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

- Testes
- Ovaries
- Potassium perchlorate ($\text{KClO}_4$)
- Sperm motility
- Hind-limb length

**Testes**
- Relative mRNA level / reference gene
- Deiodinase type 1 ($dio1$)

**Ovaries**
- Relative mRNA level / reference gene
- $5\alpha$-reductase type 2 ($sr5\alpha2$)

**Graphs**
- $\text{KClO}_4$ (μg/L) on the x-axis
- Relative mRNA level on the y-axis
- Data points and statistical significance indicated
Abstract

Perchlorate (ClO₄⁻) 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 ClO₄⁻ in adult male and female Western clawed frogs (*Silurana tropicalis*). Frogs were spawned and raised from fertilized embryo until sexual maturity in potassium perchlorate (KClO₄)-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 KClO₄ resulting in females with morphometric indices similar to those of control males – indicating potential sex-specific sensitivities to KClO₄. 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 KClO₄ 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 ClO₄⁻ 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 ClO₄⁻ exposure throughout the frog’s lifecycle, a subset of *S. tropicalis* from the previous experiment were continually exposed to environmentally relevant levels of KClO₄ 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 KClO₄ 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 KClO₄ 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 ClO₄⁻ (≤ 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 KClO₄ 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 KClO₄ (≥ 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 KClO₄ 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 CaCl₂, 2.0 mM KCl, and 3.6 mM NaHCO₃) 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 (srd5a2), 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
amplicon. The threshold for each gene was assessed automatically by the Bio-Rad CFX Manager Software 3.0. Pooled cDNA samples from each treatment were serial diluted (1:4) to produce a standard curve of six points with a starting concentration of 50 ng. Each assay required a reaction efficiency of 100 ± 15% and an $R^2 \geq 0.989$. The standard curve, control reactions, and samples were run in duplicate for further quality control. Gene expression data is presented as fold change relative to the mean control treatment. Fold change data of ovary and testis tissue samples were normalized to the mean fold change of the reference genes $ef1\alpha$ and $odc$, respectively. The expression of reference genes can differ between tissue type. A series of housekeeping genes were therefore profiled for ovary and testis samples (data not shown) and were only considered once the absence of treatment effects was confirmed.

2.4 Sperm analysis

The right testis from each frog was transferred to a clean Kimwipe™ and gently rolled to remove fat bodies and blood vessels from the surface. The cleaned testes were placed in 500 μL of 2X SAR and carefully macerated using long-nosed dissecting scissors to release sperm into solution. The diluted testicular macerate was centrifuged at 1000 rpm for 2 min to remove large cellular debris and the supernatant was collected for sperm video analysis. Placed on ice, the spermatozoa in the supernatant (sperm stock) remained inert until activated with water.

The procedure for the analysis of the sperm videos was adapted from Burness et al. (2004). Two sub-samples of the sperm stock were analyzed per testis. A drop of distilled water at room temperature (Morrow et al. 2017; Larroze et al. 2014) was added to two drops of each sub-sample of sperm stock to activate motility on a disposable Sperm Count CELL-VU Cytometer (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 KClO₄ 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 KClO₄ concentrations (*F*₁,₃₂ = 9.3, *p* = 0.005; Fig. 1E). Exposure to 107 μg/L of KClO₄ 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 KClO₄ concentration. Exposure to 107 μg/L KClO₄ 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 KClO₄-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 KClO₄ 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 KClO₄. Sperm counts were however variable within treatments and thus the effect of KClO₄ 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 KClO₄ 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 ClO₄⁻ 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., *tr$\alpha$, tr$\beta$, 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 KClO₄. 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 *dios*. 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 ClO₄⁻ 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, KClO₄ altered sex steroid-related gene expression in ovary tissue, possibly indicating that KClO₄ 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 ClO₄⁻ 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 ClO₄⁻ 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 KClO₄ action, and a two-fold increase in *cyp19* expression was observed with increasing KClO₄ concentrations in ovary tissue. The enzyme *cyp19* is responsible for the conversion of T to estradiol. Several studies have previously shown that exposure to KClO₄ (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 KClO₄ 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 *srd5a2* increased two-fold in ovary tissue of females exposed to 107 μg/L of KClO₄. The *srd5a2* 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 KClO₄-mediated increase in *srd5a2* 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 KClO₄ mediated changes to *srd5a2* expression, exposure to the thyroid hormone triiodothyronine (T3) was shown to decrease *srd5a2* 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 KClO₄ can induce long-term increases in *cyp19* and...
srd5α2 expression in female *S. tropicalis*, but the functional significance of these KClO₄-mediated transcriptional modifications requires further investigation.

Male-biased traits such as plasma androgen content and sperm motility were differently impacted by long-term exposure to *environmentally relevant* concentrations of KClO₄. Androgen concentrations (T and 5α-DHT) were significantly higher in male control and KClO₄-exposed frogs than their female counterparts. The testosterone concentrations for male and female *S. tropicalis* in the present study fall proportionately at the low end of the range of mean values reported for *S. tropicalis* by Olmstead et al. (2009). Studies however have observed plasma testosterone levels as low as 0.5 ng/L in *X. laevis* (Lee and Veeramachaneni, 2005; Kang et al. 1995). Sperm motility was affected by chronic exposure to KClO₄. It is noteworthy that sperm motility (measured as progressiveness (VAP, VSL), vigor (VCL), and straightness (STR)) was comparable to that of Larroze et al. (2014), a study on the validity of computer-assisted sperm analysis (CASA) for *S. tropicalis*. The *spermatozoa* of KClO₄-treated males were characterized by a slower swimming speed (< VAP) and less lateral head displacement (< ALH) than the sperm of control males. Inhibition of flagellar motility could produce these motility patterns. The observed increase in VSL and STR further suggest a decrease in flagellar bending. Romano et al. (2017) observed a significant decrease in mitochondrial activity in spermatozoa of hypothyroid male Wistar rats; less energy would be generated for the flagellum and movement would be impaired. Sperm density and sperm velocity are considered to be primary determinants of male fertility in externally fertilizing aquatic species, thus *in vivo* exposure to KClO₄ may negatively affect male reproductive success however further study would be required. The sperm of externally-fertilizing amphibians may also come in direct contact with ClO₄⁻ ions in aquatic environments during spawning. Since sperm activity is strongly influenced by the surrounding
aqueous chemical conditions, the effect of direct ClO₄⁻ 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 KClO₄ 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 KClO₄. 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 KClO₄. Changes in reproductive indices (i.e., androgen plasma levels, gonadal thyroid hormone- and sex steroid-related transcript levels, and sperm motility) possibly indicate that KClO₄ 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 ClO₄⁻ in natural amphibian populations.
Acknowledgements: We thank Almira Siew, Colin Campbell, and Sarah Wallace for their contributions to the project. This work was supported by Discovery Grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada to RDM (RGPIN 2349-08) and VSL (RGPIN 418576-2012), a Canada Research Chair (CRC 950-230442) to VSL, and an NSERC-PGS (PGS D2-460059-2014) to DEKC.
References


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Table 1. Effects of chronic KClO₄ treatments (for one year from fertilization) on mean body mass (g), snout-vent length (mm), and hind limb length (mm) of adult *S. tropicalis*.

<table>
<thead>
<tr>
<th>KClO₄ (µg/L)</th>
<th>Body mass (g)</th>
<th>Snout-vent length (mm)</th>
<th>Hind limb length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9.5 [7.9, 11.2]</td>
<td>14.7 [13.1, 16.4]†</td>
<td>44.9 [42.1, 47.7]</td>
</tr>
<tr>
<td>20</td>
<td>9.3 [7.6, 10.9]</td>
<td>11.5 [9.9, 13.2]*</td>
<td>45.2 [42.4, 48.0]</td>
</tr>
<tr>
<td>53a</td>
<td>9.6 [7.9, 11.2]</td>
<td>9.0 [7.3, 10.8]*</td>
<td>44.4 [41.6, 47.2]</td>
</tr>
<tr>
<td>107</td>
<td>8.8 [7.2, 10.5]</td>
<td>10.2 [8.6, 11.9]*</td>
<td>43.0 [40.2, 45.8]</td>
</tr>
</tbody>
</table>

Model: $F_{7, 71} = 4.1, p < 0.0008; R^2 = 0.29$

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 (KClO₄ = 0 µg/L)

† sexes are significantly different within treatment ($p < 0.05$; Tukey HSD tests)
Table 2. Effects of chronic KClO₄ treatments (for one year from fertilization) on mean T (pg/mL) and 5α-DHT (pg/mL) plasma levels in male and female *S. tropicalis*.

<table>
<thead>
<tr>
<th>KClO₄ (μg/L)</th>
<th>Male T (pg/mL)ᵃ</th>
<th>Female T (pg/mL)ᵇ</th>
<th>Male 5α-DHT (pg/mL)</th>
<th>Female 5α-DHT (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>991.3 [607.1, 1618.8]†</td>
<td>63.3 [36.6, 109.5]</td>
<td>1277.9 [975.5, 1580.2]†</td>
<td>577.6 [275.2, 879.9]</td>
</tr>
<tr>
<td>20</td>
<td>1096.3 [671.3, 1790.2]†</td>
<td>79.3 [45.8, 137.2]</td>
<td>1301.2 [998.8, 1603.6]†</td>
<td>487.6 [185.2, 790.0]</td>
</tr>
<tr>
<td>53</td>
<td>716.5 [438.8, 1170.0]†</td>
<td>82.8 [47.9, 143.3]</td>
<td>1026.7 [724.3, 1329.1]†</td>
<td>407.1 [104.7, 709.4]</td>
</tr>
<tr>
<td>107</td>
<td>540.8 [331.1, 883.0]†</td>
<td>71.4 [41.3, 123.6]</td>
<td>1099.4 [797.1, 1401.8]†</td>
<td>347.2 [44.8, 649.6]</td>
</tr>
</tbody>
</table>

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, $F_{3, 28} = 0.97, p < 0.42$

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.

Following log-transformation to normalize residuals.

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)
Fig. 1. Relative expression of *tra, trβ, dio1, dio2 and dio3* in testis (A, C, E, G, and I, respectively) and ovary (B, D, F, H, and J, respectively) tissues of *Silurana tropicalis* frogs chronically exposed to different aqueous concentrations of KClO₄ (0, 20, 53, and 107 μg/L; n = 7-9 frogs per treatment) for one year after fertilization. Testis and ovary gene expression data are normalized to average expression of the reference genes *odc* and *ef1α*, respectively, and presented as fold change relative to the control treatment. Note that the scales of the y-axes vary.
Figure 1.

- **A** Testis
- **B** Ovary
- **C**
- **D**
- **E**
- **F**
- **G**
- **H**
- **I**
- **J**

**Relative m RNA level / reference gene**

**Testis**

- **trα**
- **trβ**
- **dio1**
- **dio2**
- **dio3**

**Ovary**

- **trα**
- **trβ**
- **dio1**
- **dio2**
- **dio3**

**K ClO₄ (µg/L)**

0 25 50 75 100
Fig. 2. Relative expression of *ar, srd5a2, era,* and *cyp19* genes in testis (A, C, E, and G, respectively) and ovary (B, D, F, and H, respectively) tissues of *Silurana tropicalis* frogs chronically exposed to KClO₄ (0, 20, 53, and 107 μg/L; n = 7-9 frogs per treatment) for one year after fertilization. Testis and ovary gene expression data are normalized to average expression of the reference genes *odc* and *ef1α,* respectively, and presented as fold changes relative to the control treatment. Note that the scales of the y-axes vary.
Figure 2.

A. Testis

B. Ovary

C. srd5α2

D. srd5α2

E. erα

F. erα

G. cyp19

H. cyp19

Relative mRNA level / reference gene

KClO₄ (µg/L)
Table 3. Effects of chronic KClO₄ exposure on mean sperm swimming speed (VAP, VSL, and VCL), linearity (STR), head displacement (ALH), and sperm count of male S. tropicalis.

<table>
<thead>
<tr>
<th>KClO₄ (μg/L)</th>
<th>VAP (μm s⁻¹)</th>
<th>VSL (μm s⁻¹)</th>
<th>VCL (μm s⁻¹)</th>
<th>STR (%)</th>
<th>ALH (μm)</th>
<th>Sperm countb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F₃,₅₅₀.₇ = 3.16</td>
<td>F₃,₂₁₁.₂ = 3.74</td>
<td>F₃,₅₅₇.₇ = 0.64</td>
<td>F₃,₅₄₈.₆ = 4.17, p = 0.006, R² = 0.03</td>
<td>F₃,₄₈₃.₈ = 6.05, p = 0.0005, R² = 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p = 0.03, R² = 0.04</td>
<td>p = 0.01, R² = 0.004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>23.9 [21.7, 26.0]</td>
<td>7.8 [7.0, 8.6]</td>
<td>46.2 [42.1, 50.3]</td>
<td>34.6 [30.9, 38.3]</td>
<td>3.5 [3.2, 3.8]</td>
<td>6.3 [4.5, 8.2]</td>
</tr>
<tr>
<td>20a</td>
<td>21.8 [19.7, 23.9]</td>
<td>8.9 [8.1, 9.7]</td>
<td>44.1 [40.1, 48.1]</td>
<td>40.9 [37.3, 44.4]*</td>
<td>3.0 [2.7, 3.3]*</td>
<td>5.9 [4.0, 7.7]</td>
</tr>
<tr>
<td>53</td>
<td>22.2 [20.0, 24.3]</td>
<td>9.1 [8.3, 9.9]*</td>
<td>44.5 [40.4, 48.5]</td>
<td>41.3 [37.7, 44.9]*</td>
<td>2.6 [2.3, 2.9]*</td>
<td>6.6 [4.6, 8.6]b</td>
</tr>
<tr>
<td>107</td>
<td>19.8 [17.5, 22.1]*</td>
<td>7.1 [6.2, 8.1]</td>
<td>42.8 [38.4, 47.2]</td>
<td>38.8 [34.9, 42.8]</td>
<td>2.8 [2.4, 3.1]*</td>
<td>5.5 [3.5, 7.5]b</td>
</tr>
</tbody>
</table>

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 KClO₄ = 0 μg/L)