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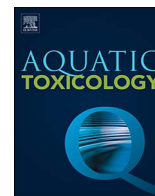Chen, Hongxing
Tian, Chang-En
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Alterations of secondary sex characteristics, reproductive histology and behaviors by norgestrel in the western mosquitofish (*Gambusia affinis*)

Li-ping Hou[a], Hongxing Chen[b], Chang-en Tian[a,*], Ye Liang[a], Rong-rong Wu[a], Xing-mei Zhang[a], Xu-wen Fang[a], Cui-ping Zhang[a], Jun-jie Hu[a], Li-ying Song[a], Yan-qiu Liang[c], Daniel Schlenk[d], Lingtian Xie[b,*]

[a] *School of Life Sciences, Guangzhou University, Guangzhou 510655, PR China*
[b] *The Environmental Research Institute, MOE Key Laboratory of Theoretical Chemistry of Environment, South China Normal University, Guangzhou 510006, PR China*
[c] *Faculty of Chemistry and Environmental Science, Guangdong Ocean University, Zhanjiang 524088, PR China*
[d] *Department of Environmental Sciences, University of California Riverside, Riverside, CA 92521, USA*

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).

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

Table 1
Measured concentrations of norgestrel (NGT) on exposure days 1, 7, 21 and 42. The concentrations of NGT were determined by gas chromatography-mass spectrometry (GC–MS). nd: not detectable (n = 3 for each treatment).

Treatment	Day	ng L^{-1}	Mean $\pm$ SEM
Control	1, 7, 21, 42	nd, nd, nd, nd	nd
5 ng L^{-1}	1, 7, 21, 42	2.8, 3.8, 4.0, 3.7	3.6 $\pm$ 0.5
50 ng L^{-1}	1, 7, 21, 42	34, 37, 42, 30	35.8 $\pm$ 5.1
500 ng L^{-1}	1, 7, 21, 42	342, 378, 402, 350	368.0 $\pm$ 27.4

using a Rotary Microtome Cut 4055 (Olympus American, Melville, NY, USA) before being placed on slides.

The sections were obtained from three different levels along the gonad-axis. The prepared slides were examined under a microscope (Nikon, Eclipse E600, Japan). Images were taken using a digital camera (Leica DFC420) and acquired using the Imagic IMS (Imagic Bildverarbeitung AG) software. Ovaries from 20 females were selected from each replicate, and two sections (out of three) from different levels were examined for each individual. In the ovaries, the oocyte developmental stage was assigned to one of the following stages: perinuclear oocyte (PO), corticolar alveolar (CO), early vitellogenic oocyte (EV), late vitellogenic oocyte (LV), atretic follicles (AF), and postovulatory follicles (POF). The oocyte developmental stages in two different layers per treatment were obtained and used for the calculation of their relative frequency.

2.6. Measured concentrations of NGT during exposure

The exposure medium was renewed daily during the exposure period. The concentration of NGT in the exposure aquarium was measured on exposure day 1, 7, 21, and 42. At each sampling date, 500 mL of the exposure medium from each aquarium were collected and subjected to the following processing procedures for the determination of the NGT concentration, according to the method in a previous study (Liang et al., 2015b). Briefly, a solid phase extraction (SPE) method was applied to enrich the target compound in the water sample by using a preconditioned CNWBOND LC-C18 SPE cartridge (200 mg of the sorbent, 3 cc) (Germany). After the SPE extraction, the cartridge was eluted with 10 mL of ethyl acetate and the eluate was collected in a glass tube, and dried under a gentle stream of nitrogen. The dried extract was dissolved in 1 mL of methanol. The target compounds in the extract were analyzed by rapid resolution liquid chromatography-electrospray ionization-tandem mass spectrometry (Agilent 1200 LC-ESI-Agilent 6460 Triple Quadrapole, Santa Clara, CA, USA), according to the method described previously (Liu et al., 2011). For quality assurance and quality control (QA/QC), a solvent blank and a procedural blank were run for each batch of samples to check background contamination and instrument performance. The limit of detection for NGT was 0.04 ng L^{-1}. The recovery determined by spiking surface waters with known amounts of NGT (100 ng L^{-1}) was 87.5–96.8% (Liu et al., 2011).

2.7. Statistical analyses

Data were presented as means $\pm$ standard error of the mean (SEM) unless stated otherwise. Data analyses were performed on SPSS (version 13.0, IBM, Chicago, IL, USA) and Origin 165 (version 8.0, OriginLab, Northampton, MA, USA) software. Prior to ANOVA analyses, Kolmogorov–Simirnov and Levene's tests were used to test for normality and homogeneity of variances, respectively. The behavioral data met the assumptions for ANOVA after log-transformation. The differences among the treatments were tested using one-way analysis of variance with specific mean comparisons performed by Dunnett's test. A p value < 0.05 was considered to be significantly different

3. Results

The measured concentrations for the control and the three norgestrel treatments (i.e., 5, 50, and 500 ng L^{-1}) are presented in Table 1. Norgestrel was not detectable in the control treatment, while the measured concentrations of NGT for 5, 50, and 500 ng L^{-1} treatments were approximately 71.6 $\pm$ 10.5%, 71.5 $\pm$ 9.5% and 73.6 $\pm$ 5.4% of the their nominal concentrations, respectively (Table 1). Therefore, the average measured concentrations of NGT (i.e., 3.6, 35.8, and 368.0 ng L^{-1}) were used for the presentation of the data throughout this study.

3.1. Effects of NGT on the growth and anal fin morphometrics

After 42 d of exposure, the standard length and the weight of the adult female fish from three NGT treatments were not affected (F$_{(3356)}$ = 0.404, P = 0.528 for length; F$_{(3356)}$ = 0.95, P = 0.342 for weight. n = 360) (Table 2). In general, exposure to 3.6 ng L^{-1} of NGT for 42 days did not affect the morphology of the anal fin of the female *G. affinis*. However, relative to the control female fish, the length ratio of ray4/ray 6 was significantly increased (F$_{(3356)}$ = 15.56, P < 0.0001, n = 360) by 27.9% (Dunnett's test, P = 0.031) and 44.3% (P < 0.001) respectively for the female fish exposed to 35.8 and 368.0 ng L^{-1} of NGT (Table 2). Meanwhile, compared to that of the control female fish, the width of ray 3 was significantly increased (F$_{(3356)}$ = 81.35, P < 0.0001, n = 360) by 200.1% (Dunnett's test, P = 0.023) and 280.4% (P < 0.001) for female fish exposed to 35.8 and 368.0 ng L^{-1} of NGT, respectively (Table 2). In addition, relative to the control females, approximately 2.0 fold (Dunnett's test, P = 0.035) and 3.9 fold (P < 0.001) of increase in the number of segments were observed in ray 3 of female fish exposed to 35.8 and 368.0 ng L^{-1} of NGT (F$_{(3356)}$ = 158, P < 0.0001, n = 360), respectively (Table 2, Fig. 1C and D).

3.2. Effects of NGT on the ovary of mosquitofish

Overall, ovarian cell development was adversely affected by NGT at the two higher concentrations of NGT (i.e., 35.8 and 368.0 ng L^{-1}). The control ovaries had several development stages of follicular cells from late vitellogenic oocytes, perinuclear oocyte and early vitellogenic
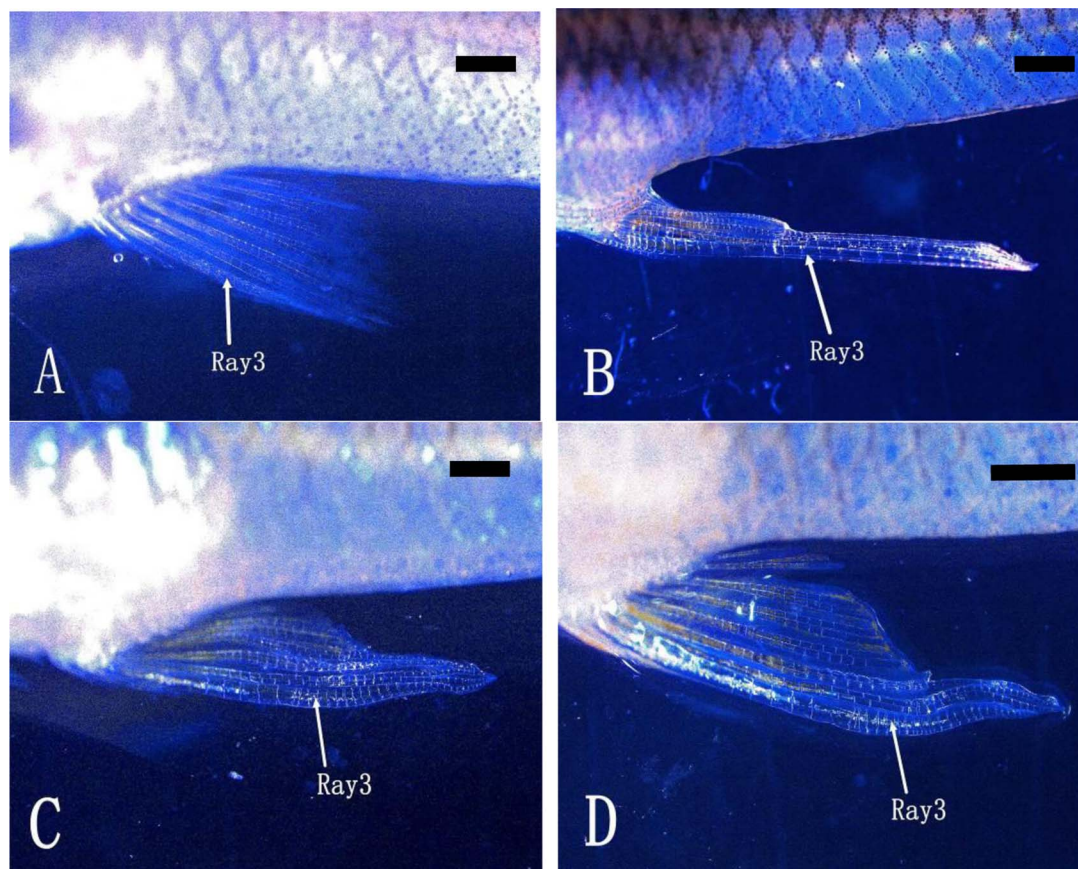
Table 2
Effects of norgestrel on the growth and anal fin morphology in the adult female western mosquitofish (*Gambusia affinis*) after 42 days of exposure to norgestrel (NGT) at 3.6, 35.8, and 368.0 ng L^{-1}. Data were expressed as mean (standard error) (n = 90 for each treatment). One-way analysis of variance (ANOVA) followed by Bonferroni's test was used for the test of significant differences among treatments. Significant differences at p < 0.05 and p < 0.001 are indicated by * and ***, respectively.

NGT conc. (ng L^{-1})	0	3.6	35.8	368.0
Standard length (mm)	24 (1.05)	25.8 (1.3)	22.35 (1.5)	22.03 (1.7)
Weight (g)	0.17 (0.01)	0.18 (0.04)	0.15 (0.07)	0.15 (0.13)
Ray4/ray 6	1.22 (0.02)	1.23 (0.03)	1.56 (0.05) [*]	1.76 (0.05) [***]
Width of ray 3 (mm)	0.15 (0.01)	0.23 (0.03)	0.45 (0.04) [*]	0.57 (0.02) [***]
Number of segment in ray 3	8 (3.03)	15 (5.04)	24 (4.04) [*]	37 (5.04) [***]

Fig. 1. Morphology of the anal fin of western mosquitofish (*Gambusia affinis*). Panel A: the anal fin of a control adult female fish with ray 3 labeled; Panel B: the anal fin of a control adult male; Panel C: the anal fin of a masculinized adult female exposed to 35.8 ng L^{-1} of norgestrel (NGT) for 42 days; Panel D: the anal fin of a masculinized adult female exposed to 368.0 ng L^{-1} of NGT. Scale bar = 1 mm.

oocyte (Fig. 2A). Norgestrel at 35.8 and 368.0 ng L^{-1} induced spermatogenesis in ovaries of the exposed females (Fig. 2B and C). Spermatogonia were observed in the ovary of fish exposed to NGT at 35.8 ng L^{-1}, while spermatogonia and early spermatocytes (ES) were seen in the ovary of fish exposed to NGT at 368.0 ng L^{-1}. Approximately 5.0 ± 0.8% and 25.0 ± 5.3% of the female fish had cyproterone acetate in their ovaries (Fig. 2D). In addition, the relative frequency of the ovarian cells in the ovaries of mosquitofish is summarized in Fig. 3. NGT caused a significant increase in the relative frequency of atretic follicular cells at all exposure concentrations and post-ovulatory follicular cells and a significant decrease of late vitellogenic oocytes at concentrations of 35.8 and 368.0 ng L^{-1} (Fig. 3).

3.3. Effects of NGT on the reproductive behavior

Results on the reproductive behavior exhibited by the control male fish paired with the NGT treated females for 42 d are shown in Fig. 4. Control male fish did not differ in spending time exhibiting no reproductive behavior and attending behavior at all exposed NGT concentrations (F$_{(3116)}$ = 5.5, P = 0.024, n = 120) (Fig. 4A and B), except that they spent significantly less time on the attending behavior to the females exposed to 368.0 ng L^{-1} (P = 0.013). However, the control males had an approximately 39.2% (Dunnett's test, P = 0.021), 43.2% (P = 0.019), and 59.8% (P < 0.001) of decrease (F$_{(3116)}$ = 12.19, P < 0.0001, n = 120) in time spending for following behavior when paired a female mosquitofish exposed to NGT at 3.6, 35.6 and 368.0 ng L^{-1}, respectively, relative to that of the control male paired with a control female fish (Fig. 4C). The control males had an approximately 58.4% (Dunnett's test, P < 0.001), 65.7% (P < 0.001), and 76.4% (P < 0.001) of decrease (F$_{(3116)}$ = 54.12, P < 0.0001,

n = 120) in number of close following behavior when paired a female mosquitofish exposed to NGT at 3.6, 35.6 and 368.0 ng L^{-1}, respectively, relative to that of the control male paired with a control female fish (Fig. 4D).

4. Discussion

The impact of progestins on aquatic organisms has gained increasing attention in recent years and a few studies have documented the masculinization of female fish by exposure to different progestins (Frankel et al., 2016a; Hou et al., 2018; Hou et al., 2017). In this study, the effects of NGT at environmentally relevant concentrations in the western mosquitofish *Gambusia affinis* were evaluated using histological analysis and behavioral assay. The results demonstrated that exposure to NGT caused the masculinization of females, which was confirmed by histopathological alterations of the ovary (e.g., including spermatogenesis in the ovary).

4.1. Masculinization of female mosquitofish by NGT

Exposure to 35.6 and 368.0 ng L^{-1} for 42 d increased the length ratio of ray 4/ray 6, the width of ray 3, and number of segments of ray 3 of the anal fin in the female mosquitofish. In mosquitofish, during normal gonopodium development, the fourth and sixth fin rays elongate and new bones are added to ray 3 (Doyle and Lim, 2002). Therefore, the length ratio of ray 4/ray 6 and width of ray 3 provides a convenient index for gonopodium formation (Angus et al., 2001; Doyle and Lim, 2002). Following treatment with NGT, masculinization of female mosquitofish fins was observed at 35.6 and 368.0 ng L^{-1}. Masculinization (i.e, increased segments and basal width in ray 3 of the anal
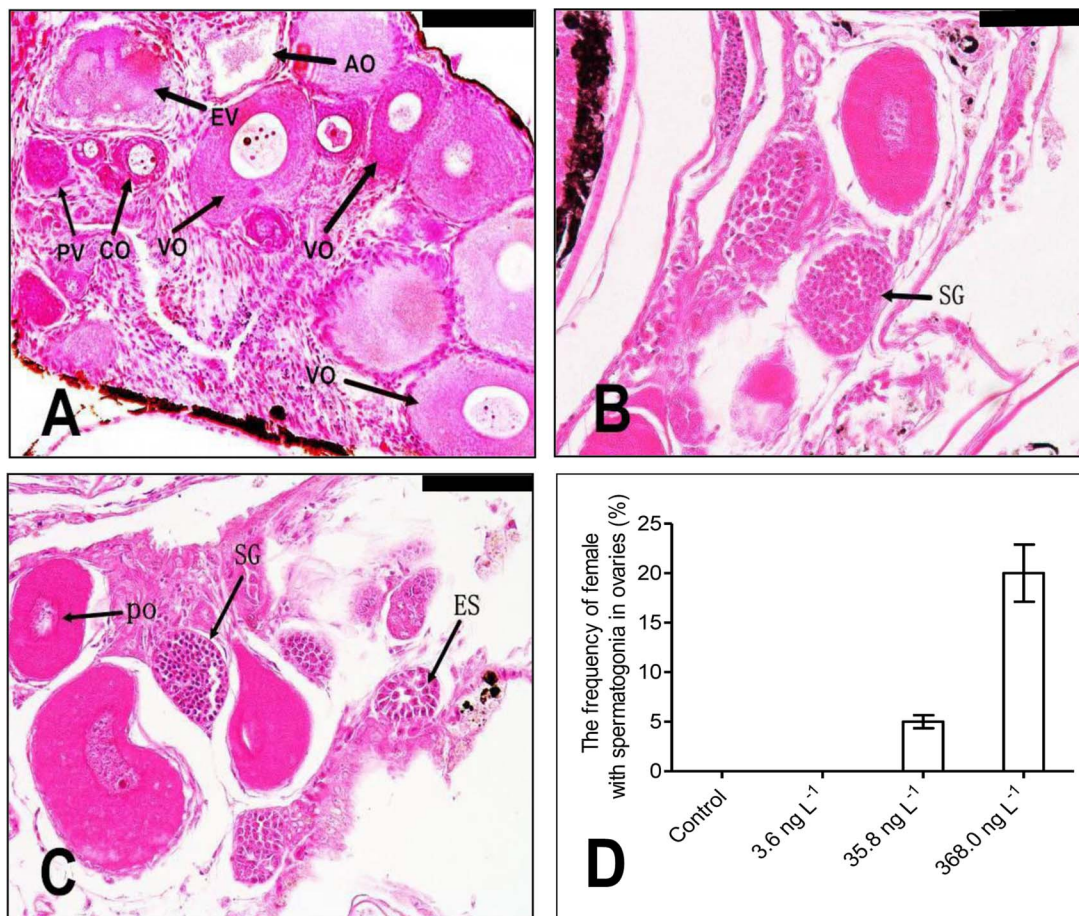
Fig. 2. Histological sections of the ovary of female mosquitofish exposed to norgestrel for 42 d. Scale bar = 50 μm. Panel A: control ovary showing several development stages of follicular cells from perinuclear oocyte (PO), corticolar alveolar (CO), early vitellogenic oocyte (EV), vitellogenic oocyte (VO) and atretic oocytes (AO). Panel B: ovary of fish exposed to 35.8 ng L^{-1} of NGT, SG: spermatogonia; Panel C: ovary of fish exposed to 368.0 ng L^{-1} of NGT, ES: early spermatocytes; Panel D: Frequency of female with spermatogonia in ovaries. Bars with different letters denote significant differences.
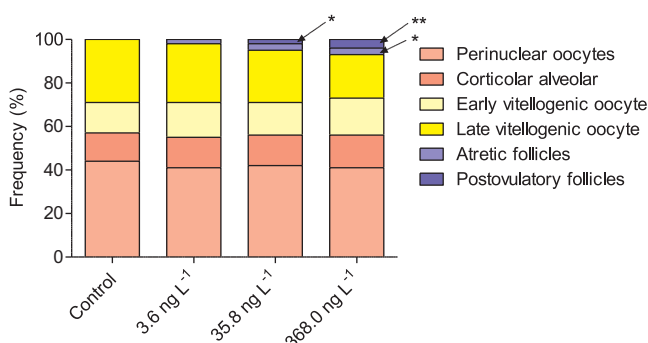

Fig. 3. Relative percentages of stages of oocytes in ovary. Perinuclear oocyte (PO), corticolar alveolar (CO), early vitellogenic oocyte (EV), late vitellogenic oocyte (LV), atretic follicles (AF), and postovulatory follicles (POF). Asterisks denote significant difference compared to the control at p < 0.05.

fin) of female mosquitofish *G. affinis* was similarly observed after exposure to 410 ng L^{-1} of progestrone for 42 d (Hou et al., 2017). Some previous studies have also documented the masculinization of female fish by exposure to different progestins (Kumar et al., 2015; Runnalls et al., 2013). For example, the length ratios of ray 4/ray 6 anal fin of the female eastern mosquitofish *Gambusia holbrooki* were significantly increased by exposure to 10 and 100 ng L^{-1} of levonorgestrel (a progestin) for 8 d (Frankel et al., 2016b). Another study from the same lab found that after 8 d of exposure to 10 and 100 ng L^{-1} of gestodene (a progestin), female fathead minnow *Pimephales promelas* showed male-

typical secondary sex characteristics including nuptial tubercles (Frankel et al., 2016a). More than 30% of the exposed female fathead minnows had a dark spot on the dorsal fin (a male secondary sexual characteristic) after 21 d of exposure to 1.2, 16.0 and 85.0 ng L^{-1} of norethindrone, a synthetic progstin (Paulos et al., 2010). The results in this study, together with the results from the above-mentioned studies, have demonstrated that progestins can masculinize fish, at environmentally relevant concentrations.

Consistent with fin ray indices, spermatogonia were observed in the ovaries of female mosquitofish exposed to NGT at 35.6 and 368.0 ng L^{-1} for 42 d, which further corroborated the occurrence of masculinization of the female mosquitofish. The induction of spermatogenesis in the fish ovary by exposure to pregestins has seldom been reported in the literature. The underlying mechanisms for the presence of spermatogenesis in the NGT exposed female mosquitofish remain unknown. A recent study showed that after larval zebrafish (20 days post fertilization) were exposed to NGT at 34 and 77 ng L^{-1} for 40 d, an all-male population was resulted, accompanied by a significant down-regulation of *Cyp11a1*, *Cyp17*, *Cyp19a1a* and *Hsd3b* (keys genes involved in steroidogenesis in teleost). Similarly, exposure to 86.9 ng L^{-1} of Levonorgestrel for 21 d significantly down-regulated 3β-*Hsd*, 20β-*Hsd* and *Cyp19a* in the larvae of fathead minnow (*P. promelas*) (Overturf et al., 2014). These results indicated NGT and levonorgestrel impair steroidal biosynthesis which may impair sexual differentiation of fish (Liang et al., 2015a; Overturf et al., 2014). In addition, given that the hypothalamus/pituitary gland is likely a major site of action of synthetic progestins (Runnalls et al., 2013), the possibility that genes involved in the HPG axis which can cause alterations in the production of
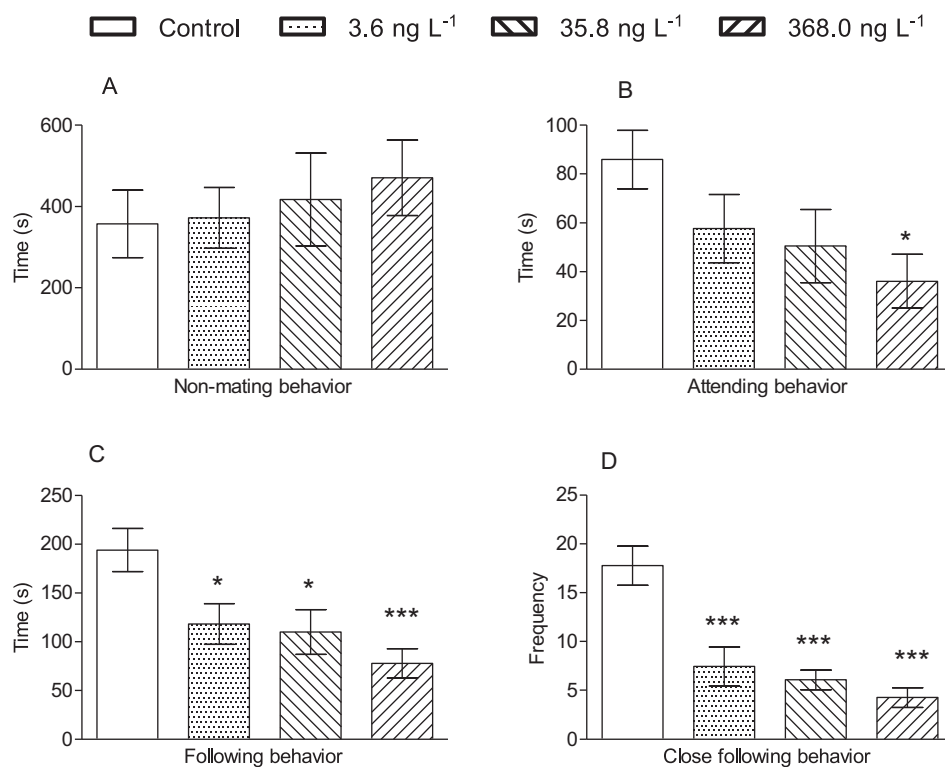
Fig. 4. Reproductive behavior exhibited by male mosquitofish paired with the norgestrel exposed female (mean ± standard error of the mean, n = 10). Panel A: Non-mating behavior; Panel B: Attending behavior; Panel C: Following behavior; Panel D: Close following behavior. Bars with * denote significant differences from the control.

hypothalamic and pituitary hormones are responsible for the observed spermatogenesis in the NGT-exposed female mosquitofish cannot be excluded (Fent, 2015; Liang et al., 2015b). Since NGT induced spermatogenesis in female mosquitofish after 42 d of exposure to environmentally relevant concentrations and exposure to progestins at environmentally relevant concentrations at early life stage of zebrafish may result in all male populations (Liang et al., 2015a; Svensson et al., 2016), intersex and gonadal histology was evaluated in adult female mosquitofish.

NGT caused a significant increase in the relative frequency of atretic folliculcar oocyte and post-ovulatory oocyte in the ovaries of fish, suggesting that long-term exposure to NGT caused histopathological alterations in the ovaries. The presence of post-ovulatory and atretic follicles in reproductive tissue of fish exposed to various progestins is considered as a sensitive endpoint for exposure to endocrine disruption chemicals (Blüthgen et al., 2013). In fish, follicular atresia tends to become more frequent following exposure to contaminants (Chen et al., 2016; Tyler and Sumpter, 1996) and might be associated with inadequate levels of steroid hormones (for instance, 17β-estradiol) controlling the normal development of the ovary (Nagahama et al., 1994). Indeed, it was reported that adult female zebrafish exposed to 3124 ng L^{-1} of levonorgestrel had significantly lower plasma 11-keto-testosterone (11-KT) and estradiol (E2) concentrations than the control ones (Blüthgen et al., 2013). The same might hold for the NGT-exposed mosquitofish though the concentrations of these two hormones were not measured in this study.

4.2. Effects of NGT on the reproductive behavior of mosquitofish

Since control of copulation is ultimately maintained by the female and her willingness to allow male encounters (Bisazza and Marin, 1991), only NGT-treated females paired with untreated males were used in the reproductive behavior test in this study. In addition, the androgenic effects of NGT can be better disentangled when the NGT-exposed female mosquitofish were used in the behavior test since the

behavior of male fish was controlled. There was a significant decrease in the time spent following breeding males and the number of encounters with males paired with a female mosquitofish from each of the three NGT treatments. An early study using a similar experimental design for the behavior test found that control male mosquitofish paired with a female treated with 100 ng L^{-1} of levonorgestrel for 8 d had decreased attending behavior, and a lower number of gonopodial thrusts (Frankel et al., 2016b). Other studies also found that the reproductive behaviors of mosquitofish were altered by paper mill effluent containing androgenic compounds (Saaristo et al., 2013; Toft et al., 2004). Collectively, these results indicate that reproductive behavior can be altered by exposure to progestins and be considered as sensitive endpoints for the effects of NGT and other synthetic progestins in mosquitofish. In addition, as suggested in other studies (Doyle and Lim, 2002; Weis et al., 2001), the reproductive behavior may provide a cost-effective screening tool for assessing the EDC exposure under field conditions.

Taken together, this study demonstrated that exposure to environmentally relevant concentrations of NGT induced masculinization of female mosquitofish, resulted in spermatogenesis in the ovary, caused histopathological alteration of the ovary and the behavioral response (e.g., following behavior) of unexposed males to NGT-exposed females was altered. These results imply that wild populations of mosquitofish could be similarly affected in NGT contaminated environments. Given that the known effects of synthetic progestins at relatively low concentrations on fish, the ecological risk of these chemicals on fish and other wildlife species deserve more attention.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2018.03.014.

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