations of *Pgr* are in temporal expression patterns, as well as in developmental stages. Such time-course transcriptional profiles of P4 have also been described by Zucchi et al. (2013).

As known, *Vtg1* is widely accepted as a biomarker in fish in response to estrogens by an ESR-mediated pathway (Muncke and Eggen, 2006; Kim et al., 2010). In the present study, a significant up-regulation of *Cyp19a1a* mRNA expression by P4 treatment at 96 and 144 hpf would increase the levels of estrogens, which might be reasonable to explain the significant induction of *Vtg1* transcript. However, no significant alterations were observed for *Esr1* transcription at 96 and 144 hpf in zebrafish following exposure to P4. A previous study (Blüthgen et al., 2013) also demonstrated that P4 had no significant effects on transcriptional expression of *Esr1* gene in adults and embryos (F1 generation) of zebrafish, which further implies that *Esr1* is not sensitivity to P4 exposure.

The signaling pathway of androgens is similar to that of estrogens in fish. Androgens regulate genes through binding to androgen receptors and ultimately leading to changes of genes expression (Yarrow et al., 2010). P4 significantly induced the transcript of *Ar* gene in vivo in the present study, which is in accordance with the previous report that P4 showed activation of the hAR in vitro (Blüthgen et al., 2013). However, Blüthgen et al. (2013) reported that P4 significantly down-regulated the mRNA expression levels of *Ar* in adults and embryo (F1 generation) of zebrafish, indicating different toxicological effects at different developmental stages. *Ar* transcript was found in the presumptive

pronephros and diencephalon of zebrafish embryos by whole-mount RNA in situ hybridization (Gorelick et al., 2008), suggesting that *Ar* plays an important role in embryonic kidney and brain. Therefore, the alterations of *Ar* mRNA expression in the present study indicate that P4 may affect embryonic development, in particular the kidney and brain.

Mr and *Gr* play the essential role of translating the corticosteroid signal in HPA axis (Alsop and Vijayan, 2009). In addition, *Cyp11b* is involved in the production of cortisol in early development of zebrafish (Wilson et al., 2013). Therefore, the significant up-regulation of *Mr*, *Gr* and *Cyp11b* might come along with an induction of cortisol formation following exposure to P4 in the present study. It was reported that cortisol could affect spontaneous hatching, swim activity and stress response in the early embryonic development of fish (Wilson et al., 2013), and thus the present results indicate that P4 may influence embryonic development and survival by interfering with the cortisol signaling pathway.

Cyp11a1 involves in catalyzing the first step of the steroidogenic pathways and it plays an important part in the synthesis of all steroids. In the present study, P4 had contrary effects on the expression levels of *Cyp11a1*, suggesting that P4 interferes with the onset of steroidogenesis during the early stage of zebrafish. A statistically significant up-regulation of *Cyp19a1a*, *Cyp11b* and *Hsd3b* mRNAs would result in increase in the levels of estradiol (E2), cortisol, 11β-OH-androstenedione or progesterone. Additionally, *Hsd17b3* and *Hsd11b2* are involved in the production of main androgens (testosterone (T) and 11-ketotestosterone (11-KT)). The observed transcriptional changes of *Hsd17b3* and *Hsd11b2* genes would lead to alterations of androgens, and thus disrupt the sexual differentiation of the brain and gonads. In previous studies, it was reported that progestins could significantly decrease the concentrations of pregnenolone, DHP, E2, T or 11-KT in fish (Paulos et al., 2010; Runnalls et al., 2013; Han et al., 2014; Overturf et al., 2014). Therefore, it is reasonable to believe that P4 may impair synthesis of steroid hormones by interfering with the transcripts of steroidogenic genes (e.g., *Cyp11a1*, *Cyp19a1a*, *Cyp11b*, *Hsd3b*, *Hsd17b3* and *Hsd11b2*), and consequently translate to reproductive impairment in zebrafish.

In adult fish, synthesis and release of sex hormones are known to be regulated primarily by follicle stimulating hormone (FSH) and luteinizing hormone (LH). Similarly, adrenocorticotropic hormone (ACTH) mainly stimulates cortisol steroidogenesis. In addition, the ACTH sequence has been confirmed within the *Pomc* gene in zebrafish (Alsop and Vijayan, 2009). In the present study, P4 treatment modulated the transcriptional expression of *Fshb*, *Lhb* and *Pomc* genes, which may change FSH, LH and ACTH production, and thus regulating the transcriptional alterations of steroidogenic genes. The transcripts of *Fshb* and *Lhb* genes were altered by P4 in zebrafish embryos–larvae in the present study. In a previous study, however, P4 did not show significant alterations in the transcriptional levels of *Fshb* and *Lhb* genes in zebrafish brain and ovary (Zucchi et al., 2013). The differences may be due to different development of zebrafish. It is known that gonadotropin-releasing hormone (GNRH) and corticotropin releasing hormone (CRH) could regulate synthesis and release of FSH, LH and ACTH from the pituitary in an adult brain, respectively. In the present study, the transcriptional alterations of *Gnrh2*, *Gnrh3* and *Crh* occurring at P4 exposure groups may change GNRH2, GNRH3 and CRH hormone levels, and further regulate FSH, LH and ACTH production.

5. Conclusions

The present results on gene transcription following 144 hpf exposure of zebrafish embryos to environmental concentrations of P4, have demonstrated that P4 could alter transcriptional expression of target genes associated with HPG and HPA axes, including receptor signaling pathways (*Pgr*, *Esr1*, *Vtg1*, *Ar*, *Mr* and *Gr*), the steroidogenic pathways (*Cyp11a1*, *Cyp19a1a*, *Cyp11b*, *Hsd3b*, *Hsd17b3* and *Hsd11b2*) and other target genes (*Gnrh2*, *Gnrh3*, *Fshb*, *Lhb*, *Crh* and *Pomc*). These results imply that P4 could cause potential effects on reproductive and adrenal endocrine systems by altering transcriptional expression of target genes along with HPG and HPA axes during the early development of zebrafish.

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