EPA Public Access

Author manuscript

Toxicol Sci. Author manuscript; available in PMC 2019 December 01.

About author manuscripts

Submit a manuscript

Published in final edited form as:

Toxicol Sci. 2018 December 01; 166(2): 318–331. doi:10.1093/toxsci/kfy203.

Evaluating iodide recycling inhibition as a novel molecular initiating event for thyroid axis disruption in amphibians

Jennifer H. Olker^{*,1}, Jonathan T. Haselman^{*}, Patricia A. Kosian^{*}, Kelby G. Donnay^{*}, Joseph J. Korte^{*}, Chad Blanksma[†], Michael W. Hornung^{*}, and Sigmund J. Degitz^{*}

^{*}U.S. Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, Duluth, MN 55804, USA

[†]Badger Technical Services, U.S. Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, Duluth, MN 55804, USA

Abstract

The enzyme iodotyrosine deiodinase (dehalogenase, IYD) catalyzes iodide recycling and promotes iodide retention in thyroid follicular cells. Loss of function or chemical inhibition of IYD reduces available iodide for thyroid hormone synthesis, which leads to hormone insufficiency in tissues and subsequent negative developmental consequences. IYD activity is especially critical under conditions of lower dietary iodine and in low iodine environments. Our objective was to evaluate the toxicological relevance of IYD inhibition in a model amphibian (*Xenopus laevis*) used extensively for thyroid disruption research. First, we characterized IYD ontogeny through quantification of IYD mRNA expression. Under normal development, IYD was expressed in thyroid glands, kidneys, liver, and intestines, but minimally in the tail. Then, we evaluated how IYD inhibition affected developing larval *X. laevis* with an *in vivo* exposure to a known IYD inhibitor (3-nitro-L-tyrosine, MNT) under iodine-controlled conditions; MNT concentrations were 7.4–200 mg/L, with an additional ‘rescue’ treatment of 200 mg/L MNT supplemented with iodide. Chemical inhibition of IYD resulted in markedly delayed development, with larvae in the highest MNT concentrations arrested prior to metamorphic climax. This effect was linked to reduced glandular and circulating thyroid hormones, increased thyroidal sodium-iodide symporter gene expression, and follicular cell hypertrophy and hyperplasia. Iodide supplementation negated these effects, effectively rescuing exposed larvae. These results establish toxicological relevance of IYD inhibition in amphibians. Given the highly conserved nature of the IYD protein sequence and scarcity of environmental iodine, IYD should be further investigated as a target for thyroid axis disruption in freshwater organisms.

¹Corresponding author: US EPA, 6201 Congdon Blvd, Duluth, MN 55804 USA. olker.jennifer@epa.gov. Tel: 218-529-5119. Fax: 218-529-5003.

Disclaimer: The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency, nor does the mention of trade names or commercial products indicate endorsement by the federal government.

Conflict of Interest: The authors claim no conflicts of interest.

Keywords

iodotyrosine deiodinase; thyroid; endocrine disruption; *Xenopus laevis*; amphibian metamorphosis; iodine deficiency

Introduction

The thyroid axis is essential for amphibian metamorphosis where the morphological and physiological changes during the transition from larvae to adult are primarily controlled by thyroid hormones (TH) (Brown and Cai, 2007; Denver, 2013). Maintaining appropriate TH levels require multiple proteins, enzymes, and processes for synthesis, secretion, transport, and control (Denver, 2013; Rousset et al., 2015; Zoeller et al., 2007). Whereas many components could be molecular targets for chemical disruption of the thyroid axis, few have been thoroughly investigated *in vivo* for adverse effects. Thyroid peroxidase (TPO) and the sodium-iodide symporter (NIS) are two toxicologically relevant targets proximal to thyroid hormone synthesis that have been well-characterized in amphibians (Hornung et al., 2015; Tietge et al., 2010, 2013). Other potential targets, including TH transporters and iodothyronine deiodinases, are less well-understood both in terms of susceptibility to chemical perturbation and characterization of organismal adverse effects. Iodotyrosine deiodinase (IYD) is another enzyme proximal to TH synthesis that, along with NIS, plays an essential role in maintaining high levels of free iodide in the thyroid gland (Friedman et al., 2006; Rokita et al., 2010; Rousset et al., 2015; Thomas et al., 2009). Conservation of iodide is especially important for amphibians and other freshwater organisms due to limited environmental availability of this raw material that is necessary for TH synthesis.

IYD is a reductive dehalogenation enzyme that catalyzes iodide recycling from monoiodotyrosine (MIT) and diiodotyrosine (DIT) (Rokita et al., 2010; Rousset et al., 2015). During thyroglobulin proteolysis, MIT and DIT not incorporated into 3,5,3',5'-tetraiodothyronine (thyroxine, T4) or 3,5,3'-triiodothyronine (T3) are liberated into the follicular cell where they are rapidly deiodinated by IYD, providing free iodide for TH synthesis. The biological importance of IYD was first documented in humans where failure of IYD (identified as DEHAL1) leads to iodotyrosine deiodinase deficiency (ITDD) disorder with negative developmental consequences including hypothyroidism, goiter, and mental retardation (Afink et al., 2008; Medeiros-Neto and Stanbury, 1994; Moreno et al., 2008; Moreno and Visser, 2010). Since initial clinical diagnosis in the 1950s, ITDD has been studied in 25 families, with analyses of genetic mutations and the role of dietary iodide content on phenotypic variation (Moreno and Visser, 2010). The IYD amino acid sequence is highly conserved and homologous proteins have been identified from a wide range of organisms (Phatarphekar et al., 2014). In amphibians, IYD has been documented in three frog species (*Xenopus laevis*, *Xenopus tropicalis*, and *Microhyla ornata*) (Fujimoto et al., 2012; Gaupale et al., 2009).

IYD inhibition has been partially evaluated in the mammalian system with several *in vivo* and *in vitro* studies. Short-term exposures of rats to nitrotyrosines show clear impacts on the thyroid system concordant with IYD inhibition, including reduced circulating TH levels,

increased thyroid gland size, and increased thyroid-stimulating hormone (TSH) levels (Green, 1968, 1971; Meinhold and Buchholz, 1983). IYD inhibition by nitrotyrosines has also been demonstrated in thyroid gland slices and perfusions, and homogenates of thyroid, kidney, and liver of several mammals (Green, 1968; Greer and Grimm, 1968; Renko et al., 2016; Solis-S et al., 2004). In addition, in an *in vitro* assay with recombinant human IYD, 12 of 44 tested environmentally relevant chemicals inhibited IYD (Shimizu et al., 2013). These studies suggest that IYD is important for thyroid homeostasis and is a toxicologically relevant target for chemical disruption of the thyroid axis, however, there is limited understanding of the adverse organismal effect of IYD inhibition.

The objective of this study was to evaluate the toxicological relevance of IYD as target in a model amphibian used extensively for thyroid disruption research. Given the limited information on IYD in amphibians, we first quantified IYD mRNA expression in multiple tissues during normal development of *X. laevis* larvae under standard culture conditions. To evaluate the effects of IYD inhibition, *X. laevis* larvae were exposed to a known IYD inhibitor (3-nitro-L-tyrosine, MNT) with an iodine-controlled diet to gain resolution on effects caused through this mode of action. In addition, a ‘rescue’ treatment, MNT exposure supplemented with iodide, was included to demonstrate the importance of iodine availability. We hypothesized that IYD inhibition would decrease TH synthesis by reducing free iodide in the thyroid gland, thus reducing levels of circulating T4 and T3 and delaying development through metamorphic stages with high demand for TH. We also expected increased levels of circulating MIT and DIT, based on lack of deiodination of these molecules, which is supported by the human clinical diagnosis for ITDD disorder (Moreno and Visser, 2010).

Materials and Methods

Experimental Animals

Xenopus laevis larvae used in these studies were obtained from an in-house culture at the U.S. EPA Mid-Continent Ecology Division (MED) in Duluth, Minnesota, USA. Larvae were reared in filtered and UV sterilized Lake Superior water (LSW), maintained at 21 ± 1 °C on a 12:12 light:dark cycle, and fed a blended diet of Trout Starter Crumble (Skretting North America, Tooele, UT) with *Spirulina* algae disks (The Wardley Corporation, Secaucus, NJ), and Tetrafin Goldfish Fish Food crisps (Tetra Sales, Blacksburg, VA). Starting 8 days post-fertilization, 24 h old live brine shrimp (Bio-Marine Brand, Bio-Marine Inc, Hawthorne, CA) were added to this diet. For the IYD gene expression study, larvae were collected directly from this culture when they reach the desired developmental stages, with sampling across multiple spawns. For the IYD inhibition study, larvae from a single spawn were transferred into the exposure system described below. Developmental staging was based on the method of Nieuwkoop and Faber (NF stage, 1994). Animal use was carried out according to the Animal Care and Use Guidelines approved by the MED Animal Care and Use Committee.

IYD Gene Expression: Ontogeny Under Normal Development

Tissue collection.—To quantify developmental patterns in IYD gene expression, thyroid gland, kidney, liver, intestine, hind limb, and tail samples were collected from stages across

metamorphosis (NF54-66) and IYD mRNA expression was measured with quantitative real-time reverse transcription polymerase chain reaction (qPCR). Larvae were collected from multiple spawns at selected stages and euthanized with 200 mg/L tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA) buffered with 400 mg/L sodium bicarbonate. Dissected tissues were flash frozen on dry ice and stored at -80°C . A minimum of three replicates for each stage were collected with tissue samples from five individuals pooled for each replicate. Tissue collections at each stage were based on expected timing of metamorphic changes in each tissue. Hind limb samples were collected at NF54, NF56, and NF58, with collections of one entire limb at NF54 and a small muscle portion from one limb for NF56 and NF58. Tail (small portion of end) and thyroid glands (pair of intact glands) were collected at NF54, NF56, NF58, NF60, and NF62. A small portion of the intestine, liver, and kidney were each collected at NF54, NF56, NF58, NF60, NF62, NF64, and NF66. Total RNA was extracted and qPCR performed as described below.

IYD Inhibition Study

Experimental design.—*Xenopus laevis* larvae were exposed to four different concentrations of MNT, an iodide-supplemented rescue treatment, and control water (LSW) from premetamorphosis (NF50) until metamorphic climax (NF62) in a static renewal waterborne exposure system. Each treatment had three replicate tanks with 26 larvae per tank at initiation; tanks were distributed randomly within the exposure unit. Tanks were monitored daily for environmental conditions and evaluated for evidence of acute toxicity (mortality), disease, and abnormal behavior. On days 7 and 13, and at NF58, sub-samples of six organisms per tank were collected for: growth and development; circulating and thyroidal iodothyronines and iodotyrosines; and thyroidal NIS and IYD mRNA expression. The remaining larvae ($n = 6-8$ organisms per tank) were collected at NF62 for growth, histological analysis of the thyroid glands, and circulating iodothyronines and iodotyrosines. The study was terminated on day 42, which was 1 week after all larvae in the control and iodide-supplemented treatments reached NF62.

Test concentrations and chemical stock preparation—Stock solution was prepared by dissolving MNT (CAS No. 621-44-3, Alfa Aesar, Thermo Fisher Scientific Chemicals, Ward Hill, MA) in 0.1 M sodium hydroxide (NaOH) and then transferring this solution to LSW. The pH of this 2 g/L stock solution was adjusted with hydrochloric acid (HCl) to match the pH of LSW (pH 7.4–7.8). New stock bottles were prepared approximately every 10 days. Exposure concentrations of MNT were selected to cause a measurable effect on development, but had been shown in range-finding experiments to not produce overt toxicity. In these range-finding experiments, exposure to 100–400 mg/L MNT delayed metamorphosis and caused glandular hypertrophy (unpublished data). Therefore, the highest target test concentration for this study was set at 200 mg/L with a 0.33 dilution factor resulting in nominal concentrations of 7.4, 22.2, 66.7, and 200 mg/L (32.7, 98.1, 294.9, 884.2 μM).

For the iodide-supplemented treatment, potassium iodide (KI) was added to 200 mg/L MNT as a ‘rescue’ for IYD inhibition. For this supplementation, KI (CAS No. 7681-11-0, Sigma-Aldrich Co, St. Louis, MO) was prepared at 200 mg/L in MilliQ water and added to the

designated solution bottle for a final concentration of 10 µg/L KI. This KI concentration was determined based on pilot study in which 10 and 100 µg/L KI were added to control (LSW) and 200 mg/L MNT treatments. MNT exposed larvae with supplementation of 10 µg/L KI developed in sync with the control larvae, demonstrating that 10 µg/L KI was sufficient to negate the developmental effects of MNT exposure; however, supplementation with 100 µg/L KI was toxic to both control and MNT exposed larvae (unpublished data).

Exposure system.—The exposure system consisted of metering pumps (Fluid Metering, Inc, Syosset, NY) for controlled dilution of stock solutions of test chemical and multichannel peristaltic pumps for daily delivery of solutions to test chambers (glass tanks), each with 6.25 L of test solution or control water. Exposures were 24 h static renewal with solutions for each test concentration mixed daily. For the daily 85% solution renewal, tanks were siphoned to 0.94 L and then refilled to 6.25 L. Exposure tanks were replaced with clean tanks with 100% renewal every 4–7 days. Control water and dilution water was LSW that was filtered through sand, UV sterilized, and filtered again to 5 µm. All wetted surfaces were either PTFE, stainless steel, or glass.

Test animals, test initiation, and feeding.—Larvae from a single spawning pair of *X. laevis* were anesthetized 15 days post-fertilization in 100 mg/L MS-222 buffered with 200 mg/L sodium bicarbonate in LSW and then gently sorted according to NF stage. After recovery in LSW, 468 NF50 larvae were randomly distributed into the 18 exposure tanks at 26 larvae per tank.

An iodine-controlled diet was important for evaluating the effect of IYD inhibition; therefore, larvae in this experiment were fed a diet of rodent pellets (AIN76-A, iodine content = 0.2 mg/kg, Research Diets, New Brunswick, NJ) blended in LSW to make a slurry. This diet was selected in effort to provide adequate but not excessive iodine. The reported iodine content of these rodent pellets is much lower than the standard larval diets recommended in the Amphibian Metamorphosis Assay (AMA, OECD, 2009) and Larval Amphibian Growth and Development Assay (LAGDA, OECD, 2015) protocols, such as Sera Micron (50 mg/kg iodine, personal communication), Trout Starter Crumble (2.4 mg/kg iodine, personal communication), and Nasco Frog Brittle (1.2 mg/kg iodine, reported value). Through pilot studies using only LSW, the AIN76-A rodent diet was shown to support normal growth and development in larvae compared with diets of Nasco Frog Brittle and the LAGDA recommended mix of Trout Starter Crumble, *Spirulina* algae disks, and goldfish crisps. Therefore, we assumed that these rodent pellets provide sufficient iodine, however actual iodine requirements for larval development are unknown and diets with even lower iodine content have not yet been tested. Larvae were fed this rodent pellet diet twice daily along with 24 h old hatched live brine shrimp (Bio-Marine Brand, Bio-Marine, Inc).

Environmental conditions.—For each exposure tank, temperature, pH, and dissolved oxygen (DO) was checked daily. Temperature was maintained at $21 \pm 0.7^\circ\text{C}$ by placing the exposure tanks in a temperature-controlled water bath. Temperature measurements were taken daily before renewal; mean tank temperatures were within 0.2°C of each other and variability across tanks within a day was less than 0.7°C . DO was measured with a YSI 550A (Yellow Springs, OH) before and after renewal from test initiation to day 11. In freshly

prepared solution, DO was at or above 8 mg/L (approximately 90% saturation). Due to declining levels of DO (< 3 mg/L before renewal), air stones were added on day 11; thereafter, DO was only measured before renewal. Before renewal, mean ($\pm$ SD) DO was 6.67 ($\pm$ 1.94) mg/L, with 4.18 ($\pm$ 1.49) mg/L for days 1–11 and 7.83 ($\pm$ 0.49) mg/L for days 12–42. On days 1–11 (previous to adding air stones), mean ($\pm$ SD) DO was 6.75 ($\pm$ 1.94) after solution renewal. pH was checked daily and was within the expected range (pH 7.2–7.8), with variability across tanks within a day less than 0.4. Conductivity, hardness, and alkalinity were measured for each treatment solution twice during the exposure. Hardness was within the expected range across all treatments (range: 43–47 mg CaCO₃ L⁻¹). Conductivity and alkalinity were within acceptable ranges across all treatments, but were higher in the two highest MNT concentrations (136–205 μ S cm⁻¹ and 55–79 CaCO₃ L⁻¹) compared with the dilution water and lowest two MNT concentrations (105–117 μ S cm⁻¹ and 44–50 mg CaCO₃ L⁻¹). The exposure was maintained at a 12:12 light:dark photoperiod with fluorescent lamps.

Exposure verification.—Water samples (20 μ l or 100 μ l, depending on MNT concentration) were collected weekly from each tank before and after renewal and diluted to 1 ml with liquid chromatograph (LC) mobile phase A (see below) in amber colored LC vials. Samples were also collected once from each prepared stock bottle for analysis at a 1:1000 dilution. Samples were vortex-mixed and analyzed using an Agilent 1260 series high performance liquid chromatograph (HPLC) with diode array detection (λ = 278 nm, Santa Clara, CA). Twenty μ l of diluted sample was injected into an Agilent Poroshell 120 EC-C₁₈ column (2.7 μ m, 50 mm $\times$ 3.0 mm, Santa Clara, CA) that was maintained at 25°C. MNT was eluted under gradient conditions with mobile phase A (5% acetonitrile:95% H₂O with 10 mM acetic acid) and mobile phase B (95% acetonitrile:5% H₂O with 10 mM acetic acid) at a flowrate of 0.5 ml/min. The starting mobile phase composition was 100% A. Mobile phase B was increased to 50% from 0 to 3 min and then held constant for 5 min. The mobile phases were returned to the starting conditions and the column was re-equilibrated for 5 min. MNT chemical concentrations were quantified using linear regression with a 7-point calibration curve (R^2 = 0.99). MNT standards ranged from 0.1 to 6 mg/L and were prepared in mobile phase A.

The mean measured aqueous concentrations of MNT are reported in Table 1. The stock solutions of MNT were within 3% of the target 2.0 g/L (mean = 2.02 g/L, SD = 0.05 g/L). LSW blanks, duplicate samples, and matrix spiked samples, at corresponding treatment concentrations, were prepared with each sample set. MNT was not detected in the LSW blanks (n=5). The analyses of duplicates were within 2% with one exception that had 9% difference and spike recoveries ranged from 100% to 107%. No MNT was detected in the control treatments. The instrument limit of detection was 0.05 mg/L.

Biological endpoints.—At the three sub-sampling points and at test termination, tanks were randomized (*a priori*) for collection of larvae across treatments. On days 7 and 13, six larvae in each tank were randomly selected for sub-sampling. For the NF58 sub-sampling, each exposure tank was divided and individuals that had reached NF58 were separated from the rest of the larvae. The date on which each larva reached NF58 was recorded. Of every

two larvae reaching NF58, one was sampled and the other continued in the exposure until six larvae had been collected from each tank. The remaining larvae were collected when they reached NF62 or at test termination ($n = 6-8$ organisms per tank). At each sampling point, larvae were euthanized in 150 mg/L MS-222 buffered with 300 mg/L sodium bicarbonate in LSW, with wet weight measured and NF stage recorded. Blood samples were collected directly from the aorta into 10 μ l heparinized microcapillary tubes (Kimble Chase, Vineland, NJ) and centrifuged to collect plasma for circulating hormone analysis (described below). For sub-samples on days 7 and 13, and at NF58, both thyroid glands were collected, with one gland flash frozen for mRNA expression analysis and the other frozen on wet ice for glandular hormone analysis (described below). Blood and glands from each tank were pooled ($n = 6$ larvae per pool for day 7, day 13, and NF58; $n = 3-8$ larvae per pool at NF62, depending on the number to reach this stage). Samples from larvae that did not reach NF62 were not pooled and were instead analyzed as individual samples.

Analytical determination of iodotyrosines and iodothyronines in plasma and thyroid glands.—Thyroid hormones (T3, rT3, T4) and mono- and diiodotyrosine (MIT, DIT) were measured in amphibian plasma (20 μ l) and thyroid glands using liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods previously described (Hassan et al., 2017; O'Shaughnessy et al., 2018) with an Agilent 1290 ultra-high pressure LC with a 6490 triple quadrupole mass spectrometer. However, chromatographic conditions were modified to separate MNT, which was present at high concentrations in the sample extract, from the target analytes. Chromatographic separation was conducted using an Agilent Zorbax SB-C₁₈ RRHT column (1.8 μ m, 100 mm $\times$ 2.1 mm, Santa Clara, CA). Compounds were eluted under gradient conditions with mobile phase A (5% acetonitrile: 95% H₂O with 0.1% formic acid) and mobile phase B (90% acetonitrile:10% H₂O with 0.1% formic acid) at a flowrate of 0.3 ml/min. The starting mobile phase composition was 100% A and was held constant for 1.5 min. Between 1.5 min and 9.0 min mobile phase B was linearly increased to 72%. Mobile phase B was then increased to 100% in 0.5 min and held constant for 0.5 min. The mobile phases were returned to the starting conditions and the column was re-equilibrated for 4 min. At the beginning of each sample run, the LC column effluent was diverted to waste to avoid high concentrations of MNT from entering the mass spectrometer. At 3.4 min the LC column effluent was directed into the mass spectrometer for target compound analysis.

Procedural blanks for the plasma analysis ($n = 4$) included only reagents and were below the lower limit of quantification (LLOQ). Plasma LLOQs were 0.5 ng/ml (MIT), 0.05 ng/ml (DIT), 0.125 ng/ml (T3), 0.125 ng/ml (rT3), and 0.125 ng/ml (T4). Technical duplicate samples ($n = 15$) were run to assess relative percent difference or precision (100% times the difference of the duplicates divided by the mean of the duplicates). Duplicate samples were distributed across each sample set with all treatments represented for each sampling point, if there was sufficient volume of plasma. Mean ($\pm$ SD) precision of duplicate samples was $7.9 \pm 4.9\%$ (MIT), $6.8 \pm 3.3\%$ (DIT), $3.7 \pm 3.9\%$ (T3), $24.9 \pm 32\%$ (rT3), and $9.8 \pm 8.1\%$ (T4). Poor precision in rT3 was due to instrument drift for rT3 values near the LLOQ. Mean percent recovery of matrix spiked samples ($n=2$) was $90.3 \pm 9\%$ (MIT), $94.4 \pm 13.7\%$ (DIT), $100.5 \pm 1.8\%$ (T3), $90.1 \pm 11.1\%$ (rT3), and $97.2 \pm 3.1\%$ (T4).

Procedural blanks for the thyroid gland analysis (n = 6) included only reagents and were below LLOQs of 0.1 ng/gland (MIT), 0.02 ng/gland (DIT), 0.05 ng/gland (T3), 0.02 ng/gland (rT3), and 0.02 ng/gland (T4). Mean ($\pm$ SD) precision of duplicate samples (n = 11) was $2.7 \pm 2.7\%$ (MIT), $6.7 \pm 7.2\%$ (DIT), $23.4 \pm 16.1\%$ (T3), $11.1 \pm 4.4\%$ (rT3), and $4.4 \pm 3.1\%$ (T4). A quality control thyroglobulin reference sample, prepared in-house, was analyzed with each set of samples (n = 4). Coefficient of variation (%) of the reference samples was 29.4% (MIT), 15.3% (DIT), 7.4% (T3), 13% (rT3), and 7.8% (T4).

Histological analysis of the thyroid glands.—Thyroid glands from larvae at NF62 or test termination were preserved and evaluated histologically following standard methods (Grim et al., 2009; OECD, 2015). Briefly, heads with arms attached were preserved by immersion in Davidson's fixative for 48 h and then stored in 10% neutral buffered formalin. All larvae from the 66.7 and 200 mg/L MNT treatments and a randomly selected subset of six larvae each from the untreated control and the iodide-supplemented rescue treatments were processed and prepared for histopathological evaluation. This set of 47 larvae included all larvae that did not reach NF62 by test termination. Tissues were trimmed, processed in a Sakura Tissue-Tek Vacuum Infiltration Processor (Torrance, CA), embedded in paraffin, transversely step-sectioned with 50 μ m steps until the largest cross-sectional area of at least one gland was reached, and stained by a standard hematoxylin and eosin staining protocol. For each larva, two bilateral gland sections at least 50 μ m apart representing the widest region of at least one gland were evaluated according to Grim et al. (2009), with histopathologic findings scored according to the following grading system: Not remarkable = 0%–20% of tissues affected; Mild = 20%–50% of tissue affected; Moderate = 50%–80% of tissues affected; Severe = >80% of tissues affected. The baseline control morphology for the diagnosis of follicular cell hypertrophy and follicular cell hyperplasia is based on NF59 control *X. laevis* which were examined in Grim et al. (2009).

RNA Extraction and Quantitative Real-Time Reverse Transcription PCRs

RNA extraction and qPCRs were conducted following methods that have been described previously for *X. laevis* NIS (Sternberg et al., 2011; Tietge et al., 2013), with additional information provided below for the *X. laevis* IYD qPCR assay. Total RNA was extracted from thyroid glands and other tissues following manufacturer's instructions for the RNeasy Plus Micro Kits (Qiagen, Germantown, MD), with RNA quality and concentration determined with a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Thermo Fisher Scientific, Waltham, MA). All samples had A260 nm/A280 nm ratios of 1.59 or greater. Samples were diluted 1:3 (hind limb and tail), 1:4 (thyroid gland), or 1:25 (intestine, liver, kidney) in 0.1 mM EDTA before qPCR.

Xenopus laevis IYD sequence information, acquired from the National Center for Biotechnology Information (NCBI) (accession number NM_001093860.2, *X. laevis* iodotyrosine deiodinase L homeolog (*iyd.L*), mRNA; note: there is no annotated .S paralog of this gene), was used to develop a quantitative PCR assay. IYD-specific primers and probe (Table 2) were selected using Primer Express 2.0 Software (Applied Biosystems, Foster City, CA) and purchased from Integrated DNA Technologies (Coralville, IA). The qPCR forward primer spans an exon-exon boundary so it should only bind to the sequence

corresponding to mRNA. The qPCR probe was dual labeled with 5' FAM and 3' Iowa Black FQ. An IYD RNA standard was made with the outer and middle sets of primers in a manner similar to that described in Sternberg et al. (2011).

For *X. laevis* NIS (NCBI accession number BC077614, *X. laevis* solute carrier family 5 [sodium iodide symporter], member 5, mRNA), primer and probe sequences were the same as described by Tietge et al. (2013).

QPCR was completed with TaqMan RNA-to-Ct 1 step kits (Applied Biosystems) on a 7500 Real Time PCR Detection System (Applied Biosystems). Copies of ribosomal protein L32 (RPL32) mRNA were also quantified as a reference gene, with primer and probe sequences as described in Stenberg *et al.* (2011) and Tietge et al. (2013). The amplification of each target gene and RPL32 was done in duplexed qPCR reactions, with separate assays for IYD and NIS. In addition to kit reagents, each reaction contained 900 nM IYD or NIS qPCR forward and reverse primers, 100 nM IYD or NIS probe, 300 nM RPL32 qPCR forward primer, and 100 nM RPL32 qPCR reverse primer and RPL32 probe. The total reaction volume was 30 µl and contained 5 µl of diluted sample or standard. Each 96-well plate included samples (tested in duplicate), a no template control (0.1 mM EDTA), and RNA standards from 10² to 10⁸ copies. IYD assays also included an aliquot of standard RNA with a known copy number for interassay control. The thermal cycler program was 48°C for 15 min, 95°C for 10 min, and 40 cycles of: 95°C for 15 sec/58°C for 1 min. All the standard curves met quality criteria, with 90%–100% efficiency and slopes of -3.3 ± 0.3 . In both assays, the target gene and RPL32 standard curves were parallel. The number of copies present in the samples was determined with the absolute quantification method by comparing the amplification response to that from known amounts of each standard. All samples were tested in duplicate, with the mean of the duplicates for each sample reported here. In the ontogeny samples, the mean (SD) coefficient of variation (CV) was 8.0 (9.1) %. For samples from the IYD inhibition experiment, with mean (SD) CV was 4.2 (6.1) % for NIS and 6.3 (6.5) % for IYD. Although RPL32 expression was intended as a reference gene for both IYD and NIS it could not be used as such because RPL32 expression in thyroid glands increased across developmental stages. Thus, gene expression data was instead normalized as copies mRNA of each gene per ng of extracted total RNA. Samples below the limit of detection (100 copies in the assay well, based on the lowest point on the standard curve) were excluded from the analysis.

Statistical Analyses

All quantitative data were tested for normality and homogeneity of variance using Shapiro-Wilk's and Levene's Median tests, respectively. When the parametric assumptions were not met, non-parametric analyses were used. Statistical analyses were completed with R data analysis software (version 3.3.1; R Core Team, 2016). Statistical analyses used $p < 0.05$ as the level of significance.

For the IYD ontogeny data, parametric assumptions were met for IYD mRNA expression in the liver, kidney, and intestine, and a one-way analysis of variance (ANOVA) was used for each tissue type to test for differences across developmental stages, with a Tukey's post-hoc test to evaluate pairwise differences in expression. Because IYD mRNA expression in

thyroid glands and tail samples did not meet parametric assumptions, a nonparametric Kruskal-Wallis one-way analysis on ranks was used with a Dunn's post hoc test for pairwise comparisons (R package `dunn.test`, version 1.3.5, Dinno, 2017).

For the quantitative data from the *in vivo* IYD inhibition experiment, parametric assumptions were met for most endpoints and data were analyzed by one-way ANOVA followed by Dunnett's pairwise comparison to the controls using the R package `multcomp` (version 1.4-6, Hothorn et al. 2008). Plasma T4 was log transformed before this analysis to meet homogeneity of variance and normality assumptions. Plasma MIT and DIT did not meet parametric assumptions and thus were analyzed with the non-parametric Kruskal-Wallis one-way analysis with Dunn's post hoc test of multiple comparisons using rank sums (R package `dunn.test`, version 1.3.5, Dinno, 2017). For biochemical measures that were below the limits of quantification, the values were estimated at one-half the detection limit. Time-to-NF58 and time-to-NF62 data were analyzed with a Cox mixed-effects proportional hazard model (R package `coxme`, version 2.2-5, Therneau, 2015). Larvae that did not reach NF62 by the end of the study were censored from the analyses of time-to-NF62, wet weight at NF62, and plasma biochemical measurements at NF62. For gene expression data, samples below the limit of detection (100 copies in the assay well) were excluded from the analyses, as described above. Each replicate tank was the experimental unit from which treatment means and standard deviations (SDs) were calculated (n=3 tanks per treatment).

Results

Expression of IYD During Metamorphic Development

Expression of IYD varied widely across tissue types and developmental stages (Figure 1). As expected, IYD was highly expressed in the thyroid gland, with significant differences across developmental stages, and the highest expression occurred at NF58-60. IYD was also expressed in the liver, kidney, and intestine, with some developmental patterns observed. In the liver, expression levels were fairly consistent across all stages with the highest expression at NF60. However, in the kidney and thyroid gland, IYD showed a developmental pattern of expression with expression peaking in the thyroid gland at NF60 and in the kidney at NF62. In the intestine, IYD expression was lower than in the thyroid and kidney, with peak expression around stage 60. In the tail, IYD expression was transient in nature with low levels of expression (2.9–20.8 copies IYD mRNA per ng extracted total RNA) only being observed at stages 60 and 62. There was no measurable IYD expressed in the limb, with the number of copies below the detection limit (100 copies per assay well) in all samples.

IYD Inhibition Study

Survival, Growth, and Development—Survival and growth were unaffected by exposure to MNT, regardless of concentration. Overall, mortalities were minimal and not related to MNT exposure. However, on day 9, a single tank of the 200 mg/L MNT treatment was removed from the study as a precaution due to a potential fungal infection. This issue was not observed in any other tanks in the study. Of the 442 larvae that started in the remaining 17 tanks, five larvae were lost due to human error (injury or death during

siphoning). An additional two mortalities occurred that were not attributed to any specific cause (one each in 7.4 and 22.2 mg/L treatments on days 11 and 13).

Body weight and time-to-NF58 was not significantly affected by MNT exposure. All larvae in the control, 7.4 and 22.2 mg/L MNT, and rescue (200 mg/L MNT + 10 µg/L KI) reached NF62 and there were no differences observed in the time to reach this stage. However, in the two highest treatments not all of the larvae reached NF62 before the study was terminated (1 week after all larvae in control and iodide-supplemented rescue treatments had reached NF62).

In the 66.7 and 200 mg/L MNT treatments, 20% and 60% of the larvae, respectively, did not reach NF62 (Table 3). Atypical development was observed in these larvae, with a continuum of apparent asynchronous developmental anomalies in which some tissues continued through metamorphosis (eg, skin and snout), whereas other tissues did not progress (eg, gills). In these larvae, the skin on the torso was more pigmented and patterned (adult-like) and the anterior portion of head narrowed. However, these larvae had obvious gill structure remaining, the posterior portion of the head was not undergoing normal remodeling with the space caudal to the eyes remaining enlarged, and relative position of the forelimbs remained posterior to the heart, as observed in normal NF60 larvae. This effect was not seen in any of the larvae in the iodide-supplemented rescue treatment.

Iodotyrosines and Iodothyronines in Plasma and Thyroid Glands

T4 and T3: Glandular T4 was significantly reduced in a concentration-dependent pattern, whereas glandular T3 was consistently low (<0.12 ng/gland) across all sampling points and treatments (Figure 2 A, B). On days 7 and 13, T4 in the iodide-supplemented rescue treatment was significantly higher than control whereas the other treatments did not differ from control. At NF58, T4 in the 66.7 and 200 mg/L MNT treatments was significantly lower than control, whereas T4 in the iodide-supplemented rescue treatment was significantly higher than control. The greatest differences were in the 200 mg/L MNT treatment where the mean glandular T4 was reduced to 28% of control and in the iodide-supplemented rescue treatment where the mean glandular T4 was increased to 169% of control.

On days 7 and 13, circulating levels of T4 and T3 were below 0.77 and 0.125 ng per ml, respectively, with no differences across treatments (Figure 2 C, D). At NF58, circulating T3 remained below 0.125 ng per ml but circulating T4 increased and was more variable across treatments. At this stage, mean plasma T4 was higher in the controls compared with the treatments; however, this was not a statistically significant effect, likely due to variability within each treatment. At NF62, there was a significant concentration-dependent reduction in circulating T4 and T3 (Figure 2 C, D). This effect was greatest in the 66.7 and 200 mg/L MNT treatments, which had mean plasma concentrations reduced to 2.9 and 2.0% of control, respectively, for T4, and 8.5 and 7.9 % of control, respectively, for T3. In the larvae that did not reach NF62, T4 was 3.5% of control or lower and T3 was below the LLOQ (0.125 ng/ml). The mean circulating concentrations of both T4 and T3 in iodide-supplemented rescue treatment were nearly identical to controls.

MIT and DIT: On days 7 and 13, glandular MIT was below the LLOQ for all treatments and glandular DIT was comparable to the control in all treatments except the iodide-supplemented rescue treatment (Figure 3 A, B). At NF58, glandular MIT and DIT were reduced in the MNT treatments, with notable but not statistically significant differences in the 200 mg/L MNT treatment, where DIT was 53% of control and MIT was 86% of control. In the iodide-supplemented rescue treatment, glandular DIT was significantly increased at all sampling points, with DIT concentration 3-, 2.5-, and 1.7-fold higher than controls at day 7, day 13, and NF58, respectively.

Circulating MIT and DIT were below the LLOQ (0.50 ng/ml and 0.05 ng/ml, respectively) for all controls at all sampling points (Figure 3 C, D). Mean MIT and DIT plasma concentrations were significantly higher than controls for one or more MNT concentration at each sampling point. On day 7, the iodide-supplemented rescue was the only treatment with plasma MIT and DIT above the LLOQ. On day 13, plasma MIT and DIT were significantly higher than controls in the iodide-supplemented rescue treatment, and plasma DIT was also significantly higher than controls in the 66.7 and 200 mg/L MNT treatments. At NF58 and NF62, all MNT treatments resulted in quantifiable levels of circulating MIT and DIT, with concentrations significantly higher than control for multiple treatments at each sampling point. The greatest MNT treatment effect was at NF58 where exposure to 200 mg/L MNT resulted in MIT and DIT plasma concentrations 6- and 20-fold higher than the LLOQs, respectively. In the iodide-supplemented rescue treatment, MIT and DIT in the plasma were both significantly higher than controls at all sampling points.

Histological Analysis of the Thyroid Glands—Thyroid gland histopathological diagnoses included diffuse hypertrophy of the gland, follicular cell hypertrophy and hyperplasia, changes in the follicle size, and colloid depletion, following criteria defined by Grim et al. (2009). Glands from all larvae in the 66.7 and 200 mg/L MNT treatments had follicular cell hypertrophy and hyperplasia, and colloid depletion (Table 4, Figure 4). Thyroid gland hypertrophy and follicular lumen decrease were also observed in a subset of the MNT exposed glands. Iodide supplementation prevented the occurrence of most of these histologically observed effects. In the iodide-supplemented rescue treatment, mild thyroid gland hypertrophy, mild follicular cell hypertrophy, and mild colloid depletion were observed in only one individual. Control glands showed mild to moderate follicular cell hypertrophy and hyperplasia, but no other pathologic observations.

NIS and IYD mRNA Expression in Thyroid Glands—Expression of NIS and IYD mRNA increased in the thyroid gland over development, with the highest expression occurring at NF58 (Figure 5). On day 7, only the 22.2 mg/L MNT treatment resulted in significantly higher glandular NIS expression than controls (Figure 5A). On day 13, however, exposure to 22.2, 66.7, and 200 mg/L MNT resulted in significantly higher glandular NIS expression, with 1.75- to 9.1-fold more copies of NIS mRNA per ng extracted total RNA than the controls. In contrast, the iodide-supplemented rescue treatment resulted in glandular NIS expression reduced to 48% of controls on day 13 and 66% of controls at NF58, although these were not statistically different from the control. Expression of IYD mRNA varied by less than 2.5-fold within each sampling point, with significantly higher

IYD expression in MNT treatments compared with controls only on day 13. At this sampling point, exposure to 22.2 or greater mg/L MNT (including the iodide-supplemented rescue treatment) resulted in 1.6- to 2.3-fold higher glandular IYD mRNA expression than controls (Figure 5B).

Discussion

Multiple priority thyroid targets have been identified in amphibians using the adverse outcome pathway (AOP) framework described by Ankley et al. (2010), with molecular initiating events such as TPO and NIS fairly well-characterized with regard to toxicological relevance and resulting adverse outcomes. IYD, however, has received little attention as a molecular target for chemical disruption of the thyroid axis (see reviews Boas et al., 2006, 2012; and Fort et al., 2007). We developed a putative IYD inhibition AOP (Figure 6, AOP #188, <https://aopwiki.org/aops/188>, last accessed 03 August 2018) as part of the network of amphibian thyroid AOPs. Characterization of the apical adverse outcome (delayed metamorphosis) in combination with the biochemical responses to IYD inhibition presented here establish the foundation for a more formal description of this AOP in amphibians.

The IYD ontogeny presented here is the first that we are aware of that documents amphibian IYD expression in kidney and liver and for a developmental series in thyroid glands. These results are consistent with mammalian studies in which IYD was present in the kidney and liver as well as the thyroid gland (Gnidehou et al., 2006; Sun et al., 2015). In the thyroid gland, rapid deiodination of MIT and DIT provides free iodide for immediate use in TH production (Gnidehou et al., 2004). Fairly constant IYD expression across development suggests continuous activity of the IYD enzyme in the thyroid gland. Given the vital physiological role of the liver and kidney in general metabolism and excretion of waste, IYD in these tissues could be essential for preservation of iodide by deiodinating MIT and DIT that might otherwise be lost from the organism. In the intestine, IYD expression was consistent with the reported up-regulation at metamorphic climax in *X. tropicalis* (Fujimoto et al., 2012). However, based on the lower expression levels, iodide recycling in the intestine may be less critical than in the thyroid glands, kidney, and liver.

Chemical inhibition of IYD resulted in altered larval development and decreased circulating TH. At the two highest MNT concentrations larval development was arrested at NF60-61, stages in metamorphosis that require the highest concentrations of TH. Reduced circulating TH, increased NIS expression, and changes in the thyroid gland morphology support thyroid disruption as the cause of this arrested development. These adverse effects were consistent with previous rodent studies demonstrating that IYD inhibition reduced serum T4 and T3 levels (Meinhold and Buchholz, 1983) and the human clinical data, where failure of IYD resulted in negative developmental consequences (eg, hyperthyroidism, goiter, and mental retardation) (Afink et al., 2008; Medeiros-Neto and Stanbury, 1994; Moreno et al., 2008; Moreno and Visser, 2010).

This study showed significant changes in the thyroid gland following MNT exposure, including follicular cell hypertrophy and hyperplasia, changes in follicular cell size, and depletion of the follicular colloid. These results, in addition to glandular NIS up-regulation,

are consistent with the well-documented compensatory response reported in previous studies following exposure to model thyroid axis disruptors that inhibit TPO or NIS (Degitz et al., 2005; Hornung et al., 2015; Tietge et al., 2005, 2010, 2013). However, in contrast to TPO and NIS inhibitors, which are capable of delaying development and causing adverse biochemical changes early in the metamorphic process, the effects of IYD inhibition were not observed until NF60 or later. This difference was not anticipated and points to the uniqueness of IYD inhibition. It is quite possible that iodine uptake from the diet and water are sufficient to support development when TH demand is low (early in the metamorphic process), whereas iodine recycling is necessary to achieve appropriate levels of TH synthesis when demand increases at metamorphic climax. We also observed a very unique phenotype in these studies. In addition to arrest at NF60-61, larvae appeared to undergo asynchronous development with various aspects of development appearing normal (narrowing of snout) and others appearing delayed (gill resorption). We cannot speculate on the underlying cause of this phenomenon, but it is reasonable to suggest that this would have significant consequences on survival.

Detection of MIT and DIT in the plasma may be a specific consequence of IYD inhibition. MIT and/or DIT in the blood or urine is well described as the human clinical diagnostic marker of mutation in the IYD gene resulting in ITDD (Medeiros-Neto and Stanbury, 1994; Moreno et al., 2008) and has been documented in rat exposures to nitrotyrosines (Green, 1968, 1971; Meinhold and Buchholz, 1983). In this study, plasma MIT and DIT were the earliest endpoints at which a treatment effect was detected (Day 13 and NF58). In larvae exposed to 200 mg/L MNT, plasma DIT at NF58 was increased at least 22-fold, which is comparable to increases demonstrated in rats exposed to MNT (Meinhold and Buchholz, 1983) and humans with ITDD (Meinhold and Buhholz, 1983; Moreno et al., 2008). According to the profiles in controls, MIT and DIT are either rarely released to the plasma or have extremely short half-lives in the plasma. Presumably, most of the MIT and DIT byproducts from thyroglobulin proteolysis are immediately processed by IYD in thyroid follicular cells and recycled back to TPO for TH synthesis. Given the high expression of IYD in the liver and kidney, it is reasonable to suggest that MIT or DIT that escapes the thyroid gland would be rapidly acted on by these sources.

In this study, appearance of MIT and DIT in the plasma demonstrates that inhibition of IYD results in these compounds accumulating in the plasma. Furthermore, plasma MIT and DIT had an interesting temporal pattern in the 66.7 and 200 mg/L MNT treatments where both analytes increased through development and then decreased at NF62. This pattern was consistent with continued TH synthesis (and thus MIT and DIT production) until increasing systemic demand for TH could not be met due to available free iodide becoming rate-limiting, at which point glandular T4, MIT, and DIT would be depleted. This explanation was supported by the continuously high plasma MIT and DIT in larvae from the iodide-supplemented rescue treatment.

The effects of IYD inhibition are largely dependent on iodine intake. It is well documented in humans that TH-homeostasis and TH-dependent development relies entirely on environmental abundance of iodine (Zimmermann, 2009). For the IYD inhibition experiment, conditions and diet were selected to reflect environmentally relevant iodine

levels, with dilution and control culture water of approximately 0.5 µg/L iodine (unpublished data) and larvae fed the lower iodine diet. Iodide supplementation negated the effects of IYD inhibition, which is concordant with results in both humans and rats, where sufficient iodine intake via diet or drinking water has been shown to ameliorate the effect of IYD failure or inhibition. In human ITDD, the severity of documented phenotypes varies widely and is thought to be dependent on dietary iodine, with mild or euthyroid phenotypes documented in several families with high seafood diets (reviewed in Moreno and Visser, 2010). In rats, iodine supplementation prevented or reversed adverse effects, including goiter, TSH increase, and serum protein-bound iodine decrease (Green, 1971). Additional testing under an iodine-deficient diet would expand the understanding of the sensitivity to thyroid disruption via inhibition of IYD.

Whereas IYD inhibition is unlikely to be a concern for human populations with iodine-supplemented diets, this pathway for thyroid disruption is highly relevant for many freshwater organisms in low iodine environments with limited dietary iodine. Most freshwater ecosystems have low concentrations of iodine, typically ranging from 0.1 to 40 µg/L, with variability based on geologic setting, proximity to the ocean, and evapotranspiration (Hutchinson, 1957; Moran et al., 2002; Oktay et al., 2001; Rau and Fehn, 1999). Iodine content of food sources for aquatic species such as fish and amphibians are rarely measured; however, these food sources could be reasonably estimated to have iodine concentrations similar to the water, assuming no external iodine inputs. Whereas minimum iodine requirements for wildlife are largely unknown, the scarcity of iodine in freshwater systems can be exemplified with two mammalian examples (recognizing that mammalian intake of water is not directly comparable to aquatic organisms' exposure to water over the gills, skin, and gut). Adult human recommended daily intake is 120–150 µg iodine per day (from Dietary Reference Intakes tables developed by the Institute of Medicine of the National Academies) and the rodent iodine 'sufficient' diet is approximately 2–3 µg iodine per day (U.S. National Research Council Subcommittee on Laboratory Animal Nutrition, 1995). These requirements could not be met in a low iodine freshwater ecosystem as they are equivalent to 500–1,500 L (for humans) or 10–30 L (for rats) of water per day (estimated based on 0.1–0.2 µg/L iodine, Rau and Fehn, 1999).

This study supports the toxicological relevance of IYD inhibition in amphibians and provides support to previous mammalian research on IYD inhibition. Through exposure to a model inhibitor, this study characterized the developmental and thyroidal effects of IYD inhibition. Testing under controlled-iodine conditions combined with an iodide-supplemented rescue treatment demonstrated the environmental relevance of IYD inhibition in low iodine freshwater ecosystems. Although few chemicals have been tested for thyroid disruption via this pathway, the results from *in vitro* testing suggest that chemicals of environmental concern (including several agrichemicals, antiparasitics, pharmaceuticals, and food colorants) can produce IYD inhibition (Shimizu et al., 2013). These results provide a basis for further investigation of IYD inhibition, including differential species and life stage sensitivities, minimum iodine requirements for normal thyroid function, and identification of environmentally relevant chemicals that disrupt the thyroid system through this pathway.

Acknowledgments:

The authors would like to thank Carsten Knutsen, Jessica Christensen, and Paige Kent for laboratory technical support with exposure systems, sampling, and sample analysis. We also thank Dr. Steve Rokita (Johns Hopkins University) for advice and sharing of IYD knowledge, Dr. Mary Gilbert (U.S. EPA, NHEERL) for advice on and source of iodine-limited diet, Joe Swintek (Badger Technical Services) for statistical advice, and Joseph Tietge for comments and suggestions on an earlier version of this manuscript. The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency, nor does the mention of trade names or commercial products indicate endorsement by the federal government.

Funding Information: This work was supported by the US Environmental Protection Agency.

References

- Afink G, Kulik W, Overmars H de Randamie J, Veenboer T, van Cruchten A, Craen M, and Ris-Stalpers C (2008). Molecular characterization of iodotyrosine dehalogenase deficiency in patients with hypothyroidism. *J. Clin. Endocrinol. Metab* 93, 4894–4901.
- Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrano JA, Tietge JE, and Villeneuve DL (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicological research and risk assessment. *Environ. Toxicol. Chem* 29, 730–741. [PubMed: 20821501]
- Boas M, Feldt-Rasmussen U, Skakkebaek ND, and Main KM (2006). Environmental chemicals and thyroid function. *Euro. J. Endocrinol* 154, 599–611.
- Boas M, Felt-Fasmussen U, and Main KM (2012). Thyroid effects of endocrine disrupting chemicals. *Mol. Cell. Endocrinol* 355, 240–248. [PubMed: 21939731]
- Brown DD and Cai L (2007). Amphibian metamorphosis. *Dev. Biol* 306, 20–33. [PubMed: 17449026]
- Degitz SJ, Holcombe GW, Flynn KM, Kosian PA, Korte JJ, and Tietge JE (2005). Progress towards development of an amphibian-based thyroid screening assay using *Xenopus laevis*: Organismal and thyroidal responses to the model compounds 6-propylthiouracil, methimazole, and thyroxine. *Toxicol. Sci* 87, 353–364. [PubMed: 16002479]
- Denver RJ (2013). Neuroendocrinology of Amphibian Metamorphosis Current Topics in Developmental Biology, Vol. 103, Academic Press, New York, NY, pp 195–227. [PubMed: 23347520]
- Dinno A (2017). dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums. R package version 1.3.5. <https://CRAN.R-project.org/package=dunn.test>, last accessed on 08/03/2018.
- Friedman JE, Watson JA, Lam DW-H, and Rokita SE (2006). Iodotyrosine deiodinase in the first mammalian member of the NADH oxidase/Flavin reductase superfamily. *J. Biol. Chem* 281, 2812–2819. [PubMed: 16316988]
- Fort DJ, Degitz S, Tietge J, and Touart LW (2007). The hypothalamic-pituitary-thyroid (HPT) axis in frogs and its role in frog development and reproduction. *Crit. Reviews in Toxicol* 37, 117–161.
- Fujimoto K, Matsuura K, Das B, Fu L, and Shi YB (2012). Direct activation of *Xenopus* iodotyrosine deiodinase by thyroid hormone receptor in the remodeling intestine during amphibian metamorphosis. *Endocrinol.* 152, 5082–5089
- Gaupale TC, Mathi AA, Ravikumar A, and Bhargava SY (2009). Localization and enzyme activity Iodotyrosine dehalogenase 1 during metamorphosis of frog *Microhyla ornata*. *Trends Comp. Endocrinol. Neurobiol.* 1163, 402–406.
- Gnidehou S, Caillou B, Talbot M, Ohayon R, Kaniewski J, Noel-Hudson MS, Morand S, Agnangji D, Sezan A, Courtin F, Virion A, and Dupuy C (2004). Iodotyrosine dehalogenase 1 (DEHAL1) is a transmembrane protein involved in the recycling of iodide close to the thyroglobulin iodination site. *FASEB J.* 18, 1574–1576. [PubMed: 15289438]
- Gnidehou S, Lacroix L, Sezan A, Ohayon R, Noel-Hudson M-S, Morand S, Francon J, Courtin F, Virion A, and Dupuy C (2006). Cloning and characterization of a novel isoform of iodotyrosine deiodinase 1 (DEHAL1) DEHAL1C from human thyroid: Comparisons with DEHAL1 and DEHAL1B. *Thyroid* 16, 715–724. [PubMed: 16910871]

- Green WL (1968). Inhibition of thyroidal iodotyrosine deiodination by tyrosine analogues. *Endocrinol.* 83, 336–347.
- Green WL (1971). Effects of 3-nitro-L-tyrosine on thyroid function in the rat: an experimental model for the dehalogenase defect. *J. Clin. Invest* 50, 2472–2484.
- Greer MA and Grimm Y (1968). Changes in thyroid secretion produced by inhibition of iodotyrosine deiodinase. *Endocrinol.* 83, 405–410.
- Grim KC, Wolfe M, Braunbeck T, Iguchi T, Ohta Y, Tooi O, Touart L, Wolf DC, and Tietge J (2009). Thyroid histopathology assessments for the amphibian metamorphosis assay to detect thyroid-active substances. *Toxicol. Pathol.* 37:415–424. [PubMed: 19387088]
- Hassan I, El-Masri H, Kosian P, Ford J, Degitz S, and Gilbert M (2017). Neurodevelopment and thyroid hormone synthesis inhibition in the rat: Quantitative understanding within the adverse outcome pathway framework. *Toxicol. Sci* 160, 57–73. [PubMed: 28973696]
- Hornung MW, Kosian PA, Haselman JT, Korte JJ, Challis K, Macherla C, Nevalainen E, and Degitz SJ (2015). *In vitro*, *ex vivo*, and *in vivo* determination of thyroid hormone modulating activity of benzothiazoles. *Toxicol. Sci.* 146, 254–264. [PubMed: 25953703]
- Hothorn T, Bretz F and Westfall P (2008). Simultaneous Inference in General Parametric Models. *Biometrical J.* 50, 346–363.
- Hutchinson GE (1957). *A Treatise on Limnology*, v.1. Geography, Physics, and Chemistry. John Wiley and Sons, New York, New York 1015 pp.
- Medeiros-Neto G and Stanbury JB (1994). The Iodotyrosine Deiodinase Defect. *Inherited Disorders of the Thyroid System*. CRC Press, Boca Raton, FL, pp. 139–159.
- Meinhold H and Buchholz R (1983). Effects of iodotyrosine deiodinase inhibition on serum concentrations and turnover of diiodotyrosine (DIT) and thyroxine (T4) in the rat. *Acta Endocrinol.* 103, 521–527. [PubMed: 6613496]
- Moran JE, Oktay SD, and Santschi PH (2002). Sources of iodine and iodine 129 in rivers. *Water Resour. Res* 38, 24–1–24–10.
- Moreno JC and Visser TJ (2010). Genetics and phenomics of hypothyroidism and goiter due to iodotyrosine deiodinase (DEHAL1) gene mutations. *Mol. Cell. Endocrinol.* 322, 91–98. [PubMed: 20298747]
- Moreno JC, Klootwijk W, van Toor H, Pinto G, D'Alessandro M, Leger A, Goudie D, Polak M Gruters A Visser TJ (2008). Mutations in the iodotyrosine deiodinase gene and hypothyroidism. *N. Engl. J. Med* 358, 1811–1818. [PubMed: 18434651]
- Nieuwkoop PD and Faber J (1994). *Normal table of Xenopus laevis* (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis, third ed. Garland, New York, NY, USA.
- Organisation for Economic Co-operation and Development (OECD). (2009). Test No. 231: Amphibian Metamorphosis Assay. OECD Publishing, Paris, France.
- Organisation for Economic Co-operation and Development (OECD). (2015). Test No. 241: The Larval Amphibian Growth and Development Assay (LAGDA). OECD Publishing, Paris, France.
- Oktay SD, Santschi PH, Moran JE, and Sharma P (2001). 129I and 127I transport in the Mississippi River. *Environ. Sci. Technol* 35, 4470–4476. [PubMed: 11757603]
- O'Shaughnessy K, Kosian P, Ford J, Oshiro W, Degitz S, and Gilbert ME (2018). Developmental thyroid hormone insufficiency induces a cortical brain malformation and learning impairments: a cross-fostering study. *Toxicol. Sci* 163, 101–115. [PubMed: 29385626]
- Phatarphekar A, Buss JM, and Rokita SE (2014). Iodotyrosine deiodinase: A unique flavoprotein present in organisms of diverse phyla. *Mol. Biosyst* 10, 86–92. [PubMed: 24153409]
- R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria URL <https://www.R-project.org/>, last accessed on 08/03/2018.
- Rau U and Fehn U (1999). Sources and reservoirs of anthropogenic iodine-129 in western New York. *Geochim. Cosmochim. Ac* 63, 1927–1938.
- Renko K, Hoefig CS, Dupuy C, Harder L, Schwiebert C, Kohrle J, and Schomburg L (2016). A non-radioactive DEHAL assay for testing substrates, inhibitors and monitoring endogenous activity. *Endocrinol.* 157, 4516–4525.

- Rokita SE, Adler JM, McTamney PM, and Watson JA Jr. (2010). Efficient use and recycling of the micronutrient iodide in mammals. *Biochimie* 92, 1227–1235. [PubMed: 20167242]
- Rousset B, Dupuy C, Miot F, and Dumont J (2015). Thyroid Hormone Synthesis and Secretion [Updated 2015 Sep 2] In: De Groot LJ, Chrousos G, Dungan K, et al., editors. *Endotext* [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-2015. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK285550/>, last accessed on 08/03/2018.
- Shimizu R, Yamaguchi M, Uramaru N, Kuroki H, Ohta S, Kitamura S, and Sugihara K (2013). Structure-activity relationships of 44 halogenated compounds for iodotyrosine deiodinase-inhibitory activity. *Toxicology* 314, 22–29. [PubMed: 24012475]
- Solis-S JC, Villalobos P, Orozco A, and Valverde-R C (2004). Comparative kinetic characterization of rat thyroid iodotyrosine dehalogenase and iodothyronine deiodinase type 1. *J. Endocrinol* 181, 385–392. [PubMed: 15171686]
- Sternberg RM, Thoemke KR, Korte JJ, Moen SM, Olson JM, Korte L, Tietge JE, and Degitz SJ Jr. (2011). Control of the pituitary thyroid-stimulating hormone synthesis and secretion of thyroid hormones during *Xenopus* metamorphosis. *Gen. Comp. Endocrinol* 173, 428–437. [PubMed: 21803044]
- Sun X, Zhang X, Jiang Y, Bao S, Shan Z and Teng W (2015). Expression of iodotyrosine deiodinase in thyroid and other organs in iodine-deficient and iodine-excess rats. *Biol. Trace Elem. Res* 167, 272–279. [PubMed: 25850545]
- Therneau TM (2015). *coxme: Mixed Effects Cox Models*. R package version 2.2-5. <https://CRAN.R-project.org/package=coxme>, last accessed on 08/03/2018.
- Thomas SR, McTamney PM, Adler JM, LaRonde-LeBlanc N, and Rokita SE (2009). Crystal structure of iodotyrosine deiodinase, a novel flavoprotein responsible for iodide salvage in thyroid glands. *J. Biol. Chem* 284, 19659–19667. [PubMed: 19436071]
- Tietge JE, Holcombe GW, Flynn KM, Kosian PA, Korte JJ, Anderson LE, Wolf DC, and Degitz SJ (2005). Metamorphic inhibition of *Xenopus laevis* by sodium perchlorate: effects on development and thyroid histology. *Environ. Toxicol. Chem* 24, 926–933. [PubMed: 15839568]
- Tietge JE, Butterworth BC, Haselman JT, Holcombe GW, Hornung MW, Korte JJ, Kosian PA, Wolfe M, and Degitz SJ (2010). Early temporal effects of three thyroid hormone synthesis inhibitors in *Xenopus laevis*. *Aquat. Toxicol* 98, 44–50. [PubMed: 20153061]
- Tietge JE, Degitz SJ, Haselman JT, Butterworth BC, Korte JJ, Kosian PA, Lindberg-Livingston AJ, Burgess EM, Blackshear PE, and Hornung MW (2013). Inhibition of the thyroid hormone pathway in *Xenopus laevis* by 2-mercaptobenzothiazole. *Aquat. Toxicol* 126, 128–136. [PubMed: 23178179]
- U.S. National Research Council Subcommittee on Laboratory Animal Nutrition. (1995). Chapter 2, Nutrient Requirements of the Laboratory Rat In *Nutrient Requirements of Laboratory Animals*, 4th ed., pp. 11–79. National Academies Press (US), Washington, DC Available from <https://www.ncbi.nlm.nih.gov/books/NBK231925/>, last accessed on 08/03/2018.
- Zimmermann MB (2009). Iodine Deficiency. *Endocrinol. Rev* 30, 376–408.
- Zoeller RT, Tan SW, and Tyl RW (2007). General background on the hypothalamic-pituitary-thyroid (HPT) axis. *Crit. Rev.Toxicol* 37, 11–53. [PubMed: 17364704]

Figure 1.

Iodotyrosine deiodinase (IYD) mRNA expression measured by qPCR in select *Xenopus laevis* tissues (thyroid gland [A], liver [B], kidney [C], intestine [D]) across a developmental series from NF54 to NF66, copies IYD mRNA per ng extracted total RNA. Mean (bars) and standard error (whiskers), n=3 biological replicates except where noted (n), with each replicate containing pooled tissue samples from 5 individuals. Letters indicate expression levels that were significantly different ($p < 0.05$). NA = not applicable (no tissue collected at that stage).

Figure 2.

The effect of aqueous exposure to 3-nitro-L-tyrosine (MNT) on glandular and circulating thyroxine (T4) [A, C] and triiodothyronine (T3) [B, D] in *Xenopus laevis* larvae. Mean (bars) and standard error (whiskers), n=3 replicate tanks per treatment (except for 200 mg/L where n=2 tanks) with samples containing pooled glands or plasma from larvae within each tank. Asterisks indicate statistical significance (p<0.05) from control. Samples below the limits of quantification were estimated at one-half the detection limit for analysis and display. Note that scale of the y-axis varies for each panel. NA = not applicable (no samples collected at sampling point).

Figure 3.

The effect of aqueous exposure to 3-nitro-L-tyrosine (MNT) on glandular and circulating diiodotyrosine (DIT) [A, C] and monoiodotyrosine (MIT) [B, D] in *Xenopus laevis* larvae. Mean (bars) and standard error (whiskers), n=3 replicate tanks per treatment (except for 200 mg/L where n=2 tanks) with samples containing pooled glands or plasma from larvae within each tank. Asterisks indicate statistical significance (p<0.05) from control. Samples below the limits of quantification were estimated at one-half the detection limit for analysis and display. Note that scale of the y-axis varies for each panel. NA = not applicable (no samples collected at sampling point).

Figure 4.

Histopathological effects of exposure to 3-nitro-L-tyrosine (MNT) on *Xenopus laevis* thyroid glands. Control glands [A] showed mild to moderate follicular cell hypertrophy and hyperplasia (n=6 larvae). Exposure to 66.7 or 200 mg/L MNT [B] resulted in glands with mild to severe follicular cell hypertrophy and hyperplasia and colloid depletion in all specimens (n=20 larvae for 66.6 mg/L, n=15 larvae for 200 mg/L). In the rescue treatment (200 mg/L MNT + 10 µg/L KI) [C] mild diffuse hypertrophy, mild follicular cell hypertrophy, and mild colloid depletion were only observed in one individual (n=6 larvae). Scale bars indicate 100 µm.

Figure 5.

Sodium iodide symporter (NIS) and iodotyrosine deiodinase (IYD) mRNA expression measured by qPCR in *Xenopus laevis* thyroid glands after exposure to 3-nitro-L-tyrosine (MNT): copies mRNA for each gene per ng total RNA extracted for NIS [A] and IYD [B]. Bars = mean, whiskers=standard error, n=3 replicate tanks per treatment except for 200 mg/L where n=2 replicate tanks. Asterisks indicate statistical significance ($p < 0.05$) from control.

Figure 6.

Representation of the putative Adverse Outcome Pathway (AOP): Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis [A] with the supporting results for several key events and the adverse outcome from the *in vivo* exposure of larval *Xenopus laevis* to 3-nitro-L-tyrosine (MNT) during metamorphosis [B]. White box is molecular initiating event; light gray boxes are key events; dark gray box is organismal adverse outcome

Table 1.

Measured aqueous concentrations of 3-nitro-L-tyrosine (MNT) in stock solution and exposure tanks.

Treatment	Nominal concentration of MNT (mg/L)	Measured concentration of MNT (mg/L)				Percentage (%) of chemical remaining at 24h, mean $\pm$ SD
		Pre-exposure	Stock and Solution in exposure tanks ^a , mean $\pm$ SD	Solution in exposure tanks after 24h ^a , mean $\pm$ SD	NA	
Stock solution	2000	NA	2017.11 $\pm$ 45.93	NA	NA	NA
Control	-	0	0	0	NA	NA
7.4 mg/L	7.4	6.62	7.87 $\pm$ 1.36	4.27 $\pm$ 1.83	53 $\pm$ 18%	
22.2 mg/L	22.2	22.21	22.68 $\pm$ 0.46	20.36 $\pm$ 0.74	90 $\pm$ 3%	
66.7 mg/L	66.7	65.87	69.41 $\pm$ 0.48	67.21 $\pm$ 1.81	97 $\pm$ 2%	
200 mg/L	200	199.38	211.24 $\pm$ 2.22	212.33 $\pm$ 4.92	101 $\pm$ 2%	
Rescue: 200 mg/L MNT + 10 μg/L KI	200	204.98	211.25 $\pm$ 1.91	212.18 $\pm$ 5.16	100 $\pm$ 2%	

^a n = 4 sampling events for stock, control, 7.4 mg/L, 22.2 mg/L, and Rescue; n=5 sampling events for 66.7 mg/L and 200 mg/L; MNT concentration was measured in all exposure tanks at each sampling event, with mean concentration calculated for each treatment (n=3 tanks for each treatment except 200 mg/L where n=2 tanks).

NA = not applicable

Table 2. *Xenopus laevis* iodotyrosine deiodinase (IYD) primers and probes used to for RNA standard generation and qPCR.

Primer/Probe	Sequence (5'-3')
RNA standard generation; outer forward	AAGGAAGAGTGGCAAGACCTAGAG
RNA standard generation; outer reverse	AAGATAAGGAATGGAGCTGTGTCC
RNA standard generation; middle forward	TAATACGACTCACTATAGGGAGGTGTTGTCACGTTCCATTGTC
RNA standard generation; middle reverse	CTAACCACCTTGCTCTCCCATCTT
qPCR forward	CATCAGAACAGCAGGGACATCTC
qPCR reverse	TGGGTCTTGCACAAACAACAAA
qPCR probe	CAGTGGAGCCCATACTGAACCCCTGGA

Table 3.

Development of *Xenopus laevis* larvae following exposure to 3-nitro-L-tyrosine (MNT), with Nieuwkoop and Faber (NF) developmental stages observed on days 7 and 13, percent of larvae that reached NF58 and NF62, and the median, minimum, and maximum number of days it took larvae to reach NF58 and NF62; n=3 tanks for each treatment except 200 mg/L where n=2 tanks.

Treatment	Day 7 NF Stages	Day 13 NF Stages	NF58 % of larvae to reach stage	NF58 # of days to reach stage, median (min-max)	NF58 % of larvae to reach stage	NF62 % of larvae to reach stage	NF62 # of days to reach stage, median (min-max)
Control	53-55	55-56	100%	21 (18-24)	100%	100%	29 (26-34)
7.4 mg/L	53-54	55-56	100%	21 (19-25)	100%	100%	29 (26-34)
22.2 mg/L	53-54	55-56	100%	21 (17-25)	100%	100%	29 (25-34)
66.7 mg/L	53-54	55-56	100%	21 (18-25)	100%	80% ^a	- ^a
200 mg/L	53-54	55-56	100%	22 (19-25)	100%	40% ^a	- ^a
Rescue: 200 mg/L MNT + 10 µg/L KI	53-54	55-56	100%	21 (17-25)	100%	100%	29 (25-35)

^aThe remaining larvae did not reach NF62 within 7 days after all larvae in control and rescue treatments had reached NF62. In 66.7 mg/L MNT treatment, 20% of larvae did not reach NF62. In 200 mg/L MNT treatment, 60% of larvae did not reach NF62. Those that did not reach NF62 before this time were censored from days-to-NF62 and analyses. Development appeared delayed and altered in these larvae as described in the Results section.

Table 4.

Prevalence (%) and severity of select histological observations in *Xenopus laevis* thyroid glands after larval exposure to 3-nitro-L-tyrosine (MNT), with a subset from control (n=6 larvae), a subset from the rescue treatment (n=6 larvae), and all larvae from the highest two MNT concentrations (66.7 and 200 mg/L MNT) were collected at NF62 or study termination.

Observation	n	Severity	Control		66.7 mg/L MNT		200 mg/L MNT		Rescue: 200 mg/L MNT + 10 µg/L KI	
			NF62		NF62	<NF62 ('arrested')	NF62	<NF62 ('arrested')	NF62	
	6		16	4	6	9	6	9	6	
Thyroid gland hypertrophy	100	Not remarkable	13	0	67	0	83			
	0	Mild	75	0	33	11	17			
	0	Moderate	13	25	0	22	0			
	0	Severe	0	75	0	67	0			
Follicular cell hypertrophy	100	Not remarkable	0	0	0	0	83			
	0	Mild	0	0	0	0	17			
	0	Moderate	0	0	0	0	0			
	0	Severe	100	100	100	100	0			
Follicular cell hyperplasia	17	Not remarkable	0	0	0	0	100			
	67	Mild	0	0	0	0	0			
	17	Moderate	0	0	0	0	0			
	0	Severe	100	100	100	100	0			
Follicular lumen decrease	100	Not remarkable	0	0	0	11	100			
	0	Mild	13	50	0	11	0			
	0	Moderate	0	25	0	44	0			
	0	Severe	88	25	100	33	0			
Colloid depletion	100	not remarkable	0	0	0	0	83			
	0	Mild	0	25	0	33	17			
	0	Moderate	6	50	0	33	0			
	0	Severe	94	25	100	33	0			