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

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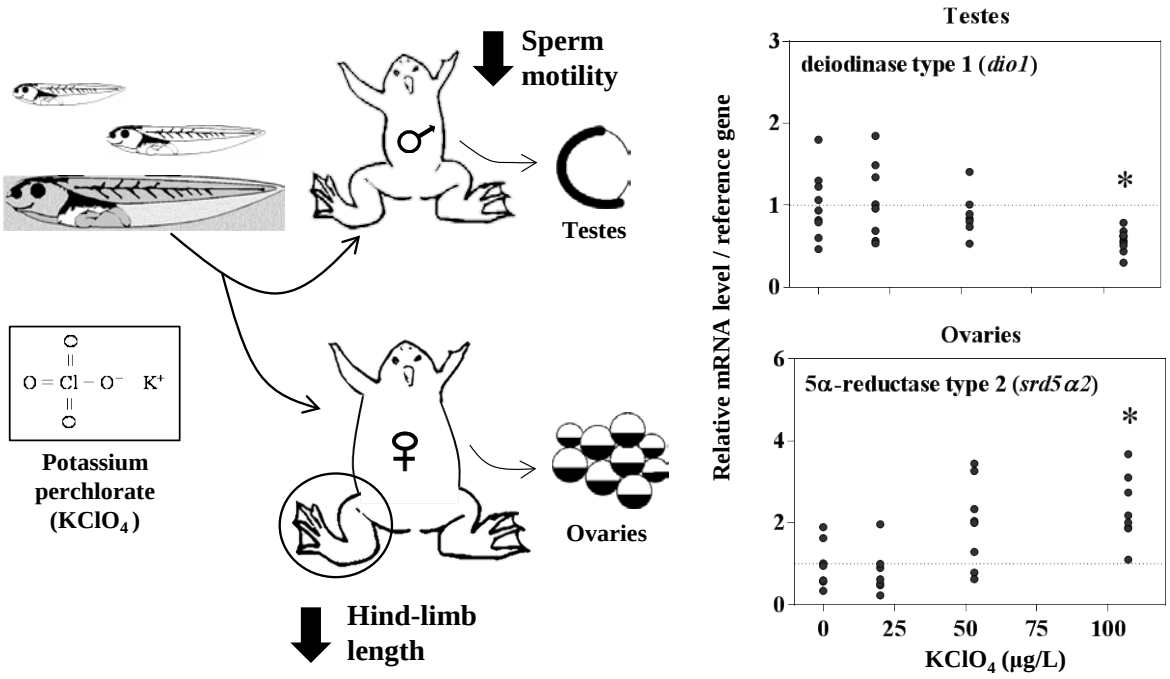
Visual abstract



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 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.

Capsule

Developmental and reproductive indices – including adult morphology, androgen plasma levels, gonadal thyroid hormone- and sex steroid-related transcript levels, and sperm motility – were altered in adult *Silurana tropicalis* frogs following chronic exposure (fertilized embryo to sexual maturity) to environmentally relevant concentrations of potassium perchlorate.

Keywords

Perchlorate; Amphibian; Morphology; Gene Expression; Sperm Motility; Western clawed frog

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; Rajagopalan et al., 2009; Parker et al., 2008; Rao et al., 2007; Dasgupta 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; Reviewed in Trumpolt 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 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 ≤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 effects of ClO_4^- exposure throughout the frog's 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^- (≤ 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 $KClO_4$ (≥ 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 (Life Technologies, Burlington, ON, CA) following the manufacturer's protocol and purified using the TURBO DNA-free™ Kit (Ambion; ThermoFisher Scientific, Ottawa, ON). The quantity of RNA was determined on a NanoDrop-2000 spectrophotometer (Thermofisher, Ottawa, ON, Canada). First strand cDNA was synthesized following the GoScript Reverse Transcription kit protocol using random primers (Promega, Madison, WI, USA) in a Mastercycler Pro S Thermocycler (Thermo Fisher, Ottawa, ON, Canada). The cDNA products were diluted 80-fold prior to qPCR amplification. We included negative control reactions for quality control (i.e., no reverse-transcriptase (noRT); no-template-controls (NTC)).

Primer sequences for aromatase (*cyp19*), estrogen receptor (*erα*), androgen receptor (*ar*), 5α-reductase type 2 (*srd5α2*), deiodinases (*dio1*, *dio2*, and *dio3*), thyroid hormone receptors (*trα* and *trβ*), and the reference genes ornithine decarboxylase (*odc*) and elongation factor-1 alpha (*ef1α*) were previously designed and validated by Langlois et al. (2010). We performed all qPCR assays using a CFX 96 Real-Time System (Bio-Rad Laboratories Inc, Mississauga, ON) and GoTaq qPCR MasterMix (Promega, Madison, WI, USA). The thermocycler program included an enzyme activation step at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, and 1 min at a gene-specific annealing temperature of 58 °C, 60 °C, or 62 °C, followed by a denaturation step of 1 min at 95 °C. Next, a dissociation curve was generated to confirm the presence of a single

155 amplicon. The threshold for each gene was assessed automatically by the Bio-Rad CFX Manager
156 Software 3.0. Pooled cDNA samples from each treatment were serial diluted (1:4) to produce a
157 standard curve of six points with a starting concentration of 50 ng. Each assay required a reaction
158 efficiency of 100 ± 15% and an $R^2 \geq 0.989$. The standard curve, control reactions, and samples
159 were run in duplicate for further quality control. Gene expression data is presented as fold
160 change relative to the mean control treatment. Fold change data of ovary and testis tissue
161 samples were normalized to the mean fold change of the reference genes *ef1α* and *odc*,
162 respectively. The expression of reference genes can differ between tissue type. A series of
163 housekeeping genes were therefore profiled for ovary and testis samples (data not shown) and
164 were only considered once the absence of treatment effects was confirmed.
165
166 2.4 Sperm analysis
167 The right testis from each frog was transferred to a clean Kimwipe™ and gently rolled to remove
168 fat bodies and blood vessels from the surface. The cleaned testes were placed in 500 μL of 2X
169 SAR and carefully macerated using long-nosed dissecting scissors to release sperm into solution.
170 The diluted testicular macerate was centrifuged at 1000 rpm for 2 min to remove large cellular
171 debris and the supernatant was collected for sperm video analysis. Placed on ice, the
172 spermatozoa in the supernatant (sperm stock) remained inert until activated with water.
173 The procedure for the analysis of the sperm videos was adapted from Burness et al.
174 (2004). Two sub-samples of the sperm stock were analyzed per testis. A drop of distilled water at
175 room temperature (Morrow et al. 2017; Larroze et al. 2014) was added to two drops of each sub-
176 sample of sperm stock to activate motility on a disposable Sperm Count CELL-VU Cytometer
177 (Fisher Scientific, Ottawa, ON). Sperm motility was recorded for 120 s on a high-resolution

monochrome CCD camera (Sony model XC-ST50) mounted on a negative phase contrast CH30 microscope (Olympus, Tokyo) at 100X. The swimming paths of all spermatozoa were quantified for each sample for 0.5 s sometime between 30 s and 60 s post activation. Five sperm parameters were measured using a CEROS (v.12) video analysis system (Hamilton-Thorne Research, Beverly, Maine, USA): 1) average path velocity (VAP) is the velocity over a smoothed path; 2) straight-line velocity (VSL) is the straight-line distance between the first and last sample point of the sperm's path divided by the total track time; 3) curvilinear velocity (VCL) is the total distance moved between successive frames on the video recording divided by the time taken for the sperm to move that total distance; 4) straightness (STR) is an estimate of the sperm's departure from a straight line while swimming, and is calculated by dividing VSL by VAP; and 5) amplitude of lateral head displacement (ALH) is the average value of the extreme side-to-side movement of the sperm head in each flagellar beat cycle.

An additional sub-sample (15 μL) was taken from the sperm stock to calculate the total number of sperm in the right testis from each male. At high magnification (400X), all spermatozoa in the four large corner squares and the large center square on a haemocytometer were counted (five squares total). The sperm density of the sperm stock was then calculated by dividing by the total spermatozoa count by the volume under these five squares (0.02 μL).

2.5 Statistical analysis

Statistical analyses were performed using Prism 6 (GraphPad Software Inc., San Diego, CA, USA) and JMP (Version 12; SAS, Cary, NC, USA). Data and residuals were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene tests, respectively. Data were log transformed when necessary to improve the fit to normality. Outlier analysis was performed

using the Grubbs Test. Morphometric data and plasma sex steroid data are presented as means (least squares means [95% CL]) calculated from a two-way ANOVA (linear model) for each variable (with sex, treatment and their interaction as predictors). Sperm motility data are presented as means (least squares means [95% CL]) and comparisons were calculated from a restricted maximum likelihood method, including male identity as a random factor to account for multiple measurements per male. Testis and ovary gene expression data are presented as standardized means ± 95% CL relative to *odc* and *ef1α* expression, respectively. Treatments were compared to controls using one-way ANOVAs and Dunnett's *post hoc* tests or *post hoc* contrast analysis for models that include random effects.

3. Results

3.1 Morphometric indices

Chronic exposure to $KClO_4$ during development generally resulted in smaller adult female frogs (Table 1). For example, all treatments significantly reduced female BM ($p < 0.05$) and the 53 and 107 µg/L treatments significantly decreased both SVL and HLL of females ($p < 0.05$). The magnitudes of the effects of all treatments on females were similar, with an approximately 40% reduction in BM, and a 10% reduction in both SVL and HLL. Developmental exposure to $KClO_4$; however, did not affect either HSI or GSI in females or males (Dunnett's tests, $p > 0.05$; data and analyses not shown). The statistically significant interaction terms in all linear models (Table 1) suggests that $KClO_4$ exposure during development has a different effect on male and female size, for example reducing the degree of sexual size dimorphism compared to frogs developing without such exposure. In one case, sexual dimorphism was reversed such that adult males were larger than females (e.g., at 53 µg $KClO_4$/L; Table 1).

3.2 Plasma sex steroid hormone levels

Plasma androgen content (T and 5α-DHT concentrations) was significantly different between the sexes of *S. tropicalis* in every treatment (Table 2). Males produced 7–15 times as much T and 2–3 times as much 5α-DHT as females across treatments, as expected in normal conditions. In males, exposure to 53 and 107 µg/L $KClO_4$ produced a slight decrease in T, though the data are very variable and the 95% CL are overlapping. Levels of 5α-DHT production by female and male frogs were not affected by chronic exposure to $KClO_4$.

3.3 Gene expression

A yearlong exposure to $KClO_4$ resulted in distinct thyroid hormone- and sex steroid-related gene expression patterns in the reproductive tissues of male and female *S. tropicalis* frogs. Differences in the mean values for the reference gene transcripts did not vary with treatment. Expression of *dio1* decreased with increasing $KClO_4$ concentrations ($F_{1, 32} = 9.3$, $p = 0.005$; Fig. 1E). Exposure to 107 µg/L of $KClO_4$ decreased the expression of *dio1* by 40% in the testis of males compared to control males (Dunnett's test, $p < 0.05$). In female frogs, expression of both *srd5α2* ($F_{1, 29} = 16.9$, $p = 0.0003$; Fig. 2D) and *cyp19* ($F_{1, 29} = 15.9$, $p = 0.0004$; Fig. 2H) in ovarian tissue were positively related to $KClO_4$ concentration. Exposure to 107 µg/L $KClO_4$ increased by twofold, on average, both ovarian *srd5α2* and *cyp19* transcripts (Dunnett's tests, $p < 0.05$). Transcript levels of the remaining thyroid hormone- or sex steroid-related genes did not change in male and female frogs ($p > 0.05$; Fig. 1 and 2).

3.4 Sperm motility

Sperm motility parameters were differentially affected between treatments (Table 3). In comparison to control males the spermatozoa of $KClO_4$-exposed males were characterized by a lower VAP (107 µg/L; $p < 0.05$) and a higher VSL (53 µg/L; $p < 0.05$) resulting in an increase in the STR of spermatozoa (20 and 53 µg/L; $p < 0.05$). The ALH was also reduced in spermatozoa of males from all $KClO_4$ treatments (20, 53, and 107 µg/L; $p < 0.05$) compared to control males. Other parameters – including VCL and sperm count – were unaffected by chronic exposure to $KClO_4$. Sperm counts were however variable within treatments and thus the effect of $KClO_4$ on sperm count might be worth further study with larger sample sizes.

4. Discussion

Perchlorate contamination has been reported in ground and surface waters across North America (ASTSWMO 2011; Blount et al. 2010; GAO 2010; Parker et al. 2008; Backus et al. 2005; Reviewed in Trumpolt et al. 2005) and the anion has been found to compete with iodide at the sodium-iodide symporter in the thyroid (Carr et al. 2008). However, few studies have examined the effects of prolonged exposure to the thyroid hormone disrupting chemical particularly at environmentally relevant concentrations in lower vertebrates, such as amphibians. This study examined the effects of a yearlong chronic exposure to $KClO_4$ in *S. tropicalis*. 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 female and male adult frogs.

The frogs used in this study were sacrificed once they were one year old, which facilitated the study of potential sex specific differences in developmental and reproductive indices. During the period of metamorphosis, male and female frogs are the same size – sex differences in body size emerge only 10 to 20 weeks after metamorphosis in *S. tropicalis* (Olmstead et al. 2009). Circulating sex steroid levels moreover develop sexually-dimorphic patterns as males and females differentiate in body size (Olmstead et al. 2009). Both sex steroid- and thyroid hormone-related gene expression are characterized by sexually dimorphic patterns in testicular and ovarian tissues of adult *S. tropicalis* (Duarte-Guterman and Trudeau, 2011). Disruption of sex-specific morphological, biochemical, and transcriptional dimorphisms would therefore not likely be evident until after the completion of metamorphosis. Previous studies examining the developmental effects of ClO_4^- on aquatic species often focused on a single sex, pooled male and female individuals, or simply did not examine gender differences.

Developmental and reproductive data on the differential effects of ClO_4^- as a function of gender are lacking in the literature.

Female control *S. tropicalis* were significantly larger (BM and SVL) than control males – as expected in normal conditions (in the absence of $KClO_4$) – but that difference was reduced at every concentration of $KClO_4$ tested. At 53 µg $KClO_4$/L, this pattern was even reversed where the males were larger than the females. However, since this does not follow a concentration-dependent response and the values are within the 95% CI, this difference may be a result of biological variability. We previously documented that the HLL of female stage NF 60 tadpoles exposed to 107 µg/L of $KClO_4$ were shorter than control females prior to the completion of metamorphosis (*S. tropicalis*: Flood and Langlois, 2014). Other short-term studies have confirmed that ClO_4^- at concentrations ≤ 100 µg/L can alter BM, hind leg growth, as well as tail resorption in developing tadpoles (*X. laevis*: Hu et al. 2006; Goleman et al. 2002a, 2002b). The morphometric data of the present study highlights, for the first time, both the possible permanency of $KClO_4$-induced developmental effects in amphibian ontogeny and the potential for sex differences in the developmental effects of ClO_4^-.

Amphibian development and growth is dependent on thyroid hormones, and thus we examined the effects of environmentally relevant concentrations of $KClO_4$ on thyroid hormone-related gene expression. ClO_4^- competitively inhibits the uptake of I^- via the NIS limiting the synthesis of the iodine-rich thyroid hormones (T4 and T3) by the thyroid gland (Carr et al. 2008). A direct relationship between thyroid hormone status and thyroid hormone-related gene expression (e.g., *trα*, *trβ*, *dio1*, *dio2* and *dio3*) in larval and adult gonadal tissues has been demonstrated in *S. tropicalis* (T3, iopanoic acid: Flood and Langlois, *Under review*; $KClO_4$: Flood and Langlois, 2014; T3: Duarte-Guterman and Trudeau, 2011). Among the five thyroid

hormone biomarkers examined in the present study, transcript levels of *dio1* decreased by 40% in testicular tissue following exposure to $KClO_4$. Thyroid hormones have been shown to play an important role in testicular development and function (Reviewed in Flood et al. 2013; Wagner et al. 2008; Maran 2003), with thyroid hormone-related genes demonstrating a male-biased pattern of expression in reproductive tissues of adult *S. tropicalis* (Duarte-Guterman and Trudeau, 2011). The activation or deactivation of thyroid hormones are mediated by *dio*s. The *dio1* enzyme can activate T4 to produce T3 via outer (5′)-ring deiodination as well as inactivate T4 or T3 via inner (5)-ring deiodination. As plasma thyroid hormone levels were not monitored in the present study, the functional significance of changes to this biomarker of peripheral thyroid hormone metabolism in the testicular tissues of *S. tropicalis* remains to be determined.

Chronic exposure to ClO_4^- has been reported to disrupt embryonic androgen synthesis and the subsequent reproductive development of threespine stickleback (*G. aculeatus*) without changing whole-body levels of thyroid hormones (Petersen et al. 2014). In the present study, $KClO_4$ altered sex steroid-related gene expression in ovary tissue, possibly indicating that $KClO_4$ may have indirect secondary effects on the sex steroid axis. The targeted disruption of thyroid hormone synthesis has been shown to indirectly mediate the effects of ClO_4^- on other endocrine pathways, including the hypothalamus–pituitary–gonad axis (reviewed in Duarte-Guterman et al. 2014; Flood et al. 2013). A direct relationship between thyroid hormone status and sex steroid-related molecular responses in larval and adult gonadal tissues has moreover been established in *S. tropicalis* (T3, iopanoic acid: Flood and Langlois, *Under review*; T3: Duarte-Guterman and Trudeau, 2011). The feminizing effects of ClO_4^- and other thyroid hormone disrupting chemicals have been extensively reported in a wide range of vertebrate species (Vertebrates: Reviewed in Duarte-Guterman et al. 2014; teleost fish: Reviewed in Habibi et al. 2012; *Oryzias latipes*: Liu et

al. 2011; *D. rerio*: Mukhi et al. 2007*; X. laevis*: Goleman et al. 2002a). We therefore examined potential estrogenic modes of $KClO_4$ action, and a two-fold increase in *cyp19* expression was observed with increasing $KClO_4$ concentrations in ovary tissue. The enzyme *cyp19* is responsible for the conversion of T to estradiol. Several studies have previously shown that exposure to $KClO_4$ (Flood and Langlois, 2014) or T3 (Duarte-Guterman and Trudeau, 2011) does not affect *cyp19* mRNA levels in gonadal mesonephros tissue of pre-metamorphic *S. tropicalis*. Exposure to thyroid hormone disruptors have however been reported to increase *cyp19* expression in ovary tissue of adult fish (thiourea, *C. gariepinus*: Rasheeda et al. 2005) and mammals (propylthiouracil, rats: Hapon et al. 2010). In further support of $KClO_4$ mediated changes to *cyp19* expression, exposure to T3 has been shown to significantly decrease *cyp19* mRNA levels and activity in ovary tissues of a wide range of adult vertebrate species (chicken: Sechman 2013; rat: Hatsuta et al. 2004; mouse: Cecconi et al. 1999; pig: Gregoraszczuk et al. 1998; Chan and Tan 1986). The present study demonstrated that the expression of *srd5α2* increased two-fold in ovary tissue of females exposed to 107 µg/L of $KClO_4$. The *srd5α2* enzyme converts T to the more potent and non-aromatizable androgen, 5α-DHT – actively competing with *cyp19* for T as a substrate. We first documented a $KClO_4$-mediated increase in *srd5α2* mRNA levels in hepatic tissue (an important tissue for androgen metabolism) of *S. tropicalis* tadpoles treated during sexual differentiation, earlier in development (Flood and Langlois, 2014). In support of $KClO_4$ mediated changes to *srd5α2* expression, exposure to the thyroid hormone triiodothyronine (T3) was shown to decrease *srd5α2* transcripts by 50% *ex vivo* in ovary tissue (Campbell and Langlois, *Under review*). Taken together these findings indicate that chronic exposure to environmentally relevant concentrations of $KClO_4$ can induce long-term increases in *cyp19* and

345 *srd5α2* expression in female *S. tropicalis*, but the functional significance of these $KClO_4$
346 mediated transcriptional modifications requires further investigation.

347 Male-biased traits such as plasma androgen content and sperm motility were differently
348 impacted by long-term exposure to environmentally relevant concentrations of $KClO_4$. Androgen
349 concentrations (T and 5α-DHT) were significantly higher in male control and $KClO_4$-exposed
350 frogs than their female counterparts. The testosterone concentrations for male and female *S.*
351 *tropicalis* in the present study fall proportionately at the low end of the range of mean values
352 reported for *S. tropicalis* by Olmstead et al. (2009). Studies however have observed plasma
353 testosterone levels as low as 0.5 ng/L in *X. laevis* (Lee and Veeramachaneni, 2005; Kang et al.
354 1995). Sperm motility was affected by chronic exposure to $KClO_4$. It is noteworthy that sperm
355 motility (measured as progressiveness (VAP, VSL), vigor (VCL), and straightness (STR)) was
356 comparable to that of Larroze et al. (2014), a study on the validity of computer-assisted sperm
357 analysis (CASA) for *S. tropicalis*. The spermatozoa of $KClO_4$-treated males were characterized
358 by a slower swimming speed (< VAP) and less lateral head displacement (< ALH) than the
359 sperm of control males. Inhibition of flagellar motility could produce these motility patterns. The
360 observed increase in VSL and STR further suggest a decrease in flagellar bending. Romano et al.
361 (2017) observed a significant decrease in mitochondrial activity in spermatozoa of hypothyroid
362 male Wistar rats; less energy would be generated for the flagellum and movement would be
363 impaired. Sperm density and sperm velocity are considered to be primary determinants of male
364 fertility in externally fertilizing aquatic species, thus *in vivo* exposure to $KClO_4$ may negatively
365 affect male reproductive success however further study would be required. The sperm of
366 externally-fertilizing amphibians may also come in direct contact with ClO_4^- ions in aquatic
367 environments during spawning. Since sperm activity is strongly influenced by the surrounding

aqueous chemical conditions, the effect of direct ClO_4^- exposure on sperm motility and fertilization success should also be investigated in future studies.

This is the first study to examine the effects of chronic exposure to environmentally relevant concentrations of $KClO_4$ in frogs from fertilized embryo to sexual maturity. Responses in thyroid hormone-sensitive metamorphic processes (i.e., body size, SVL and HLL) indicate targeted disruption of the thyroid hormone axis caused by prolonged exposure $KClO_4$. Moreover, exposed females had morphometric indices similar to those of control males indicating a possible loss of natural sexual dimorphism and highlights for the first-time potential sex-specific sensitivities to $KClO_4$. Changes in reproductive indices (i.e., androgen plasma levels, gonadal thyroid hormone- and sex steroid-related transcript levels, and sperm motility) possibly indicate that $KClO_4$ exposure may also have indirect secondary effects on the reproductive axes in male and female adult frogs. Whether these changes are functionally important to higher-level processes (population, community) remains to be elucidated. This study nonetheless provides a framework for future investigations to examine the effects of chronic exposure to ClO_4^- in natural amphibian populations.

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References

ASTM. ASTM E1439-98: Standard guide for Conducting the Frog Embryo Teratogenesis Assay – Xenopus (FETAX). ASTM, Philadelphia. **1998**.

ASTSWMO. *Perchlorate Policy Update*. Washington, DC: Federal Facilities Research Center Emerging Issues Focus Group. **2011**.

Backus, S. M., Klawuun, P., Brown, S., D'sa, I., Sharp, S., Surette, C., Willams, D. J. Determination of perchlorate in selected surface waters in the Great Lakes Basin by HPLC/MS/MS. *Chemosphere* **2005**, *61* (6), 834–843; DOI 10.1016/j.chemosphere.2005.04.054.

Bernhardt, R. R., von Hippel, F. A., Cresko, W. A., Perchlorate induces hermaphroditism in threespine sticklebacks. *Environ. Toxicol. Chem.*, **2006**, *25* (8), 2087–2096; DOI 10.1897/05-454R.1

Blount, B. C., Alwis, K. U., Jain, R. B., Solomon, B. L., Morrow, J. C., Jackson, W. A. Perchlorate, nitrate, and iodide intake through tap water. *Env. Sci. Technol.* **2010**, *44* (24), 9564–9570; DOI 10.1021/es1025195.

Bulaeva, E., Lanctôt, C., Reynolds, L., Trudeau, V. L., Navarro-Martín, L. Sodium perchlorate disrupts development and affects metamorphosis-and growth-related gene expression in tadpoles of the wood frog (*Lithobates sylvaticus*). *Gen. Comp. Endocrinol*. **2015**, *222* (1), 33–43; DOI 10.1016/j.ygcen.2015.01.012.

Burness, G., Casselman, S. J., Schulte-Hostedde, A. I., Moyes, C. D., Montgomerie, R. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). *Behav. Ecol. Sociobiol.* **2004**, *56* (1), 65–70; DOI 10.1007/s00265-003-0752-7.

Campbell, D. E. K., Langlois, V. S. L. Thyroid hormones and androgens differentially regulate gene expression in testes and ovaries of sexually mature *Silurana tropicalis. Gen. Comp. Endocrinol*. *Under Review*.

Cardone, A., Angelini, F., Esposito, T., Comitato, R., Varriale, B. The expression of androgen receptor messenger RNA is regulated by tri-iodothyronine in lizard testes. *J. Steroid Biochem. Mol. Biol.* **2000**, *72* (3), 133–141; DOI 10.1016/S0960-0760(00)00021-2.

Carr, D. L., Carr, J. A., Willis, R. E., Pressley, T. A. A perchlorate sensitive iodide transporter in frogs. *Gen. Comp. Endocrinol.* **2008**, *156* (1), 9–14; DOI 10.1016/j.ygcen.2008.01.005.

Cecconi, S., Rucci, N., Scaldaferri, M. L., Masciulli, M. P., Rossi, G., Moretti, C., D'Armiento, M., Ulisse, S. Thyroid hormone effects on mouse oocyte maturation and granulosa cell aromatase activity. *Endocrinology* **1999**, *140* (4), 1783–1788; DOI 10.1210/en.140.4.1783.

Chan, W. K., Tan, C. H. Inhibition of follicle-stimulating hormone induction of aromatase activity in porcine granulosa cells by thyroxine and triiodothyronine. *Endocrinology* **1986**, *119* (5), 2353–2359; DOI 10.1210/endo-119-5-2353.

427 Dasgupta, P. K., Dyke, J. V., Kirk, A. B., Jackson, W. A. Perchlorate in the United States.
428 Analysis of relative source contributions to the food chain. *Env. Sci. Technol.* **2006**, *40*
429 (21), 6608–6614; DOI 10.1021/es061321z.
430 Duarte-Guterman, P., Ryan, M. J., Trudeau, V. L. Developmental expression of sex steroid-and
431 thyroid hormone-related genes and their regulation by triiodothyronine in the gonad–
432 mesonephros of a Neotropical frog, *Physalaemus pustulosus*. *Gen. Comp. Endocrinol*.
433 **2012**, *177*, 195–204; DOI 10.1016/j.ygcen.2012.03.011.
434 Duarte-Guterman, P., Trudeau, V. L. Transcript profiles and triiodothyronine regulation of sex
435 steroid-and thyroid hormone-related genes in the gonad–mesonephros complex of
436 *Silurana tropicalis*. *Mol. Cell. Endocrinol.* **2011**, *331* (1), 143–149; DOI
437 10.1016/j.mce.2010.09.004.
438 Duarte-Guterman. P., Navarro-Martín, L., Trudeau, V. L. Mechanisms of crosstalk between
439 endocrine systems: regulation of sex steroid hormone synthesis and action by thyroid
440 hormones. *Gen. Comp. Endocrinol.* **2014**, *203* (1), 9–85; DOI
441 10.1016/j.ygcen.2014.03.015.
442 Flood, D. E. K., Fernandino, J. I., Langlois, V. S. Thyroid hormones in male reproductive
443 development: evidence for direct crosstalk between the androgen and thyroid hormone
444 axes. *Gen. Comp. Endocrinol*. **2013**, *192* (1), 2–14; DOI10.1016/j.ygcen.2013.02.038.
445 Flood, D. E. K., Langlois, V. S. Crosstalk between the thyroid hormone and androgen axes
446 during reproductive development in *Silurana tropicalis*. *Gen. Comp. Endocrinol.* **2014**,
447 *203* (1), 232–240; DOI 10.1016/j.ygcen.2014.03.037.
448 GAO (U.S. Government Accountability Office). *Perchlorate: Occurrence is widespread but at*
449 *varying levels; Federal agencies have taken some actions to respond to and lessen*
450 *releases*. **2010**. http://www.gao.gov/products/GAO-10-769 (accessed 17 November
451 2015).
452 Goleman, W. L., Carr, J. A., Anderson, T. A. Environmentally relevant concentrations of
453 ammonium perchlorate inhibit thyroid function and alter sex ratios in developing
454 *Xenopus laevis*. *Environ. Toxicol. Chem.* **2002a**, *21* (3), 590–597; DOI
455 10.1002/etc.5620210318.
456 Goleman, W. L., Urquidi, L. J., Anderson, T. A., Smith, E. E., Kendall, R. J., Carr, J. A.
457 Environmentally relevant concentrations of ammonium perchlorate inhibit development
458 and metamorphosis in *Xenopus laevis*. *Environ. Toxicol. Chem.* **2002b**, *21* (2), 424–430;
459 DOI 10.1002/etc.5620210227.
460 Gregoraszczuk, E. L., Slomczynska, M., Wilk, R. Thyroid hormone inhibits aromatase activity in
461 porcine thecal cells cultured alone and in coculture with granulosa cells. *Thyroid* **1998**, *8*
462 (12), 1157–1163; DOI 10.1089/thy.1998.8.1157.
463 Habibi, H. R., Nelson, E. R., Allan, E. R. O. New insights into thyroid hormone function and
464 modulation of reproduction in goldfish. *Gen. Comp. Endocrinol.* **2012**. *175* (1), 19–26;
465 DOI 10.1016/j.ygcen.2011.11.003.

Hapon, M. B., Gamarra-Luques, C., Jahn, G. A. Short term hypothyroidism affects ovarian function in the cycling rat. *Reprod Biol Endocrinol*. **2010**, *8* (1), 14; DOI 10.1186/1477-7827-8-14.

Hatsuta, M., Tamura, K., Shimizu, Y., Toda, K., Kogo, H. Effect of thyroid hormone on CYP19 expression in ovarian granulosa cells from gonadotropin treated immature rats. *J. Pharmacol. Sci.* **2004**, *94* (4), 420–425; DOI 10.1254/jphs.94.420.

Hu, F., Sharma, B., Mukhi, S., Patiño, R., Carr, J. A. The colloidal thyroxine (T4) ring as a novel biomarker of perchlorate exposure in the African Clawed Frog *Xenopus laevis*. *Toxicol. Sci.* **2006**, *93* (2), 268–277; DOI 10.1093/toxsci/kfl053.

Jackson, W. A., Böhlke, J. K., Gu, B., Hatzinger, P. B., Sturchio, N. C. Isotopic composition and origin of indigenous natural perchlorate and co-occurring nitrate in the southwestern United States. *Env. Sci. Technol.* **2010**, *44* (13), 4869–4876; DOI 10.1021/es903802j.

Johnson, K. M., Lema, S. C. Tissue-specific thyroid hormone regulation of gene transcripts encoding iodothyronine deiodinases and thyroid hormone receptors in striped parrotfish (*Scarus iseri*). *Gen. Comp. Endocrinol.* **2011**, *172* (3), 505–517; DOI 10.1016/j.ygcen.2011.04.022.

Kang, L., Marin, M., Kelley, D. Androgen biosynthesis and secretion in developing *Xenopus laevis*. *Gen. Comp. Endocrinol.* **1995**, *100* (3), 293–307; DOI 10.1006/gcen.1995.1160.

Kloas, W., Lutz, I. Amphibians as model to study endocrine disrupters. *J. Chromatogr. A.* **2006**, *1130* (1), 16–27; DOI 10.1016/j.chroma.2006.04.001.

Langlois, V. S., Duarte-Guterman, P., Ing, S., Pauli, B. D., Cooke, G. M., Trudeau, V. L. Fadrozole and finasteride exposures modulate sex steroid-and thyroid hormone-related gene expression in *Silurana (Xenopus) tropicalis* early larval development. *Gen. Comp. Endocrinol*. **2010**, *166* (2), 417–427; DOI 10.1016/j.ygcen.2009.11.004.

Larroze, S., Pickford, D. B., Holt, W. V. Validation of computer-assisted sperm-motility analysis in the amphibian *Silurana tropicalis*. *Reprod. Fertil. Dev.* **2014**, *27* (7), 1049–1056; DOI 10.1071/RD14015.

Lee, S. K., Veeramachaneni, D. R. Subchronic exposure to low concentrations of di-n-butyl phthalate disrupts spermatogenesis in *Xenopus laevis* frogs. *Toxicol. Sci*. **2005**, *84* (2), 394–407; DOI 10.1093/toxsci/kfi087.

Liu, C., Zhang, X., Deng, J., Hecker, M., Al-Khedhairy, A., Giesy, J. P., Zhou, B. Effects of prochloraz or propylthiouracil on the cross-talk between the HPG, HPA, and HPT axes in zebrafish. *Environ. Sci. Technol.* **2010**, *45* (2), 769–775; DOI 10.1021/es102659p.

Maran, R. R. M. Thyroid hormones: their role in testicular steroidogenesis. *Arch. Androl.* **2003**, *49* (5), 375–388; DOI 10.1080/01485010390204968.

Morrow, S., Gosalvez, J., Lopez-Fernandez, C., Arroyo, F., Holt, W. V., Guille, M. J. Effects of freezing and activation on membrane quality and DNA damage in *Xenopus tropicalis* and *Xenopus laevis* spermatozoa. *Reprod. Fertil. Dev*. **2017**, *29* (8), 1556–1566. DOI 10.1071/RD16190.

Mukhi, S., Torres, L., Patiño, R. Effects of larval–juvenile treatment with perchlorate and co-treatment with thyroxine on zebrafish sex ratios. *Gen. Comp. Endocrinol*. **2007**, *150* (3), 486–494; DOI 10.1016/j.ygcen.2006.11.013.

Nieuwkoop, P. D., Faber, J. *Normal Table of Xenopus laevis (Daudin)*. New York, NY: Garland, **1994**.

Olmstead, A. W., Korte, J. J., Woodis, K. K., Bennett, B. A., Ostazeski, S., Degitz, S. J. Reproductive maturation of the tropical clawed frog: *Xenopus tropicalis*. *Gen. Comp. Endocrinol.* **2009**, *160* (2), 117–123; DOI 10.1016/j.ygcen.2008.10.025.

Opitz, R., Schmidt, F., Braunbeck, T., Wuertz, S., Kloas, W. Perchlorate and ethylenethiourea induce different histological and molecular alterations in a non-mammalian vertebrate model of thyroid goitrogenesis. *Mol. Cell. Endocrinol.* **2009**, *298* (1), 101–114.

Parker, D. R., Seyfferth, A. L., Reese, B. K. Perchlorate in groundwater: a synoptic survey of "pristine" sites in the coterminous United States. *Env. Sci. Technol.* **2008**, *42* (5), 1465–1471; DOI 10.1021/es7021957.

Petersen, A. M., Dillon, D., Bernhardt, R. R., Torunsky, R., Postlethwait, J. H., von Hippel, F. A., Buck, C. L., Cresko, W. A. Perchlorate disrupts embryonic androgen synthesis and reproductive development in threespine stickleback without changing whole-body levels of thyroid hormone. *Gen. Comp. Endocrinol.* **2015**, *210* (1), 130–144; DOI 10.1016/j.ygcen.2014.10.015.

Rajagopalan, S., Anderson, T. A., Fahlquist, L., Rainwater, K. A., Ridley, M., Jackson, W. A. Widespread presence of naturally occurring perchlorate in high plains of Texas and New Mexico. *Env. Sci. Technol.* **2006**, *40* (10), 3156–3162. DOI 10.1021/es052155i.

Rao, B., Anderson, T. A., Orris, G. J., Rainwater, K. A., Rajagopalan, S., Sandvig, R. M., Scanlon, B. R., Stonestrom, D. A., Walvoord, M. A., Jackson, W. A. Widespread natural perchlorate in unsaturated zones of the southwest United States. *Env. Sci. Technol.* **2007**, *41* (13), 4522–4528. DOI 10.1021/es062853i.

Rasheeda, M. K., Sreenivasulu, G., Swapna, I., Raghuveer, K., Wang, D. S., Thangaraj, K., Gupta, A. D., Senthilkumaran, B. Thiourea-induced alteration in the expression patterns of some steroidogenic enzymes in the air-breathing catfish *Clarias gariepinus*. *Fish. Physiol. Biochem.* **2005**, *31* (2-3), 275–279; DOI 10.1007/s10695-006-0036-z.

Romano, R. M., Gomes, S. N., Cardoso, N. C. S., Schiessl, L., Romano, M. A., Oliveira, C. A. New insights for male infertility revealed by alterations in spermatic function and differential testicular expression of thyroid-related genes. *Endocrine* **2017**, *55* (2), 607–617; DOI 10.1007/s12020-016-0952-3.

Sambroni, E., Gutieres, S., Cauty, C., Guiguen, Y., Breton, B., Lareyre, J. J. Type II iodothyronine deiodinase is preferentially expressed in rainbow trout (*Oncorhynchus mykiss*) liver and gonads. *Mol. Reprod. Dev.* **2001**, *60* (3), 338–350; DOI 10.1002/mrd.1096.

Sechman, A. The role of thyroid hormones in regulation of chicken ovarian steroidogenesis. *Gen. Comp. Endocrinol.* **2013**, *190*, 68–75; DOI 10.1016/j.ygcen.2013.04.012.

Sharma, P., Patiño, R. Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in zebrafish (*Danio rerio*). *Gen. Comp. Endocrinol.* **2013**, *184* (1), 111–119; DOI 10.1016/j.ygcen.2012.12.018.

Supriya, A., Raghuveer, K., Swapna, I., Rasheeda, M. K., Kobayashi, T., Nagahama, Y. Thyroid hormone modulation of ovarian recrudescence of air-breathing catfish *Clarias gariepinus*. *Fish. Physiol. Biochem.* **2005**, *31* (2), 267–270; DOI 10.1007/s10695-006-0034-1.

Swapna, I., Rajasekhar, A., Supriya, A., Raghuveer, K., Rasheeda, M. K., Majumdar, K. C., Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus*. *Comp. Biochem. Physiol. Part A.* **2006**, *144* (1), 1–10; DOI 10.1016/j.cbpa.2006.01.017.

Tietge, J. E., Holcombe, G. W., Flynn, K. M., Kosian, P. A., Korte, J. J., Anderson, L. E. Metamorphic inhibition of *Xenopus laevis* by sodium perchlorate: effects on development and thyroid histology. *Environ. Toxicol. Chem.* **2005**, *24* (4), 926–933; DOI 10.1897/04-105R.1.

Trumpolt, C. W., Crain, M., Cullison, G .D., Flanagan, S. J. P., Siegel, L., Lathrop, S. Perchlorate: sources, uses and occurrences in the environment. *Remed. J.* **2005**, *16* (1), 65–89; DOI 10.1002/rem.20071.

Urbansky, E. T. Perchlorate chemistry: implications for analysis and remediation. *Bioremediat. J.* **1998**, *2* (2), 81–95. DOI 10.1080/10889869891214231.

Wagner, M. S., Wajner, S. M., Maia, A. L. The role of thyroid hormone in testicular development and function. *J. Endocrinol.* **2008**, *199* (3), 351–365; DOI 10.1677/JOE-08-0218.

Table 1. Effects of chronic $KClO_4$ treatments (for one year from fertilization) on mean body mass (g), snout-vent length (mm), and hind limb length (mm) of adult *S. tropicalis*.

$KClO_4$ (µg/L)	Body mass (g)		Snout-vent length (mm)		Hind limb length (mm)	
	Male	Female	Male	Female	Male	Female
0	9.5 [7.9, 11.2]	14.7 [13.1, 16.4]†	44.9, [42.1, 47.7]	51.5 [48.7, 54.2]†	47.2 [44.6, 49.8]	52.0 [49.4, 54.6]
20	9.3 [7.6, 10.9]	11.5 [9.9, 13.2]*	45.2, [42.4, 48.0]	46.9 [44.1, 49.7]	46.0 [43.4, 48.6]	47.5 [44.9, 50.1]
53[a]	9.6 [7.9, 11.2]	9.0 [7.3, 10.8]*	44.4, [41.6, 47.2]	43.0 [39.4, 45.2]*	46.8 [44.2, 49.4]	43.8 [41.0, 46.5]*
107	8.8 [7.2, 10.5]	10.2 [8.6, 11.9]*	43.0, [40.2, 45.8]	45.6 [42.8, 48.4]*	45.2 [42.6, 47.8]	45.7 [43.1, 48.3]*
	Model: $F_{7, 71} = 5.7$, $p < 0.0001$; $R^2 = 0.36$ **Effects:** Sex, $F_{1, 71} = 12.8$, $p = 0.0006$; Treatment, $F_{3, 71} = 4.8$, $p = 0.004$; Sex x Treatment, $F_{3, 71} = 4.1$, $p = 0.01$		**Model:** $F_{7, 71} = 4.1$, $p < 0.0008$; $R^2 = 0.29$ **Effects:** Sex, $F_{1, 71} = 5.0$, $p = 0.03$; Treatment, $F_{3, 71} = 4.6$, $p = 0.005$; Sex x Treatment, $F_{3, 71} = 3.2$, $p = 0.03$		**Model:** $F_{7, 71} = 3.3$, $p < 0.004$; $R^2 = 0.25$ **Effects:** Sex, $F_{1, 71} = 1.0$, $p = 0.32$; Treatment, $F_{3, 71} = 4.6$, $p = 0.006$; Sex x Treatment, $F_{3, 71} = 2.9$, $p = 0.04$	

Means (least squares means [95% CL]) and comparisons were calculated from a linear model for each morphological variable with sex, treatment, and their interaction as predictors. ANOVA statistics for each model are presented below the least squares statistics.

[a] Sample size for each sex per treatment is 10 except for females in this treatment where n = 9

* treatment is significantly different ($p < 0.05$; Dunnett's tests) from the control ($KClO_4$ = 0 µg/L)

† sexes are significantly different within treatment ($p < 0.05$; Tukey HSD tests)

Table 2. Effects of chronic $KClO_4$ treatments (for one year from fertilization) on mean T (pg/mL) and 5α-DHT (pg/mL) plasma levels in male and female *S. tropicalis*.

$KClO_4$ (µg/L)	T (pg/mL)[a]		5α-DHT (pg/mL)	
	Male	Female [b]	Male	Female
0	991.3 [607.1, 1618.8]†	63.3 [36.6, 109.5]	1277.9 [975.5, 1580.2]†	577.6 [275.2, 879.9]
20	1096.3 [671.3, 1790.2]†	79.3 [45.8, 137.2]	1301.2 [998.8, 1603.6]†	487.6 [185.2, 790.0]
53	716.5 [438.8, 1170.0]†	82.8 [47.9, 143.3]	1026.7 [724.3, 1329.1]†	407.1 [104.7, 709.4]
107	540.8 [331.1, 883.0]†	71.4 [41.3, 123.6]	1099.4 [797.1, 1401.8]†	347.2 [44.8, 649.6]
	Model: $F_{7, 28} = 26.2$, $p < 0.0001$; $R^2 = 0.87$ **Effects:** Sex, $F_{1, 28} = 177.2$, $p < 0.0001$; Treatment, $F_{3, 28} = 0.86$, $p = 0.47$; Sex x Treatment, $F_{3, 28} = 0.97$, $p < 0.42$		**Model:** $F_{7, 32} = 7.3$, $p < 0.0001$; $R^2 = 0.61$ **Effects:** Sex, $F_{1, 32} = 47.2$, $p < 0.0001$; Treatment, $F_{3, 32} = 1.12$, $p = 0.36$; Sex x Treatment, $F_{3, 28} = 0.15$, $p < 0.93$	

Means (least squares means [95% CL]) and comparisons were calculated from a linear model for each androgen with sex, treatment, and their interaction as predictors. ANOVA statistics for each model are presented below the least squares statistics.

[a] T was log-transformed to normalize residuals.

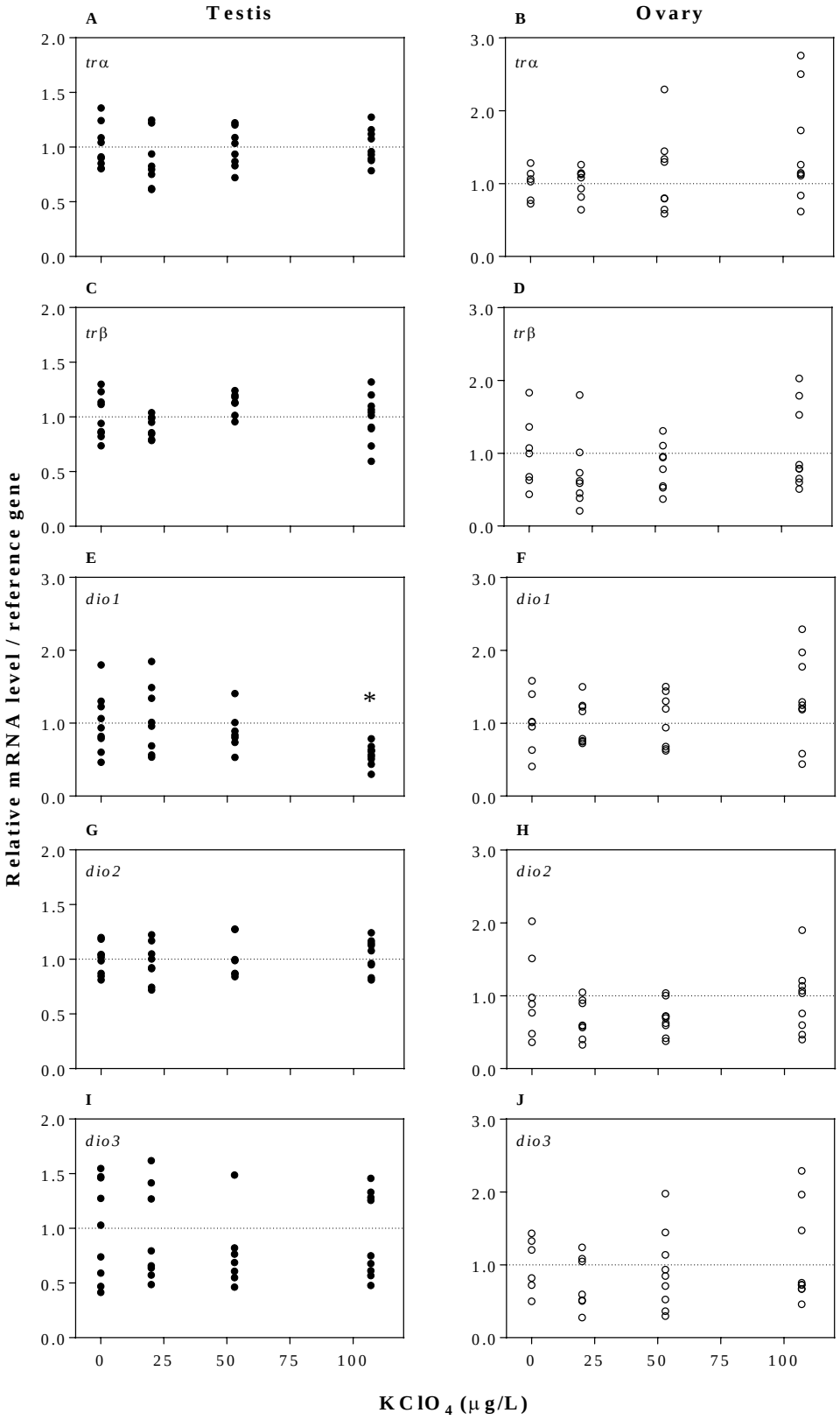
[b] For each sex per treatment n = 6, except n = 5 for levels of T in females in all treatments.

† sexes are significantly different within treatment ($p < 0.05$; Tukey HSD tests)

589 **Fig. 1.** Relative expression of *trα, trβ, dio1, dio2 and dio3* in testis (A, C, E, G, and I,
590 respectively) and ovary (B, D, F, H, and J, respectively) tissues of *Silurana tropicalis* frogs
591 chronically exposed to different aqueous concentrations of $KClO_4$ (0, 20, 53, and 107 μg/L; n =
592 7-9 frogs per treatment) for one year after fertilization. Testis and ovary gene expression data are
593 normalized to average expression of the reference genes *odc* and *ef1α,* respectively, and
594 presented as fold change relative to the control treatment. Note that the scales of the y-axes vary.

595

596 **Figure 1.**

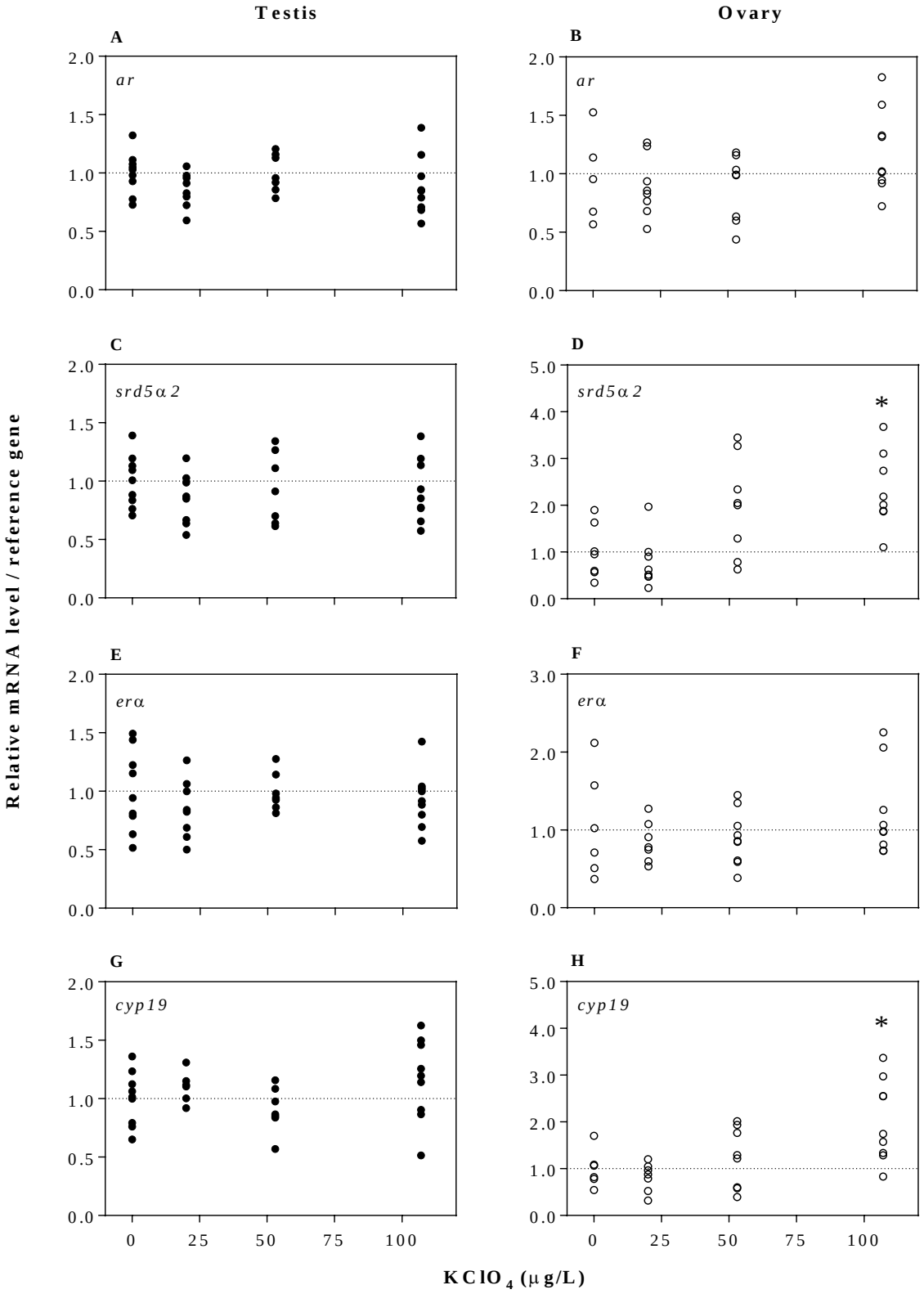


597

598 **Fig. 2.** Relative expression of *ar, srd5α2, erα,* and *cyp19* genes in testis (A, C, E, and G,
599 respectively) and ovary (B, D, F, and H, respectively) tissues of *Silurana tropicalis* frogs
600 chronically exposed to $KClO_4$ (0, 20, 53, and 107 μg/L; n = 7-9 frogs per treatment) for one year
601 after fertilization. Testis and ovary gene expression data are normalized to average expression of
602 the reference genes *odc* and *ef1α,* respectively, and presented as fold changes relative to the
603 control treatment. Note that the scales of the y-axes vary.

604

605 **Figure 2.**



606

Table 3. Effects of chronic $KClO_4$ exposure on mean sperm swimming speed (VAP, VSL, and VCL), linearity (STR), head displacement (ALH), and sperm count of male *S. tropicalis*.

$KClO_4$ (µg/L)	VAP (µm s^{-1}) $F_{3, 550.7} = 3.16$ $p = 0.03$, $R^2 = 0.04$	VSL (µm s^{-1}) $F_{3, 211.2} = 3.74$ $p = 0.01$, $R^2 = 0.004$	VCL (µm s^{-1}) $F_{3, 557.7} = 0.64$ $p = 0.59$	STR (%) $F_{3, 548.6} = 4.17$, $p = 0.006$, $R^2 = 0.04$	ALH (µm) $F_{3, 483.8} = 6.05$ $p = 0.0005$, $R^2 = 0.03$	Sperm count[b] (x 10^6 mL^{-1}) $F_{3, 26.1} = 0.24$ $p = 0.87$
0	23.9 [21.7, 26.0]	7.8 [7.0, 8.6]	46.2 [42.1, 50.3]	34.6 [30.9, 38.3]	3.5 [3.2, 3.8]	6.3 [4.5, 8.2]
20[a]	21.8 [19.7, 23.9]	8.9 [8.1, 9.7]	44.1 [40.1, 48.1]	40.9 [37.3, 44.4]*	3.0 [2.7, 3.3]*	5.9 [4.0, 7.7]
53	22.2 [20.0, 24.3]	9.1 [8.3, 9.9]*	44.5 [40.4, 48.5]	41.3 [37.7, 44.9]*	2.6 [2.3, 2.9]*	6.6 [4.6, 8.6][b]
107	19.8 [17.5, 22.1]*	7.1 [6.2, 8.1]	42.8 [38.4, 47.2]	38.8 [34.9, 42.8]	2.8 [2.4, 3.1]*	5.5 [3.5, 7.5][b]

Abbreviations: VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; STR, straightness; ALH, lateral head displacement. Means (least squares means [95% CL]) and comparisons were calculated from a linear model for each sperm parameter with treatment as predictor (statistics for each of the fixed effects shown at top of each column) and male identity as a random effect (to account for several sperm being measured from each male).

Sample size for each sperm parameter per treatment is six unless noted otherwise. Sample size for sperm count per treatment is 10 unless noted otherwise ([a] n = 7; [b] n = 9).

* treatment is significantly different ($p < 0.05$; *post hoc* contrast analyses) from the control (where $KClO_4$ = 0 µg/L)