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Impaired Anterior Swim Bladder Inflation Following Exposure to the Thyroid Peroxidase Inhibitor 2-Mercaptobenzothiazole Part I: Fathead Minnow

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Abstract

In the present study, a hypothesized adverse outcome pathway linking inhibition of thyroid peroxidase (TPO) activity to impaired swim bladder inflation was investigated in two experiments in which fathead minnows (*Pimephales promelas*) were exposed to 2-mercaptobenzothiazole (MBT). Continuous exposure to 1 mg MBT/L for up to 22 d had no effect on inflation of the posterior chamber of the swim bladder, which typically inflates around 6 days post fertilization (dpf), a period during which maternally-derived thyroid hormone is presumed to be present. In contrast, inflation of the anterior swim bladder, which occurs around 14 dpf, was impacted. Specifically, at 14 dpf, approximately 50% of fish exposed to 1 mg MBT/L did not have an inflated anterior swim bladder. In fish exposed to MBT through 21 or 22 dpf, the anterior swim bladder was able to inflate, but the ratio of the anterior/posterior chamber length was significantly reduced compared to controls. Both abundance of thyroid peroxidase mRNA and thyroid follicle histology suggest that fathead minnows mounted a compensatory response to the presumed inhibition of TPO activity by MBT. Time-course characterization showed that fish exposed to MBT for at least 4 d prior to normal anterior swim bladder inflation had significant reductions in anterior swim bladder size, relative to the posterior chamber, compared to controls. These results, along with similar results observed in zebrafish (see part II, this issue) are consistent with hypothesis that thyroid hormone signaling plays a significant role in mediating anterior swim bladder inflation and development in cyprinids and that role can be disrupted by exposure to thyroid hormone synthesis inhibitors. Although anterior swim bladder inflation has not been directly linked to survival as posterior swim bladder inflation has, potential links to adverse ecological outcomes are plausible given involvement of the anterior chamber in sound production and detection.

Abbreviations

ANOVA, analysis of variance; AOP, adverse outcome pathway; CVs, coefficients of variance; dpf, days post-fertilization; FELS, fish early life stages; hpf, hours post-fertilization; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LSW, Lake Superior water; MS-222, tricaine methanesulfonate; MBT, 2-mercaptobenzothiazole; RSCABS, Rao-Scott adjusted Cochran-Armitage test for trend by slices; T₃, triiodothyronine; T₄, thyroxine; TPO, thyroid peroxidase

Keywords: adverse outcome pathway; cyprinid; endocrine disruption; swim bladder; fish early life stage

1. Introduction

In ecological risk assessment, one of the primary tools used to assess chemical hazard is the OECD 210 fish early-life stage (FELS) test. Following the guidelines published in 1992 (OECD, 1992), the FELS test generally involves exposing over 360 fish to a test chemical for 1 to 3 months, depending on species (Oris et al., 2012). There are a range of possible test species, including rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*) and fathead minnows (*Pimephales promelas*). Key endpoints include hatching success, growth, and survival, all of which can be related to potential population-level impacts. However, despite its widespread use, the FELS test has important limitations. First, because of its minimum 30 d duration, the test is low throughput and requires substantial resources in terms of exposure characterization, maintenance, and husbandry. Additionally, because it focuses only on apical outcomes, the FELS test provides little or no information concerning chemical mechanisms or modes of action (MOA). This limits the utility of the results for potentially inferring impacts of related chemicals acting through the same MOA. Consequently, there is an interest in developing alternatives to the FELS test that would require fewer resources, increased throughput, and provide relevant mechanistic information that could help support the development and application of more predictive approaches to risk assessment (Volz et al., 2011; Villeneuve et al., 2014).

One proposed alternative to the FELS would utilize information from in vitro, high-throughput screening assays to identify a chemical's molecular target(s). Once a chemical is known to target a certain protein or pathway, potential downstream effects associated with that perturbation could be inferred based on previous knowledge, using adverse outcome pathways (AOPs) as conceptual frameworks to guide this prediction (Ankley et al., 2010). Adverse outcome pathways link molecular initiating events (MIEs) to adverse outcomes of demographic significance through measurable key events at increasing levels of biological organization (Villeneuve et al. 2014). Pairing molecular target information with corresponding AOP(s) has potential to provide a rapid and low-cost indication of chemical hazard. This could help inform prioritization of chemicals for testing based on the most relevant hazards.

A workshop which focused on strategies related to the development of AOPs relevant to FELS testing was held in 2012. An overall strategy for AOP development built around key morphological events during fish development was illustrated using the event of swim bladder inflation (Villeneuve et al., 2014). The fathead minnow swim bladder is relatively easy to observe in semi-transparent larval fish under a light microscope. The swim bladder begins as a single posterior chamber which inflates soon after hatching, around 5-6 days post fertilization (dpf). Around 14 dpf, a second chamber is formed anterior to the original chamber by evagination (Robertson et al., 2007). Inflation of the posterior swim bladder has been shown to be important for maintaining buoyancy and is considered critical for young-of-year survival (Czesny et al., 2005; Robertson et al., 2007; Woolley and Gin, 2010). However, in a laboratory setting where food resources are not limited and efficient swimming is not required to avoid predation and/or capture prey, impacts of disrupted swim bladder inflation may not manifest as apical impacts on either growth or survival. Development of AOPs that link specific MIEs, which could be detected in either high throughput in vitro assays or shorter-term fish embryo tests, to the key event of

impaired swim bladder inflation, would therefore have useful predictive value and application in ecological risk assessment.

The example provided by Villeneuve et al. (2014) proposed a linkage between glycogen synthase kinase 3 beta (GSK3B) inhibition and impaired swim bladder development and inflation. However, it was noted that this MIE, which can impair WNT signaling, is not necessarily specific to swim bladder development and, in all likelihood, would lead to a variety of other adverse effects that would be lethal to an embryo prior to swim bladder inflation. Here we consider disruption of thyroid hormone signaling as a possibly more relevant mechanism through which swim bladder inflation may be perturbed. Thyroid hormones are known to regulate vertebrate development and its role in processes such as amphibian metamorphosis has been well studied. Fish also go through a transition in development from embryos to mobile larval fish that is thought to be thyroid-mediated. Previous work has shown that disruption of thyroid hormone levels can affect the embryo to larval transition in multiple ways, including inhibiting swim bladder maturation (Liu and Chan, 2002).

The present research, conducted with a known thyroid disrupting chemical, 2-mercaptobenzothiazole (MBT), was designed to evaluate a hypothesized AOP linking thyroid axis disruption and impaired swim bladder inflation. This chemical has been shown to disrupt the thyroid axis both in vitro and in vivo by inhibiting thyroid peroxidase (Tietge et al., 2013; Hornung et al., 2015). Thyroid peroxidase (TPO) activity is localized in thyroid follicles and plays a critical role in the conversion of iodide to iodine in order to support synthesis of thyroid hormones. Two types of thyroid hormone are produced: thyroxine (T_4) and triiodothyronine (T_3), which is generally the more active form, generated through peripheral deiodination of T_4 . Consequently, based on knowledge of thyroid function, we hypothesized that the MIE of TPO inhibition would lead to decreased concentrations of T_4 and T_3 (key events; KEs), which in turn could impair swim bladder inflation (another KE). Depending on the chamber affected, impaired inflation may be either directly or indirectly linked to the adverse outcome of

reduced survival (an adverse outcome of regulatory significance; e.g., Wooly and Qin, 2010; Czesny et al. 2005).

This hypothesized AOP was tested in a series of experiments conducted with two cyprinid fish species, fathead minnow (detailed in the present paper) and zebrafish (Stinckens et. al., this issue). Here we report results of two complementary experiments conducted to examine links between MBT exposure and impaired anterior and/or posterior swim bladder inflation in early-life stage fathead minnows. The first experiment was designed to test the hypothesis that continuous exposure to MBT would reduce circulating T_3 and T_4 concentrations during fish early life stages, leading to dose-dependent impairment of swim bladder inflation. This was followed by a second experiment designed to pinpoint the specific developmental window(s) in which early life stage fathead minnows were susceptible to the effects of TPO inhibition on swim bladder inflation. In addition to measuring the direct effects of exposure on KEs, we assessed endpoints reflecting the ability of the fish to compensate for perturbation of the system, a key consideration in the context of AOP development and endpoint interpretation (e.g., Ankley and Villeneuve 2015). Vertebrates are known to compensate for thyroid hormone deficiencies through increases in serum thyroid stimulating hormone concentrations and proliferation of thyroid tissue (e.g., Degitz et al., 2005; Tietge et al., 2005; Opitz et al., 2006; Zoeller et al., 2007; Tietge et al., 2013).

2. Materials and methods

2.1. Exposure Design and Analytical Verification (Experiment 1)

Our first experiment was designed to evaluate the impacts of MBT on fathead minnows exposed continuously from just hours post fertilization (hpf) through 21 dpf. Three concentrations of MBT along with a control were tested using four replicate tanks per treatment. Solvent-free stock solutions (80

mg/L) of MBT (CAS 149-30-4, >99% pure, Sigma-Aldrich, St. Louis, MO) were prepared by dissolving neat chemical in filtered, UV treated, Lake Superior water (LSW). Stock solution was delivered to a mixing cell, diluted, and continuously distributed to exposure tanks (38 ± 6 ml/min) at nominal concentrations of 0, 0.25, 0.5, and 1 mg MBT/L over the 21 d study. Flow-rate of stock chemical delivery to the mixing cells and tank temperatures was recorded daily. Every 4 d pH, dissolved oxygen, conductivity, and flow rates to each tank were recorded. MBT concentrations in the stock solution and test tanks were determined at least every 4 d using high performance liquid chromatography (HPLC). Daily measurements over the first 9 days of exposure showed stock concentrations to be highly stable, thereafter stock concentrations were verified only when a new stock solution was prepared. The exposure unit was kept on a 16/8h light/dark cycle at $25 \pm 1^\circ\text{C}$ over the duration of the study.

In order to verify exposure concentrations, water from the stock or exposure tanks was collected and directly injected as 50 μL aliquots onto a Kinetex C18 50 x 2.1 mm column (Phenomenex Inc., Torrance, CA) using an Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) HPLC with diode-array detection at 235 nm. Analysis was performed under isocratic conditions (65:35 water: acetonitrile+10 mM acetic acid) at 0.4 mL/min. The retention time of MBT was 1.2 min. The method demonstrated acceptable accuracy and precision through analysis of spiked samples ($n=6$, $102 \pm 1.3\%$ [mean $\pm$ SD]) and tank duplicates ($n=21$, $98.8 \pm 1.3\%$). The method detection limit was 0.001 mg/L.

2.2. Embryo Collection and Exposure Initiation (Experiment 1)

Newly fertilized (< 8 hpf) fathead minnow embryos were collected from breeding pairs maintained at an on-site culture at the Duluth EPA laboratory. Viable embryos were pooled and then randomly distributed in groups of 40 to glass jars with a coarse (ca. 1000 μm) polyester mesh bottom, hereafter referred to as baskets. To obtain enough newly fertilized embryos the study was initiated over

a span of 2 d, loading replicates 1 and 2 on the first day and replicates 3 and 4 on the second day. The two groups received consistent treatment throughout the 21 d exposure.

Four baskets were placed in each tank to initiate the exposure. Baskets were removed from tanks after all fish had hatched. Any dead embryos and larval fish were removed and counted.

Following hatching, larval fish were fed to satiation with newly hatched brine shrimp twice daily (Bio-Marine, Inc., Hawthorne, CA) except on the day of sampling.

2.3. Sampling (Experiment 1)

Fish from experiment 1 were sampled at four different time points (2, 6, 14, and 21 dpf). Time points were selected to correspond with anticipated timing of swim bladder inflation and increases in thyroid hormone concentrations (Crane et al., 2004). Sampling occurred by tank with replicates 1 and 2 of each MBT concentration and controls sampled one day and the last two replicates for each concentration and associated controls sampled on the subsequent day due to accommodate the staggered start dates.

After 2 days of exposure, 10 embryos were removed from each of the four baskets in a tank and combined into a group of 40 embryos from each tank. Each embryo was observed for general health and development and 20 individuals were allocated for RNA isolation and the remaining 20 fish allocated for whole-body T_3/T_4 analysis. All samples were flash-frozen in liquid nitrogen and stored at -80°C until subsequent analyses.

On days 6, 14, and 21, larval fish were sampled from each tank ($n=20, 14$, and 10 , respectively). Fish were anesthetized with MS-222 (100 mg/L buffered with $200\text{ mg NaHCO}_3/\text{L}$), observed for general health and swim bladder inflation, and photographed. Individuals were split into two even groups, one for RNA isolation and the other for whole-body T_3/T_4 analysis. On days 14 and 21 fish allocated for RNA isolation were sectioned to allow the subsequent RNA sample to be more specific to the thyroid (located

in head section) or swim bladder (located in abdomen section; Figure S1). All samples were flash-frozen in liquid nitrogen and stored at -80°C.

2.4. Histopathological Analysis of Thyroid Follicles and Swim Bladder (Experiment 1)

A subset of larval fish from the control and each treatment ($n = 8$ per treatment) were sampled on days 14 and 21 for histopathological assessment of the thyroid follicles. Euthanized fish were fixed for 24 hours in Bouin's fixative, processed according to routine paraffin histology methods, and embedded to provide 4 μm thick H&E stained sagittal sections of the whole fish. Each fish was evaluated for histopathology of the swim bladder and other tissues in the visceral cavity, as well as the thyroid follicles. Pathology evaluations compared histomorphology in treated fish to that of controls. Thyroid follicles were evaluated for follicular cell hypertrophy (cell size), hyperplasia (cell number), lumen area, colloid depletion, colloid quality, and the presence of ectopic follicles. Observed histopathology was scored for severity based on criteria adapted from the Amphibian Metamorphosis Assay (Grim et al., 2009; Blackshear USEPA). A description of the adapted diagnostic criteria for scoring each pathology is provided in the supplementary text (Text S1).

2.5. Whole-body T_3 and T_4 Measurements (Experiment 1)

Whole-body T_3 and T_4 were extracted from embryos (day 0) and larval fish (6, 14, 21 dpf) following a modified procedure described by (Crane et al., 2004). Briefly, samples were spiked with 0.5 ng of both $^{13}\text{C}_6\text{-T}_3$ and $^{13}\text{C}_6\text{-T}_4$ to serve as internal standards for quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Hornung et. Al., 2015). Samples were homogenized with 1 mL of 1 mM propylthiouracil in ethanol using a ball mill homogenizer (3 min, 30 Hz), centrifuged (20800 g, 10 min), and the supernatant collected. The extraction was repeated and supernatants were combined. The extract was diluted with 300 μL LC-MS grade water, washed with 1 mL hexane, and then evaporated under nitrogen. Extracts were resuspended in 2 mL 90:10:0.2 water:acetonitrile:formic acid

and quantitatively loaded onto preconditioned Evolute CX (Biotage, Charlotte, North Carolina) solid phase extraction (SPE) cartridges (50 mg, 3 mL). Cartridges were rinsed sequentially with 3 mL 2% formic acid in water followed by 3 mL of methanol. Thyroid hormones were eluted from the column with 2.4 mL (2 x 1.2 mL) of freshly prepared 5% NH₄OH in methanol. Extracts were evaporated to dryness under nitrogen, reconstituted in 100 µL 75:25:0.1 water:acetonitrile:formic acid, and transferred to amber autosampler vials for analysis. Thyroid hormone analysis and quantification was performed by LC-MS/MS as previously detailed (Hornung et. al., 2015). Method blanks (n = 8), non-spiked larval fish (n = 6), and spiked larval fish (n = 6, spiked with 0.25 ng T₃ and T₄) were analyzed with each sample batch to ensure accurate quantification of target analytes. Spike concentrations were calculated by subtracting endogenous hormone concentration determined in non-spiked larval fish from spiked larval fish. All method blanks remained below detection for both T₃ and T₄ while recoveries from the spiked larval fish averaged 112±7% and 104±7% for T₃ and T₄, respectively. For statistical analysis, concentrations of T₃ and T₄ below detection limits were assigned a value of one-half the limit of quantitation (0.25 ng) before calculating final the concentration on a ng/g basis.

2.6. Exposure Design and Analytical Verification (Experiment 2)

For experiment 2, early-life stage fathead minnows were exposed to MBT for varying periods with three replicate tanks per treatment group and four replicate tanks for both the MBT positive and negative LSW controls based on a design previously employed by Hagenaaars et al. (2014; Figure S2). The MBT stock solution (in this case 200 mg/L) was prepared as described for experiment 1 and delivered continuously to a mixing cell at a target flow rate of 3.6 mL/min. Diluted stock solution (final concentration 1 mg/L) or control LSW water was continuously dispensed into sixteen 10 L exposure tanks at 45±6mL/min. The exposure unit was kept on a 16/8h light/dark cycle at 25±1°C throughout the 22 d duration of the study.

On days when chemical exposure started (Figure S2), the control LSW was drained from the tank until the volume was ~1 L. Tanks were then moved to a new location in the exposure unit and allowed to fill with MBT exposure water, which reached the target concentration of 1 mg MBT/L within 4 hours (Table S1). On days when a chemical exposure ended, the tanks were similarly drained and transferred to LSW. The concentration in the tanks was below 0.1 mg MBT/L within 2 h and below the detection limit of 0.001 mg/L within 21 h.

Flow-rate of stock chemical delivery to the mixing cell, flow-rate of test solution to tanks, and tank temperatures were monitored and recorded daily. Dissolved oxygen, pH, and conductivity were recorded for each tank every 5 d. The MBT concentration in the test tanks was determined every 3-5 days and stock concentrations were analyzed periodically using the same methods outlined for experiment 1. Tank duplicates (n=12) had 99.3±0.7% agreement throughout the study.

2.7. Embryo Collection and Exposure Initiation (Experiment 2)

Test initiation was similar to that described for experiment 1, with slight modifications. In experiment 2, the mesh on the baskets was replaced with a finer polyester mesh (500 µm) to visualize the embryos more readily and prevent larval fish from swimming out of the baskets upon hatch. Viable embryos up to 12 hpf were used. Once embryos were pooled, they were randomly distributed to fill each basket with 15 embryos and all replicates were initiated on the same day. Once all baskets were loaded, two were placed in each tank. Larval fish were fed to satiation with newly hatched brine shrimp (Bio-Marine, Inc., Hawthorne, CA) twice daily except on days where fish were to be photographed or sampled.

2.8. Assessment of Swim Bladder Inflation (Experiment 2)

To assess development of the swim bladder, all fish were removed from each tank on days 14, 18, and 22. Imaging occurred by tank starting with all tanks in replicate 1, followed by replicate 2, and so

on. Images were rapidly captured via video using an Alpha NEX 7 Digital Camera (Sony Electronics Inc., San Diego, CA), followed by immersing the fish immediately in the appropriate treatment water and returning them to their original tank. On day 22, fish were anesthetized with MS-222 and images were taken. Fish were then flash-frozen in liquid nitrogen, and stored at -80°C for gene expression analysis.

2.9. Swim Bladder Inflation Analysis (Experiments 1 and 2)

Morphological differences in inflated anterior swim bladders were quantified using Image-Pro Premier v6.2 software (Media Cybernetics, Inc., Rockville, MD). For fish that inflated their anterior swim bladder, the longest portion of the anterior and posterior swim bladder was measured to calculate a ratio for each fish by dividing the length of the anterior by that of the posterior swim bladder.

2.10. RNA Extraction and Quantitative Real-time PCR Analyses (Experiments 1 and 2)

Relative abundance of thyroid peroxidase (*tpo*) mRNA transcripts was evaluated using quantitative real-time polymerase chain reaction (qPCR). For each time point in experiment 1, embryos or larval fish were pooled from each tank for qPCR analyses resulting in four biological replicates per treatment. Due to increasing size, the number of fish pooled from each tank decreased as sampling continued (i.e., N= 20, 10, 7, and 5 on day 2, 6, 14, and 21 respectively). RNeasy Micro and Mini Plus kits (Qiagen, Valencia, CA), which include on-column genomic DNA removal, were used for RNA isolation from embryos and larval fish, respectively. The manufacturer's protocols were followed with a slight modification to the RNeasy Plus Micro kit that included a second 80% ethanol wash immediately after the first 80% ethanol wash to further remove salts and improve RNA purity. RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and RNA was diluted to 10 ng total RNA/μL with RNase-free water for use in qPCR assays. Quality of 24 randomly selected RNA samples (n=12 from experiments 1 and 2, respectively) were evaluated using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) with an RNA 6000 Nano Kit (Agilent). RNA

integrity numbers ranged from 7.2-9.8 with a mean of 8.3. All RNA was stored at -80°C until subsequent use.

All *tpo* qPCR assays were single-step, real-time PCR assays conducted in a 96-well format using TaqMan RNA-to-C_T 1-Step Kit (Life Technologies, Grand Island, NY). Each 20 µL reaction well contained 20 ng total RNA, 6 pmol of each primer (forward GGAAGTGGGAAGCCGTTGTAT [Sense] and reverse AGATGGATCCCTGCGGAGTA [AntiSense]), and 0.3 pmol of probe (CGTTCAGCCTTTGGGTTGGTTT [Sense]). Amplicon size was 86 base pairs and sequences were obtained from draft fathead minnow genome assemblies (Burns et al. 2015) using reciprocal best hit Blast against *Danio rerio* sequences to confirm sequence identity (E value 2e-44; 91% identity relative to NCBI Accession XM_009292775.1, Predicted: *Danio rerio* thyroid peroxidase (*tpo*), mRNA).

Real-time qPCR assays were conducted using a 7500 Real-Time PCR system with a cycling protocol of 48°C for 15 min, and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A standard curve of known molar quantities of *tpo* mRNA standard was used to interpolate quantity values. Replicate (n=2) analyses were conducted for a minimum of six samples per assay to estimate intra-assay variability. Coefficients of variation (CVs) were generally < 10% and never exceeded 20%. Samples from each time point in experiment 1 were analyzed in the same assay, with one 96-well plate containing samples from day 2 and day 6 and the other plate containing samples from day 14 and day 21 to eliminate the need to consider inter-assay variability. Samples from experiment 2 were analyzed in a separate assay on one 96-well plate. Amplification efficiencies ranged from 96-98%.

2.11 Statistical Analysis

Data for survival, T₃ and T₄ measurements, swim bladder inflation, and gene expression were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test to determine significant differences among treatments within each time point. Data normality and homogeneity of

variance were assessed using the Kolmogorov-Smirnov and Levene's tests, respectively. Data were log-transformed if they did not meet the assumptions of the ANOVA, except for swim bladder inflation (percent) data which were arcsin transformed. Differences among treatments were considered significant at $p < 0.05$. Statistical analyses were conducted using Statistica 12 (Statsoft, Inc., Tulsa, OK) and GraphPad Prism v 5.02 (GraphPad Software, La Jolla, CA). Statistical analyses of the histopathological findings, were performed using the Rao-Scott adjusted Cochran-Armitage test for trend by Slices (RSCABS; Green et al., 2014) to identify significant differences in the prevalence at specified severities of each pathology across treatments at days 14 and 21. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Exposure Verification (Experiments 1 and 2)

There was no detectable MBT in the control water throughout experiments 1 and 2. Measured MBT concentrations over the course of experiment 1 were generally within 80-118% of nominal (Table S2). However, on day 20 (day 19 for replicates 3 and 4) a power outage led to a sequencer failure. This resulted in no MBT solution being delivered to the test tanks for a period of approximately 12 hours. Following this delivery outage, concentrations fell to approximately 3-6% of nominal before recovering to target levels within 8 hours of chemical delivery being reinitiated (Table S2). Measured MBT concentrations over the course of experiment 2 were consistently within 97-107% of nominal (Table S3).

3.2. Survival (Experiments 1 and 2)

On a per tank basis, survival over the course of experiment 1 ranged from 68%-84%. In experiment 2 survival ranged from 43% to 80%. There were no significant treatment-related differences in survival for either experiment (Figure S3). Mortality was greatest within the first 48 h after the initiation of the experiments suggesting handling stress to the embryos was the likely cause of most mortality.

3.3. *tpo* mRNA Expression (Experiments 1 and 2)

Based on the hypothesis that *tpo* expression levels would be increased as part of a compensatory feedback response to TPO inhibition, *tpo* mRNA expression levels were analyzed as an indirect measure of TPO inhibition by MBT (Figure 1). There were no significant differences in *tpo* mRNA abundance between treatment groups on days 2 or 6 in experiment 1. However, on sampling day 14, there was a significant concentration-dependent increase in abundance of *tpo* mRNA, with fish exposed to 1 mg/L MBT having 8-fold higher levels of *tpo* mRNA than the control fish (Figure 1A). On sampling day 21, fish exposed to the 1 mg/L MBT treatment continued to have significantly higher *tpo* mRNA levels compared to the control fish, but the concentration-dependence was no longer evident (Figure 1A). This discrepancy in the apparent concentration-dependence of the response may be related to the system shutdown that occurred on day 20/19 (see “exposure verification” section above).

In experiment 2, levels of *tpo* mRNA on day 22 were evaluated. The abundance of *tpo* transcripts was significantly greater in fish that had been exposed to MBT continuously from 0-22 dpf or 6-22 dpf (Figure 1B). The *tpo* mRNA levels generally appeared to increase with increasing duration of MBT exposure. However, in all groups where MBT treatment ceased prior to day 22, no significant increase in *tpo* mRNA levels was detected.

3.4. Histopathology (Experiment 1)

Histopathological examination revealed a concentration-dependent increase in prevalence and severity of follicular cell hypertrophy and hyperplasia in fish exposed to MBT for 14 d (Figure 2; Tables S4, S5). Fish exposed to 0.5 mg/L and 1.0 mg/L MBT had a statistically greater prevalence of both follicular cell hypertrophy and hyperplasia at both mild and moderate severity as compared to the control fish. Histopathological examination also revealed a statistically significant impact on decreased lumen area and colloid depletion in fish exposed to 1 mg/L MBT for 14 d with both mild and moderate

decreases in lumen area and depletion of colloid. Qualitative assessment of the colloid identified pale, lacy, and granular appearing colloid in less than half of the fish exposed to 0.5 mg MBT/L and nearly all fish exposed to 1 mg MBT/L. rather than the homogenous, brightly staining colloid observed in the control fish. There were no ectopic follicles observed in any of the fish exposed to MBT for 14 d. Likewise, examination of the liver and the epithelium of the swim bladder did not reveal any significant pathology in fish exposed to MBT for 14d compared to controls.

The histopathological examination identified significant impacts on follicular cell hypertrophy and hyperplasia in fish exposed to 0.5 or 1 mg/L MBT for 21 d, but overall there were less pronounced effects observed at 21 d compared to 14 d (Tables S6, S7). For example, fish exposed to 0.5 mg/L for 21 d displayed statistically significant follicular cell hypertrophy and follicular cell hyperplasia with mild severity. Two fish exposed to 1 mg/L MBT for 21 d had follicular cell hypertrophy that was of moderate severity while most (6 of 8) were mild. Fish exposed to either 0.5 mg/L MBT or 1 mg/L MBT also had a statistically greater prevalence of increased follicular lumen area with moderate severity. Changes in colloid quality were also less prevalent at 21 d than 14 d with less than half of the fish exposed to 1 mg/L MBT displaying a qualitative change in the colloid. Ectopic thyroid follicles were observed in 50% of fish exposed to 1 mg/L for 21 d with the follicles developing adjacent to the blood vessels in the gill arches as opposed to the follicles developing near the ventral aorta in the controls (Figure S4). Three of eight fish in the group exposed to 1 mg MBT/L for 21d exhibited some liver pathology which consisted of a relatively small area of hepatocyte hypertrophy, apoptosis, and cystic degeneration. Similar to d 14, histological examination of the epithelium of the swim bladder did not reveal any pathology in fish exposed to MBT for 21 d, relative to control fish.

3.5. Thyroid Hormone Concentrations (Experiment 1)

Whole-body T_3 and T_4 concentrations were examined to determine the direct impacts of TPO inhibition on thyroid hormone synthesis, a critical KE in the AOP. There were no differences in whole-body T_3 in embryos at the initiation of the experiment (Figure 3A). Whole-body T_3 concentrations were significantly reduced in fish exposed to 1 mg MBT/L at 14 d compared to the controls; however, by 21 d the T_3 had recovered, with no significant effect of MBT exposure on whole-body T_3 levels.

There were no differences in whole-body T_4 in embryos at the initiation of the experiment (Figure 3B). At 6 dpf, there was a significant reduction in whole-body T_4 in fish exposed to 1 mg MBT/L compared to the controls. Whole-body T_4 could not be reliably quantified for the fish exposed through 14 dpf due to poor recovery of $^{13}C_6$ - T_4 internal standard. However, whole-body T_4 concentrations in the 1 mg MBT/L exposed fish had recovered by 21 dpf, when all MBT treatments had significantly higher whole-body T_4 than the controls.

3.6. Swim bladder Inflation (Experiment 1)

In experiment 1, there were no significant treatment effects on posterior swim bladder inflation, with 95-100% of fish inflating their posterior swim bladder on or before 6 dpf (Figure 4A). However, an effect on anterior swim bladder inflation was observed at 14 dpf with significantly fewer fish exposed to 1 mg MBT/L having inflated their anterior swim bladder compared to control fish (Figure 4B). However, by day 21 all fish had inflated their anterior swim bladder (Figure 4C). Among the fish exposed to 0.5 and 1 mg MBT/L that did inflate their swim bladder, many had anterior swim bladders that were significantly smaller relative to the size of the posterior swim bladder than those of control fish (Figure 5A). For example, the average length (mean $\pm$ SD) of the anterior swim bladder in 14 dpf fish exposed to 1 mg MBT/L was $26 \pm 2\%$ of the posterior swim bladder length, as opposed to $49 \pm 12\%$ in control fish.

By day 21, although all fish had inflated their anterior swim bladder, some of the MBT-exposed fish continued to exhibit anterior swim bladders that were significantly smaller than the controls in

relative size (Figure 5B). The average length (mean $\pm$ SD) of the anterior swim bladder in fish exposed to 1 mg MBT/L MBT was $46\pm 8\%$ of the posterior swim bladder length, while the control fish anterior swim bladders had average length of $78\pm 4\%$ of the posterior swim bladder length.

3.7. Swim Bladder Inflation (Experiment 2)

Treatment groups exposed to MBT from the time of test initiation or from 6 dpf through 14 dpf (i.e., treatments 0-14, 0-18, 0-22, and 6-22 dpf) had significantly fewer individuals with inflated anterior swim bladders than control fish on day 14 of the study (Figure 6A). The effect was most pronounced for fish continuously exposed to MBT starting at study initiation. However, similar to the first experiment, the difference in inflation seen on day 14 was no longer evident by the end of the study. No significant differences in anterior swim bladder inflation percentages were detected by 18 or 22 dpf (Figure 6B and 6C). Nonetheless, a significant decrease in anterior swim bladder size, relative to posterior chamber size, was observed for fish either continuously exposed to MBT or those for which exposure began no later than 10 dpf. The effect was consistent throughout the study (i.e., at 14, 18, or 22 dpf; Figure 7).

4. Discussion

The present study was designed to test a hypothesized AOP linking the molecular initiating event of TPO inhibition to the adverse outcome of reduced survival, with the key events including a reduction in whole-body thyroid hormone levels and impaired swim bladder inflation. The AOP was tested using MBT, an environmentally relevant compound, which has been reported as a TPO inhibitor *in vivo* and *in vitro*. In addition, we evaluated the occurrence of potential compensatory changes in thyroid system of fish exposed to the putative TPO inhibitor.

4.1. MBT Inhibits TPO in the Fathead Minnow

MBT has been shown to inhibit TPO *in vitro* using porcine or rat thyroid glands (Paul et al., 2013; Hornung et al., 2015) and *in vivo* with *Xenopus laevis* (Tietge et al., 2013; Hornung et al., 2015), but has not been shown to inhibit TPO in any fish species. Methods for direct measurement of TPO inhibition in fathead minnow embryos or larval fish have not been developed. In contrast to porcine, rat, and *X. laevis* which have thyroid glands, fathead minnows, like most teleost fish, have individual thyroid follicles distributed close to the ventral aorta in the pharyngeal region (Wabuke-Bunoti and Firling, 1983). Consequently, it is technically difficult to dissect the diffuse follicles for *in vitro* measurements of TPO activity. Yet, it has been shown that compensatory changes in gene expression with complementary thyroid histopathology provide useful endpoints to detect thyroid-axis disruption by TPO inhibitors when direct measurements of TPO activity in thyroid tissue is impractical (Opitz et al., 2006). Therefore, changes in *tpo* mRNA levels and thyroid follicular histopathology were used as indirect measures of TPO inhibition by MBT in the fathead minnow.

Tpo mRNA levels in MBT-exposed fish in experiment 1 did not vary from control fish at day 2. The lack of effect of MBT on levels of *tpo* mRNA at 2 dpf is consistent with the embryos not producing endogenous T₄, as thyroid follicles are not detected in the fathead minnows until after 2 dpf (Wabuke-Bunoti et al., 1983). Previous results suggest that fathead minnows produce endogenous T₄, and likely have TPO activity before hatching (Crane et al., 2005), but we observed no effect on *tpo* mRNA expression at 6 dpf. We hypothesize that even if MBT were inhibiting TPO at this stage, a compensatory response (manifested in part as increased *tpo* mRNA abundance) may not be elicited due to the availability of maternally-derived T₄ (Power et al., 2001). Only after the maternally-derived T₄ was depleted would compensatory responses be invoked via negative feedback along the hypothalamic-pituitary-thyroid (HPT) axis. Alternatively, the fathead minnow hypothalamic-pituitary-thyroid (HPT) axis may not be well developed at this time point, and may not have a wholly effective feedback mechanism to compensate for the TPO inhibition. Compensatory responses to the model TPO inhibitor,

methimazole, have been reported in the fathead minnow, but were not examined prior to 28 dpf (Crane et al., 2006).

At 14 dpf a dose-dependent increase in *tpo* mRNA levels was observed, suggesting a compensatory response to the MBT exposure. Studies with *X. laevis* have shown significant increases in gene expression for genes involved in the thyroid axis following exposure to TPO inhibitors (Opitz et al., 2006; Tietge et al., 2013). Because *tpo* expression could not be normalized on a per cell basis, it is unclear whether increased *tpo* mRNA reflects activation of increased *tpo* expression at the cellular level, increased numbers of *tpo* expressing cells, or a combination of both. However, the histopathological analysis of the thyroid follicles also supports a compensatory response at day 14. The size and number of thyroid follicles often reflects the activity of the thyroid, with an increase in tall columnar cells rather than cuboidal epithelium indicating an increase in thyroid activity. There was a concentration-dependent increase in the follicular cell hypertrophy, hyperplasia, and colloid depletion (Table S4). Similar histopathological responses, suggestive of a compensatory response to thyroid axis disruption have also been observed in amphibians exposed to TPO inhibitors (Degitz et al., 2005; Tietge et al., 2005; Opitz et al., 2006; Tietge et al., 2013).

By day 21 there was no longer a concentration-dependent effect on *tpo* mRNA abundance and only fish exposed to the highest concentration of MBT showed an increase in *tpo* mRNA levels. The histopathological analyses also showed less impact on the prevalence and severity of many of the endpoints. It is not clear if the interruption in MBT delivery due to power failure on day 19-20 of the exposure contributed to the lack of concentration-dependent *tpo* mRNA response at day 21. However, it appears that the fish exposed to the lower concentrations of MBT were more readily able to compensate for the TPO inhibition by 21 d as compared to the fish exposed to the 1 mg MBT/L.

To better understand the timing of susceptibility of fathead minnow embryos or larvae to MBT exposure and to explore the temporal nature of putative compensatory responses, a second experiment was performed in which fish were exposed to 1 mg MBT/L for various times during a 22 d period. *Tpo* mRNA levels in experiment 2 were only elevated when fish were exposed to MBT through the day they were sampled. Additionally, levels of *tpo* mRNA were only increased in fish exposed to MBT for at least 16 days or more, suggesting a compensatory response is only observed when fish are continually exposed to the TPO inhibitor for a long period of time, and only after the HTP is active. For fish exposed to MBT from 0-6, 0-10, 0-14, and 0-18 days, 4 or more d of depuration/recovery in control LSW appeared to be enough to eliminate the compensatory response observed when fish were continuously exposed to 1 mg MBT/L.

4.2. MBT Reduces Thyroid Hormone Levels

The next hypothesized KE downstream of the inhibition of TPO by MBT, was a decrease in T_3 and T_4 concentrations. The whole-body thyroid measurements made on embryos and larval fish did not include a protease digestion for extracting the thyroid hormones present in the follicles. Therefore, similar to what has been reported previously (Crane et al., 2006), the measurements reflect both tissue and circulating levels of the hormones.

Both T_3 and T_4 were detected in fertilized eggs prior to the start of the exposure, indicating the presence of maternally-derived hormone. Concentrations were lower than what has been previously reported in fathead minnow or other teleost embryos, but the measurements in this study were made using LC-MS/MS rather than a radioimmunoassay method used in other studies (see Power et al., 2001 for review; Crane et al., 2005). Arguably, MS/MS should provide more robust quantification of the analytes.

A decrease in T_4 was observed in fathead minnows exposed to 1 mg MBT/L through 6 dpf, suggesting that thyroid follicles are actively synthesizing T_4 and MBT is inhibiting this process at this stage. Consistent results were observed in zebrafish, where whole body T_4 concentrations were significantly lower than those of controls following exposure to 0.4 or 0.7 mg MBT/L through 120 hpf (Stinckens et al., this issue). In contrast, there was no observed effect on T_3 in fathead minnows exposed to MBT through 6 dpf. Experiments in zebrafish similarly saw no effect on T_3 concentration in fish exposed through 120 hpf (Stinckens et al., this issue). These observations, together with the lack of increase in *tpo* mRNA levels at 6 dpf, suggests that at this stage, shortly after hatch, maternally derived hormone was likely being converted to T_3 in the peripheral tissues by outer ring deiodinase enzymes and was sufficient for maintaining thyroid-dependent processes without eliciting a detectable compensatory response. A significant decrease in T_3 was observed only in the fish exposed to 1 mg MBT/L for 14 d. Unfortunately, we were unable to acquire T_4 measurements in fish at this time point, but the decreased T_3 levels in fish exposed to the highest concentration of MBT suggests T_4 synthesis was likely reduced at a time when maternally derived T_4 would no longer be expected to be present. Interestingly, there was no effect on T_3 levels by the 0.25 or 0.5 MBT/L exposures. This suggests the treated fish were able to effectively compensate and maintain T_4 and T_3 levels similar to that of control fish. At day 21, the T_3 concentrations in fish exposed to MBT were not different than those of control fish, but T_4 levels were significantly higher in fish exposed to MBT than in the controls. Given the interruption in MBT delivery on exposure day 20/19 it is plausible that the rebound in whole body T_3 and T_4 reflects rapid recovery and accumulation of stored T_4 as TPO inhibition temporarily ceased. It is notable that zebrafish exposed to 0.35 mg MBT/L through 32 dpf continued to show decreased whole body T_4 (Stinckens et al., this issue). Rapid recovery following temporary cessation of MBT delivery could be facilitated by the compensatory response observed, including proliferation of the T_4 synthesizing cells. Indeed, similarly rapid recovery has been observed fathead minnows following cessation of exposure to other endocrine

active chemicals, often in conjunction with strong evidence for compensatory responses (Ankley and Villeneuve, 2015).

4.3. MBT Delays Anterior Swim Bladder Inflation

The primary tissue/organ-level KE we focused on the present study was impaired swim bladder inflation. Inflation of the swim bladder has been suggested to be thyroid regulated in multiple teleost species (Brown et al., 1988; Winata et al., 2009) and the lack of swim bladder inflation has been linked to a reduction in larval fish survival (Czesny et al., 2005; Robertson et al., 2007; Woolley and Gin, 2010; Kurata et al., 2014). In the fathead minnow the swim bladder has two chambers, a posterior and anterior chamber that inflate at approximately 6 dpf and 14 dpf, respectively (Devlin et al., 1996). Evaluation of posterior swim bladder inflation at 6 dpf showed no effect of MBT exposure on posterior swim bladder inflation. This result is consistent with similar observations in zebrafish (Stinckens et al., this issue). We hypothesize that the posterior chamber is able to inflate at 6 dpf due to the presence of maternally-derived T_4 and that conversion of T_4 to T_3 by outer ring deiodination was sufficient to inflate the posterior swim bladder. Type II iodothyronine deiodinase has been shown to be expressed in the posterior swim bladder of zebrafish (Dong et al., 2013). Furthermore, the morpholino knockdown of type I and type II deiodinase enzymes together and type III deiodinase enzyme alone led to a significant reduction in posterior swim bladder inflation (Heijlen et al., 2014; Bagci et al., 2015). The hypothesized role of maternally-derived T_4 in facilitating inflation of the posterior swim bladder could be examined further in the fathead minnow, for example, through exposing embryos to a chemical inhibitor of outer ring deiodinase activity.

The anterior swim bladder is expected to inflate in the fathead minnow around 14 dpf. However, we observed that about half the fish exposed to 1 mg MBT/L did not inflate their anterior swim bladder. Other investigators have speculated that the inflation of the anterior chamber may be

regulated by thyroid hormones (Winata et al., 2009); however, to date, this connection had not been definitively shown. In the present study, we did not observe a complete lack of anterior chamber inflation, because by day 21 all of the anterior chambers had inflated. Due to the interruption in MBT stock delivery on day 19-20 of experiment 1 it could not be deduced from that experiment alone whether exposure to MBT simply delays anterior inflation, or whether the anterior chamber inflated only because the fish were able to recover during the period of minimal MBT exposure. The fact that whole-body T_4 concentrations in the MBT treated fish exceeded those of control fish, tend to support the possibility of a rapid recovery which could have allowed anterior inflation, assuming thyroid hormone-dependence. However, experiment 2 suggested that eventual inflation (i.e., by 21 dpf) was not simply due to the interruption in MBT exposure. In experiment 2, although anterior swim bladder inflation was impaired at 14 dpf, fish exposed continuously through 18 or 22 dpf were able to inflate their anterior chambers. This suggests the possibility that compensatory responses along the HPT axis facilitated eventual inflation, even though systemic T_3 , T_4 , may have been insufficient to support inflation during the normal developmental period. Indeed, the *tpo* mRNA, histopathological, and whole-body thyroid hormone measurements all suggest compensation for the TPO inhibition.

Similar impacts on anterior swim bladder inflation were observed in zebrafish exposed to MBT (Stinckens et al., this issue). However, the effects in zebrafish were more severe, with most exposed fish lacking inflation rather than just showing a delay in inflation. Furthermore, anterior swim bladder inflation in zebrafish was impacted at lower MBT concentrations than those needed to impact anterior inflation in fathead minnow (Stinckens et al. this issue). It is possible, but at present uncertain, whether the differing outcome among species reflects differing capacities to mount a compensatory response to the TPO inhibition, however, Zoeller et al. (2007) have noted some clear species differences in the relevance of specific proteins in specific thyroid-mediated events and potential differences in vulnerability to different stressors as a result.

Regardless of whether impaired anterior chamber inflation persisted, exposure to MBT consistently reduced relative anterior chamber size throughout the experiments. Overall, there was a concentration-dependent decrease in size of the anterior swim bladder of fish exposed to MBT for 14 d. The concentration-dependence was not as clear at 21 d when only the fish exposed to 1 mg MBT/L still had a smaller anterior chamber. Nonetheless, the observation that MBT-exposed fathead minnows had smaller inflated anterior swim bladders compared to control fish was consistent with effects in zebrafish (Stinckens et al., this issue). Overall, the anterior swim bladders were smaller when exposed to 1 mg MBT/L for any length of time as long as exposure occurred between 6 and 14 dpf, and the effect was observed through 22 days of exposure. Consequently, it appears that while compensation may allow the fathead minnows to ultimately inflate the anterior swim bladder, the anterior swim bladders do not develop normally.

Because of the observed impairment and delay in anterior swim bladder inflation by MBT, we also wanted to determine if there is a sensitive exposure window during which exposure to a TPO inhibitor would lead to delayed and/or impaired anterior swim bladder inflation. Only the fish exposed to MBT starting at 0 or 6 dpf, prior to endogenous thyroid hormone synthesis, had a significant reduction in the percentage of fish observed at day 14 with inflated anterior swim bladder. For fish exposed to MBT starting day 10, inflation rates were similar to the control group. This result suggests that once endogenous thyroid hormone is being synthesized, there is likely sufficient hormone available to inflate the anterior swim bladder. However, even if exposure to MBT did not start until 10 dpf, after endogenous T_4 production is thought to begin, reduced anterior chamber size was observed. Indeed, it appeared that as long as TPO inhibition occurred prior to 14 dpf, anterior swim bladder size was affected even though inflation could occur. Thus, while compensatory responses may allow for inflation of the anterior chamber, ultimate function may still be impacted by exposure during certain developmental

windows, even if that exposure is transient (e.g., in the case of fish exposed from days 0-10 only, and then assessed on 14, 18, or 22 dpf.; Figure 7).

The present data do not rule out the possibility that MBT is impacting swim bladder inflation through a non-thyroid-related mechanism that may simply correlate with some of the systemic changes in T₃ and T₄ concentrations and apparent compensatory responses. Analysis of MBT in a broad array of pathway-based assays as part of US EPA's ToxCast program illustrates that MBT can elicit a diversity of pathway-based biological activities in vitro, in addition to its effect on TPO (actor.epa.gov/dashboard). Thus, exposures to additional thyroid-disrupting agents, ideally with and without the addition of a compensatory dose of exogenous T₄ are needed to more conclusively evaluate the link between hypothyroidism and impaired anterior swim bladder inflation. Nonetheless, results of the present study are consistent with a hypothesized role of thyroid hormone signaling in facilitating anterior swim bladder inflation and as such provide initial support for the proposed AOP.

4.4. Uncertainty of AOP Relevance to Risk Assessment

While our data are consistent with the hypothesis that anterior swim bladder inflation is thyroid-mediated and can be impacted by exposure to a TPO inhibitor like MBT, the ecological relevance of impaired anterior swim bladder inflation is not well defined. Unlike the posterior chamber whose function has been clearly linked to buoyancy control and survival traits (Czesny et al., 2005; Robertson et al., 2007; Woolley and Gin, 2010; Kurata et al., 2014), the link between anterior chamber inflation and impaired survival and/or successful reproduction are less clear. The anterior swim bladder marginally contributes to the buoyancy of fish, but appears to be most important for producing and transducing sound through the Weberian Apparatus (Popper, 1974; Finney et al., 2006; Lechner and Ladich, 2008). While it is plausible that impaired anterior swim bladder inflation or reduced size of the anterior chamber could lead to a reduction in fish survival because fish would be less likely to respond to their

surrounding environment through behaviors such as predator avoidance or prey seeking (Wisenden et al. 2008; Fay 2009), direct evidence supporting that connection is lacking. Consequently, while our investigation provides initial support for a potential linkage between TPO inhibition and impacts on the swim bladder, the ultimate ecological relevance of this remains unclear.

Ecological relevance aside, assuming the impacts of MBT on anterior swim bladder inflation are the result of TPO inhibition and not extrathyroidal MBT actions, the observations from the present study may offer near term utility relative to screening and evaluation of HPT-axis disrupting chemicals. Specifically the identification of improper and delayed anterior swim bladder inflation may provide a rapid screening tool for HPT disruption in the fathead minnow and other small fish species. This is significant given that there currently are no biomarkers that are used to screen for thyroid axis disruption in any fish species. Our study suggests the exposure to potential TPO inhibitors would only have to occur for 8 days (i.e., from 6 dpf to 14 dpf) to be able to see a potential effect on the anterior swim bladder. Additionally, if our hypothesis that posterior inflation around 6 dpf is insensitive to TPO inhibition due to presence of maternally derived thyroid hormones is correct, a 14 day exposure (from 0 dpf to 14 dpf) may offer further diagnostic potential for discriminating chemicals causing direct inhibition of endogenous thyroid hormone synthesis (e.g., through inhibition of TPO) from those impacting peripheral deiodination. That is, thyroid hormone synthesis inhibitors would be expected to impact anterior but not posterior inflation, while peripheral deiodinase inhibitors should impact inflation of both chambers. While additional characterization of specificity and sensitivity of the responses are needed, short term experiments with larval fish may prove useful as a complementary assay for detecting and discriminating thyroid disrupting chemicals, for example, to support programs focused on the identification of endocrine-disrupting chemicals (e.g., USEPA 2014).

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Figure Legends

Figure 1. Effects of MBT on relative expression of thyroid peroxidase (*tpo*) mRNA in pooled embryo or larval fathead minnows (n=4 pools per treatment). A) Experiment 1. Data represent relative transcript abundance for pools of 20, 10, 7, or 5 individuals per pool for 2, 6, 14, and 21 days post fertilization (dpf), respectively. For pools from 2 and 6 dpf whole body RNA was extracted. For pools from 14 and 21 dpf only the anterior-most (head) section (see Figure S1) of the larval fish was extracted. B) Experiment 2. Relative transcript abundance from pools of whole body 22 dpf larval fish (n=5/pool). Error bars indicate standard error of the mean. Different letters denote significant differences among treatments within each time point ($p<0.05$).

Figure 2. Histopathology of thyroid follicles of larval fathead minnows exposed to MBT from 0-14 days post fertilization (dpf). A) Follicles from a control fish. The follicular epithelium was arranged as a single layer of low cuboidal follicular cells with nuclei in close proximity to others in adjacent cells. Colloid is homogenous and brightly eosinophilic. B) Fish exposed to 0.25 mg/L showing mild follicular cell hypertrophy. C) Fish exposed to 1.0 mg/L with moderate follicular cell hypertrophy, moderate follicular cell hyperplasia, increased follicular lumen area, and lightly-staining, granular colloid. D) Fish exposed to 1.0 mg/L with moderate follicular cell hypertrophy, moderate follicular cell hyperplasia, decreased follicular lumen area and decreased colloid. Bar = 25 microns (for all panels).

Figure 3. Effects of MBT exposure on whole-body thyroid hormone concentrations measured in fathead minnows exposed to MBT for 0, 6, 14, or 21 d. A) triiodothyronine (T_3) concentrations; B) thyroxine (T_4) concentrations. Error bars indicate standard error of the mean. Different letters denote significant

differences among treatments within each time point ($p < 0.05$). N=4 pools of 20, 10, 7, or 5 individuals on days 0, 6, 14, and 21, respectively.

Figure 4. Effects of MBT exposure on the mean percent inflation of the anterior or posterior swim bladder in fathead minnows exposed through A) 6 days post fertilization (dpf); B) 14 dpf; C) 21 dpf. Error bars indicate standard error of the mean. Different letters denote significant differences among treatments within each time point ($p < 0.05$). N=4 groups of 20, 14, and 10 individuals on days 6, 14, and 21, respectively.

Figure 5. Effects of MBT exposure on the size of the anterior and posterior swim bladder. Mean length of anterior swim bladder in relation to posterior length ($n=4$) in fathead minnows exposed to MBT for A) 14 days post fertilization (dpf); B) 21 dpf. Error bars indicate standard error of the mean. Different letters denote significant differences among treatments within each time point ($p < 0.05$).

Figure 6. Effects of the timing of a 1 mg MBT/L exposure on anterior swim bladder inflation. Mean percent of fathead minnows on A) day 14, B) day 18 or C) day 22 with an inflated anterior swim bladder following exposure to MBT for varying number of days. All treatments had $n=3$ tanks, except the control and 0-22 days post fertilization window which had $n=4$ tanks. Error bars indicate standard error of the mean. Different letters denote significant differences among treatments within time-point ($p < 0.05$).

Figure 7. Effects of timing of 1 mg MBT/L exposure on the size of the anterior and posterior swim bladder. Mean length of anterior swim bladder in relation to posterior length on A) day 14, B) day 18, or C) day 22 for fathead minnows exposed to MBT for varying number of days. Error bars indicate standard error of the mean. Different letters denote significant differences among treatments within each time point ($p < 0.05$).

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Figure 1

Figure 2

Figure 3

Figure 4

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Figure 6

Figure 7