Environmental Pollution 232 (2018) 322–328

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Chronic nitrate exposure alters reproductive physiology in fathead minnows☆

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ARTICLE INFO

Article history:
Received 31 March 2017
Received in revised form
27 July 2017
Accepted 1 August 2017
Available online 3 October 2017

Keywords:
Endocrine disruption
Vitellogenin
11-Ketotestosterone
Nutrients
Chronic exposure
Aquatic toxicology

ABSTRACT

Nitrate is a ubiquitous aquatic pollutant that is commonly associated with eutrophication and dead zones in estuaries around the world. At high concentrations nitrate is toxic to aquatic life but at environmental concentrations it has also been purported as an endocrine disruptor in fish. To investigate the potential for nitrate to cause endocrine disruption in fish, we conducted a lifecycle study with fathead minnows (*Pimephales promelas*) exposed to nitrate (0, 11.3, and 56.5 mg/L (total nitrate-nitrogen (NO_3-N)) from <24 h post hatch to sexual maturity (209 days). Body mass, condition factor, gonadal somatic index (GSI), incidence of intersex, and vitellogenin induction were determined in mature male and female fish and plasma 11-keto testosterone (11-KT) was measured in males only. In nitrate-exposed males both 11-KT and vitellogenin were significantly induced when compared with controls. No significant differences occurred for body mass, condition factor, or GSI among males and intersex was not observed in any of the nitrate treatments. Nitrate-exposed females also had significant increases in vitellogenin compared to controls but no significant differences for mass, condition factor, or GSI were observed in nitrate exposed groups. Estradiol was used as a positive control for vitellogenin induction. Our findings suggest that environmentally relevant nitrate levels may disrupt steroid hormone synthesis and/or metabolism in male and female fish and may have implications for fish reproduction, watershed management, and regulation of nutrient pollution.

1. Introduction

Endocrine disrupting chemicals (EDCs) including steroid hormones, pharmaceuticals, and pesticides are commonly detected in rivers that receive municipal wastewater effluent (Desbrow et al., 1998; Kolpin et al., 2002; Ternes et al., 2004) and can disrupt estrogenic, androgenic or thyroid hormone-dependent mechanisms including reproduction, metabolism, growth and development. A sizeable and growing body of literature describes effects of EDCs, such as estrogens, in fish, including induction of vitellogenin, an egg yolk protein, in males (Purdom et al., 1994; Folmar et al., 2001), altered circulating sex hormone concentrations (Folmar et al., 2001; Tetreault et al., 2011), decreased sperm count and motility (Jobling et al., 2002a, 2002b), decreased fertilization success (Harris et al., 2011) reduced gonad size (Tetreault et al., 2011), and development of testicular oocytes (i.e., intersex) (Folmar et al., 2001; Jobling et al., 1998; Vajda et al., 2008). As a result of these and other findings, the effects of EDCs on aquatic organisms have received considerable attention in popular media as well as scientific literature (Blazer et al., 2007; Colborn et al., 1996, 1993; Guillette and Edwards, 2008; Hinck et al., 2009).

The number of documented and purported EDCs continues to grow and currently includes nitrate (NO_3), one of the most ubiquitous aquatic pollutants worldwide (Kaiser, 2001; Fields, 2004). Between 2006 and 2012 approximately 42,000 waterways in the U.S. were categorized as 'impaired' under Section 303(d) of the Clean Water Act (U.S. Environmental Protection Agency, 1987). Of the 42,000 listings, nutrient pollution (nitrate and phosphorous) is the cause of approximately 17% (7140) of the listings, surpassed only by pathogens and metals among the top reasons for impairment. High nitrate loading is commonly associated with both point and nonpoint source pollution of anthropogenic origin such as use of fertilizers in agriculture and suburban settings, animal waste from confined production facilities and releases in municipal wastewater effluents. Nitrate in surface waters draining

☆ This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

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http://dx.doi.org/10.1016/j.envpol.2017.08.004
0269-7491/

agricultural areas can be as high as 59.7 mg/L NO_3-N (McCoy, 1972) and has been reported at concentrations of 0.7–38.0 mg/L NO_3-N in artesian springs in agricultural areas (Katz, 1999). The U.S. Environmental Protection Agency (USEPA) limit for nitrate in drinking water is 10 mg/L NO_3-N (U.S. Environmental Protection Agency, 1986); however, no such criterion has been established for protection of aquatic life.

Although nitrate toxicity to fish and other aquatic organisms is well-documented (Carmago et al., 2005; Hickey and Martin, 2009; Rodrigues et al., 2011; Scott and Crunkilton, 2000), Guillette and Edwards (2005) only recently suggested that nitrate could be an EDC. The authors hypothesized that altered circulating steroid hormone levels in juvenile American alligators (*Alligator mississippiensis*) living in highly eutrophic lakes in Florida may have been related to nitrate exposure (Guillette and Edwards, 2005). Since 2005, nitrate has been further implicated as an EDC in other aquatic species, including fish and amphibians (see Poulsen et al., 2017). Edwards and Guillette (2007) found that decreased sperm counts and increased testicular weight in male mosquito fish (*Gambusia holbrooki*) were significantly correlated with increased nitrate concentrations, up to 5 mg/L NO_3-N, in Florida springs. In Siberian Sturgeon (*Acipenser baeri*), 57 mg/L NO_3-N was associated with increases in plasma testosterone, 11-Keto testosterone (11-KT), and estradiol after 30 days of exposure (Hamlin et al., 2008). Similarly, in Atlantic salmon (*Salmo salar*), nitrate was found to result in increased plasma testosterone at concentrations as low as 10.3 mg/L NO_3-N after 27 days of exposure, though 11-KT and thyroxine were unaffected (Freitag et al., 2015). Northern Leopard frogs (*Rana pipiens*) exposed to 2.26 mg/L NO_3-N during early developmental stages had testicular oocytes, female skewed sex-ratios, and less-developed testicular tissue (Orton et al., 2006). Southern toad tadpoles (*Bufo terretris*) exposed to nitrate levels of 30 mg/L NO_3-N in spring water showed reduced growth and altered thyroxine concentration (Edwards et al., 2006). Results of these studies generally support the hypothesis that nitrate is an EDC and may alter steroid hormone concentrations or activity.

The purpose of this study was to further investigate the endocrine-disrupting effects of chronic nitrate exposure in fish by examining reproductive physiology, including plasma 11-KT and vitellogenin concentrations, body condition, and gonadal somatic index, of male and female fathead minnows (*Pimephales promelas*), a model freshwater fish species, following exposure from hatch to sexual maturity.

2. Methods

2.1. Study design

Four replicates were used for each of the four treatments (control, 11.3 mg/L nitrate-nitrogen (NO_3-N) (50 mg/L total nitrate), 56.5 mg/L nitrate-nitrogen (NO_3-N) (250 mg/L total nitrate), and 50 ng/L estradiol) for a total of 16 experimental units. Estradiol (E2) was included as a positive control for estrogenic effects. Stock nitrate solutions were prepared weekly from reagent grade $NaNO_3$ (J.T. Baker, Center Valley, PA) dissolved in deionized water. Stock E2 solution was prepared weekly in acetone and stored in the dark at 4 °C. Temperature, pH and dissolved oxygen were measured daily with a Hach HQ40d water quality meter. Nitrate levels were measured weekly with a nitrate test kit (Lamotte 3689-SC) and colorimeter (Lamotte model SMART2).

Fathead minnow embryos were purchased from Aquatic Bio-Systems, Inc. (Fort Collins, CO) and allowed to hatch during transport. Fifteen <24 h old larval fathead minnows of unknown sex were transferred into each 5-L beaker with 2-L of aerated dechlorinated tap water. The beakers were then spiked with appropriate volumes of stock solution to maintain target nitrate or E2 concentrations. Fish were fed *ad libitum* with *Artemia* nauplii twice daily. A 90% water change was completed in the beakers every 48 h to maintain the target nitrate concentrations and consistent water quality. Fish were maintained in the 5-L beakers for the first 60 days of the exposure then transferred to 20-L glass aquaria with 15-L of water. At this point the fish were transitioned from *Artemia* to flake food (TetraMin) and fed *ad libitum* twice daily. Each aquarium was aerated and equipped with a charcoal filter. A 50% water change was conducted weekly while the fish were in the 20-L aquaria to maintain target nitrate levels in all treatments. Fish remained in the 20-L aquaria within a 3500-L water bath (set to 25 °C) until study termination after 209 total days of exposure. The fish were sexually mature at study termination.

2.2. Fish processing

Upon study termination, fish were euthanized by overdose (100 mg/L) of neutral buffered MS-222 (Argent, Redmond WA). Total lengths (nearest mm) and weights (nearest 0.01 g) were recorded for each fish. Final weights were averaged per experimental unit for each treatment. Lengths and weights were used to calculate Fulton's condition factor (K) by the formula:

$$K = (\text{Weight (g)} / \text{Length}^3 \text{ (mm)}) * 100{,}000$$

Males and females were bled individually from the caudal vein into heparinized glass microcapillary tubes (Fisher, #22-362-566) by severing the caudal peduncle with a scalpel. Sex was confirmed by examination of the dissected gonads under a stereo microscope. Blood from six males was pooled to create one sample for males for each replicate and the same was performed for samples from six females in each replicate. Pooled blood samples were centrifuged (2000 rpm) at 4 °C for 10 min to obtain plasma, which was transferred to a clean vial and stored at −80 °C until 11-KT and vitellogenin analyses for males and vitellogenin analysis for females. Approximately 600 μl - 800 μl of plasma was obtained for each replicate. About 250 μl of plasma was required per assay.

Gonads were weighed to determine gonadal somatic index (GSI), calculated by dividing gonad weight (g) by total fish weight (g) and multiplying by 100. Testes from three haphazardly selected males per treatment group were placed into histology cassettes and fixed in 10% neutral buffered formalin for histological processing at the Veterinary Medicine Diagnostic Lab at the University of Georgia. Briefly, the preserved testes were dehydrated in alcohol, embedded in paraffin wax, sectioned longitudinally at 6 μm, mounted on glass microscope slides, and stained with hematoxylin and eosin. A single longitudinal tissue section from the center region allowed for examination of maximal surface area of testicular tissue. Sectioned and stained testes were examined by an experienced reader under a light microscope for presence of oocytes. A subset (10%) of the sections was examined by a second reader to assess quality control.

2.3. 11-Keto testosterone and vitellogenin analysis

Plasma samples were allowed to thaw on ice and each sample was separated into two glass tubes. One of the duplicate samples was used for 11-KT analysis and the other for quantification of vitellogenin. Plasma from male fish only was used for 11-KT analysis. Plasma was extracted three times with a 50:50 mixture of ethyl acetate/hexane. Plasma 11-KT was analyzed according to the protocol provided with the commercial enzyme immunoassay 11-KT kit (Cayman Chemical, item #582751, Ann Arbor, MI). Briefly, the 11-KT assay is a competitive binding assay between 11-KT and