Title

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.

62	Keywords
63	masculinization
64	birth control pill
65	environmental gestagen
66	environmental androgen
67	phallus development
68	intersex
69	

70 **Abbreviations**

71	ethanol	EtOH
72	levonorgestrel	LNG
73	secondary sex characteristic	SSC
74	liquid chromatography tandem mass spectrometry	LC/MS/MS
75	wastewater treatment plant effluent	WTPE

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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 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 17 α -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

105 lagoons (Fent, 2015; Orlando and Ellestad, 2014; Yost et al., 2013; Yost et al., 2014).
106 Nevertheless, the number of environmental studies on gestagens is relatively few compared to
107 EE₂ and other EDCs.

108 Human contraceptive progestins were first approved for use in the U.S. in the 1960s
109 (Sitruk-Ware et al., 2013). Most of the first progestins were relatively nonspecific and not only
110 bound human PR but also androgen and glucocorticoid receptors, resulting in off-target and
111 undesirable side effects including unwanted weight gain, hirsutism, and acne (Africander et al.,
112 2011; Sitruk-Ware et al., 2013). Specific progestins have been categorized into generations
113 based upon the parent compound the progestins are derived from, with newer generation
114 serving as more specific ligands for PR (Orlando and Ellestad, 2014). However, it has been
115 shown through multiple *in-vitro* assays that some progestins activate fathead minnow and
116 other fish androgen receptors, while producing little to no activation of progesterone receptors,
117 suggesting that exposure responses to these chemicals may differ greatly between humans and
118 other vertebrates (Ellestad et al., 2014; Runnalls et al., 2013). Studies examining the exposure
119 effects of gestagens on reptiles have found evidence of reduced steroid hormone synthesis and
120 yolk development in alligators (Austin, 1991) and altered sex ratios in red-eared slider turtles
121 (Wibbels and Crews, 1995). Changes in gonadal structure, final maturation of eggs,
122 metamorphosis, and reproductive behavior have also been observed in African clawed frogs
123 (Hoffmann and Kloas, 2012; Kvarnryd et al., 2011; Lorenz et al., 2011; Ogawa et al., 2011). In
124 several species of fish, gestagens decrease fecundity, increase ovarian apoptosis, and alter
125 development of secondary sexual characteristics (Paulos et al., 2010; Runnalls et al., 2013;
126 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) in males 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 17 β -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 two 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 TetraMin® 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-17 α -ETHYNYL-18-HOMO-17 β -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 (Figure 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.

INSERT FIGURE 1

2.3 Water chemistry analysis

Water samples were collected at 15:00 on days 1, 4, and 8 and frozen at -20°C. Samples were then shipped overnight on wet ice to the Organic Geochemical Research Laboratory (US Geological Survey, KS). The samples were logged in, thawed, and sample aliquots were pipetted into glass 2 mL chromatography vial to which, 5 µL of a 5% tetrasodium EDTA solution was added. The bulk sample and the vial samples were refrozen until analysis. Samples were analyzed using a Waters Corp. (Milford, MA) Acquity H-Class Bio ultrahigh pressure liquid chromatograph (UPLC) and AB/Sciex (Ontario, Canada) API 5500 tandem mass spectrometer (MS/MS). Samples were analyzed for LNG and progesterone-d10 as an internal standard. To compensate for potential matrix effects, each sample was analyzed unspiked and then spiked

using stacked direct aqueous injection of 95 μ L of sample and 5 μ L of a solution (A mix) containing 1.9 ng/mL of internal standard and then with 5 μ L of a solution (SA mix) containing 1.9 ng/mL of the internal standard and of LNG, respectively. Addition of 5 μ L of the 1.9 ng/ μ L A and SA solutions provided the equivalent mass of the injection of 95 μ L of a sample with an analyte concentration of 100 ng/L. The compounds were separated using a 20 min gradient separation. Specific UPLC conditions are presented in Table 1.

The samples were analyzed by MS/MS using scheduled multiple reaction monitoring (SMRM) in positive-ion mode. The SMRM LNG method was set with a 1 min acquisition window centered on the retention time for each analyte. The declustering potential (DP), entrance potential (EP), collision energy (CE), and collision exit potential (CXP) was optimized for each MRM transition pair. Specific MS/MS conditions are presented in Table 1.

INSERT TABLE 1

An 8-point standard curve of solutions ranging from 0.5 to 1000 ng/L was analyzed with the samples using stacked injections drawing up 95 μ L of standard solution and 5 μ L of A mix. The experimental samples then were analyzed as A-SA pairs as previously stated. A laboratory water blank sample was analyzed after the standard curve and after the analysis of each A-SA paired sample. Two MRM (protonated molecule-daughter ion) transitions were monitored for each analyte. Analytes were identified based on their analyte retention time, the average ratio of the two daughter ions of the protonated analyte calculated from the standard curve $\pm$ 25%, and their retention time to each other ($\pm$ 1 s).

Sample runs were processed using AB/Sciex Multiquant 3.0 and samples were initially quantitated using the 8-point standard curve. The resulting processed file was exported to Excel (Microsoft, 15.0) and quantitation was performed using the method of standard addition.

2.4 Behavioral trials

Behavior trials were conducted using a setup consisting of six, 38 L glass aquariums (Figure 2). To obtain behavior recordings, a high quality USB webcam (Logitech C920 HD Pro, 960-000764) was mounted above each tank so that the entire surface area was visible in a single frame. To provide sufficient contrast and prevent effects from external stimuli, the sides and bottom of each tank were covered with white paper. Opaque dividers were placed between each tank to prevent visual interaction between individuals in adjacent tanks, and blackout curtains were hung around the perimeter of the system to prevent interference from external stimuli. Care was taken in positioning the lighting units so that the tanks were illuminated indirectly to prevent shadowing and provide equal lighting across the entire surface area of the tank.

INSERT FIGURE 2

For each trial, two individuals were randomly selected from the treatment populations based on the various male-female experimental combinations (n = 5 per treatment combination). Combinations tested included control males x control females, control males x 10 ng/L LNG exposed females, and control males x 100 ng/L LNG exposed females. To minimize

contamination, both fish were removed from the exposure system and briefly placed in separate 500 mL beakers containing pure dechlorinated H₂O for 20 s, after which they were immediately moved into the experimental tank. The beakers were then cleaned and refilled with fresh water to be used for the following trial. Once introduced to the experimental tank, both fish were allowed a 10 min acclimation period, during which they were allowed to freely interact with one another. After the acclimation period, all courtship and reproductive interactions were recorded for a period of 10 min using iSpy recording software (<http://www.ispyconnect.com/>, ver. 6.1.2.0). Videos were later analyzed for the presence of several courtship and reproductive behaviors (Table 2), and the time and/or frequency of each quantified using JWatcher™ (<http://www.jwatcher.ucla.edu/>, ver. 0.9).

INSERT TABLE 2 HERE

2.5 Anal fin morphometrics

At the conclusion of each behavior recording, both individuals were euthanized by immersion in excess MS-222 (300 ppm). For each fish, body mass (g) and standard length (mm) were recorded, and a photograph of the anal fin obtained using a Nikon DS Fi1 5 megapixel color digital camera mounted on a Nikon SMZ-2T Stereo Zoom Dissecting Scope. Images were imported and analyzed using ImageJ (<http://imagej.nih.gov/ij/>, ver. 1.48). Fin ray lengths were measured in pixels, with each pixel representing 18.5 µm as determined by a stage micrometer. Anal fin morphometrics were measured following the protocol by Angus et al. (2001), where

the ratio of the length of the 4th and 6th anal fin ray (4:6 ratio) was calculated and rounded to the nearest micron.

2.6 Statistical analysis

All data were analyzed using SAS Studio (version 3.3, Cary, NC). Reproductive behaviors and 4:6 ratio anal fin measurements were checked for homogeneity of variance and normality, and any data that did not meet these requirements were log transformed. Differences were determined by one-way analysis of variance (ANOVA) followed with post-hoc analysis by Tukey's HSD method. Differences were considered significant if $p < 0.05$. Unless otherwise noted, all data are presented as the mean $\pm$ SEM.

3. Results and Discussion

The goal of this study was to examine the exposure effects of LNG on mosquitofish anal fin and reproductive behavior. As a consequence of 100 ng/L LNG exposure, male were supermasculinized, as their anal fins were elongated compared to control males. Female mosquitofish anal fins were masculinized at both the 10 ng/L and 100 ng/L LNG concentrations. In addition, control males responded behaviorally to the 100 ng/L LNG exposed females very differently than how they responded to control females, although no differences were observed between control males and 10 ng/L females. Overall, we found that control male reproductive behaviors were diminished when paired with LNG exposed females relative to control female mosquitofish.

3.1 Water chemistry analysis

Measured concentrations of LNG in each treatment on days 1, 4, and 8 are shown in Table 3. No LNG was detected in the ethanol control samples. Measured concentrations for the 10 ng/L treatment ranged from 2.8 - 18 ng/L, with an overall average of 8.6 ± 5 ng/L. Concentrations for the 100 ng/L ranged from 14 - 83 ng/L, with an average of 39 ± 22.1 ng/L.

INSERT TABLE 3

Measurement of all environmental gestagens is comparatively limited relative to measurements of environmental estrogens. Available results, however, suggest that concentrations are generally in the single to low hundreds of ng/L in WTPE and downstream surface waters and are more variable in animal agricultural sources. In animal agriculture wash water lagoons, environmental gestagens have been reported at greater than 10,000 ng/L and are in low to hundreds of ng/L in runoff and receiving surface waters (Fent, 2015). The progestin used in this study, LNG, has been measured in WTPE up to 40 ng/L. In static exposure studies using relatively lipophilic steroid compounds such as LNG, comparisons of nominal to measured concentrations commonly result in lower measured versus nominal concentrations, where the compound may partition out of the water and adhere to tank walls, tubing surfaces, and may be bioaccumulated by the fish or biodegraded by the biota (fish and microbes) within the system (OPPTS/USEPA, 2009). For this reason, flow-through exposure systems are preferred. While our actual average concentration (39 ± 22.1 ng/L) for the 100 ng/L treatment

departed from nominal levels, both LNG treatments are environmentally relevant concentrations.

3.2 Morphology and anal fin development

Exposure to LNG caused significant increases in the 4:6 anal fin ratios of female mosquitofish at both the 10 ng/L (1.381 ± 0.088 , $p = 0.002$) and 100 ng/L (1.533 ± 0.067 , $p = 0.001$), although these differences were not significant between the two treatments ($p = 0.081$) (Figure 3).

INSERT FIGURE 3

Exposure to 100 ng/L LNG resulted in significant increases in the 4:6 ratios of male mosquitofish (3.142 ± 0.308 , $p < 0.05$) (Figure 4a and 4b). No differences were found between the control (2.530 ± 0.113) and 10 ng/L LNG (2.981 ± 0.159) treated males, $p = 0.132$.

INSERT FIGURE 4

Female mosquitofish developed elongated anal fins after just 8 d of exposure to both treatments of LNG, similar in 4:6 anal fin ray ratio to those exhibited by normal mosquitofish males. This anal fin elongation in female mosquitofish has been observed in other lab studies examining exposure effects of known androgens such as the anabolic steroid, 17β -trenbolone (Brockmeier et al., 2013); the fish androgen, 11-ketotestosterone (Angus et al., 2001); and the

relatively weak androgen found in all vertebrates, androstenedione (Stanko and Angus, 2007). Complex environmental mixtures such as paper mill effluents also masculinize female mosquitofish anal fins (Orlando et al., 2007; Parks et al., 2001; Toft et al., 2004). While this is the first study to examine the effects of a progestin on eastern mosquitofish, female masculinization and development of male secondary sexual characteristics has been noted in previous exposure studies utilizing other teleost species (Kumar et al., 2015; Orlando and Ellestad, 2014)

While the mechanisms that mediate progestogenic endocrine disruption are poorly understood, exposure of several aquatic species to LNG has been shown to cause changes in mRNA expression of several key steroidogenic enzymes, including decreases in FHM larval *hsd3b*, *hsd20b*, and *cyp17* expression (Overturf et al., 2014). Our lab and others have reported that 19-nortestosterone derived progestin pharmaceuticals, including LNG, activate fish androgen receptors *in vitro* (Bain et al., 2015; Ellestad et al., 2014a; Orlando and Ellestad, 2014). Additionally, LNG exposure of several aquatic species has been shown to mediate changes in the production and concentration of multiple sex steroids, such as decreases in ex-vivo ovarian 11-KT, pregnenolone, 17, 20-DHP, and T concentrations in female FHMs (Overturf et al., 2014), as well as decreases in 11-KT plasma levels in male pubertal roach (Kroupova et al., 2014) and decreases in plasma E2 concentrations in female pubertal roach (Kroupova et al., 2014). Taken together, it appears that LNG and similar progestins act through changes in gonadotropin expression in the pituitary, as well as changes in plasma sex steroid levels (Kumar et al., 2015).

While not examined as part of this study, it is interesting to note that the normal development of the mosquitofish gonopodium is mediated by an androgen-dependent

induction of the sonic hedgehog (*shh*) gene in the distal portion of outgrowing anal fin rays (Ogino et al., 2004a), which has also been shown to be required for penial morphogenesis (Podlasek et al., 2003) and initial outgrowth of the genital tubercle and cloaca (Haraguchi et al., 2001). As such, our results further support the androgenic actions of LNG. As *shh* signaling has been shown to also play a role in cell proliferation, fin regeneration, and the formation of various skeletal elements (Avaron et al., 2007), it is possible that exposure to LNG and other progestins may cause developmental deformities and decreased regeneration abilities.

In our study, male mosquitofish were also affected by LNG exposure, as evidenced by the increased anal fin ray length found in the 100 ng/L treatment (Figure 2). Similarly, field studies examining anal fin morphology of male mosquitofish exposed to paper mill effluent *in situ* were also supermasculinized (i.e., exposed males had longer anal fins, without differences in body length). Previous studies examining the effects of progestins have found many adverse effects on female fish morphology in fathead minnows and minimal effects on male morphology (Paulos et al., 2010; Runnalls et al., 2013), indicating that responses to progestin exposure by males and females may be species dependent. In the wild, males display a large amount of variation in regards to gonopodial length and structure (Kahn et al., 2010). Because female mosquitofish have been shown to preferentially associate with males possessing longer gonopodial structures (Kahn et al., 2010; Langerhans et al., 2005), it is possible that they may gravitate towards and preferentially mate with LNG exposed males compared to unexposed males. As LNG exposure has been shown to alter survival, growth, and steroidogenic enzyme expression in fathead minnows (Overturf et al., 2014), such a selection by females could cause widespread disruptions in the overall health and survivability of wild mosquitofish populations

due to a decrease in offspring from more fit, unexposed males. Furthermore, both males and females that develop an elongated anal fin as a result of exposure to LNG may also be at greater risk of predation due to the increased energetic costs associated with predator avoidance and swimming dynamics (Langerhans et al., 2005; Oufiero and Garland, 2007).

3.3 Behavioral trials

The reproductive behavior of control males were altered when paired with 100 ng/L LNG exposed female mosquitofish. Control males paired with exposed females spent more time exhibiting no reproductive behavior, 7.916 ± 0.753 min for 100 ng/L LNG, compared to 5.371 ± 0.765 min for control males paired with control females, $p = 0.021$. Control males paired with LNG exposed females spent less time exhibiting attending behavior, 1.839 ± 0.661 min for 100 ng/L LNG, compared to 4.386 ± 0.757 min for control males paired with control females, $p = 0.032$. Also, there was a dramatic decrease in the number of gonopodial thrusts with 0.6 ± 0.6 in control males paired with for 100 ng/L LNG exposed females compared with 6 ± 1.549 thrusts for control males paired with control female mosquitofish, $p = 0.036$ (Figure 3a, 3b, and 3d). No differences in following behavior were observed for both 10 ($p = 0.899$) and 100 ng/L ($p = 0.432$) treatments (Figure 3c), and no differences were observed for any of the observed behaviors as a result of the 10 ng/L LNG treatment, $p > 0.05$ (Figures 5a-5d).

INSERT FIGURE 5

Normal reproductive behavior of mosquitofish has been previously described (Pyke, 2005), and their descriptions were adopted for our study. Although research by others has shown that both male and female mosquitofish can display their own selection preferences based upon physical characteristics such as body size (Bisazza and Marin, 1991; Bisazza and Marin, 1995; McPeck, 1992; Pilastro et al., 1997) and gonopodium length (Kahn et al., 2010), reproductive biology theory says that control of copulation is ultimately maintained by the female and her willingness to allow for male approaches (Bisazza and Marin, 1991; Rosen and Tucker, 1961). Although the effects of LNG on teleost behavior have not been published, supermasculinization of calling behavior in male African clawed frogs (*Xenopus laevis*) has been reported (Hoffmann and Kloas, 2012). However, alteration of normal reproductive behaviors in both eastern and western mosquitofish have been reported in studies of the exposure effects of androgenic paper mill effluent (Ellis et al., 2003; Toft et al., 2004), phytosterols (Denton et al., 1985; Krotzer, 1990), and 17 β -trenbolone (Saaristo et al., 2013).

Because only females were exposed to LNG treatments in this study before being utilized in the paired social interaction trials with untreated males, the observed alterations to reproductive behavior provide support to the control of reproduction by females. That said, there is much work that needs to be done to determine the full extent of the effects of LNG and other progestins on behavior. Because reproductive behavior is arguably one of the most sensitive endpoints that can currently be measured, our results document that LNG and other gestagens can affect reproductive behavior and merit further investigation.

In conclusion, this is the first known report documenting that aqueous exposure to the LNG affects the reproductive health of a viviparous fish, the eastern mosquitofish. Adult female

410 mosquitofish exposed for eight days to environmentally relevant concentrations of LNG
411 developed elongated anal fins similar to males and to females exposed to other known
412 androgenic substances. Such changes may have drastic influences on sexual selection in this
413 and other teleost species that use fin morphology as a selective marker for mate choice.
414 Furthermore, control males reproductive behaviors were diminished when paired with females
415 that had been exposed to 100 ng/L LNG. The observed disruption of normal reproductive
416 behavior due to LNG exposure is also noteworthy and provides further evidence for the use of
417 behavioral assays as a sensitive indicator of environmental impact. Taken together, alterations
418 in reproductive development and behavior mirrors what is being documented in oviparous fish
419 species and other aquatic vertebrates. Future research is needed to fully understand the
420 potential impact of LNG exposure on male mosquitofish, gametogenesis in both sexes, and if
421 development within the adult viviparous mosquitofish female protects the next generation
422 during embryogenesis.

Acknowledgements

This work was supported by grants from the Cosmos Club Foundation (TEF, Cosmos Scholars - 2015) and the Morris Animal Foundation (EFO, D14ZO-010). Analytical chemistry support was from the United States Geological Survey (USGS), Toxics Substances Hydrology Program. We thank Amanda Gillis, Jacob Held, Zoe Bailey, and Matt Levitas for their help with fish collections and general husbandry. Thanks also to Grace DeWitt and Elizabeth Lavin for their help with performing the behavioral trials. We sincerely appreciate the editorial suggestions provided by the USGS reviewer. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the authors.

432 **Figure Captions**

433 Figure 1. Diagram of static replacement exposure system.

434 Figure 2. Diagram of behavioral recording system utilized for paired social interaction trials.

435 Figure 3. Mean $\pm$ SEM of 4:6 ratios of female mosquitofish in each treatment (3A). Letters
436 denote a significant difference ($p < 0.05$). Anal fins of females after 8 days of exposure to
437 ethanol control, 10 ng/L levonorgestrel, and 100 ng/L levonorgestrel (nominal concentrations)
438 treatments (3B).

439 Figure 4. Mean $\pm$ SEM of 4:6 ratios of male mosquitofish in each treatment (4A). Letters denote
440 a significant difference ($p < 0.05$). Anal fins of males after 8 days of exposure to ethanol control,
441 10 ng/L levonorgestrel, and 100 ng/L levonorgestrel (nominal concentrations) treatments (4B).

442 Figure 5. Mean $\pm$ SEM of ethanol control male mosquitofish exhibiting no behavior (5A),
443 attending behavior (5B), following behavior (5C), and gonopodial thrusts paired with
444 levonorgestrel exposed females (5D). Letters denote a significant difference ($p < 0.05$).

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616

1 Table 1. Analysis conditions for liquid chromatography, tandem mass spectrometry
2

Water Chemistry Analysis Conditions	
Ultra-Pressure Liquid Chromatography (UPLC) Conditions	
Analytical Column	Waters BEH 50 x 2mm with 1.7 µL packing with matching BEH guard column
Mobile Phase	A, 0.1% formic acid; B, methanol
Sample Temperature	5 °C
Column Temperature	40 °C
Flow Rate	0.4 mL/min
Tandem Mass Spectrometry (MS/MS) Conditions	
Curtain Gas	20 L/h
Gas 1	40 L/h
Ionspray Voltage	2500 V
Collision Gas	8
Source Temperature	650 °C
Target SMRM cycle	0.25 s
Resolution	Unit

3

4 Table 2. Reproductive behaviors exhibited by adult mosquitofish (adapted from Pyke (2005))

5

Behavior	Description	Classification	Unit
No Behavior	Male and female do not show any physical interactions during this time period	N/A	Time (s)
Attending Behavior	Male remains close to static female facing her. Male may circle female or slightly adjust his position every few seconds	Courtship	Time (s)
Following Behavior	Male maintains a close, ≤ 5 cm, distance to female while she is in motion	Courtship	Time (s)
Gonopodial Thrust	Male positions himself behind female and quickly darts forward, simultaneously swinging his gonopodium towards the female's urogenital opening.	Mating	Frequency

6

Table 3. Concentrations of levonorgestrel (LNG) on days 1, 4, and 8 determined by liquid chromatography, tandem mass spectrometry.

Treatment	Day	ng/L	Mean $\pm$ SEM
Ethanol control	1	nd*	
	4	nd	nd
	8	nd	
10 ng/L LNG	1	2.8	
	4	18	8.6 $\pm$ 5
	8	3.4	
100 ng/L LNG	1	14	
	4	83	39 $\pm$ 22.1
	8	20	

nd* = no detection

Figure 1

Figure 2

Figure 3a

Figure 3b

EtOH Control Female Anal Fin

10 ng/L LNG Female Anal Fin

100 ng/L LNG Female Anal Fin

Figure 4

Figure 4a

Figure 4b

EtOH Control Male Anal Fin

10 ng/L LNG Male Anal Fin

100 ng/L LNG Male Anal Fin