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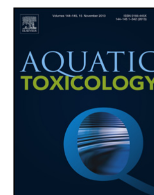

Long-term exposure to environmentally relevant concentrations of progesterone and norgestrel affects sex differentiation in zebrafish (*Danio rerio*)

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ABSTRACT

The aim of this study was to investigate the effects of progestins on the sex differentiation of zebrafish by measuring the sex ratio and transcriptions of genes related to sex differentiation (*Amh*, *Dmrt1*, *Figa*, *Sox9a* and *Sox9b* genes) as well as sex hormone levels and transcriptional expression profiles along the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes in juvenile zebrafish. Exposure of zebrafish to 4, 33, 63 ng L^{-1} progesterone (P4) or 4, 34, 77 ng L^{-1} norgestrel (NGT) started at 20 days post fertilization (dpf) and ended at 60 dpf. The results showed that exposure to P4 caused a significant increase in proportion of females as well as significant down-regulation of *Amh* gene and up-regulation of *Figa* at a concentration of 63 ng L^{-1}. However, the shift in the sex ratio toward males was observed following exposure to 34 and 77 ng L^{-1} NGT, which came along with the significant induction of *Dmrt1* gene and inhibition of *Figa* gene. The sex hormones in exposed fish were measured with estrone being detected only in the fish exposed to the highest P4 concentration; whereas estradiol and androstenedione were detected only in the fish of the control and lowest NGT concentration. Furthermore, the increase in females was associated with the significant up-regulation of several key genes controlling the synthesis of sex hormones (i.e., *Cyp17*, *Cyp19a1a* and *Hsd3b*) following exposure to 63 ng L^{-1} P4 whereas the significant down-regulation of *Cyp11a1*, *Cyp17*, *Cyp19a1a* and *Hsd3b* genes was observed in the male-biased populations caused by 34 and 77 ng L^{-1} NGT. The overall results imply that both P4 and NGT could significantly affect sex differentiation in zebrafish, and that changes may be reflected by altered sex hormone levels and transcriptional expression profiles of genes related to synthesis of sex hormones.

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1. Introduction

Human pharmaceuticals present in the aquatic environment have drawn widespread concerns as some of them may cause adverse effects on aquatic organisms (Corcoran et al., 2010; Fent et al., 2006; Kloas et al., 2009). Natural and synthetic steroids have been known to be one of the most active pharmaceutical classes, but most studies focus on the adverse effects of estrogens and androgens, for example, feminization of fathead minnow and suppression of sexual behavior in male Japanese medaka by estrogens (Kidd et al., 2007; Oshima et al., 2003), and irreversible masculinization in zebrafish caused by androgens (Morthorst et al., 2010). However, very little attention has been paid to ecotoxicological effects caused by progestins.

Natural progesterone (P4) is mainly derived from the excretion (feces and urine) of humans and animals, and it is an important

Abbreviations: NGT, norgestrel; P4, progesterone; *Amh*, anti-Mullerian hormone; *Dmrt1*, doublesex and mab-3 related transcription factor 1; *Figa*, factor in the germline alpha; *Sox9a*, sry-related high mobility group box-9a; *Sox9b*, sry-related high mobility group box-9b; *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; *Cyp19a1a*, ovarian cytochrome P450 aromatase; *Cyp11b*, 11-beta-hydroxylase; *Hsd3b*, hydroxysteroid 3-beta dehydrogenase; *Hsd20b*, hydroxysteroid 20-beta dehydrogenase; *Hsd17b3*, hydroxysteroid 17-beta 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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regulator of oocyte growth and maturation. P4 is also used in hormone replacement therapy in humans. The synthetic progestins, including norgestrel (NGT), have been used widely in oral contraceptives, contraceptive implants, intrauterine devices, vaginal ring and also as active pharmaceutical ingredients for clinical use in humans (Han et al., 2000; Kejuan et al., 2007; Qureshi and Attaran, 1999). Natural and synthetic progestins occur in the environment due to their incomplete removal in wastewater treatment plants and direct excretion of animals and direct discharge of wasters (Liu et al., 2012b). P4 and NGT were found in surface waters at concentrations up to 199 ng L^{-1} and 22 ng L^{-1}, respectively (Kolpin et al., 2002; Liu et al., 2011). In municipal wastewater treatment plant effluents, P4 and NGT were detected at a concentration of 1 and 11 ng L^{-1}, respectively (Liu et al., 2012b). In addition, P4 and NGT were detected in the receiving surface waters of swine farms with concentrations up to 30.5 and 465 ng L^{-1}, respectively (Liu et al., 2012c). Furthermore, P4 and NGT were also detected in flush waters of swine and dairy cattle farms with relatively high concentrations up to 3470–11900 ng L^{-1} and 10800–6460 ng L^{-1}, respectively, since these chemicals are often used to control pregnancy time of animals in the livestock industry (Liu et al., 2012a,c). Environmental residues of P4 and NGT may pose potential risks to aquatic organisms in the receiving aquatic environment.

Until recently, some studies have reported the reproductive effects of P4 (Blüthgen et al., 2013a; DeQuattro et al., 2012; Murack et al., 2011) and synthetic progestins (Han et al., 2014; Paulos et al., 2010; Runnalls et al., 2013; Zeilinger et al., 2009) in fish at low concentrations. However, the effects of progestins on sex differentiation in fish remain unclear. In developing fish, the balance between estrogens and androgens plays an important role in the course of sex differentiation. For example, exposure to estrogens or androgens during the early development of zebrafish resulted in a change of the sex ratio toward females or males, respectively (Baumann et al., 2013; Holbech et al., 2006). The synthetic progestin levonorgestrel has been reported to be a potent androgenic chemical in the three-spined stickleback, including induction of the normally male specific glue protein spiggin and suppression of liver vitellogenin in females (Svensson et al., 2013), and disruption of androgen-dependent reproductive cycle in males (Svensson et al., 2014). Yeast-based in vitro assay further demonstrated some synthetic progestins (e.g., levonorgestrel, norethindrone, gestodene, drospirenone, desogestrel and medroxyprogesterone acetate) showed androgenic activity (Runnalls et al., 2013). NGT is a racemic mixture of two optical isomers called enantiomers, i.e., levonorgestrel and dextronorgestrel. Furthermore, levonorgestrel is the only bioactive isomer in the racemic mixture NGT. Therefore, we hypothesize that NGT is also a chemical with androgenic activity, similar to levonorgestrel. P4 also has a weak androgenic activity as it weakly binds to the androgen receptor (Ellestad et al., 2014). In addition, 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 effects of P4 and NGT on sex differentiation are largely unknown. Therefore, it is necessary to assess and understand adverse effects of P4 and NGT on sex differentiation in fish.

As a protogynic fish, zebrafish is an important vertebrate model for the investigation of chemical effects on sex differentiation. Approximately 10 days post fertilization (dpf) the differentiation of gonads begins and zebrafish develops ovary-like gonads, regardless of chromosomal background. At approximately 20 dpf, around 50% ovaries will be transformed into testes simultaneously with ovarian apoptosis. In the following development period, ovarian development and maturation of fish continue (von Hofsten and Olsson, 2005). Development of gonads is completed at approximately 60 dpf under hormone control. Several studies reported that exposure of zebrafish to estrogens or androgens in the sensitive phase of gonadal transformation led to the alterations of the sex ratio (Holbech et al., 2006; Orn et al., 2003). Therefore, sex ratio is used as an essential endpoint for the evaluation of sex differentiation following exposure to progestins from 20 to 60 dpf.

Sex differentiation in zebrafish is distinct from mammals, since no sex chromosomes and sex determination genes have been identified in zebrafish until now. However, several genes, such as *Amh*, *Dmrt1*, *Figa*, *Sox9a* and *Sox9b*, have been associated with the process of sex differentiation in zebrafish. In mammals, anti-Mullerian hormone (AMH), produced by Sertoli cells, initiates degeneration of the Mullerian ducts and inhibits the aromatase enzyme that converts androgens to estrogens in the gonads (von Hofsten and Olsson, 2005). Although zebrafish does not have Mullerian ducts, other AMH functions may be important for male differentiation. The sex determining gene *Dmy* (also referred to as *Dmrt1y*) is located on the Y chromosome in Japanese medaka and originates from *Dmrt1* duplication, which suggests that *Dmrt1* gene is also crucial for testis development (Jorgensen et al., 2008). Furthermore, *Amh* and *Dmrt1* are expressed at high levels during testicular differentiation and are expressed at low levels or not expressed or down-regulated during ovarian differentiation in zebrafish (Jorgensen et al., 2008; Schulz et al., 2007; von Hofsten and Olsson, 2005). *Figa* plays an important role in the formation of ovarian follicle and is a stable and efficient gene expressed specifically in the zebrafish female germline, and thus it is useful for the assessment of female development (Jorgensen et al., 2008; Onichtchouk et al., 2003). The *Sox9* (*Sry*-related high mobility group box-9) gene family encodes a diverse range of developmental regulators involved in sex differentiation, which are characterized by the presence of a DNA-binding high mobility group (HMG) domain with at least 50% similarity to that of *Sry*, the sex determining factor on the Y chromosome (Jorgensen et al., 2008; Wegner, 1999). In adult zebrafish, *Sox9a* transcript was observed in testis while abundant *Sox9b* expression was seen in the ovary. The different expression patterns of two *Sox9* genes suggest they have unique functions during sex differentiation (Chiang et al., 2001; Jorgensen et al., 2008). Since *Amh*, *Dmrt1*, *Figa*, *Sox9a* and *Sox9b* are involved in sex differentiation, the transcriptional expression levels of these five genes are investigated in the present study.

The objective of this study was to assess the effects of exposure of juvenile zebrafish to environmental levels of P4 or NGT on sex differentiation. The sex ratio and transcriptional expression of genes related to sex differentiation were used as the endpoints for the evaluation of sex differentiation. In addition, the concentrations of sex hormones and transcriptional expression profiles of target genes along the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes in zebrafish were also measured in order to identify potential modes of actions of P4 and NGT on sex differentiation.

2. Materials and methods

2.1. Chemicals

Chemical standard progesterone (P4, CAS 57-83-0, purity 98%) was purchased from Tokyo Chemical Industry (Shanghai, China). Norgestrel (NGT, CAS 6533-00-2, purity 100%) was obtained from US Pharmacopeia (Rockville, MD). Stock solutions (1 mg mL^{-1}) for each compound were dissolved in 100% ethanol and then stored at $-20\,^{\circ}C$ in the dark.

2.2. Test species and experimental design

Juvenile zebrafish (*Danio rerio*) were obtained from spawning adults placed in groups of 6 males and 3 females. Newly fertilized eggs were collected and transferred into petri dishes, and kept at

26 ± 1 °C with a photoperiod of 14 h:10 h light:dark. The embryos normally hatch about 3 days post fertilization (dpf) but do not need to be fed until 4 dpf. At that time, larvae were fed with a nutrient solution twice daily until 18 dpf where the food was replaced with newly hatched baby brine shrimp (Tianjin, China). The nutrient solution was prepared by tissue homogenate of brine shrimp and filtration.

Exposure of zebrafish to the test chemicals started at 20 dpf and ended at 60 dpf. For each replicate, 20 fish were randomly placed in 5 L glass tanks containing 1–2 L of filtered and aerated tap water (1 L from 20 to 40 dpf, 2 L from 40 dpf to 60 dpf). Four replicate tanks were included for each treatment. The zebrafish were exposed to P4 or NGT at the nominal concentrations of 0, 5, 50 and 100 ng L^{-1} (0, 15.9, 159, 318 pmol L^{-1} P4; or 0, 16, 160, 320 pmol L^{-1} NGT). Ethanol was used as the solvent for the test chemicals at a concentration of 0.001%, so the solvent control and exposure groups received 0.001% (v/v) ethanol only. The experiment was performed in a semi-static system at 26 ± 1 °C with a 14 h:10 h light/dark cycle. The treatment water of the tanks was renewed daily. The conductivity (146–164 us cm^{-1}), pH (6.84–7.26) and oxygen concentration (≥75%) were recorded every five days to ensure exposure water quality. Dead fish in all the treatment tanks were recorded and removed daily during the exposure phase. At 60 dpf, 12 fish for each replicate ($n = 4$) were randomly taken and anesthetized with 0.01% tricaine methanesulfonate (MS-222, Sigma–Aldrich). Total body length (cm) and wet weight (g) were measured immediately to calculate condition factor (K-factor = (wet weight/total body length^3) × 100). Then, 5 fish per replicate were stored at −80 °C for sex hormone assay and 4 fish per replicate were homogenized using a pestle and mortar in liquid nitrogen for subsequent RNA extraction, and other 3 fish per replicate were also stored at −80 °C for backup. Although sexual differentiation of juveniles was basically complete at 60 dpf, final sex ratio was determined until zebrafish reached their adult stage at 140 dpf to see the effects of exposure to the progestins at juvenile stage. So after 60 dpf, the remaining fish were transferred to the clean water and reared under the same conditions until 140 dpf. At that time, adult zebrafish were anaesthetized and dissected for the investigations of sex ratio. The sex ratio was determined morphologically according to a previous method by Brion et al. (2004). The ovary is a bilobed structure that is suspended in the body cavity by a vascularized mesovarium in a female fish, and the testes are long, while, paired organs that are attached to the dorsal body wall in a male fish (Gupta and Mullins, 2010).

2.3. Transcriptional expression analysis

RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR) were performed according to our previous methods (Liang et al., 2015a). Briefly, total RNA was extracted from 4 fish from each tank using Trizol reagent (Invitrogen) as described by the manufacturer. The quality of total RNA was determined using the electrophoresis on agarose gel stained with gelred (Biotium, USA). RNA concentration was determined at 260 nm by using a SmartSpec™ Plus Spectrophotometer (Bio-Rad USA), and the purity was analyzed from absorbance at 260 nm and 280 nm (the 260/280 ratio 1.83 ∼ 2.03 for all RNA samples). All RNA samples were diluted with nuclease-free water to 80 μg mL^{-1} for synthesis of complementary DNA (cDNA). Approximately 500 ng of total RNA was reverse transcribed to cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo) in a total volume of 50 μL according to the manufacturer's instructions. The first strand cDNA was diluted to a final volume of 200 μL by adding 150 μL nuclease-free water, and stored at −20 °C for subsequent qRT-PCR.

All qRT-PCR reactions were analyzed on the Applied Biosystems ViiA™ 7 Dx (ABI) using the THUNDERBIRD SYBR® qPCR Mix (Toyobo) in a final volume of 20 μL, containing 10 μL of THUNDERBIRD SYBR® qPCR Mix 0.4 μL of each of forward and reverse primer (10 μM), 2.5 μL of cDNA sample and 6.7 μL nuclease-free water. Table 1 listed the primer sequences of target and housekeeping genes. All primers were designed by using Primer Premier 5.0 program and synthetized by Invitrogen (China). The amplification efficiencies of all primers were between 95% and 107%. The

Table 1
Primers for quantitative real-time PCR in zebrafish.

Gene	Sense primer (5′-3′)	Antisense primer (5′-′3′)	GenBank number
Amh	TTCCTCCACGCCGACTGTAT	CCTGCCTCCTGCTGTTTGAC	NM_001007779.1
Dmrt1	TTTACCAGCCCACTCCATACTC	AGGCGGCCATTTCCACTAG	AF439562.1
Figa	CGACACGGAGGACCGAGATA	GGCACAATACGCCGAAGATAC	NM_198919.2
Sox9a	GCCAGGCAAAGCGGATCT	GCGGGAGGTATTGGTCAAACT	NM_131643.1
Sox9b	CGAGAAGCGTCCGTTTGTG	CCGTCTGGGCTGGTATTTGTA	NM_131644.1
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

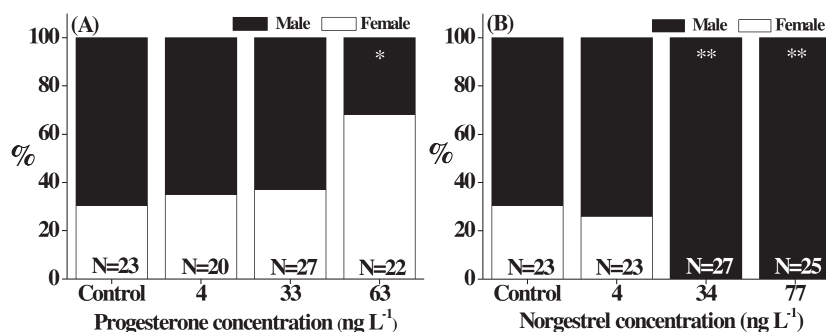

Fig. 1. The sex ratio of zebrafish after exposure to progesterone (A) or norgestrel (B) from 20 to 60 days post fertilization. The sex ratio was measured by identification of the gonadal sex in 140-day-old fish. The *N* indicates the total number of fish from four replicate tanks in each treatment group. Compared to the solvent control, statistically significant differences are marked with asterisks (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

qRT-PCR amplification was carried out for 1 min at 95 °C, followed by 40 cycles of 15 and 60 s at 95 °C and 60 °C, respectively. Melting curve analysis from 60 °C to 95 °C was performed to ensure the specificity of each amplicon. The β-*actin*, *RpL13α* and *EF1-α* were selected as reference genes for normalization because of their stable expression between the solvent control and treatment groups (ANOVA, $p > 0.05$). In addition, normalization by multiple reference genes is necessary in order to measure transcriptional levels accurately (Vandesompele et al., 2002). Therefore, the average Ct value (threshold cycle) of β-*actin*, *RpL13α* and *EF1-α* was employed as reference to normalize the mRNA expression of target genes. The relative transcriptional expression levels of each gene to solvent control were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4. Sex hormone assay

Five individual fish from each replicate were homogenized and extracted with 15 mL methanol/0.1 M acetic acid–sodium acetate buffer solution (1:1, v/v) by ultrasonication for 10 min and then centrifuged at 9391 × g for 10 min at 4 °C. The supernatant was transferred to 250 mL round-bottom flask. The fish samples were extracted again and the supernatants from the two extractions were combined. The extract in the round-bottom flask from per replicate was diluted to a final volume of 200 mL with Milli-Q water in order to keep the concentration of methanol in the solution below 5%. The aqueous solutions of fish sample extracts were analyzed according to our previous method (Liu et al., 2011). Briefly, the aqueous solutions of fish sample extracts were enriched through solid-phase extraction using HLB cartridges (6 mL, 500 mg). The cartridges were then dried under air for about 2 h before elution with 10 mL ethyl acetate. The eluted solutions were dried under a gentle stream of nitrogen and re-dissolved in 0.4 mL of methanol for instrumental analysis. Androstenedione and testosterone concentrations were measured by rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry (RRLC–MS/MS) (Agilent 1200 LC-Agilent 6460 QQQ, USA). To improve the sensitivity, a pentafluorobenzoyl chloride derivatization method was applied to the quantification of estrone and estradiol in the extracts prior to the analysis by gas chromatography–mass spectrometry with negative chemical ionization (GC–NCI–MS) (Agilent 6890N/5975B, USA) as described by Zhao et al. (2009). The limit of detection (LOD) of androstenedione, testosterone, estrone and estradiol in fish samples were 0.10 ng g^{-1}, 0.10 ng g^{-1}, 0.20 ng g^{-1} and 0.20 ng g^{-1}, respectively. The recoveries achieved for androstenedione, testosterone, estrone and estradiol in spiked biota samples (50 ng g^{-1}) were 103%, 86%, 116% and 88%, respectively.

2.5. Measurement of P4 and NGT concentrations in exposure solutions

Since the exposure solutions were renewed daily, water samples (500 mL, $n = 4$ replicate tanks) were randomly collected once at 50 dpf from all treatment groups at the beginning of exposure (0 h) and prior to water renewal (24 h). Tanks 1 and 2 were mixed together as the first composite sample at every time point. Similarly, tanks 3 and 4 were also mixed together as the second composite sample. Target compounds P4 and NGT were extracted from water samples using solid phase extraction (SPE) and then analyzed by RRLC–MS/MS (Liu et al., 2011). The LODs for P4 and NGT were 0.05 ng L^{-1} and 0.04 ng L^{-1}, respectively (Liu et al., 2011). The recoveries determined by spiking surface waters with known amounts of P4 and NGT (5 and 100 ng L^{-1}), were 92.4–102% and 87.5–96.8%, respectively (Liu et al., 2011).

2.6. Statistical analysis

Statistical software SPSS (version 13.0) was used for all statistical analyses. Kolmogorov–Simirnov and Levene's tests were used to test for normality and homogeneity of variances, respectively. If necessary, raw data were transformed to meet the assumptions of one-way analysis of variance (ANOVA). The significance of differences in relative mRNA expression and sex hormone production

Table 2
The nominal and measured concentrations of the two progestins in the exposure experiment.

Compound	Exposure time (h)[a]	Nominal concentration (ng L^{-1})			
		0	5	50	100
Progesterone	0	0	5.27 ± 0.03[b]	43.34 ± 0.30	82.53 ± 0.06
	24	0	3.68 ± 0.36	21.71 ± 1.46	43.90 ± 7.50
	Average	0	4.48 ± 0.20	32.53 ± 0.88	63.22 ± 3.78
Norgestrel	0	0	4.42 ± 0.20	34.76 ± 0.71	78.36 ± 4.43
	24	0	4.00 ± 0.06	32.66 ± 0.71	76.18 ± 2.81
	Average	0	4.21 ± 0.13	33.71 ± 0.71	77.27 ± 3.62

[a] Exposure time (0 h and 24 h).
[b] Measured concentrations are given as mean ± S.D. ($n = 2$ replicates).

Table 3
Changes in the concentrations of sex hormones following exposure to progesterone or norgestrel from 20 to 60 days post fertilization. Data are represented as mean ± SEM of five individual juvenile zebrafish per replicate (n = 4 replicates).

Steroid hormone (ng g^{-1})	Progesterone concentration (ng L^{-1})				Norgestrel concentration (ng L^{-1})			
	0	4	33	63	0	4	34	77
Estrone	N.D.[a]	N.D.	N.D.	6.05 ± 0.53	N.D.	N.D.	N.D.	N.D.
Estradiol	0.49 ± 0.10	0.52 ± 0.10	0.40 ± 0.06	0.40 ± 0.07	0.49 ± 0.10	0.52 ± 0.06	N.D.	N.D.
Androstenedione	5.18 ± 0.39	4.67 ± 0.29	4.74 ± 0.16	4.54 ± 0.37	5.18 ± 0.39	4.71 ± 0.56	N.D.	N.D.
Testosterone	3.19 ± 0.25	2.61 ± 0.28	2.41 ± 0.07	3.87 ± 0.47	3.19 ± 0.25	3.29 ± 0.21	2.85 ± 0.29	2.52 ± 0.27

[a] N.D. – not detected.

were analyzed by ANOVA followed by Tukey multiple comparison tests. The chi-square test was used to examine significant differences in the sex ratio between the solvent control and exposure groups. When the expected values in any of the cells of a contingency table are below 5, the Fisher's exact test was used instead of chi-square test. A $p \le 0.05$ was considered statistically significant differences relative to the solvent control.

3. Results

3.1. Measured concentrations of P4 and NGT

The measured concentrations of P4 and NGT at the beginning of exposure (0 h) and prior to water renewal (24 h) are given in Table 2. The measured concentrations of NGT were stable during the exposure period and average concentrations were 4, 34 and 77 ng L^{-1} in the three exposure solutions, respectively. The measured concentrations of P4 at T_0 were found very close to the nominal concentrations but were further decreased over the 24 h period, with average concentrations of 4, 33 and 63 ng L^{-1} for the three nominal concentrations, respectively. In addition, P4 or NGT was not detected in the solvent control. All exposure concentration data are presented in their average measured concentrations hereafter.

3.2. Fish survival and growth after exposure to P4 or NGT

Exposure to P4 or NGT had no significant effects on fish survival and the survival rates in all treatments were greater than 85% (data not shown). There were no significant changes in the total body length, wet weight and K-factor in fish between the solvent control and exposure groups (P4 or NGT) (data not shown).

3.3. Sex ratio after exposure to P4 or NGT

As shown in Fig. 1, P4 and NGT had different effects on the sex ratio. Exposure to P4 resulted in a significant increase in the proportion of females at a concentration of 63 ng L^{-1}. However, NGT treatment had a very strong effect on sex differentiation with the sex ratio shifting toward males, and no females could be identified at concentrations of 34 and 77 ng L^{-1}.

3.4. Concentrations of sex hormones after exposure to P4 or NGT

Table 3 shows the concentrations of sex hormones in whole fish collected at termination of the exposure experiment. For P4 treatments, estrone was only detected at a concentration of 6.05 ng g^{-1} in the fish exposed to 63 ng L^{-1}. Estradiol and androstenedione were detected in all P4-exposed groups and remained unaffected in comparison with the solvent control ($p > 0.05$). However, for NGT treatments, estradiol and androstenedione were only detected in the lowest concentration treatment (4 ng L^{-1}) and also showed no significant difference in their levels when compared with the solvent control ($p > 0.05$). But no estradiol and androstenedione were detected in the fish following exposure to the middle and highest concentrations of NGT. No significant alterations for the production of testosterone were observed following exposure to P4 or NGT ($p > 0.05$).

3.5. Gene transcriptional expression profiles after exposure to P4 or NGT

Fig. 2 shows the gene transcriptional expression profiles involved in the sex differentiation. P4 exposure significantly decreased the transcript of *Amh* gene but increased the expression of *Figa* mRNA in the 63 ng L^{-1} group (Fig. 2A). The significant up-regulation of *Dmrt1* transcription and down-regulation of *Figa* transcription were observed in the 34 and 77 ng L^{-1} NGT-exposed groups (Fig. 2B).

Gene transcriptional expression profiles along the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes are shown in Fig. 3. For the receptor signaling pathway, exposure to 63 ng L^{-1} P4 and 77 ng L^{-1} NGT significantly induced the expression levels of *Pgr*

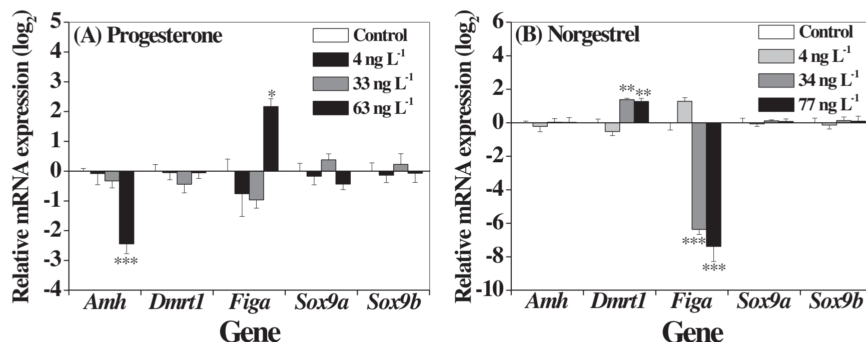

Fig. 2. Gene transcriptional expression profiles involved in the sex differentiation in juvenile zebrafish after exposure to progesterone (A) or norgestrel (B) from 20 to 60 days post fertilization. The results are represented as mean ± SEM (n = 4 replicates). Compared to the solvent control, statistically significant differences are marked with asterisks (*$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$).

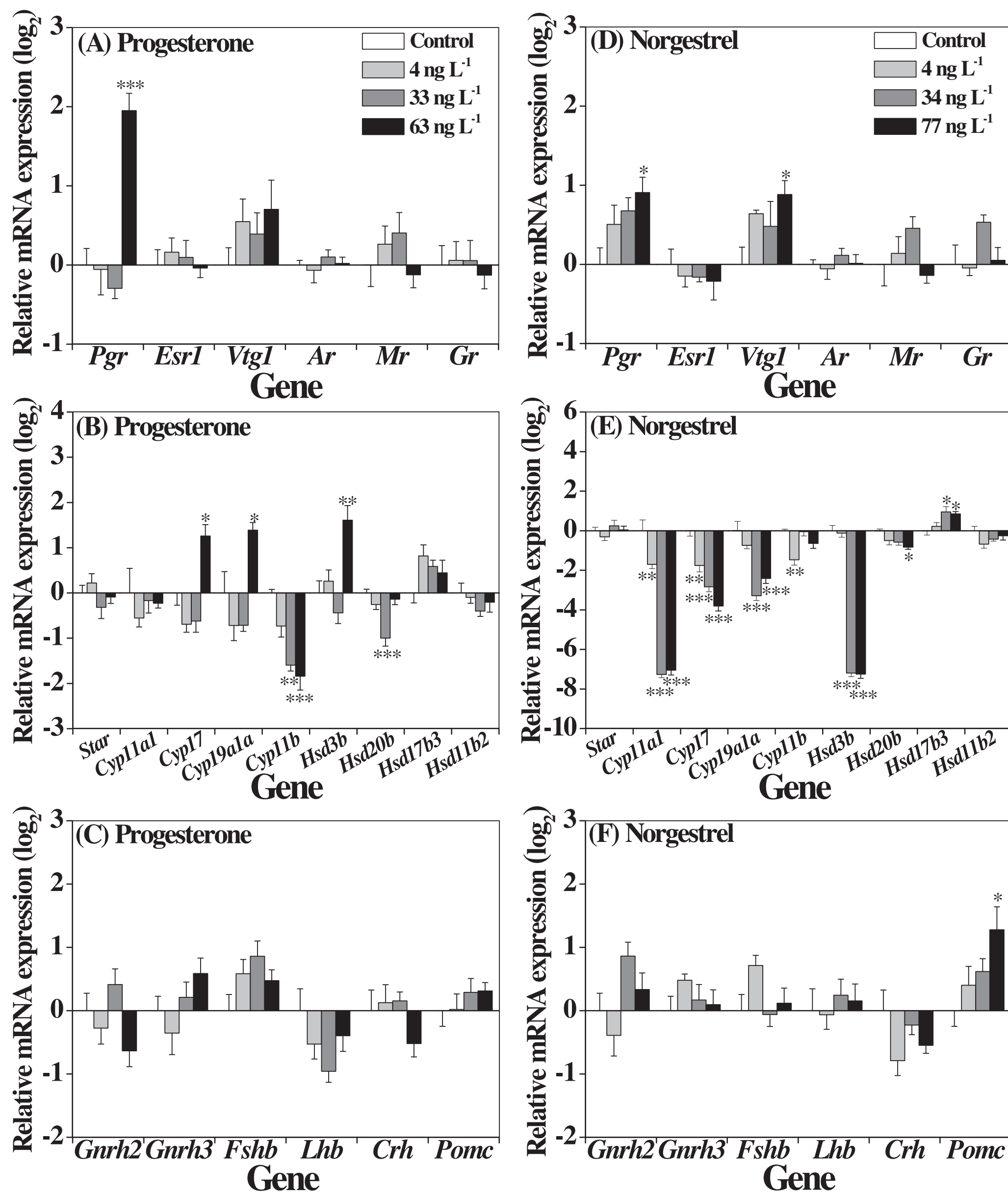

Fig. 3. Gene transcriptional expression profiles along the hypothalamic-pituitary-gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes in juvenile zebrafish after exposure to progesterone (A–C) or norgestrel (D–F) from 20 to 60 days post fertilization. The results are represented as mean ± SEM ($n = 4$ replicates). Compared to the solvent control, statistically significant differences are marked with asterisks (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

mRNA (Fig. 3A and D). In addition, P4 exposure had no significant effects on the expression of *Vtg1* mRNA (Fig. 3A), while 77 ng L^{-1} NGT significantly up-regulated the transcription of *Vtg1* gene (Fig. 3D). For the steroidogenic pathway, the expression levels of *Cyp11a1* mRNA remained unaffected by P4 (Fig. 3B), while NGT at all exposure concentrations significantly suppressed *Cyp11a1* mRNA expression (Fig. 3E). Transcripts of *Cyp17*, *Cyp19a1a* and *Hsd3b* were all significantly up-regulated following exposure to 63 ng L^{-1} P4 (Fig. 3B), while they were significantly down-regulated by NGT at 34 and 77 ng L^{-1} (Fig. 3E). The expression levels of *Cyp11b* mRNA were significantly decreased by 33 and 63 ng L^{-1} P4, and 4 ng L^{-1} NGT (Fig. 3B and E). Significant down-regulation of *Hsd20b* mRNA was also observed in the 33 ng L^{-1} P4 and 77 ng L^{-1} NGT groups (Fig. 3B and E). In addition, the transcription of *Hsd17b3* gene was significantly suppressed following exposure to 34 and 77 ng L^{-1} NGT, but not for P4 (Fig. 3B and E). For the hypothalamic and pituitary hormones, P4 treatment had no significant effects on the expression levels of *Gnrh2*, *Gnrh3*, *Fshb*, *Lhb*, *Crh* and *Pomc* mRNAs, and only the transcription of *Pomc* gene was significantly increased by NGT at 77 ng L^{-1} (Fig. 3C and F).

4. Discussion

The present study demonstrated that exposure to environmentally relevant concentrations of P4 and NGT could affect the sex differentiation of zebrafish. Previous studies showed that sex ratio could be an effective biomarker for the assessment of endocrine disrupting chemicals (EDCs) (Baumann et al., 2013; Holbech et al., 2006); therefore, it was used in the present study to evaluate the effects of progestins on sex differentiation. Exposure of juvenile zebrafish to P4 from 20 to 60 dpf led to a significant shift in sex ratio toward females in the 63 ng L^{-1} group, which is consistent with the significant inhibition of *Amh* gene (related to male differentiation) and induction of *Figa* gene (related to female differentiation). A

change in sex ratio after P4 exposure is supported by a previous study, which has shown that P4 during the critical time for sex determination could cause biased sex ratio toward females in birds (Correa et al., 2005).

As for NGT, the present study found that it caused a very strong effect on sex differentiation, leading to the shift of sex ratio toward males. At the same time, the transcriptional expression of key genes involved in sex determination and differentiation were also altered, with a significant up-regulation for *Dmrt1* gene and down-regulation for *Figa* gene. The previous reports showed that levonorgestrel (or L-norgestrel) showed strong androgenic effects in three-spined sticklebacks, including induction of male specific glue protein spiggin and suppression of liver vitellogenin in females, and disruption of androgen-dependent reproductive cycle in males (Svensson et al., 2013, 2014), and also in fathead minnows causing masculinization of females (Zeilinger et al., 2009). Furthermore, levonorgestrel proved to be a strong agonist of androgen receptor (Ellestad et al., 2014). Taken together, these results suggest that NGT (or D,L-norgestrel) is a chemical with potent androgenic activity by binding to androgen receptor, which could account for the observed male-biased population. We previously demonstrated that the transcriptional expression of *Ar* gene varied during different developmental stages of zebrafish (Liang et al., 2015b). Therefore, the present study found *Ar* remained unaffected by NGT, which may not contradict the fact that NGT can alter sex ratio through an androgen receptor-mediated pathway. It is known that the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish is mediated through apoptosis (von Hofsten and Olsson, 2005). Furthermore, previous studies (Meresman et al., 2002; Rodriguez et al., 2002) provided evidences that progestins could induce apoptosis in the macaque ovarian epithelium and human eutopic endometrium. Consequently, the present study possibly implies that NGT could induce oocyte apoptosis and accelerate the formation of testicular tissues, and finally resulting in a male-biased sex ratio.

It is known that sex hormones regulate normal sex differentiation of zebrafish (Baumann et al., 2013; Holbech et al., 2006). For the first time, we demonstrated that P4 and NGT could alter the sex ratio of the fish population and transcriptional expression of key genes involved in sex differentiation. Furthermore, exposure to P4 or NGT could regulate sex hormone levels in juvenile zebrafish. In the present study, estrone could be only measured in the highest concentrations of P4, which is concomitant with more females compared with the solvent control. It is interesting that P4 had no significant effects on the levels of estradiol, androstenedione and testosterone, compared with sex ratio showing a female-biased pattern in the 63 ng L^{-1} group. Therefore, the present results possibly suggest that the balance of sex hormones was disrupted by P4, which may account for the observed female-biased population. In contrast, estradiol could not be detected after exposure to 34 and 77 ng L^{-1} NGT, which is consistent with a change of sex ratio toward to males. However, androstenedione and testosterone levels were not increased by NGT. It is more likely to imply that the disruption of the balance of estrogens and androgens could be a major factor contributing to the observed change in sex ratio toward males following exposure to NGT. The findings of sex hormone assay provide evidence that P4 and NGT may alter the balance of sex hormones in juvenile zebrafish at low concentrations as measured in the aquatic environment, thereby leading to disruption of sex differentiation in zebrafish.

As is known, the reproductive and adrenal endocrine systems are primarily controlled by HPG and HPA axes, respectively. Progestins have been reported to modulate HPG and HPA axes (Blüthgen et al., 2013b; Han et al., 2014; Overturf et al., 2014). In humans and fish, progestins could activate progesterone receptors (*Pgr*) to elicit their specific physiological responses (Africander et al., 2011). In the present study, the significant transcriptional induction of *Pgr* gene clearly reflects the progestogenic activity of P4 in juvenile zebrafish, which was also found in zebrafish eleutheroembryos (Zucchi et al., 2012). The significant up-regulation of *Cyp17*, *Cyp19a1a* and *Hsd3b* genes was observed in the 63 ng L^{-1} P4 group, which may provide a plausible mechanism for the observed change in sex ratio toward females. For the NGT exposure, transcripts of *Cyp11a1*, *Cyp17*, *Cyp19a1a* and *Hsd3b* genes were strongly inhibited in the 34 and 77 ng L^{-1} groups; at the same time, no females observed. Alterations of these genes may also contribute to male differentiation following exposure to NGT. The effects of P4 and NGT on transcriptional expression of genes involved in the hypothalamic and pituitary hormones were relatively low. Therefore, we believe a direct P4 or NGT action on steroidogenic genes, not an indirect effect via changing the production of hypothalamic and pituitary hormones (Baron et al., 2005; Laughlin et al., 2010; Weltzien et al., 2004). The overall results from the transcriptional data in the present study showed that P4 and NGT could mainly alter transcripts of steroidogenic genes, and may finally translate to the observed disruption in sex differentiation.

5. Conclusions

The results from this study have showed that P4 and NGT could cause a disruption of sex differentiation at environmentally relevant concentrations based on the skewed sex ratio in the subsequent adult population. P4 and NGT were demonstrated to significantly induce more females and more males, respectively, which were accompanied with the regulation of the transcriptional levels of several genes (i.e., *Amh*, *Dmrt1* and *Figa*) related to sex differentiation. In addition, the balance of estrogens and androgens in fish might be altered following exposure to the two chemicals. Meanwhile, P4 and NGT could mainly mediate the transcriptional expression of steroidogenic genes. The modulating hormones and genes associated with steroid synthesis could contribute to disruption of sex differentiation in zebrafish.

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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.2015.01.006.

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