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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 (E₂, 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($\pm$ 3) and 17($\pm$ 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 E₂ 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 *trb* 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.

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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; E₂, 17 β -estradiol; EE₂, 17 α -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; T₃, triiodothyronine; T₄, thyroxine; *trb*, 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 ($TR\alpha$ and $TR\beta$) 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. 17 β -Estradiol, sodium perchlorate and reduced feeding exposures

Tadpoles were exposed to the following treatments: 17 β -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 μ l 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 μ l 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 sphenoccephalus*), 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).

2.3. Growth, development and tissue sampling

To monitor for early effects on tadpole development, 80 tadpoles per treatment were staged during the exposures and up to 2 weeks later (on days 4, 10, 17 and 24). In addition, tadpoles were sampled ($n = 20$) when the median developmental stage reached Gs28 (during treatment), Gs30 (end of treatment and during pre-metamorphosis), Gs36 (prometamorphosis), and Gs42 (metamorphic climax). Sampled tadpoles for each targeted developmental stage were taken from one of the four replicates (i.e., Gs28, Gs30, Gs36 and Gs42 were sampled from different replicates). This allowed the maintenance of equal densities in each replicate and avoided changes in growth and development of tadpoles due to sampling. Tadpoles were euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma) dissolved in water. After euthanasia, morphometrics (snout-vent length [SVL], tail length [TL] and weight) and developmental stage were recorded for each individual. SVL and weight measurements were used to calculate the condition factor of tadpoles [Condition factor (k) = $100(\text{weight}/\text{SVL}^3)$]. Whole bodies with a ventral incision were preserved in RNAlater solution (Ambion Inc., Austin, TX) and stored at 4 °C for a maximum of 3 days (validated by Navarro-Martín et al., 2012). Livers and tails were then dissected and immediately frozen and stored at –80 °C until further analysis.

2.4. Cloning of *L. sylvaticus* candidate genes involved in growth

Recent studies carried out in our laboratory have characterized several genes involved in wood frog (*L. sylvaticus*) metamorphosis (Navarro-Martín et al., 2012). From those, we chose to examine *trβ* as it is a TH direct-response gene with levels that increase in parallel with TH to peak at metamorphosis. In contrast, *trα* levels remain relatively constant throughout development in *X. laevis* (Yaoita and Brown, 1990; Shi et al., 1996; Damjanovski et al., 2002; Das et al., 2010). Therefore, studying *trβ* levels provided us with a better indication of the effects of TH withdrawal. *Dio2* was chosen as a candidate gene as *Dio2* converts T_4 into the more potent T_3 (Becker et al., 1997; Dubois et al., 2006). In contrast, the other deiodinases either convert THs to biologically inactive forms (*Dio3*) or are not well-studied in the context of amphibian metamorphosis (*Dio1*) (Becker et al., 1997; Dubois et al., 2006; Kuiper et al., 2006). *Igf1* was chosen because it is a well-conserved gene involved in growth and known to be modulated by developmental age and nutritional status in many vertebrates (Reinecke and Collet, 1998; Rousseau and Dufour, 2007). The isoform *igf2* has a sixfold lower affinity to the *igf1-r* which mediates the growth effects of the *igfs* (Annunziata et al., 2011) and in mice has been reported to affect fetal growth but not growth rates after birth (DeChiara et al., 1990), and therefore was not examined here. Sequences for GH-IGF1 axis genes were not available for *L. sylvaticus* prior to this study so we used homology cloning for the growth-related genes insulin-like growth factor 1 (*igf1*) and insulin-like growth factor 1 receptor (*igf1r*). Degenerate primers were designed based on gene sequence alignments of well-conserved regions for various vertebrate species (amphibians, fish, and birds) using the Primer 3 program (<http://frodo.wi.mit.edu/primer3/>). Sequences were obtained through NCBI Pubmed (<http://www.ncbi.nlm.nih.gov/>). The designed cloning primers can be found in Table 1.

Total RNA was extracted from adult *L. sylvaticus* liver using the RNeasy Mini Kit (with DNase digestion step) following the manufacturer's instructions (Qiagen, Hilden, Germany). Extracted RNA was eluted in RNase-free water and stored at –80 °C. Five micrograms of total RNA were reverse-transcribed to cDNA using Superscript II (Invitrogen, Carlsbad, CA) and 200 ng of random hexamer primers following the manufacturer's instructions. Resulting

cDNA was used to amplify the desired genes using native Taq DNA polymerase (Invitrogen) and the respective primers. The cycling conditions used were: 2 min at 94 °C, 40 cycles of 45 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C, then 10 min at 72 °C (Eppendorf Mastercycler). The RT-PCR products were resolved on 1% agarose gel stained with ethidium bromide. Bands fitting the approximate size of expected products were excised and purified from the electrophoresis gel using the QIAquick gel extraction kit, according to the manufacturer's instructions (Qiagen). When the amplified product was not evident or the band was very faint, a segment of the gel at the expected size was excised and purified using the QIAquick gel extraction kit (Qiagen). The purified product was then submitted to a second RT-PCR reaction using the same cycling conditions as described above. Purified products were ligated into the P-GEM-T Easy Vector (Promega, Fitchburg, WI) and transformed into One Shot Top 10 Competent Cells (Invitrogen). Plasmid DNA was purified from the cells using the QIAprep Miniprep plasmid purification kit (Qiagen). The purified DNA was sent for sequencing to the Ontario Genomics Innovation Centre (Ottawa Health Research Institute, Ottawa, Canada). Sequences from at least three clones per gene were aligned to obtain the final consensus sequence. The obtained sequences were analyzed by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and ClustalW multiple alignment (<http://www.ch.embnet.org/software/ClustalW.html>) programs to determine nucleotide identities with corresponding genes of other species in the database. Protein sequences were predicted for the cloned genes using the Expasy translate tool (<http://web.expasy.org/translate/>).

2.5. Analysis of *igf1*, *igf1r*, *trβ* and *dio2* mRNA levels by real-time RT-PCR

2.5.1. RNA extraction and cDNA synthesis

Total RNA was isolated from livers of Gs28, Gs30 and Gs42 tadpoles using the RNeasy Micro Kit (Qiagen), whereas total RNA from tail tissue of Gs42 tadpoles was isolated using a combined protocol (Qiagen supplementary protocol: <http://www.qiagen.com/literature/render.aspx?id=582>) of TRIzol (Invitrogen) and Qiagen RNeasy Mini Kit (Qiagen). Navarro-Martín and collaborators (2012) have previously optimized this method. On-column DNase digestion was performed in all cases. The extracted RNA was resuspended in RNase-free water, and the concentration of total RNA, the ratio of absorbance at 260 and 280 nm and the ratio of absorbance at 260 and 230 nm were determined using a spectrophotometer (NanoDrop-2000, Thermo Fischer Scientific, Waltham, MA). The RNA integrity number (RIN) of select samples was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to confirm the high quality of the samples. All quality parameters were optimal (ratios of absorbance of ~2 and RIN numbers ≥8). Samples were then stored at –80 °C. Total cDNA of liver and tail samples was synthesized from 1 to 2.5 µg of total RNA respectively (because less RNA was isolated from livers than tails due to the smaller size of these organs). This was done using 200 ng of random hexamer primers (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). All procedures followed manufacturer protocols. In the case of liver samples (for which 1 µg of starting RNA was used), the incubation period at 42 °C was increased to 1.5 h (while tails were incubated at 42 °C for 50 min). Synthesized cDNA was stored at –20 °C until further analysis.

2.5.2. Real-time RT-PCR reactions

Gene specific primers for *trβ* and *dio2* were previously designed and optimized (Navarro-Martín et al., 2012). Gene-specific primers for *igf1* and *igf1r* were developed based on the *L. sylvaticus* sequences cloned as described in Section 2.4. Real-time RT-PCR pri-

Table 1Cloning primers and nucleotide percent identity of *Lithobates sylvaticus* *igf1* and *igf1r* cDNA sequences compared to other frog species.

Gene	Cloning primers nucleotide sequence 5' → 3'	Accession number	Length (bp)	<i>Siluranatropic alis</i>	<i>Xenopus laevis</i>	Predicted protein length (aa)
<i>igf1</i>	F: CCSTCTCTGTTGCGWAAATC R: GGHGRCAGTACATCTCYAG	KF819506	515	81 (95) <i>XM002936829</i>	81 (100) NM001163393	110
<i>igf1r</i>	F: CHTCTGTSAAACCBGARTAY R: CCWGCCATYTGRATCATCTT	KF819507	431	81 (99) <i>XM002933305</i>	83 (99) NM001088265	132

F and R refer to forward and reverse primers, respectively.

Bold numbers indicate percentage of identity.

Numbers in brackets represent the percentage of the *L. sylvaticus* sequence coverage when compared to the other frog sequences. Accession numbers for sequences of other species are indicated in italics.

mers used are summarized in Table 2. Real-time RT-PCR assays using SYBR Green I Chemistry were conducted to measure the relative mRNA levels of *igf1*, *igf1r* and *trβ*. For *dio2*, real-time RT-PCR assays were performed using fluorogenic 5' nuclease chemistry. Primer concentrations were optimized for each gene in each tissue analyzed (see Table 2). Levels of *igf1*, *igf1r* and *trβ* were analyzed in both the liver and the tail and *dio2* was analyzed in the tail only. Each sample ($n = 8$ per treatment group) was run in duplicate in optically clear 96-well plates in a final volume of 25 μ l. For every gene and real-time RT-PCR run, 'no template' and 'no reverse transcriptase' controls were included to verify that there was no genomic contamination and to confirm the specificity of target cDNA amplification. Each reaction contained 5.2 ng (tail) or 6.25 ng (liver) of RNA transformed to cDNA, 2.50 mM MgCl₂ (Qiagen), 1 $\times$ Q-PCR buffer (Qiagen), 0.20 mM dNTPs (Invitrogen), 0.1 μ M ROX reference dye (Stratagene, La Jolla, CA), and 1.25 U HotStarTaq (Qiagen). For the real-time RT-PCR reactions using the SYBR Green I chemistry, 0.25 $\times$ SYBR Green I dye (Invitrogen) and gene-specific primers to a concentration of 112.5–800 nM (see Table 2) were added to the reaction. The thermal cycling parameters for SYBR Green reactions consisted of a 15-min enzyme activation step at 98 $^{\circ}$ C, followed by 40 cycles of denaturation (95 $^{\circ}$ C for 15 s), annealing (60 $^{\circ}$ C for 5 s), elongation (72 $^{\circ}$ C for 54 s), and data acquisition (81 $^{\circ}$ C for 18 s). The 40 cycles were followed by denaturation for 1 min at 95 $^{\circ}$ C, 30 s at 55 $^{\circ}$ C and finally 30 s at 95 $^{\circ}$ C. Alternatively, the reactions measured by fluorogenic 5' nuclease chemistry (*dio2*) used PrimeTime PCR assays that contained 400 nM of primers and 200 nM of dual-labeled fluorescent probes (IDT). In this case the thermal cycling parameters were: an initial cycle Taq activation at 95 $^{\circ}$ C for 15 min, followed by 40 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min. Relative mRNA abundance of target and reference genes was interpolated using the relative standard curve method (Applied Biosystems, 1997). Standard curves for each gene and tissue were generated using serial dilutions of cDNA pooled from all samples. Assays were performed using an Mx3000P real-time RT-PCR system (Stratagene). The threshold for each gene was calculated using the MxPro-Mx3000P v4.10 software (Stratagene, 2007). Efficiencies

of relative standard curves were $100 \pm 10\%$, slope between -3.1 and -3.6 and $R^2 > 0.985$ in all cases, in accordance with the stringent conditions outlined in the manufacturer's instructions (Mx3000P System, Stratagene).

2.5.3. Real-time RT-PCR data analysis

MxPro-Mx3000P v4.10 software (Stratagene, 2007) was used to analyze the results of each real-time RT-PCR run. Gene expression was averaged for duplicates. As the common housekeeping gene *rpl8* was found to be affected by the treatments and due to the difficulty of finding a housekeeping gene that remains stable during tadpole metamorphosis, a data-driven normalization algorithm (NORMA-Gene, developed by Heckmann et al., 2011) was used to normalize mRNA levels. This method has been used for *L. sylvaticus* samples previously (Lancôt et al., 2013, 2014; Navarro-Martín et al., 2012, 2014). Normalization was performed using several target genes analyzed for each tissue. Duplicate data obtained for each sample, considered technical replicates, were averaged prior to normalization. Biological replicates ($n = 8$) were averaged to obtain the mean fold change in mRNA levels $\pm$ standard error of the mean (SEM).

2.6. Statistical analysis

Differences between treatments in development and growth (as measured by SVL, TL, weight and condition factor) were analyzed using non-parametric Kruskal–Wallis test with pairwise multiple comparisons, as this data did not meet parametric assumptions. Gene expression data were first analyzed for normality (Kolmogorov–Smirnov) and homogeneity of variance (Levene's test) and log₁₀ transformed to meet parametric assumptions. To analyze tail gene expression data a one-way ANOVA was performed to examine differences between treatments. In the case of the liver gene expression data, a two-way ANOVA was performed with treatment and Gs as independent factors and gene expression level as the dependent variable. Tukey's HSD post hoc multiple comparisons were used in both cases to check for statistical differences in gene expression levels between treatments (tail and liver) and

Table 2

Real-time RT-PCR primer and probes sets and conditions.

Gene	Primer	Nucleotide sequence 5' → 3'	Concentration (nM) liver	Concentration (nM) tail
<i>igf1</i>	Forward Reverse	CTACTCACTCTAACCCACTCAGCA AGCCTCTGTCTCCACATACAAAC	450	600
<i>igf1r</i>	Forward Reverse	TCCGTCGTGTAGGCGTTGT AGGGTTGTTCTCGGCATCT	112.5	450
<i>trβ</i>	Forward Reverse	AAGGAACCACTGCCAAGAATGT AACGCTTGCTGCTGCCAAA	800	450
<i>dio2</i>	Forward Reverse Probe	CACCTTTTAGACTTTGCCAGC GCTTATAAAGGAGGTCAAGTG /56-FAM/AGCGCCCTCTTGTCTGT/3IABkFQ/	–	400 200

developmental stages (liver). Analyses were performed using SPSS 20.0.0 (IBM Corp.) and differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Survival, development and growth

Survival from the start of the experiment (Gs25) to metamorphic climax (Gs42) in the treatment groups was as follows: Control: 97.5%; SP: 95.0%; E₂: 97.5% and RF: 90%. No significant differences were detected between any of the treatments or with respect to the control group (Chi-square, $P > 0.05$). At the end of the experiment the percentage of surviving animals that reached metamorphic climax (Gs42) ranged between 86% and 100% in all treatments with no significant differences observed between any of the treatments or controls (Chi-square, $P > 0.05$).

In order to track the development of tadpoles during and post-exposure, the mean Gs was determined on days 4, 10, 17, and 24 of the experiment. It is important to note that although treatments were exposed for the same developmental window (Gs25 to Gs30), RF animals were exposed for 6 days longer than control, E₂ and SP-treated animals due to delays in development. Statistical analysis showed significant differences in mean Gs between the different analyzed days regardless of the treatment (Fig. 1, Kruskal–Wallis test, $H = 1175.43$, $df = 3$, $P < 0.001$). Comparisons of mean Gs between treatments (regardless of the day) also revealed significant differences between treatments (Kruskal–Wallis test, $H = 19.31$, $df = 3$, $P < 0.001$). Multiple pairwise comparisons were carried out to examine possible significant differences between treatments for each of the experimental days (Fig. 1, Kruskal–Wallis test; day 4: $H = 46.81$, $df = 3$, $P < 0.001$; day 10: $H = 85.44$, $df = 3$, $P < 0.001$; day 17: $H = 105.91$, $df = 3$, $P < 0.001$; day 24: $H = 134.43$, $df = 3$, $P < 0.001$). Multiple pairwise comparisons showed that as

early as day 4 of exposure, the development of tadpoles was significantly delayed in the SP ($P = 0.008$) and RF ($P < 0.001$) groups when compared to controls. This delay was maintained and particularly pronounced at the other experimental days tested (up to 2 weeks after the end of the exposure). At day 17, SP-treated and RF tadpoles were 3–4 developmental stages behind controls (SP: $P < 0.001$; RF: $P < 0.001$). At day 24, SP and RF groups were 3–4 and 4–5 developmental stages behind controls, respectively (SP: $P < 0.001$; RF: $P < 0.001$). The development of E₂-treated tadpoles was not significantly different from controls on day 4 ($P = 0.804$). However, when tadpoles reached Gs30 (experimental day 10 and end of exposure), a significant delay in development was evident in the E₂-treated tadpoles relative to controls ($P = 0.014$). Although mean Gs appeared lower in E₂-treated tadpoles at days 17 and 24, this was not statistically different ($P > 0.05$).

Mean SVL, weight, TL and k were compared between all treatments at Gs 28, 30, 36, and 42. In addition, the mean time (measured in experimental days from the start of the experiment) taken by the tadpoles to reach these median stages was also compared between the different treatments. Kruskal–Wallis tests revealed several significant differences in growth parameters between treatments as well as within treatments at the various developmental stages examined (Fig. 2). At Gs28, the Kruskal–Wallis test revealed significant differences in SVL (Kruskal–Wallis test, $H = 14.14$, $df = 3$, $P = 0.003$) and k (Kruskal–Wallis test, $H = 18.45$, $df = 3$, $P < 0.001$) between the different treatments. Multiple pairwise comparisons revealed that animals in the SP treatment were shorter than controls ($P = 0.002$), but also had a significantly higher condition factor (k) ($P < 0.001$). At Gs30, significant differences in TL (Kruskal–Wallis test, $H = 10.26$, $df = 3$, $P = 0.017$), k (Kruskal–Wallis test, $H = 9.88$, $df = 3$, $P = 0.020$), and experimental days (Kruskal–Wallis test, $H = 76.00$, $df = 3$, $P < 0.001$) were found between the treatments. At Gs30, RF and E₂-treated animals were found to have shorter tails than controls (RF: $P = 0.003$, E₂: $P = 0.033$). E₂-treated animals had higher k values than controls ($P = 0.005$). RF tadpoles were significantly delayed in reaching median Gs30 compared to controls ($P < 0.001$) and all the other treatments (E₂: $P < 0.001$; SP: $P < 0.001$), in agreement with their delayed development described above. Significant differences in experimental days (Kruskal–Wallis test, $H = 74.81$, $df = 3$, $P < 0.001$), TL (Kruskal–Wallis test, $H = 8.84$, $df = 3$, $P = 0.032$), weight (Kruskal–Wallis test, $H = 8.70$, $df = 3$, $P = 0.034$), and k (Kruskal–Wallis test, $H = 26.33$, $df = 3$, $P < 0.001$) were present between the treatments at Gs36. SP-treated Gs36 tadpoles were heavier than all other treatments and controls ($P < 0.05$). SP and RF tadpoles had longer tails than control tadpoles ($P < 0.05$). The condition factor k was increased in SP-treated and RF-treated animals, relative to controls (SP: $P < 0.001$; RF: $P = 0.029$). Finally, all treatments were significantly delayed in reaching median Gs36 compared to controls (E₂: $P = 0.006$; RF: $P < 0.001$; SP: $P < 0.001$). At Gs42, significant differences between treatments were detected in SVL (Kruskal–Wallis test, $H = 10.67$, $df = 3$, $P = 0.014$), TL (Kruskal–Wallis test, $H = 11.66$, $df = 3$, $P = 0.009$) weight (Kruskal–Wallis test, $H = 12.08$, $df = 3$, $P = 0.007$) and experimental days (Kruskal–Wallis test, $H = 21.48$, $df = 3$, $P < 0.001$). At this stage, multiple pairwise comparisons also revealed that SP-treated animals were significantly longer in SVL than controls ($P = 0.012$), and heavier than all the treatments ($P < 0.05$). The mean number of days for tadpoles to reach Gs42 was as follows: 40.08 (± 1.63) for controls, 51.12 (± 3.08) for SP, 46.35 (± 2.19) for E₂, and 57.27 (± 3.16) for RF. The SP and RF treatments took respectively 11.12 (± 3.08) and 17.39 (± 3.16) days longer than controls to reach Gs42 (SP: $P = 0.006$; RF: $P < 0.001$). In contrast, significant differences in the number of days to reach Gs42 were not found in E₂-treated tadpoles compared to controls ($P > 0.05$).

Fig. 1. Effects of sodium perchlorate (SP, 14 mg/L), 17 β -estradiol (E₂, 200 nM), and reduced feeding (RF) on the development of *Lithobates sylvaticus* tadpoles. Treatments were applied covering developmental stages Gs25 to Gs30. The solid arrow represents the exposure time for Control, SP, and E₂ treatment. The gray arrow represents the additional experimental time for RF treatment (since a delay in development was observed). Data points represent the mean Gosner stage + SEM ($n = 80$ for each day and treatment). Data were analyzed using non-parametric Kruskal–Wallis test with pairwise multiple comparisons ($P < 0.05$). Letters indicate significant differences between treatments and experimental days. The order of letters from top to bottom refers to the following treatments: Ctrl – Control; E₂ – 17 β -estradiol; SP – sodium perchlorate; RF – reduced feeding.

Fig. 2. Snout-vent length (a; SVL; mm), tail length (b; mm), weight (c; g), and condition factor (d; k) of *Lithobates sylvaticus* tadpoles at Gs28, 30, 36 and 42, during and after exposures to sodium perchlorate (SP, 14 mg/L), 17 β -estradiol (E₂, 200 nM), reduced feeding (RF), and control (Ctrl). The exposure duration (Gs25–Gs30) is represented as a gray double-headed arrow. Data points represent the mean $\pm$ SEM ($n = 20$). Treatment effects on the different growth parameters were compared individually per each stage using non-parametric Kruskal–Wallis test with pairwise multiple comparisons. Significance was set at $P < 0.05$. Letters indicate significant differences between treatments at each stage. Condition factor (k) = $100 \times (\text{weight}/\text{SVL}^3)$.

3.2. Gene cloning

Partial cDNA sequences of *L. sylvaticus* *igf1* and *igf1r* were cloned as described in the Section 2.4. These sequences were submitted to the NCBI GenBank and the accession numbers, length, and percent identity to corresponding genes in other frog species are presented in Table 1. The *igf1* cDNA fragment translated into a 110-amino acid sequence, which represents ~72% of the full predicted IGF1 protein from *Silurana tropicalis* and *X. laevis*. The *igf1r* cDNA fragment translated into a 132-amino acid sequence representing ~22% of the full length of the *X. laevis* IGF1 receptor protein.

3.3. Gene expression analysis by real-time RT-PCR

3.3.1. Gene expression in tail at Gs42

One-way ANOVA was used to compare the effect of the treatments on *trb*, *dio2*, *igf1* and *igf1r* mRNA levels in tails at metamorphic climax (Gs42, Fig. 3). Results showed that tail *trb* and *igf1r* mRNA levels were affected by the treatments (ANOVA, $F_{(3,28)} = 7.762$, $P = 0.001$; $F_{(3,28)} = 6.742$, $P = 0.001$; respectively). Thus, *trb* expression in the tail of SP-treated tadpoles was reduced to just over half the levels of controls (Tukey, $P < 0.05$). No significant changes were observed in tail *trb* for any of the other treatments (Tukey, $P > 0.05$). A similar trend was observed for tail *dio2* expression, which was seemingly decreased in the SP treatment. However, like the doubling in *dio2* mRNA levels observed in the RF treatment, this was not significant (ANOVA, $F_{(3,28)} = 1.538$,

$P = 0.227$). The expression of *igf1r* was increased ~0.5-fold in the E₂-treated group compared to controls (Tukey, $P < 0.05$). Although *igf1* gene expression levels followed the same trend in the E₂ treatment, significant differences were not detected for this gene in the tail (ANOVA, $F_{(3,28)} = 1.888$, $P > 0.05$).

3.3.2. Liver gene expression at Gs28, Gs30 and Gs42

Two-way ANOVA, with treatment and Gs as independent factors and gene expression level as the dependent variable, revealed significant interactions in all genes studied: *igf1* ($F_{(6,79)} = 15.466$, $P < 0.001$), *igf1r* ($F_{(6,79)} = 9.338$, $P < 0.001$) and *trb* ($F_{(6,79)} = 5.441$, $P < 0.001$). Consequently, a one-way ANOVA comparing all stages and treatments together was performed. There were significant differences in all genes studied (Fig. 4): *igf1* ($F_{(11,79)} = 25.717$, $P < 0.001$), *igf1r* ($F_{(11,79)} = 13.852$, $P < 0.001$) and *trb* ($F_{(11,79)} = 161.143$, $P < 0.001$). We observed that the normal developmental progression (from Gs28 to Gs42) in the specific hepatic mRNAs analyzed in the control group were altered by the treatments in different ways, as explained below.

The levels of hepatic *trb* increased progressively in control animals through development, with a 3-fold increase between Gs28 and Gs30, and a 60-fold overall increase in expression between Gs28 and Gs42 (Tukey's HSD post hoc test, $P < 0.05$). The 3-fold increase observed between Gs28 and Gs30 (mid- to end of exposure) in control animals was suppressed in SP-, E₂-, and RF-treated tadpoles (Tukey's HSD post hoc test, $P < 0.05$), with tadpoles showing similar levels of *trb* at both developmental stages. Despite this

Fig. 3. Fold change in tail *trb*, *dio2*, *igf1* and *igf1r* mRNA in Gs42 *Lithobates sylvaticus* tadpoles after exposure to sodium perchlorate (SP, 14 mg/L), 17 β -estradiol (E₂, 200 nM), reduced feeding (RF) and control as determined by real-time RT-PCR. Data have been normalized using the NORMA-Gene method and are presented as the normalized fold change in mRNA levels relative to the mean of control. Bars represent the mean $\pm$ SEM of biological replicates ($n = 8$). One-way ANOVA was used to analyze for differences between treatments, followed by a Tukey's HSD post hoc test. Letters indicate significant differences between treatments. Significance is indicated if $P < 0.05$.

Fig. 4. Fold change in liver *igf1*, *igf1r*, and *trb* mRNA in Gosner stage (Gs) 28, Gs30 and Gs42 *Lithobates sylvaticus* tadpoles after exposure to sodium perchlorate (SP, 14 mg/L), 17 β -estradiol (E₂, 200 nM) and reduced feeding (RF) as determined by real-time RT-PCR. Data have been normalized using the NORMA-Gene method and are presented as the normalized fold change in mRNA levels relative to the mean of control of Gs28. Data points represent the mean $\pm$ SEM of biological replicates ($n = 6-8$). Data were analyzed for differences between treatments and/or developmental stages by two-way ANOVA followed by Tukey's HSD post hoc tests. Letters represent statistical differences between treatments and stages ($P < 0.05$).

altered pattern of expression during the exposures, by Gs42 the levels of hepatic *trb* in all treatments were not significantly different from the levels of controls. An overall significant increase in hepatic *trb* seen in the controls between Gs28 and Gs42 was also observed in all the treatments.

The levels of hepatic *igf1* increased from a low level at Gs28 to a maximum at Gs30, before decreasing to levels observed at Gs28 by Gs42 in the control animals. At Gs28, the liver *igf1* mRNA levels of SP-treated tadpoles were approximately 10 times that of controls (Tukey's HSD post hoc test, $P < 0.05$), but otherwise not significantly different at other developmental stages (Tukey's HSD post hoc test, $P > 0.05$). The 5-fold increase in *igf1* expression between Gs28 and Gs30 in control tadpole livers was lessened in E₂-treated tadpoles, due to significantly higher starting *igf1* levels (Gs28) in this treatment (Tukey's HSD post hoc test, $P < 0.05$). The natural increase in liver *igf1* mRNA levels observed in control tadpoles at Gs30 (the end of the exposure) was decreased approximately 6-fold in RF tadpoles (Tukey's HSD post hoc test, $P < 0.05$).

Levels of *igf1r* increased during development in control tadpole livers (Tukey's HSD post hoc test, $P < 0.05$), reaching maximum levels at Gs42, with a 3.5-fold increase over Gs28 expression. This increase at Gs42 was suppressed in SP-treated tadpoles that showed similar *igf1r* levels in all developmental stages studied. The developmental expression pattern of *igf1r* in E₂-treated tadpoles was similar to that of controls. Levels of liver *igf1r* were altered at the end of the exposure (Gs30) in RF tadpoles. At that stage tadpoles showed 2-times higher levels of liver *igf1r* than controls (Tukey's HSD post hoc test, $P < 0.05$), prematurely reaching levels seen in Gs42 tadpoles.

4. Discussion

Our results demonstrate that exposure to SP, E₂ and RF resulted in delayed *L. sylvaticus* tadpole development, particularly during the early stages of pre-metamorphosis. This delay persisted until tadpoles reached metamorphic climax at Gs42 (approximately 40 days post-exposure) in the SP-treated and RF tadpoles, but not in the E₂-exposed tadpoles. Perchlorate is known to competitively inhibit iodide uptake by the thyroid gland, and because iodide is indispensable for thyroid hormone (TH) production, the chemical

effectively lowers or blocks the production of TH (Wolff, 1998). Thyroid hormones play a crucial roles in directing development and initiating the transformations of metamorphosis (Buchholz et al., 2006; Tata, 2006), and hence perchlorate can be a powerful inhibitor of these processes in amphibians. Perchlorate salts such as sodium, ammonium, or potassium perchlorate have variously been shown to prevent metamorphosis and/or inhibit developmental features such as hindlimb growth, forelimb emergence, and tail resorption in exposed *X. laevis* (Goleman and Carr, 2006; Goleman et al., 2002a,b; Tietge et al., 2005) and *L. sphenoccephalus* (Ortiz-Santaliestra and Sparling, 2007). To study a possible disruption of the thyroid pathway, we analyzed the levels of the *trβ* mRNA in the tails and livers of *L. sylvaticus* tadpoles. The *trβ* gene encodes one of two TH receptors, which mediate the actions of T3, the active form of TH (Buchholz et al., 2006; Tata, 2006). We found that tail *trβ* mRNA levels were significantly lower in the SP treatment compared to control and other treatments at metamorphic climax (Gs42). These results were particularly striking as they were observed approximately 40 days after the treatment was discontinued. This suggests incomplete recovery or long-lasting repercussions of chemically inhibiting thyroid hormone production during early development in *L. sylvaticus* tadpoles. Similar effects have been observed in this species and other anurans exposed to endocrine-disrupting chemicals, supporting the idea that the expression of *trβ* can be a good indicator of endocrine disruption (Langlois et al., 2010; Lanctôt et al., 2013; Mortensen et al., 2006; Opitz et al., 2006; Zhang et al., 2006).

Levels of *trβ* mRNA are known to be low during pre- and pro-metamorphosis and to peak during metamorphic climax in parallel with circulating TH (Shi et al., 1996; Tata, 2006). When the levels of *trβ* were examined in the liver of tadpoles over Gs28 (mid-exposure), Gs30 (end of exposure) and Gs42 (metamorphic climax), a progressive increase in *trβ* was evident in the control group, as expected. This pattern of change was modified in all the treatments by the suppression of the 3-fold increase in *trβ* observed in the controls between Gs28 and Gs30, supporting other studies indicating the dependence of *trβ* expression on TH (Duarte-Guterman and Trudeau, 2010; Hogan et al., 2007), even at these early stages of development. Indeed, whereas levels of *trβ* increase significantly between Gs28 and Gs30 in the control treatment, they remain unchanged between the two stages in all the other treatments, even as individually, the time points are not always significantly different from the matched control time points. Differences in average developmental stage were observed between all treatments and controls during early development. Progression through developmental stages and metamorphosis is mediated by TH, partially through *trβ*. If the transient upset of hepatic *trβ* observed between Gs28 and Gs30 in all treatments is representative of gene expression patterns in other tissues, the developmental delays observed could be explained by a generalized disruption of *trβ* in the tadpoles. In the SP treatment, which specifically inhibited TH, the decrease in *trβ* mRNA still observed at Gs42 in the tails of tadpoles further suggests that the developmental delay observed in that treatment may be related to *trβ* availability.

The treatments affected growth during development and at metamorphic climax, prompting us to examine the expression of growth-related genes. This first required the cloning and sequencing of *igf1* and *igf1r* in *L. sylvaticus*. The hormone IGF1 mediates somatic growth in vertebrates via IGF1R signaling (Jones and Clemmons, 1995; Yakar et al., 2000), and is released by the liver in response to growth hormone stimulation (Le Roith et al., 2001; Reinecke and Collet, 1998). To our knowledge, no other study has explored the changes in *igf1* and *igf1r* over the course of amphibian larval development. We observed that in untreated control tadpoles, liver *igf1* mRNA levels reached a peak during mid-pre-metamorphosis, increasing approximately 6-fold from

Gs28 to Gs30. A possible explanation of this observation is that during pre- and pro-metamorphic developmental stages, tadpoles are actively growing in order to reach a certain size before being able to metamorphose, as proposed by Wilbur and Collins (1973). On the other hand, decreasing growth rates are observed in tadpoles at metamorphic climax, and likely due to energetic resources being used to allow metamorphosis to complete. Supporting this idea, hepatic *igf1* at Gs42 decreased to levels similar to those measured at Gs28. On the other hand, liver *igf1r* mRNA levels did not follow the same developmental pattern as *igf1*. Our results showed liver *igf1r* mRNA levels increasing during development, and reaching maximum levels (~3.5-times higher than Gs28) at Gs42. These results demonstrate that liver *igf1* and *igf1r* do not necessarily correlate in developing *L. sylvaticus* tadpoles.

Treatments with the natural estrogen 17β-estradiol (E₂) or its synthetic counterpart 17α-ethinylestradiol (EE₂) have been traditionally used to disrupt the sex differentiation of amphibians, with variable results ranging from complete feminization to complete masculinization, depending on the species, dosage, and window of exposure (reviewed in Hayes, 1998). However, several studies in amphibians and fish have also documented the effects of these estrogens on both development and growth. For example, early development or embryonic exposure to E₂ accelerated the development and increased growth as assessed by body weight and/or length in *X. laevis* at concentrations of 1 μM (Nishimura et al., 1997) or 0.01 nM (Bauer-Dantoin and Meinhardt, 2010). Delayed metamorphosis was also reported in E₂-treated *X. laevis* males (Sharma and Patiño, 2010) and EE₂-treated *X. laevis* (Tompsett et al., 2012; Pettersson and Berg, 2006). A study carried out by Hogan and collaborators (2008) showed an increase in body weight and size in *L. pipiens* tadpoles exposed to 5 nM of EE₂ during early metamorphosis (from Gs27 to Gs30). An earlier study also demonstrated increased weight in *L. sylvaticus* tadpoles exposed to 500 nM of E₂ and above for 2 weeks at Gs26 (Hogan et al., 2006). Though we did not see any significant effects of 10 days of exposure to 200 nM E₂ on the weight or SVL of the animals, our E₂-exposed wood frog tadpoles had smaller TLs than controls at Gs30. In addition, at Gs30 a significantly increased *k* was observed for tadpoles in the E₂ treatment. Fulton's condition factor (*k*) is used as a fitness index, typically taken to indicate a better energetic status of the animal (Stevenson and Woods, 2006). The increase in *k* observed in the E₂ treatment suggests that growth and/or fitness was somehow altered at Gs30. The absence of significant effects on weight or SVL could be due to the lower dose and shorter exposure time used in the present study, compared to that of Hogan and collaborators (2006). The effects of estrogens on the somatic growth axis genes and *igf1* in particular have been previously reported in fish. For example, an inhibitory effect of E₂ or EE₂ on the GH-IGF1 axis and related genes was noted in several species of fish (tilapia: Davis et al., 2007; Shved et al., 2007, 2008, 2009; fathead minnow: Filby et al., 2007; rainbow trout: Norbeck and Sheridan, 2011). Our results showed that E₂ treatment up-regulated rather than inhibited *igf1* in the tadpole liver, though this effect was only observed mid-treatment (4 days in, Gs28). Insofar as they may indicate a stimulatory effect of E₂ on the GH-IGF1 axis as a whole, these results are in agreement with studies in goldfish showing 3–5-fold increases in serum GH levels in response to intraperitoneal silastic implants of E₂ (Trudeau et al., 2000; Zou et al., 1997). Increased *igf1r* mRNA levels were also observed at Gs42 in the tails of E₂-treated animals. Our findings suggest a potentiating interaction between E₂ and elements of the growth pathway in tadpoles; however its exact nature remains to be explored in future studies.

We exposed tadpoles to a RF regimen to alter growth and development in a chemically-independent manner. Tadpoles deprived of food were delayed in their development, but were otherwise not

significantly different from the controls in weight and SVL. This is in agreement with the findings of Audo and collaborators (1995) who found that a 10-day food deprivation during early development (Gs25 onwards) in *Hyla chrysoscelis* tadpoles resulted in delayed metamorphosis. The weight and size of the food-restricted tadpoles in that study similarly did not differ from the well-fed controls. This suggests that tadpoles submitted to food restriction early in development were able to catch up in terms of body size, but were nevertheless at a disadvantage because of delayed metamorphosis. We have also found decreased levels of hepatic *igf1* in *L. sylvaticus* tadpoles at Gs30, immediately after food deprivation. Decreased serum IGF1 levels have been reported in humans, rats and fish in response to fasting or protein deficiency (Beauloye et al., 2002; Thissen et al., 1990, 1999; Yamamoto et al., 2011). It has been postulated that this effect is due to GH pathway inhibition (Beauloye et al., 2002). A similar mechanism may be responsible for the decreased liver *igf1* mRNA levels seen in our tadpole study after 17 days of RF regime (Gs30). At the same stage, liver *igf1r* was increased to ~2-times the control level, prematurely reaching Gs42-levels of expression in that treatment. Hepatic *igf1* and *igf1r* mRNA levels were inversely correlated after 17 days in the RF treatment. However, these effects were no longer detected at metamorphic climax. Since growth differences are not present between controls and RF tadpoles at Gs42, we propose that the disruption of liver gene expression patterns may be part of a compensatory mechanism at play in the fasting liver.

We also report growth effects in the tadpoles treated with the thyroid inhibitor SP (14 mg/L). These tadpoles showed extremely high liver *igf1* mRNA levels (~10-fold higher than controls) during treatment with SP (Gs28) and were heavier and/or longer than controls at later stages of development (Gs36 and 42). Treatment with TH has previously been shown to be potentiating to *igf1* expression. Thus, the expression of zebrafish and tilapia hepatic *igf1* or amphioxus hepatic caeca *igf1* homolog increased in response to T₃ both *in vivo* and *in vitro* in these organisms (Schmid et al., 2003; Wang and Zhang, 2011). Conversely, inhibition of TH by the T₃ inhibitor desethylamiodarone (DEA) resulted in an inhibition of the T₃-induced *igf1* (or *igf1* homolog) expression in zebrafish liver (or amphioxus hepatic caeca) (Wang and Zhang, 2011). In contrast to these studies, we have found that the inhibition of TH synthesis by SP resulted in an increase in liver *igf1* mRNA levels. We suggest that the hepatic *igf1* upregulation observed in *L. sylvaticus* tadpoles at Gs28 could be due to the effect of SP on growth rates (as those animals were also smaller than controls). The SP-specific effect is also supported by the absence of growth effects and *igf1* upregulation at Gs28 in the RF tadpoles, despite the similar developmental delays observed in the two treatments. The larger size and weight of tadpoles at Gs36 and metamorphic climax may be a result of increased hepatic *igf1* levels in early development. Overall, the normal pattern of hepatic *igf1* and *igf1r* expression is disrupted in the SP treatment. Coupled with the effect of treatment on growth parameters in late development, these effects indicate that thyroid axis inhibition by SP also affects somatic growth, with underlying disruption of growth genes.

The distinction between the processes of TH-dependent metamorphosis and purely somatic growth is well illustrated by reports of athyroid tadpoles spontaneously arising in laboratory populations of *X. laevis* (Rot-Nikcevic and Wassersug, 2004). Lacking normal TH production, these tadpoles do not metamorphose and instead remain in larval form for years. Their development is arrested but their growth is not, and they can reach giant sizes of 4 times the length and over 50 times the weight of normal *X. laevis* tadpoles (Rot-Nikcevic and Wassersug, 2004). The existence of this gigantic phenotype may suggest a moderating influence of TH on somatic growth in amphibians. Metamorphosis is timed and hormonally regulated in amphibians, and it is likely that the metamor-

phic development and growth programs are balanced or otherwise linked. The effects of perchlorate on somatic growth or related genes has not, to our knowledge, been described so far, with the majority of studies focusing on its inhibitory effects on development and metamorphic markers. Our study demonstrated that TH inhibition by SP early during development was able to disrupt *igf1* and *igf1r* gene expression patterns as well as growth parameters in *L. sylvaticus* tadpoles. We suggest that these findings add to the evidence of a relationship between development and somatic growth in amphibians, with disruptions of the thyroid pathway affecting growth and growth genes. In addition, a direct effect of SP itself on one or several components of the GH-IGF1 axis cannot be dismissed. Further investigations are required to elucidate whether the effects observed are direct or indirect and to understand possible interactions between the thyroid and growth pathways in amphibians.

5. Conclusions

This study demonstrated the specific effects of sodium perchlorate, estrogen and reduced feeding regimes on the metamorphosis-related gene thyroid hormone receptor beta (*trβ*) and growth-related genes insulin-like growth factor 1 (*igf1*) and insulin-like growth factor 1 receptor (*igf1r*). We have also described the normal expression of these genes during *L. sylvaticus* development. We have found that the treatments delayed the development of *L. sylvaticus* tadpoles but affected growth variably. Estradiol treatment affected growth minimally, and had transient effects on *igf1* expression in the liver. The reduced feeding regime also did not have long-lasting effects on morphology or gene expression. However, reduced feeding markedly affected the expression of all 3 genes examined immediately post-treatment. Finally, sodium perchlorate exposure resulted in immediate effects on *trβ*, *igf1* and *igf1r* in the liver and long-lasting effects on body weight and size during later development. Our findings call attention to the distinction and complex interactions between the development and somatic growth axes in wood frog tadpoles. We also highlight the long-lasting developmental effects of transient insults to the various endocrine axes and the inherent complexity of these endocrine systems.

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