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Sodium perchlorate disrupts development and affects metamorphosis- and growth-related gene expression in tadpoles of the wood frog (*Lithobates sylvaticus*)

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ABSTRACT

Numerous endocrine disrupting chemicals can affect the growth and development of amphibians. We investigated the effects of a targeted disruption of the endocrine axes modulating development and somatic growth. Wood frog (*Lithobates sylvaticus*) tadpoles were exposed for 2 weeks (from developmental Gosner stage (Gs) 25 to Gs30) to sodium perchlorate (SP, thyroid inhibitor, 14 mg/L), estradiol (E_2, known to alter growth and development, 200 nM) and a reduced feeding regime (RF, to affect growth and development in a chemically-independent manner). All treatments experienced developmental delay, and animals exposed to SP or subjected to RF respectively reached metamorphic climax (Gs42) approximately 11(±3) and 17(±3) days later than controls. At Gs42, only SP-treated animals showed increased weight and snout-vent length ($P < 0.05$) relative to controls. Tadpoles treated with SP had 10-times higher levels of liver *igf1* mRNA after 4 days of exposure (Gs28) compared to controls. Tadpoles in the RF treatment expressed 6-times lower levels of liver *igf1* mRNA and 2-times higher liver *igf1r* mRNA ($P < 0.05$) at Gs30. Tadpoles treated with E_2 exhibited similar developmental and growth patterns as controls, but had increased liver *igf1* mRNA levels at Gs28, and tail *igf1r* at Gs42. Effects on tail *trβ* mRNA levels were detected in SP-treated tadpoles at Gs42, 40 days post-exposure, suggesting that the chemical inhibition of thyroid hormone production early in development can have long-lasting effects. The growth effects observed in the SP-exposed animals suggest a relationship between TH-dependent development and somatic growth in *L. sylvaticus* tadpoles.

1. Introduction

Anurans have complex lifecycles characterized by the remodeling of their body during metamorphosis. Their development is driven by numerous hormones (Kikuyama et al., 1993; Shi et al., 1996), and punctuated by morphological milestones or stages (Gosner, 1960). The relationship between amphibian somatic growth and development is complex. A popular model by Wilbur and Collins (1973) postulates that the two are intricately linked and that a minimal size must be achieved by the tadpole to be able to undergo metamorphosis. At the same time, the observation that *Xenopus laevis* tadpoles unable to metamorphose can reach giant sizes suggests that the development and growth of amphibians can be uncoupled (Rot-Nikcevic and Wassersug, 2004). Body size at metamorphosis and developmental rates has also been shown to be affected by food restriction (Audo et al., 1995) and exposure to various pollutants (Brodeur et al., 2013; Stepanyan et al., 2011), further demonstrating the complexity of this interaction. While the development of amphibians has been studied in depth, the relationship between development (e.g., progress through metamorphic stages) and somatic growth (e.g., body size) has been comparatively less well explored, especially at a molecular level.

Amphibian metamorphosis, which transforms the larval tadpole into an adult frog, is controlled by the thyroid hormone (TH)

Abbreviations: dio2, deiodinase2; E_2, 17β-estradiol; EE_2, 17α-ethinylestradiol; GH, growth hormone; HPT, hypothalamus–pituitary–thyroid; *igf1*, insulin-like growth factor 1; *igf1-r*, insulin-like growth factor 1 receptor; RF, reduced feeding; SP, sodium perchlorate; SVL, snout-vent length; TH, thyroid hormone; T_3, triiodothyronine; T_4, thyroxine; *trβ*, thyroid receptor β; TL, tail length.

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through the hypothalamus–pituitary–thyroid (HPT) axis (Buchholz et al., 2006; Tata, 2006). The hormone is released from the thyroid gland into the bloodstream mostly in the form of thyroxine (T_4), which is transformed into its more active form triiodothyronine (T_3) by deiodination through the action of deiodinase enzymes in peripheral tissues (Fort et al., 2007). At the level of target organs and tissues, TH binds to its nuclear thyroid receptors (TRα and TRβ) and acts as a transcription factor activating TH-responsive genes that drive the changes of metamorphosis (Buchholz et al., 2006; Damjanovski et al., 2002; Das et al., 2010; Fort et al., 2007; Shi et al., 1996; Tata, 2006). The absence of TH completely blocks metamorphosis (Goleman and Carr, 2006; Rot-Nikcevic and Wassersug, 2004).

The growth hormone (GH)-insulin-like growth factor 1 (IGF1) endocrine axis is known to mediate somatic growth in fish, birds and mammals, but has been comparatively less studied in amphibians. In mammals, the liver is the main source of circulating IGF1, which is produced in response to growth hormone (GH) signaling (Le Roith et al., 2001; Reinecke and Collet, 1998). At the cellular level, the binding of IGF1 to IGF1-R activates signaling cascades in the cell that result in downstream cell proliferation and differentiation (Jones and Clemmons, 1995; Yakar et al., 2000). The GH-IGF system is also conserved in fish (Wood et al., 2005). For example, IGF1-R signaling modulates the accelerated catch-up growth observed in post-hypoxic zebrafish embryos (Kamei et al., 2011). Both IGF1 and its receptor IGF1-R are present in amphibians (Bautista et al., 1990; Kajimoto and Rotwein, 1990; Reinecke and Collet, 1998). Furthermore, the gigantic phenotype of GH-overexpressing transgenic tadpoles generated by Huang and Brown (2000) suggests that the function of the GH-IGF1 pathway is conserved in amphibians.

Amphibian development and growth can be affected by various endocrine disruptors. Perchlorate, a chemical used to make solid propellants and explosives, which can also be an aquatic pollutant, inhibits TH synthesis by blocking the thyroid sodium-iodide symporter (Wolff, 1998). Treatment of *X. laevis* tadpoles with sodium perchlorate (SP) has been previously shown to inhibit metamorphosis (Goleman and Carr, 2006; Tietge et al., 2005), but also affect growth by reducing the snout-vent length of exposed tadpoles (Goleman et al., 2002a). Long-term natural blockage of metamorphosis in athyroid *X. laevis* tadpoles results in excessive growth (Rot-Nikcevic and Wassersug, 2004), indicating a relationship between development and somatic growth.

The effects on amphibian larval growth and/or development have been reported in a growing number of studies as an unintended and sometimes unexpected effect of endocrine disruptors that have been used primarily to disrupt either the thyroid or the sexual differentiation endocrine axis (Bauer-Dantoin and Meinhardt, 2010; Goleman et al., 2002a; Tompsett et al., 2012; Nishimura et al., 1997; Hogan et al., 2006, 2008), however the nature of any interactions are at present unclear. This study aims to specifically examine the effect of endocrine disruptors on the growth and development of wood frog (*Lithobates sylvaticus*) tadpoles, a native North American species. Specifically, we investigated the effects of a two-week exposure to SP, a known thyroid inhibitor with possible growth effects (Goleman et al., 2002a), and E_2, shown to affect growth and development in some studies (Nishimura et al., 1997; Hogan et al., 2006, 2008), on the development and growth of tadpoles. A reduced feeding (RF) regime was employed to disrupt development in a chemically-independent manner. We studied the expression of selected metamorphosis- and growth-related genes to investigate the underlying molecular effects of these treatments and reveal any interactions between development and somatic growth.

2. Materials and methods

2.1. Animals and rearing conditions

Five clutches of wild wood frog (*L. sylvaticus*) fertilized eggs were collected from natural wetlands located on Canadian Forces Base Gagetown, New Brunswick, Canada (45°40′N, 66°29′W). The clutches were combined in the laboratory and kept in aerated water in glass tanks until hatching. Upon reaching developmental Gosner stage 25 (Gs; Gosner, 1960), tadpoles ($n = 160$ per treatment) were randomly assigned to 10 L aquaria (4 tadpoles/L). Four replicates were assigned per each treatment. Once tadpoles reached Gs30, animals from each replicate were split ($n = 20$) into two aquaria of 20 L to reduce densities to 1 tadpole/L. Tadpoles housed in the same tank during the treatment period ($n = 40$) were sampled together and considered as a treatment replicate. Tadpoles were kept on a 12 h light/dark photoperiod. Temperature and pH were recorded regularly (approximately 3–5 times per week), and were maintained between 20.7–23.0 °C and 6.14–7.80, respectively, throughout the experiment. Tadpoles were fed rabbit pellets (Rolf C. Hagen, Inc.) *ad libitum* daily (with the exception of the reduced feeding treatment, see details below) and boiled kale leaves once weekly. The care, treatment and sampling of animals used in this study followed the guidelines and standards of the Animal Care Committee of the University of New Brunswick and the Canadian Council on Animal Care.

2.2. 17β-Estradiol, sodium perchlorate and reduced feeding exposures

Tadpoles were exposed to the following treatments: 17β-estradiol (E_2, 200 nM, equivalent to 0.05 mg/L), sodium perchlorate (SP, 14 mg/L), reduced feeding (RF) and control. Exposures were carried out in a static-renewal system. SP was first dissolved in 1 L of water and then added to the aquariums. In the case of the E_2 exposure, a stock solution of 10 mM E_2 was prepared in ethanol and 200 µl of the stock solution was added to each aquarium to reach a final exposure concentration of 200 nM, or 0.05 mg/L. Furthermore, 200 µl of clean ethanol was added to the other treatment aquaria (Control, SP, and RF) to control for any effect of ethanol addition. In all cases the total amount of ethanol added was 0.002% of the final tank volume. Tadpoles in the RF treatment were fed half the amount of rabbit pellets (Rolf C. Hagen, Inc.) given to the other treatments for the duration of the exposure and then fed normally as described in Section 2.1.

Seventy-five percent of the water was renewed every 48 h during exposures to maintain chemical concentrations and once a week thereafter. After exposures were stopped, tadpoles were reared in clean water until they reached metamorphic climax (Gs42). Exposures were carried out from developmental stage Gs25 to Gs30, which occurred at day 10 for controls, E_2, and SP-treated animals, and at day 16 for the RF animals. Treatment duration was chosen to target the same developmental window (Gs25–30) in all treatments, which resulted in a longer chronological exposure of the RF treatment (due to its developmental delay). The Gs25–Gs30 window of exposure was chosen because *Lithobates pipiens* are most sensitive to EE_2 at this time (Hogan et al., 2008) and because this is the equivalent period of thyroid gland differentiation in *X. laevis* (NF 35–53) (Denver et al., 2002; Fort et al., 2007). The 14 mg/L SP dosage was chosen as comparable concentrations of perchlorate were used in similar studies as sublethal to tadpoles (Ortiz-Santaliestra and Sparling, 2007; *Lithobates sphenocephalus*), inhibitory, but not suppressive to metamorphosis during short-term 14-day exposure (Goleman et al., 2002a; *X. laevis*), as well as environmentally relevant (Goleman et al., 2002a,b).

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