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Aqueous exposure to the progestin, levonorgestrel, alters anal fin development and reproductive behavior in the eastern mosquitofish (*Gambusia holbrooki*)

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ABSTRACT

Endogenous progestogens are important regulators of vertebrate reproduction. Synthetic progestins are components of human contraceptive and hormone replacement pharmaceuticals. Both progestogens and progestins enter the environment through a number of sources, and have been shown to cause profound effects on reproductive health in various aquatic vertebrates. Progestins are designed to bind human progesterone receptors, but they also have been shown to strongly activate androgen receptors in fish. Levonorgestrel (LNG) activates fish androgen receptors and induces development of male secondary sex characteristics in females of other species. Although behavior has been postulated to be a sensitive early indicator of exposure to certain environmental contaminants, no such research on the reproductive behavior of gestagen-exposed fish has been conducted to date. The goal of our study was to examine the exposure effects of a human contraceptive progestin, LNG, on the reproductive development and behavior of the viviparous eastern mosquitofish (*Gambusia holbrooki*). Internal fertilization is a requisite characteristic of viviparous species, and is enabled by an androgen driven elongation of the anal fin into the male gonopodium (i.e., phallus). In this study, we exposed adult mosquitofish to ethanol (EtOH control), 10 ng/L, and 100 ng/L LNG for 8 d using a static replacement exposure design. After 8 d, a subset of males and females from each treatment were examined for differences in the 4:6 anal fin ratio. In addition, paired social interaction trials were performed using individual control males and control females or females treated 10 ng/L or 100 ng/L LNG. Female mosquitofish exposed to LNG were masculinized as evidenced by the elongation of the anal fin rays, a feature normal to males and abnormal to females. LNG caused significant increases in the 4:6 anal fin ratios of female mosquitofish in both the 10 ng/L and 100 ng/L treatments, although these differences were not significant between the two treatments. LNG caused significant increases in the 4:6 anal fin ratio of males exposed to 100 ng/L, with no effects observed in the 10 ng/L treatment. In addition, the reproductive behavior of control males paired with female mosquitofish exposed to 100 ng/L LNG was also altered, for these males spent more time exhibiting no reproductive behavior, had decreased attending behavior, and a lower number of gonopodial thrusts compared to control males paired to control female mosquitofish. Given the rapid effects on both anal fin morphology and behavior observed in this study, the mosquitofish is an excellent sentinel species for the detection of exposure to LNG and likely other 19-nortestosterone derived contraceptive progestins in the environment.

1. Introduction

Over the past several decades, there has been much interest in the exposure effects of endocrine disrupting chemicals whose biological effects are mediated by estrogen, androgen, and thyroid hormone receptors. Recently researchers have begun to examine the exposure effects of environmental gestagens on aquatic

Abbreviations: EtOH, ethanol; LNG, levonorgestrel; SSC, secondary sex characteristic; LC/MS/MS, liquid chromatography tandem mass spectrometry; WTPE, wastewater treatment plant effluent.

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vertebrates, including natural progestogens (e.g., progesterone) and synthetic progestins (e.g., levonorgestrel). Progestogens are known to mediate physiological processes through nuclear progesterone receptors PR, membrane PR, and perhaps PR membrane components. Progestogens play critical roles in the control of vertebrate reproduction, including regulation of gestation, gamete maturation, and reproductive behaviors (Boni et al., 2007; Tubbs et al., 2010; Wasserman et al., 1982; Zhu et al., 2003). Synthetic progestins are designed to activate human nuclear PRs and are often combined with an estrogen, commonly 17α-ethinylestradiol, in oral contraceptives and hormone replacement therapies (Sitruk-Ware and Nath, 2013). Progestins are also commonly utilized as growth promoters and to synchronize reproduction in at least cattle, dairy cows, and pigs (Cavallin et al., 2014; Kolok and Sellin, 2008). Gestagens and their metabolites are excreted from all vertebrate animals, and as a result, are released into the environment via a myriad of pathways (e.g., wastewater treatment and paper mill plant effluent, runoff from agricultural fields where animal manure has been applied as fertilizer, and runoff from animal feeding operations (Kolodziej et al., 2004; Kolodziej and Sedlak, 2007; Kolok and Sellin, 2008; Orlando and Ellestad, 2014). Research examining the extent of gestagen pollution in the environment has detected up to 188 ng/L in wastewater treatment plant effluent, 34 ng/L in surface waters, and progestogen concentrations in excess of 10,000 ng/L in agricultural runoff lagoons (Fent, 2015; Orlando and Ellestad, 2014; Yost et al., 2013, 2014). Nevertheless, the number of environmental studies on gestagens is relatively few compared to EE_2 and other EDCs.

Human contraceptive progestins were first approved for use in the U.S. in the 1960s (Sitruk-Ware et al., 2013). Most of the first progestins were relatively nonspecific and not only bound human PR but also androgen and glucocorticoid receptors, resulting in off-target and undesirable side effects including unwanted weight gain, hirsutism, and acne (Africander et al., 2011; Sitruk-Ware et al., 2013). Specific progestins have been categorized into generations based upon the parent compound the progestins are derived from, with newer generation serving as more specific ligands for PR (Orlando and Ellestad, 2014). However, it has been shown through multiple *in vitro* assays that some progestins activate fathead minnow and other fish androgen receptors, while producing little to no activation of progesterone receptors, suggesting that exposure responses to these chemicals may differ greatly between humans and other vertebrates (Ellestad et al., 2014; Runnalls et al., 2013). Studies examining the exposure effects of gestagens on reptiles have found evidence of reduced steroid hormone synthesis and yolk development in alligators (Austin, 1991) and altered sex ratios in red-eared slider turtles (Wibbels and Crews, 1995). Changes in gonadal structure, final maturation of eggs, metamorphosis, and reproductive behavior have also been observed in African clawed frogs (Hoffmann and Kloas, 2012; Kvarnryd et al., 2011; Lorenz et al., 2011; Ogawa et al., 2011). In several species of fish, gestagens decrease fecundity, increase ovarian apoptosis, and alter development of secondary sexual characteristics (Paulos et al., 2010; Runnalls et al., 2013; Svensson et al., 2013; Zeilinger et al., 2009; Zucchi et al., 2012). While previous studies examining the exposure effects of EDCs on the fecundity, morphology, and physiology in fish, only a minority of studies have examined effects on reproductive behavior (Söffker and Tyler, 2012).

The mosquitofish is an excellent model for aquatic toxicology/endocrine disruption studies due to its wide native range in the United States, exotic range throughout the world, distinct courtship and reproductive behaviors, and the development of dimorphic secondary sex characteristics (SSC) (Orlando et al., 2005). An example of a SSC is the development of the phallus called a gonopodium in males from an unspecialized, anal fin containing 10 rays in juveniles. In females, the anal fin simply enlarges as the body size increases. In males, there is androgen dependent elongation of anal fin rays 3–5 (gonopodium) which functions as a phallus and allows for the use of internal fertilization (Ogino et al., 2004b; Rosa-Molinar et al., 1996). Previous studies examining the exposure effects of paper mill effluent (Bortone et al., 1989; Howell et al., 1980; Orlando et al., 2007; Toft et al., 2004), androgenic growth promoters such as 17β-trenbolone (Brockmeier et al., 2013; Saaristo et al., 2013; Sone et al., 2005), 11-ketotestosterone (Angus et al., 2001), and androstenedione (Stanko and Angus, 2007) have noted both the modification of female anal fins, as well as altered reproductive behavior due to exposures to such contaminants. Knowing that select progestins masculinize SSCs in female fishes, and that mosquitofish are an excellent model for studying androgenic endocrine disruptors, we hypothesized that exposure to the human contraceptive progestin LNG would masculinize anal fin development and alter normal reproductive behavior in the mosquitofish.

2. Materials and methods

2.1. Research organisms

Feral adult mosquitofish were netted from five sites on Paint Branch Creek on the campus of the University of Maryland, College Park, under permit #SCP201531, and transported in aerated buckets to our lab. Individuals were sorted into groups based on sex and reproductive status (as determined by body length and the presence of a gonopodium) and housed in a recirculating aquaculture system consisting of ~38 L glass tanks. All fish were quarantined for 2 weeks under optimal water conditions (temperature = 25 ± 1.8 °C, pH = 7.1–7.4, dissolved oxygen, DO > 5 ppm), and individuals displaying developmental deformities or signs of illness were euthanized using excess MS-222 (300 ppm). Throughout the quarantine and experiment, fish were fed twice with Tetra-Min® tropical flake food and once daily with freshly hatched brine shrimp. After quarantine, adult mosquitofish were transferred from the breeding colony to the exposure system. This research was conducted under an approved University of Maryland IACUC protocol (R-14-60).

2.2. LNG stock preparation and fish exposure

Superstock solutions were prepared by dissolving LNG (Steraloids Inc., Rhode Island, CAS 797-63-7, 4-ESTREN-17α-ETHYNYL-18-HOMO-17β-OL-3-ONE(−)) in 95% ethanol, EtOH (Fisher Scientific), aliquoted, and stored at −20 °C. Each day, working stock solutions were made by a 1:2000 dilution of the superstock solution with 95% EtOH into a sterilized, aluminum foil covered glass container. Fish were exposed using a static replacement system to three treatments consisting of the control (EtOH only), 10 ng/L LNG, and 100 ng/L LNG (nominal concentrations) for 8 d (Fig. 1). EtOH concentrations for all treatments did not exceed 0.0000095%. During the exposure period, dechlorinated freshwater was delivered to a 19 L glass, mixing vessel using a single channel peristaltic pump (Cole Parmer Masterflex pump model 7553-50 w/ Masterflex Easyload II head model 77200-62) at a rate of 500 mL/min. For each treatment, another peristaltic pump (Gilson Minipuls 3) was used to add LNG at a rate of 1 mL/min to achieve the appropriate final LNG concentrations of 10 and 100 ng/L. For each 19 L tank, a smaller peristaltic pump (APT instruments model SP200VOHD) was used to move water from the appropriate mixing vessel into the treatment tank. Using these pumps, water changes were performed twice a day between 09:00 and 15:00. Fish were kept in same-sex groups, with stocking rates of 4 adults per tank.