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The progestin norethindrone affects sex differentiation and alters transcriptional profiles of genes along the hypothalamic–pituitary–gonadal and hypothalamic–pituitary–adrenal axes in juvenile zebrafish *Dario renio*

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ABSTRACT

Natural and synthetic progestins may pose a threat to wild fish populations living in receiving waters. In this study, the effects of norethindrone (NET) on the sex differentiation of zebrafish (*Dario renio*) and the mechanisms underlying these effects were investigated. Juvenile zebrafish (20 days post fertilization, pdf) were exposed to environmentally relevant concentrations (5, 50, 500, and 1000 ng L^{-1}) for 45 d. Sex ratio of the NET-exposed populations, the histology of the gonads and the transcriptional profile of the regulatory genes involved in sex differentiation and steroidogenesis were examined. The results showed that a significantly higher ratio of male/female was induced in the zebrafish populations exposed to NET at concentrations higher than 32.3 ng L^{-1}. Exposure to NET caused acceleration of sexual mature in males and a delay in ovary maturation in female zebrafish. Among the genes regulating sexual differentiation, transcripts of *Dmrt1* showed a dose-dependent increase while transcripts of *Figa* and *Fox12* showed a dose-dependent decrease in response to exposure to NET. For genes regulating the steroidogenesis, the expressions of *Cyp11a1*, *Cyp17*, *Cyp19a1a*, and *Cyp11b* were significantly down-regulated by exposure to NET, while *Hsd17b3* expression was significantly up-regulated by exposure to NET at 421.3 and 892.9 ng L^{-1}. For the receptor genes in the gonads, the transcriptional expression of *Pgr*, *Ar*, and *Mr* was significantly up-regulated at 421.3 and 892.9 ng L^{-1} of NET. For genes involved in the hypothalamic–pituitary axis, the transcriptional expression of *Gnrh3* and *Pomc* was significantly up-regulated by exposure to NET with the exception for *Gnrh3* at 4.2 ng L^{-1}. The results demonstrated that exposure to NET at the juvenile stage could affect gonad differentiation and sex ratio, which might be accounted for by the alterations of the transcriptional expressions of genes along the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes.

1. Introduction

The occurrences of natural and synthetic progestins in the aquatic environments and their potential adverse effects on wildlife populations have been receiving increasing attention in the past two decades (Fent, 2015; Kumar et al., 2015). There are over 20 synthetic progestins used in a variety of medical applications including oral contraceptives, hormonal therapy, contraceptive implants, intrauterine devices, and vaginal ring (Kumar et al., 2015). Natural and synthetic progestins enter the aquatic environments mainly via the discharge of effluents from wastewater treatment plants, the domestic effluents containing the excretion of the urine and feces from humans, and the effluents from livestock and poultry farms (Fent, 2015; Kumar et al., 2015; Runnalls et al., 2013; Svensson et al., 2016). Synthetic progestins have been detected in various waters worldwide at the range of low ng L^{-1} to a few hundred ng L^{-1} at locations receiving wastewater effluents (Fent, 2015; Kumar et al., 2015). Various studies have demonstrated that synthetic progestins at the environmentally relevant concentrations (< μg L^{-1}) cause many adverse effects including increased vitellogenin production in male or juvenile fish, altered reproduction, impaired egg maturation, diminished embryonic development, ovotestes/intersex, and skewed sex ratio of the progestin-exposed populations in a variety of fish species (Ellestad et al., 2014; Frankel et al., 2016a; Hou et al., 2018b; Kumar et al., 2015; Liang et al., 2015a; Paulos

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et al., 2010; Sanchez et al., 2011; Svensson et al., 2016). These adverse effects are probably due to the disruption of the normal endocrine system in fish by progestins since they could bind to different receptors including nuclear progestin receptors (PRs), the androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) with different affinities (Fent, 2015; Kumar et al., 2015), therefore affecting the expressions of genes involved in steroidogenesis and eventually the hormone levels in fish. However, the exact mechanisms underlying these adverse effects might be species-specific and might differ among different types of progestins (Fent, 2015; Jobling et al., 1998; Kumar et al., 2015).

Norethindrone (NET), as well as noretynodrel, norethisterone, norethisterone acetate, etynodiol acetate, and lynestrenol, is one of the ingredients used in the first generation of oral contraceptive pills made in the 1960 s (Nelson and Cwiak, 2007; Runnalls et al., 2013). NET is used extensively due to its availability as a nonprescription drug and has been prioritized as being "dangerous for the environment (R 51/53)" (Carlsson et al., 2006). Data on the concentration of NET in different waters are scarce. A few studies have shown that it is in the range of 0.1 ng L^{-1} in the surface waters (Wu et al., 2015) to 224 ng L^{-1} in the influent of WTP (Vulliet et al., 2007). A maximal value of 872 ng L^{-1} was reported in a US stream (Kolpin et al., 2002). Norethindrone is shown to be androgenic and its effects at different biological levels in fish have been documented (Kumar et al., 2015; Runnalls et al., 2013). NET has been shown to interfere with the steroidogenesis in fish (Petersen et al., 2015). Exposure to 168 ng L^{-1} of NET lowered expression of luteinizing hormone levels in the brain along with significantly down-regulation of membrane progesterone receptor in ovary tissue in the adult fathead minnow, which could modulate the hormone levels in fish and ultimately affect sexual differentiation, sex determination, and reproduction in fish (Petersen et al., 2015). In addition, it has been shown that NET produces a significant decrease in fecundity of Japanese medaka *Oryzias latipes* at concentrations ≥ 25 ng L^{-1} in a 28 day static-renewal reproduction study and in the fathead minnow *Pimephales promelas* in a 21 day flow-through reproduction study in the low ng L^{-1} range (i.e., 1, 10, and 100 ng L^{-1}) (Paulos et al., 2010). Morphological changes induced by NET in *P. promelas* implies that NET exposure is likely to have a potent androgenic effect (Paulos et al., 2010). Moreover, discrete immersions at 1000 μg L^{-1} of NET for 3 h each on the second, fifth and eighth day post-hatching (dph) caused 92% masculinization and appearance of secondary sexual characteristics in the fighting fish *Betta splendens*, suggesting an androgenic potency of NET (Balasubramani and Pandian, 2008). The above mentioned studies have demonstrated that the adverse effects caused by exposure to NET are closely related to the disruption of fish endocrine systems.

Sex determination and sexual differentiation play important roles in the maintenance of fish populations in nature. In zebrafish, early developmental stages are the period for sex determination and gonad differentiation (Luzio et al., 2015) and are more sensitive to contaminants. Previously we showed that exposure to 63 ng L^{-1} of P4 at early developmental stages of zebrafish caused increased proportion of females in the population, while exposure to 34 and 77 ng L^{-1} of norgestrel resulted in 100% of males in the exposed population (Liang et al., 2015a). Other studies have shown that exposure to levonorgestrel at environmentally relevant concentrations (as low as 5.5 ng L^{-1}) caused 100% males (Hua et al., 2016; Svensson et al., 2016), whereas P4 did not affect the sex ratio (Svensson et al., 2016). It seems that the effects of progestins on the sex determination and gonad differentiation in fish remain inconsistent and inconclusive. Therefore, the aims of this study were to investigate the effects of NET on gonad differentiation and sex determination and to elucidate the underlying mechanisms for these effects. To achieve these, juvenile zebrafish at 20 dpf were exposed to NET for 45 days, the sex ratio of the exposed populations and genes involved in the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes were evaluated.

2. Materials and methods

2.1. Chemicals

Norethindrone (NET, purity: 98%) and dimethyl sulfoxide (DMSO, purity 99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solution of NET was prepared by dissolving the appropriate amount of NET in DMSO to achieve a final concentration of 1 mg mL^{-1}. The stock solutions were freshly prepared weekly and stored at −20 °C prior to use during the exposure.

2.2. Maintenance of zebrafish

Adult zebrafish *Danio rerio* were purchased from a local pet market (Guangzhou Huadiwan market, China) and maintained in 30 L glass aquaria with 25 L of filtered dechlorinated tap water in the laboratory. The temperature was kept at 28 ± 1 °C and photoperiod was maintained at 14 h light:10 h dark. The fish were fed commercially available red worm flakes twice daily (Haisheng Co., Shanghai, China).

Juvenile zebrafish (*D. rerio*) were obtained from spawning adults placed in groups of 12 males and 6 females. Approximately 1000 fertilized eggs were collected. They were transferred to petri dishes and allowed for hatching. The hatched larvae (after approximately 3 days) were transferred to beakers (1 L capacity) and fed a nutrient solution (filtrated solution from the homogenate of brine shrimp) twice daily until 18 days post fertilization (dpf). After that, the juvenile fish were fed newly hatched brine shrimp nauplii (Tianjin, China) twice daily.

2.3. Experimental design on exposure to NET

Juvenile zebrafish at 20 dpf were exposed to NET at five nominal concentrations: 0 (solvent control), 5, 50, 500, and 1000 ng L^{-1} (i.e., 0.017, 0.17, 1.68, and 3.35 nM). Each treatment had four replicates (n = 4). For each replicate, 30 fish were randomly selected and transferred to 30 L glass aquaria containing 25 L of filtered and well-aerated dechlorinated tap water. For the solvent control, DMSO was added to a final concentration of 0.01% (v/v). For other the treatment groups, stock solutions of NET were properly diluted with exposure medium to designated working solutions to ensure the four nominal concentrations and the concentration of DMSO at each replicate of these treatments was 0.01% (v/v). The experiment was performed in a semi-static system at 26 ± 1 °C with a 14 h : 10 h (light : dark) photoperiod. The exposure medium in each aquarium was renewed daily. The conductivity (146–164 u s cm^{-1}), pH (6.8–7.3) and dissolved oxygen concentration (≥ 75%) were recorded using a multi-parameter water quality meter (YSI Model 85 m; Yellow Springs, OH) every five days. Dead fish in the aquaria were recorded and removed daily during the exposure. The juvenile fish were fed newly hatched brine shrimp nauplii twice daily.

At 42 dpf (22 d after the exposure), 5 fish for each replicate were randomly removed and anesthetized with 0.01% tricaine methanesulfonate (MS-222, Sigma–Aldrich). The fish were homogenized using a pestle and mortar in liquid nitrogen for subsequent RNA extraction (see below). The remaining fish were exposed to NET until 65 dpf when sexual differentiation of zebrafish was completed.

2.4. Histology and sex determination in zebrafish

After the exposure, the fish were anesthetized with 0.01% MS-222. All survived individuals were used for histological analysis for the determination of sex. 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).

The gonads (ovaries and testes) of zebrafish (n = 96, 94, 90, 85, 82