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Environmental Toxicology and Chemistry, Vol. 34, No. 1, pp. 112–119, 2015

Printed in the USA

A TIME-COURSE TRANSCRIPTIONAL KINETICS OF THE HYPOTHALAMIC–PITUITARY–GONADAL AND HYPOTHALAMIC–PITUITARY–ADRENAL AXES IN ZEBRAFISH ELEUTHEROEMBRYOS AFTER EXPOSURE TO NORGESTREL

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(Submitted 7 May 2014; Returned for Revision 4 August 2014; Accepted 29 September 2014)

Abstract: The objective of the present study was to investigate the effects of norgestrel on the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes in zebrafish eleutheroembryos. Zebrafish embryos were exposed to different concentrations of norgestrel (0 ng L^{-1}, 5 ng L^{-1}, 50 ng L^{-1}, and 100 ng L^{-1}) for 144 h post fertilization (hpf), and the transcriptional profiles of the HPG and HPA axes were examined every day. Norgestrel modulated the expression of *Pgr* and *Vtg1* messenger (m)RNAs mainly at 96 hpf for all treatment groups. In addition, norgestrel strongly altered the expression of *Cyp11a1* mRNA above 5 ng L^{-1} (significant upregulation from 48 hpf to 120 hpf and significant downregulation for 144 hpf). Norgestrel treatment could significantly induce expression of *Cyp19a1a*, *Cyp11b*, *Gnrh2*, *Gnrh3*, and *Lhb* mRNAs but inhibit transcripts of *Hsd11b2* and *Crh* genes above 5 ng L^{-1} at different time points. The transcriptional expression levels of *Esr1*, *Ar*, *Star*, *Hsd17b3*, *Fshb*, and *Pomc* were also mediated by 5 ng L^{-1} norgestrel or higher during different exposure periods. Taken together, the overall results imply that the transcriptional changes in zebrafish eleutheroembryos may pose a potential effect on embryonic development, in particular in the brain and gonadogenesis. *Environ Toxicol Chem* 2015;34:112–119. © 2014 SETAC

Keywords: Gene transcription Hypothalamic–pituitary–adrenal axis Hypothalamic–pituitary–gonadal axis Norgestrel Zebrafish

INTRODUCTION

Natural and synthetic steroids are the main active compounds present in aquatic environments that can impair the endocrine system of aquatic vertebrates such as fish [1]. Estrogens such as 17β-estradiol and 17α-ethinylestradiol have been demonstrated to interfere with sexual behavior and sexual development in male fish [2,3]. Also, the adverse effects of androgens, including testosterone and 17β-trenbolone, have been extensively researched [4,5]. However, very little attention has been paid to the toxicological effects of progestins (progestogens), especially for the effects on the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes, which primarily control the reproductive and adrenal endocrine systems.

Synthetic progestins, including norgestrel, have been used widely in oral contraceptives, contraceptive implants, intrauterine devices, and vaginal rings and as active pharmaceutical ingredients for clinical use in humans [6–8]. Progestins have been detected in surface waters [9–11] and wastewaters [12–14]. Norgestrel was found in surface water at concentrations up to 22 ng L^{-1} [11] and in municipal wastewater-treatment plant effluents at concentrations up to 11 ng L^{-1} [13]. In addition, norgestrel is often used to control the pregnancy time of animals in the livestock industry and has detected in wastewaters of swine and dairy cattle farms with concentrations up to 10 800 ng L^{-1} and 6460 ng L^{-1}, respectively [12,14]. Furthermore, discharge of these wastewaters from domestic wastewater-treatment plants and animal farms could lead to contamination of the receiving aquatic environments [14].

Recent studies have reported that progestins could cause endocrine disruption in the reproductive system of fish at the nanogram per liter concentration range [15,16]. More importantly, Zucchi et al. [17] determined the effects of 4 different progestins on transcriptional changes of steroid hormone receptors in zebrafish and found that progestins may disrupt sex differentiation and consequently impair reproduction by modulating some gene transcripts. Progestins are widely known to bind to progesterone receptors; and they also have some interactions with other steroid hormone receptors—such as androgen, estrogen, glucocorticoid, and mineralocorticoid receptors—and exhibit different mechanisms of action [17–19]. In addition, levonorgestrel has been reported to interfere with the messenger (m)RNA expression of steroidogenic genes in fathead minnows [20]. Steroid biosynthesis begins with a regulator protein called the steroidogenic acute regulatory protein (Star), which can transfer cholesterol from the outer to the inner mitochondrial membrane (Figure 1). Subsequently, 2 classes of steroidogenic enzymes carry out biosynthesis of all steroid hormones: the cytochrome P450 family and hydroxysteroid dehydrogenases (Figure 1). Any disruption in transcriptional expression of these enzymes may lead to alteration in the synthesis of steroid hormones, which are crucial to reproduction in fish [21]. However, it is not known whether norgestrel at environmental concentrations could have effects on receptor signaling pathways, steroidogenic pathways, and other target genes in fish at the molecular level.

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Published online 15 October 2014 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.2766

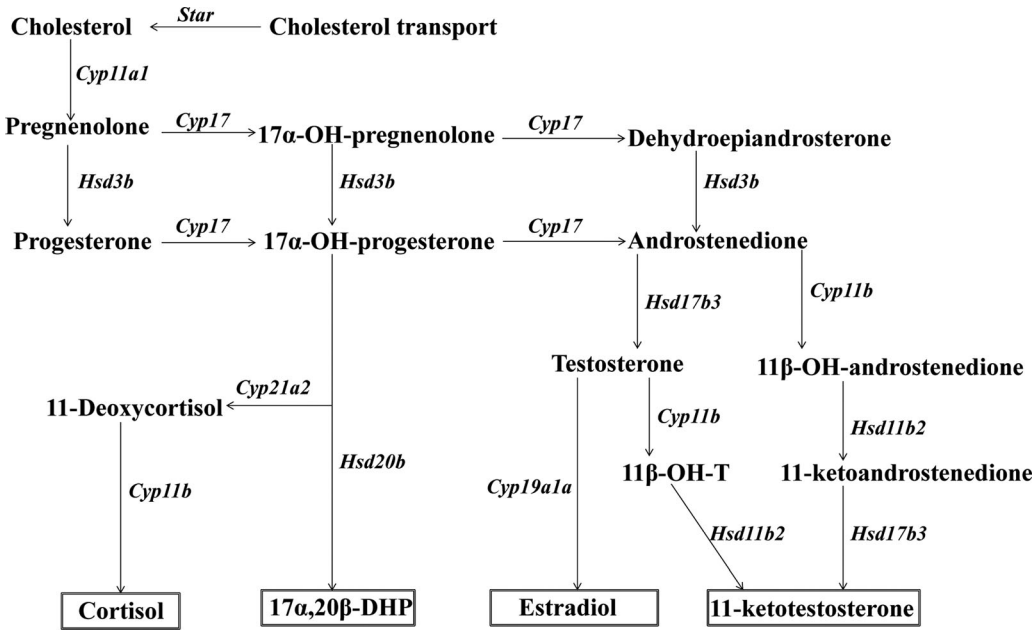

Figure 1. The main pathway of steroid synthesis in zebrafish. Biosynthetic enzymes have been identified to date, except for Cyp21a2. Major circulating steroids are framed. Source data from Busby et al. [40] and Tokarz et al. [41].

The aim of the present study was to assess the effects of norgestrel on the HPG and HPA axes in zebrafish eleutheroembryos. Zebrafish embryos were exposed to norgestrel for 144 h post fertilization (hpf), and the transcriptional expression of target genes, including receptor signaling pathways (*Pgr*, *Esr1*, *Vtg1*, *Ar*, *Mr*, and *Gr*), steroidogenic pathways (*Star*, *Cyp11a1*, *Cyp17*, *Cyp19a1a*, *Cyp11b*, *Hsd3b*, *Hsd20*b, *Hsd17b3*, and *Hsd11b2*), and other target genes (*Gnrh2*, *Gnrh3*, *Fshb*, *Lhb*, *Crh*, and *Pomc*), was detected daily (see Table 1 for descriptions of genes). The results from the present study can help us understand the potential impact of norgestrel on the development of the reproductive and adrenal endocrine systems in fish.

MATERIALS AND METHODS

Test organisms and test chemical

Adult zebrafish (*Danio rerio*) were obtained from a local supplier and bred in the laboratory for at least 1 mo before exposure experiment. The fish were held in a flow-through system with aerated tap water (pH 7–8) and fed with red worms (*Chironomid* larvae) once a day. The water temperature was at $26 \pm 1\,^{\circ}C$ with a natural photoperiod. The test chemical norgestrel (CAS number 6533-00-2; purity 100%) was obtained from US Pharmacopeia, with its stock solution ($1\,mg\,mL^{-1}$) prepared in ethanol.

Exposure of embryos to norgestrel

Embryos in the tests were obtained by spawning adult zebrafish put in groups of 6 males and 3 females in tanks overnight. The spawning was stimulated by the onset of light the following morning. Then, the embryos were collected within 30 min and rinsed with aerated embryonic rearing water, which was prepared according to International Organization for Standardization standard 7346/3. The embryos that had developed normally at the early blastula stage (at 2–4 hpf) were used in the further exposure experiments. Fertilized embryos ($n = 160$) were randomly distributed into each glass breaker including 500 mL of norgestrel exposure solutions at different nominal concentrations ($5\,ng\,L^{-1}$, $50\,ng\,L^{-1}$, and $100\,ng\,L^{-1}$). The water control and solvent control (0.001% ethanol) were also included. Each exposure concentration had 4 replicates. In total, 20 beakers were used in the tests. The embryos were placed in an illuminated incubator at $26 \pm 1\,^{\circ}C$, with a 14:10-h light:dark photoperiod. The experiment was conducted for 144 hpf. During the experimental period, the exposure solutions were renewed daily. Embryos were examined daily under an inverted biological microscope until 144 hpf, and dead embryos were recorded and removed during the exposure phase. At different developmental stages (24 hpf, 48 hpf, 72 hpf, 96 hpf, 120 hpf, and 144 hpf), 15 eleutheroembryos were randomly sampled per replicate and placed in RNAlater (Ambion) at $-20\,^{\circ}C$ for subsequent RNA isolation.

Isolation of RNA and reverse transcription

Isolation of total RNA was carried out using Trizol reagent (Invitrogen) as described by Huang et al. [18]. Total RNA concentration was determined at 260 nm using a SmartSpec™ Plus Spectrophotometer (Bio-Rad), and the purity and quality were analyzed by measuring 260/280 nm ratios. The synthesis of first-strand complementary (c)DNA was performed from 500 ng RNA of each sample using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo) as described by the manufacturer. Prior to quantitative real-time polymerase chain reaction (qRT-PCR), each cDNA sample was diluted 4-fold with nuclease-free water and stored at $-20\,^{\circ}C$.

Quantitative real-time polymerase chain reaction

According to our previous methods [18], quantitative real-time PCR was performed on the Applied Biosystems ViiA™ 7 Dx (ABI) using the THUNDERBIRD SYBR® qPCR Mix (Toyobo). The Primer Premier 5.0 program was used to design the primer sequences for the target and housekeeping genes according to the following criteria (Table 1). The length of primers was usually 15 base pairs (bp) to 30 bp. In addition, amplicons of 70 bp to 200 bp were strongly recommended. If

Table 1. Primers for quantitative real-time polymerase chain reaction in zebra fish eleutheroembryos

Gene	GenBank number	Sense primer (5′–3′)	Antisense primer (5′–3′)	Product size (bp)	Efficiency (%)
Pgr	NM_001166335.1	AGGCTTCTGGTTGTCATTATGG	TCCGGCGCACAGGTAGTTA	98	103
Esr1	NM_152959.1	ACTCTCACCCATGTACCCTAAGG	CGGGTAGTATCCCACTGAAGC	151	100
Vtg1	NM_001044897.2	CCTTGGAGAAAATTGAGGCTATC	CTGAATGAACTCGGGAGTGGTA	161	107
Ar	NM_001083123.1	CCACGAACCCCCGTTTATCT	TCCATCCATTCGCCCATCT	165	99
Mr	EF567113.1	ATCGGCTTGGATGCTATGG	TGGCTCGGTCTTAATGTGAGTTA	135	95
Gr	EF567112.1	TCACCACTTCAAGCGGACAG	GCTCCTGCTCCAACCATCTC	137	104
Star	NM_131663.1	GCCTGAGCAGAAGGGATTTG	CCACCTGGGTTTGTGAAAGTAC	170	98
Cyp11a1	AF527755.1	GAGGGGTGGACTCGGTTACTT	GCAATACGAGCGGCTGAGAT	109	99
Cyp17	AY281362.1	CTGCTCTGTTTAAGCCTGTTCTC	GCTGGCACAAATCCATTCATC	80	98
Cyp19a1a	NM_131154.2	CGGGACTGCCAGCAACTACT	TGAAGCCCTGGACCTGTGAG	264	103
Cyp11b	DQ650710.1	CTGGGCCACACATCGAGAG	AGCGAACGGCAGAAATCC	171	106
Hsd3b	AY279108.1	AGCCCATTCTGCCCATCTT	TGCCTCCTCCCAGTCATACC	200	99
Hsd20b	AF298898.1	TGGAGAACAGGCTGAGGTGAC	CGTAGTATCGGCAGAAGAGCAT	81	98
Hsd17b3	AY551081.1	ACATTCACGGCTGAGGAGTTT	ATGCTGCCATACGTTTGGTC	74	102
Hsd11b2	NM_212720.2	CAACCCCAGGTGCGATACTAC	GCACGAGGCATCACTTTCTTCT	131	100
Gnrh2	NM_181439.4	GGTCTCACGGCTGGTATCCT	TGCCTCGCAGAGCTTCACT	89	104
Gnrh3	NM_182887.2	TGGTCCAGTTGTTGCTGTTAGTT	CCTGAATGTTGCCTCCATTTC	116	99
Fshb	NM_205624.1	GCAGGACTATGCTGGACAATG	CCACGGGGTACACGAAGACT	151	98
Lhb	NM_205622.2	GGCTGGAAATGGTGTCTTCTT	GGAAAACGGGCTCTTGTAAAC	202	99
Crh	BC085458.1	GTCTGTTGGAGGGGAAAGTTG	CATTTTGCGGTTGCTGTGAG	200	98
Pomc	AY158003.1	GAGGGGAGTGAGGATGTTGTGT	TCGGAGGGAGGCTGTAGATG	216	96
β-actin	AF057040.1	TCTGGCATCACACCTTCTACAAT	TGTTGGCTTTGGGATTCAGG	93	97
RpL13α	NM_212784.1	CCCTTCCCGTGGATCATATC	TTTGCGTGTGGGTTTCAGAC	208	96
EF1-α	BC064291.1	GAGGAAATCACCAAGGAAGTCA	AATCTTCCATCCCTTGAACCAG	147	96

bp = base pair; *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.

absolutely necessary, product size could be increased to 300 bp at most. The GC content of primers was kept within 40% to 60%, and melting temperature was kept as close as possible to 60 °C. The last 5 bases at the 3′ end avoided 3 continuous nucleotides, especially for G and C. Finally, primer pairs were selected with the minimal number of potential primer dimers and primer hairpins if possible. All designed primers were synthetized by Invitrogen. Conditions of qRT-PCR were as follows: initial denaturation at 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The melt curves were used to validate the specificity of PCR products. Based on a previous study [22], *β-Actin*, ribosomal protein L13α (*RpL13α*), and elongation factor 1 alpha (*EF1-α*) were used as reference genes for the zebrafish developmental course. The average mRNA expression levels of the 3 housekeeping genes were therefore used to normalize the expression of mRNA for each target gene. The changes in the relative mRNA expression of target genes in zebrafish eleutheroembryos were analyzed by the $2^{-\Delta\Delta Ct}$ method [23].

Measurement of norgestrel in exposure solutions

Because the exposure solutions were renewed daily, the actual exposure concentrations of norgestrel 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; $n = 4$ replicate breakers). The extraction of water samples and instrumental analysis of the target chemical were performed according to our previous method [11]. Briefly, solid-phase extraction was applied for the collected water samples using CNWBOND LC-C18 solid-phase extraction cartridges (200 mg, 3 mL). Then, the cartridges were dried under air for approximately 2 h, and the target compound was eluted from each cartridge with 10 mL ethyl acetate. The eluted solutions were dried by a gentle stream of nitrogen, and the final extracts were redissolved in 1 mL methanol for later analysis by rapid resolution liquid chromatography-electrospray ionization tandem mass spectrometry (Agilent 1200 LC-Agilent 6460 QQQ). The limit of detection of norgestrel was 0.04 ng L^{-1} [11]. The recoveries determined by spiking surface waters with known amounts of norgestrel (5 ng L^{-1} and 100 ng L^{-1}) were 87.5% and 96.8%, respectively [11].

Data analysis and statistics

The basic transcriptional levels of target genes were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison, and $p \leq 0.05$ was considered statistically significantly different between 24 hpf and all other time points. The effects of norgestrel treatment and time on the relative mRNA expression of target genes were analyzed by two-way ANOVA, and statistically significant differences between means were determined using Tukey's test at the $p \leq 0.05$ level. Statistical analyses were carried out using SPSS (Ver 13.0). All data are expressed as mean ± standard error of the mean.

RESULTS

Analytical results for the concentrations of norgestrel

During the exposure experiment, the actual concentrations of norgestrel at the beginning and after 24 h were very close to the nominal concentrations, which implies that norgestrel is relatively stable (Table 2). The average measured concentrations of norgestrel were 6.23 ng L^{-1}, 45.08 ng L^{-1}, and 86.63 ng L^{-1} in the 3 exposure solutions, respectively. Norgestrel was not detected in the solvent control. For simplicity, all exposure concentration data are presented using the nominal concentrations.

Table 2. Nominal and measured concentrations of norgestrel in the exposure experiment

Nominal concentration	Measured Concentrations ($ng\,L^{-1}$)										
	0 h exposure time					24 h exposure time					
	Tank 1	Tank 2	Tank 3	Tank 4	Average[a]	Tank 1	Tank 2	Tank 3	Tank 4	Average[a]	Average[a]
$0\,ng\,L^{-1}$	0	0	0	0	0	0	0	0	0	0	0
$5\,ng\,L^{-1}$	6.82	6.48	6.18	5.90	6.35 ± 0.20	6.09	6.10	6.13	6.08	6.10 ± 0.01	6.23 ± 0.11
$50\,ng\,L^{-1}$	49.00	45.70	48.40	44.83	46.98 ± 1.01	43.21	43.38	43.09	42.99	43.17 ± 0.08	45.08 ± 0.55
$100\,ng\,L^{-1}$	88.13	85.59	90.35	87.79	87.97 ± 0.97	86.23	83.22	88.96	82.69	85.28 ± 1.45	86.63 ± 1.21

[a]Measured concentrations are given as mean ± standard error of the mean ($n = 4$ replicates).

Basic transcriptional levels of target genes involved in the HPG and HPA axes in zebrafish eleutheroembryos without exposure to norgestrel

There were no significant differences in the survival rates for exposed embryos between the solvent controls and norgestrel treatments. The survival rates were more than 96.67% in all exposure groups, including the solvent controls. The mRNA expression levels of the target genes involved in the HPG and HPA axes were measured by qRT-PCR in unexposed eleutheroembryos at 24 hpf, 48 hpf, 72 hpf, 96 hpf, 120 hpf, and 144 hpf (Figure 2). Compared with levels at 24 hpf, the transcripts of *Pgr*, *Esr1*, *Vtg1*, *Ar*, *Mr*, and *Gr* were significantly increased at other time points (from 48 hpf to 144 hpf for the *Vtg1* and *Mr* genes; from 72 hpf to 144 hpf for the *Esr1* gene; from 96 hpf to 144 hpf for the *Ar* gene; at 72 hpf, 120 hpf, and 144 hpf for the *Gr* gene; at 96 hpf for the *Pgr* gene) ($p < 0.05$; Figure 2A). The mRNA expression levels of *Star*, *Cyp11b*, and *Hsd11b2* from 48 hpf to 144 hpf were significantly increased compared with those at 24 hpf, wheresas the transcripts of *Cyp11a1*, *Hsd3b*, and *Hsd17b3* were significantly decreased ($p < 0.05$). The transcript of *Hsd20b* was significantly upregulated from 96 hpf to 144 hpf in comparison with that from 24 hpf to 72 hpf, whereas the expression levels of *Cyp17* mRNA were significantly downregulated at 144 hpf compared with the level at 24 hpf. There were no significant differences in the transcription of *Cyp19a1a* between 24 hpf and all other time points ($p > 0.05$; Figure 2B). Compared with levels at 24 hpf, the transcripts of *Gnrh2*, *Gnrh3*, *Crh*, and *Pomc* were significantly increased from 48 hpf to 144 hpf, and significant

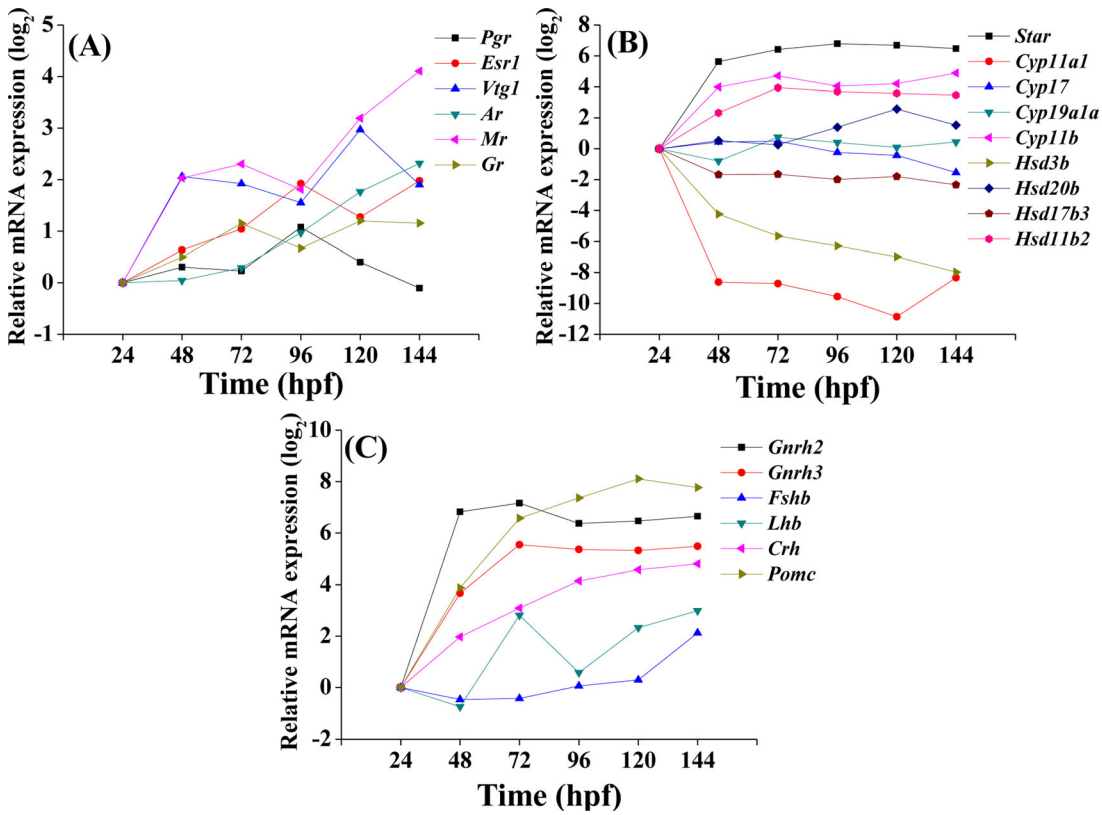

Figure 2. Basic transcriptional changes of the target genes involved in the hypothalamic–pituitary–gonadal and hypothalamic–pituitary–adrenal axes during the early development of zebrafish. Experimental controls were without exposure to norgestrel. The transcriptional expression levels of target genes at 48 h postfertilization (hpf), 72 hpf, 96 hpf, 120 hpf, and 144 hpf are expressed relative to the values at 24 hpf by using the $2^{-\Delta\Delta Ct}$ method. Data are expressed as mean ± standard error of the mean of 4 replicate samples.

induction of *Fshb* mRNA expression occurred at 144 hpf. The expression of *Lhb* mRNA also was upregulated at 72 hpf, 120 hpf, and 144 hpf ($p < 0.05$; Figure 2C).

Transcriptional expression of target genes involved in HPG and HPA axes in zebrafish eleutheroembryos following exposure to norgestrel

In the present study, no significant differences in transcriptional expression of target genes were observed between the water control and solvent control. A significant interaction could be found between norgestrel exposure concentration and time on the transcriptional expression levels of a series of genes involved in the HPG and HPA axes, except for *Gr* and *Hsd20b* (two-way ANOVA, $p < 0.05$; data not shown). The effects of norgestrel on mRNA expression levels of target genes in zebrafish eleutheroembryos are shown in Figure 3. Norgestrel at all exposure concentrations significantly suppressed *Pgr* mRNA expression at 96 hpf, whereas a significant upregulation of *Vtg1* mRNA was observed at 48 hpf and 96 hpf. The expression levels of *Esr1* and *Ar* mRNAs were also altered by norgestrel at different exposure concentrations and time points.

A significant upregulation of *Cyp11a1* mRNA was observed for 5 ng L^{-1} norgestrel or higher concentrations from 48 hpf to 120 hpf, whereas it was significantly suppressed at 144 hpf at all selected concentrations. For the mRNA expression of the *Cyp19a1a* gene, it was significantly upregulated at 5 ng L^{-1} norgestrel or higher from 96 hpf to 144 hpf. Similarly, a significant induction was observed at 50 ng L^{-1} and 100 ng L^{-1} norgestrel for the transcript of *Cyp11b*. Norgestrel in all treatment groups displayed contrary effects on the expression of *Hsd17b3* mRNA, with a significant decrease at 24 hpf, but a significant increase at 144 hpf. A significant decrease in *Hsd11b2* mRNA expression occurred at 24 hpf, 48 hpf, and 144 hpf exposed to the nominal concentration of 5 ng L^{-1} norgestrel or higher compared with the solvent control.

The expression levels of *Gnrh2*, *Gnrh3*, and *Lhb* mRNAs were significantly increased at 5 ng L^{-1} norgestrel or higher at different time points. Norgestrel exposure could significantly induce or inhibit transcripts of the *Fshb* and *Pomc* genes above 5 ng L^{-1} at different time points. In addition, transcript of the *Crh* gene was significantly decreased at 24 hpf exposed exposure to 50 ng L^{-1} or 100 ng L^{-1} norgestrel compared with the solvent control.

DISCUSSION

The overall results from the present study showed that the basic transcription of the target genes related to the HPG and HPA axes significantly differed in different developmental stages of zebrafish, especially for some steroidogenic genes (*Star*, *Cyp11b*, *Hsd11b2*, *Cyp11a1*, and *Hsd3b*) and other target genes (*Gnrh2*, *Gnrh3*, *Crh*, and *Pomc*) in the solvent controls. The transcript of the *Star* gene was significantly induced during embryogenesis, indicating onset of the steroid biosynthetic pathway. Because *Cyp11b* is the final enzyme in the pathway of cortisol biosynthesis, this could limit cortisol production during the early stages of development [24]. Moreover, *Hsd11b2* has been suggested to be involved in metabolizing cortisol to cortisone to protect embryos from excess cortisol exposure by eliminating receptor binding [24,25]. The expression of *Cyp11b* and *Hsd11b2* mRNAs significantly increased from 48 hpf to 72 hpf in the present study, which is in accordance with previous results [25]. The transcripts of *Cyp11a1* and *Hsd3b* were reported as maternal transcripts during embryogenesis [26,27], which could be used to explain that zebrafish *Cyp11a1* and *Hsd3b* mRNAs were expressed abundantly at 24 hpf and then dropped subsequently. The expression of *Gnrh2* and *Gnrh3* transcripts was significantly increased at 48 hpf, indicating that the embryonic expression of these 2 genes started between 24 hpf and 48 hpf. It is known that the HPA axis operates by secreting corticotropin-releasing hormone from the hypothalamus, which in return stimulates the production of adrenocorticotropic hormone (ACTH) that regulates synthesis and release of cortisol hormones in adult fish. Moreover, the ACTH sequence is derived from the *Pomc* gene in zebrafish [28]. In the present study, the expression of *Crh* and *Pomc* mRNAs was significantly increased from 48 hpf onward, which leads us to propose that cortisol steroidogenesis may be activated during the development of zebrafish. Actually, the rise in cortisol was observed

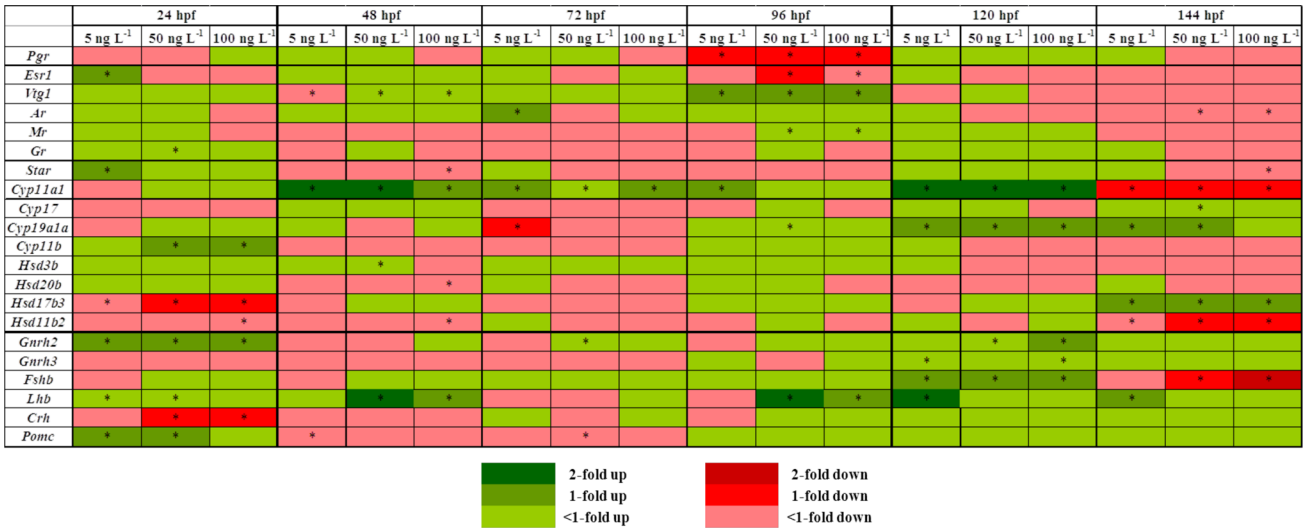

Figure 3. Heat map depicting the relative messenger (m)RNA expression of the target genes involved in the hypothalamic–pituitary–gonadal and hypothalamic–pituitary–adrenal axes in zebrafish eleutheroembryos following exposure to 5 ng L^{-1}, 50 ng L^{-1}, and 100 ng L^{-1} of norgestrel. Shades of green represent upregulation, and shades of red represent downregulation (related to $\log_2$ expression). * Asterisk indicates significant differences between the exposure group and the solvent control ($p \leq 0.05$). Data are expressed as mean ± standard error of the mean of 4 replicate samples.

from 48 hpf to 144 hpf [25], and the transcript of the *Cyp11b* gene was also increased in the present study, which confirm that biosynthesis of the cortisol pathway becomes active from 48 hpf.

It is well known that progestins regulate genes by binding to the specific palindromic *Pgr* response elements and activating *Pgr* signaling pathways to elicit specific physiological effects in fish and mammals [19,29]. In the present study, norgestrel significantly inhibited *Pgr* mRNA expression levels at 96 hpf. Han et al. [16] demonstrated that the inhibition of reproduction is the result of downregulation of several important genes, including *Pgr*. In addition, the reproductive results were combined with the downregulation of *Pgr* [30]. Consequently, we believe that alteration of *Pgr* mRNA expression in response to norgestrel may result in a disruption of gonadal development and finally translate to adverse effects of norgestrel on the reproductive system of zebrafish. In addition, the basic transcriptional expression levels of *Pgr* apparently displayed a significant increase at 96 hpf, whereas it was significantly downregulated in all norgestrel-treatment groups. This could imply that treatment with norgestrel causes the missing of an important peak in the *Pgr* transcript at 96 hpf.

In general, expression of the *Vtg* gene is known to be regulated with estrogens by estrogen receptors [31]. It was surprising to find *Vtg1* transcript in unexposed embryonic zebrafish, which is consistent with a previsous study [31]. Maternal transfer of estradiol to egg yolk occurred in avian species [32]. It is resonable to assume that the presence of estrogens of maternal origin could result in the expression of *Vtg1* mRNA in untreated embryonic zebrafish, though this has not been shown so far. The present study showed a significant increase of *Vtg1* mRNA expression by norgestrel exposure at 96 hpf in comparison with the solvent control. However, *Esr1* mRNA expression was clearly inhibited by norgestrel treatment at 96 hpf in the present study. Therefore, it is reasonable to suppose that *Esr1* was not the main regulating estrogen receptor at this time point, which is in accordance with previous findings [33]. Moreover, the basic transcriptional expression of *Vtg1* was significantly induced at 48 hpf, but 50 ng L^{-1} and 100 ng L^{-1} norgestrel inhibited its transcript. The *Vtg1* transcript would suggest that norgestrel exposure results in an over-expression peaking at 48 hpf.

It was reported that progestin exposure led to inhibition of reproduction, masculinization of females, changes of estrogen and androgen levels, delay of oocyte development, oviductal agenesis, and changes in male mating behavior in fish and amphibians [15,16,34–36]. Those previous results suggested that progestins could impair the synthesis of steroid hormones in fish and amphibians. Moreover, progestins decreased *Star* and *Cyp11a1* mRNA levels in male *Xenopus laevis* [37] and had no effects on the transcripts of the *Star* and *Cyp11a1* genes in fathead minnows [20]. However, our present results demonstrate that norgestrel displayed contrary effects on the expression of *Star* and *Cyp11a1* mRNAs in zebrafish eleutheroembryos, depending on the exposure time. The *Cyp11a1* gene encodes an enzyme that is involved in catalyzing the first step of the steroidogenic pathway, and this enzyme plays a crucial role in the synthesis of all steroids. In the present study, transcript of *Cyp11a1* (up to 2.66-fold change) was strongly affected by norgestrel treatment. The basic transcriptional expression levels of *Cyp11a1* could be used to explain these changes following exposure to norgestrel. The transcript of the *Cyp11a1* gene remained unaffected at 24 hpf in response to norgestrel because of its abundant basic expression resulting in a lack of sensitivity to norgestrel exposure. The basic transcriptional expression levels of *Cyp11a1* were significantly downregulated from 48 hpf to 120 hpf, but its decline was depressed by norgestrel. As development proceeded, the basic expression of *Cyp11a1* mRNA started to increase again at 144 hpf, but norgestrel treatment was inclined to inhibit its elevation. The transcriptional changes of the *Cyp11a1* gene at all time points except 24 hpf clearly reflect that norgestrel could interfere with the synthesis of steroid. In addition, the transcripts of the *Cyp17*, *Cyp19a1a*, *Cyp11b*, and *Hsd3b* genes were upregulated at various time points in zebrafish eleutheroembryos in response to norgestrel, which further implies that norgestrel has potential effects on embryonic development by interfering with the steroidogenic pathway. Both *Hsd17b3* and *Hsd11b2* are involved in the production of androgens (testosterone and 11-ketotestosterone). Therefore, our results suggest that norgestrel has potential effects on the production of androgens by affecting *Hsd17b3* and *Hsd11b2* transcripts, which could interfere with embryonic brain development and sexual differentiation of gonads. Furthermore, biosynthesis of cortisol may also be changed because *Hsd11b2* has been suggested to be associated with the metabolism of cortisol [24,25].

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are known mainly to stimulate sex steroid synthesis and release. In addition, it has previously been reported that synthesis of sex hormones is independent of FSH and LH stimulation [38]. In the present study, the expression levels of *Fshb* and *Lhb* mRNAs were not completely consistent with transcriptional alterations of steroidogenic genes following the exposure to norgestrel at all selected concentrations during the course of development. Therefore, we believe that at least 2 mechanisms existed to interpret the expression of steroidogenic genes under the norgestrel exposure: an indirect effect of norgestrel on steroidogenesis via changing FSH and LH production, and a direct norgestrel action on steroidogenic genes. As is known, gonadotropin-releasing hormone can activate the synthesis and release of FSH and LH from the pituitary in an adult brain. The present results showed that norgestrel had direct or indirect effects (by altering gonadotropin-releasing hormone production) on the expression of *Fshb* and *Lhb* mRNAs during the early stage of zebrafish. More importantly, the significant increase of *Gnrh2* and *Gnrh3* mRNA expression suggests that norgestrel could disrupt the development of the brain and eyes in zebrafish eleutheroembryos [39]. Similarly, it was hypothesized that norgestrel had at least 2 modes of action on the mRNA expression of cortisol synthetic genes: a direct effect and an indirect effect. Furthermore, the present results showed that ACTH is independent of corticotropin-releasing hormone during early development because the transcript of *Pomc* does not change with mRNA expression of *Crh* in response to norgestrel.

The present results demonstrated that norgestrel could affect transcriptional expression of genes related to receptor signaling pathways, steroidogenic pathways, and hypothalamic and pituitary hormones at very low concentrations (5–100 ng L^{-1}), which could regulate embryonic development and homeostasis [17,39]. Environmental concentrations of norgestrel far exceed the test concentrations of the present study [12–14], and thus, the present data imply that norgestrel in the aquatic environment could have potential effects on embryonic development, especially for the brain and gonadogenesis.

CONCLUSIONS

The results presented herein show that norgestrel could modulate transcriptional expression profiles of genes associated

with the HPG and HPA axes during the early development of zebrafish. Norgestrel could mediate multiple receptor signaling pathways in zebrafish eleutheroembryos by altering the expression of *Pgr*, *Esr1*, *Vtg1*, *Ar*, *Mr*, and *Gr* mRNAs. Moreover, norgestrel could also impair synthesis of steroid hormones by changing *Star*, *Cyp11a1*, *Cyp17*, *Cyp19a1a*, *Cyp11b*, *Hsd3b*, *Hsd20b*, *Hsd17b3*, and *Hsd11b2* mRNA expression in steroidogenic pathways. In the HPG axis, transcriptional expression of *Fshb* and *Lhb* is independent of the expression of *Gnrh2* and *Gnrh3* mRNAs after norgestrel exposure in zebrafish eleutheroembryos. Similarly, in the HPA axis, the transcript of *Pomc* is also independent of *Crh* mRNA expression following exposure to norgestrel in zebrafish development. These results indicate that norgestrel at environmental concentrations may affect embryonic brain development and the sexual differentiation of gonads, resulting in potential risks to the sexual differentiation of juveniles and reproduction of adults, which remains to be shown in further experiments.

Acknowledgment—The authors acknowledge financial support from the National Natural Science Foundation of China (41273119, U1133005, 41201522, and 41121063). This is Contribution No. 1974 from GIG CAS.

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