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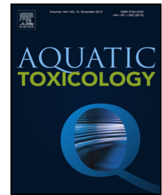

Environmental hormones and their impacts on sex differentiation in fathead minnows

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ABSTRACT

Runoff from lands fertilized with animal manure from concentrated animal feeding operations (CAFOs) is a source of hormones to surface water. In this study we tested the hypothesis that larval fathead minnows exposed to sex steroids singly or in a "typical" CAFO mixture during sex differentiation would respond with changes in the expression of a set of target genes, leading to gonadal abnormalities later in life. In the first experiment, a static daily-renewal system was used to expose larvae during the period of 10–20 days post-hatch (dph) to either 5 ng/L 17β-trenbolone (17β-TRB) or 5 ng/L 17α-ethinylestradiol (EE_2). In a second experiment, fish were exposed from 0 to 45 dph in a flow-through system to a CAFO mixture composed of steroids and degradates (2–16 ng/L), atrazine and degradates (15–250 ng/L), and nitrate (3–11 mg/L). In the single hormone experiment, expression of genes involved in steroidogenesis (*cyp19a*, *cyp17*, and *star*) was decreased in females. In contrast, no differences in gene expression were observed in fish exposed to the CAFO mixture. However, the majority (84%) of treated males had testes containing an ovarian cavity, indicative of feminization, compared to 0% in the control males. Overall, our results show that: (1) changes in gene expression after single hormone exposures are sex-specific, with females more responsive than males; and (2) phenotypic alterations in testicular development can be elicited by a simulated "CAFO" mixture when fathead minnows are exposed during the first 45 days of development. More research is needed to further discern the complex response of fish to steroid mixtures, especially those associated with runoff from land-applied CAFO waste.

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1. Introduction

Natural and synthetic hormones have been detected in aquatic environments. Important sources of hormones to surface waters are runoff from land on which animal manure has been applied as a fertilizer and discharge of treated municipal wastewater (Gall et al., 2011). Concentrated animal feeding operations (CAFOs) produce large amounts of animal waste that contain nutrients, as well as natural and synthetic hormones (Burkholder et al., 2007). In addition, CAFOs are often associated with corn fields that commonly apply herbicides during late spring-early summer (April–June), coinciding with fish spawning (Stoeckel et al., 2012). One herbicide of interest is atrazine, which is widely used and reported as an endocrine disruptor (Weber et al., 2013). Midwest agricultural fields have subsurface tile-drain networks that facilitate transport of excess water from agricultural fields to a ditch network system. As a result, excess nutrients, herbicides, and hormones can be quickly mobilized into ditches (Gall et al., 2011), ultimately reaching streams and rivers.

Effects of sex steroids on fish and wildlife reproduction have been widely studied (Orberdorster and Cheek, 2000; Tyler et al.,

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1998). However, there is significantly less known about the effects of androgens compared to estrogens (Ankley et al., 2003; Jensen et al., 2006), and almost no studies are available that have tested androgen and estrogen hormones and degradates in a mixture. Using information on the estrogenic potency (indexed to 17α-ethinylestradiol, $EE_2 = 1$, maximum) and androgenic potency (indexed to 17β-trenbolone, 17β-TRB = 1 maximum) of the mixture (Thorpe et al., 2003) and assuming concentration addition (Brian et al., 2005; Silva et al., 2002; Zhang et al., 2009), predictions of biological effects of steroid mixtures could be determined. However, there has been little exploration into the testing of mixtures containing endocrine disrupting compounds with dissimilar modes of action. Furthermore, to our knowledge, no controlled studies have been conducted exposing fish larvae or juveniles to a mixture of hormones, pesticides, and nutrients similar to those found in the environmental matrices, such as CAFO runoff.

Using fathead minnows we tested the overarching hypothesis that larvae exposed to sex steroids singly or in a "typical" CAFO mixture while undergoing sex differentiation (10–45 days post hatch, dph) would respond with changes in the expression of genes involved in this process, leading to gonadal abnormalities later in life. We tested this hypothesis in two experiments: one that evaluated molecular responses after exposure to single synthetic sex steroids; and a second experiment that evaluated molecular and organ (gonad) responses after exposure to a CAFO mixture (containing sex steroids and degradates; atrazine and degradates; and nitrate). Specifically, we hypothesized that activation of genes critical to ovarian development (*cyp19a* and *esr1*) would be observed in fish exposed to EE_2 or an "estrogenic" mixture. Analogously, an activation of genes critical for testicular development (*dmrt1* and *ar*) was expected after exposure to 17β-TRB or an "androgenic" mixture. We also expected a skewed phenotypic sex ratio toward males in larva exposed to the CAFO mixture, based on our previous studies showing this response in fathead minnows exposed from 0 to 45 dph to ditch water from a CAFO site containing a similar chemical mixture (Leet et al., 2012).

2. Methods

2.1. Animal model and experimental design

Fathead minnows used for all experiments were spawned at the Purdue University Aquatic Research Laboratory from fish reared at USEPA's Mid-Continent Ecology Division Laboratory in Duluth, MN. These fish contain a sex-linked DNA marker used for genotyping gender (Olmstead et al., 2011).

In the first experiment, a static, daily-renewal system was used to expose fathead minnow larvae (10–20 dph) to either 5 ng/L (18 pM) 17β-TRB, a synthetic androgen or 5 ng/L (17 pM) EE_2, a synthetic estrogen. These treatments were chosen because they are environmentally relevant concentrations that are sublethal (Shved et al., 2008). Fathead minnows were exposed during this developmental period to target gonadal differentiation (van Aerle et al., 2002; Uguz, 2008). Exposures were conducted in 18 6-well plastic plates with mesh well inserts (Corning, Inc., NY, USA) to facilitate daily water changes. Embryos (<24 h post fertilization, hpf) were removed from breeding substrates, triple rinsed in reverse osmosis (R/O) water reconstituted to a hardness of 3 meq with Replenish® (Seachem, Inc., Madison, GA, USA), and individually placed in a well containing a 10 mL total volume of clean reconstituted R/O. Fish were reared in clean water during the first 10 dph and those larvae were (total $N = 101$) split into groups to either be used as ($N = 30$ larvae) or exposed to the synthetic hormones from 10 to 20 dph (EE_2 $N = 35$ larvae; 17β-TRB $N = 36$). Survival and abnormalities were recorded daily. At the end of the exposure, fish were euthanized with MS-222, measured (±0.1 mm), and processed for gene expression analyses and genotyping (controls $N = 29$; EE_2 $N = 28$; 17β-TRB $N = 32$). In order to match genetic and phenotypic sex, RNA was extracted from the midsection of each larva and gene expression data matched to genetic sex. This also allowed for the determination of sex-specific body measurements for these fish. Water samples were pooled from the exposed and control wells daily after water exchange for confirmatory chemical analysis.

The second experiment consisted of a flow-through 45 days exposure of fathead minnows to a contaminant mixture that mimicked chemicals detected at an Indiana CAFO field site (Gall et al., 2011; Leet et al., 2012). The mixture contained the following nominal hormone concentrations in ng/L: 20 estrone (E_1; 74 pM), 10 17α-estradiol (17α-E_2; 37 pM), 10 17β-estradiol (17β-E_2; 37 pM), 10 estriol (E_3; 35 pM), 30 testosterone (*T*; 104 pM), 10 androstenedione (AND; 35 pM), 10 17α-trenbolone (17α-TRB; 37 pM), and 10 17β-TRB (37 pM). In addition, 0.110 μg/L (510 pM) atrazine and 10 mg/L nitrate were included. The exposure system consisted of 24, 9.5-L glass tanks, half of which received the chemical mixture in R/O reconstituted water a multiposition, microelectric valve actuator system (Valco Instruments Co. Inc., Houston, TX). Control tanks were set-up in the same system only receiving R/O water. Each tank received a flow of 3 mL/min and continuous aeration through a small aerator stone. One clutch of eggs still attached to the breeding substrate was placed in each tank (control $N = 12$; exposed $N = 12$). All but 150 eggs were removed from the substrate after eggs eyed to ensure enough eggs hatched for determination of sex ratios at 45 dph, using a combination of gonadal histology and genotyping ($n = 20$–50 juveniles/tank). Juveniles were weighed (±0. 1 mg wet) and measured (±1 mm) at the end of the study. Fish ($n = 20$) from three control and three exposed tanks were chosen for examination of gene expression changes at 20 dph. During the exposure, water samples were periodically collected for chemical analysis from the outflow of random tanks using 1 L amber glass bottles.

For both experiments, embryos/juveniles were incubated in a temperature-controlled chamber (24 ± 1 °C) with a photoperiod of 16 L:8 D. Dissolved oxygen was maintained at a minimum of 6 mg/L. From 0 to 4 dph fish were fed diluted Rotifeast (Reed Mariculture, Inc., Campbell, CA, USA); *ad libitum* live brine shrimp larvae (Artemia, Brine Shrimp Direct, Ogden, UT, USA) three times daily (twice daily on weekends) from 5 to 20 dph; and adult brine shrimp twice daily from 21 to 45 dph. All Institutional Animal Care and Use Procedures were approved through the Purdue University Animal Care and Use Committee (protocol # 11-038).

2.2. Exposure solutions and water chemistry analysis

Hormones were dissolved in 1 mL methanol and diluted into 1 L sterile deionized water for stock solution preparation, then sonicated for 30 min and stirred (on ice) overnight to ensure complete dissolution. Working solutions were prepared with sterile R/O water. The final concentration of methanol was 0.0000005%, negating the need for a vehicle control. Hormones and pesticides were quantified using solid phase extraction, eluting with methanol, evaporating eluant, and reconstituted residues in methanol (0.5 mL) followed by high performance reverse-phase liquid chromatography–tandem electrospray ionization mass spectrometry (HPLC/ESI–MS/MS) for the hormones (details provided Supplemental Material of Gall et al., 2011) and GC/MS for the pesticides.

For the CAFO mixture study, water chemistry is presented as the average concentration of the different analytes during three developmental periods representing pre- (0–9 dph), early (10–20 dph) and late (21–45 dph) sex differentiation for the fathead

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