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Lifecycle exposure to perchlorate differentially alters morphology, biochemistry, and transcription as well as sperm motility in *Silurana tropicalis* frogs☆

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ABSTRACT

Perchlorate (ClO_4^-) contamination has been reported in ground and surface waters across North America. However, few studies have examined the effects of prolonged exposure to this thyroid hormone disrupting chemical, particularly at environmentally relevant concentrations in lower vertebrates, such as amphibians. The aim of this study was to examine the effects of a yearlong chronic exposure to ClO_4^- in adult male and female Western clawed frogs (*Silurana tropicalis*). Frogs were spawned and raised from fertilized embryo until sexual maturity in potassium perchlorate ($KClO_4$)-treated water at different concentrations (0, 20, 53, and 107 μg/L). Developmental and reproductive indices – including adult morphology, androgen plasma levels, gonadal thyroid hormone- and sex steroid-related transcript levels, and sperm motility – were evaluated in male and female adult frogs. Female growth (e.g., body mass, snout-vent length, and hind limb length) was significantly reduced following chronic exposure to environmentally relevant concentrations of $KClO_4$ resulting in females with morphometric indices similar to those of control males – indicating potential sex-specific sensitivities to $KClO_4$. Changes to reproductive indices (i.e., plasma androgen levels, gonadal thyroid hormone- and sex steroid-related transcript levels, and sperm motility) were also observed in both sexes and suggest that $KClO_4$ exposure may also have indirect secondary effects on the reproductive axes in male and female adult frogs. These effects were observed at concentrations at or below those reported in surface waters contaminated with ClO_4^- suggesting that this contaminant may have developmental and reproductive effects post-metamorphosis in natural amphibian populations.

1. Introduction

Perchlorate (ClO_4^-) contamination has been reported in aquatic environments in North America as a result of various anthropogenic applications, including solid propellants, munitions, pyrotechnics and fertilizers (GAO, 2010; Reviewed in Dasgupta et al., 2006; Reviewed in Trumpolt et al., 2005). The anion can also be introduced to and accumulate in the environment naturally via atmospheric deposition (Jackson et al., 2010; Parker et al., 2008; Rao et al., 2007; Dasgupta et al., 2006; Rajagopalan et al., 2006). Since it is highly water soluble, ClO_4^- accumulates in ground and surface waters (Urbansky, 1998); thereby, placing aquatic vertebrates (e.g., fish, amphibians, and birds) at a high risk of exposure. The majority of surface and ground waters contaminated by ClO_4^- in the United States of America and Canada are characterized by concentrations less than 100 μg/L (ASTSWMO, 2011; Blount et al., 2010; GAO, 2010; Parker et al., 2008; Backus et al., 2005). Therefore, it is important to examine the effects of environmentally relevant concentrations of ClO_4^- in aquatic species.

The effects of ClO_4^- are mainly mediated through the targeted disruption of thyroid function. Amphibians are highly susceptible to endocrine disruptors that target thyroid function, as metamorphosis is dependent upon thyroid hormones. Exposure to ClO_4^- has been shown to impede tail reabsorption and hind leg growth in

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developing tadpoles (*Lithobates sylvaticus*: Bulaeva et al., 2015; *Silurana tropicalis*: Flood and Langlois, 2014; *Xenopus laevis*: Opitz et al., 2009; Hu et al., 2006; Tietge et al., 2005; Goleman et al., 2002a, 2002b). Therefore, metamorphosis serves as a critical developmental window for evaluating exposure to thyroid hormone disruptors (Kloas and Lutz, 2006) and the effects of ClO_4^- have been well studied in this context (*L. sylvaticus*: Bulaeva et al., 2015; *S. tropicalis*: Flood and Langlois, 2014; *X. laevis*: Hu et al., 2006; Opitz et al., 2009; Tietge et al., 2005; Goleman et al., 2002a, 2002b). To date, however, the lasting effects of a developmental exposure (fertilized embryo to sexual maturity) to thyroid hormone-disrupting chemicals on adult amphibians after metamorphosis have received relatively little attention.

The primary mechanism of ClO_4^- is the competitive inhibition of iodide (I^-) uptake via the Na^+/I^- symporter (NIS) limiting the synthesis of the iodine-rich thyroid hormones, tetraiodothyronine (T4) and triiodothyronine (T3), by the thyroid gland (Carr et al., 2008). Thyroid hormones, however, have been shown to integrate with various endocrine axes and the targeted disruption of thyroid hormone synthesis can indirectly mediate the effects of ClO_4^- on other signalling pathways. For example, thyroid hormone-disrupting chemicals (e.g., ClO_4^-, methimazole, propylthiouracil, and thiourea) have been shown to alter aspects of the hypothalamus–pituitary–gonad axis (i.e., steroidogenesis, gonadal cellular differentiation, and development (Flood et al., 2013)). Disruption of thyroid function during sexual differentiation can consequently result in observable changes in sex steroid hormone levels, gonadal morphology, and population-level sex ratios in both fish and amphibians (*Danio rerio*: Sharma and Patiño, 2013; Mukhi et al., 2007; *Gasterosteus aculeatus*: Bernhardt et al., 2006; *Clarias gariepinus*: Swapna et al., 2006; Supriya et al., 2005; *X. laevis*: Goleman et al., 2002a). Transcripts of thyroid hormone-related machinery have moreover been detected in testicular and ovarian tissues of numerous species (*Physalaemus pustulosus*: Duarte-Guterman et al., 2012; *S. tropicalis*: Duarte-Guterman and Trudeau, 2011; *Scarus iseri*: Johnson and Lema, 2011; *Oncorhynchus mykiss*: Sambroni et al., 2001; *Podarcis sicula*: Cardone et al., 2000). A direct relationship between thyroid hormone status and sex steroid-related molecular responses in larval gonadal tissues has also been found in *S. tropicalis* (Duarte-Guterman and Trudeau, 2011). We previously observed that *S. tropicalis* exposed to $KClO_4$ at environmentally relevant concentrations $\leq$100 μg/L from embryo to sexual differentiation (Nieuwkoop–Faber stage 56 and 60 (NF); Nieuwkoop and Faber, 1994) induced changes in the transcription of sex steroid-related genes in gonadal and liver tissues (Flood and Langlois, 2014). To further investigate the chronic effects of ClO_4^- exposure throughout the frogs' lifecycle, a subset of *S. tropicalis* from the previous experiment were continually exposed to environmentally relevant levels of $KClO_4$ until they reached sexual maturity. Developmental and reproductive indices were assessed, including adult morphology, androgen plasma levels, gonadal thyroid hormone- and sex steroid-related transcript levels, and sperm motility.

2. Material and methods

2.1. Animals and exposure

Larval *S. tropicalis* (stage NF 10–12) were previously exposed to environmentally relevant concentrations of $KClO_4$ in 1-L glass jars until the climax of metamorphosis (stage NF 60; ~12 weeks post-hatch; for details, refer to Flood and Langlois, 2014). For the present study, a subset of *S. tropicalis* from the previous study was allowed to develop to sexual maturity (1 year after egg fertilization). Exposure to one of four concentrations of $KClO_4$ of which the average measured concentrations were <1, 20, 53, and 107 μg/L was maintained (Flood and Langlois, 2014). Measured concentrations were close to the nominal target concentrations of 0, 25, 50 and 100 μg/L (Flood and Langlois, 2014). Studies have confirmed that environmentally relevant concentrations of ClO_4^- ($\leq$100 μg/L) can have measurable effects on thyroid histology and morphometric indices in developing tadpoles (*X. laevis*: Hu et al., 2006; Tietge et al., 2005; Goleman et al., 2002a, 2002b), without completely inhibiting metamorphosis – facilitating the study of long-term exposure to $KClO_4$ at sexual maturity. Specifically, with the completion of tail reabsorption (~14 weeks after hatch), metamorphs were transferred to glass 10-L treatment tanks where exposure to the same concentrations of reagent-grade $KClO_4$ ($\geq$99.0%; Sigma Canada Ltd., Oakville, ON, Canada) was continued in dechlorinated, aerated water. Density was maintained at the appropriate body weight per liter for the duration of the experiment (ASTM, 1998) and tank size was adjusted as required over the course of the yearlong exposure. We completely replaced water and $KClO_4$ every 3 d, maintaining a water temperature of 25 ± 1 °C and a light:dark regime of 12:12 h (light commencing at 0700 h local time) for the duration of the experiment. Metamorphs were fed once daily with the same amount of commercially available Nasco *Xenopus* Frog Brittle (Nasco, California, USA) with the essential nutrients for proper *Xenopus* development, including 1.2 ppm of iodine. Animals were housed in the Queen's University Animal Care Facility (Kingston, ON, Canada) in accordance with the guidelines of the Queen's University Animal Care Committee and the Canadian Council on Animal Care.

One year after fertilization, frogs were anaesthetized by immersion in a 2% w/v solution of ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma Canada Ltd., Oakville, ON, Canada), after which individual body mass (BM), snout-vent length (SVL), and hind limb length (HLL) was recorded. Animals were then euthanized by decapitation. Blood samples (200–500 μL) were collected via exsanguination for sex steroid hormone analyses (1 sample per animal; 8 animals per treatment), immediately centrifuged and the plasma fraction (the main medium for sex steroid hormones) was collected and stored at −80 °C. The whole left testis ($n = 10$ males per treatment) and an ovary section (10–30 mg from each of 10 females per treatment) were dissected, weighed, and stored at −80 °C for further gene expression analysis. The whole right testis of each male was also dissected and weighed, then placed in 2X Simplified Amphibian Ringers (SAR; 113.0 mM NaCl, 1.0 mM $CaCl_2$, 2.0 mM KCl, and 3.6 mM $NaHCO_3$) on ice for immediate sperm analysis.

2.2. Sex steroid analysis

Plasma concentrations of testosterone (T) and 5α-dihydrotestosterone (5α-DHT) were measured using commercially available ELISAs (T: Cayman Chemical, Cedarlane, Burlington, ON, Canada; 5α-DHT: IBL America, Cedarlane, Burlington, ON, Canada). Plasma samples were thawed on ice and diluted in the immunoassay buffer. The quality criteria for the application of commercial kits were verified as instructed by the manufacturer and their immunoassay protocols were followed. All plasma samples were measured in duplicate (2 samples per animal; 6 animals per treatment). The absorbance of samples was measured using an Infinite® M1000 PRO plate reader (Tecan, Montreal, QC, Canada) at 405 nm for T and 450 nm for 5α-DHT. The limit of detection according to the manufacturer was 6 pg/mL for both T and 5α-DHT.

2.3. Gene expression analysis

Total RNA from ovary and testis tissue was isolated using TRIzol