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Developmental timing of sodium perchlorate exposure alters angiogenesis, thyroid follicle proliferation and sexual maturation in stickleback

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ABSTRACT

Perchlorate, a common aquatic contaminant, is well known to disrupt homeostasis of the hypothalamus–pituitary–thyroid axis. This study utilizes the threespine stickleback (*Gasterosteus aculeatus*) fish to determine if perchlorate exposure during certain windows of development has morphological effects on thyroid and gonads. Fish were moved from untreated water to perchlorate-contaminated water (30 and 100 mg/L) starting at 0, 3, 7, 14, 21, 42, 154 and 305 days post fertilization until approximately one year old. A reciprocal treatment (fish in contaminated water switched to untreated water) was conducted on the same schedule. Perchlorate exposure increased angiogenesis and follicle proliferation in thyroid tissue, delayed gonadal maturity, and skewed sex ratios toward males; effects depended on concentration and timing of exposure. This study demonstrates that perchlorate exposure beginning during the first 42 days of development has profound effects on stickleback reproductive and thyroid tissues, and by implication can impact population dynamics. Long-term exposure studies that assess contaminant effects at various stages of development provide novel information to characterize risk to aquatic organisms, to facilitate management of resources, and to determine sensitive developmental windows for further study of underlying mechanisms.

1. Introduction

The perchlorate ion (ClO_4^-) is a common aquatic contaminant in the United States (U.S.) and has been classified as a contaminant of concern by the U.S. Environmental Protection Agency (EPA), which has committed to setting drinking water limits under the Clean Water Act (USEPA, 2011). The U.S. Department of Defense (DoD) uses a majority of the commercially available perchlorate for ammunition and solid rocket propellant and most contamination originates from military and manufacturing sites (Morrison et al., 2006; Trumpolt et al., 2005; Urbansky, 2002). Naturally produced perchlorate salts persist at low levels in arid regions such as Antarctica, the Atacama Desert in Chile, and the Southwestern U.S. (Kounaves et al., 2010; Rao et al., 2007; Urbansky et al., 2001).

Perchlorate salts are highly water soluble and the ion is kinetically stable, nonreactive and resistant to adsorption in solution (Brown and Gu, 2006; Morrison et al., 2006). These properties make perchlorate highly mobile and readily available to aquatic organisms that are exposed through ambient water (via respiratory and gastrointestinal epithelia, and integument) and/or ingested food (Furin et al., 2013; Huber et al., 2011). The widespread occurrence and persistence of this known endocrine disruptor in water sources is a concern for the health of wildlife and humans. Understanding the adverse effects of exposure throughout an organism's lifecycle (fertilization to maturation) is crucial to elucidating fitness and population level consequences as well as identifying cohorts and stages of concern.

The toxic effects of perchlorate stem from its ionic similarity to iodide. It competitively inhibits the uptake of iodide via the

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sodium/iodide symporter (NIS, alias SLC5a5) in the basolateral membrane of thyroid follicle cells (Carr et al., 2005; Wolff, 1998). In sufficient concentration and with chronic exposure, perchlorate effectively inhibits the synthesis of thyroid hormones (TH: thyroxine, T4 and triiodothyronine, T3), which are essential regulators of metabolism, growth, development and metamorphosis in vertebrates (Carr et al., 2005; Choksi et al., 2003; Wolff, 1998). Lack of iodide uptake halts production of T4 and can lead to increased thyroid stimulating hormone due to lack of negative feedback to the hypothalamus and pituitary as well as hypothyroidism and changes in thyroid tissue morphology; these changes include follicle cell hypertrophy, proliferation of follicles and surrounding tissue, increased vasculature, and reduced colloid (Blanton and Specker, 2007; Choksi et al., 2003; Mukhi et al., 2005; Tietge et al., 2010).

Perchlorate interferes with normal development and reproduction in multiple taxa, including amphibians, birds, and mammals (Kendall and Smith, 2006). Studies utilizing zebrafish (*Danio rerio*) (Mukhi et al., 2005; Patiño and Mukhi, 2007; Patiño et al., 2007, 2003; Schmidt et al., 2012), eastern mosquitofish (*Gambusia holbrooki*) (Bradford et al., 2005; Park et al., 2006), threespine stickleback (*Gasterosteus aculeatus*; hereafter, stickleback) (Bernhardt and von Hippel, 2008; Bernhardt et al., 2006, 2011; Furin et al., 2015; Petersen et al., 2015), fathead minnows (*Pimephales promelas*) (Crane et al., 2005; Pickford et al., 2005), and short-finned molly (*Poecilia sphenops*) (Burcu et al., 2009) have all found detrimental effects on development and/or reproduction from perchlorate exposure. These detrimental effects include: reduced growth rates (Bernhardt et al., 2011; Crane et al., 2005; Liu et al., 2008; Mukhi and Patiño, 2007; Mukhi et al., 2007; Park et al., 2006; Schmidt et al., 2012), skeletal abnormalities (Bernhardt et al., 2011; Furin et al., 2015), abnormal thyroid histomorphology (Bradford et al., 2005; Crane et al., 2005; Liu et al., 2006, 2008; Mukhi et al., 2005; Mukhi and Patiño, 2007; Patiño et al., 2003; Petersen et al., 2015; Schmidt et al., 2012), reduced reproductive output (Bernhardt and von Hippel, 2008; Bernhardt et al., 2006; Mukhi and Patiño, 2007; Patiño et al., 2003), skewed sex ratio (Mukhi et al., 2007), and intersex gonads (Bernhardt et al., 2006).

Increasing evidence shows that thyroid hormones play a critical role in regulating gonadal development in teleosts (Flood et al., 2013; Liu et al., 2011). Thyroid hormone receptors are present on teleost ovarian and testicular cells (Nelson and Habibi, 2009) and goitrogens affect gonadal development in fishes including increased numbers of Sertoli and germ cells in tilapia (*Oreochromis niloticus*) exposed to thiouracil (PTU) (Matta et al., 2002) and impaired testicular and ovarian recrudescence in catfish (*Clarias gariepinus*) exposed to thiourea (Supriya et al., 2005; Swapna et al., 2006). Perchlorate causes a female bias in the sex ratio of zebrafish (Mukhi et al., 2007; Sharma and Patiño, 2013), while evidence of masculinization was found in stickleback (Bernhardt et al., 2006). A critical window for sex determination in stickleback was determined to be within the first 14 days post hatch (dph) (Hahlbeck et al., 2004). Lewis et al. (2008) found that sex determination in stickleback was accompanied by proliferation of primordial germ cells in genetic females, and no proliferation in genetic males, before 11 days post fertilization (dpf). Perchlorate has the potential to cause perturbations during these sensitive early developmental periods.

To determine the sensitive developmental phases, we studied the morphological effects of perchlorate exposure on stickleback thyroid and gonads using various exposure durations and time points during development. Changes in thyroid tissue histomorphology are a sensitive endpoint for some endocrine disruptors, such as perchlorate, that directly alter iodide disposition (Carr and Patiño, 2011). Due to the importance of TH during development, the masculinizing effects of perchlorate on stickleback and their early sex determination, we hypothesized that perchlorate exposure during early development and metamorphosis (through 21 dpf) would cause significant histological changes to thyroid tissue such as thyrocyte hypertrophy, colloid depletion and follicle hyperplasia, as well as abnormal gonads and male-biased sex ratios.

2. Materials and methods

2.1. Experimental design

In a chronic static renewal test, fish were either introduced or removed from sodium perchlorate (>98% purity, Sigma–Aldrich, St. Louis, MO, USA) contaminated (30 or 100 mg/L) water at varying times during development. This was done to determine critical developmental windows (timing) of exposure. Specifically two exposure regimes were carried out in which fish were either moved from control water to perchlorate-treated water (upshift) or from perchlorate-treated water to control water (downshift) at 0, 3, 7, 14, 21, 42, 154 or 305 dpf (Fig. 1). Once they reached approximately one year old, stickleback were euthanized and processed for histological analysis of thyroid and gonads.

2.2. Fish collection and husbandry

Stickleback were collected from Rabbit Slough (61.534°N, 149.266°W) in the Matanuska-Susitna Valley, Alaska on 4 June 2008. Rabbit Slough fish were chosen to represent the ancestral oceanic ecotype (Cresko, 2000; Hohenlohe et al., 2010). Fish were kept in outdoor pools filled with de-chlorinated city water with 3 g/L Instant Ocean© added. A mass cross using eggs stripped from 40 females and sperm collected from 40 males was performed on 10 June 2008. Embryo medium consisted of reverse osmosis purified water to which Instant Ocean© was added to 4 g/L. Contaminated (treatment) water was produced by adding sodium perchlorate dried at 90 °C before weighing, at a nominal 30 and 100 mg/L. Clutches of eggs from the 40 females were combined in one container in order to randomize them before dividing them into 38 Petri dishes (100 × 20 mm) with a previously determined water treatment (control: no perchlorate added, 30 or 100 mg/L of perchlorate). The treatments were divided among the 38 Petri dishes as follows: control = eight, 30 mg/L upshift = eight, 100 mg/L upshift = eight, 30 mg/L downshift = seven, and 100 mg/L downshift = seven. The eggs were subsequently fertilized with mixed sperm from the 40 males. Each Petri dish of embryos was then subdivided into three Petri dishes with approximately 100 embryos per dish for three replicates of each treatment (114 total Petri dishes). Embryos were incubated at 20 ± 0.5 °C. Water was changed daily and dead embryos were removed for the first 10 dpf.

By 10 dpf, most embryos had hatched and embryos from each Petri dish were transferred to their own 56.8 L aquarium (60 cm × 31 cm × 32 cm) with aerated AZOO© multi sponge filters (65 mm diameter). All aquaria started with 6 L of water because of the small size of the fish. The water level was proportionally increased as the fish grew to maintain fish density at appropriate and consistent conditions (1 L water per 1 cm fish). Water changes (15% of total volume) were carried out biweekly and as needed. Two to three milliliters of Bacta-pur© N3000 live bacteria (IET-Aquaresearch Ltd., Quebec, Canada) were added to each tank once a week to limit nitrate concentrations. Reverse osmosis purified water was added weekly to compensate for evaporative loss. A YSI photometer model 9100 (Yellow Springs Instrument Co., Yellow Springs, OH, USA) was used to periodically check salinity (4–5 g/kg), pH (7.0–8.0), and ammonia (<2 mg/L total nitrogen); no out of range levels were detected. Perchlorate concentrations in aquaria were measured with an Acorn Ion 6 meter (Oakton