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Deiodinase inhibition impairs the formation of the three posterior swim bladder tissue layers during early embryonic development in zebrafish

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Abstract

Thyroid hormone system disruption (THSD) negatively affects multiple developmental processes and organs. In fish, inhibition of deiodinases, which are enzymes crucial for (in)activating thyroid hormones (THs), leads to impaired swim bladder inflation. Until now, the underlying mechanism has remained largely unknown. Therefore, the objective of this study was to identify the process during swim bladder development that is impacted by deiodinase inhibition. Zebrafish embryos were exposed to 6 mg/L iopanoic acid (IOP), a model deiodinase inhibitor, during 8 different exposure windows (0–60, 60–120, 24–48, 48–72, 72–96, 96–120, 72–120 and 0–120 hours post fertilization (hpf)). Exposure windows were chosen based on the three stages of swim bladder development: budding (24–48 hpf), pre-inflation, i.e., the formation of the swim bladder tissue layers (48–72 hpf), and inflation phase (72–120 hpf). Exposures prior to 72 hpf, during either the budding or pre-inflation phase (or both), impaired swim bladder inflation, while exposure during the inflation phase did not. Based on our results, we hypothesize that DIO inhibition before 72 hpf leads to a local decrease in T3 levels in the developing swim bladder. Gene transcript analysis showed that these TH level alterations disturb both Wnt and hedgehog signaling, known to be essential for swim bladder formation, eventually resulting in impaired development of the swim bladder tissue layers. Improper development of the swim bladder impairs swim bladder inflation, leading to reduced swimming performance. This study demonstrates that deiodinase inhibition impacts processes underlying the formation of the swim bladder and not the inflation process, suggesting that these processes primarily rely on maternal rather than endogenously synthetized THs since TH measurements showed that THs were not endogenously synthetized during the sensitive period.

Keywords

Thyroid hormone system disruption; iopanoic acid; swim bladder development; zebrafish embryo; Wnt/hedgehog signaling; deiodinase inhibition

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1. Introduction

Thyroid hormones (THs) are critical for normal vertebrate development. They are involved in regulating processes such as growth, energy metabolism and brain development (Blanton and Specker, 2007; de Escobar et al., 2004). Environmental pollutants can interfere with the thyroid hormone system (THS). In fish for example, it is known that thyroid hormone system disruption (THSD) can lead to impaired development of eyes and fins, and to impaired swim bladder inflation (Bagci et al., 2015; Baumann et al., 2016; Reider and Connaughton, 2014; Sharma et al., 2016; Stinckens et al., 2016; Stinckens et al., 2018; Stinckens et al., 2020). The swim bladder is a gas-filled organ necessary for regulating buoyancy (Lindsey et al., 2010). The organ consists of two different chambers: a posterior and anterior chamber, with the latter having an additional role in hearing (Robertson et al., 2007). Development and inflation of both chambers is separated in time. In zebrafish, the posterior chamber inflates during embryonic development around 4–5 days post fertilization (dpf). The anterior chamber, which buds from the posterior chamber during early development, inflates during the juvenile stage around 21 dpf. An adverse outcome pathway (AOP) network was previously developed linking THSD to impaired swim bladder inflation (Knapen et al., 2020; Stinckens et al., 2016; Stinckens et al., 2018; Stinckens et al., 2020). The AOPs that are part of this network have recently been endorsed and published by the OECD (Vergauwen et al., 2022a, 2022b, 2022c, 2022d, 2022e). The swim bladder AOP network describes how thyroperoxidase (TPO) inhibition and iodothyronine deiodinase (DIO) type 1 and 2 inhibition affect swim bladder inflation. TPO is an enzyme crucial for TH synthesis, whereas DIO enzymes are required for both activation and inactivation of THs. DIO isoforms type 1 and 2, which are mainly regarded as the activating DIO enzymes, are required for TH activation through the conversion of the prohormone thyroxine (T4) to its more active form 3,5-triiodothyronine (T3). DIO isoform type 3 on the other hand causes TH inactivation by removing iodine from the inner ring of T4 and T3, yielding reverse T3 (rT3) and 3,5-diiodo-L-thyronine (T2) respectively. TPO and DIO1/2 inhibition have been shown to affect the swim bladder in a life-stage specific manner. TPO inhibition prevents proper anterior chamber inflation in the juvenile life stage, while DIO1/2 inhibition affects the development and inflation of both chambers (Bagci et al., 2015; Cavallin et al., 2017; Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2018; Stinckens et al., 2020). However, the precise underlying mechanisms through which THSD impacts swim bladder inflation during these different life stages remains unclear.

In the present study we aimed to advance our mechanistic understanding of how deiodinase inhibition impacts formation and inflation of the posterior swim bladder. The development of the posterior chamber takes place during early embryonic development and can be subdivided into three different stages: the budding, pre-inflation and inflation phase. At around 30–42 hours post fertilization (hpf) during the budding phase, the swim bladder primordium evaginates from the foregut epithelium (Hagenaars et al., 2014; Winata et al., 2009). During the pre-inflation phase (48–72 hpf), Wnt/β-catenin and hedgehog signaling are required for the proper organization and development of the three tissue layers that make up the swim bladder wall: epithelium, a mesenchymal smooth muscle layer, and the outer mesothelium. Hedgehog signaling is involved in the differentiation of the epithelial

and mesenchymal layer, and in the organization of the outer mesothelium. Wnt/β-catenin is required for the growth of all three layers. All three layers are present at 60 hpf (Winata et al., 2009; Yin et al., 2011). As there is crosstalk among TH signaling and both the Wnt and hedgehog signaling pathways, it is plausible that DIO inhibition can interfere with these crucial pathways (Desouza et al., 2011; Skah et al., 2017). During the inflation phase (72–120 hpf), the eleutheroembryos exhibit a sequential series of upward movements in order to reach the air-water interface and gulp air (Lindsey et al., 2010). This air is transported through peristaltic movements into the swim bladder via the pneumatic duct (Goolish and Okutake, 1999). To inflate the swim bladder, surfactant proteins are needed to maintain surface tension. As reduced TH levels have been found to decrease surfactant proteins, it is also possible that DIO inhibition disrupts the inflation phase through this mechanism (Zheng et al., 2011). Following inflation, the volume of the swim bladder is maintained and regulated by release of oxygen from the blood. Oxygen is released from hemoglobin through local acidification of the blood through lactic acid production by epithelial cells (Finney et al., 2006; Umezawa et al., 2012). It has been hypothesized that specific solute transporters required for local blood acidification are additional potential molecular targets for THSD (Villeneuve et al., 2014).

To elucidate the time frame during which the posterior swim bladder can be directly impacted by DIO inhibition, zebrafish embryos were exposed to iopanoic acid (IOP), a DIO inhibitor, during specific time windows corresponding to the different phases in swim bladder development (budding, pre-inflation and inflation phase). Gene transcript analysis was then performed to gain insight in which processes in the sensitive window might be affected. Finally, the effect of DIO inhibition on whole-body TH levels during early embryonic development was assessed to provide further insight into the link between THSD and impaired swim bladder inflation.

2. Material and methods

2.1 Egg production

Broodfish (adult wild type, AB strain) were housed in a ZebTEC standalone system (Tecniplast, Buguggiate, Italy) at the University of Antwerp. All broodfish were kept in reconstituted fresh water (basis reverse osmosis (RO) water, pH 7.5 ± 0.3 ($NaHCO_3$), conductivity 500 ± 50 μS (Instant Ocean® Sea Salt, Blacksburg, USA)) with constant temperature of 28.0 ± 0.2 °C and in a 14/10h light/dark cycle. Ammonia, nitrite and nitrate levels were measured twice a week using Tetratest kits (Tetra, Melle, Germany) and remained below 0, 0.3 and 12.5 mg/L respectively. On weekdays, fish were fed twice with granulated food (1.5% of their mean wet weight, Zebrafeed, Sparos, Olhão, Portugal) and once with Chironomidae or Chaoboridae larvae, *Daphnia* sp. or *Artemia* sp. nauplii (Aquaria Antwerp bvba, Aartselaar, Belgium). During weekends, fish were fed once a day with granulated food (3% of their mean wet weight, Gemma Micro, Skretting, Stavanger, Norway). The evening prior to spawning, 1 female and 1–2 males were placed in a breeding tank and separated by a divider. The divider was removed the following day as soon as the lights turned on. Embryos were collected 30–40 minutes after spawning in reconstituted fresh water. Using a stereomicroscope (Leica S8APO, Leica Microsystems

GmBH, Germany), the quality of the embryos was assessed. Coagulated, unhealthy and unfertilized eggs were removed. Adult zebrafish maintenance and handling were carried out in strict accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Zebrafish embryos are not protected under this Directive until the age of 120 hpf. The embryo exposure experiments did therefore not require approval by an Ethical Committee for Animal Testing.

2.2 Exposure

Iopanoic acid (IOP) (CAS: 96–83-3, MW: 570.93 g/mol, TCI Europe, >98% purity) stock solution (100 mg/L; 175.2 μM) was prepared in control medium (reconstituted fresh water, see 2.1) containing 0.1M NaOH and sonicated until fully dissolved (± 15 min). pH of the stock solution was then adjusted to 7.5 ± 0.1 with 1N HCl. The stock solution was kept in the dark at room temperature and exposure solutions were made fresh daily. Analytical measurements from a previous zebrafish exposure study under similar circumstances have shown that this method works well as IOP concentrations remained between 84–110% of the nominal concentration (Stinckens et al., 2020). One final test concentration of 6 mg/L (10.5 μM) IOP was chosen based on both concentrations used in literature for exposure in *Xenopus* tadpoles (Cai and Brown, 2004; Haselman et al., 2022) and fathead minnow embryos (Cavallin et al., 2017) and on a series of preliminary experiments (Table S1). Eight different exposure windows between 0–120 hpf (0–60, 60–120, 24–48, 48–72, 72–96, 96–120, 72–120 and 0–120 hpf, illustrated in Figure 1A) were chosen based on a set of preliminary experiments exploring different exposure windows (Table S1). The continuous exposure (0–120 hpf) was used as a reference exposure scenario where impaired swim bladder inflation was expected based on previous research. Embryos were kept in 24-well plates (sterile tissue culture plates, Greiner Bio-One, Frickenhausen, Germany) containing either the exposure medium (6 mg/L IOP) or control medium for unexposed embryos. Plates were filled 24 hours in advance to saturate the plastic with the chosen medium. Each well contained 1 embryo and 2 mL of medium. Embryos were exposed immediately after collection; they were transferred to two consecutive volumes of exposure solution (to minimize dilution of the final test concentration) and finally to the 24-well plates. The same method was used at the start and end of each exposure window (Figure 1) to transfer the embryos to their new medium (IOP or control medium). The medium was renewed daily. In each plate, the first column (4 wells) served as an internal control and a plate was valid if mortality in the internal control was ≤25% (OECD, 2013). Two plates were used per test condition (n=40). An additional 24-well plate filled with 4 mg/L 3,4-dichloroanilline (DCA, CAS: 95–76-1, Sigma-Aldrich) was used as a reference for mortality. An experiment was deemed valid if mortality was <10% in the control plate and ≥30% in the DCA plate at the end of the experiment (OECD, 2013). Mortality was assessed daily and scored based on four parameters: coagulation, heartbeat absent, lack of somite formation and non-detachment of the tail (OECD, 2013). Throughout the experiment, embryos were kept in an incubator (MIR-254-PE, Panasonic, TCPS, Rotselaar, Belgium) at a constant temperature of 28.5 ± 0.2°C and a 14/10h light/dark cycle. Plates were sealed with parafilm to prevent evaporation. Chorions of exposed embryos were manually removed using forceps (5 mm, Dumont), when the majority (>90%) of the control embryos had hatched (around 55 hpf), to reduce hatching variation as this influences swim bladder inflation (Stinckens et al., 2016).

One additional exposure experiment was performed to investigate whether an inability to reach the water surface contributes to the observed impact on swim bladder inflation. Zebrafish embryos were exposed to a lower concentration of IOP of 3 mg/L (5.3 μM) because only one continuous exposure duration of 120 hpf was used rather than a series of shorter exposure windows which require a slightly higher exposure concentration to cause similar effects. During the inflation phase, the water column depth was adjusted (2.5, 5, 7.5 and 10 mm) to allow eleutheroembryos to reach the water surface more easily. Detailed information about this experiment can be found in the Supporting Information (1.2).

2.3 Analysis of swimming activity

Five exposure windows (0–60, 60–120, 24–48, 72–120 and 0–120 hpf) were chosen for analysis of swimming activity. A selection was made to limit the time between the first and last plate on a given measuring day to four hours, as diurnal rhythm can affect behavior. The selection included controls and the continuously exposed group (0–120 hpf) as well as two shorter exposures where effects on the swim bladder were observed (0–60, 24–48 hpf), and two windows where no effects were present (60–120, 72–120 hpf). One plate per condition (20 test embryos) was analyzed using a Zebrabox 3.0 video tracking device (ViewPoint, Lyon, France). Each plate was recorded during 40 min (first 15 min were omitted from the analysis as acclimation period) in light (1200 lux) and analyzed using the ZebraLab software (v3.20.5.104; ViewPoint, > 1 mm/s counted as movement). Swimming activity was defined as the sum of the swimming distance (mm) and of the time moved (s) and was compared among treatments and within treatments (inflated compared to uninflated). Assessment of swimming activity of the internal plate controls showed that no plate effect was present (Figure S1).

2.4 Morphological assessment

At 96 and 120 hpf, swim bladder inflation was scored. A binary scoring system was used, where 1 corresponds to an uninflated swim bladder and 0 to an inflated swim bladder. Embryos that showed malformations prior to exposure (for time windows starting after 24 hpf), were excluded as the observed uninflated swim bladder was not due to the exposure. At 120 hpf, a full morphological scoring was performed. Eleutheroembryos were anesthetized in 100 mg/L ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich, CAS: 886–86-2, buffered to pH 7.5) and transferred to a microscope slide. Presence or absence of multiple sublethal effects was assessed for each individual: oedema, circulation tail (disturbed or absent), blood accumulations, deviations of the head (including malformation eyes, ears and mouth), and deviating pigmentation. No significant increase in these sublethal effects were observed (data not shown). All embryos were photographed (Canon EOS 600D, 18 megapixels) with a calibrator. These images were used to determine larval length and swim bladder surface using the free image processing software ImageJ (v1.53e, https://imagej.nih.gov/ij/). Swim bladder surface was only assessed for those with inflated swim bladders.

2.5 T3 and T4 measurements

Separate exposures were carried out to collect samples for whole-body T3 and T4 measurements. Exposures were performed as explained above (daily medium renewal,

dechorionation at 55 hpf, see section 2.2), except that 960 mL polypropylene containers (DECA, Herentals, Belgium) were used instead of 24-well plates, due to the large number of embryos required for TH analysis. Four independent exposures were carried out to collect four replicate samples (one exposure = 1 replicate). Each test chamber was filled with 200 mL exposure medium and 75 embryos. The same exposure windows used for the analysis of swimming activity (0–60, 60–120, 24–48, 72–120 and 0–120 hpf), were chosen for TH level measurements. First, unexposed embryos were sampled at 48, 60, 72, 96 and 120 hpf to construct a detailed TH ontogeny. Second, for exposure windows 0–60 hpf and 24–48 hpf, sampling was done at the end of exposure (60 and 48 hpf respectively) and at 120 hpf for exposed embryos and corresponding controls. For the other three exposure windows (60–120, 72–120, 0–120 hpf) sampling only took place at 120 hpf (for details on the sampling moments see Figure S2). For each replicate, 150 embryos were pooled and euthanized in 1 g/L MS-222 and subsequently washed three times using control medium. Embryos were placed in a cryovial (VWR International bv, Leuven, Belgium) and snap frozen in liquid nitrogen. Samples were stored at −80°C until further analysis. Samples were extracted as previously described in Nelson et al. (2016) and Stinckens et al. (2016). Extracts were analyzed for T3 and T4 concentration using liquid chromatography mass spectrometry (LC-MS) using a triple quadrupole mass spectrometer with electrospray ionization. Full methodological details and method performance metrics are included in the Supporting Information (1.3).

2.6 qPCR

Transcription of nine genes involved in the development of the swim bladder (Wnt signaling and hedgehog signaling) and markers of the different tissue layers was analyzed at 60 and 120 hpf (see Table S2). These markers included *hb9* (marker epithelium), *fgf10a* (marker mesenchym), *acta2* (marker smooth muscle present in mesenchym) and *anxa5b* (marker outer mesothelium). The timepoints were chosen based on the developmental timeline of the posterior swim bladder: the three tissue layers are present at 60 hpf, and the effect on swim bladder inflation can be assessed at 120 hpf. At 60 hpf, both continuously exposed and unexposed embryos were sampled. At 120 hpf, unexposed embryos and embryos exposed between 0–60 hpf were sampled, and a distinction was made between embryos with inflated and those without inflated swim bladders. Separate exposures in polypropylene containers were carried out to collect the samples, each container contained 200 mL medium and housed 40 embryos (i.e., one replicate). Exposures were carried out as described in section 2.2 with daily renewal of the medium and dechorionation at 55 hpf. Five replicates were used and each replicate was derived from an independent batch of embryos. At both timepoints (60 and 120 hpf), embryos were sampled from each replicate and each sample consisted of 10 pooled embryos. Embryos were euthanized in 1 g/L MS-222 and rinsed three times with control medium and were then transferred to diethyl pyrocarbonate (DEPC, Sigma-Aldrich, CAS: 1609–47-8) treated water and dissected on ice using 55 mm forceps (Dumont). Cuts were made between the otolith and heart and at the level of the cloaca (Figure S3) resulting in 3 different parts: head, abdomen and tail. Samples were snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. Only abdomen samples were used for further gene transcription analysis because they include the swim bladder tissue. RNA was extracted from 5 biological replicate samples for each condition using the

Nucleospin® RNA isolation kit (Macherey-Nagel, Düren, Germany). Purity and integrity of the RNA was confirmed using a BioDrop spectrophotometer and Fragment Analyzer (Agilent Technologies, Diegem, Belgium). Purity was determined using A260/230 and A260/280 ratios and integrity was based on the RNA quality number (RQN). RNA integrity was always 9.2 or higher (Table S3). While the purity of the RNA was low for some samples (Table S3), the resulting cDNA was of high purity and no issues were detected in the subsequent qPCR analysis. cDNA was constructed starting from 250 ng RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions, using random hexamer primers. qPCR analysis was performed using QuantStudio3 (ThermoFisher Scientific, Waltham, MA, USA). Each qPCR reaction contained 1 μL of both 10 μM forward and 10 μM reverse primer (sequences can be found in Table S2), 8 μL DEPC-treated water and 5 μL CAPITAL qPCR Green Mix LRox (Westburg, Leusden, The Netherlands) combined into a master mix. To each reaction 350 ng cDNA in 5 μL was added. The thermal cycling program consisted of: initial denaturation phase of 3 min at 95°C, followed by 45 cycles of 20s at 95°C, 15s at annealing temperature (Table S2) and 30s at 72°C. Melting curves were analyzed to detect potential unspecific amplification. Primer efficiencies were determined for each run using duplicate standard curves consisting of 6 sequential dilutions of a reference sample (mixture of all cDNA samples) and was used to determine relative transcript abundances. Stability of the candidate reference genes was analyzed for each timepoint separately, as transcription levels of housekeeping genes can be time-dependent (Tang et al., 2007). Out of a candidate list of 5 potential reference genes, *hprt1* and *rpn2* were chosen as the most stable genes at 60 hpf and *hprt1* and *β-actin* at 120 hpf using GeNorm (Figure S4) (Vandesompele et al., 2002). Fold changes were calculated against the appropriate control samples (60 or 120 hpf) for each exposure sample and normalized using the geometric mean of the two selected reference genes (Vandesompele et al., 2002).

2.7 Data analysis

Statistical analyses were performed in GraphPad (version 9.3.1) or R (version 4.1.2). Data are reported as average ± standard deviation (SD) and were considered significantly different if p-values were < 0.05. Percentages of uninflated swim bladders and mortality were compared to the control using a binary logistic regression (Michiels et al., 2017). Normality of the data was assessed using the Shapiro-Wilks test. Normally distributed data (larval length, gene transcription at 120 hpf, T3 and T4 levels, and T4/T3 ratios) were analyzed using a one-way analysis of variance (ANOVA) with a Tukey's multiple comparisons test. Gene transcription data at 60 hpf were analyzed using an unpaired student t-test. Swim bladder surface and swimming activity data were not normally distributed. A Kruskal-Wallis test with a Dunn's multiple comparisons was therefore used to analyze swim bladder surface and to compare swimming activity among treatments for those with either inflated or uninflated swim bladders. Differences in swimming activity within a treatment (inflated vs. non-inflated) was analyzed using a Mann-Whitney test.

3. Results

3.1 Effects on the posterior swim bladder

Effects on swim bladder inflation are summarized in Figure 1B. Exposure to IOP during exposure windows of 0–60 hpf (48.7% non-inflated), 24–48 hpf (27.8% non-inflated), 48–72 hpf (25.7% non-inflated) and 0–120 hpf (85.7% non-inflated) all resulted in impaired posterior swim bladder inflation when compared to the control group. For time windows 60–120 hpf (13.2% non-inflated), 72–96 hpf (5.6% non-inflated), 96–120 hpf (7.7% non-inflated) and 72–120 hpf (5.0% non-inflated), no significant effect on posterior swim bladder inflation was found. There were no significant differences in mortality among the treatments (Figure S5) nor were there malformations other than impaired swim bladder inflation (data not shown), indicating that there was no systemic toxicity.

In embryos with inflated swim bladders, both continuous exposure (0–120 hpf) and exposure between 0–60 hpf caused a significant reduction of posterior swim bladder surface area (Figure 2A). For all the other exposure windows, no significant effects on posterior swim bladder surface were observed.

3.2 Morphological and physiological effects

No significant effects on growth measured as body length were observed (Figure 2B). Swimming activity was analyzed for a selection of exposure windows (see section 2.3). Individuals with uninflated swim bladders travelled a significantly shorter distance when compared to the embryos with inflated swim bladders (Asterisks in Figure 2C). Furthermore, the group exposed between 60–120 hpf with inflated swim bladders moved significantly less distance than the controls (Figure 2C). A significant decrease in time moved was found for organisms exposed between 24–48 hpf with uninflated swim bladders compared to the same group with inflated swim bladders (Asterisks in Figure 2D).

No significant alterations in the total time moved were found within both the group with inflated and uninflated swim bladders (Figure 2D).

3.3 Gene transcription analysis

Gene transcription analysis was performed for 60 and 120 hpf embryos exposed to IOP from 0–60 hpf. IOP exposure significantly decreased transcript levels of all tested genes at 60 hpf (Figure 3A). At 120 hpf, transcript levels of *acta2* (marker of smooth muscle and mesenchymal layers) and *anxa5b* (marker of outer mesothelial layer) were significantly decreased following IOP exposure (Figure 3B). Furthermore, *anxa5b* mRNA levels for eleutheroembryos with uninflated swim bladders were significantly lower than those with inflated swim bladders. Although not significant (p-value = 0.0507), a similar trend was observed for *acta2*. Transcript levels of the Wnt signaling molecule *wnt5b* were significantly reduced due to IOP exposure. A significant decrease in mRNA levels was also observed for the Wnt receptor *fzd2*, but only for those with inflated swim bladders. With regard to hedgehog signaling, transcript levels of the hedgehog signaling molecule *ihha* were significantly reduced due to IOP exposure. For the IOP exposed group with inflated swim

bladders, mRNA levels of the hedgehog receptor *ptc1* were significantly lower than those of the control group.

3.4 Impact of water column depth on inflation

No significant differences in swim bladder inflation were found among the different water depths for both control and IOP exposed groups (Figure S6). Irrespective of the water column depth, a significantly higher percentage of uninflated swim bladders was found in the IOP exposed groups compared to their respective controls (Figure S6). Swim bladder surface was significantly reduced for the group that was exposed in only 2.5 mm of IOP-containing solution compared to their respective controls and compared to the other IOP exposed groups (Figure S7A). No effects on larval length were observed (Figure S7B).

3.5 T4 and T3 measurements

First, T4 and T3 levels were measured in control embryos at multiple timepoints between 48 and 120 hpf to construct a detailed TH ontogeny (Figure 4A-B, Table S4). T4 levels (Figure 4A) were low between 48 hpf (0.128 ± 0.050 ng/g) and 72 hpf (0.213 ± 0.041 ng/g), and increased significantly after 72 hpf, reaching its highest values at 96 and 120 hpf. The opposite pattern was observed for T3 (Figure 4B). T3 levels remained stable between 48 (0.409 ± 0.204 ng/g) and 72 hpf (0.365 ± 0.097 ng/g). From 72 to 96 hpf (0.129 ± 0.032), there was a three-fold decrease in T3 levels. This decrease continued until 120 hpf, as T3 levels in three out of four samples dropped below the limit of quantification. As a result, the T4/T3 ratio showed a strong increase at 96 and 120 hpf (Figure S8).

Second, the effect of IOP exposure on TH levels was assessed for a selection of exposure windows (24–48, 0–60, 60–120, 72–120 and 0–120 hpf) (Figure 4C, D). For exposure windows 24–48 and 0–60 hpf, TH levels were measured both at the end of exposure (48 hpf and 60 hpf, respectively) as well as at 120 hpf. TH levels were only assessed at 120 hpf for the remaining exposure windows. Both at 48 and 60 hpf, no effect of IOP exposure on whole-body T4 or T3 levels was observed (Figure 4C, D; Table S5). At 120 hpf, a significant decrease in T4 was noted for both the continuous exposure (0–120 hpf) and exposure between 0–60 hpf compared to the control (Figure 4C; Table S6). T3 levels were higher following IOP exposure, with T3 levels being the highest for the continuously exposed embryos (0–120 hpf) (Figure 4D, Table S6). As T3 levels in three out of four control samples were below the quantification limit, no statistical comparison could be performed. However, this does show that whole-body T3 levels of IOP exposed groups are higher than those of the control group.

4. Discussion

4.1 DIO inhibition affects the formation but not the inflation of the posterior swim bladder

DIO inhibition during the budding (24–48 hpf) and the pre-inflation phase (48–72 hpf) both led to a decrease of about 25% in the proportion of inflated swim bladders at 120 hpf (Figure 1). Exposure that covers the budding and the early pre-inflation phase (0–60 hpf) did result in a stronger effect on swim bladder inflation (50% uninflated) than exposures during both phases separately. These results show that DIO inhibition during the pre-inflation phase

directly contributes to the observed effects, suggesting that it interferes with the formation of the three different tissue layers. In addition, DIO inhibition during the budding phase could play a role as well, although the response observed after exposure during this time window (24–48 hpf) could also, at least in part, be caused by continued presence of the chemical during the pre-inflation phase. Further research into the elimination kinetics of IOP in zebrafish embryos might help clarify this issue. Similarly, the stronger effect observed in the 0–60 hpf exposure window may either be caused by impacting both the budding and pre-inflation phase, and/or by a higher internal dose during the pre-inflation phase as a result of a longer exposure duration.

In any case, our results clearly show that the effect of DIO inhibition on the swim bladder originates prior to 72 hpf, as none of the exposure windows targeting the inflation phase caused significant effects (Figure 1). The absence of effects in the 60–120 hpf scenario specifically, further suggests that the disruption may even occur before 60 hpf, although some uncertainty related to the time that is required for reaching a sufficiently high internal IOP dose needs to be taken into account. It is important to note that the effect on swim bladder inflation was not due to an overall growth delay as no effects on larval length, an indicator of growth, were found (Figure 2B). Also, previous research has shown that exposure to IOP until 7 dpf still results in uninflated swim bladders (Stinckens et al., 2018), demonstrating that our observations do not merely reflect a delay in inflation, but constitute a permanent adverse effect. In summary, our results suggest that DIO inhibition affects the formation and subsequent development of the posterior swim bladder, rather than the inflation process itself.

Finally, our results show that swimming activity decreased for eleutheroembryos with uninflated swim bladders compared to those with inflated swim bladders (Figure 2C,D). This highlights that impaired swim bladder inflation does lead to ecologically relevant effects at the organismal and eventually the population level, confirming similar findings in this respect in previous studies (Hagenaars et al., 2014; Stinckens et al., 2016; Stinckens et al., 2020). More subtle effects on swim bladder surface area were only observed for two exposure windows: 0–60 and 0–120 hpf (Figure 2A). These are the longest exposure windows leading to the most pronounced effects on the proportion of inflated swim bladders. The effect on swim bladder surface area was not clearly reflected in the swimming behavior (Figure 2C, D).

4.2 DIO inhibition interferes with the development of the swim bladder tissue layers

To further understand the mechanism through which DIO inhibition affects swim bladder development, a gene transcription analysis was performed for key genes involved in swim bladder formation in 0–60 hpf exposed embryos at both 60 and 120 hpf. At 60 hpf, the timepoint at which all three swim bladder layers should be present, mRNA levels of genes involved in Wnt and hedgehog signaling were significantly decreased (Figure 3A). Transcript levels of the markers of all three tissue layers were also reduced, indicating improper development of the different swim bladder tissue layers (Figure 3A).

In general, similar observations were made at 120 hpf. Transcript levels of Wnt and hedgehog signaling were still decreased. Transcription of both the Wnt receptor, *fzd2*,

and hedgehog signaling receptor, *ptc1*, was only significantly decreased for the group with inflated swim bladders due to slightly higher variation in the non-inflated group. Transcript levels of *hb9* (marker of the epithelial layer) and *fgf10a* (marker of the mesenchymal layer) were no longer decreased at 120 hpf (Figure 3B). On the other hand, mRNA levels of *acta2* (marker of smooth muscle present in mesenchymal layer) remained significantly decreased (Figure 3B). For the marker of the outer mesothelium, *anxa5b,* transcript levels remained decreased at 120 hpf. In fact, they were even lower in the group with uninflated swim bladders compared to inflated swim bladders (Figure 3B).

Despite a few subtle differences between the gene transcription patterns at 60 and 120 hpf, the overall gene transcription responses are relatively consistent across these time points. First, both hedgehog and Wnt signaling, which are crucial in swim bladder development (Winata et al., 2009; Yin et al., 2011), were disrupted following IOP exposure. As there is considerable crosstalk between these signaling pathways (Yin et al., 2011; Yin et al., 2012), it is difficult to conclude at this point which of these responses is directly resulting from THSD, in particular since there is evidence of interaction between TH and hedgehog signaling (Dentice et al., 2007; Hasebe et al., 2008; Ishizuya-Oka et al., 2001; Xing et al., 2014), and between TH and Wnt signaling (Dentice et al., 2012; Ely et al., 2018) in different species. Second, gene transcription of the markers of all three tissue layers was affected, indicating that the development of these layers was disrupted. The reduced mRNA levels of *acta2* at both 60 and 120 hpf suggests a lack of differentiation into smooth muscle tissue in the mesenchymal layer, while the outer mesothelial layer seemed to be the most severely affected. Similar effects on the markers of the different swim bladder tissue layers have previously been reported for *ihha* morphants (Korzh et al., 2011) and following Wnt signaling knockdown (Yin et al., 2011). In summary, we hypothesize that DIO inhibition affects swim bladder formation by perturbing the formation of the different swim bladder tissue layers through impacting both hedgehog and Wnt signaling leading to altered TH levels (Figure 5).

To further confirm that the improper development of the swim bladder is the primary cause of the uninflated swim bladders rather than a failure to reach the water surface, an additional experiment using different water column heights during the inflation phase (72–120 hpf) was performed. The results of this experiment show that regardless of the distance required to reach the water surface, the IOP exposed embryos were unable to inflate their swim bladders. This further confirms our hypothesis that disrupted formation of the swim bladder lies at the basis of the inability to inflate the swim bladder.

4.3 The formation of the tissue layers relies on maternally derived THs

To understand how THSD might affect different processes and organ systems during early development, it is not only important to consider the development of the organ system itself, but also to take the development and activation of the hypothalamus-pituitary-thyroid (HPT) axis and resulting endogenous alterations of TH levels into account (Figure 6). During early embryonic development, the HPT-axis is not yet functional. Embryos therefore rely on maternally derived THs and transcripts of key thyroid signaling genes in the first days of their development (Chang et al., 2012; Vergauwen et al., 2018; Walter et al., 2019). In

zebrafish, endogenous TH synthesis is thought to be initiated between 60–72 hpf (Chang et al., 2012; Walter et al., 2019), as the first thyroid follicle is formed at 55 hpf (Alt et al., 2006). Furthermore, Walter et al. (2019) showed that exposure to the TPO inhibitor 6-propyl-2-thiouracil resulted in a decrease of T4 at 72 hpf, confirming that the thyroid gland is indeed functional at this timepoint. However, information about earlier timepoints is currently lacking. We therefore performed a TH level analysis during development with a higher time resolution.

In the present study, T4 and T3 levels between 48–120 hpf were measured. Both T4 and T3 levels remained stable until 72 hpf (Figure 4A-B), confirming previous findings (Chang et al., 2012; Walpita et al., 2007). From 96 hpf onwards, opposite patterns were observed for the two hormones. T4 concentrations increased at 96 hpf, reaching a highest value at 120 hpf (Figure 4A). This increase after 72 hpf indicates that endogenous TH synthesis starts around this timepoint. THs that are present before 72 hpf therefore represent the maternally derived pool. Since we showed that DIO inhibition affects the development of the posterior swim bladder before 72 hpf, it is clear that the impact on swim bladder development takes place prior to the start of endogenous TH synthesis (see overview in Figure 5). In a broader perspective, this likely explains why no effects of TPO inhibition (Knapen et al., 2020; Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020) or TPO knockout (Fang et al., 2022) on the posterior swim bladder have been observed, as no systemic effects of TPO inhibition are to be expected prior to the start of endogenous TH synthesis (Figure 6). On a side note, effects of local, extrathyroidal TPO inhibition might occur prior to 72 hpf, as has been suggested to be the case for eye development (Li et al., 2012).

In contrast to T4, T3 levels decreased drastically after 72 hpf (Figure 4B). Walter et al. (2019) also reported decreasing endogenous T3 levels until 120 hpf. This decline probably reflects the depletion of the maternal T3 pool. In contrast however, Chang et al. (2012) reported peak T3 levels around 120 hpf, which the authors linked to the embryo-to-larval transition which takes place around this timepoint. As more TH data become available such variation between studies might be clarified.

4.4 Alterations in whole-body TH levels following IOP exposure are life-stage specific

For two IOP exposure scenarios where impaired posterior swim bladder inflation was observed, i.e. 24–48 and 0–60 hpf, whole-body T4 and T3 levels at the end of the exposure period were unaltered in the exposed embryos compared to the controls. This is not unexpected, since both the thyroid gland (Chang et al., 2012; Walter et al., 2019) and the liver (Chu and Sadler, 2009; Tao and Peng, 2009) are not yet fully formed and functional at those time points. T4 and T3 represent maternally derived pools, and systemic (hepatic) activation of T4 into T3 is not expected to occur at a physiologically relevant rate. However, although DIO inhibition did not result in decreased whole-body T3 levels in the period where swim bladder development is affected (< 72 hpf), these T3 levels do not necessarily represent local T3 levels in the different peripheral tissues. In fact, tissue-specific TH dynamics are tightly regulated through a complex interplay of different TH metabolizing pathways (Bianco and Kim, 2006; van der Spek et al., 2017). Differences in local versus systemic TH levels have been previously reported for humans, rodents, and *Xenopus laevis*

(Campos-Barros et al., 2000; Huang et al., 2001; Liu et al., 2008; Muller et al., 2014; Peeters et al., 2005). *Dio1* and *dio2* expression was previously reported in the swim bladder at 96 hpf (Dong et al., 2013), suggesting that they are indeed involved in regulating swim bladder development.

In contrast, exposure during early embryonic development did alter T3 and T4 levels at 120 hpf (Figure 4C, D). T4 levels at 120 hpf were significantly reduced after IOP exposure during early development (0–60 hpf) but not after IOP exposure during late development (Figure 4C), suggesting that the early life stages when the HPT-axis itself is developing are more sensitive to THSD, leading to an improperly functioning HPT-axis during later development.. Unexpectedly, T3 levels in continuously exposed embryos were higher than those of the controls at 120 hpf (Figure 4D). In fact, they were similar to those observed in control embryos aged 48–72 hpf (Figure 4) and thus seem to reflect an inhibition of the normal systemic decrease of maternally derived T3 (Figure 4B). Interestingly, Cavallin et al. (2017) reported an almost identical effect of IOP exposure on TH levels in fathead minnows in the embryonic stage, while T3 levels have been reported to decrease after IOP exposure during larval and juvenile life stages in both zebrafish and fathead minnow (Cavallin et al., 2017; Stinckens et al., 2020) further illustrating that the effect on TH levels depends on the life stage (Figure S9B). As IOP has been suggested to also inhibit DIO3 (Becker et al., 1997; Bouzaffour et al., 2010; Huang et al., 2011; Kaplan and Yaskoski, 1980; Renko et al., 2015), in addition to DIO1/2 (Haselman et al., 2022; Renko et al., 2015; Stinckens et al., 2018), DIO3 inhibition could be one possible mechanism underlying the higher T3 levels in embryos. DIO3 activity results in inactivation of THs and is considered important during early development, where it protects differentiating tissues from the detrimental effects of excess TH (Darras et al., 1999). Also, deiodinases are involved in feedback mechanisms within the HPT-axis (Arrojo et al., 2013), where DIO1/2 activity in the pituitary is involved in the negative feedback of THs on the secretion of thyroid stimulating hormone (Arrojo et al., 2013; Jones et al., 2017) and IOP might inhibit DIO1/2 locally in the pituitary.

Regardless of the mechanisms underlying the higher whole-body T3 and lower whole-body T4 levels following IOP exposure at 120 hpf, it is important to highlight that these TH level changes take place after the events that adversely affect posterior swim bladder development: we have shown that disruption of swim bladder development takes place prior to 72 hpf, and this is likely the result of local DIO inhibition rather than of systemic effects.

4.5 Conclusion

Exposure to IOP prior to 72 hpf, during the formation (budding and pre-inflation phase) of the swim bladder led to a significant increase of uninflated swim bladders at 120 hpf. Based on our results, we hypothesize that DIO inhibition before 72 hpf leads to a local decrease in T3 levels in the developing swim bladder (Figure 5). The alteration in TH levels then perturbs hedgehog and Wnt signaling, leading to impaired development of all three tissue layers of the swim bladder. Improper development of the swim bladder impairs inflation, eventually resulting in reduced swimming performance. It should be noted that a histological assessment of the developing swim bladder tissue layers in a follow-up study would strengthen the weight of evidence supporting this hypothesis.

This study investigated how the sensitivity of the developing target organ, the swim bladder, to THSD depends on the simultaneous development of the HPT-axis (Figure 6). As such, our results aid in understanding why swim bladder inflation is less sensitive to THSD mechanisms that specifically inhibit TH synthesis, such as TPO inhibition, while mechanisms impacting processes downstream of TH synthesis, such as DIO inhibition, do clearly impair swim bladder inflation (Figure 6). In general, this information is helpful for better understanding other adverse effects caused by THSD as well, such as impaired eye development (Gölz et al., 2022). In conjunction with a deeper understanding of endogenous TH dynamics, and the timing and activation of the different components of the HPT axis, this will ultimately allow for more accurate predictions related to the relationships between different THSD mechanisms and specific adverse effects at different timepoints. In that context, it would be valuable if follow-up studies would also focus on more environmentally realistic exposure scenarios, such as environmentally relevant exposure concentrations or mixture toxicity assessments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

All data are available through https://data.gov (DOI: 10.23719/1529156).

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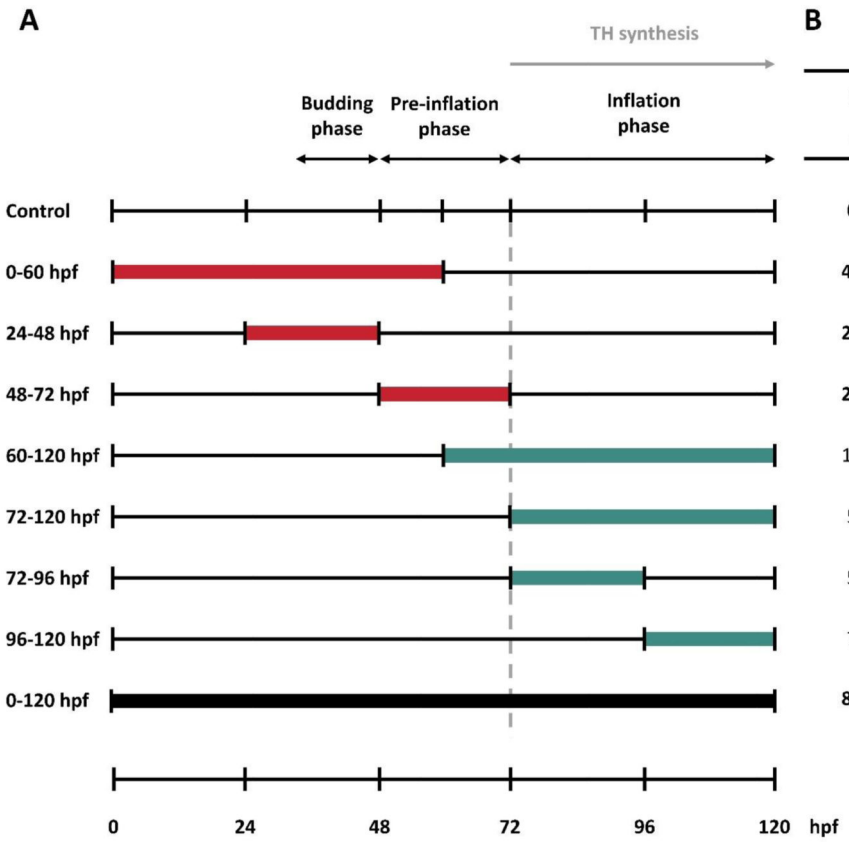

B

	Non inflated SB		SB surface	Larval length	T4 level	T3 level
	%	Fraction				
Control	0.0	0/37	-	-	-	-
0-60 hpf	**48.7**	**19/39**	↓	ns	↓	/
24-48 hpf	**27.8**	**10/36**	ns	ns	ns	/
48-72 hpf	**25.7**	**9/35**	ns	ns	NA	NA
60-120 hpf	13.2	5/38	ns	ns	ns	/
72-120 hpf	5.0	2/40	ns	ns	ns	/
72-96 hpf	5.6	2/36	ns	ns	NA	NA
96-120 hpf	7.7	3/39	ns	ns	NA	NA
0-120 hpf	**85.7**	**30/35**	↓	ns	↓	/

Figure 1: Overview of the experimental setup and main results

(A) Overview of the experimental setup showing the different exposure windows. Thicker lines indicate the periods of IOP exposure (6 mg/L). Red lines show the exposure windows that result in effects on the swim bladder, and green lines show the exposure windows that have no effect on the swim bladder. Continuous exposure to IOP is marked in black and was used as a reference exposure scenario where impaired swim bladder inflation was expected based on previous research. All experiments were ended at 120 hpf for assessment of swim bladder inflation. (B) Summary of the effects of the different exposure windows on swim bladder (SB) inflation, SB surface, larval length and whole body T4 and T3 levels at 120 hpf. Effect on SB inflation is given both as the percentage of eleutheroembryos with uninflated SB and the fraction of eleutheroembryos with non-inflated swim bladders compared to the total number of surviving eleutheroembryos. Eleutheroembryos that showed malformations prior to exposure (for exposure windows starting after 24 hpf), were excluded from the total number as the observed uninflated swim bladder was not due to the exposure. Values that differ significantly from the control are indicated in bold. For all other parameters significant effects are indicated by bold arrows. ‘ns’ indicates no significant results. NA symbolizes the exposure windows for which no TH level measurements were performed. ‘/’ indicates that no statistical comparison could be performed because T3 levels in three out of four replicate samples of the controls had dropped below the limit of quantification at 120 hpf. Detailed information about the effects on SB surface, larval length and thyroid hormone levels can be found in Figures 2 and 4.

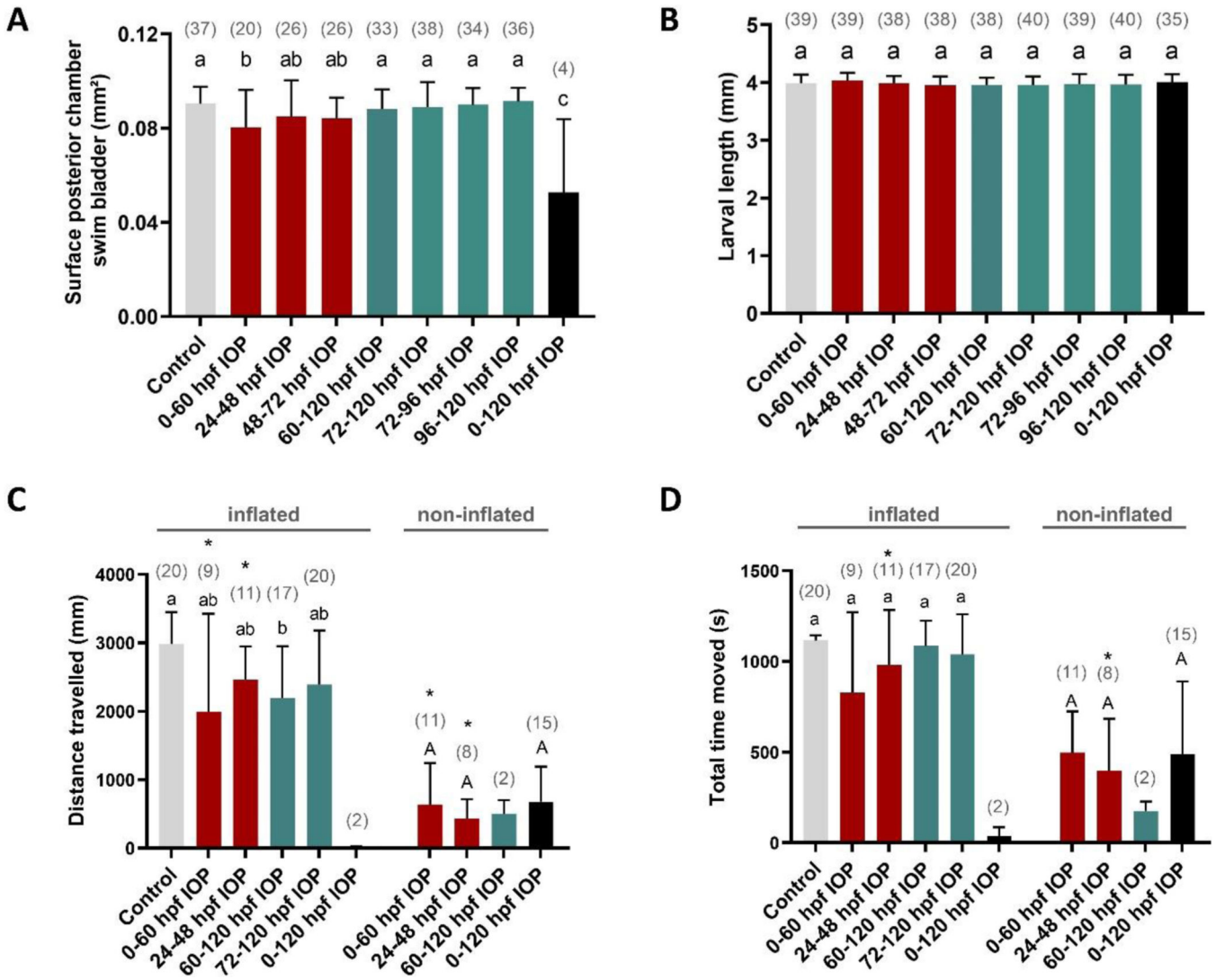

Figure 2: Effects of IOP exposure on morphology and swimming activity.

Effects of IOP exposure (6 mg/L) during different phases in development on swim bladder surface (A), larval length (B) and swimming behaviour (C,D) at 120 hpf. Swimming activity was analysed as the total distance travelled (C) and the total duration of movements during the behavioural test (D) in light conditions (1200 lux). Error bars show standard deviation. Sample sizes (n) are shown between parentheses. Significant differences are denoted by different letters. For swimming activity lowercase and capital letters indicate the significant differences within inflated or uninflated swim bladders respectively. Bars without letters represent the conditions where sample size was too small for statistical analysis. Differences within exposure windows (inflated compared to non-inflated) are indicated with an asterisk and could only be assessed for exposure windows 0–60 and 24–48 hpf due to sample size.

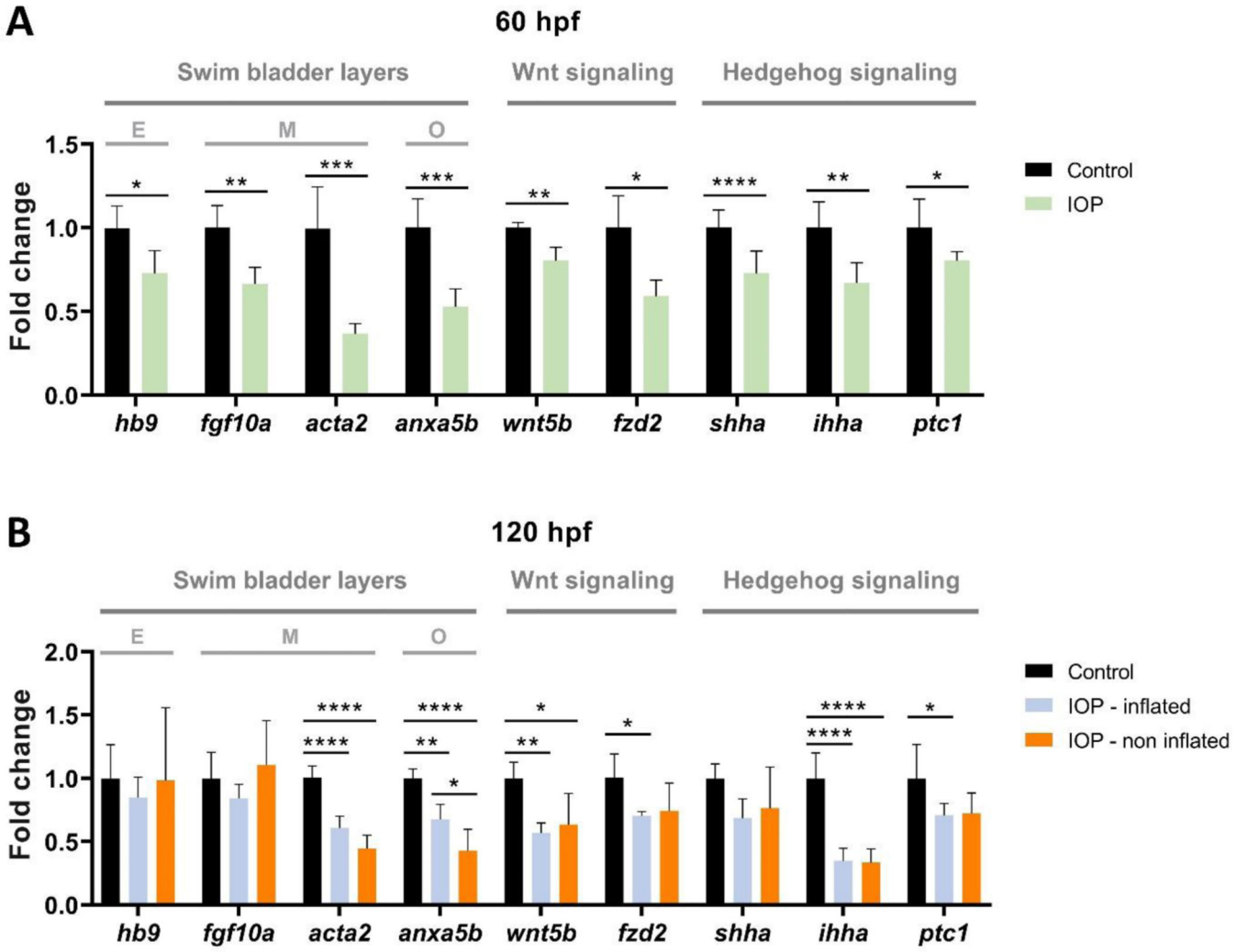

Figure 3: Effect of IOP exposure on gene transcript levels of key swim bladder related genes.

Relative gene transcript levels (relative to controls) for the genes of interest at (A) 60 hpf and (B) 120 hpf, both following IOP exposure (6 mg/L) between 0–60 hpf. Five replicate samples (each sample consisting of 10 abdomens) were used for each condition and statistically significant differences are denoted by asterisks. Error bars show standard deviation. E = marker epithelial layer, M = markers mesenchymal layer, O = marker outer mesothelial layer.

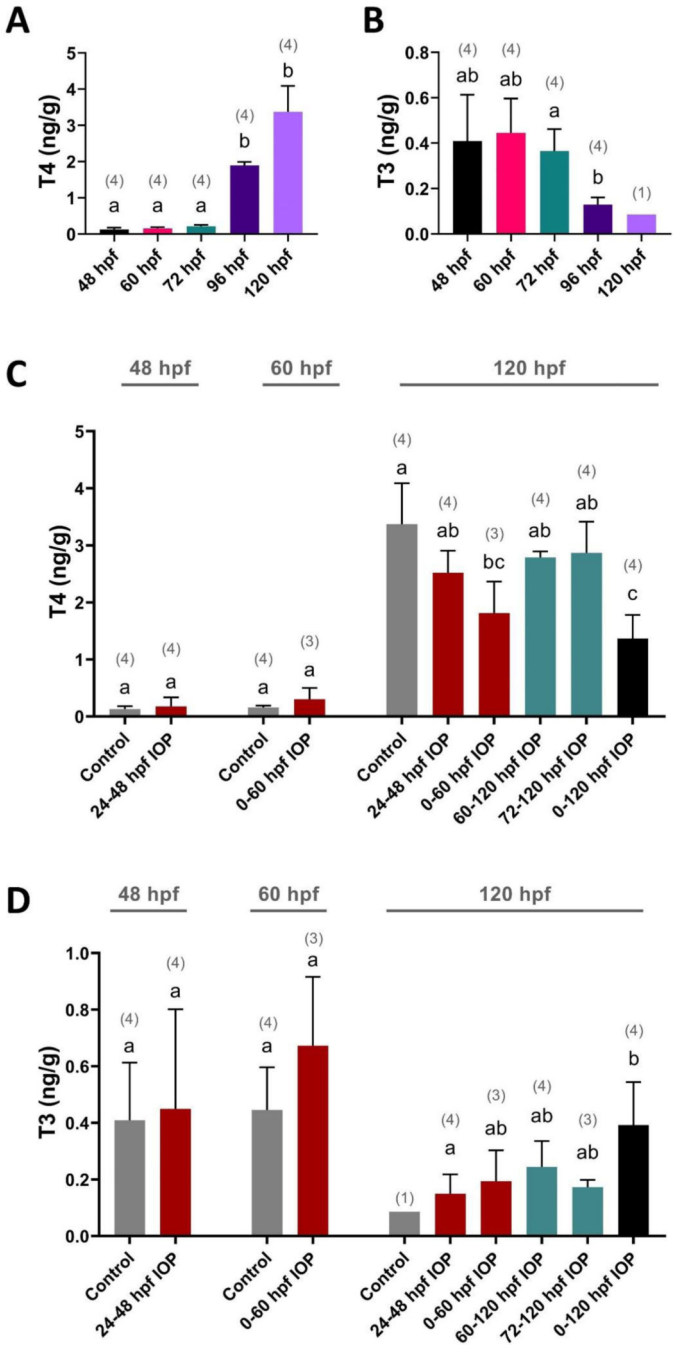

Figure 4: Whole-body T4 and T3 levels.

Levels of T4 (A) and T3 (B) in control embryos at multiple timepoints between 48 and 120 hpf. Effects on T4 (C) and T3 (D) levels after IOP exposure (6 mg/L) during specific time windows (X-axis) measured at 48, 60 and 120 hpf. T4 levels at 48, 60 and 120 hpf in (A) correspond to control data in (C), and T3 levels at 48, 60, 120 hpf in (B) correspond to control data in (D). Error bars show standard deviation. Sample sizes (n) are given between parentheses, each sample consisted of 150 pooled eleutheroembryos. Statistically significant differences are indicated by different letters. Bars without letters represent the conditions

where sample size was too small for statistical analysis. In (C) and (D), statistical analysis was performed within each sampling time point. In (B) and (D) T3 levels in three out of four replicate samples of the controls had dropped below the limit of quantification at 120 hpf.

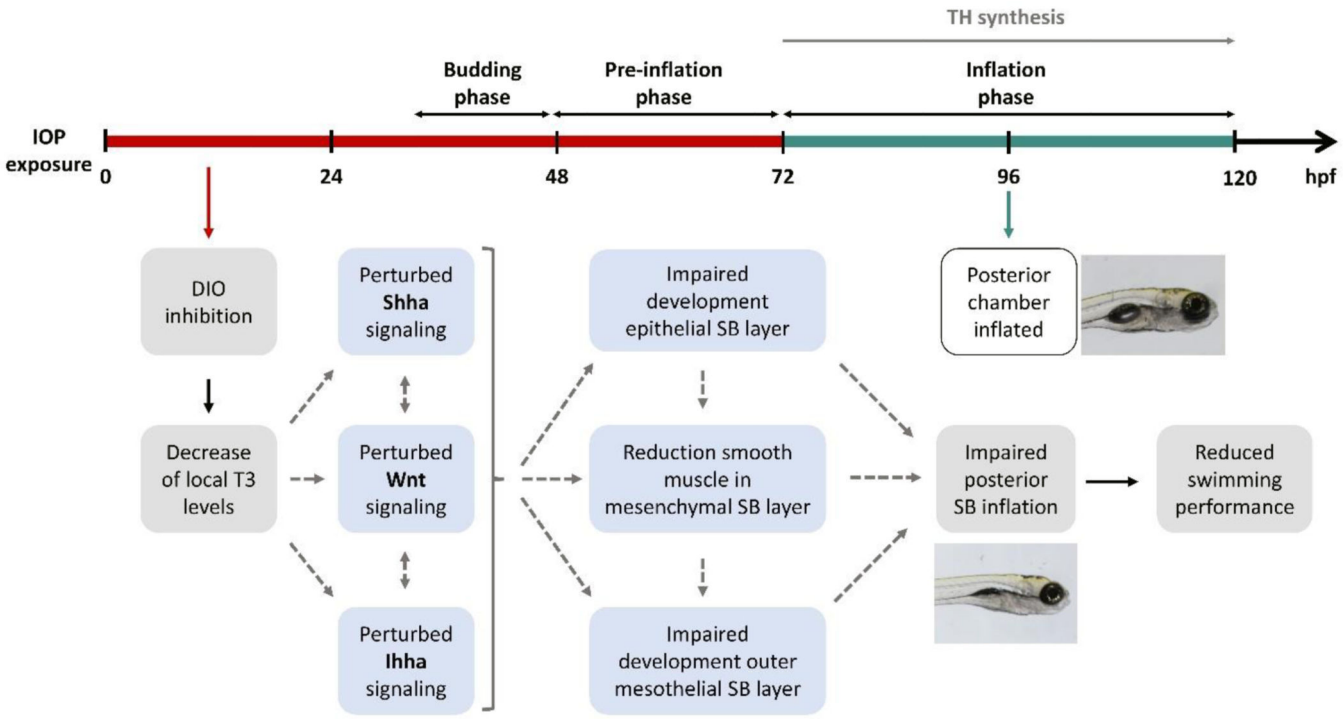

Figure 5: Hypothesized mechanism of how DIO inhibition affects the posterior swim bladder during embryonic development.

Exposure to IOP during the formation of the swim bladder (SB) (red bar and red arrow) leads to impaired inflation of the posterior chamber through perturbation of Sonic hedgehog a (Shha), Wnt and Indian hedgehog a (Ihha) signaling. This process takes place prior to endogenous thyroid hormone (TH) synthesis, suggesting that it primarily relies on maternally transferred THs. Exposure during the inflation phase (green bar and green arrow) does not affect the swim bladder. Grey boxes show events that are currently included in AOPs 155 and 157 linking DIO inhibition to impaired posterior chamber inflation (https://aopwiki.org/). Blue boxes and dotted lines indicate the hypothesized mechanism that is presented in the current study.

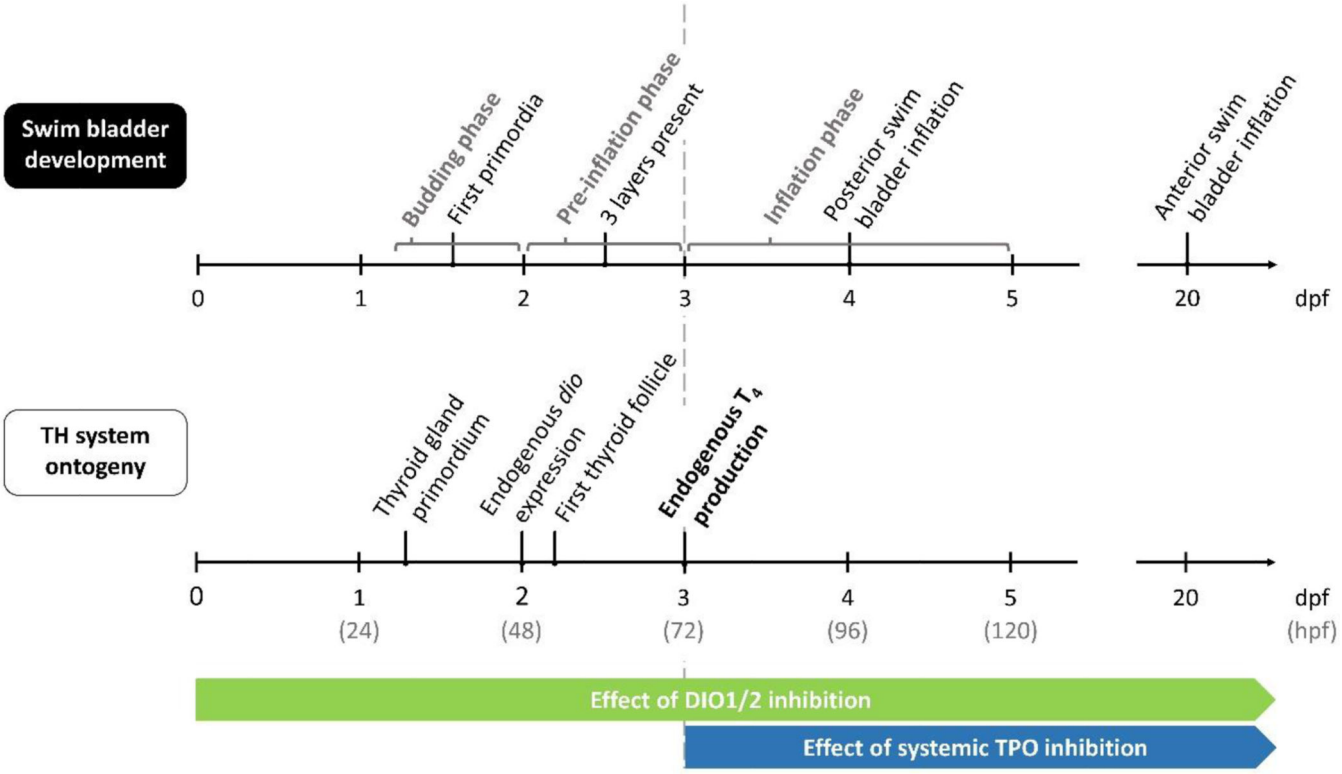

Figure 6: Ontogeny of the swim bladder and thyroid hormone system.

The timelines represent swim bladder development and development of the thyroid hormone (TH) system between 0–20 days post fertilization (dpf). The green arrow shows the time period during which deiodinase (DIO) inhibition might affect the zebrafish development. The blue arrow shows when thyroperoxidase (TPO) inhibition might affect the embryo. As TPO is crucial for TH synthesis, effects of inhibition only take place after initiation of endogenous TH synthesis (grey dashed line).