Gonadal histology of adult western mosquitofish (*Gambusia affinis*) following early life exposure to 17 α -ethynylestradiol, 17 β -trenbolone, and/or atrazine

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Research Article

Keywords: runoff, cafo, development, compounds, simulated, gonadal

Posted Date: September 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-916070/v1>

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Abstract

Confined animal feeding operations (CAFOs) are a source of endocrine disrupting compounds (EDCs) in the aquatic environment and introduced there through agricultural runoff and insufficient waste storage systems. This study used the three compounds 17 α -ethynylestradiol (EE₂), 17 β -trenbolone (TB), and atrazine (Atr) alone and in a mixture representing a simulated CAFO runoff. Laboratory exposures were performed in order to assess if early life exposure during the period of sexual differentiation to CAFO runoff altered gonadal development in western mosquitofish (*Gambusia affinis*). Juveniles were exposed to low and high concentrations of EE₂ (50 or 250 ng/L), TB (10 or 50 ng/L), Atr (150 or 1,500 ng/L), or simulated CAFO runoff (50 ng/L EE₂, 10 ng/L TB, and 150 ng/L Atr or 250 ng/L EE₂, 50 ng/L TB, and 1,500 Atr) for 30-days followed by a 46-day recovery period. Gonadal histology was measured in adult males and females to assess stages of sexual development (i.e., oogenesis or spermatogenesis) and gonadal histopathology. All of the ovaries analyzed were in the maturing or mature stages of oogenesis, and all of the testes examined were in the intermediate or advanced stages of spermatogenesis. Intersex was observed in one individual exposed to 1,500 ng/L Atr. Ovarian histopathology was normal in all females, however testicular histopathology showed a loss of cyst structure leading to unbound interstitial sperm cells and unsynchronized cyst development in all males except the control fish. This study provided some evidence that exposure to simulated CAFO runoff and its individual compounds impaired testicular development.

1. Introduction

The United States (US) is the largest producer of livestock products, and over the past few decades the farming in the US has shifted heavily towards concentrated animal feeding operations (CAFOs), in order to meet global demand (FAO, 2016; Long et al., 2018). The US Department of Agriculture (USDA) estimated that livestock and poultry produced over 1.1 billion tons of wet weight manure in 2007 (US EPA, 2013). Manure produced on CAFOs is typically stored in wastewater lagoons which function as holding reservoirs or anabolic reactors, and the resultant wastewater generally does not receive any treatment prior to its land application (Bradford et al., 2008). Manure applied as fertilizer has been shown to improve soil condition for plant growth (Kapkiyai et al., 1999) and to increase the organic content of soil (Sommerfeldt & Chang, 1987). Although manure generated by CAFOs can be used as fertilizer, its runoff from agricultural fields presents environmental risks with regard to contaminants in these wastes, including excessive nutrients (Jongbloed & Lenis, 1998), salts (Hao & Chang, 2003), heavy metals (Barker & Zublena, 1995), microbial pathogens (Schets et al., 2005), antibiotics (Gilchrist et al., 2007), and natural and synthetic hormones (Liu et al., 2012).

The use of natural and synthetic hormones in CAFO agricultural practices is common, with over 90% of cattle on feedlots in the US treated with one or more growth-promoting steroids or steroid-like compounds (Gadd et al., 2010; USDA, 2000). For example, implants containing the anabolic steroid trenbolone acetate are subcutaneously inserted into cows, and two active metabolites, 17 α -trenbolone (17 α -TB) and 17 β -trenbolone (TB), are excreted from the cow (Schiffer et al., 2001). The synthetic estrogen, 17 α -

ethynylestradiol (EE₂), is used to increase livestock productivity (i.e. feed use efficiency), to treat diseases, and to regulate breeding in cattle (Gadd et al., 2010). While EE₂ is released from CAFOs, the major source of EE₂ in the environment is from human urine, as EE₂ is the active ingredient in most birth control pills (Pauwels et al., 2008). Animal waste containing TB and EE₂ can enter waterways through runoff from land application of manure or through leakage or spillage from manure lagoons (Burkholder et al., 2007). Analyses of water samples from a beef CAFO reported concentrations of 125 ng/L 17α – TB and 20 ng/L TB in a discharge drain that collects runoff, and 50 ng/L 17α – TB and 7 ng/L TB in a receiving stream located 381 meters downstream (Durhan et al., 2006). In waste storage lagoons, EE₂ was detected at 195 ± 59.8 ng/L but was not detected in receiving streams (Liu et al., 2012). Although the present study focuses on the synthetic hormones EE₂ and TB, farmers often use several other pharmaceuticals (i.e. methyl testosterone, dexamethasone, medroxyprogesterone, and norgestrel), based on their needs and desired production outcomes (Liu et al., 2012).

Another chemical of concern involving CAFO operations is atrazine (Leet et al., 2012). Atrazine is one of the most commonly used herbicides in agricultural practices and is frequently detected in streams in the US (Gilliom et al., 2006). Since CAFOs are often located close to agricultural fields, those fields are likely to have received both atrazine and CAFO manure, and runoff is likely to discharge both veterinary pharmaceuticals and herbicides into surface waters - especially following rain events (Stoeckel et al., 2012). Thus, CAFO runoff varies in chemical nature, yet the introduction of compounds into the environment occurs routinely (Bradford et al., 2008).

Exposure to compounds present in CAFO runoff can interfere with the hypothalamus-pituitary-gonadal axis leading to dysregulation of spermatogenesis and oogenesis (Bringolf et al., 2004; Tillitt et al., 2010). For example, fathead minnows (*Pimephales promelas*) exposed to 20 ng/L EE₂ had fewer mature spermatozoa, with mainly spermatogonia and spermatocytes in the testes (Salierno & Kane, 2009). Additionally, ovotestis formation was reported in female western mosquitofish (*Gambusia affinis*) exposed to 1 or 10 µg/L TB for 28 days when exposure started at the fry stage, but not when exposure started at the adult stage, suggesting a crucial window of sensitivity to androgenic compounds (Sone et al., 2005). Lastly, fathead minnows exposed to 5 and 50 µg/L atrazine during active reproduction exhibited decreased egg production and induced gonadal abnormalities such as genetic males with testicular oocytes and ovaries with undifferentiated germ cells (Tillitt et al., 2010).

Traditionally, studies on aquatic organisms have analyzed the effects of individual compounds of CAFO runoff, however only a few investigations have studied the results for multiple constituents of CAFO runoff (Huang et al., 2012; Leet et al., 2015). Chemicals occur in the environment as mixtures, thus highlighting the necessity to conduct laboratory studies with mixtures in order to simulate real world environmental conditions. The present study used environmentally relevant concentrations of EE₂, TB, and atrazine and their mixture to simulate CAFO runoff to assess whether exposure during the period of sexual differentiation in western mosquitofish (starting at 2–7 days post hatch [DPH]) would affect

gonadal development. To evaluate gonadal development, the fish were dissected as adults and the ovaries and testes were histologically analyzed and compared to those of unexposed fish.

2. Methods

2.1 Chemicals

17 α -Ethinylestradiol (17 α -Ethinyl-1,3,5(10)-estratriene-3,17 β -diol, $\geq$ 98% purity, Lot WXBC0533), atrazine (2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, $\geq$ 98% purity, Lot SzBD304XV), and 17 β -trenbolone (17 β -Hydroxyestra-4,9,11-trien-3-one, $\geq$ 93% purity, Lot FE03131501) were obtained from Sigma-Aldrich, St. Louis, MO. 17 α -Ethinylestradiol and atrazine were dissolved in methanol and diluted with deionized water (DiH₂O) to nominal concentrations of 50 or 250 ng/L EE₂, and 150 or 1,500 ng/L Atr. 17 β -Trenbolone was dissolved in acetonitrile and diluted with DiH₂O to nominal concentrations of 10 or 50 ng/L. Final concentrations of solvents were 0.001% in EE₂ and TB treatment groups and 0.085% in Atr treatment groups.

2.2 Western Mosquitofish.

A stock population of western mosquitofish was obtained from Mosquito Control Contractors Incorporated in New Iberia, LA. The fish were housed in a greenhouse at the University of Louisiana's Ecology Center (EC) under natural light conditions using tray systems, each with two trays and a 150-gallon filtering tank with recirculating well water. The stock population was used to produce the juvenile fish used in all experiments. Fish were bred during the summer months in 2019. The main stock population and newborn offspring were fed fish flakes (Tetramin, Blacksburg, VA) every other day, while the breeding fish were fed daily. Water temperature and water quality parameters (pH, nitrates, alkalinity, etc.) were monitored weekly with Tetramin EasyTest Strips (Blacksburg, VA) and they were always within the acceptable limits (nitrate < 40 ppm, nitrite < 0.5 ppm, hardness < 150 ppm, alkalinity 120 to 300 ppm, pH 6.8 to 7.6). All husbandry and exposure experiments with fish were approved by the University of Louisiana at Lafayette Institutional of Animal Care and Use Committee (IACUC 8717-056).

2.3 Experimental Design

Juvenile western mosquitofish (2–7 DPH) were used in a 76-day experiment: a 30-day static renewal exposure to simulated CAFO runoff low, high, or individual compounds, followed by a 46-day recovery period (Fig. S1). Juveniles from four broods were selected for the experiment and then acclimated for 24-hours in moderately hard reconstituted fresh water (Eaton et al., 1995) in laboratory conditions at US Geological Survey (USGS) Wetlands and Aquatic Research Center (WARC): 16 h light: 8 h dark photoperiod and 20 $\pm$ 1°C water temperature. After the acclimation period, four juveniles were randomly placed into 400 mL glass beakers (constituting a single replicate) containing moderately hard reconstituted fresh water (four replicates, n = 16 individuals per treatment). The sex ratio per treatment was unknown at the start of the experiment, because the sex cannot be determined phenotypically prior to sexual maturation (Lamatsch et al., 2015). Juveniles were exposed for 30 days to simulated CAFO

runoff at a low concentration mixture (50 ng/L EE₂, 10 ng/L TB, and 150 ng/L Atr), simulated CAFO runoff at a high concentration mixture (250 ng/L EE₂, 50 ng/L TB, and 1,500 ng/L Atr), and single compounds exposures: EE₂ low (50 ng/L EE₂), EE₂ high (250 ng/L EE₂), TB low (10 ng/L TB), TB high (50 ng/L TB), Atr low (150 ng/L atrazine), or Atr high (1,500 ng/L atrazine) (See exposure table, Table S1). Solutions were changed every 72 hours to maintain favorable water quality conditions and to maintain consistent concentrations of compounds (Hayes et al., 2003; Jackson et al., 2019). Preliminary experiments concluded water quality was acceptable for four juveniles in 400 mL of moderately hard reconstituted fresh water with 100% water changes every 72 hours (unpublished data). After the exposure, juveniles were placed in pure moderately hard reconstituted fresh water, transported to the greenhouse at the EC, and placed in EC well water to acclimate for 24 hours. Each replicate was then placed in a 1-quart paint bucket (Lowes, Mooresville, NC) within a tray system filled with recirculating well water for a 46-day recovery period at the EC.

During both the exposure and recovery periods, fish were monitored daily for mortality and fed daily either brine shrimp (Brine Shrimp Direct, Ogden, UT) 2-3x per week or fish flakes (Tetramin, Blacksburg, VA) on the other days of the week. Experiments were concluded on day 76 when 60% of all the fish were sexually mature. To euthanize, fish were overdosed with a 0.1% MS-222 solution and dissected (AFS Committee, 2014). Assessed endpoints were stages of sexual development and gonadal histopathology.

2.4 Chemical Analyses

During the 30-day exposure period, the concentrations of exposure compounds were analytically quantified three times (in duplicate) in the exposure solutions just after preparation. Analyses for chemical quantification were also attempted for water samples taken just prior to water change (at 72 hours), but were not successful (see Results section). Water samples were concentrated and purified using solid phase extraction (SPE), then quantified by high performance liquid chromatography (HPLC). The chemical mixtures (Table S1) were quantified at low and high levels. Strata C18-E cartridges (Phenomenex, Torrance, CA) were used in SPE. Cartridges were conditioned with methanol, washed with DiH₂O, and the treatment sample (400 mL) was applied under vacuum at a flow rate of 5–10 mL/min. Following treatment sample application, the cartridge was washed with DiH₂O under vacuum to remove unbound chemicals. The column was then eluted using two aliquots (1 mL) of methanol, under vacuum, and the resulting sample was brought to complete dryness under nitrogen gas. Each sample was rehydrated with 50 µL acetonitrile and DiH₂O (50:50 v/v). After SPE, the eluent was injected as two aliquots (25 µL) into a Shimadzu HPLC system (Columbia, MD) with a control unit (CBM-20A) and pump (LC20AD) coupled to a Dionex detector (AD20, Sunnyvale, CA). A Pinnacle DB Biphenyl column (3 µm 50 x 4.6 mm) (Restek, Bellefonte, PA) was used to separate EE₂, TB, and Atr with the gradient (Table S2) containing DiH₂O for solvent A and acetonitrile for solvent B using a flow rate of 1 mL/min. The HPLC gradient used in this project was adapted from a method for separating estrogens developed by Gall (2011).

2.5 Gonadal Histology

Sample preparation

A subset of individual fish ($n = 8$; 4 females, 4 males) from each treatment group were dissected and gonads were processed for histopathology. Testes and ovaries were weighed for morphometric endpoints; ovaries < 0.1 mg were not processed for histology. Treatment groups with less than four males were: control ($n = 2$), simulated CAFO runoff ($n = 2$), EE₂ high ($n = 2$), and TB low ($n = 0$). Ovaries and testes were directly placed in McDowell Trump's Fixative (Electron Microscopy Sciences, Hatfield, PA), and then placed in 4° C for 24 hours. Tissues were placed in sodium cacodylate buffer, rinsed with DiH₂O, and then postfixed with 2% Osmium Tetroxide (Electron Microscopy Sciences, Hatfield, PA). Tissues were rinsed with DiH₂O, dehydrated in a graded ethanol series (35%-100%) and in 100% acetone, and then infiltrated with Spurr's Low Viscosity Resin (Electron Microscopy Sciences, Hatfield, PA) (Spurr, 1969). Semi-thin sections (4–5 μ m) were mounted on a microscope slide, and stained with Methylene Blue Borax (Bloch & Korkmaz, 2005). At least five histological sections from each fish were examined to classify the stage of gonadal development as well as to assess potential cellular damage of testes and ovaries (See gonadal cellular structure subsection for scale).

Stages of Sexual Development

Histological sections of ovaries ($n = 4$ individuals per treatment) and testes ($n = 4$ individuals per treatment) were visualized microscopically and analyzed to classify the stage of oogenesis or spermatogenesis as a measure of sexual development (Jackson et al., 2019). Sex was confirmed by the sole presence of testicular or ovarian tissue, while gonads with both tissue types were classified as intersex (Huang et al., 2016). The stage of oogenesis in female western mosquitofish was categorized as immature (primary oocytes [O1]), maturing (cortical [O2] and/or early vitellogenic oocytes [O3]), or mature (mature ovum [O4]) based on the classification by Leusch et al. (2006). The stage of spermatogenesis in male western mosquitofish was categorized as immature, intermediate, or mature based on the classification by Uribe et al. (2014). Testes filled primarily with spermatogonia and primary spermatocytes were classified as immature, testes with all of the sperm cell types (spermatogonia, primary spermatocytes, spermatids, and spermatozoa) were classified as intermediate, and mature testes contained predominately spermatozoa.

Gonadal Cellular Structure

Cellular structure of the ovary and testis was qualitatively analyzed for morphological alterations. Ovaries were assessed as affected on a scale of 0–3 (0 changes absent, 1 minimal, 2 moderate, or 3 severe) (Blazer et al., 2013). Testes were evaluated with three metrics: (1) extent of being affected, on a scale of 0–3 (0 changes absent, 1 minimal, 2 moderate, or 3 severe); (2) cyst arrangement on a scale of 0–3 (0 normal, 1 minimal disruption, 2 moderate disruption, or 3 severe disruption); and (3) presence of interstitial unbound sperm cells on a scale of 0–1 (0 absent or 1 present) (Blazer et al., 2013; Velasco-Santamaría et al., 2013).

2.6 Statistical Analyses

Statistical analyses were performed at $\alpha = 0.05$ using statistical software: SAS and R (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). Stages of sexual development were analyzed with Fisher's Exact Test followed by Bonferroni's adjustment. Gonadal cellular structure alterations were analyzed with logistic regression.

3. Results

3.1 Chemical Analyses

The measured initial EE₂ concentration in the simulated CAFO water with the nominal EE₂ of 50 ng/L was 71.5 ± 8.4 ng/L (mean $\pm$ SE) while it was 266.8 ± 19.5 ng/L EE₂ in the water with a nominal concentration of 250 ng/L. The initial TB concentration measured in the simulated CAFO water with the nominal concentration of 10 ng/L was 7.4 ± 2.1 ng/L while it was 40.5 ± 4.2 ng/L TB in the water with the nominal concentration of 50 ng/L. The initial measured Atr concentration in the simulated CAFO water with the nominal concentration of 150 ng/L was 133.8 ± 16.3 ng/L and it was 976.9 ± 87.1 ng/L Atrazine in the water for nominal concentration 1,500 ng/L. Quantification at 72 hours was unsuccessful due to a lack of separation of the peaks between synthetic and natural hormones; concentrations were assumed to be within 70% of initial concentrations for this study based on validation completed by another study with similar experimental design (Jackson et al., 2019).

3.2 Gonadal Histology

Stages of Sexual Development

Stage of oogenesis did not differ significantly among treatment groups ($P = 0.120$) (Table 1). Histological observations of female fish in all treatment groups showed ovaries in maturing (cortical alveoli [O2] and/or early vitellogenic oocyte [O3]) or mature (vitellogenic oocyte [O4]) stages of oogenesis (Fig. 1). No female fish had immature ovarian development (i.e., only primary oocytes [O1]). All the individuals in Atr low ($n = 4$) and Atr high ($n = 4$) were in the mature stage, while this was observed for 50% of the fish in the control group. Embryos were present in the following treatment groups: simulated CAFO runoff low ($n = 1$ individual), simulated CAFO runoff high ($n = 1$), EE₂ high ($n = 1$), and Atr low ($n = 2$) (Fig. 2). Both histologically-analyzed females exposed to TB high appeared histologically mature (only two fish were analyzed in this case as the two others had ovaries that were too small for processing). The stages of spermatogenesis did not differ significantly among treatment groups, following Bonferroni's adjustment ($P = 0.026$, $\alpha = 0.006$) (Table 1). Intersex was observed in one individual: a fish exposed to Atr high in which the testis contained ovarian tissue (Fig. 3).

Gonadal Cellular Structure

All ovaries were scored as non-affected (changes absent), thus statistical analysis was not warranted. For the testicular cell structure endpoints, the logistic models did not converge, due to the small sample size ($n = 2$) in control, simulated CAFO runoff low, and EE₂ low treatment groups (Table 2). Treatment

groups were then combined to yield a model which fitted the data; however, this adjusted model also failed to converge. Testes from the control group showed intact cyst structure, meaning that cells were enclosed within cysts and displayed synchronized mitosis and meiosis (Fig. 4). Cyst degradation (thinning or absence of germinal epithelium) and unbound interstitial sperm cells were observed in males of all other treatment groups (Fig. 5, 6, and 7). Fish in the simulated CAFO runoff low treatment displayed severely affected testis (100%), severe cyst degradation (100%, Fig. 7), and interstitially unbound sperm cells (100%, Table 2, Fig. 7) while the control fish showed no alterations of the testis. Unsynchronized cyst development (Fig. 6) or irregular cyst formation (Fig. 7) were observed in male fish in all treatment groups except for the control group.

4. Discussion

Exposure to simulated CAFO runoff or its individual compounds did not result in differences in the stages of sexual development among treatment groups in male or female fish (Table 1). Gonadal histology confirmed all female fish sampled displayed ovaries in the maturing or the mature stage of oogenesis, and all male fish sampled showed testes in the intermediate or advanced stages of spermatogenesis (Table 1). Intersex was confirmed in a single male mosquitofish exposed to Atr high; the presence of ovarian tissue located within the testis indicated that this involved a feminization of a male fish (Fig. 3). Atrazine exposure has been reported to feminize zebrafish by increasing the expression of aromatase, leading to decreased levels of testosterone and increased levels of estrogen (Weber et al., 2013). Although not statistically significant, female fish exposed to simulated CAFO runoff (low and high), EE₂ high, and Atr low treatment groups produced embryos while zero embryos were observed in the ovaries of the control fish, thus providing some evidence for accelerated oogenesis in the former treatment groups. Initial effects of EE₂, TB, and Atr on sexual development could have been negligible or absent, or were present and then reversed during the recovery period. For example, in zebrafish (*Danio rerio*) the effects of EE₂ were reversible following a 40-day recovery period (Baumann et al., 2014). However, irreversible masculinization (i.e., skewed sex ratio [all male population]) was shown after a 60-day exposure to TB followed by a 170-day recovery period (Morthorst et al., 2010). In light of the latter study, these results may suggest that an irreversible masculinization by TB in fish may require an exposure period over 30-days.

Histological examinations are valuable indicators of sublethal effects because they are often the result of the integration of several physiological processes (van der Ven et al., 2003). The cellular structure of the testes was altered by exposure to simulated CAFO runoff and its individual compounds, however the differences among treatment groups were not statistically significant - potentially due to a low sample size and lack of variation within treatment groups (Table 2). In contrast to the observations of abnormalities in testicular cellular structure, ovarian cell structure was not altered, thus gonadal malformations due to exposure to simulated CAFO runoff appeared to be sex-specific. Testes from the control group displayed organized and defined cysts, with clearly defined boundaries (Fig. 4), yet males in all treatment groups had various levels of thin and degenerated germinal epithelium surrounding the

cysts (Fig. 5, 6, and 7). The germinal epithelium was severely damaged in fish exposed to simulated CAFO runoff low, EE₂ low, TB high, and Atr low, and showed sperm cells that were not bound in cyst but instead released and suspended in the organ's matrix (Fig. 7). This is consistent with a study reporting thinning of the germinal epithelium in fathead minnows exposed to TB (Leino et al., 2005). These alterations in testicular cell structure suggest that the unbound interstitial sperm cells can exit the efferent duct prior to completion of spermatogenesis, which may cause a decreased fertilization rate.

Mixtures of synthetic estrogens, androgens, and herbicides commonly occur in surface waters, yet only a few studies have attempted to assess the interactions of the compounds and their potential adverse health effects in fish (Leet et al., 2015; Velasco-Santamaría et al., 2010, 2013). The results of the present study do not provide a consistent picture with respect to the toxicity interactions (i.e., potentiation, antagonism, or additive) among the components of the simulated CAFO runoff. However, alterations in only testicular cellular structure, not ovarian cellular structure, were observed which may suggest sex-specific effects of simulated CAFO runoff and its individual compounds. Future research is needed to get a more complete picture of the interactions of EE₂, TB, and Atr in fish.

In conclusion, this study provided evidence that exposure to simulated CAFO runoff during sexual differentiation followed by a recovery period did have an adverse effect on gonadal development in male western mosquitofish. The presence of high variability among individuals indicates that future studies would benefit from an increase in the number of replicates. Moreover, starting the exposure period earlier (*in utero*) would provide a more complete picture of the effects of early life exposure to CAFO runoff on the reproductive health in fish.

Declarations

- Funding: This research did not receive any specific grant from funding agencies.
- Conflict of Interest Disclosure: The authors declare no competing financial interests.
- Data Availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.
- Code availability: Not applicable.
- Ethics approval statement: All husbandry and exposure experiments with fish were approved by the University of Louisiana at Lafayette Institutional of Animal Care and Use Committee (IACUC 8717-056) prior to the start of this study.
- Permission to reproduce material from other sources: All material presented in this manuscript is original content.

Acknowledgements

The authors acknowledge Dr. Karl Hasenstein of UL Lafayette for assisting in the HPLC gradient method development. Funds from the Dr. Granger Professorship of Pre-Medicine awarded to Dr. Felgenhauer were used to purchase chemicals and other supplies used in the experiments.

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Tables

Table 1: Histological stage of oogenesis and spermatogenesis in western mosquitofish (*Gambusia affinis*) determined on day 76 after exposure to treatments¹ for 30 days followed by a 46-day recovery period. Stages of oogenesis: immature (primary oocytes [O1]), maturing (cortical [O2] and/or early vitellogenic oocytes [O3]), and mature (vitellogenic oocytes [O4]). Stages of spermatogenesis: immature (spermatogonia, primary spermatocytes), intermediate (all sperm cell types), and advanced (predominately spermatozoa). EE₂, 17 α -ethinylestradiol; TB, 17 β -trenbolone; Atr, Atrazine. *n* = number of individuals, n.d. = no data.

Treatment	Females $P = 0.120^2$			Males $P = 0.026^2$		
	<i>n</i>	Maturing	Mature	<i>n</i>	Intermediate	Advanced
Control	4	50%	50%	2	0%	100%
Simulated CAFO runoff low	5	40%	40%	2	100%	0%
Simulated CAFO runoff high	4	75%	25%	4	75%	25%
EE ₂ low	4	75%	25%	4	25%	75%
EE ₂ high	4	25%	75%	2	50%	50%
TB low	4	25%	75%	n.d.	n.d.	n.d.
TB high	2	100%	0%	4	100%	0%
Atr low	4	0%	100%	4	75%	25%
Atr high	4	0%	100%	4	0%	100%

¹ Treatments:

Simulated CAFO runoff low = 50 ng/L EE₂, 10 ng/L TB, 150 ng/L Atrazine

Simulated CAFO runoff high = 250 ng/L EE₂, 50 ng/L TB, 1,500 ng/L Atrazine

EE₂ low = 50 ng/L EE₂

EE₂ high = 250 ng/L EE₂

TB low = 10 ng/L TB

TB high = 50 ng/L

Atr low = 150 ng/L

Atr high = 1,500 ng/L

² Differences in the stage of sexual development were assessed with Fisher's exact test, followed by Bonferroni's adjustment ($\alpha = 0.006$).

Table 2: Histological observations on lesions in testicular tissue in western mosquitofish (*Gambusia affinis*) on day 76 after exposure to treatments¹ for 30 days followed by a 46-day recovery period. Scale adopted from Blazer (2013) and Velasco-Santamaría (2013). Values are expressed as percentages. EE₂, 17 α -ethynylestradiol; TB, 17 β -trenbolone; Atr, Atrazine. TB low had zero males present in the treatment group on day 76, thus the treatment was excluded from the table. *n* = number of individuals.

Lesion	Score	Control <i>n</i> = 2	Mix low <i>n</i> = 2	Mix high <i>n</i> = 4	EE ₂ low <i>n</i> = 4	EE ₂ high <i>n</i> = 2	TB high <i>n</i> = 4	Atr low <i>n</i> = 4	Atr high <i>n</i> = 4
Affected	0 (absent)	100	0	0	0	0	0	0	0
	1 (minimal)	0	0	50	50	100	25	0	25
	2 (moderate)	0	0	50	50	0	50	75	50
	3 (severe)	0	100	0	0	0	25	25	25
Cyst arrangement	0 (normal)	100	0	0	0	0	0	0	25
	1 (minimal disruption)	0	0	50	50	100	25	0	25
	2 (moderate disruption)	0	0	50	25	0	50	75	50
	3 (severe disruption)	0	100	0	25	0	25	25	0
Interstitial unbound sperm	0 (absent)	100	0	0	25	0	0	0	50
	1 (present)	0	100	100	75	100	100	100	50

¹ Treatments:

Mix low = 50 ng/L EE₂, 10 ng/L TB, 150 ng/L Atr

Mix high = 250 ng/L EE₂, 50 ng/L TB, 1,500 ng/L Atr

EE₂ low = 50 ng/L

EE₂ high = 250 ng/L

TB low = 10 ng/L

TB high = 50 ng/L

Atr low = 150 ng/L

Atr high = 1,500 ng/L

Figures

Figure 1

Ovarian histological section of a female western mosquitofish (*Gambusia affinis*) from the control group. This mature ovary contains primary (O1), cortical (O2), early vitellogenic (O3), and vitellogenic (O4) oocytes. Scale bar = 50 μm ; * indicates primordial germ cells

Figure 2

Histological section of an embryo in the ovary of a female western mosquitofish (*Gambusia affinis*) exposed to Atr low (150 ng/L) for 30 days during early life, and then a 46-day recovery period. Scale bar = 50 μ m

Figure 3

Histological section of a male western mosquitofish (*Gambusia affinis*) showing intersex condition. The male was exposed to Atr high (1,500 ng/L) for 30 days and followed by a 46-day recovery period. Ovarian tissue (arrow head) is located in a cyst on the periphery of the testis. Scale bar = 50 μ m. Atr, Atrazine

Figure 4

Histological section of control male western mosquitofish (*Gambusia affinis*) testis: (A) Spermatogonia (Sg) on the testis periphery, 4x (B) Germ cells in cysts are in several stages of spermatogenesis: primary spermatocyte (1Sc), early spermatid (eSt), late spermatid (lSt), and spermatozeugmata (Szm). The cell types in each cyst undergo synchronous development, and the sperm cells are bound in cysts with the matrix absent of sperm cells. The nuclei of spermatozoa (SP) heads are associated with Sertoli cells (arrow head), 10x. (C) Szm are released into the efferent duct (*), 4x. (D) SP nuclei are outward facing and each Szm will be ejected through the efferent duct, 10x. A and C scale bar = 50 μ m, B and D scale bar = 20 μ m

Figure 5

Histological section of a testis in western mosquitofish (*Gambusia affinis*) that was minimally affected by exposure to Atr high (1,500 ng/L). (A) Cyst arrangement was minimally disrupted (area in a rectangle), 4x. Scale bar = 200 μm. (B) Area of the rectangle at 10x showing the minimal cyst disruption with thinning of the germinal epithelium (arrow) resulting in congestion and interstitial unbound sperm cells were present in the matrix (arrow head). Scale bar = 50 μm. Atr, Atrazine

Figure 6

Histological section of a testis in western mosquitofish (*Gambusia affinis*) that was moderately affected by exposure to simulated CAFO runoff high (250 ng/L EE2, 50 ng/L TB, and 1,500 ng/L Atr). (A) Cyst arrangement was moderately disrupted, 4x. Scale bar = 200 μm. (B) Unsynchronized cyst development (in rectangle) and cyst degradation (arrows) was present with unbound interstitial sperm cells (arrow heads), 10x. Scale bar = 50 μm. (C) Cyst degradation (arrows) and unbound interstitial cells (arrow heads), 10x. Scale bar = 50 μm EE2, 17α -ethynylestradiol; TB, 17β- trenbolone; Atr, Atrazine

Figure 7

Histological section of a testis in western mosquitofish (*Gambusia affinis*) that was severely affected by exposure to simulated CAFO runoff low (50 ng/L EE2, 10 ng/L TB, and 150 ng/L Atr). Cyst arrangement was severely disrupted with total cyst degradation, unbound interstitial sperm cells (arrow heads) were observed, and irregular cyst formation (arrows) was present. * = efferent duct. Scale bar = 50 μ m. EE2, 17 α -ethynylestradiol; TB, 17 β -trenbolone; Atr, Atrazine

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