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Alterations of secondary sex characteristics, reproductive histology and behaviors by norgestrel in the western mosquitofish (*Gambusia affinis*)

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ABSTRACT

Synthetic hormones in wastewater effluents released into the aquatic environments may interfere with the normal endocrine systems of fish in receiving streams. Norgestrel (NGT) is a synthetic progestin widely used in oral contraceptives and frequently detected in wastewater effluents. In this study, adult female mosquitofish (Gambusia affinis) were exposed to three environmentally relevant concentrations of norgestrel (NGT) (i.e., 3.6, 35.8, and 368.0 ng L−1) for 42 d, fin morphology, histology of the ovary, and reproductive behaviors were evaluated. The results showed that NGT at all three concentrations caused an increased frequency of atretic follicular cells in ovaries and impaired mating behaviors exhibited by males toward the NGT-exposed females. In mosquitofish exposed to NGT at 35.8 and 368 ng L^{-1}, the anal fin of females had an increased length ratio of ray4/ray 6, an increased width of ray 3, and increased number of segments in ray 3. The histopathological analysis showed that exposure to NGT increased the incidence of spermatogenesis in ovaries. Mating behavior was impaired 58.4%, 65.7%, and 76.4% ($P < 0.01$ in all cases) when mosquitofish were exposed to NGT at 3.6, 35.6 and 368.0 ng L^{-1}, respectively. The rapid masculinization, the increased frequency of atretic follicles, the incidence of spermatogenesis in the ovary of female fish, and the altered reproductive behaviors suggest that wild populations of mosquitofish could be similarly affected inhabiting in NGT contaminated environments.

1. Introduction

Endocrine disrupting chemicals (EDCs) have been receiving increasing attention due to their deleterious effects on aquatic organisms and potential adverse effects on human beings (Kortenkamp, 2007; Sonnenschein and Soto, 1998; Tyler et al., 1998). EDCs include natural and synthetic compounds that mimic or block activation of steroid hormone receptors, and impair signaling by hormones (Fent, 2015; Sumpter and Johnson, 2005). Natural and synthetic steroid hormones have attracted more interest than other EDCs due to their relatively high potential of disrupting the endocrine system in vertebrates such as fish and amphibians at relatively low concentrations (Jobling et al., 2006; Kumar et al., 2015; Zhang et al., 2017). The steroid hormones can cause a suite of adverse effects including vitellogenin synthesis in male or juvenile animals, impaired egg maturation, ovo-testes/intersex, altered reproduction, and diminished embryonic development in fish which may reduce populations of fish and amphibians (Kidd et al., 2007; Sumpter and Johnson, 2005).

Norgestrel (NGT) is a synthetic progestin widely used in oral contraceptives, contraceptive implants, intrauterine devices, and vaginal rings (Fang et al., 2007; Liang et al., 2015a). NGT enters aquatic environments mainly through the discharge of effluents from wastewater treatment plants from the urine and feces of humans and livestock. Due to the inefficient removal of NGT by treatment plants, it occurs in waters at concentrations ranging from 22 ng L^{-1} in surface water to 10088 ng L^{-1} in discharge from a swine farm (Liu et al., 2012a, b). Transcriptional expression of genes related to the hypothalamic-pituitary-gonadal (HPG) and the hypothalamic-pituitary-adrenal (HPA) axes was altered by NGT when zebrafish embryos were exposed to nominal concentrations of 5, 50, and 100 ng L^{-1} for 144 h post fertilization, implying that NGT may pose a potential effect on embryonic development, in the brain and may alter gonadogenesis (Liang et al., 2015b).

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Furthermore, after 40 d of exposure to NGT at 4, 34 and 77 ng L^{-1}, the expression of *Dmrt1* and *Figa*, two genes related to sex differentiation in fish, were markedly altered (Liang et al., 2015a). In addition, another synthethic progestin, levonorgestrel induced the *de novo* synthesis of nuptial tubercles (a secondary sexual characteristics in males) in female fathead minnow *Pimephales promelas* after the adult fish were exposed to NGT at concentrations higher than 29.7 ng L^{-1} for 21 d, indicating androgenic effects (Zeilinger et al., 2009).

Alterations of fish behavior at the level of the individual organism offers a clear link between biochemical/molecular levels (e.g., the transcriptional expressions of receptor regulated genes) and effects at the population and community levels of biological organization (Tierney et al., 2010; Weis et al., 2001). Altered fish behaviors such as courtship and mating, may have profound effects on reproduction (Bayley et al., 1999; Munakata and Kobayashi, 2010). The effects of natural and synthetic hormones on fish behavior have been reported and sexual behavior has been considered as a sensitive biomarker to the exposure to estrogen mimics (Melvin and Wilson, 2013; Söffker and Tyler, 2012). For example, after dietary exposure to 3 and 30 μg g^{-1} of 17-β estradiol for two weeks, mating behaviors such as following breeding males, dancing, floating, and crossing of male fish were suppressed markedly (Oshima et al., 2003). A recent study showed that acute exposure to 10 and 100 ng L^{-1} of levonorgestrel caused masculinization of adult female mosquitofish (*Gambusia holbrooki*) (Frankel et al., 2016b). In addition, male fish exposed to 100 ng L^{-1} of levonorgestrel spent more time exhibiting no reproductive behavior, had decreased attending behavior, and a lower number of gonopodial thrusts (Frankel et al., 2016b). However, the chronic effects of NGT on the reproductive behaviors in fish have never been reported.

The mosquitofish has been frequently used in ecotoxicological studies on the effects of endocrine disrupting chemicals (Hou et al., 2017; Xie et al., 2010), particularly for androgenic and antiandrogenic compounds, due to its marked sexual dimorphism and external secondary sexual characteristics (Pyke, 2005). The objectives of this study were to evaluate the effects of NGT on the morphology of the anal fin and to examine the impact of NGT on the reproductive behavior of adult female mosquitofish. Adult female fish were exposed to three environmentally relevant concentrations of NGT (nominal concentrations: 5, 50, and 500 ng L^{-1}) for 42 d and morphometrics of the anal fin, histology of the ovary, and reproductive behaviors were examined.

2. Materials and methods

2.1. Test organisms

Feral adult female mosquitofish (*Gambusia affinis*) were purchased from a local pet store (Guangzhou Huadiwan market, Guangzhou, China) providing fish originally collected from the Liuxi River, which is used as the drinking water source for Guangzhou city (Xie et al., 2010). This river is very pristine and free of contamination. The fish were maintained in 40 L glass aquaria with 30 L of well aerated dechlorinated tap water. They were acclimated to laboratory conditions for more than 2 months prior to exposure to NGT. During the acclimation, the fish were fed commercially available red worm flakes twice daily (Haisheng Co., Shanghai, China). Uneaten food and fecal matter were siphoned out from the aquaria each day. The conductivity, water hardness, dissolved oxygen (DO) and pH were recorded each day with a multiparameter water quality meter (YSI Model 85 m; Yellow Springs, OH). The total water hardness was 150 ± 3.5 mg L^{-1} (as $CaCO_3$). The DO was 7.5 ± 0.1 mg L^{-1}. The conductivity was 20 ± 0.2 μS cm^{-1}. pH was 7.6 ± 0.5. The photoperiod was maintained as 14 h:10 h (light: dark).

2.2. Exposure to norgestrel

Norgestrel (> 99.9% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Three nominal concentrations (i.e., 5, 50, and 500 ng L^{-1}, ~0.0016, 0.016, and 0.16 mM, respectively) were used in this study. A stock solution (1 mg/mL) for each nominal exposure concentration was prepared by dissolving appropriate amount of NGT in 100% ethanol. Each stock solution was further diluted to the final working concentrations with the exposure medium. For the exposure, adult female mosquitofish were treated with NGT at nominal concentrations of 0 (with ethanol at 0.001%, v/v), 5, 50, 500 ng L^{-1}. Each treatment had three replicates with 30 fish per replicate. The exposure medium was renewed each day. The experiment was conducted in a semi-static system at 25 ± 1 °C. The water chemistry of the exposure media was maintained similarly as that during the acclimation. The water samples were taken weekly and the NGT concentration in each aquarium was measured (see Section 2.6 for details). The exposure lasted for 42 d.

2.3. Behavioral assays

Behavioral assays were performed according to previous methods (Chen et al., 2016). Briefly, after the exposure NGT for 42 d, ten female fish (3, 3, and 4 fish from each replicate) from each treatment were randomly selected and used for the behavior test. A light box was placed under four glass aquaria (10 cm × 10 cm × 10 cm) with 400 mL of well aerated dechlorinated tap water. The light box was positioned so that the aquaria were illuminated indirectly to prevent shadowing and provide equal lighting across the surface area of the aquaria. The sides and bottom of each aquarium were covered with white paper to provide sufficient contrast and prevent effects from external stimuli. Black dividers were placed between each aquarium to prevent visual interaction between individuals in adjacent aquaria.

For each trial, one randomly selected NGT-treated female fish was paired with a randomly selected control male fish in one aquarium. Combinations tested included a control male × a control females, a control male × a NGT (at the measured concentrations three concentrations, i.e., 3.6, 35.8, and 368.0 ng L^{-1}) exposed female. The fish were transferred to the aquarium and allowed 10 min of acclimation. The activity of the fish was recorded for 10 min with a digital camcorder (Sony, Japan) centrally positioned 150 cm above the light box. The digital video was analyzed with an animal movement tracking software Noldus EthoVision® XT (Noldus Information Technology, Netherlands). Fish reproduction behavior parameters, including attending behavior, following behavior and close following behavior were quantified from the video with a tracking rate of 25 frames/s by the software according to the manufacturer's instructions. The definitions of these behaviors were modified from previous studies (Frankel et al., 2016b; Pyke, 2005; Toft et al., 2004) (See Table S1 for details of their definitions). The number of female fish from each treatment used for the behavior assay was 30.

2.4. Biometrics and fin morphometrics

After 42 d of exposure and the behavior test, the fish were anesthetized using an excess of anesthesia (MS-222, 300 ppm). The standard length of the fish (± 0.1 mm) and their mass (± 0.1 mg) were recorded. The width of the third ray of the anal fin (± 0.1 mm), the number of segments on the identical ray, and the length ratio of ray 4/ray 6 were determined. The anal fins were photographed using a calibrated dissecting microscope (Olympus SZH-ILLK).

2.5. Histopathological analysis

The ovaries of the adult female fish (n = 20 for each replicate) were dissected and fixed in Bouin's solution (composition: 75 mL of saturated picric acid, 25 mL of 40% formaldehyde, and 5 mL of glacial acetic acid) for histological evaluation, according to the method as described previously (Hou et al., 2017). Sequential portions (5 mm) were acquired