General and Comparative Endocrinology 243 (2017) 60–69

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

# Exogenous iodide ameliorates perchlorate-induced thyroid phenotypes in threespine stickleback

Alison M. Gardell [a], Frank A. von Hippel [b,*], Elise M. Adams [b], Danielle M. Dillon [c], Ann M. Petersen [d], John H. Postlethwait [e], William A. Cresko [f], C. Loren Buck [c,*]

[a] Department of Biological Sciences, University of Alaska Anchorage, Anchorage, AK 99508, USA
[b] Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA
[c] Center for Bioengineering Innovation & Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA
[d] Department of Integrative Biology, Oregon State University, Cascades, Bend, OR 97703, USA
[e] Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA
[f] Institute of Ecology and Evolution, University of Oregon, Eugene, OR 97403, USA

ARTICLE INFO

*Article history:*
Received 2 May 2016
Revised 7 October 2016
Accepted 30 October 2016
Available online 1 November 2016

*Keywords:*
Endocrine disruption
Histopathology
NIS
Obesogen
Sodium-iodide symporter
Thyroxine

ABSTRACT

Perchlorate is a ubiquitous environmental contaminant that has widespread endocrine disrupting effects in vertebrates, including threespine stickleback (*Gasterosteus aculeatus*). The target of perchlorate is thyroid tissue where it induces changes in the organization, activation, and morphology of thyroid follicles and surrounding tissues. To test the hypothesis that some phenotypes of perchlorate toxicity are not mediated by thyroid hormone, we chronically exposed stickleback beginning at fertilization to perchlorate (10, 30, 100 ppm) or control water with and without supplementation of either iodide or thyroxine ($T_4$). Stickleback were sampled across a one-year timespan to identify potential differences in responses to treatment combinations before and after sexual maturation. We found that most thyroid histomorphological phenotypes induced by perchlorate (follicle proliferation, reduced follicle area (adults only), colloid depletion, thyrocyte hypertrophy (subadults only)) were significantly ameliorated by exogenous iodide supplementation. In contrast, treatment with exogenous $T_4$ did not correct any of the thyroid-specific histopathologies induced by perchlorate. Whole-body thyroid hormone concentrations were not significantly affected by perchlorate exposure; however, supplementation with iodide and $T_4$ significantly increased $T_4$ concentrations. This study also revealed an increased erythrocyte area in the thyroid region of perchlorate-exposed adults, while lipid droplet number increased in perchlorate-exposed subadults. Increased erythrocyte area was ameliorated by both iodide and $T_4$, while neither supplement was able to correct lipid droplet number. Our finding on lipid droplets indicates that exposure to perchlorate in early development may have obesogenic effects.



## 1. Introduction

Perchlorate ($ClO_4^-$) is an endocrine disrupting compound that adversely affects the health of animals. The use of perchlorate as an oxidizer for a suite of consumer and industrial applications has introduced it into the environment, and it is now detectable in surface and ground waters, many agricultural products, and human breast milk and urine (De Groef et al., 2006; Urbansky, 2002). Perchlorate competitively inhibits the transport of iodide into thyroid follicles via blockade of the sodium-iodide symporter (NIS) of thyroid epithelial cells (thyrocytes)—one of the initial steps of *de novo* thyroid hormone biosynthesis. The competition of iodide with perchlorate at the NIS can ultimately reduce the downstream production of thyroid hormones, thyroxine ($T_4$) and triiodothyronine ($T_3$), which has widespread consequences for the organism because these hormones are integral to numerous physiological functions (Carr and Patiño, 2011; Wolff, 1998). Due to perchlorate's known mechanism of action in vertebrates (De la Vieja et al., 2000; Dohán et al., 2007; Wolff, 1998), empirical work on this contaminant has largely focused on effects on the thyroid and corresponding thyroid hormone levels.

Fish are particularly susceptible to exposure to perchlorate because it is a water soluble and highly stable compound, persisting for decades or longer in surface waters (Motzer, 2001; Urbansky, 2002). Uptake of perchlorate occurs primarily via the gills, intestines, and skin of fish (Theodorakis et al., 2006), although

* Corresponding authors.
*E-mail addresses:* Frank.vonHippel@nau.edu (F.A. von Hippel), Loren.Buck@nau.edu (C.L. Buck).

http://dx.doi.org/10.1016/j.ygcen.2016.10.014

it is rapidly excreted (Furin et al., 2013). Teleosts have a well-developed endocrine system that includes a hypothalamic-pituitary-thyroid (HPT) axis with molecular components that correspond closely to those of other vertebrates (Blanton and Specker, 2007). However, differences exist in the morphology of the thyroid of mammals compared to fish. In mammals, the thyroid is a discrete encapsulated gland while in fish it is a diffuse tissue composed of discrete follicles. Morphological responses of the thyroid in fish exposed to goitrogens (e.g., perchlorate) appear to be especially plastic, exhibiting profound structural and organizational changes. In teleosts, several robust and thyroid-specific biomarkers of histopathology have been established for perchlorate exposure including thyrocyte hypertrophy, proliferation of thyroid follicles, and colloid depletion (Bradford et al., 2006; Crane et al., 2005; Furin et al., 2015; Liu et al., 2006, 2008; Mukhi et al., 2005; Mukhi and Patiño, 2007; Patiño et al., 2003; Petersen et al., 2015; Schmidt et al., 2012).

The thyroid is the most vascularized mammalian endocrine gland and is comprised of a dynamic network of blood vessels (Gérard et al., 2008). Under low iodide conditions, the vascular bed can expand up to two-fold, and is accompanied by an increase in microvessel density and blood flow (Tseleni-Balafouta et al., 2006). Fish exposure to perchlorate increases blood vessel proliferation in the tissue surrounding the thyroid follicles (Furin et al., 2015; Mukhi et al., 2005; Patiño et al., 2003). Furin et al. (2015) found that angiogenesis in the thyroid region was a rapidly induced and permanent biomarker for perchlorate exposure; the transfer of perchlorate-exposed fish to clean water did not result in a reversal of this phenotype unless the fish were transferred to perchlorate-free water prior to 42 days post fertilization (dpf).

The evaluation of additional perchlorate-induced changes in other neighboring tissues that support the thyroid follicles of fish, such as adipose tissue, is relatively understudied. Thiouracil, another goitrogen, was shown to increase the size of adipose tissue pads on the thyroid of rats (*Rattus norvegicus*) (Smeds and Wollman, 1983; Wollman et al., 1982). Expression of transcripts for *AdipoR1* and *AdipoR2* genes in the adipose tissue of rats is regulated by thyroid hormones (Seifi et al., 2013). To date, we are not aware of any study that has documented changes in adiposity of the thyroid region following exposure to perchlorate.

Here, we selected threespine stickleback (*Gasterosteus aculeatus*; hereafter, 'stickleback'), a well-studied teleost, as our model organism for investigating the effects perchlorate on the thyroid. In the first objective of this study, we set out to identify the histological phenotypes within and surrounding the thyroid follicles, as well as changes in thyroid hormone content, that stem from perchlorate exposure. Secondly, we aimed to test the hypothesis that perchlorate has effects other than its action on thyroid hormone production. This hypothesis predicts that overcoming perchlorate's NIS blockade by supplementation of perchlorate-exposed stickleback with either exogenous iodide or $T_4$ would rescue expression of thyroid hormone-specific phenotypic effects but not those effects that are mediated via some other mechanism of perchlorate toxicity. Previous research on zebrafish (*Danio rerio*) demonstrated that the effects of perchlorate can be ameliorated by the addition of exogenous thyroid hormone (Manzon and Youson, 1997; Mukhi et al., 2007). If we find that exogenous iodide rescues perchlorate-induced alterations of the thyroid tissue in stickleback, it would provide strong evidence that perchlorate acts directly on the thyroid only by interfering with NIS. In contrast to iodide supplementation, treatment with exogenous $T_4$ bypasses the thyroid because thyroid hormones are produced downstream of NIS. If both exogenous iodide and $T_4$ rescue thyroid-specific phenotypes, this finding would provide compelling support that perchlorate acts on thyroid morphology through NIS, but the effects are mediated by $T_4$. If neither iodide nor $T_4$ rescue thyroid-specific phenotypes, then perchlorate acts directly on target tissues independent of its effect on NIS. The evaluation of these hypotheses is important to our understanding of the fundamental mode of action of perchlorate.

## 2. Methods

### *2.1. Field collections & housing conditions*

Adult anadromous stickleback were collected from Rabbit Slough, Alaska (61.5595°N, 149.2583°W) during the reproductive seasons (May-July) of 2010 and 2011. Eggs and sperm were removed from fish for *in vitro* mass crosses (each cross comprised of sperm from one euthanized male mixed with eggs stripped from six live females) using protocols described in Cresko et al. (2004) to produce approximately 2,250 embryos per treatment group, totaling approximately 800,000 embryos (Petersen et al., 2015). Embryos were incubated in 1 L Pyrex jars and monitored daily, and mortalities were removed. Following hatching, larvae were maintained in 113.6 L glass aquaria filled with Instant Ocean-fortified reverse osmosis water (6 ppt). Aquaria were housed within indoor animal facilities at the University of Alaska Anchorage (UAA). Each tank was aerated with a 15 cm diameter biofilter (Aquatic Ecosystems). Water quality measures including ammonia, iodide, nitrate, pH, temperature, salinity and specific conductivity were assessed weekly using a YSI multiprobe (Yellow Springs, OH) and API water testing kits (Mars Fishcare, Chalfont, PA). Tank water changes (partial volume) were performed as needed to maintain water quality parameters within an acceptable range. All animal protocols were approved by the UAA Institutional Animal Care and Use Committee (IRB reference #159870-1). Field work was conducted under Alaska Department of Fish & Game scientific collection permits (SF2010-029 and SF2011-025).

### *2.2. Experimental design and exposures*

Immediately upon fertilization, stickleback embryos were chronically exposed to sodium perchlorate ($NaClO_4$, Acros Organics, ⩾99% purity, Pittsburgh, PA) with and without iodide or $T_4$ supplements; all substances were dissolved in biologically conditioned reverse osmosis water. Control fish were exposed to biologically conditioned reverse osmosis water alone at the same salinity (6 ppt). All fish were exposed to a photoperiod and temperature (12.5–19.6 °C) that mimicked their natural environment for the duration of the experiment. The experiment was conducted in a factorial fashion, with three levels of perchlorate concentration (10, 30, 100 ppm) plus the control and three supplementation options (no supplement, iodide, $T_4$) for a total of twelve treatment conditions. Each treatment employed ten tank replicates and embryos were randomly assigned across each of the treatments (225 embryos per tank). Perchlorate concentration was monitored weekly using an Acorn Ion 6 meter (Oakton Instruments, Vernon Hills, IL) equipped with a perchlorate ISE electrode (Cole-Parmer, Vernon Hills, IL). For the supplemented treatment tanks, $T_4$ and iodide were maintained at 6 nM and 0.473 nM, respectively. Supplement concentrations were selected based on similar studies in fish, which added either exogenous $T_4$ (Lam et al., 2005; Mukhi et al., 2007) or exogenous iodide (Mustafa and MacKinnon, 1999) to tank water. $T_4$ in tanks was monitored biweekly using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Total $T_4$, MP Biomedicals, Santa Ana, CA). A commercially available iodine/iodide multitest (Seachem, Madison, GA) was used to monitor iodide concentrations in tanks on a biweekly basis. Perchlorate, iodide, and $T_4$ concentrations in treatment tanks were kept within a targeted range by the addition of water or the compound of interest. Fish were euthanized at ten time points: 84, 112, 140, 168, 196, 224, 252, 280, 308, or 336 dpf. On each collection day, whole fish were either snap-frozen in liquid nitrogen and stored at −80 °C until analyzed for hormones or euthanized with an overdose of pH neutral MS-222 and fixed in Bouin's solution and held at room temperature until analyzed for histology.

### 2.3. Histomorphological analyses

#### 2.3.1. Tissue preparation

Tissues were prepared for histological analyses according to Petersen et al. (2015). Briefly, each fish that had been fixed in Bouin's solution was bisected into anterior and posterior portions, which were then separately processed; here we present results from the anterior portion only (thyroid histology). Tissues were dehydrated, embedded in paraffin blocks, and sectioned using a microtome. The tissue sections (5 μm) were stained with hematoxylin and eosin and visualized at 100× and 400× magnifications under a Leica DM4500B microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DFC420C camera (Leica Microsystems, Wetzlar, Germany). Two sections of the thyroid region were imaged for each fish in order to control for artifacts introduced by the sectioning process. All measurements were made in duplicate or triplicate after calibration to the image scale bar using an Intuos touch pad (Wacom, Vancouver, WA) and Image J (NIH) software. Fish representing treatment groups containing 30 ppm perchlorate were excluded from the analysis of histomorphological parameters because of low sample size.

#### 2.3.2. Thyroid

The stickleback anterior region (snout to the pectoral fins) was sectioned horizontally to visualize the thyroid region. The branchial arteries were used as a reference point to target a common plane of sectioning for imaging of the thyroid follicles. Sections within this region were selected based on the number and quality of follicles (emphasis on the colloid). Methods similar to those described by Petersen et al. (2015) were utilized for quantifying thyroid-related histological parameters. At low (100×) magnification, the total number of thyroid follicles was counted. Follicle and colloid area were quantified for five randomly selected follicles per fish. To randomize follicle selection, each follicle was assigned a number and then a random number generator (random.org) was used to assign which of the follicles to measure. Thyrocyte height was measured at the anterior, posterior, left, and right positions on the image for each of the selected follicles.

#### 2.3.3. Blood vessels

Using the same image region from Section 2.3.2, but at 400× magnification, total erythrocyte area was quantified within a specific region bounded by skeletal muscle and branchial artery/cartilage as a proxy for vasculature in the thyroid region (Fig. 1). The ventral aortic vessel was not included in the measured region. Additionally, discrete blood vessels in the region surrounding the thyroid follicles were counted and the total number is reported. Vessels that did not contain erythrocytes were not included in the analysis. The mean blood vessel area was quantified for up to five haphazardly selected vessels per image (observed 0–7 vessels/fish).

#### 2.3.4. Lipid droplets

Lipid droplets were defined as unstained, circular or ovular structures (Genten et al., 2009; Lee et al., 2015; Schmidt et al., 2012) found in the thyroid region. The total number of lipid droplets was counted in the same 400× histological images used in Section 2.3.3. The mean area of an individual lipid droplet was quantified for up to five randomly selected lipid droplets per image (observed 0–9 droplets/fish).

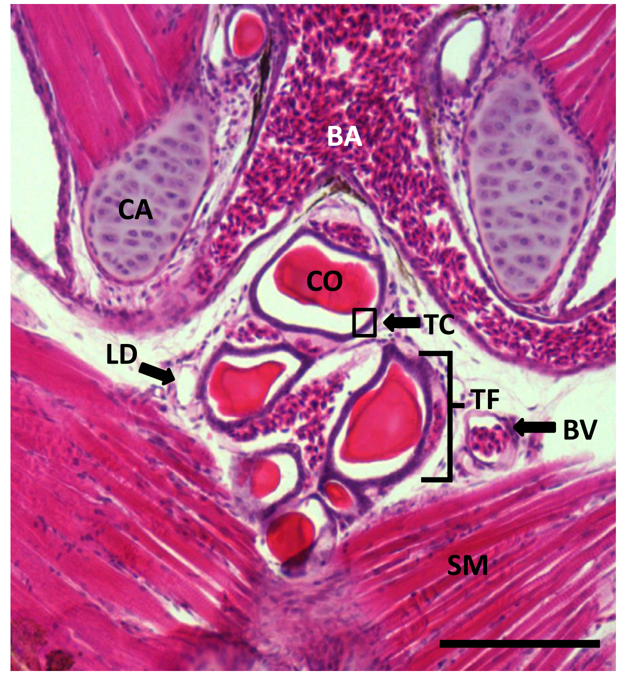


**Fig. 1.** Representative histological image from an adult stickleback depicting the targeted region for measuring parameters related to the thyroid follicles, blood vessels, and lipid droplets. LD = Lipid Droplet, BV = Blood Vessel, CA = Cartilage, CO = Colloid, SM = Skeletal Muscle, TC = Thyrocyte, TF = Thyroid Follicle. Scale bar is 100 μm.

### 2.4. Endocrinology

Methods described in Petersen et al. (2015) were used to prepare whole-body homogenates for thyroid hormone extraction. Briefly, whole-body homogenates were extracted twice using barbital. Extracts were resuspended in enzyme immunoassay buffer (0.1M PBS, 0.15M NaCl, 0.1% BSA, pH 7.4) and then assayed for $T_3$ and $T_4$ concentrations using commercially available ELISA kits (Total $T_3$ and $T_4$, MP Biomedicals, Santa Ana, CA). Manufacturer-provided standards were assayed in parallel with samples on 96-well plates. Both $T_3$ and $T_4$ assays were validated by tests of parallelism and standard addition. Samples were assayed in triplicate for $T_3$ and in duplicate for $T_4$. Absorbance was measured on a plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm and hormone concentrations were determined by extrapolation from a standard curve. $T_3$ standards ranged from 0–6 ng/ml while $T_4$ standards ranged from 0–100 ng/ml. The intra- and inter-assay variation for $T_3$ was 6.9% and 12.2%, respectively. The intra- and inter-assay variation for $T_4$ was 3.6% and 13.0%, respectively.

### 2.5. Statistical analyses

Data were initially tested for normality and homogeneity of variance using Levene's test and the Shapiro-Wilk test, respectively. When the assumption of normality was not met, a logarithmic transformation was applied to the data. For histological parameters, transformed data were analyzed using analysis of variance (ANOVA). Separate ANOVA tests were performed on subadult (84–196 dpf) and adult (300–350 dpf) age categories because many of the response variables were age-dependent. First, a one-way ANOVA was performed on treatment groups with no supplementation to determine whether there was a significant effect of perchlorate. Dunnett's T3 post hoc multiple comparisons of means was used to identify significant differences between the levels of perchlorate. Next, a two-way ANOVA with perchlorate concentration and iodide or $T_4$ supplementation/no supplementation as fixed factors was performed. The significance level of the interaction term was deemed the "rescue" effect for each of the supplementation groups. For endocrine data, limited samples sizes prevented separate ANOVA analyses for subadult and adult age categories. Thus, endocrine data (whole-body hormone concentrations standardized per unit tissue weight) from 84–336 dpf fish were combined into a single group per treatment. All statistical analyses were performed in SPSS (v. 25, IBM, Chicago, IL, USA). Differences were considered statistically significant at the $P < 0.05$ level.

## 3. Results

### 3.1. Iodide supplementation ameliorates most perchlorate-induced effects on thyroid histomorphology

#### 3.1.1. Subadults

Perchlorate significantly impacted the number of thyroid follicles in subadults in a dose dependent fashion ($F_{2,25} = 8.048$, $P = 0.002$, Fig. 2A). Subadults from the 100 ppm exposure group had significantly more follicles than fish from the 10 ppm group (Dunnett's T3, $P = 0.002$). Follicle area was not significantly altered by perchlorate in subadult fish (Fig. 2B). Perchlorate had a significant effect on colloid area ($F_{2,25} = 14.825$, $P < 0.001$, Fig. 2C); fish exposed to 100 ppm perchlorate had significantly less colloid than 10 ppm exposed fish (Dunnett's T3, $P = 0.001$). Thyrocyte height was also significantly affected by perchlorate exposure ($F_{2,25} = 9.450$, $P = 0.001$, Fig. 2D); 100 ppm perchlorate-exposed fish had larger thyrocytes than 10 ppm exposed fish (Dunnett's T3, $P = 0.001$). Iodide supplementation decreased mean follicle number ($F_{1,51} = 15.985$, $P < 0.001$, Fig. 2A), increased mean colloid area ($F_{2,51} = 12.156$, $P = 0.001$, Fig. 2C), and decreased mean thyrocyte height ($F_{1,51} = 36.187$, $P < 0.001$, Fig. 2D). The interaction between perchlorate and iodide was significant for follicle area ($F_{2,51} = 4.861$, $P = 0.012$), colloid area ($F_{2,51} = 8.253$, $P = 0.001$), and thyrocyte height ($F_{2,51} = 17.305$, $P < 0.001$). $T_4$ supplementation decreased mean follicle number ($F_{1,63} = 5.561$, $P = 0.021$, Fig. 2A), decreased mean follicle area ($F_{1,63} = 9.736$, $P = 0.003$, Fig. 2B), and decreased mean thyrocyte height ($F_{1,63} = 12.984$, $P = 0.001$, Fig. 2D). The interaction between perchlorate and $T_4$ was only significant for follicle area ($F_{2,63} = 3.393$, $P = 0.040$).

#### 3.1.2. Adults

Perchlorate also had a significant effect on the number of thyroid follicles in adults ($F_{2,19} = 13.036$, $P < 0.001$, Fig. 2A; Fig. 3). The lifetime exposure to 100 ppm perchlorate significantly increased the mean number of follicles compared to control (Dunnett's T3, $P = 0.004$) and 10 ppm fish (Dunnett's T3, $P = 0.011$). Perchlorate significantly decreased both follicle area ($F_{2,19} = 37.003$, $P < 0.001$, Fig. 2B) and colloid area ($F_{2,19} = 31.129$, $P < 0.001$, Fig. 2C); comparisons of mean follicle and colloid areas were

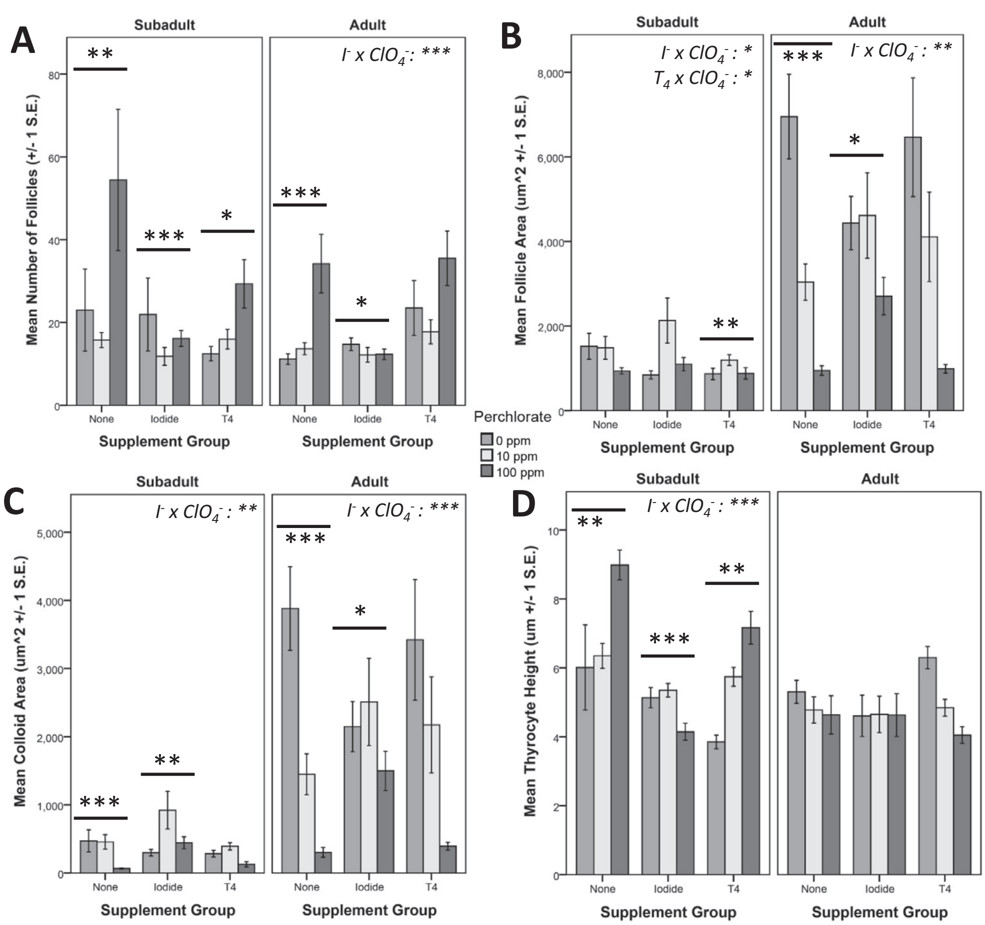


**Fig. 2.** A–D Iodide supplementation ameliorates most perchlorate-induced effects on thyroid histomorphology. Mean follicle number (A), follicle area (B), colloid area (C), and thyrocyte height (D) for subadult and adult stickleback exposed to perchlorate alone or co-exposed to perchlorate and iodide or $T_4$. Asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) denote significant main effects and/or interactions identified in one- and two-way ANOVAs.

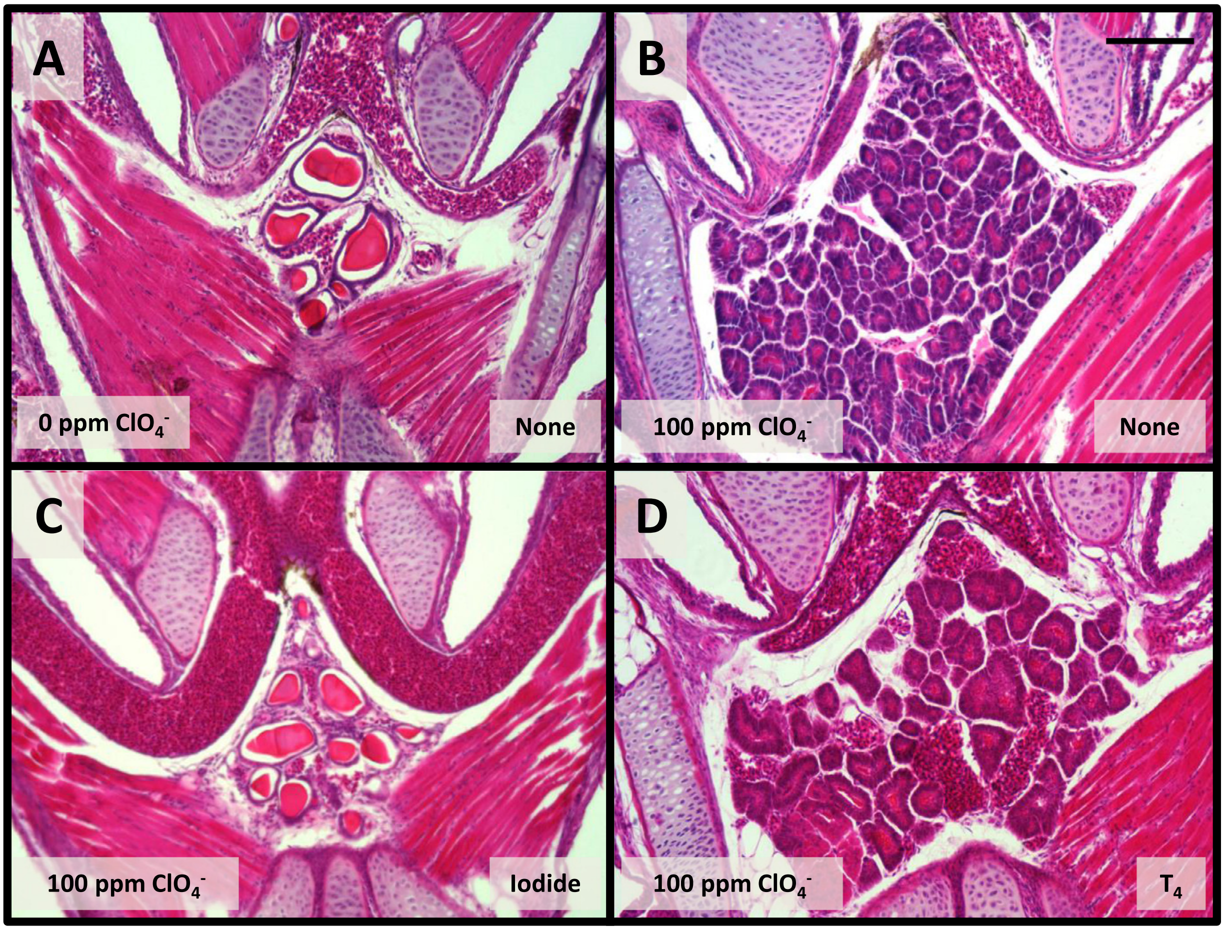


**Fig. 3.** A–D: Iodide supplementation ameliorates perchlorate-induced thyroid histopathologies while $T_4$ does not. Representative histological images for targeted thyroid-specific phenotypes in each of the following treatments: 0 ppm perchlorate + no supplement (A), 100 ppm perchlorate + no supplement (B), 100 ppm perchlorate + iodide (C), 100 ppm perchlorate + $T_4$ (D). Concentration of perchlorate and supplement type are noted on the lower left and lower right of each image panel, respectively. Scale bar is 100 μm.

significant at all levels of perchlorate (Dunnett's T3). Perchlorate did not significantly affect mean thyrocyte height. Iodide supplementation decreased mean follicle number ($F_{1,41}$ = 5.707, P = 0.022, Fig. 2A), increased mean follicle area ($F_{1,41}$ = 4.185, P = 0.047, Fig. 2B), and increased mean colloid area ($F_{1,41}$ = 7.372, P = 0.010, Fig. 2C). The interaction between perchlorate and iodide was significant for follicle number ($F_{2,41}$ = 10.595, P < 0.001), follicle area ($F_{2,41}$ = 7.599, P = 0.002), and colloid area ($F_{2,41}$ = 10.909, P < 0.001). $T_4$ supplementation did not significantly alter any of the thyroid histomorphological parameters, and the interaction between perchlorate and $T_4$ was also not significant for these parameters.

### 3.2. Perchlorate increased erythrocyte area in adults, but not subadults

In subadults, perchlorate did not have a significant effect on vessel number, vessel area, or total erythrocyte area (Fig. 4). Vessel number of subadults showed a significant interaction between perchlorate and iodide supplementation ($F_{2,95}$ = 6.163, P = 0.003, Fig. 4A). Iodide supplementation decreased vessel area in subadults ($F_{1,95}$ = 7.257, P = 0.008, Fig. 4B), but did not significantly affect the other parameters. $T_4$ supplementation did not show a main effect on any of the blood parameters for subadults, but there was a significant interaction between perchlorate and $T_4$ on total erythrocyte area ($F_{2,102}$ = 7.248, P = 0.001). In adults, perchlorate significantly influenced total erythrocyte area ($F_{2,18}$ = 4.790, P = 0.021, Fig. 4C); fish exposed to 10 ppm perchlorate exhibited greater total erythrocyte area than control fish (Dunnett's T3, P = 0.009). Although neither iodide nor $T_4$ supplementation showed significant main effects, the interactions between perchlorate and iodide ($F_{2,37}$ = 4.848, P = 0.013) and perchlorate and $T_4$ ($F_{2,33}$ = 6.838, P = 0.003) were both significant for total erythrocyte area in adults.

### 3.3. Perchlorate increased lipid droplet number in subadults, but not adults

Perchlorate exerted a significant effect on the number of lipid droplets in subadults, with the greatest number seen in 10 ppm exposed fish ($F_{2,25}$ = 3.530, P = 0.045, Fig. 5A); such an effect was not significant in adults, though some individual perchlorate exposed adult fish appeared to show a similar effect (Fig. 6). The main effects of iodide and $T_4$ on lipid droplet number were non-significant in both subadults and adults. Perchlorate, iodide, and $T_4$ did not significantly alter individual lipid droplet area (in contrast to lipid droplet number) in subadults or adults. However, results showed a significant interaction between perchlorate and iodide for subadult individual lipid droplet area ($F_{2,51}$ = 3.870, P = 0.027, Fig. 5B).

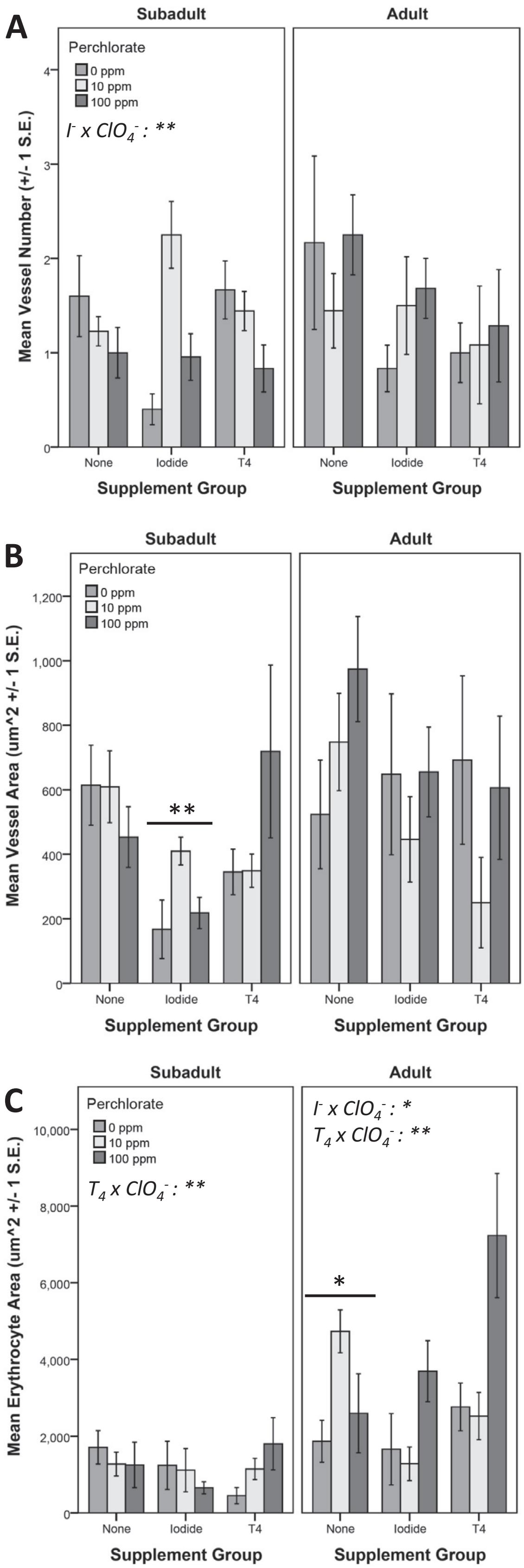


**Fig. 4.** A–C: Perchlorate increased erythrocyte area in adults, but not subadults. Mean vessel number (A), vessel cross-sectional area (B), and total erythrocyte area (C) for subadult and adult stickleback exposed to perchlorate alone or co-exposed to perchlorate and iodide or $T_4$. Asterisks ($^*P < 0.05$, $^{**}P < 0.01$) denote significant main effects and/or interactions identified in one- and two-way ANOVAs.

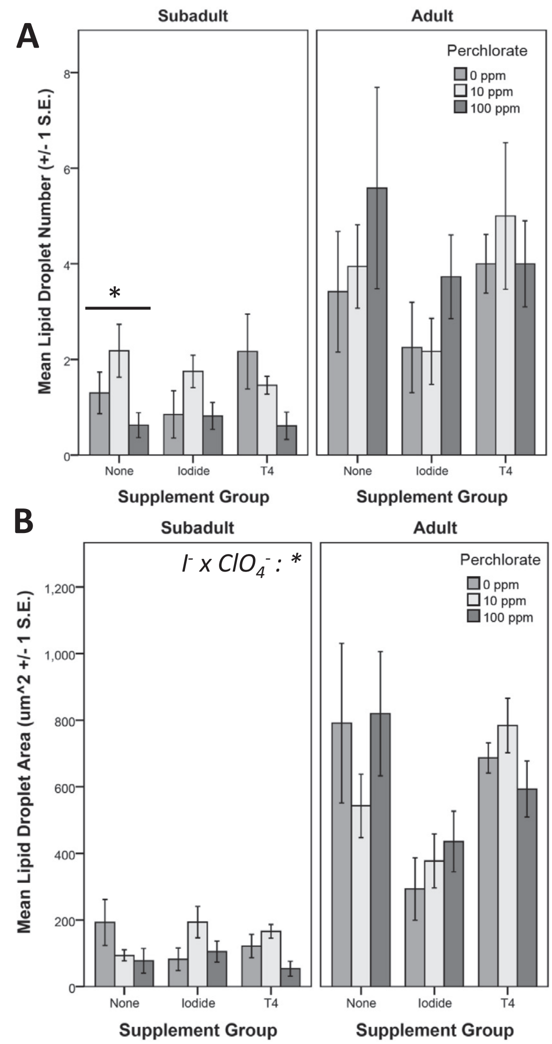


**Fig. 5.** A–B: Perchlorate increased lipid droplet number in subadults, but not adults. Mean lipid droplet number (A) and individual droplet area (B) for subadult and adult stickleback exposed to perchlorate alone or co-exposed to perchlorate and iodide or $T_4$. Asterisks ($^*P < 0.05$) denote significant main effects and/or interactions identified in one- and two-way ANOVAs.

### *3.4. Supplementation with iodide and $T_4$ increased whole-body thyroid hormone concentrations*

Perchlorate did not have a significant effect on whole-body $T_3$ or $T_4$ concentrations. Supplementation with iodide significantly increased $T_4$ concentrations ($F_{1,55} = 11.366$, $P = 0.001$, Fig. 7A) but not $T_3$ (Fig. 7B). $T_4$ supplementation significantly increased both $T_4$ ($F_{1,52} = 37.069$, $P < 0.001$, Fig. 7A) and $T_3$ concentrations ($F_{1,45} = 13.489$, $P = 0.001$, Fig. 7B). The interaction between perchlorate and $T_4$ supplementation was significant for whole-body $T_3$ concentration ($F_{3,45} = 2.972$, $P = 0.042$, Fig. 7B). Co-exposure to perchlorate (particularly, 10 ppm) and $T_4$ tended to increase whole-body $T_3$ concentration.

## 4. Discussion

### *4.1. Perchlorate modifies thyroid architecture without changing whole-body thyroid hormone content*

We found that perchlorate induces the proliferation of smaller, more numerous thyroid follicles in both subadult and adult stickleback. These findings are consistent with earlier studies

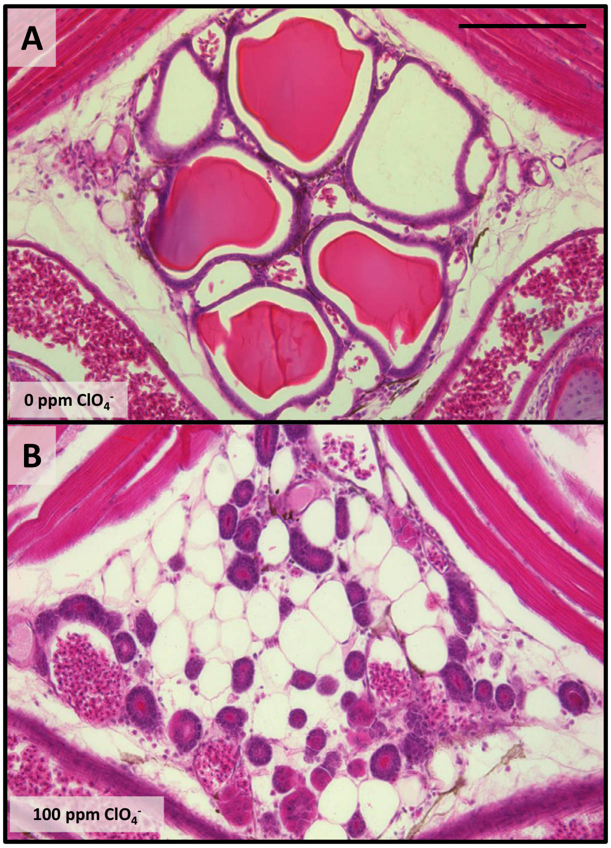


**Fig. 6.** A–B: Representative histological images of lipid droplet accumulation surrounding the thyroid follicles in a control adult (A) compared to a perchlorate-treated adult (B). Neither supplementation with iodide nor $T_4$ were able to rescue this perchlorate-induced phenotype. Scale bar is 125 μm.

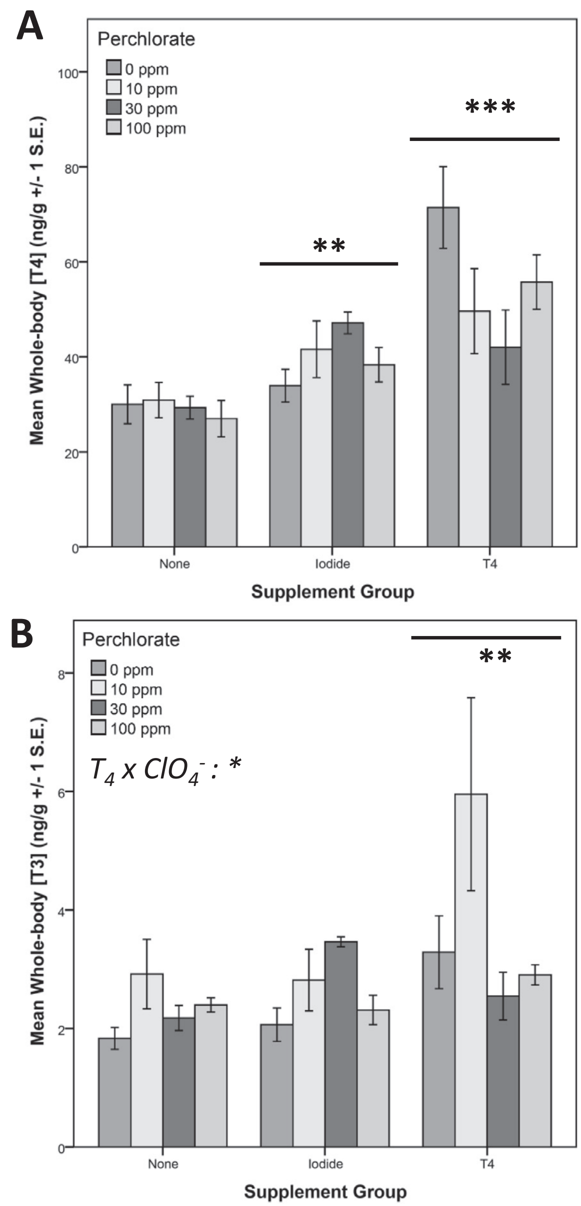


**Fig. 7.** A–B: Supplementation with iodide and $T_4$ increased whole-body thyroid hormone concentrations. Mean whole-body $T_4$ (A) and $T_3$ (B) concentrations in stickleback following exposure to perchlorate alone or co-exposure to perchlorate and iodide or $T_4$. Asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) denote significant main effects and/or interactions identified in one- and two-way ANOVAs.

conducted in stickleback (Furin et al., 2015; Petersen et al., 2015) and zebrafish (Patiño et al., 2003; Schmidt et al., 2012). This modification in thyroid architecture likely increases the surface area to volume ratio of this tissue, thereby allowing for the more effective import of iodide via NIS (Petersen et al., 2015) and subsequent secretion of $T_4$ from the follicle. Follicle area was significantly decreased by perchlorate exposure in adults, while no effect was observed in subadults. However, follicle area was marginally reduced in subadult stickleback at high dose (100 ppm) perchlorate treatment. A larger sample size would likely reveal that there are indeed significantly smaller follicles in subadults exposed to perchlorate, as seen in adults. Both adult and subadult stickleback chronically exposed to perchlorate decreased their mean colloid area, a phenotype that was also observed following perchlorate exposure in zebrafish (Mukhi and Patiño, 2007; Patiño et al., 2003) and African clawed frog (*Xenopus laevis*) (Hu et al., 2006). Our observed decrease in colloid area followed a classical dose-dependent response in adults while the effect was only detected at the 100 ppm perchlorate level in subadults. Decreased colloid area is likely a result of depleting stores of thyroglobulin, a precursor protein of thyroid hormone, and the primary protein component of colloid. We suspect that perchlorate is outcompeting iodide at NIS, leading to the depletion of colloid of perchlorate-exposed stickleback.

Our histological findings in the thyroid tissue were not accompanied by decreases in whole-body thyroid hormone concentrations. Although one might expect perchlorate to dampen whole-body thyroid hormone concentrations, it is likely that other features revealed through histology of the thyroid (overall infrastructure, depletion of colloid) compensated to provide constant levels of these hormones. The stability of whole-body thyroid hormone concentrations in perchlorate-exposed individuals is consistent with previous work in stickleback (Furin et al., 2015; Gardell et al., 2015; Petersen et al., 2015), zebrafish (Mukhi et al., 2005), and fathead minnow (*Pimephales promelas*) (Crane et al., 2005). However, it is possible that perchlorate-exposed stickleback experience hypothyroidism and that it could be detected with another proxy (e.g., circulating thyroid hormone levels). Whole-body concentrations are generally less informative than plasma levels because the majority of thyroid hormone is stored as the prohormone, $T_4$ (Eales and Brown, 1993). In contrast, $T_3$ whole-body and circulating levels should more closely mirror each other because the majority of $T_4$ deiodination occurs at peripheral tissues (Blanton and Specker, 2007) and $T_3$ is rapidly used for physiologic functions (Van der Geyten et al., 2005). Our results of a lack of effect on whole-body concentrations of $T_3$ reinforce the notion that thyroid histological measures (e.g., follicle number, colloid area) are more sensitive biomarkers for perchlorate exposure in stickleback than are whole-body thyroid hormone levels.

Hypertrophy of thyrocytes is a notable perchlorate-induced phenotype that could serve as an index of hypothyroidism. Thyroid stimulating hormone (TSH) induces thyrocyte hypertrophy in thyroid cell cultures (Kimura et al., 2001). The pituitary-derived TSH is a major signaling molecule responsible for driving feedback to various components of the HPT axis, particularly the thyroid follicles (Blanton and Specker, 2007). Thyrocyte hypertrophy in response to perchlorate exposure was detected only in subadult stickleback in this study, suggesting that the extended exposure in adults allowed for compensatory recovery from this phenotype. This difference may also be explained by the fact that younger fish have a higher demand for thyroid hormone synthesis because of a variety of physiological functions (e.g., growth, development, metamorphosis) (Carr and Patiño, 2011), which could result in TSH-induced hypertrophy of thyrocytes in perchlorate-exposed subadults. Thyrocyte hypertrophy appears to be a robust biomarker of perchlorate exposure in a broad spectrum of vertebrates including zebrafish (Liu et al., 2006, 2008; Mukhi et al., 2005, 2007; Patiño et al., 2003; Sharma and Patiño, 2013), eastern mosquitofish (*Gambusia holbrooki*) (Bradford et al., 2005; Park et al., 2006), fathead minnow (Crane et al., 2005), African clawed frog (Hu et al., 2006), and rat (York et al., 2004).

### *4.2. Iodide supplementation ameliorates most perchlorate-induced thyroid histopathologies*

We found that exogenous iodide supplementation effectively ameliorated perchlorate-induced thyroid histological effects in subadult and adult stickleback. With only one exception, exogenous iodide was able to at least partially rescue the phenotype in every case where we detected a significant effect of perchlorate (Fig. 3). For some of the thyroid phenotypes (e.g., thyrocyte height), we observed a similar, albeit non-significant trend when supplementing with exogenous $T_4$. This finding is not surprising because stickleback appear to maintain normal thyroid hormone content when exposed to perchlorate (Gardell et al., 2015; Petersen et al., 2015), which suggests that $T_4$ is not limiting, at least at the perchlorate concentrations we tested. Collectively, these results suggest that iodide limitation (supply-based) could explain most of perchlorate's effects on thyroid histomorphology. Because a single concentration of iodide was used in this study, we are unable to determine if the histopathological phenotypes observed in the thyroid were solely due to iodide limitation. The much stronger amelioration of perchlorate-induced phenotypes with iodide supplementation compared to $T_4$ supplementation may indicate that exogenous iodide is preferred by fish for *de novo* thyroid hormone biosynthesis. However, this may not be true for all fish species and requires further investigation. For example, Manzon and Youson (1997) found that treatment with exogenous $T_4$ ameliorated perchlorate-induced decreases in the thyroid hormones of sea lamprey (*Petromyzon marinus*) larvae. Iodine supplementation to drinking water has been evaluated as a solution for preventing goitrogenic effects of perchlorate in humans (Lewandowski et al., 2015). However, an excess of iodide has been shown to induce toxic effects including inflammation, oxidative stress, and even autoimmune disease (Luo et al., 2014).

### *4.3. Stickleback can use exogenous iodide for thyroid hormone biosynthesis in a high perchlorate environment*

Our endocrine results indicate that stickleback can effectively use exogenous iodide for the *de novo* biosynthesis of thyroid hormones, even in the presence of a high perchlorate environment. This result is somewhat surprising given that perchlorate has a higher affinity for NIS than iodide and the transport of perchlorate is electroneutral (De la Vieja et al., 2000; Dohán et al., 2007). However, it is possible that an excess of exogenous iodide may overwhelm and outcompete perchlorate (up to 100 ppm) at the thyroidal NIS. We observed a notable increase in whole-body $T_4$ following supplementation with iodide while $T_3$ remained unchanged. In contrast, Mustafa and MacKinnon (1999) found that salmonids treated with iodized feed and iodinated water had higher levels of $T_4$ and $T_3$ in the plasma. This discrepancy may be attributed to the fact that our study measured whole-body thyroid hormone concentrations. Another study by Van der Geyten et al. (2005) found that the half-life of exogenous $T_3$ in the plasma of Nile tilapia (*Oreochromis niloticus*) was only $\sim$1.25 h. We hypothesize that the higher stability of $T_4$ compared to that of $T_3$ may have contributed to the sole increase in $T_4$ concentration observed in stickleback supplemented with iodide.

In our study, whole-body $T_4$ concentration of stickleback increased following exposure to exogenous $T_4$. Although this finding was not surprising, it provides empirical evidence that stickleback are able to take up $T_4$ from the water. Our data also suggest that stickleback are able to deiodinate exogenous $T_4$ to the more physiologically-relevant form, $T_3$; a concurrent increase was observed in both thyroid hormones following treatment. However, this effect was largely driven by a few individuals in the 10 ppm perchlorate + $T_4$ supplement group with high concentrations of whole-body $T_3$. Manzon et al. (1998) found that treatment of larval sea lamprey with exogenous $T_4$ resulted in increased serum $T_4$ levels while serum concentrations of $T_3$ remained constant. In contrast, tilapia that were fed $T_4$-enriched food did not increase either $T_4$ or $T_3$ plasma levels; however, $T_4$ concentration in the liver increased and was accompanied by greater deiodinase activity (Van der Geyten et al., 2005). It is unclear as to how stickleback are able to effectively convert exogenous $T_4$ to $T_3$, but not $T_4$ that had been synthesized from exogenous iodide. Future studies are needed to investigate this question through evaluation of deiodinase activity at specific peripheral target tissues.

### *4.4. Perchlorate increases erythrocyte area in adult stickleback, and this phenotype is ameliorated by both iodide and $T_4$ supplementation*

Our study found that the total erythrocyte area surrounding the thyroid follicles increased in adult stickleback when chronically exposed to perchlorate. This effect, however, was significant only at low dose (10 ppm) perchlorate. Non-monotonic dose responses, such as the one reported here, are commonly observed in studies on endocrine disruptors (Vandenberg et al., 2012). The induction of angiogenesis by perchlorate has been observed previously in stickleback (Furin et al., 2015), zebrafish (Mukhi et al., 2005; Patiño et al., 2003), and rats (Rodriguez et al., 1991). Furin et al. (2015) found that increased angiogenesis is a robust and irreversible phenotype observed in stickleback transferred from clean water to 100 ppm perchlorate, unless they are transferred back to clean water prior to 42 dpf. Increased vascularization around the thyroid follicles would be expected under perchlorate conditions because iodide is delivered to the tissue via the bloodstream, and thus increased vascularization may mechanistically increase transport of iodide at the thyroidal NIS. Gérard et al. (2008) found a suite of proangiogenic factors and their receptors were induced under iodine deficient conditions in mice (*Mus musculus*). We suggest that vascular endothelial growth factor (VEGF) would be an excellent molecular target for follow-up studies on angiogenesis in perchlorate-exposed stickleback. As a regulator of angiogenesis, VEGF has implications for pathological physiologies such as tumor formation (Ferrara et al., 2003; Ramsden, 2000). A mechanistic understanding of VEGF's role in perchlorate-induced angiogenesis may also identify new connections between perchlorate and tumorigenesis.

We found that perchlorate-induced vascularization of the thyroid region was ameliorated by both exogenous iodide and $T_4$. This is the only histological parameter affected by perchlorate that was ameliorated by exogenous $T_4$. We also found that iodide significantly decreased blood vessel area in subadult stickleback compared to groups not exposed to supplemental iodide. This finding is not surprising because iodide has been shown to suppress thyroid function and blood flow (Arntzenius et al., 1991). Iodide is delivered to the thyroid region via the bloodstream, so one might expect that smaller vessels surrounding the thyroid, and thus decreased blood flow, would occur if iodide was in excess. If plasma iodide exceeds a critical threshold, a transient blockade of organification occurs in the thyroid (Wolff-Chaikoff effect) and serves to prevent damage from free radicals (Wolff and Chaikoff, 1948; Wolff et al., 1949). The Wolff-Chaikoff effect is largely disabled by vasoconstriction and the downregulation of NIS (Eng et al., 1999). Two DNA binding proteins located on the NIS flanking and promoter regions were identified in rat thyroid cells and were found to be sensitive to iodide and to have the capability of modulating NIS promoter activity (Suzuki et al., 2010).

### 4.5. Perchlorate as a putative obesogen in subadult stickleback

Lipid droplet number was found to increase in subadult stickleback exposed to low dose (10 ppm) perchlorate, a phenotype that was not ameliorated by exogenous iodide or $T_4$ (Fig. 5). This is the first study that we know of that has demonstrated increased adiposity surrounding the thyroid follicles of fish following perchlorate exposure, or due to any contaminant. Meador et al. (2011) found that Chinook salmon (*Oncorhynchus tshawytscha*) exposed to tributyltin, a well-studied endocrine disruptor, exhibited increased lipid-associated plasma parameters, while some parameters only increased at the low dose treatment. Our observation that lipid droplets increased only in perchlorate-treated subadult stickleback suggests that lipid deposits are mobilized for metabolism in reproductively mature fish, which may explain the absence of this phenotype in adults.

Because of perchlorate's adipose-inducing effects, this contaminant could be acting as an obesogen. The obesogen hypothesis postulates that environmental contaminants contribute to the contemporary obesity epidemic observed in human populations (Decherf and Demeneix, 2011). Many environmental contaminants (e.g., tributyltin) are being touted as obesogens because of their influence on adiposity in various tissues. A recent study in zebrafish documented increased hepatic triglyceride levels coupled with altered transcriptional regulation of lipid metabolism genes in the liver and brain following exposure to tributylin (Lyssimachou et al., 2015). Another study demonstrated increased intrathyroidal adipose tissue and lipid droplets in the thyroid gland of obese human patients and mice (Lee et al., 2015). Our results highlight the need for additional studies to better understand perchlorate's role as a putative obesogen. For example, the use of whole-body or tissue-specific lipid content measurements would be good candidates for initial screening of obesogenic effects in stickleback and other model organisms.

## 5. Conclusions

Our results suggest that perchlorate-induced thyroid histopathologies in stickleback are likely driven by the interference of perchlorate at the thyroidal NIS. Our work provides empirical support for the hypothesis that perchlorate's mode of action for altering the morphology of the thyroid follicles and surrounding tissues (but not lipid droplet number) is largely caused by a limitation of iodide. Further work is necessary to understand the specific biochemical pathways and signaling molecules responsible for producing thyroid histopathologies. Additionally, this work has revealed an interesting novel phenotype of perchlorate exposure in stickleback, the induction of adipose tissue in individuals that have not yet reached reproductive maturity. Our work highlights a new and exciting avenue of investigation for perchlorate toxicity—evaluating perchlorate's putative role as an obesogen.


## Acknowledgments

Richard Bernhardt and Lauren Smayda are thanked for performing fish collections in the field, animal husbandry, and experimental exposures. Funding was provided by NIH Grant number 1RO1ES017039.


## References


Arntzenius, A.B., Smit, L.J., Schipper, J., Heide, D.V.D., Meinders, A.E., 1991. Inverse relation between iodine intake and thyroid blood flow: color doppler flow imaging in euthyroid humans. J. Clin. Endocrinol. Metab. 73, 1051–1055.

Blanton, M.L., Specker, J.L., 2007. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. Crit. Rev. Toxicol. 37, 97–115.

Bradford, C.M., Park, J.W., Rinchard, J., Anderson, T.A., Liu, F., Theodorakis, C.W., 2006. Uptake and elimination of perchlorate in eastern mosquitofish. Chemosphere 63, 1591–1597.

Bradford, C.M., Rinchard, J., Carr, J.A., Theodorakis, C., 2005. Perchlorate affects thyroid function in eastern mosquitofish (*Gambusia holbrooki*) at environmentally relevant concentrations. Environ. Sci. Technol. 39, 5190–5195.

Carr, J.A., Patiño, R., 2011. The hypothalamus–pituitary–thyroid axis in teleosts and amphibians: endocrine disruption and its consequences to natural populations. Gen. Comp. Endocrinol. 170, 299–312.

Crane, H.M., Pickford, D.B., Hutchinson, T.H., Brown, J.A., 2005. Effects of ammonium perchlorate on thyroid function in developing fathead minnows, *Pimephales promelas*. Environ. Health Perspect. 113, 396–401.

Cresko, W.A., Amores, A., Wilson, C., Murphy, J., Currey, M., Phillips, P., Bell, M.A., Kimmel, C.B., Postlethwait, J.H., 2004. Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. Proc. Natl. Acad. Sci. U.S.A. 101, 6050–6055.

De Groef, B., Decallonne, B.R., Van der Geyten, S., Darras, V.M., Bouillon, R., 2006. Perchlorate versus other environmental sodium/iodide symporter inhibitors: potential thyroid-related health effects. Eur. J. Endocrinol. 155, 17–25.

De la Vieja, A., Dohan, O., Levy, O., Carrasco, N., 2000. Molecular analysis of the sodium/iodide symporter: impact on thyroid and extrathyroid pathophysiology. Physiol. Rev. 80, 1083–1105.

Decherf, S., Demeneix, B.A., 2011. The obesogen hypothesis: A shift of focus from the periphery to the hypothalamus. J. Toxicol. Environ. Health B, Crit. Rev. 14, 423–448.

Dohán, O., Portulano, C., Basquin, C., Reyna-Neyra, A., Amzel, L.M., Carrasco, N., 2007. The $Na^+/I^-$ symporter (NIS) mediates electroneutral active transport of the environmental pollutant perchlorate. Proc. Natl. Acad. Sci. 104, 20250–20255.

Eales, J.G., Brown, S.B., 1993. Measurement and regulation of thyroidal status in teleost fish. Rev. Fish Biol. Fisheries 3, 299–347.

Eng, P.H.K., Cardona, G.R., Fang, S.L., Previti, M., Alex, S., Carrasco, N., Chin, W.W., Braverman, L.E., 1999. Escape from the acute Wolff-Chaikoff effect is associated with a decrease in thyroid sodium/iodide symporter messenger ribonucleic acid and protein. Endocrinology 140, 3404–3410.

Ferrara, N., Gerber, H.P., LeCouter, J., 2003. The biology of VEGF and its receptors. Nat. Med. 9, 669–676.

Furin, C.G., von Hippel, F.A., Hagedorn, B., O'Hara, T.M., 2013. Perchlorate trophic transfer increases tissue concentrations above ambient water exposure alone in a predatory fish. J. Toxicol. Environ. Health A 76, 1072–1084.

Furin, C.G., von Hippel, F.A., Postlethwait, J.H., Buck, C.L., Cresko, W.A., O'Hara, T.M., 2015. Developmental timing of sodium perchlorate exposure alters angiogenesis, thyroid follicle proliferation and sexual maturation in stickleback. Gen. Comp. Endocrinol. 219, 24–35.

Gardell, A.M., Dillon, D.M., Smayda, L.C., von Hippel, F.A., Cresko, W.A., Postlethwait, J.H., Buck, C.L., 2015. Perchlorate exposure does not modulate temporal variation of whole-body thyroid and androgen hormone content in threespine stickleback. Gen. Comp. Endocrinol. 219, 45–52.

Genten, F., Terwinghe, E., Danguy, A., 2009. Atlas of Fish Histology. CRC Press.

Gérard, A.C., Poncin, S., Caetano, B., Sonveaux, P., Audinot, J.N., Feron, O., Colin, I.M., Soncin, F., 2008. Iodine deficiency induces a thyroid stimulating hormone-independent early phase of microvascular reshaping in the thyroid. Am. J. Pathol. 172, 748–760.

Hu, F., Sharma, B., Mukhi, S., Patiño, R., Carr, J.A., 2006. The colloidal thyroxine ($T_4$) ring as a novel biomarker of perchlorate exposure in the African clawed frog *Xenopus laevis*. Toxicol. Sci. 93, 268–277.

Kimura, T., Van Keymeulen, A., Golstein, J., Fusco, A., Dumont, J.E., Roger, P.P., 2001. Regulation of thyroid cell proliferation by TSH and other factors: a critical evaluation of in vitro models. Endocr. Rev. 22, 631–656.

Lam, S.H., Sin, Y.M., Gong, Z., Lam, T.J., 2005. Effects of thyroid hormone on the development of immune system in zebrafish. Gen. Comp. Endocrinol. 142, 325–335.

Lee, M.H., Lee, J.U., Joung, K.H., Kim, Y.K., Ryu, M.J., Lee, S.E., Kim, S.J., Chung, H.K., Choi, M.J., Chang, J.Y., Lee, S.H., Kweon, G.R., Kim, H.J., Kim, K.S., Kim, S.M., Jo, Y. S., Park, J., Cheng, S.Y., Shong, M., 2015. Thyroid dysfunction associated with follicular cell steatosis in obese male mice and humans. Endocrinology 156, 1181–1193.

Lewandowski, T.A., Peterson, M.K., Charnley, G., 2015. Iodine supplementation and drinking-water perchlorate mitigation. Food Chem. Toxicol. 80, 261–270.

Liu, F.J., Cobb, G.P., Anderson, T.A., Cheng, Q.Q., Theodorakis, C.W., 2006. Uptake, accumulation and depuration of sodium perchlorate and sodium arsenate in zebrafish (*Danio rerio*). Chemosphere 65, 1679–1689.

Liu, F., Gentles, A., Theodorakis, C.W., 2008. Arsenate and perchlorate toxicity, growth effects, and thyroid histopathology in hypothyroid zebrafish *Danio rerio*. Chemosphere 71, 1369–1376.

Luo, Y., Kawashima, A., Ishido, Y., Yoshihara, A., Oda, K., Hiroi, N., Ito, T., Ishii, N., Suzuki, K., 2014. Iodine excess as an environmental risk factor for autoimmune thyroid disease. Int. J. Mol. Sci. 15, 12895.

Lyssimachou, A., Santos, J.G., Andre, A., Soares, J., Lima, D., Guimaraes, L., Almeida, C. M., Teixeira, C., Castro, L.F., Santos, M.M., 2015. The mammalian "obesogen" tributyltin targets hepatic triglyceride accumulation and the transcriptional regulation of lipid metabolism in the liver and brain of zebrafish. PLoS One 10, e0143911.

Manzon, R.G., Eales, J.G., Youson, J.H., 1998. Blocking of $KClO_4^-$ induced metamorphosis in premetamorphic sea lampreys by exogenous thyroid hormones (TH); Effects of $KClO_4^-$ and TH on serum TH concentrations and intestinal thyroxine outer-ring deiodination. Gen. Comp. Endocrinol. 112, 54–62.

Manzon, R.G., Youson, J.H., 1997. The effects of exogenous thyroxine ($T_4$) or triiodothyronine ($T_3$), in the presence and absence of potassium perchlorate, on the incidence of metamorphosis and on serum $T_4$ and $T_3$ concentrations in larval sea lampreys (*Petromyzon marinus* L.). Gen. Comp. Endocrinol. 106, 211–220.

Meador, J.P., Sommers, F.C., Cooper, K.A., Yanagida, G., 2011. Tributyltin and the obesogen metabolic syndrome in a salmonid. Environ. Res. 111, 50–56.

Motzer, W.E., 2001. Perchlorate: problems, detection, and solutions. Environ. Forensics 2, 301–311.

Mukhi, S., Carr, J.A., Anderson, T.A., Patiño, R., 2005. Novel biomarkers of perchlorate exposure in zebrafish. Environ. Toxicol. Chem. 24, 1107–1115.

Mukhi, S., Patiño, R., 2007. Effects of prolonged exposure to perchlorate on thyroid and reproductive function in zebrafish. Toxicol. Sci. 96, 246–254.

Mukhi, S., Torres, L., Patiño, R., 2007. Effects of larval–juvenile treatment with perchlorate and co-treatment with thyroxine on zebrafish sex ratios. Gen. Comp. Endocrinol. 150, 486–494.

Mustafa, A., MacKinnon, B.M., 1999. Atlantic salmon, *Salmo salar* L., and Arctic char, *Salvelinus alpinus* (L.): comparative correlation between iodine-iodide supplementation, thyroid hormone levels, plasma cortisol levels, and infection intensity with the sea louse *Caligus elongatus*. Can. J. Zool. 77, 1092–1101.

Park, J.W., Rinchard, J., Liu, F., Anderson, T.A., Kendall, R.J., Theodorakis, C.W., 2006. The thyroid endocrine disruptor perchlorate affects reproduction, growth, and survival of mosquitofish. Ecotoxicol. Environ. Saf. 63, 343–352.

Patiño, R., Wainscott, M.R., Cruz-Li, E.I., Balakrishnan, S., McMurry, C., Blazer, V.S., Anderson, T.A., 2003. Effects of ammonium perchlorate on the reproductive performance and thyroid follicle histology of zebrafish. Environ. Toxicol. Chem. 22, 1115–1121.

Petersen, A.M., Dillon, D., Bernhardt, R.A., Torunsky, R., Postlethwait, J.H., von Hippel, F.A., Buck, C.L., Cresko, W.A., 2015. Perchlorate disrupts reproductive development in threespine stickleback without changing whole-body levels of thyroid hormone. Gen. Comp. Endocrinol. 210, 130–144.

Ramsden, J., 2000. Angiogenesis in the thyroid gland. J. Endocrinol. 166, 475–480.

Rodriguez, A.F., Davidson, H.G., Villadiego, M.S., Fernandez, A.M., Lacave, I.M., Sanz, J.F., 1991. Induction of thyroid proliferative changes in rats treated with antithyroid compound. Anatomia, Histologia, Embryologia 20, 289–298.

Schmidt, F., Schnurr, S., Wolf, R., Braunbeck, T., 2012. Effects of the anti-thyroidal compound potassium-perchlorate on the thyroid system of the zebrafish. Aquat. Toxicol. 109, 47–58.

Seifi, S., Nazifi, S., Tabandeh, M., Saeb, M., 2013. AdipoR1 and AdipoR2 gene expression are regulated by thyroid hormones in adipose tissue. Mol. Cell. Biochem. 377, 55–63.

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

Smeds, S.A., Wollman, S.H., 1983. Capillary endothelial cell multiplication in adipose tissue pads on the thyroid during the feeding of thiouracil. Endocrinology 112, 1718–1722.

Suzuki, K., Kimura, H., Wu, H., Kudo, N., Bae Kim, W., Suzuki, S., Yoshida, A., Caturegli, P., Kohn, L.D., 2010. Excess iodide decreases transcription of NIS and VEGF genes in rat FRTL-5 thyroid cells. Biochem. Biophys. Res. Commun. 393, 286–290.

Theodorakis, C., Patiño, R., Snyder, E.M., Albers, E., 2006. Perchlorate effects in fish. In: Kendall, R.J., Smith, P.N. (Eds.), Perchlorate Ecotoxicology. SETAC Press, Pensacola, FL, pp. 155–184.

Tseleni-Balafouta, S., Kavantzas, N., Balafoutas, D., Patsouris, E., 2006. Comparative study of angiogenesis in thyroid glands with Graves disease and Hashimoto's thyroiditis. Appl. Immunohistochem. Mol. Morphol. 14, 203–207.

Urbansky, E., 2002. Perchlorate as an environmental contaminant. Environ. Sci. Pollut. Res. 9, 187–192.

Van der Geyten, S., Byamungu, N., Reyns, G.E., Kühn, E.R., Darras, V.M., 2005. Iodothyronine deiodinases and the control of plasma and tissue thyroid hormone levels in hyperthyroid tilapia (*Oreochromis niloticus*). J. Endocrinol. 184, 467–479.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs Jr., David R., Lee, D.H., Shioda, T., Soto, A.M., Saal, F.S.V., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455.

Wolff, J., 1998. Perchlorate and the thyroid gland. Pharmacol. Rev. 50, 89–106.

Wolff, J., Chaikoff, I.L., 1948. Plasma inorganic iodide as a homeostatic regulator of thyroid function. J. Biol. Chem. 174, 555–564.

Wolff, J., Chaikoff, I.L., Goldberg, R.C., Meier, J.R., 1949. The temporary nature of the inhibitory action of excess iodine on organic iodine synthesis in the normal thyroid. Endocrinology 45, 504–513.

Wollman, S.H., Herveg, J.P., Smeds, S., 1982. Lipolysis and blood capillary enlargement in adipose tissue pads on thyroids of rats fed thiouracil. Endocrinology 111, 1867–1873.

York, R.G., Barnett Jr., J., Brown, W.R., Garman, R.H., Mattie, D.R., Dodd, D., 2004. A rat neurodevelopmental evaluation of offspring, including evaluation of adult and neonatal thyroid, from mothers treated with ammonium perchlorate in drinking water. Int. J. Toxicol. 23, 191–214.