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IMPAIRED SWIM BLADDER INFLATION IN EARLY-LIFE STAGE FATHEAD MINNOWS EXPOSED TO A DEIODINASE INHIBITOR, IOPANOIC ACID

Running title: Effects of deiodinase inhibition on swim bladder inflation

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Abstract: Inflation of the posterior and/or anterior swim bladder are processes previously demonstrated to be thyroid-hormone regulated. We investigated whether inhibition of deiodinases, which convert thyroxine (T4) to the more biologically-active form, 3,5,3'-triiodothyronine (T3), would impact swim bladder inflation. Two experiments were conducted using a model deiodinase inhibitor, iopanoic acid (IOP). First, fathead minnow embryos were exposed to 0.6, 1.9, or 6.0 mg/L or control water until 6 days post-fertilization (dpf) at which time posterior swim bladder inflation was assessed. To examine anterior swim bladder inflation, a second study was conducted with 6 dpf larvae exposed to the same IOP concentrations until 21 dpf. Fish from both studies were sampled for T4/T3 measurements and gene transcription analyses. Incidence and length of inflated posterior swim bladders were significantly reduced in the 6.0 mg/L treatment at 6 dpf. Incidence of inflation and length of anterior swim bladder were significantly reduced in all IOP treatments at 14 dpf, but inflation recovered by 18 dpf. Throughout the larval study, whole body T4 concentrations increased and T3 concentrations decreased in all IOP treatments. Consistent with hypothesized compensatory responses, deiodinase-2 mRNA was up-regulated in the larval study, and thyroperoxidase mRNA was down-regulated in all IOP treatments in both studies. These results support the hypothesized adverse outcome pathways linking inhibition of deiodinase activity to impaired swim bladder inflation.

Keywords: Developmental toxicity, Aquatic Toxicology, Endocrine disrupting compounds, Thyroid disruption, Adverse Outcome Pathway

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INTRODUCTION

The hypothalamic-pituitary-thyroidal (HPT) axis plays an important role in fish development [1,2]. The HPT axis is a highly conserved system in which thyroperoxidase (TPO) expressed in the thyroid follicles plays a critical role in the production of thyroxine (T4), which then can be converted to the more biologically-active thyroid hormone, 3,5,3'-triiodothyronine (T3), via deiodination in peripheral tissues. Exposure to thyroid-active compounds can result in disruption of HPT-axis signaling along with adverse effects on critical thyroid-dependent developmental events in vertebrates, such as amphibian metamorphosis [3-5] and swim bladder inflation in fish [2,6,7].

In the present study, we examined the effects of deiodinase inhibition on swim bladder inflation in fathead minnows (*Pimephales promelas*). The fathead minnow swim bladder consists of two chambers, a posterior chamber that inflates around 5-6 days post-fertilization (dpf; 1-2 days post-hatch) and an anterior chamber that inflates around 14 dpf (9 days post-hatch). The anterior swim bladder is formed via evagination from the original posterior chamber [8]. Both chambers are important relative to buoyancy and swimming performance [8-10]; therefore, impairment of swim bladder inflation may result in reduced young-of-year survival when considering predator avoidance and the ability to scavenge for food. In addition to its role in buoyancy, the anterior swim bladder plays an important role in hearing [11,12]. Although information concerning the direct importance of auditory ability in fish to young-of-year survival is limited, there is at least a plausible connection that fish use auditory signals for a variety of behaviors such as spawning, predator avoidance, and feeding [13].

Potential connections between disrupted thyroid hormone synthesis and signaling and adverse outcomes associated with swim bladder non-inflation in fish were recently summarized

in a series of adverse outcome pathway descriptions (AOPs 155-159 [14-18]); combined they form an AOP network. Adverse outcome pathways are a conceptual framework intended to help organize and summarize the scientific support connecting the perturbation of a specific biomolecular target or biological pathway by a chemical or stressor with adverse effects that are considered relevant to risk assessment and regulatory decision-making [19,20]. These proposed AOPs lay out plausible connections between specific mechanisms of thyroid axis disruption and ecologically-relevant effects in fish. However, empirical evidence that helps to define the specificity, applicability, quantitative considerations, and level of confidence in application of these AOPs to regulatory decision-making is still needed.

In a previous study, exposure to a model TPO inhibitor, 2-mercaptobenzothiazole (MBT), resulted in impaired anterior swim bladder inflation (14 dpf) in larval fathead minnows. Posterior swim bladder inflation, which occurs earlier in development (6 dpf) was not affected by MBT exposure, potentially due to the availability of maternally-deposited thyroid hormone in the yolk [6]. Similar responses were observed in zebrafish, where exposure to MBT did not impair inflation of the posterior chamber at 5 dpf, but anterior chamber inflation and size were impaired at 32 dpf [7]. In the present study, we hypothesized that the molecular initiating event of deiodinase inhibition would result in a reduction in whole-body T3 concentrations leading to impaired inflation of both the posterior and anterior chamber of the swim bladder in developing fathead minnows. This hypothesis was based on the assumption that conversion of T4 to T3 would still be required to facilitate normal posterior swim bladder inflation even if the pool of maternally-derived T4 from the yolk was adequate to obviate the need for new thyroid hormone synthesis during early embryonic development.

To test this hypothesis, fathead minnows were exposed to iopanoic acid (IOP) in two complementary experiments. In the first exposure, fathead minnow embryos were continuously exposed to IOP until 6 dpf to examine the potential effects on posterior swim bladder inflation. A second IOP exposure was conducted with fathead minnow larvae (initiated at 6 dpf) to examine potential effects on anterior swim bladder inflation. As a direct measure of key events associated with the hypothesized deiodinase AOPs (AOPs 155-158 [14-17]), thyroid hormone concentrations (i.e., T3 and T4) were measured throughout the two independent studies. Additional molecular endpoints (mRNA expression) were examined to characterize compensatory responses to deiodinase inhibition that may influence dose-response, time-course behaviors along this series of AOPs.

MATERIALS AND METHODS

Experimental design: Embryo exposure

Fathead minnow embryos and larvae were obtained from adult spawning pairs from a culture facility at the US Environmental Protection Agency, Mid-Continent Ecology Division in Duluth, MN. All procedures were conducted in accordance with approved Animal Care and Use Guidelines. Embryos were deposited on spawning substrates and were kept in 20 L aquarium tanks containing control Lake Superior water (LSW) along with their respective parents for approximately 24 hours in the culture facility. After 24 h, embryos were removed from the spawning substrate and developmental stage was identified. Only embryos that were at stage 20 or 21 (tailbud stage) were used in this study [21]. Appropriately staged embryos from 8-10 spawning pairs were pooled together, and then divided into groups of 30 embryos that were placed in mesh-bottomed egg baskets. Egg baskets were randomly distributed among 16 test aquaria, with two baskets per aquarium. Fathead minnow embryos placed in the aquaria were

exposed to one of three concentrations of IOP (0.6, 1.9, or 6.0 mg IOP/L; TCI America; purity >98%) or LSW controls in a solvent-free, flow-through test system, using four replicate tanks per treatment. Concentrations were selected based on a range-finding study (data not shown). Control water was UV-treated and filtered prior to use in the exposure system. A rocker mechanism was used to gently agitate each egg basket until all embryos had hatched. Post-hatch larvae were sampled at 4 and 6 dpf, after 3 and 5 days of continuous exposure, respectively.

Tank temperatures were maintained at 25±1°C, and the photoperiod was set at 16:8 h light:dark. Water quality measures were assessed over the duration of the test (mean ± SD): temperature, 24.8ºC ± 0.1ºC; dissolved oxygen, 7.7 ± 0.1 mg/L; pH, 7.4 ± 0.3; and flow rate, 45.9 ± 2.0 mL/min. Analytical quantification of T3 and T4 was determined from pools of 25 or 20 embryos per tank at 4 and 6 dpf, respectively (n=4 pools of embryos per treatment per time point). Analyses of mRNA expression were conducted using 6 dpf embryos, pooled in groups of 10 whole embryos per tank (n=4 per treatment). Posterior swim bladder inflation was assessed at 6 dpf, as described below.

Experimental design: Larval exposure

The larval study was conducted using the same continuous flow-through testing system and IOP concentrations as the embryo study. The test was initiated with 6 dpf larval fathead minnows with posterior swim bladders already inflated. Seventy-two larvae were randomly placed in each of four replicate tanks per treatment. Mid-way through loading fish, four sets of 25 larvae were pooled for T3/T4 analyses at time 0. Larvae held in the system were fed live brine shrimp (*Artemia* sp.) to satiation twice daily throughout the study. Water quality measures assessed over the duration of the test were (mean ± SD): temperature (25.2 ± 0.3ºC), dissolved oxygen (7.5 ± 0.2 mg/L), pH (7.5 ± 0.3), and flow rate (45.5 ± 1.7 mL/min).

At 10 dpf, 10 larvae were randomly sampled and pooled per tank for gene expression analyses and an additional 10 larvae per tank were pooled for T3/T4 measurements. Anterior swim bladder inflation occurs around 14 dpf; therefore, to assess incidence of anterior swim bladder inflation and swim bladder length, additional samples were collected at 14 dpf. At this time, 7 larvae were pooled for gene expression, 7 larvae were pooled for T3/T4 measurements, and 2 larvae were preserved for histopathology per treatment tank. Additional time points were examined to evaluate whether any effects on anterior chamber inflation were persistent or simply represented a developmental delay. At 18 dpf, 3 larvae were pooled for mRNA expression, 7 larvae were pooled for T3/T4, and 2 larvae were preserved for histopathology per tank. Finally, at 21 dpf, 3 fish were pooled for mRNA expression, 5 were pooled for T3/T4, and 2 fish were preserved for histopathology. All larvae collected at 14, 18, and 21 dpf were imaged as described below for subsequent measurements of body length and swim bladder lengths (14 and 18 dpf only). All larvae were anesthetized in buffered MS-222 prior to imaging and preservation.

Analytical verification

Water samples were collected daily from all tanks during the embryo exposure and approximately every fourth day during the larval exposure to verify chemical concentrations. Water samples were diluted 1:1 with acetonitrile (ACN) and a 50 µL aliquot injected onto a Kinetex C18 50 x 2.1 mm column (Phenomenex Inc., Torrence, CA) with an Agilent 1100 Series (Agilent Technologies, Santa Clara, CA) high performance liquid chromatograph (HPLC). Separation was performed under isocratic conditions (55:45 water:ACN acidified with 10 mM acetic acid) at 0.5 mL/min and IOP was detected using diode array at 231 nm (detection limit 0.061 mg/L).

Swim bladder and body length assessments

At 6 dpf in the embryo study, 25 larvae were randomly selected from each replicate tank per treatment (n=100 per treatment) to assess swim bladder inflation and body length. For the larval study, 16 larvae were randomly selected and examined for body and swim bladder lengths from each replicate tank per treatment (n=64 per treatment) at 14 dpf. At 18 dpf, 12 larvae were examined from each replicate tank per treatment for body and swim bladder lengths (n=48 per treatment, except for 1.9 mg IOP/L where n=36 due to technical error). At 21 dpf, the musculature of the control larvae was developed to a degree in which it was no longer possible to examine the swim bladders externally; therefore, swim bladder lengths were not assessed at 21 dpf using imaging software; however, body lengths were still measured. Anesthetized larvae were photographed using a Nikon Digital Camera DXM1200 (5.24 MP) with a Nikon SMZ-U light microscope (Nikon Instruments, Inc., Melville, NY). Each photograph included a ruler for calibration in subsequent measurements. Incidence of swim bladder inflation was first assessed and calculated as a percentage of inflation of the sample population within each treatment group per time point. Standard body length (i.e., tip of the head to the last vertebra, excluding caudal fin) and the longest portion of the anterior (larval study only) and posterior swim bladder of each larvae with inflated swim bladders were measured using Image-Pro Premier v 6.2 software (Media Cybernetics, Inc., Rockville, MD). In addition to general swim bladder length comparisons, posterior swim bladder length in the embryo study and anterior swim bladder length in the larval study were normalized to body length and compared across treatments to account for possible influences of body size on swim bladder length.

mRNA expression

Due to their involvement in the HPT axis and hypothesized targets of deiodinase inhibition, relative abundance of gene transcripts coding for deiodinases 1, 2, and 3 (*dio1-3*) and thyroperoxidase (*tpo*) were measured by quantitative polymerase chain reaction (QPCR). First, total RNA was extracted from each sample of pooled organisms (n=5-10 larvae per sample as detailed above) using RNeasy Micro or Mini kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quality and concentration of the total RNA was determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Thermo Fisher Scientific, Wilmington, DE). Embryo and larval total RNA samples had $A_{260\ nm}/A_{280\ nm}$ ratios > 2.07 and $A_{260\ nm}/A_{230\ nm}$ ratios > 1.43. All samples were diluted to 10 ng/µL RNA prior to QPCR. Relative transcript abundance of *dio1-3* and *tpo* was measured using Taqman® RNA-to-C_T 1-step kits (Applied Biosystems, Foster City, CA, USA). Each 20 µL reaction contained 20 ng total RNA, 300 nM forward primer, 300 nM reverse primer, and 150 nM probe (see Supplementary Table 1 for sequences). The thermocycling program was set to 48°C for 15 min, 95°C for 10 min, and finally 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative transcript abundance was quantified using gene-specific mRNA duplicate standard curves with six concentrations, following a 10-fold dilution series (2×10^7 to 200 copies/µL). Replicate sample reactions were included within each QPCR plate with approximately 12% of the samples run in duplicate on each plate. Mean (±SD) coefficient of variation for the replicate samples was 9.56 (± 9.48). Amplification efficiencies ranged from 87.1 to 100.6%. The standards were prepared through a series of cDNA amplifications and in vitro transcription reactions (MEGAscript, Ambion) as described by Villeneuve et al. [22].

Whole body T3 and T4 measurements

Thyroid hormones, T3 and T4, were extracted from whole bodies of embryos or larval fish and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with isotope dilution. Extraction and analysis methods were previously described in detail [6,23]. Briefly, whole-body samples (ranging 0.0207 – 0.184 g) were spiked with isotopically labeled T3 and T4, homogenized, and thyroid hormones were extracted with ethanol. Extracts were defatted with hexane prior to further clean up by solid phase extraction (SPE) using Evolute CX cartridges (Biotage, Charlotte, North Carolina). Final SPE extracts were evaporated to dryness under nitrogen, reconstituted in 75:25:0.1 water:ACN:formic acid, and transferred to amber microvials for LC-MS/MS analysis. Method blanks (n=12), matrix control (larval fish, n=12), and matrix spikes (n=12; spiked with 0.25 ng T3 and T4) were analyzed with each sample set for quality assurance purposes. Spike recovery was calculated by subtracting endogenous thyroid hormone concentration (from control matrix) from matrix spikes. Matrix control duplicates showed high precision with average (SD) percent agreements of 94.1 (3.8) and 91.5 (5.4) for T3 and T4, respectively. Mean (SD) spike recovery values were 103.4 (11.9) and 104.0 (8.1) for T3 and T4, respectively. Multiple method blanks in sample batches from the larval exposure had low but quantifiable levels of T3 and T4; however, we attribute this to carry-over from standards run prior in the sample sequences. Correcting for low concentrations observed in method blanks would have no significant effect on relative reported hormone concentrations; thus, final concentrations were not blank corrected. For statistical purposes, non-detections were assigned a value of one-half the lower limit of quantitation (LLOQ = 0.01 ng) prior to calculating final concentration based on tissue mass.

Histopathology (larval study only)

Histology was examined in two larvae per tank at 14, 18, and 21 dpf in the larval study (n=8 per treatment per time point). Anesthetized whole larvae were initially fixed in Bouin's fixative for approximately 48 h and subsequently rinsed with deionized water and transferred to 10% neutral buffered formalin until processed. The fish were embedded using standard paraffin histological processing methods and were sectioned along the sagittal plane and then stained with hematoxylin and eosin. The primary tissues for evaluation were the thyroid follicles and swim bladders, but additional tissues were also assessed for possible treatment-related pathologies based on histopathology best practice guidelines [24]. Semiquantitative grading for recording histological effects was based on the estimated percentage of tissues affected and the differences from experimental and historic control larvae [25]. For thyroid effects, mid-luminal sections of the thyroid follicles were selected for evaluation using criteria developed in the Amphibian Metamorphosis Assay [26,27].

At 21 dpf, the musculature of the control fish was more developed than any of the IOP treatments, rendering it impossible to externally examine the swim bladders; therefore, swim bladder lengths were assessed histologically at 21 dpf. In order to confidently use the histological length measurements, we first directly compared the lengths of swim bladders acquired by measurements from digital images of live organisms versus histological determinations. There was no significant difference between the two techniques for any of the time points or treatments for anterior swim bladder measurements (Sup. Fig. S1), rendering it practical to use the length measurements acquired by histology to assess anterior swim bladder lengths at 21 dpf. However, in the case of the posterior chamber, there was a significant difference between the live animal and histological measurements when examining the 14 and 18 dpf posterior swim bladders (Sup. Fig. S2), with the histological measurement potentially

underestimating the live-measured size in some cases. Therefore, posterior swim bladder measurements made based on histology at 21 dpf may slightly underestimate length.

Statistical analyses

Normality of the QPCR and T3/T4 data were assessed using a Kolmogorov-Smirnov test. Data were log-transformed if they did not fit a normal distribution and were reassessed for normality. Parametric data were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. Nonparametric data that did not meet the basic assumptions of ANOVA were assessed using a Kruskal-Wallis test followed by Dunn's post-hoc analysis. Percent inflation data were arcsine transformed prior to one-way ANOVA [28]. Biological data are presented as mean (SE), and differences among treatments were considered significant at $p < 0.05$. All statistical analyses were conducted using GraphPad Prism v. 5.02 (GraphPad Software) or Statistica 10 (StatSoft, Inc.).

RESULTS

Analytical verification

During the embryo study, mean (SD) tank concentrations of IOP were 0.63 (0.0070), 1.9 (0.020), and 6.5 (0.13) mg/L (n=24 with four replicate tanks measured over the course of 6 days). Matrix spike recovery values (%) for 0.6 mg/L (n=2), 1.9 mg/L (n=2), and 6.0 mg/L (n=2) in the embryo study were 116.5 (7.3), 103.9 (1.1), and 101.1 (2.7) of the spiked concentrations, respectively, and percent duplicate agreement (n=20) was 99.3 (0.7). During the larval study, mean (SD) concentrations of IOP were 0.65 (0.061), 2.0 (0.028), 6.1 (0.11) mg/L (n=25 for each). Matrix spike recovery values (%) for 0.6 mg/L (n=5), 1.9 mg/L (n=5), and 6.0 mg/L (n=3) in the larval study were 102.9 (0.84), 101.4 (2.8), and 102.4 (0.11), respectively, and

percent duplicate agreement (n=18) was 99.2 (0.7). No IOP (<0.061 mg/L) was detected in any of the control tanks in either study.

Survival and body length

In the embryo study, there were no significant differences in mortality rate between any of the treatments, with mortality ranging from 3% to 6% at 6 dpf. In the larval study, there was significantly higher mortality in the 6.0 mg IOP/L treatment compared to all other treatments ($p=0.0037$), with mean (SE) percent mortality of 4.2 (1.5), 3.1 (0.35), 3.8 (0.35), and 9.7 (1.5) in the control, low, medium, and high IOP treatments, respectively.

There were no statistically significant differences in the body lengths of 6 dpf fathead minnows that had been exposed to IOP during embryonic development (Sup. Fig. S3). In the larval study, fish exposed to 6.0 mg IOP/L were significantly shorter compared to all other treatments at 14 and 18 dpf, but body length had recovered by 21 dpf (Sup. Fig. S4).

mRNA expression analyses

At the termination of the embryo study (6 dpf), expression of *tpo* mRNA was significantly down-regulated in a dose-dependent manner (Fig. 1). Down-regulation of *tpo* mRNA was also observed in the larval study in all three IOP concentrations at 10 and 14 dpf (Fig. 2A). However, by 18 dpf, down-regulation of *tpo* mRNA was only observed in the 1.9 and 6.0 mg IOP/L treatments, and by 21 dpf there were no longer any statistically significant differences between the treatments and controls.

In the embryo study, exposure to IOP had no significant effects on *dio1-3* mRNA expression as measured at the time of test termination at 6 dpf (Sup. Fig. S5). During the larval study, no significant differences were observed in *dio1* mRNA expression between any of the treatments (Fig. 2B). However, significant up-regulation of *dio2* was observed in IOP treatments

starting at 14 dpf, and continued to increase over the duration of the study (Fig. 2C). Compared to controls, *dio3* was also significantly up-regulated in larvae exposed to 1.9 and 6.0 mg IOP/L through 10 dpf, and 6.0 mg/L through 18 dpf (Fig. 2D).

Whole body thyroid hormone concentrations

As a direct measure of deiodinase inhibition, whole body concentrations of T3 and T4 were examined in both the embryo and larval IOP studies. Contrary to an expected decrease, pooled whole body T3 concentrations were significantly increased in a dose-dependent manner in the embryo study at both 4 and 6 dpf (Fig. 3A). A similar pattern was observed in T4 concentrations at 4 dpf; however, by 6 dpf, there were no significant differences in T4 concentrations between any of the treatments (Fig. 3B).

Thyroid hormone concentrations measured in the larval study were more consistent with hypothesized effects of exposure to a deiodinase inhibitor. Specifically, T3 concentrations were significantly reduced in all IOP treatment concentrations throughout the duration of the larval study (Fig. 4A). Decreased T3 concentrations corresponded with a dose-dependent increase in whole body T4 concentrations at all time points throughout the larval study (Fig. 4B).

Thyroid follicle and liver pathology

Histological assessment revealed no thyroid follicle pathology related to IOP exposure in the larval study. No effects on thyrocyte hyperplasia, hypertrophy, or changes in gland size or colloid quality were observed in the thyroid follicles of the larvae (data not shown).

In addition to examination of the thyroid, the liver tissues were evaluated for potential pathology. Hepatocyte vacuolization, an indirect measure of glycogen storage in hepatocytes, was found to vary between the control and treated larvae. Glycogen-filled vacuoles in hepatocytes, unlike clear and round lipid vacuoles, are irregular clear spaces filled with a lightly

stained lacy network [29]. Throughout the study, hepatocytes in control larvae were highly vacuolated, indicating high prevalence of glycogen storage, while larvae exposed to IOP had reduced vacuolization, indicating lower prevalence of glycogen storage at 14 and 21 dpf (Sup. Table 2). The reduction of glycogen storage in hepatocytes of larvae exposed to 1.9 and 6.0 mg IOP/L suggests that IOP may either interfere with hepatocyte glycogen production and storage or another aspect of energy metabolism in the exposed larvae.

Swim bladder inflation and length

Embryonic exposure to 6.0 mg IOP/L resulted in significant impairment of posterior swim bladder inflation at 6 dpf (Fig. 5A). The posterior swim bladders that were inflated in the highest treatment group were also significantly shorter compared to all other treatment groups (Fig. 5B). Normalization of posterior swim bladder length to body length did not influence the significant results on reduced swim bladder length with exposure to 6 mg IOP/L (Sup. Fig. S6).

During the larval study, exposure to all three IOP concentrations inhibited anterior swim bladder inflation and reduced anterior swim bladder length at 14 dpf, while posterior swim bladder length was significantly increased in all IOP treatments (Fig. 6A and 7A), with a significant negative correlation between anterior and posterior swim bladder length (Pearson $r = -0.3819$, $p<0.0001$). By 18 dpf, recovery of inflation was evident (no significant effect on % inflated), but anterior swim bladder length was still significantly reduced in all IOP treatments compared with controls (Fig. 6B and 7B). Percent inflation of the anterior swim bladders could not be determined at 21 dpf due to thickening of the lateral musculature preventing visualization of the swim bladders; however, swim bladder lengths were determined by histological measurement at 21 dpf as previously described. A significant reduction in the length of the anterior swim bladders persisted at 21 dpf in all IOP treatment groups, but posterior swim

bladder lengths were similar to controls (Fig. 7C). There were no significant correlations between anterior and posterior swim bladder lengths at 18 or 21 dpf. Normalization of anterior swim bladder length to body length did not influence the observed reduction in anterior swim bladder length at any of the time points (Sup. Fig. S7). There were no pathologies detected in the structure and organization of the cellular layers of the swim bladder.

DISCUSSION

The present study was designed to empirically test the AOP-based hypothesis that deiodinase inhibition as a molecular initiating event can impair anterior and posterior swim bladder inflation in early-life stage fathead minnows. Previous studies have demonstrated IOP to be a deiodinase inhibitor both in vitro and in vivo, therefore it is a suitable model chemical to investigate this hypothesis [3,30-32]. Endpoints measured in the current experiment align with key events in the related AOPs. These included measurements of whole body T3 and T4 concentrations and swim bladder inflation and morphology. Additionally, mRNA expression and histological analyses were conducted to examine potential compensatory responses to exposure to IOP, given that our previous studies with MBT (a TPO inhibitor) showed apparent compensatory up-regulation of *tpo* mRNA and thyroid follicle hypertrophy and hyperplasia in response to MBT exposure (Nelson et al., 2016).

Embryo study: Effects on posterior swim bladder

The first objective was to determine the potential effect of embryonic exposure to a model deiodinase inhibitor on posterior swim bladder inflation. Consistent with our hypothesis, despite the potential availability of maternally-derived thyroid hormones, embryonic exposure to IOP (6 mg/L) resulted in significant impairment of posterior swim bladder inflation and significantly reduced the length of inflated posterior swim bladders at 6 dpf (Fig. 5). The

observation of increased whole body T4 content in IOP-exposed animals at 4 dpf was also consistent with the expectation of less conversion of T4 to T3 in IOP-treated fish. However, although we hypothesized that the posterior swim bladder was unable to inflate due to inhibition of deiodinase activity and presumably limited availability of T3, whole body T3 concentrations were unexpectedly higher in the IOP-exposed fish, with significant increases observed in the 6.0 mg/L treatment at 4 dpf and in the 1.9 and 6.0 mg IOP/L treatments at 6 dpf. One possible explanation for the elevated T3 concentrations may be the potential impact of IOP exposure on DIO3. While deiodinase types 1 and 2 are largely regarded to be responsible for conversion of T4 to the more active T3, DIO3 is an inactivating enzyme that removes iodine from the inner ring of T4 and T3, resulting in reverse T3 (rT3) and the inactive product, 3,5-diiodo-L-thyronine (T2), respectively [33]. Maternal sources of thyroid hormones are known to include both T4 and T3 [1,34]. Consequently, reduced conversion of T3 to inactive forms may be one plausible explanation for the increase. Excess T4 could result from either reduced conversion of T4 to T3 via effects on DIO1 or DIO2 or reduced conversion to rT3 via an effect on DIO3.

An alternative explanation could involve the role of deiodinases in negative feedback. Intra pituitary conversion of T4 to T3 by DIO2 and/or clearance of T3 DIO3 are thought to play a critical role in negative feedback of thyroid hormones on thyroid stimulating hormone secretion [35,36]. Thus, it is certainly plausible that DIO inhibition by IOP could alter negative feedback regulation allowing for increased T4 and T3 production during embryonic stages. However, given numerous uncertainties regarding the developmental stages at which the negative feedback loop is operative or inoperative, the specific role of the deiodinases in the process, and the expression and activity of the various deiodinases in different tissues over the course of development, one can only speculate as to whether this explains the results observed.

Regardless of the mechanistic basis for the increased T3, the fact remains that the embryos with the greatest whole body T3 had reduced posterior swim bladder inflation compared to those organisms with lesser T3 concentrations. Thus, these results raise some question regarding the utility of whole body T3 concentrations as a potential predictor of posterior swim bladder chamber inflation as well as DIO inhibition during early life stages when maternally-derived hormones are available. Overall, the results support some, but not all aspects of the relationships depicted in AOPs 155 and 157 [14,16].

Consistent with increased T4 concentrations, *tpo* mRNA expression was significantly down-regulated in a dose-dependent manner with increasing IOP exposure concentration (Fig. 1), which may play a compensatory role in the stabilization of T4 concentrations at 6 dpf after initially increasing at 4 dpf (Fig. 3).

Larval study: Effects on anterior swim bladder

The second objective of our study was to determine the potential effect of larval IOP exposure on anterior swim bladder inflation. At test initiation (6 dpf), the posterior swim bladder was already inflated in all fish, allowing for a focused examination of effects on anterior swim bladder inflation. Exposure to all three IOP concentrations inhibited anterior swim bladder inflation and reduced anterior swim bladder length at 14 dpf (Fig. 6). These findings indicate that inflation of the anterior chamber is at least 10 times more sensitive to IOP than inflation of the posterior chamber. At present it is unclear whether this is due to depletion of the maternal sources of T3 by 14 dpf or whether it may reflect involvement of different localized DIO enzymes for anterior versus posterior inflation; nonetheless, the life-stage associated with anterior inflation appears to be significantly more sensitive than the earlier life-stage associated with posterior swim bladder inflation.

As determined histologically, the uninflated anterior swim bladders did not appear to result from a lack of development of the cellular layers of the swim bladder, but more likely was due to lack of the volume of gas required to expand the chamber. While there was evidence of recovery of inflation by 18 dpf (Fig. 6), anterior swim bladder length was still significantly decreased at 18 and 21 dpf (Fig. 7). In previous studies with cichlids, the size of the anterior swim bladder plays a role in the hearing frequency range of fish due to proximity to the ear [11]. However, among some species of thorny catfishes, smaller bladders were associated with better hearing [37]. Given the incredible species diversity in peripheral hearing structures among fish, a simple generalizable relationship between anterior swim bladder size and hearing ability in all species is unlikely. Nonetheless, there is ample evidence that the swim bladder, through its interaction with the Weberian apparatus, has an important role in hearing in otophysine species [38]. Therefore, even though anterior swim bladder inflation was only delayed, a smaller-sized chamber could potentially affect fish behavior in response to auditory stimuli, at least in some species.

Consistent with the results of MBT exposure in fathead minnows and zebrafish [6,7], posterior swim bladder length was significantly increased in all of the IOP treatments at 14 and 18 dpf, with the exception of the 6 mg IOP/L treatment group at 18 dpf (Fig. 7). One possible explanation of the increased posterior swim bladder length at 14 and 18 dpf is due to delayed evagination of the anterior swim bladder from the posterior chamber, leaving excess gas in the posterior chamber, thereby increasing the size of the posterior chamber [8]. Studies in zebrafish demonstrate that gas can be efficiently transferred between the anterior and posterior chambers, with the anterior chamber showing a much greater overall range of volume change in response to ambient pressure [39]. The exact role that thyroid signaling may play in either regulating

evagination to form the two chambers of the swim bladder or to influence gas exchange between the chambers is unknown.

In the larval study, at test initiation, any maternally-derived hormones that may have been present in the embryo study were likely no longer available, as the yolk sac has been entirely absorbed. At this point, any changes in thyroid hormone concentrations can confidently be attributed to effects of chemical exposure on endogenously synthesized hormone. Consistent with our hypothesis, whole body concentrations of T3 were significantly reduced, resulting in elevated T4 concentrations in all IOP treatments throughout the larval study (Fig. 4). At this stage of larval development, if there was an effect on DIO3 activity which would have limited the conversion of T3 to inactive forms, it was overwhelmed by the reduced conversion of endogenously-synthesized T4 to T3. This suggests that the excess T3 observed in the embryo exposure was likely from maternal sources, while the excess T4 may be from reduced conversion of maternal and/or endogenously-synthesized T4. When considering homeostatic regulation of the HPT axis, the expression patterns of gene transcripts coding for deiodinases and *tpo* align well with the thyroid hormone concentration profiles. Compensatory up-regulation of *dio2* mRNA expression, which would serve to help boost T3 concentrations, could be expected in response to the significant decrease in T3 concentrations throughout the larval study in all treatment groups. Likewise, given the excess availability of T4 in the IOP-treated fish throughout the larval study, it is not surprising that *tpo* mRNA transcript abundance was down-regulated as a compensatory measure [40,41].

Histological effects of the IOP exposures were also more consistent with reduced conversion of T4 to T3, than an overall hypothyroid state. For example, in contrast with exposure to the TPO inhibitor, MBT, no thyroid follicular cell hypertrophy or hyperplasia were

observed in fathead minnow larvae exposed to IOP. With TPO inhibition, and consequently decreased T4 concentrations, the thyroid gland is stimulated to compensate by increasing T4 production via follicular cell size and abundance [6]. In the case of deiodinase inhibition, a surplus of T4 was available to the fish; consequently, they had no need to compensate via follicular hyperplasia and/or hypertrophy. Negative feedback on thyroid-stimulating hormone secretion, the major driver of thyroid cell growth in teleosts and other vertebrates is largely regulated by plasma T4 concentrations [40,42]. Consequently, the lack of thyroid follicle proliferation under conditions of excess T4 is consistent with what would be expected based on known HPT axis physiology.

Effects of IOP exposure on deiodinase mRNA expression

Among the three forms of iodothyronine deiodinases found in teleost fish, expression of mRNAs coding for DIO2 was the most significantly impacted in the larval study, showing a time-dependent increase at all three IOP concentrations. Deiodinase 3 mRNA expression was also impacted by IOP exposure, although it did not show the time-dependence and sensitivity that was evident for *dio2;* only the fish exposed to 6 mg/L showed a significant increase in *dio3* at more than one time point. Deoidinase 1 mRNA expression was unaffected by IOP exposure.

Based on previously hypothesized AOPs, inhibition of DIO1 and DIO2, both of which are involved in conversion of T4 to T3, was postulated to plausibly result in impaired posterior and anterior swim bladder inflation [14-17]. Given that IOP has been shown to be a potent inhibitor of both DIO1 and DIO2 in vitro (unpublished data), the lack of compensatory response of *dio1* in the present study may imply that anterior swim bladder inflation is more dependent on DIO2 and DIO3 activity, or possibly a balance between the two, than DIO1 activity. Based on the whole body measurements conducted in the present study, it cannot be determined whether

this may reflect tissue specificity of *dio2* mRNA expression and activity in or around the developing anterior swim bladder or a difference in time-dependent expression of *dio1* and *dio2* mRNA during development. Dong et al. [43] reported expression of both *dio1* and *dio2* mRNA in the zebrafish swim bladder (although only the posterior chamber was inflated at the stages examined in their study). However, in a recent gene ontogeny study conducted in our laboratory, peak *dio2* mRNA expression occurs during larval development, approximately at the time when anterior swim bladder inflation occurs (unpublished data). Deiodinase 1, on the other hand, is initially expressed during embryonic development and levels out around the time of hatch and posterior swim bladder inflation (unpublished data). Our data for the fathead minnow are similar to previous results in zebrafish that showed a gradual increase in *dio1* mRNA expression over the course of development, while *dio2* expression increased markedly around the time of hatch and anterior swim bladder inflation [34, unpublished data]. Likewise, the results are consistent with those in striped parrotfish in which *dio2* and *dio3* mRNA expression was impacted by exogenous exposure to T3 or methimazole, but *dio1* mRNA expression was not [44]. The results also align well with observations on the effects of dio-specific knockdown during zebrafish development. Specifically, knockdown of *dio1* alone did not appear to have much impact on zebrafish development [45]. In contrast, knockdown of either *dio2* or *dio3* alone had profound developmental effects [45,46]. Overall, the results suggest a more constitutive expression of *dio1*, with *dio2* and *dio3* expression showing greater responsiveness to changes in thyroid status during the fathead minnow developmental stages examined in the present study. Together, these data suggest that AOPs linking DIO2 and perhaps DIO3 inhibition to impaired swim bladder inflation may be more relevant than the hypothesized AOPs involving DIO1 inhibition as the molecular initiating event.

Specificity of thyroid-disruption on swim bladder inflation

Overall, results from the present study support the hypothesis that a reduction in available T3, via inhibition of deiodination of maternally-derived and endogenous T4, results in impaired swim bladder inflation, particularly the anterior chamber, during early fathead minnow development. Based on the results of our IOP exposure alone, we cannot rule out the possibility that IOP may impact swim bladder inflation via processes independent of effects on available T3. Indeed, in the present embryo study, inflation of the posterior chamber was impaired even though whole body T3 concentrations in the IOP-treated embryos exceeded those of controls. Nonetheless, there is supporting evidence from a number of deiodinase knockdown studies that suggest a specific role for deiodinase activity in the process of swim bladder inflation (as well as numerous other developmental events in fish). For example, knockdown of *dio1* and *dio2* significantly reduced swim bladder inflation (96 hpf) in zebrafish [47], and knockout of *dio2* in zebrafish also resulted in defects in swim bladder inflation [48]. Deiodinase 3 knockdown had an even more pronounced effect of swim bladder inflation, but also impacted both larval length and hatching success, suggesting perhaps a more systemic developmental impact [46,47].

Additionally, morpholino knockdown of deiodinase 3 in zebrafish resulted in disturbed escape-response behavior, possibly a result of perturbation of thyroid-dependent gene transcription patterns involved in muscular development [46]. Delays in muscular development in *dio3* knockdown zebrafish align with our observation of delayed muscular development (i.e., lack of transparency) at 21 dpf in the IOP-exposed fathead minnows. Finally, although they did not focus on swim bladder specifically, Walpita et al. [49] showed that developmental effects of *dio2* morpholino knockdown could be offset by T3 supplementation. Thus, there is growing evidence for an important role of deiodinase activity in both swim bladder inflation and other

processes during fish development. Consequently, it is reasonable to conclude that significant impairment of deiodinase activity is likely to impact swim bladder inflation and relative chamber size. However, it does not necessarily imply that impacts of deiodinase inhibition during fish development are restricted to swim bladder, or that there are not other pathway perturbations or chemical modes of action that could cause the same non-inflation phenotype.

CONCLUSIONS

Overall, results from the present study support our hypothesis that posterior and anterior swim bladder inflation in fathead minnows was impacted by exposure to a model deiodinase inhibitor. Given the potential presence of maternal sources of T3 around the time of posterior chamber inflation, the posterior chamber appears less sensitive to effects on endogenous thyroid hormone status than the anterior chamber. The results broadly align with AOPs 155-158 [14-17] by providing further evidence that perturbation of deiodinase activity in early-life stage fish can result in critical morphological effects (e.g., swim bladder inflation) via perturbation of key events associated with the HPT-axis (e.g., thyroid hormone production); however the increased T3 abundance in the presence of decreased posterior chamber inflation in the embryo study, along with the potentially more prominent roles of *dio2* and *dio3* in mediating the effects, suggest some refinements to the existing AOPs may be needed. While further gene knockdown experiments and/or chemical exposure experiments have potential to strengthen the hypothesized causal relationship between HPT-perturbation and swim bladder effects in fathead minnows and other fish species, the present study, examining effects of deiodinase inhibition, along with previous studies investigating effects of TPO inhibition [6,7], demonstrate swim bladder inflation can be expected as a common effect of short-term early-life stage fish exposure to thyroid disrupting chemicals.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data Accessibility—The data from this manuscript can be accessed through www.data.gov.

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Figure 1. Effects of iopanoic acid (IOP) exposure on fathead minnow embryo thyroperoxidase (*tpo*) mRNA transcript abundance at 6 days post-fertilization. Bars represent mean ± SE of n=4 pooled whole body samples per treatment. Different letters indicate significant differences ($p < 0.05$) among treatments.

Figure 2. Effects of iopanoic acid exposure (IOP; 0 mg/L, 0.6 mg/L, 1.9 mg/L, and 6.0 mg/L) on fathead minnow larval expression of mRNA transcripts for (A) thyroperoxidase (*tpo*), (B) deiodinase 1 (*dio1*), (C) deiodinase 2 (*dio2*), (D) deiodinase 3 (*dio3*) at 10, 14, 18, and 21 days post-fertilization (dpf). Data points represent the mean ± SE of n=4 pooled whole body larval samples per treatment. * indicates significant differences ($p < 0.05$) between control and IOP treatments.

Figure 3. Effects of iopanoic acid exposure (IOP; 0 mg/L, 0.6 mg/L, 1.9 mg/L, and 6.0 mg/L) on whole-body thyroid hormone concentrations in pooled fathead minnows at 4 and 6 days post-fertilization (dpf). (A) 3,5,3'-triiodothyronine (T3) concentrations; (B) thyroxine (T4) concentrations. Data points represent the mean ± SE of n=4 pooled whole body samples per treatment. Different letters indicate significant differences ($p < 0.05$) among treatments.

Figure 4. Effects of iopanoic acid exposure (IOP; 0 mg/L, 0.6 mg/L, 1.9 mg/L, and 6.0 mg/L) on whole-body thyroid hormone concentrations in pooled fathead minnow larvae at 6 (T0), 10, 14, 18, and 21 days post-fertilization (dpf). (A) 3,5,3'-triiodothyronine (T3) concentrations; (B) thyroxine (T4) concentrations. Data points represent the mean ± SE of n=4 pooled whole body samples per treatment. Different letters indicate significant differences ($p < 0.05$) among treatments.

Figure 5. Effects of iopanoic acid exposure (IOP; 0 mg/L, 0.6 mg/L, 1.9 mg/L, and 6.0 mg/L) on the posterior swim bladder in fathead minnows at 6 days post-fertilization. (A) mean percent

inflation of posterior swim bladders (n=4 reps of 25 fish); (B) mean length of inflated posterior swim bladders (n=86-99). Bars represent mean ± SE. Different letters indicate significant differences ($p < 0.05$) among treatments.

Figure 6. Effects of iopanoic acid exposure (IOP; 0 mg/L, 0.6 mg/L, 1.9 mg/L, and 6.0 mg/L) on the mean percent inflation of anterior swim bladders in fathead minnow larvae at (A) 14 days post-fertilization (dpf) (n=4 reps of 16 fish), and (B) 18 dpf (n=4 reps of 12 fish). Bars represent mean ± SE. Different letters indicate significant differences ($p < 0.05$) among treatments.

Figure 7. Effects of iopanoic acid exposure (IOP; 0 mg/L, 0.6 mg/L, 1.9 mg/L, and 6.0 mg/L) on the mean length of inflated anterior (black bars) and posterior (white bars) swim bladders in fathead minnow larvae at (A) 14 days post-fertilization (dpf) (n=27-64), (B) 18 dpf (n=36-48), and (C) 21 dpf (n=8). Bars represent mean ± SE. Significant ($p < 0.05$) differences between treatments are indicated with lower case letters for anterior swim bladders and upper case letters for posterior swim bladders. Swim bladder lengths for 14 and 18 dpf samples were obtained via live measurements with a camera, and 21 dpf swim bladder lengths were obtained through histological measurements due to lack of transparency of the fish at 21 dpf.

Figure 1.

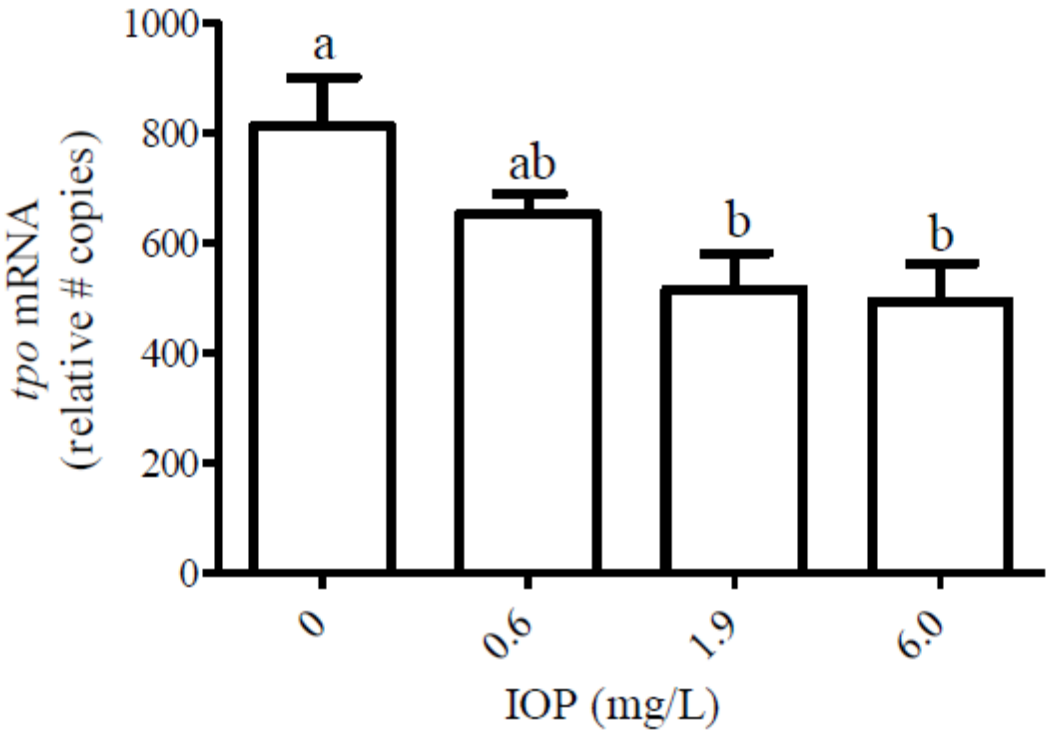


Figure 2.

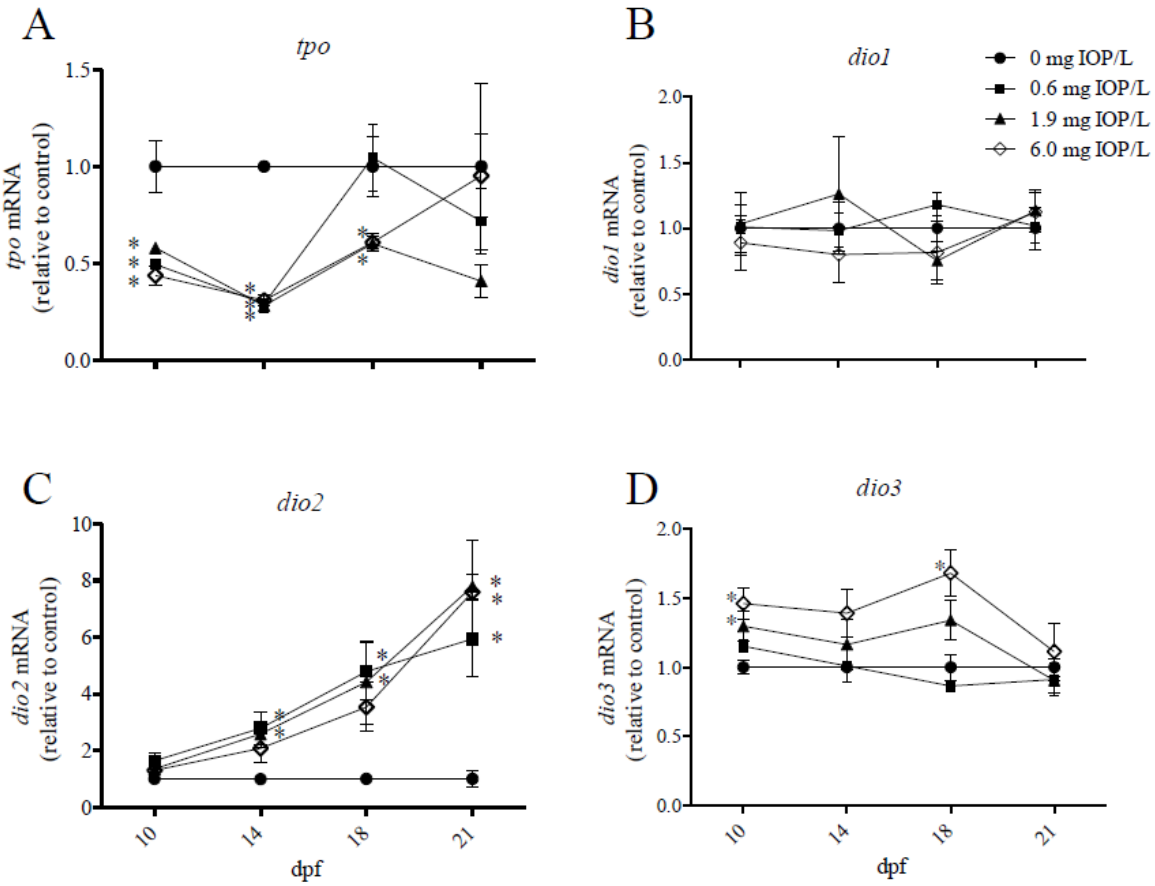
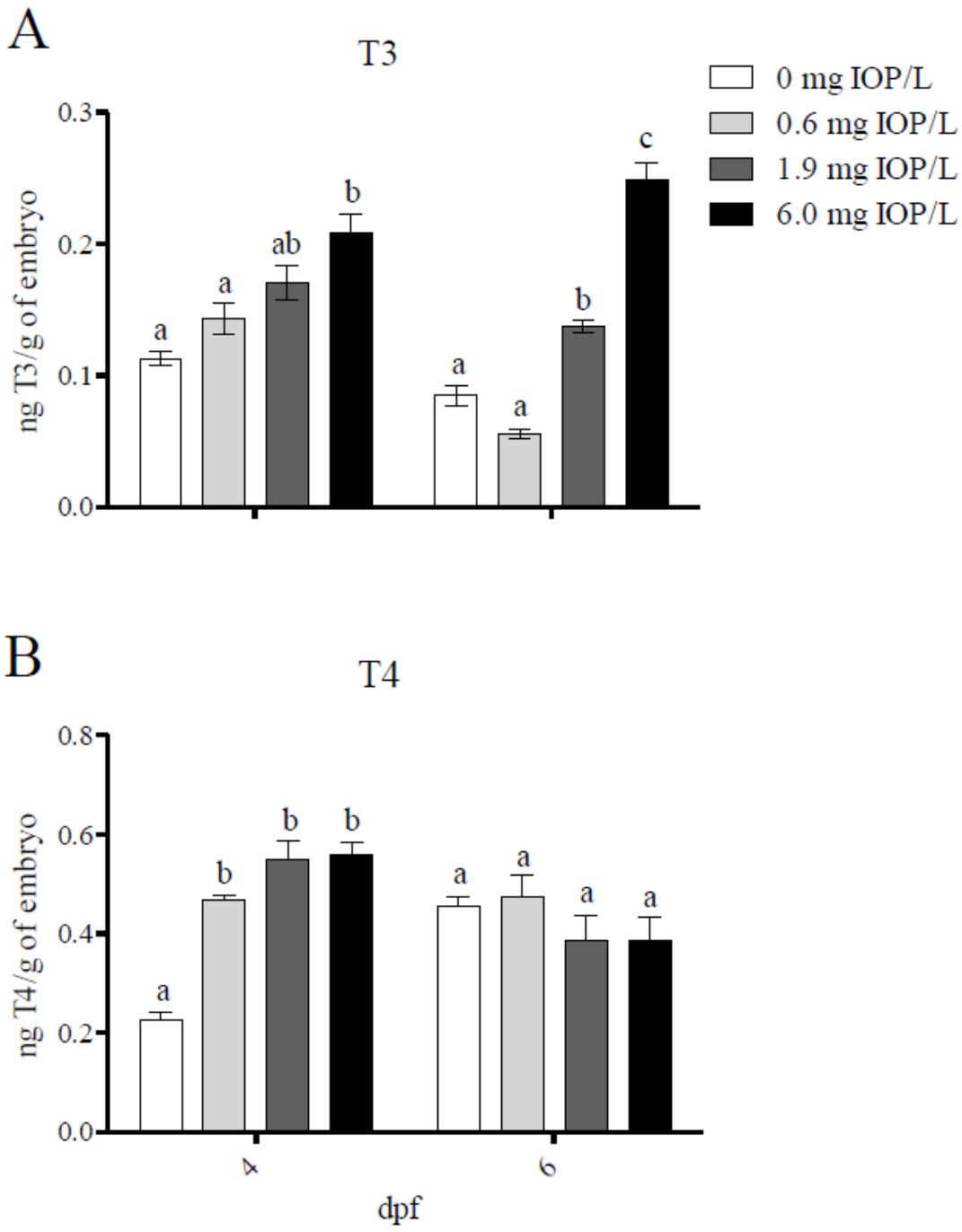


Figure 3.



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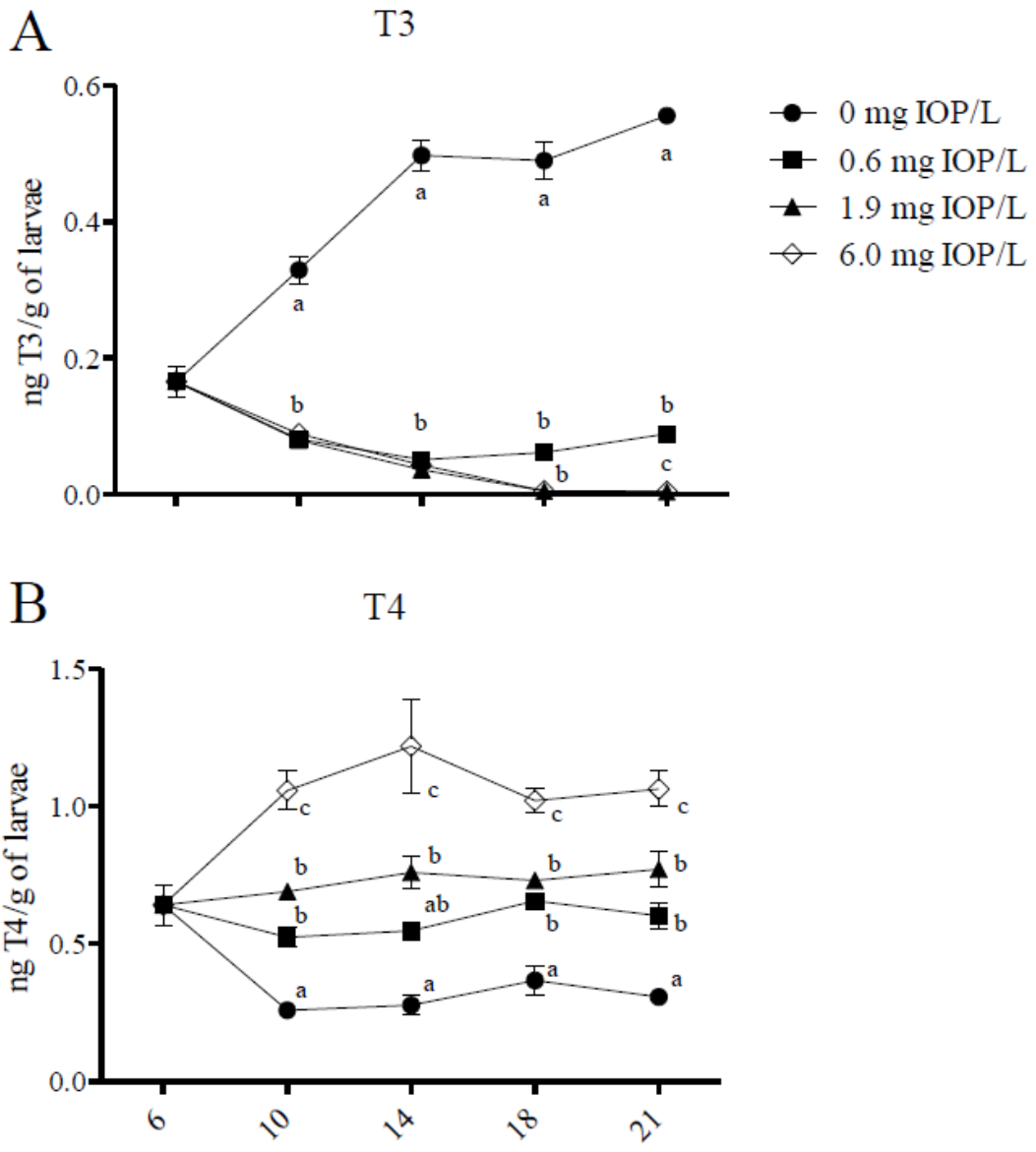


Figure 5.

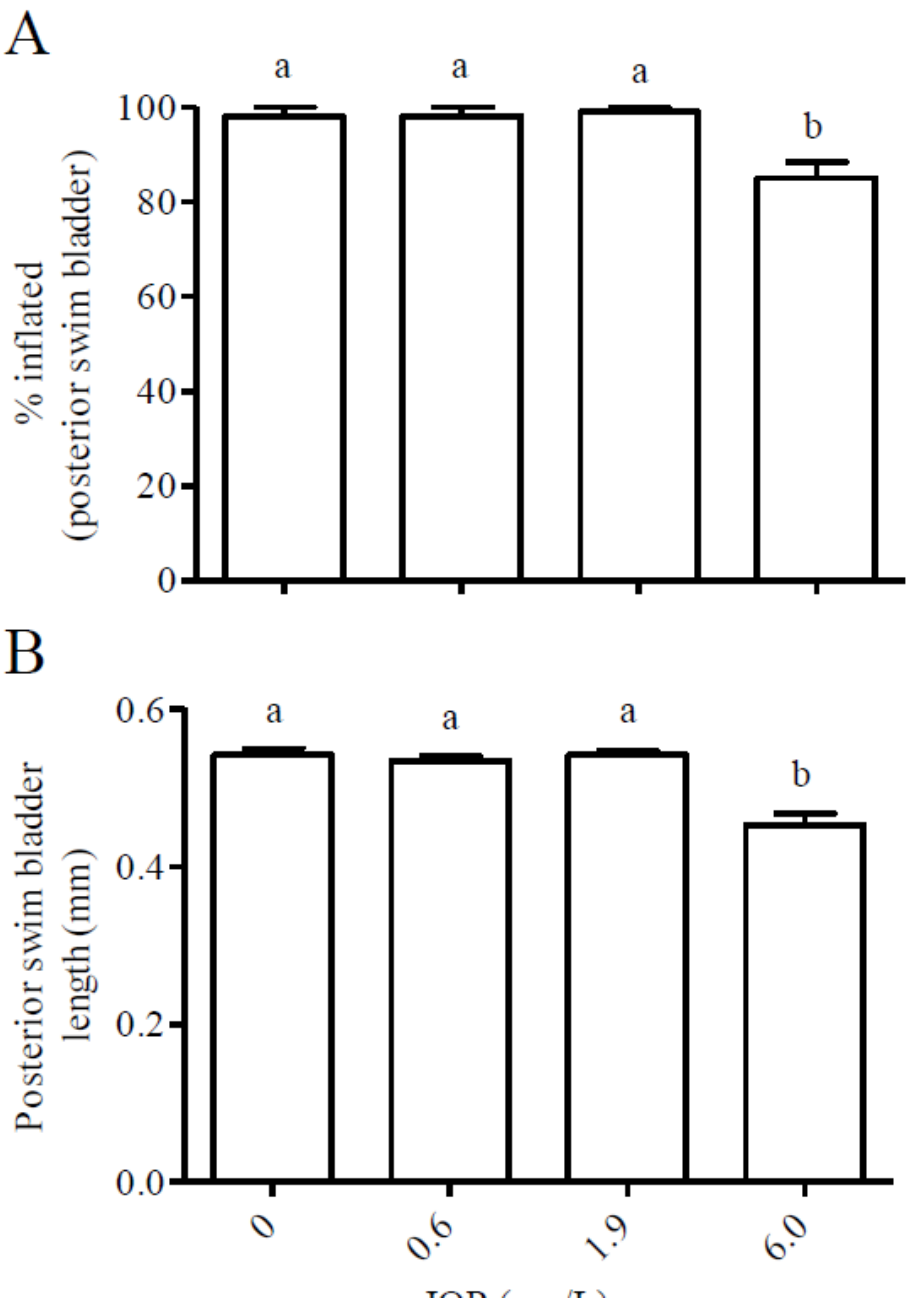
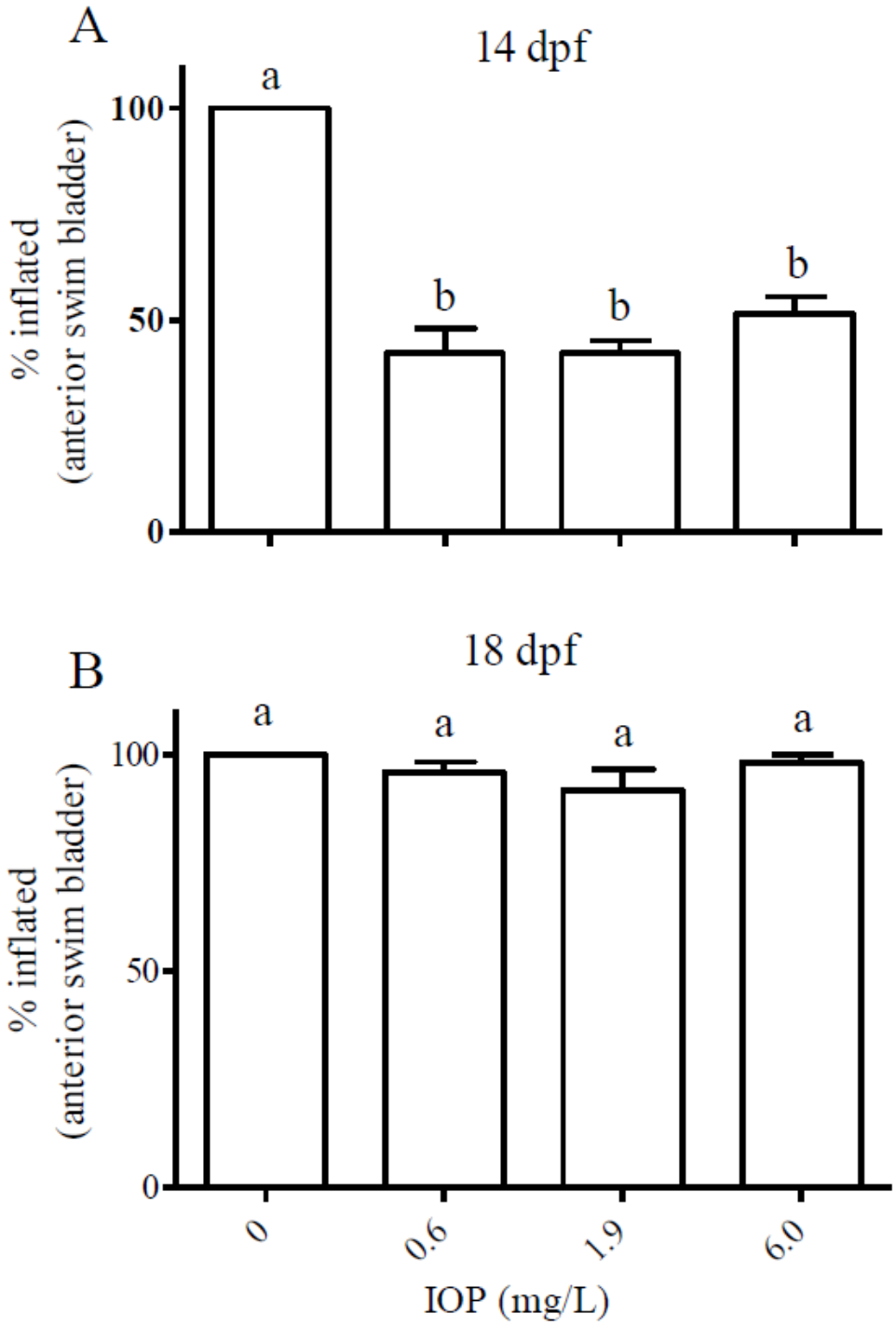


Figure 6.



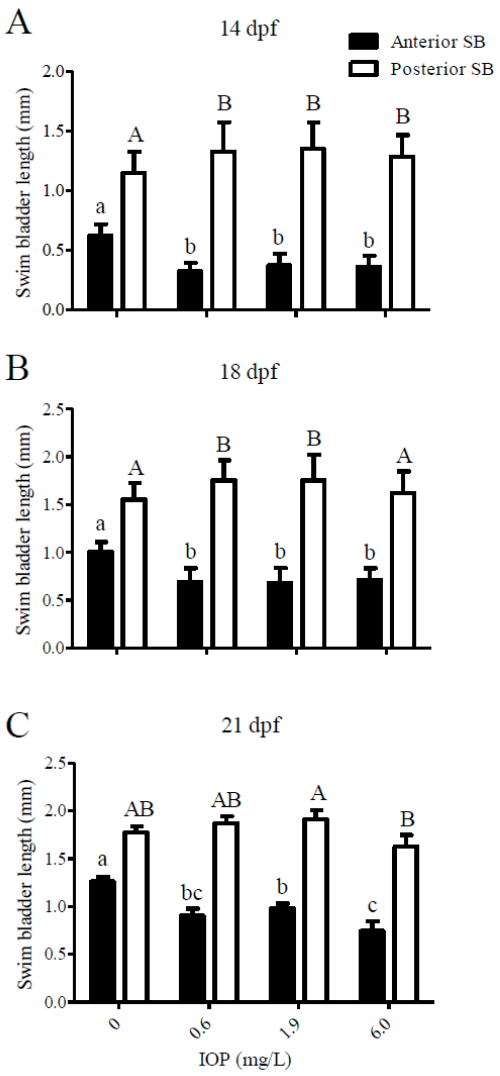


Figure 7