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The binary mixtures of megestrol acetate and 17α-ethynylestradiol adversely affect zebrafish reproduction☆

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Synthetic progesterones and estrogens are broadly used bioactive pharmaceutical agents and have been detected in aquatic environments. In the present study, we investigated the combined reproductive effects of megestrol acetate (MTA) and 17α-ethinylestradiol (EE2) on zebrafish. Adult zebrafish were exposed to MTA (33, 100 or 333 ng/L), EE2 (10 ng/L) or a mixture of both (MTA + EE2: 33 + 10, 100 + 10 or 333 + 10 ng/L) for 21 days. Results demonstrated that egg production was significantly reduced by exposure to 10 ng/L EE2, but not MTA. However, a combined exposure to MTA and EE2 caused further reduction of fish fecundity compared to EE2 exposure alone, suggesting an additive effect on egg production when EE2 is supplemented with MTA. Plasma concentrations of 17β-estradiol and testosterone in the females and 11-ketotestosterone in the males were significantly decreased in the groups exposed to EE2 or MTA alone compared with the solvent control, and the plasma concentrations of the three hormones were further reduced in the co-exposure groups relative to the MTA exposure group, but not the EE2 exposure group. These data indicate that the inhibitory effects on plasma concentrations in the co-exposures were predominantly caused by EE2. Furthermore, exposure to MTA and EE2 (alone or in combination) led to histological alterations in the ovaries (decreased vitellogenic/mature oocytes), but not in the testes. This study has important implications for environmental risk assessment of synthetic hormones that are concurrently present in aquatic systems.

1. Introduction

Synthetic progesterones (progestins) and estrogens are widely used in human and veterinary medicine, and have various therapeutic applications (Aris et al., 2014; Fent, 2015). In many countries (including USA and China), progestins are also used in agriculture as growth promoters (Fent, 2015). Progestins are generally used alone or in combination with estrogens for contraception (Erkkola and Landgren, 2005), and are therefore discharged into the aquatic environment as waste-water or agricultural waste from livestock farms (Aris et al., 2014; Fent, 2015).

Megestrol acetate (MTA) is a widely used active pharmaceutical progestin (Argiles et al., 2013), and up to 34 ng/L of MTA was reported in the surface water in China (Chang et al., 2009, 2011). The synthetic estrogen 17α-ethinylestradiol (EE2) is also used in many formulations, particularly in combined oral contraceptive pills, and is ubiquitous in the aquatic environment worldwide, and high EE2 concentrations (up to 41 ng/L) have been reported in surface waters (Valdés et al., 2015; Kolpin et al., 2002). In China, 24 ng/L of EE2 was reported in the surface water in 2009 (Lei et al., 2009), and up to 357 ng/L was reported in the flush water of a swine farm in the south (Liu et al., 2012).

Synthetic estrogens and progestins are highly potent chemicals, and exposure to these hormones may have significant adverse effects on aquatic wildlife (Caldwell et al., 2008; Runnalls et al., 2013; Säfholm et al., 2015). Several studies have indicated that exposure of fish to environmental concentrations of EE2 can lead to vitellogenin (VTG) synthesis induction, gametogenesis impairment, reproductive failure, disruption of sex differentiation, and alterations in aggression and courtship behaviors in males (Colman et al., 2009; Hill and Janz, 2003; Santos et al., 2007; Weber et al., 2003). However, progestins have attracted attention and research interests only in the past few years. Recent studies have

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demonstrated that progesterone and progestins at environmental concentrations can cause endocrine disruptions (Kroupova et al., 2014; Paulos et al., 2010; Petersen et al., 2015; Runnalls et al., 2013, 2015; Zucchi et al., 2013, 2014), affect sex differentiation (Liang et al., 2015; Hua et al., 2015), reduce egg production (DeQuattro et al., 2012; Paulos et al., 2010; Runnalls et al., 2013, 2015; Zeilinger et al., 2009; Zhao et al., 2015a, b) and disrupt reproductive cycles (Svensson et al., 2014) in fish.

Aquatic organisms are exposed to various combinations of synthetic hormones, however, a limited number of environmental risk assessment studies have been conducted (Zucchi et al., 2014; Zhao et al., 2015a; Runnalls et al., 2015). Recent studies demonstrated that exposure to a mixture of progestins can have additive effects on zebrafish reproduction (Zucchi et al., 2014; Zhao et al., 2015a), and combined exposure to synthetic estrogens and progestins can lead to alterations in plasma sex hormones and reduction in egg production in fathead minnow (Runnalls et al., 2015).

In the present study, we investigated the combined effects of different concentrations of MTA and EE2 on zebrafish reproduction, by assessing plasma sex hormones, gametogenesis, gene transcription along the hypothalamic-pituitary-gonadal (HPG) axis in females, and reproductive outcome (egg production).

2. Materials and methods

2.1. Chemicals and reagents

MTA (CAS number 595-33-5; purity >99.7%), EE2 (CAS number 57-63-6; purity >98%) and dimethyl sulfoxide (DMSO; CAS number 67-68-5; purity ≥99.5%) were purchased from Sigma-Aldrich (Fluka, Shanghai, China). Progesterone-d9 (P-d9; CAS number 15775-74-3; purity >98%) and ethynylestradiol-d4 (EE2-d4; CAS number 350820-06-3; purity ≥98%) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). All other reagents used in this study were of analytical grade. MTA and EE2 stock solutions (1 mg/mL) were dissolved in DMSO and stored at 4 °C.

2.2. Fish maintenance and experimental design

Five-month-old zebrafish (*Danio rerio*; wild-type, AB strain) were randomly placed into 30-L glass tanks containing 20 L water. The fish were maintained in a semistatic system with charcoal-filtered tap water (pH 7.0–7.4) at 28 ± 0.5 °C with a 14:10 light/dark cycle. After an acclimatization of 7 days, the experiment started with a pre-exposure period of 14 days to establish the baseline rate of fecundity for each tank (spawning group) (Ankley et al., 2001), followed by 21 days of exposure. The experimental set-up consisted of the following exposure treatments: solvent control (SC; containing 0.001% [v/v] DMSO); EE2: 10 ng/L (0.034 nM); MTA: 33, 100 and 333 ng/L (corresponding to 0.086, 0.260 and 0.866 nM, respectively); EE2 + MTA combinations: 10 ng/L EE2 + 33 ng/L MTA, 10 ng/L EE2 + 100 ng/L MTA and 10 ng/L EE2 + 333 ng/L MTA. One fixed concentration of EE2 and three concentrations of MTA were examined to determine the joint effects of them on fish reproduction. The chosen EE2 concentration (10 ng/L; 0.034 nM) is an environmentally relevant concentration at which impaired reproduction has been reported in zebrafish (Van den Belt et al., 2001, 2003). The highest MTA concentration used in the present study has been previously shown not to inhibit egg production in zebrafish (Han et al., 2014). There were 3 replicates for each exposure group and each tank contained 6 males and 6 females. During the experimental period, the exposure water was renewed once daily. The fish were fed twice daily with newly hatched brine shrimp and pellet food (Zeigler Brothers, Gardners, PA, USA). Eggs were collected daily. The fish were maintained in accordance with guidelines for the care and use of laboratory animals of the National Institute for Food and Drug Control of China.

After 21 days of exposure, the fish were euthanized using an overdose of 300 mg/L MS-222 (Sigma-Aldrich, Shanghai, China) by prolonged immersion until cessation of opercular movement. The fish were measured for body length and wet weight to calculate the condition factor ($K = [\text{wet weight [g]}/\text{body length [cm]}^3] \times 100$). Blood samples were collected from the caudal vein of the fish with a glass capillary and transferred into heparin sodium-rinsed tubes. After centrifugation (7000 × *g* for 5 min at 4 °C), the blood plasma was stored at −80 °C for hormone analysis. All the fish were dissected immediately. Testes and ovaries were collected from individuals and weighed in order to assess the gonadosomatic index ($GSI = \text{gonad weight [g]}/\text{body weight [g]} \times 100$). Two fish from each tank (n = 3 replicates; a total of 6 fish sampled for each exposure concentration) were fixed in Bouin's solution for 24 h and then kept in 70% ethanol until further histological processing.

2.3. Sex hormone assay

The plasma samples from 3 individual fish of the same sex were pooled as one sample. Plasma extraction and the determination of sex hormone concentrations were performed as previously described (Drevnick and Sandheinrich, 2003). Briefly, 10 μL of plasma from each biological replicate was diluted to 400 μL using ultrapure water (Milli-Q, Millipore, Billerica, MA, USA) and extracted twice with 2 mL ethyl ether. The ether phase was collected and evaporated under a gentle stream of nitrogen gas. The residues were redissolved with the buffer provided in the detection kits. Estradiol (E2), testosterone (T) and 11-ketotestosterone (11-KT) concentrations were measured using enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA). The detection limits were 20 pg/mL for E2, 6 pg/mL for T and 1.3 pg/mL for 11-KT. The intra- and inter-assay coefficients of variance were <20%, <15% and <15%, for E2, T and 11-KT assay, respectively.

2.4. Gonadal histology

The fixed samples were dehydrated and paraffin-embedded, sectioned into 4 μm sections along the long axis of the gonad, and mounted on slides. The slides were then stained with hematoxylin and eosin (Sigma-Aldrich, Shanghai, China). A total of 9 tissue sections per sample were collected from three steps equally spaced between the leading edge of the tissue and the midline of the gonad. Six samples from each sex and treatment group were randomly selected for histological and stereological analyses. Sections from all treatment groups were examined under an Olympus MVX10 light microscope, equipped with an Olympus Camedia C-5050 camera (Olympus Co. Ltd., Shanghai, China). Ovarian staging identified primary oocytes (PO), early vitellogenic oocytes (EVO), late vitellogenic/mature oocytes (LVO/MO), and atretic follicles (AF), and testicular staging identified spermatogonia, spermatocytes and spermatids present (Johnson et al., 2009; Wang et al., 2015). The ovarian and testicular staging were further quantified using a previously described method (Wang et al., 2015). Quantitatively staging of the ovaries was based on the relative percentages of oocytes/follicles at different development stages by counting their numbers, while quantitatively testicular staging was based on the relative percentages of sperm cells at different developmental stages by measuring their areas occupied in every fish and gonad with Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

2.5. Quantitative real-time PCR assay

Total RNA was extracted from the brains (2 pooled as one sample) and gonads of the females (n = 3 replicates), using a RNAiso plus kit (Takara, Dalian, China), in accordance with the manufacturer's instructions. The procedures for RNA extraction, purification, quantification and first-strand cDNA synthesis were carried out as previously described (Han et al., 2014). Oligonucleotide primers specific to each of the selected genes were obtained by using Primer 3 (http://frodo.wi.mit.edu/; see supporting information; Table S1). The amplification efficiencies of primers and transcriptional stability of 5 candidate housekeeping genes (*rpl8*, *18s*, *β-actin*, *gapdh*, *ef1α*) were assessed using geNorm analyses (http://medgen.ugent.be/genorm). The results of the analysis by geNorm analyses showed that *β-actin* was the most stable gene and was selected as the reference gene for the transcription assay (see supporting information; Fig. S1). The relative transcriptional abundance of each target gene (*cyp17*, *cyp19a*, *17βhsd*, *ptgs2*, *lhr*, *lhβ*) to its corresponding SC group was carried out on an ABI 7300 system (PerkinElmer, Applied Biosystems, Foster City, CA, USA) and changes in mRNA expression analysed by the $2^{-\Delta\ \Delta CT}$ method.

2.6. Chemical analysis

The exposure concentrations of MTA and EE2 were measured in the first and the third week, respectively. The water samples were collected into amber glass bottles (n = 3 tanks, each containing 500 mL exposure water), just after (T_0) and before renewal (T_{24}) of the water solution. The extraction and analysis of megestrol acetate (MTA) were carried out as previously described (Liu et al., 2011). Briefly, the water samples were passed through 0.7 μm glass-fiber filters (Whatman, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). MTA was extracted using a preconditioned 6-mL Oasis HLB solid phase extraction cartridge (500 mg Sorbent per cartridge; Waters, USA). The cartridges were then dried and eluted with ethyl acetate. The extracts were dried and redissolved in 1 mL of methanol for instrumental analysis. For EE2, the extraction and analysis were carried out as described previously (Sun et al., 2009). Briefly, the water samples were passed through 0.7 μm glass-fiber filters and EE2 was extracted using preconditioned 3-mL C18 SPE cartridge (500 mg: CNW Technologies, China). Afterwards, the cartridges were dried and eluted with acetonitrile. Then the elution was evaporated to dryness with rotary evaporator, reconstituted in acetonitrile to a final volume of 1 mL and filtered for analysis. The concentrations of MTA and EE2 were analysed by liquid chromatography-tandem mass spectrometry (Agilent 6460; Agilent Technologies, CA USA). For MTA, the limit of detection (LOD) and the limit of quantitation (LOQ) of the LC–MS/MS analysis method were 0.12 ng/L and 0.40 ng/L, respectively; For EE2, the LOD and LOQ were 0.30 ng/L and 0.82 ng/L, respectively. The percentages of recovery, determined by spiking clean water samples with known amounts of MTA (20, 200 and 800 ng/L) and EE2 (10 ng/L), were 68.0%, 79.5%, 81.5% and 82.5%, respectively. As a quality control for the used analytical techniques, 100 μL of 1 mg/L of P-d9 or EE2-d4 was added to each sample as the internal standard.

2.7. Statistical analysis

Normality and homogeneity of variance were tested by using Kolmogorov-Smirnov and Levene's tests, respectively. If necessary, raw data were transformed to meet the assumptions of parametric statistics. Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test by using SPSS version 13.0 software (Chicago, IL, USA). A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. MTA and EE2 concentrations in water

The measured MTA and EE2 concentrations are shown in Table 1. The concentrations of MTA were lower (8.9–15.5% decrease) but close to the nominal concentrations just after renewal (T_0) of the exposure solutions. However, the measured concentrations were further decreased (27.7–55.2% decrease) after 24 h of exposure (T_{24}). The concentrations of EE2 in the water were also lower (18–23% decrease) but close to nominal concentrations just after renewal (T_0) of the exposure solutions; while a further decrease in the concentrations was measured after 24 h of exposure (T_{24}; 37.0–45.0% decrease). The decreased concentrations following 24 h of exposure could be attributed to sorption to fish, tank walls, water particulates, or to degradation (Zeilinger et al., 2009). The concentrations of MTA or EE2 in the solvent control tanks were below the detection limit. For simplification, all results are presented using the nominal concentrations.

3.2. Growth index

No mortality was observed in any group during the 14 days pre-exposure and 21 days exposure period. In the females, exposure to MTA, EE2 or their mixtures did not cause significant changes in body length and weight, condition factor (K), and gonadosomatic index (GSI; supporting information, Table S2). Similarly, in the males, exposure to MTA or EE2 did not affect growth, K or GSI, however, GSI was significantly increased in the co-exposure groups compared with those exposed to 10 ng/L EE2 alone (supporting information, Table S2).

Table 1
The measured concentrations of MTA and EE2 in each single or co-exposure group by HPLC-MS/MS analysis. The co-exposure groups included: 33 ng/L MTA +10 ng/L EE2, 100 ng/L MTA +10 ng/L EE2 and 333 ng/L MTA +10 ng/L EE2 groups. "—" indicates that was not determined. LOD = limit of detection. Data are the means of six samples from two different sampling time points (at the 1st and the 3rd week, three replicates at each time). The water was sampled just after renewal (T_0) and before renewal (T_{24}). *RR* (%) = mean residual ratios of MTA after 24 h.

Exposure groups		MTA (ng/L)			EE2 (ng/L)		
		T_0	T_{24}	RR%	T_0	T_{24}	RR%
Solvent control (SC)		< LOD	< LOD	—	<LOD	<LOD	—
EE2	10	—	—	—	8.2 ± 0.2	6.5 ± 0.3	79.3
MTA	33	27.9 ± 4.3	14.8 ± 1.1	53.1	—	—	—
	100	87.7 ± 3.3	63.4 ± 2.1	72.3	—	—	—
	333	303.4 ± 17.4	240.7 ± 21.8	79.3	—	—	—
EE2+MTA	10 + 33	26.4 ± 3.1	13.6 ± 1.3	51.6	8.1 ± 0.9	6.3 ± 1.2	77.8
	10 + 100	85.6 ± 3.4	62.3 ± 2.1	72.7	7.8 ± 0.3	5.7 ± 0.4	73.1
	10 + 333	325.4 ± 12.0	229.0 ± 12.5	70.4	7.7 ± 0.9	5.5 ± 0.5	71.4

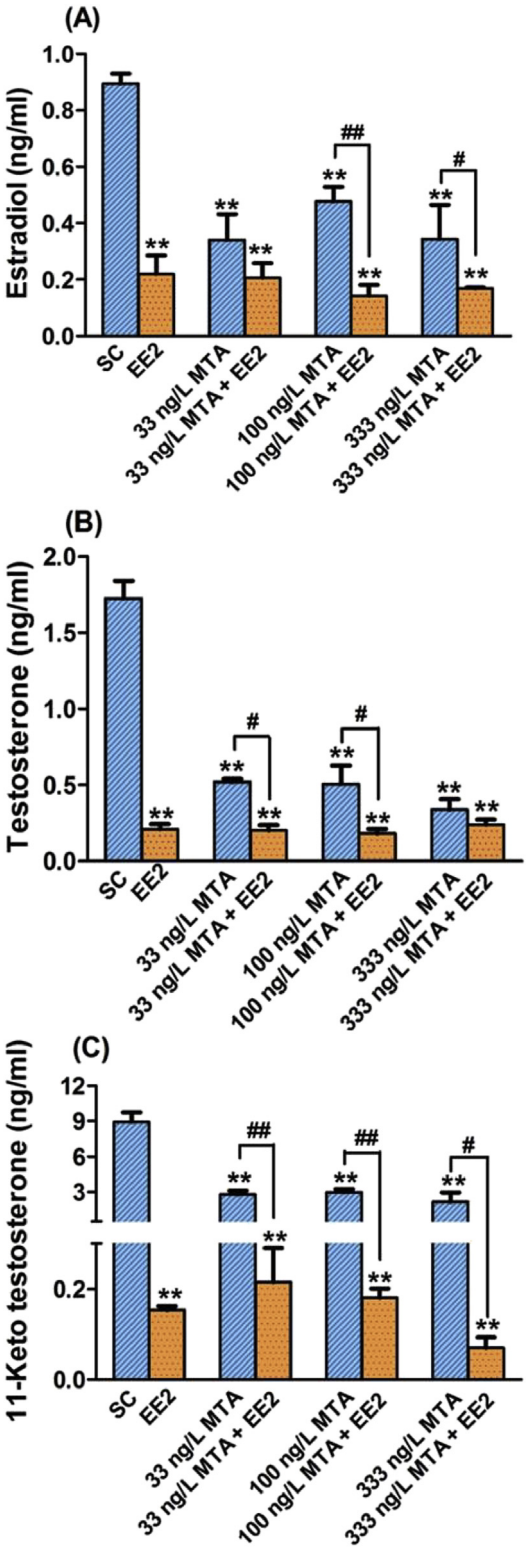

Fig. 1. Plasma concentrations of (A) 17β-estradiol (E2) and (B) testosterone (T) in female zebrafish and (C) 11-ketotestosterone (11-KT) in male zebrafish after exposure to MTA (33, 100 and 333 ng/L), EE2 (10 ng/L) and MTA/EE2 combinations (33 ng/L MTA + 10 ng/L EE2; 100 ng/L MTA + 10 ng/L EE2; 333 ng/L MTA + 10 ng/L EE2) for 21 days. Data are expressed as mean ± standard error (SEM) of three replicate samples (three fish pooled as per sample). $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences between solvent control (SC) and exposure groups. $^{\#}P < 0.05$) and $^{\#\#}P < 0.01$ indicate the statistically significant differences between the single exposure group and corresponding co-exposure groups.

3.3. Sex hormones

In the females, exposure to EE2 caused significant decrease in the plasma concentrations of 17β-estradiol (E2) by 75.4%, while the plasma E2 concentrations were significantly decreased by 62.0%, 46.7% and 61.6%, respectively, in the MTA exposure, compared with the solvent control (Fig. 1A). Co-exposure of 10 ng/L EE2 + MTA (100 ng/L and 333 ng/L MTA, respectively) further caused a significant decrease (69.9% and 50.5%, respectively) in plasma E2 concentration relative to the corresponding MTA single treatment (Fig. 1A). Likewise, a significant reduction of plasma T concentration by 87.8% was measured in EE2 single treatment group, while exposure to MTA caused a significant decrease in T concentration by 69.9%, 70.6% and 80.5%, respectively, compared with the solvent control (Fig. 1B). The plasma T concentrations were further reduced in the combined EE2 and MTA (33 ng/L and 100 ng/L MTA; 61.5% and 64.5%, respectively) co-exposure groups, compared with the corresponding MTA single treatment group (Fig. 1B).

In the males, a significant decrease in plasma 11-KT concentration (by 98.3%) was measured in EE2 single treatment group, and exposure to MTA caused a significant decrease in 11-KT by 68.8%, 66.7% and 75.8%, respectively, when compared with solvent control group (Fig. 1C). Furthermore, the plasma 11-KT concentrations were further significantly reduced (92.3%, 94.0% and 96.8%) in the EE2 and MTA (33, 100, and 333 ng/L MTA) co-exposure groups, compared with the corresponding MTA single treatment group (Fig. 1C).

3.4. Gene transcriptions

To further investigate the potential mechanisms implicated in the hormonal exposure-induced altered reproductive function, the genes along the HPG axis involving steroid production, maturation and ovulation of oocytes were evaluated in the females.

In the ovaries, exposure to 10 ng/L EE2 alone significantly downregulated the expression of *cyp17* (2.7-fold), *cyp19a* (2.9-fold), *17βhsd* (2.9-fold), *lhr* (15.9-fold), but not *ptgs2*, when compared with the solvent control group (Fig. 2A–E). Significant downregulation of *cyp17* (1.8, 2.1 and 3.2-fold) and *cyp19a* (2.3, 2.2 and 3.2-fold) was also observed in each MTA single exposure group, while no changes in expression of *17βhsd* and *lhr* were observed in any MTA single exposure groups (Fig. 2A–E). Expression of *ptgs2* was not significantly altered by exposure to 33 and 100 ng/L MTA alone, but was significantly downregulated (5.3-fold) by exposure to 333 ng/L MTA alone, compared with the solvent control group (Fig. 2D). In all the co-exposure groups, all the investigated genes were significantly downregulated (Fig. 2A–E). Moreover, co-exposure of EE2 with 33 (10.2-fold) or 100 ng/L (10.9-fold) MTA caused further downregulation of *lhr*, compared with the corresponding MTA single exposure (Fig. 2E).

In the brains, downregulation of *lhβ* was caused by exposure to EE2 (7.8-fold) or MTA (10.5, 6.6 and 6.4-fold; respectively) alone (Fig. 2F). Significant downregulation of *lhβ* was also observed in each co-exposure group relative to the solvent control group (7.4, 4.6 and 10.8-fold; respectively; Fig. 2F).

3.5. Histological alterations in gonads

Typical histological sections of ovaries are shown in the supporting information (Fig. S2). In the ovaries, no obvious pathological changes were observed in any group. The percentage of follicles at each stage of development is shown in Fig. 3. The percentages of PO, CAO or EVO were not significantly altered in the ovaries of fish exposed to EE2 and MTA, alone or in combination; the percentages of these three stages of oocytes in each co-exposure group also did

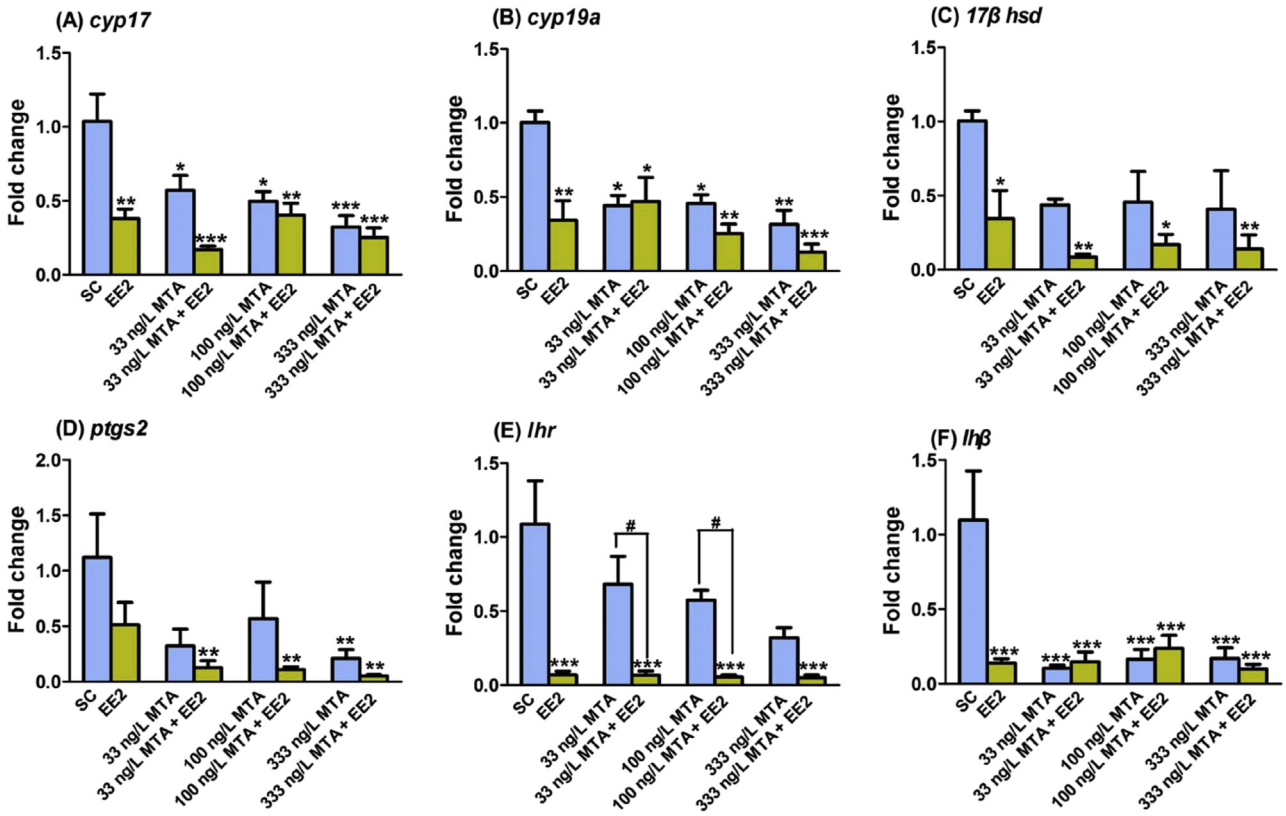

Fig. 2. Expression of (A) *cyp17*, (B) *cyp19a*, (C) *17βhsd*, (D) *ptgs2* and (E) *lhr* in ovaries, and (F) *lhβ* in brains of female zebrafish after exposure to MTA (33, 100 and 333 ng/L), EE2 (10 ng/L) and MTA/EE2 combinations (33 ng/L MTA + 10 ng/L EE2; 100 ng/L MTA + 10 ng/L EE2; 333 ng/L MTA + 10 ng/L EE2) for 21 days. Data are expressed as mean ± SEM of three replicate samples (each replicate sample contained one ovary or two pooled brains). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate statistically significant differences between the solvent control (SC) and the exposure groups or the co-exposure groups and the corresponding MTA single exposure groups. #P < 0.05 indicates statistically significant differences between the single exposure group and the corresponding co-exposure groups.

not show obvious difference when compared with the corresponding MTA single exposure group or EE2 single exposure group (Fig. 3A). EE2 single exposure did not affect the percentage of LVO/MO, however, significant decreases in percentages of oocytes at this stage were found in fish exposed to 100 and 333 ng/L MTA (Fig. 3B). In the co-exposure groups, MTA dose-dependent decrease in the percentage of LVO/MOs was observed; however, no differences were observed between the co-exposure group and the corresponding MTA single exposure group or the EE2 single exposure group (Fig. 3B). In addition, EE2 single exposure resulted in a higher percentage of AF relative to the solvent control group, while exposure to MTA alone did not affect the percentages of AF (Fig. 3C). However, when EE2 was co-exposed with 100 or 333 ng/L MTA, the percentages of AF were significantly increased relative to the solvent control (Fig. 3C). No obvious alterations of percentages of AF were observed in any of the co-exposure groups when compared with the corresponding MTA single exposure group or EE2 single exposure group (Fig. 3C). Typical histological sections of testes are shown in the supporting information (Fig. S3). In the testes, no obvious pathological changes and effects on developmental stages were observed in any treatment group (see supporting information, Fig. S4).

3.6. Reproductive outcome

Before chemical exposure, we performed a 14-day pre-exposure experiment, where egg production was found to be consistent and similar across all groups (Fig. 4A). The mean cumulative egg production during the exposure period (21 days) is shown in Fig. 4B–C. After 21 days of exposure, egg production of female fish exposed to EE2 alone was significantly reduced when compared with the solvent control (Fig. 4B–C). No significant difference in egg production was observed following exposure to any concentrations of MTA alone relative to the solvent control, although a decreasing trend was observed following exposure to 333 ng/L MTA (Fig. 4B–C). Co-exposure of EE2 with MTA (33, 100 and 333 ng/L) caused a significant and MTA-concentration-dependent decrease in egg production, when compared with the solvent control or the corresponding MTA single exposure (Fig. 4B–C). When compared to EE2 single exposure, no obvious differences were observed in egg production following co-exposure with 33 or 100 ng/L MTA, however, when co-exposed with 333 ng/L MTA, a further decrease in egg production was observed (Fig. 4C).

4. Discussion

In the current study, we found that co-exposure to MTA and EE2 at environmentally relevant concentrations could notably reduce steroid hormone concentrations in zebrafish, and was accompanied by downregulation of genes involved in steroid production, maturation and ovulation, all of which can lead to additive impairment of reproductive function. Many endocrine disruptors can influence fish reproduction by modulating sex hormone levels (Ankley et al., 2009; Ji et al., 2013; Zhou, 2015). In our study, plasma concentrations of E2 and T in females and 11-KT in males were significantly decreased in all the MTA single treatment groups, indicating that MTA is a potential endocrine disruptor. Decreases in plasma steroid hormones concentrations have been previously observed in fish

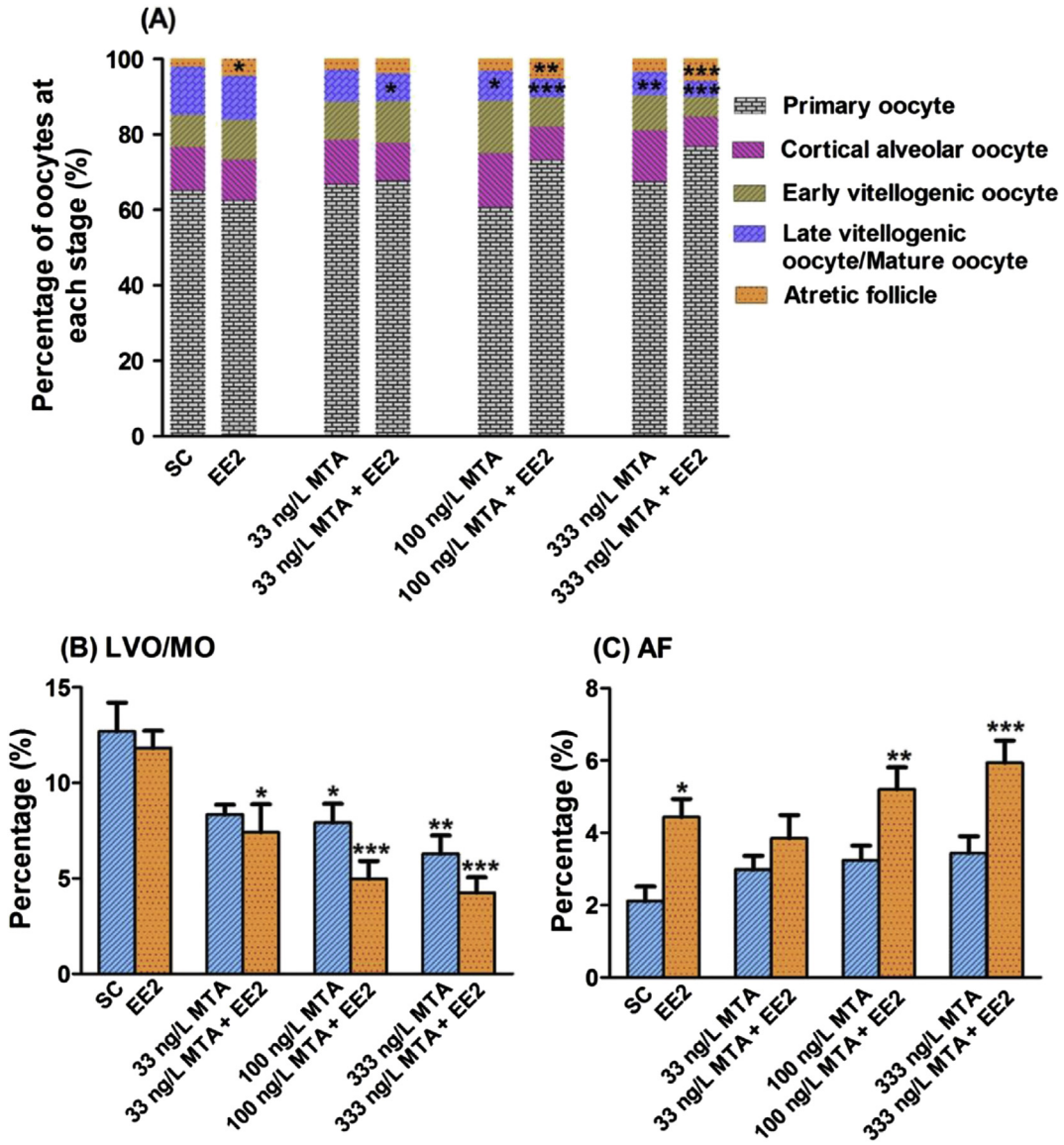

Fig. 3. Relative percentages of different stages of oocytes in female zebrafish after exposed to MTA (33, 100 and 333 ng/L), EE2 (10 ng/L) and MTA/EE2 combinations (33 ng/L MTA + 10 ng/L EE2; 100 ng/L MTA + 10 ng/L EE2; 333 ng/L MTA + 10 ng/L EE2) for 21 days. (A) Percentages of different stages of oocytes in ovaries of female zebrafish; (B) Percentages of late vitellogenic oocytes (LVO)/mature oocytes (MO); (C) Percentages of atretic follicles (AF). Data are expressed as mean ± SEM of six individual fish from 3 replicate tanks. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate statistically significant differences between the solvent control (SC) and the exposure groups.

exposed to progestins such as norethindrone (Paulos et al., 2010), gestodene (Runnalls et al., 2013) and levonorgestrel (Kroupova et al., 2014; Runnalls et al., 2013, 2015). However, several studies have reported that certain progestins such as desogestrel, drospirenone, dydrogesterone and medroxyprogesterone acetate, do not affect plasma steroid hormones (Runnalls et al., 2013; Zhao et al., 2015a). These different responses may be due to differences in chemical potency and fish species (Runnalls et al., 2013; Zhao et al., 2015a).

In the present study, EE2 exposure reduced plasma concentrations of E2 and T in females and 11-KT in males, consistent with a number of previous reports (Flores-Valverde et al., 2010; Coe et al., 2008; Schultz et al., 2003; Peters et al., 2007). A further decrease in the plasma concentrations of E2, T and 11-KT was observed in the co-exposure groups when compared with the corresponding MTA single treatment, while no alterations were observed when compared with the EE2 single treatment group, suggesting that these inhibitory effects were primarily caused by EE2. Such changes may lead to endocrine disruption and subsequently alter normal reproductive development.

We further examined several key genes along the HPG axis in the females, and significant downregulation of key steroidogenic enzyme genes (*cyp17*, *cyp19a* and *17βhsd*) was observed in ovaries of zebrafish exposed to EE2 and MTA (alone or in combination). In theca cells of fish ovaries, *cyp17* encoded enzymes are responsible for the production of and rostenedione, which is then converted to testosterone, catalysed by 17-hydroxysteroid-dehydrogenase (Hsd17b, encoded by *17βhsd*); testosterone is subsequently secreted to the granulosa cells and converted to estradiol by aromatase (Cyp19a, encoded by *cyp19a*) (Clelland and Peng, 2009). The downregulation of these genes may therefore account for the observed reduction in plasma concentrations of steroid hormones, which may result in an inhibition of follicle growth. In zebrafish, the luteinizing hormone (LH) signaling is mainly responsible for stimulating oocyte maturation and ovulation (Clelland and Peng, 2009; Chu et al., 2015). Downregulation of *lhr* and *ptgs2* in the

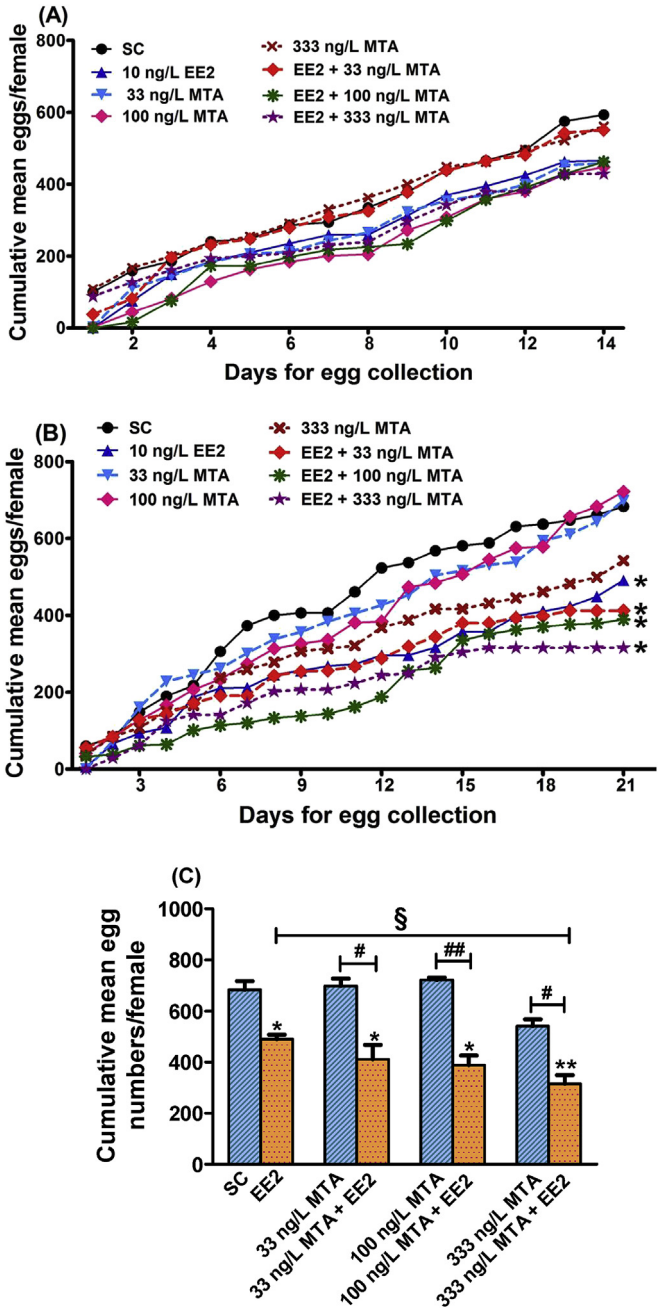

Fig. 4. Mean cumulative number of eggs spawned by each female zebrafish during (A) the 14-d pre-exposure period and (B) the 21-d exposure period. (C) Represents the mean number of eggs spawned by each female in each group at the end of the exposure period. Data are expressed as mean ± SEM of three replicate tanks (each tank contained six female zebrafish). $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences between the solvent control (SC) and the exposure groups. $^{\#/\S}P < 0.05$ and $^{\#\#/\S\S}P < 0.01$ indicate statistically significant differences between the single exposure group and the corresponding co-exposure groups.

ovary, and *lhβ* in the brain, was also observed in the present study, which may indicate the inhibited action of LH, preventing the oocytes from undergoing maturation. Moreover, downregulation of *ptgs2*, a gene encoding the key rate-limiting prostaglandin synthases for triggering ovulation (Lister and Van Der Kraak, 2008), may result in the reduction of prostaglandins, and finally affect ovulation. In the current study, all of the six investigated genes were significantly downregulated in all co-exposure groups, indicating disrupted regulation of oocyte growth, maturation and ovulation by co-exposure to MTA and EE2. Furthermore, expression of *lhr* in zebrafish has been previously shown to increase after the midvitellogenic stage and reach the highest level before final oocyte maturation (Kwok et al., 2005).

In the current study, combined exposure to MTA and EE2 resulted in decreased proportions of LVO/MO and inhibition of oocyte maturation in the ovary, suggesting that these hormones can cause synergistic effects on zebrafish fecundity. The observed lower proportions of mature oocytes correspond to *lhr* downregulation, and *lhr* expression in zebrafish has been previously reported to increase after midvitellogenic stage and reach the highest level just before final oocyte maturation (Kwok et al., 2005).

Increased proportions of AF were observed in female fish exposed to EE2 alone or in combination with MTA, but not MTA alone. Moreover, the addition of MTA to EE2 did not increase the AF percentages, indicating that EE2 plays a major role AF induction, and may imply that EE2 is a potential environmental stress factor that stimulates atresia once follicles developmental maturity, as high occurrence of AF is limited to oocytes in the later stages of development (Tyler and Sumpter, 1996). This might result in reduced spawning or fecundity. Our observations are consistent with previous studies in adult zebrafish and medaka (Van den Belt et al., 2002; Papoulias et al., 1999). Furthermore no effects on any developmental stage in the testes (testes development or spermatogenesis) were observed in our study, indicating a gender-specific effect of synthetic estrogens and progestins on gonadal development and gametogenesis, as reported in previous studies (Schindler et al., 2003; Zhao et al., 2015a, b).

Reproduction function (egg production in females) is an important ecologically relevant indicator of endocrine disruption in fish (Arcand-Hoy and Benson, 1998). While MTA alone did not affect egg production, EE2 alone caused significant reduction in egg production in the present study, which has been previously reported in zebrafish (Santos et al., 2007). Interestingly, a further reduction in egg production was observed when there combined exposure to EE2 and MTA when compared with EE2 single exposure, which may be indicative of an additive effect of these two synthetic hormones. Recent studies have reported similar additive effects of EE2 with different combinations of progestins on fish fecundity (Runnalls et al., 2015; Zhao et al., 2015a). These results may thus highlight the potential environmental risks of synthetic hormone combinations to fish species that inhabit contaminated waters.

5. Conclusion

In conclusion, environmentally relevant concentrations of EE2 and MTA (both alone and in combination) can significantly induce reproductive impairment in zebrafish. While further investigations are required to fully elucidate the underlying mechanisms of the combined hormonal effects on fish fecundity, this study highlights the necessity for the implementation of effective and reliable strategies for risk assessment of synthetic hormone combinations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.03.031.

References

Ankley, G.T., Bencic, D.C., Breen, M.S., Collette, T.W., Conolly, R.B., Denslow, N.D., Edwards, S.W., Ekman, D.R., Garcia-Reyero, N., Jensen, K.M., Lazorchak, J.M., Martinovic, D., Miller, D.H., Perkins, E.J., Orlando, E.F., Villeneuve, D.L., Wang, R.L., Watanabe, K.H., 2009. Endocrine disrupting chemicals in fish: developing exposure indicators and predictive models of effects based on mechanism of action. Aquat. Toxicol. 92, 168–178.

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A., 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20, 1276–1290.

Arcand-Hoy, L.D., Benson, W.H., 1998. Fish reproduction: an ecologically relevant indicator of endocrine disruption. Environ. Toxicol. Chem. 17, 49–57.

Argiles, J.M., Anguera, A., Stemmler, B., 2013. A new look at an old drug for the treatment of cancer cachexia: Megestrol acetate. Clin. Nutr. 32, 319–324.

Aris, A.Z., Shamsuddin, A.S., Praveena, S.M., 2014. Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. Environ. Int. 69, 104–119.

Caldwell, D.J., Mastrocco, F., Hutchinson, T.H., Lange, R., Heijerick, D., Janssen, C., Anderson, P.D., Sumpter, J.P., 2008. Derivation of an aquatic predicted no-effect concentration for the synthetic hormone, 17α-ethinyl estradiol. Environ. Sci. Technol. 42, 7046–7054.

Chang, H., Wan, Y., Hu, J.Y., 2009. Determination and source apportion of five classes of steroid hormones in urban rivers. Environ. Sci. Technol. 43, 7691–7698.

Chang, H., Wan, Y., Wu, S., Fan, Z., Hu, J., 2011. Occurrence of androgens and progestogens in wastewater treatment plants and receiving river waters: comparison to estrogens. Water Res. 45, 732–740.

Chu, L., Li, J., Liu, Y., Cheng, C.H., 2015. Gonadotropin signaling in zebrafish ovary and testis development: insights from gene knockout study. Mol. Endocrinol. 29, 1743–1758.

Clelland, E., Peng, C., 2009. Endocrine/paracrine control of zebrafish ovarian development. Mol. Cell Endocrinol. 312, 42–52.

Coe, T.S., Hamilton, P.B., Hodgson, D., Paull, G.C., Stevens, J.R., Sumner, K., Tyler, C.R., 2008. An environmental estrogen alters reproductive hierarchies, disrupting sexual selection in group-spawning fish. Environ. Sci. Technol. 2008 (42), 5020–5025.

Colman, J.R., Baldwin, D., Johnson, L.L., Scholz, N.L., 2009. Effects of the synthetic estrogen, 17α-ethinylestradiol, on aggression and courtship behavior in male zebrafish (*Danio rerio*). Aquat. Toxicol. 91, 346–354.

DeQuattro, Z.A., Peissig, E.J., Antkiewicz, D.S., Lundgren, E.J., Hedman, C.J., Hemming, J.D.C., Barry, T.P., 2012. Effects of progesterone on reproduction and embryonic development in the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 31, 851–856.

Drevnick, P.E., Sandheinrich, M.B., 2003. Effects of dietary methylmercury on reproductive endocrinology of fathead minnows. Environ. Sci. Technol. 37, 4390–4396.

Erkkola, R., Landgren, B.M., 2005. Role of progestins in contraception. Acta. Obstet.Gynecol. Scand. 84, 207–216.

Fent, K., 2015. Progestins as endocrine disrupters in aquatic ecosystems: concentrations, effects and risk assessment. Environ. Int. 84, 115–130.

Flores-Valverde, A.M., Horwood, J., Hill, E.M., 2010. Disruption of the steroid metabolome in fish caused by exposure to the environmental estrogen 17α-ethinylestradiol. Environ. Sci. Technol. 44, 3552–3558.

Han, J., Wang, Q.W., Wang, X.F., Li, Y.G., Wen, S., Liu, S., Ying, G.G., Guo, Y.Y., Zhou, B.S., 2014. The synthetic progestin megestrol acetate adversely affects zebrafish reproduction. Aquat. Toxicol. 150, 66–72.

Hill Jr., R.L., Janz, D.M., 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio):* effects on sex ratio and breeding success. Aquat. Toxicol. 63, 417–429.

Hua, J., Han, J., Guo, Y., Zhou, B., 2015. The progestin levonorgestrel affects sex differentiation in zebrafish at environmentally relevant concentrations. Aquat. Toxicol. 166, 1–9.

Ji, K., Hong, S., Kho, Y., Choi, K., 2013. Effects of bisphenol S exposure on endocrine functions and reproduction of zebrafish. Environ. Toxicol. Chem. 47, 8793–8800.

Johnson, R., Wolf, J., Braunbeck, T., 2009. OECD Guidance Document for the Diagnosis of Endocrine-related Histopathoogy of Fish Gonads (Draft of January 7). www.oecd.org/dataoecd/33/27/42140701.pdf.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: an ational reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Kroupova, H.K., Trubiroha, A., Lorenz, C., Contardo-Jara, V., Lutz, I., Grabic, R., Kocour, M., Kloas, W., 2014. The progestin levonorgestrel disrupts gonadotropin expression and sex steroid levels in pubertal roach (*Rutilus rutilus*). Aquat. Toxicol. 154, 154–162.

Kwok, H.F., So, W.K., Wang, Y., Ge, W., 2005. Zebrafish gonadotropins and their receptors: I. Cloning and characterization of zebrafish follicle stimulating hormone and luteinizing hormone receptors–evidence for their distinct functions in follicle development. Biol. Reprod. 72, 1370–1381.

Lei, B., Huang, S., Zhou, Y., Wang, D., Wang, Z., 2009. Levels of six estrogens in water and sediment from three rivers in Tianjin area, China. Chemosphere 76, 36–42.

Liang, Y.Q., Huang, G.Y., Liu, S.S., Zhao, J.L., Yang, Y.Y., Chen, X.W., Tian, F., Jiang, Y.X., Ying, G.G., 2015. Long-term exposure to environmentally relevant concentrations of progesterone and norgestrel affects sex differentiation in zebrafish (*Danio rerio*). Aquat. Toxicol. 160, 172–179.

Lister, A.L., Van Der Kraak, G., 2008. An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. Gen. Comp. Endocrinol. 159, 46–57.

Liu, S., Ying, G.G., Zhao, J.L., Chen, F., Yang, B., Zhou, L.J., Lai, H.J., 2011. Trace analysis of 28 steroids in surface water, wastewater and sludge samples by rapid resolution liquid chromatography-electrospray ionization tandem mass spectrometry. J. Chromatogr. A 1218, 1367–1378.

Liu, S., Ying, G.G., Zhou, L.J., Zhang, R.Q., Chen, Z.F., Lai, H.J., 2012. Steroids in a typical swine farm and their release into the environment. Water Res. 46, 3754–3768.

Papoulias, D.M., Noltie, D.B., Tillitt, D.E., 1999. An in vivo model fish system to test chemical effects on sexual differentiation and development: exposure to ethinyl estradiol. Aquat. Toxicol. 48, 37–50.

Paulos, P., Runnalls, T.J., Nallani, G., Point, T.L., Scott, A.P., Sumpter, J.P., Huggett, D.B., 2010. Reproductive responses in fathead minnow and Japanese medaka following exposure to a synthetic progestin, Norethindrone. Aquat. Toxicol. 99, 256–262.

Petersen, L.H., Hala, D., Carty, D., Cantu, M., Martinović, D., Huggett, D.B., 2015. Effects of progesterone and norethindrone on female fathead minnow (*Pimephales promelas*) steroidogenesis. Environ. Toxicol. Chem. 34, 379–390.

Peters, R.E.M., Courtenay, S.C., Cagampan, S., Hewitt, M.L., MacLatchy, D.L., 2007. Effects on reproductive potential and endocrine status in the mummichog (*Fundulus heteroclitus*) after exposure to 17 alpha-ethynylestradiol in a short-term reproductive bioassay. Aquat. Toxicol. 85, 154–166.

Runnalls, T.J., Beresford, N., Kugathas, S., Margiotta-Casaluci, L., Scholze, M., Scott, A.P., Sumpter, J.P., 2015. From single chemicals to mixtures – reproductive effects of levonorgestrel and ethinylestradiol on the fathead minnow. Aquat. Toxicol. 169, 152–167.

Runnalls, T.J., Beresford, N., Losty, E., Scott, A.P., Sumpter, J.P., 2013. Several synthetic progestins with different potencies adversely affect reproduction of fish. Environ. Sci. Technol. 47, 2077–2084.

Säfholm, M., Jansson, E., Fick, J., Berg, C., 2015. Mixture effects of levonorgestrel and ethinylestradiol: estrogenic biomarkers and hormone receptor mRNA expression during sexual programming. Aquat. Toxicol. 161, 146–153.

Santos, E.M., Paull, G.C., Van Look, K.J.W., Workman, V.L., Holt, W.V., van Aerle, R., Kille, P., Tyler, C.R., 2007. Gonadal transcriptome responses and physiological consequences of exposure to oestrogen in breeding zebrafish (*Danio rerio*). Aquat. Toxicol. 83, 134–142.

Schindler, A.E., Campagnoli, C., Druckmann, R., Huber, J., Pasqualini, J.R., Schweppe, K.W., Thijssen, J.H.H., 2003. Classification and pharmacology of progestins. Maturitas 61, 171–180.

Schultz, I.R., Skillman, A., Nicolas, J.M., Cyr, D.G., Nagler, J.J., 2003. Short-term exposure to 17α-ethynylestradiol decreases the fertility of sexually maturing male rainbow trout (Oncorhynchus mykiss). Environ. Toxicol. Chem. 22, 1272–1280.

Sun, L., Yong, W., Chu, X., Lin, J.M., 2009. Simultaneous determination of 15 steroidal oral contraceptives in water using solid-phase disk extraction followed by high performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1216, 5416–5423.

Svensson, J., Fick, J., Brandt, I., Brunström, B., 2014. Environmental concentrations of an androgenic progestin disrupts the seasonal breeding cycle in male three-spined stickleback (*Gasterosteus aculeatus*). Aquat. Toxicol. 147, 84–91.

Tyler, C.R., Sumpter, J.P., 1996. Oocyte growth and development in teleosts. Rev. Fish. Biol. Fish. 6, 287–318.

Valdés, M.E., Marino, D.J., Wunderlin, D.A., Somoza, G.M., Ronco, A.E., Carriquiriborde, P., 2015. Screening concentration of E1, E2 and EE2 in sewage effluents and surface waters of the "Pampas" region and the "Río de la Plata" estuary (Argentina). Bull. Environ. Contam. Toxicol. 94, 29–33.

Van den Belt, K., Verheyen, R., Witters, H., 2001. Reproductive effects of ethynylestradiol and 4t-octylphenol on the zebrafish (*Danio rerio*). Environ. Contam. Toxicol. 41, 458–467.

Van den Belt, K., Verheyen, R., Witters, H., 2003. Effects of 17a-ethynylestradiol in a partial life-cycle test with zebrafish (*Danio rerio*): effects on growth, gonads and female reproductive success. Sci. Total Environ. 309, 127–137.

Van den Belt, K., Wester, P.W., van der Ven, L.T., Verheyen, R., Witters, H., 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): time dependency and reversibility. Environ. Toxicol. Chem. 21, 767–775.

Wang, Q., Lam, J.C., Han, J., Wang, X., Guo, Y., Lam, P.K., Zhou, B., 2015. Developmental exposure to the organophosphorus flame retardant tris(1,3-dichloro-2-propyl) phosphate: estrogenic activity, endocrine disruption and reproductive effects on zebrafish. Aquat. Toxicol. 160, 163–171.

Weber, L.P., Hill Jr., R.L., Janz, D.M., 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*): II. Histological evaluation of gametogenesis and organ toxicity. Aquat. Toxicol. 63, 431–446.

Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Länge, R., 2009. Effects of synthetic gestagens on fish reproduction. Environ. Toxicol. Chem. 28, 2663–2670.

Zhao, Y.B., Castiglioni, S., Fent, K., 2015a. Synthetic progestins medroxyprogesterone acetate and dydrogesterone and their binary mixtures adversely affect reproduction and lead to histological and transcriptional alterations in zebrafish (*Danio rerio*). Environ. Sci. Technol. 49, 4636–4645.

Zhao, Y.B., Castiglioni, S., Fent, K., 2015b. Environmental progestins progesterone and drospirenone alter the circadian rhythm network in zebrafish (*Danio rerio*). Environ. Sci. Technol. 49, 10155–10164.

Zhou, B.S., 2015. Adverse outcome pathway: framework, application, and challenges in chemical risk assessment. J. Environ. Sci. 35, 191–193.
Zucchi, S., Castiglioni, S., Fent, K., 2013. Progesterone alters global transcription profiles at environmental concentrations in brain and ovary of female zebrafish (*Danio rerio*). Environ. Sci. Technol. 47, 12548–12556.
Zucchi, S., Mirbahai, L., Castiglioni, S., Fent, K., 2014. Transcriptional and physiological responses induced by binary mixtures of drospirenone and progesterone in zebrafish (*Danio rerio*) at environmental concentrations. Environ. Sci. Technol. 48, 3523–3531.