

The thyroid hormone system disrupting potential of resorcinol in fish

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ARTICLE INFO

Edited by Dr. Caterina Faggio

Keywords:

Thyroid hormone system disruption
swim bladder inflation
eye development
thyroid hormone levels
zebrafish embryo
adverse outcome pathway

ABSTRACT

Environmental pollutants capable of interfering with the thyroid hormone (TH) system increasingly raise concern for both human and environmental health. Recently, resorcinol has received attention as a compound of concern due to its endocrine disrupting properties. It is a known inhibitor of thyroperoxidase (TPO), an enzyme required in TH synthesis, and therapeutic use of resorcinol exposure has led to hypothyroidism in humans. There is limited evidence concerning ecotoxicologically relevant effects of resorcinol in fish. A set of adverse outcome pathways (AOPs) has recently been developed linking thyroid hormone system disruption (THSD) to impaired swim bladder inflation and eye development in fish. In the present study, these AOPs were used to provide the background for testing potential THSD effects of resorcinol in zebrafish eleutheroembryos. We exposed zebrafish eleutheroembryos to resorcinol and assessed TH levels, swim bladder inflation and eye morphology. As a TPO inhibitor, resorcinol is expected to affect TH levels and eye morphology but not swim bladder inflation during embryonic development. Indeed, thyroxine (T4) levels were significantly decreased following resorcinol exposure. In contrast to our hypothesis, swim bladder inflation was impaired at 5 days post fertilization (dpf) and no effects on eye morphology were detected. Therefore, *in vitro* assays were performed to identify potential additional thyroid hormone system disruption-related mechanisms through which resorcinol may act. Two new mechanisms were identified: TH receptor (TR) antagonism and transthyretin (TTR) binding inhibition. Both of these mechanisms can plausibly be linked to impaired swim bladder inflation and could, therefore, explain the observed effect. Overall, our study contributes to the knowledge of the THSD potential of resorcinol both *in vivo* in the zebrafish model as well as *in vitro*.

1. Introduction

The hypothalamus-pituitary-thyroid (HPT) axis plays an important role in normal vertebrate development and in various crucial homeostatic processes. As the thyroid hormone (TH) system is highly conserved across vertebrate taxa (Haigis et al., 2023b; Sachs and Buchholz, 2017), thyroid hormone system disruption (THSD) has been linked to a multitude of adverse effects in various vertebrate species such as impaired

brain development in mammals (Gilbert, 2011; Zoeller et al., 2002), impaired metamorphosis in amphibians (Thambirajah et al., 2019) and impaired swim bladder and eye development in fish (Baumann et al., 2016; Gözl et al., 2022; Knapen et al., 2020; Nelson et al., 2016; Stinckens et al., 2018, 2020; Van Dingenen et al., 2023).

A growing number of pollutants are reported to interfere with the proper functioning of the TH system, raising concerns for both human and environmental health. Resorcinol is a chemical that is widely

Abbreviations: AhR, aryl hydrocarbon receptor; AOP, adverse outcome pathway; DIO1, iodotyrosine deiodinase 1; DIO2, iodotyrosine deiodinase 2; DMSO, dimethylsulfoxide; Dpf, days post fertilization; DUOX, dual oxidase; Hpf, hours post fertilization; QPCR, quantitative polymerase chain reaction; HPT, hypothalamus-pituitary-thyroid; MIE, molecular initiating event; NIS, sodium/iodide symporter; SDU, University of Southern Denmark; SVHC, substance of very high concern; TH, thyroid hormone; THSD, thyroid hormone system disruption; TPO, thyroperoxidase; TR, thyroid hormone receptor; TTR, transthyretin; T3, triiodothyronine; T4, thyroxine; UA, University of Antwerp.

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<https://doi.org/10.1016/j.ecoenv.2024.116995>

Received 23 April 2024; Received in revised form 22 August 2024; Accepted 31 August 2024

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distributed in the environment due to its high production volume (ECHA, 2022) and broad field of applications, ranging from use in the wood and rubber industry (EC, 2002) to therapeutic use in different skin treatments, as well as its presence as a naturally occurring phenolic compound in some plants. Research regarding its endocrine disrupting potential has shown that resorcinol is capable of inhibiting thyroperoxidase (TPO), an enzyme required for thyroid hormone (TH) synthesis (Cooksey et al., 1985; Paul Friedman et al., 2016; Paul et al., 2014). A recent review by Pasquier et al. (2023) highlighted that therapeutic use of resorcinol (e.g., treatment of skin ulcers) in humans can induce severe hypothyroidism with goiter formation (Lynch et al., 2002), while in rodents, effects include decreased thyroxine (T4) concentrations, histological changes in the thyroid gland, increased thyroid gland weight and a decrease in iodine uptake (Arnott and Doniach, 1952; Cooksey et al., 1985; Doniach and Logothetopoulos, 1953). Pasquier et al. (2023) concluded that based on human data, resorcinol can be regarded as an endocrine disruptor according to the WHO definition (WHO/UNEP et al., 2012), even with the existing discrepancies between human and rodent data. The European Commission has attempted to identify resorcinol as an endocrine disrupting substance of very high concern (SVHC) under the European REACH legislation on the basis of its THS disrupting effects in humans, but in December 2022 the EU Member State Committee was unable to secure approval for the EU Commission's latest SVHC identification proposal. In September 2023, ECHA received a new harmonized classification and labelling intention for resorcinol in the context of the CLP (classification, labelling and packaging) legislation which has been added to the EU registry of classification and labelling intentions until outcome.

In fish, research on the effects of resorcinol has so far mainly focused on lethality, with LC50 values being determined for multiple fish species and across different developmental stages (summarized in Table S1). LC50 values differ greatly, ranging from 243 μM in adult fathead minnows up to 4995 μM in zebrafish eleutheroembryos. However, knowledge of the endocrine disrupting effects of resorcinol in fish is currently limited, with only a few studies that have linked resorcinol to THSD, showing that resorcinol disrupts the thyroid gland function (Jarque et al., 2018) and decreases the intrafollicular T4 levels (Li et al., 2012; Thienpont et al., 2011) in zebrafish eleutheroembryos. Several adverse outcome pathways (AOPs) are available that link THSD to impaired swim bladder inflation (AOPs 155–159, Vergauwen et al., 2022a, b, Vergauwen et al., 2022c, 2022d, 2022e) and impaired eye development (AOP 363, Gözl et al., 2022) in fish. These AOPs can provide the background and support for testing potential THSD effects of chemicals in fish. Two of these AOPs have TPO inhibition as their molecular initiating event (MIE) and lead to impaired inflation of the anterior, but not the posterior, chamber of the swim bladder (AOP 159), as well as an altered retinal layer structure (AOP 363) through a decrease in T4 levels during embryonic development.

In the present study, we exposed zebrafish eleutheroembryos to resorcinol from fertilization until 5, 7 or 8 days post fertilization (dpf). During this time frame, the eyes including the retinal layers develop, as does the posterior chamber of the swim bladder. The anterior chamber only inflates later during development (around 21 dpf) and was not evaluated in the current study. Given the fact that resorcinol is a known and potent TPO inhibitor, and based on our mechanistic understanding of the effects of TPO inhibition in fish as described in AOPs 159 and 363, we hypothesized that resorcinol exposure would result in decreased T4 levels and effects on the retinal layers, while no effects were expected on posterior swim bladder inflation, as previous research has shown that exposure to prototypical TPO inhibitors has no effect (Nelson et al., 2016; Stinckens et al., 2016, 2020). A decrease in whole-body T4 levels was indeed observed. However, we did observe impaired inflation of the posterior swim bladder chamber while there were no observable effects on the eye parameters investigated. We, therefore, performed a series of *in vitro* assays assessing different mechanisms involved in THSD to potentially identify new THSD-related mechanisms through which

resorcinol may act in addition to TPO inhibition, and which may help explain the observed swim bladder inflation effects.

2. Material and methods

2.1. Ethics statement

Fish are regarded as non-protected animals until they are free-feeding, 5 dpf onwards for zebrafish (Implementing decision 2012/707/EU), according to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Experiments exceeding 5 dpf were approved by the ethical committee for animal testing at the University of Antwerp (UA) (project 2021–15) and by the Danish animal ethical committee at the University of Southern Denmark (SDU) (permission No 2018–15–0201–01549). All experiments and fish husbandry were carried out in strict accordance with Directive 2010/63/EU.

2.2. Zebrafish housing and egg production

Adult wild type zebrafish were housed both at UA and SDU. Housing conditions at both facilities were similar (Table S2), with main differences in temperature: 28°C at UA and 26°C at SDU, and in feeding regime: three times a day at UA and twice a day at SDU.

2.3. Zebrafish embryo exposures

Zebrafish embryo exposures were carried out at UA and SDU (experimental conditions in Table S3) to determine the effects of resorcinol exposure on different endpoints (Table S4). Resorcinol stock solutions were prepared by dissolving resorcinol (CAS: 108–46–3, purity 99 %, Sigma-Aldrich, St. Louis, MO, USA) in reconstituted fresh water. Exposures were semi-static and solutions were made fresh each day and were renewed daily by removing as much liquid as possible without exposing the embryo to air.

2.4. Assessment of effect on swim bladder inflation

Nominal test concentrations, 0, 182, 363, 545 and 727 μM (0, 20, 40, 60, 80 mg/L), of the final experiment (until 7 dpf) (experiment 5, Table S4) performed at UA were chosen based on preliminary experiments performed at both SDU and UA (experiments 1–4 in Table S4, detailed results in Tables S5–6), to ensure minimal mortality. Embryos were exposed within 2 h post fertilization (hpf) and housed in presaturated 24-well plates. Two 24-well plates were used per concentration, each well contained 2 mL of the exposure medium and one embryo ($n=40$). The first column (4 wells) of each plate was filled with control medium (Table S3) and was used as an internal control. One additional plate filled with 4 mg/L 3,4-dichloroaniline (DCA, CAS: 95–76–1, purity 98 %, Sigma-Aldrich) was used as a reference for mortality. Plates were deemed valid if mortality of the internal control was $\leq 25\%$ and experiments were valid if mortality of the DCA plate was $> 30\%$ and survival of the controls was $> 90\%$ (OECD, 2013). All experiments reported in the study passed these validity criteria. All plates were sealed with parafilm® to prevent evaporation and kept in an incubator (MIR-254-PE, Panasonic, TCPS, Rotselaar, Belgium) with a 14/10 h light/dark cycle and a constant temperature of $28.5 \pm 0.2^\circ\text{C}$. Mortality was scored daily starting at 1 dpf. Chorions of exposed and control embryos were removed using forceps when $> 90\%$ of control embryos had hatched (around 55 hpf) to reduce hatching variation, as this influences swim bladder inflation (Stinckens et al., 2016).

2.4.1. Analysis of swimming activity and turning angles

Swimming activity was assessed at both 5 and 7 dpf using a Zebrafish 3.0 video tracking device (ViewPoint, Lyon, France). Activity was recorded during 40 min (including an acclimation period of 10 min) in

light (1200 lux). Two 24-well plates were used per test concentration ($n=40$). All plates were analyzed on the same day. Swimming activity was analyzed as the average swimming speed, the total distance moved and total time moved. Swimming activity did not significantly differ between the internal plate controls of each plate at both 5 and 7 dpf (Figures S1, S2). The frequency of 8 different turning angles was determined, normalized against the total swimming distance (average turning angles per mm) and expressed relative to the controls.

2.4.2. Morphological assessment

At 5 and 7 dpf, swim bladder inflation was scored using a binary scoring system. Presence of additional sublethal effects: oedema, circulation in the tail (disturbed or absent), blood accumulations, deviations of the head (including malformation of eyes, ears, and mouth), and deviating pigmentation, was also scored at these timepoints. eleutheroembryos were transferred to a microscope slide and were photographed (Canon EOS 600D, 18 megapixels, Tokyo, Japan) under a stereomicroscope (Leica S8APO, Leica Microsystems GmbH, Wetzlar Germany), alongside a calibrator. Using the free image processing software ImageJ (version 1.53e), larval length was determined for all eleutheroembryos and swim bladder surface was measured for those with inflated swim bladders.

2.5. T4 supplementation

An additional exposure was performed at SDU (experiment 6, Table S4) where T4 was supplemented to see if this could rescue the effect observed on the swim bladder. T4 was dissolved in dimethylsulfoxide (DMSO). DMSO concentrations in the final exposure medium were 0.01 %. Zebrafish embryos were exposed to either 727 μM (80 mg/L) resorcinol, 2.5, 5, 10 nM T4 or 727 μM resorcinol supplemented with one of the T4 concentrations from within 2 hpf until 8 dpf. One 24-well plate was used per condition ($n=20$) and each well contained 2 mL test solution. Validity criteria were identical as those mentioned in Section 2.4 and were all passed. Mortality was scored daily starting at 1 dpf and swim bladder inflation was scored daily starting at 5 dpf until the end of the experiment at 8 dpf. Well plates were kept in an incubator with a constant temperature of $26 \pm 1^\circ\text{C}$ and a 14/10 h light/dark cycle.

2.6. Thyroid hormone measurements

Separate zebrafish exposures were performed at UA to collect samples for TH measurements. Briefly, zebrafish embryos were exposed to either 0, 9, 91, 182, 363 or 545 μM (0, 1, 10, 20, 40, 60 mg/L) resorcinol. At 5 dpf, four replicate samples each consisting of 100 pooled eleutheroembryos, housed and exposed in 960 mL polypropylene containers, were collected. Detailed information about these exposures and sampling can be found in the supplementary information 1 (Section 2.5). The 9 μM samples from the second exposure were excluded from analysis of the whole-body TH levels, resulting in only two replicates for this treatment (see Section 2.9 for more detailed explanation). Samples were extracted as previously described in Nelson et al. (2016) and Stinckens et al. (2016). Extracts were analyzed for triiodothyronine (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 identical to those described in Van Dingenen et al. (2023).

2.7. Eye histology

An additional exposure was performed at SDU to determine the effects on eye histology. Briefly, zebrafish embryos were exposed to either 0, 4.5, 45 or 454 μM (0, 0.5, 5, 50 mg/L) resorcinol for 5 days from fertilization. At 5 dpf, four replicate samples consisting of each 8–12 eleutheroembryos were collected and euthanized. The

eleutheroembryos were fixed in 10 % formalin, dehydrated in an ascending series of ethanol and embedded in paraffin. Coronal Section (3 μm) were prepared and stained with the hematoxylin-eosin stain. Pictures were taken and eye diameter, as well as the thicknesses of the photoreceptor and inner plexiform layers were measured using Image J 1.52 n (Schneider et al., 2012). For further details, please refer to the supplementary information 1 (Section 2.6).

2.8. qPCR analysis

Gene transcription analysis was performed for genes corresponding to the MIEs evaluated in the *in vitro* assays (Table S7). In a separate exposure carried out at UA, both 363 and 727 μM exposed eleutheroembryos (whole body) were sampled for analysis. For 363 μM , independent samples of eleutheroembryos with inflated and uninflated swim bladders were collected (Table S8). For each treatment, 5 replicate samples were collected. Detailed information about the exposure and sampling can be found in the supplementary information 1 (Section 2.7). RNA was isolated using Trizol (ThermoFisher Scientific) and chloroform (Sigma-Aldrich) and further cleaned with the RNeasy mini kit (Qiagen, Hilden, Germany). Purity and RNA integrity was measured for all samples (S.I. 2.7, Table S8). cDNA was synthesized from 250 ng RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. A QuantStudio3 system (ThermoFisher Scientific, Waltham, MA, USA) was used for qPCR analysis. Each qPCR reaction contained 8 μL DEPC-treated water, 1 μL of the forward and reverse primer (10 μM) and 5 μL of the CAPITAL qPCR Green Mix LRoX (Westburg, Leusden, The Netherlands). To each reaction 350 ng cDNA in 5 μL was added. The thermal cycling profile consisted of: 3 min at 95°C , followed by 45 cycles of 20 s at 95°C , 15 s at the annealing temperature (Table S7) and 30 s at 72°C . Specific amplification was confirmed using the melt curves. A sequential series of 6 dilutions of the reference sample (mixture of all cDNA samples) was included in each run to determine primer efficiency. Out of a candidate list of five possible reference genes, *arnt2* and *β -actin* were identified as the two most stable genes using GeNorm (Figure S3) (Vandesompele et al., 2002). Relative fold changes were calculated against the control samples and normalized using the geometric mean of the two selected reference genes.

2.9. Analytical measurements

From each exposure, samples of the medium were taken. Samples were taken both right before and after the medium renewal at the beginning and at the end of each exposure (overview sampling timepoints and replicates in Table S9). Each sample contained 1 mL of exposure medium, samples were kept in the dark at $< 4^\circ\text{C}$ until analysis with high-performance liquid chromatography (HPLC). Detailed information about the method can be found in supplementary information 1 (Section 2.8). On average resorcinol concentrations remained above 80 % of the nominal concentrations (Table S10, Figures S4-S7). Therefore, nominal concentrations are used for reporting the data. Only for the lowest test concentration for the TH measurements (9 μM), actual concentrations remained on average around 50 % of the nominal concentration (Table S9, Figures S5A, S6A) and unexpectedly low concentrations (around 0.9 μM) were detected during the second exposure in the samples of the old medium at 5 dpf (Figure S6A). The 9 μM samples from the second exposure were, therefore, excluded from analysis of the whole-body TH levels.

2.10. In vitro assays

A battery of *in vitro* assays was performed to investigate through which mechanisms resorcinol might interfere with the TH system. A selection was made to include targets that are important in TH synthesis (TPO and the sodium/iodide symporter (NIS)), TH (in)activation

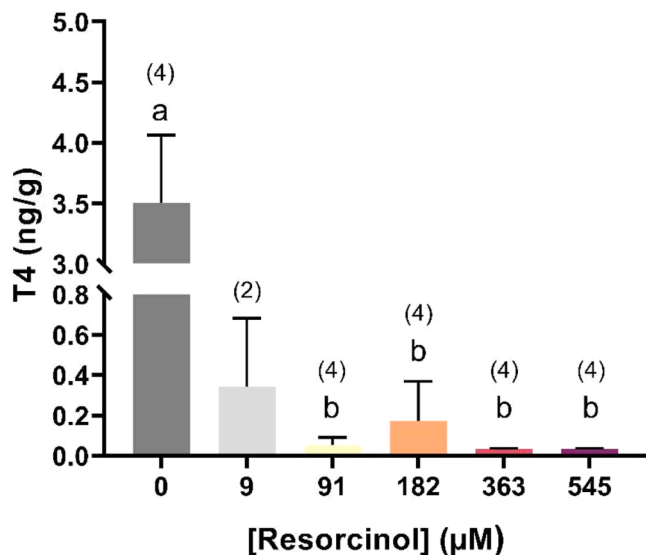


Fig. 1. : Effect of resorcinol exposure on whole-body T4 levels at 120 hpf. Average T4 levels following continuous resorcinol exposure. Error bars show standard deviation and sample sizes are given in parentheses. Each sample consisted of 100 pooled eleutheroembryos. Different letters indicate significant differences. Treatment 9 µM was left out of the statistical analysis because only two replicates were included (see Section 2.9).

(iodotyrosine deiodinase 1 (DIO1)), TH transport (transthyretin (TTR)), TH metabolism (aryl hydrocarbon receptor (AhR)) and the TH signaling (TH receptor; TR). Data for iodotyrosine deiodinase 2 (DIO2) were taken from Stinckens et al. (2018). The data were obtained from at least three independent experiments. Detailed information about each of the assays can be found in the [supplementary information 1 \(Section 2.9\)](#).

2.11. Statistical analysis

All data underlying graphs can be found in [supplementary information 2](#). All analyses were performed in either R (versions 4.1.2 and 4.2.2) or GraphPad Prism (version 9.5.0). Data were considered significantly different if the p-value < 0.05. The *in vitro* bioassay data were evaluated using a log-logistic dose-response model to estimate the half maximal effective concentrations (EC50/IC50). Swim bladder inflation and mortality was analyzed using a binary logistic regression (Michiels et al., 2017). An odds ratio was calculated in case of a 'zero cell count' (when all eleutheroembryos in a group have the same score, e.g. 100 % uninflated swim bladders) by adding a small value (0.5) to each cell (Pype et al., 2015). Data normality for larval length, swim bladder surface, swimming activity, eye histology, gene transcription and TH levels were assessed using the Shapiro-Wilks test. Levene's test was employed to assess homogeneity of variances (eye histology). Normally distributed data (swim bladder surface, eye histology, gene transcription, TH levels, swimming activity) were analyzed using a one-way analysis of variance (ANOVA) with a Tukey's multiple comparisons test. Differences in swimming activity within a given concentration (for fish with inflated vs. non-inflated swim bladder) were analyzed using a student t-test. For non-normally distributed data (larval length), a Kruskal-Wallis test with a Dunn's multiple comparisons test was used. Resorcinol concentrations in the medium were analyzed using a two-way ANOVA (two factors: day and fresh/old medium).

3. Results and discussion

3.1. Resorcinol disrupts the thyroid hormone system *in vivo* in zebrafish

Resorcinol exposure significantly and strongly reduced whole-body

T4 levels in five day old eleutheroembryos (Fig. 1, Table S11). Whole-body T3 levels were below the limit of quantification (0.046 ng/mL, data not shown). This is in line with previous reports of decreases in intrafollicular T4 content at 4 (Li et al., 2012) and 5 dpf (Thienpont et al., 2011) in zebrafish following resorcinol exposure and is most plausibly linked to TPO inhibition. In contrast, no effects on T4 levels were observed in rats exposed to up to 2725 µM resorcinol via drinking water (Welsch et al., 2008). As other TPO inhibitors, such as methimazole and 6-propyl-2-thiouracil, do affect T4 levels in rats, it was suggested that the lack of effect from resorcinol exposure could be attributed to a high resorcinol clearance rate in rats (Motonaga et al., 2016).

3.2. Resorcinol impairs posterior swim bladder inflation, but not through TPO inhibition

In contrast to what was expected, an increasing incidence of uninflated swim bladders with increasing resorcinol concentration was consistently observed (Fig. 2). This effect was not due to a general growth delay, as no effects on larval length (Figure S8, S9), an indicator of growth, were observed. The effect on the swim bladder persisted until 7 dpf (Fig. 2A), which was not the case for other TPO inhibitors such as methimazole and 2-mercaptobenzothiazole in previous studies (Stinckens et al., 2016; 2018). For the eleutheroembryos with inflated swim bladders, no effects on swim bladder surface were observed (Figure S8, S9). THSD interferes with the formation of the swim bladder, which takes place during early embryonic development (prior to 3 dpf) and relies on maternally transferred, rather than endogenously synthesized THs (Van Dingenen et al., 2023), explaining why posterior swim bladder inflation has been shown to be less responsive to TPO inhibition (Nelson et al., 2016; Stinckens et al., 2016). This was further supported by a recent study that created a zebrafish TPO knockout line and reported normally inflated posterior chambers (Fang et al., 2022). In the present study, to test the hypothesis that the observed effect is not due to TPO inhibition, an additional experiment was performed to determine if T4 supplementation could rescue the impaired inflation of the swim bladder. Regardless of the concentration of T4 added to the medium, the effect of 727 µM resorcinol on swim bladder inflation could not be rescued (Fig. 2B), confirming that it is not caused by TPO inhibition.

3.3. Resorcinol causes erratic swimming behavior in zebrafish eleutheroembryos with an uninflated swim bladder

A significant decrease in general swimming activity was observed in fish that were unable to inflate their swim bladder. Specifically, eleutheroembryos exposed to either 182 or 363 µM resorcinol with uninflated swim bladders showed a decrease in the total time moved, travelled less distance and moved slower than those with inflated swim bladders (Fig. 3A-C) at 5 dpf. As opposed to previous reports of decreased swimming activity in zebrafish eleutheroembryos subjected to THSD and failing to inflate the swim bladder (Stinckens et al., 2016; Van Dingenen et al., 2023), in the current study, within eleutheroembryos with uninflated swim bladders, the total time moved increased in a dose-dependent manner (Fig. 3B). A similar trend was observed for distance travelled, although to a lesser extent as the increase was only significant for the highest concentration (Fig. 3C). Similar changes in swimming activity could still be observed at 7 dpf (Figure S10). Morash et al. (2023) also reported an increase in time moved in zebrafish eleutheroembryos after resorcinol exposure, corroborating our observations. To further investigate these findings, a path angle analysis was performed, which can be indicative of neurobehavioral effects (Zhang et al., 2021). Two distinct patterns were observed for eleutheroembryos with and without inflated swim bladders (Fig. 3D,E). An equal distribution across all turning angles was observed for eleutheroembryos with inflated swim bladders (Fig. 3D). For eleutheroembryos with uninflated swim bladders an increase in smaller turning angles (0–15°, 15–45°,

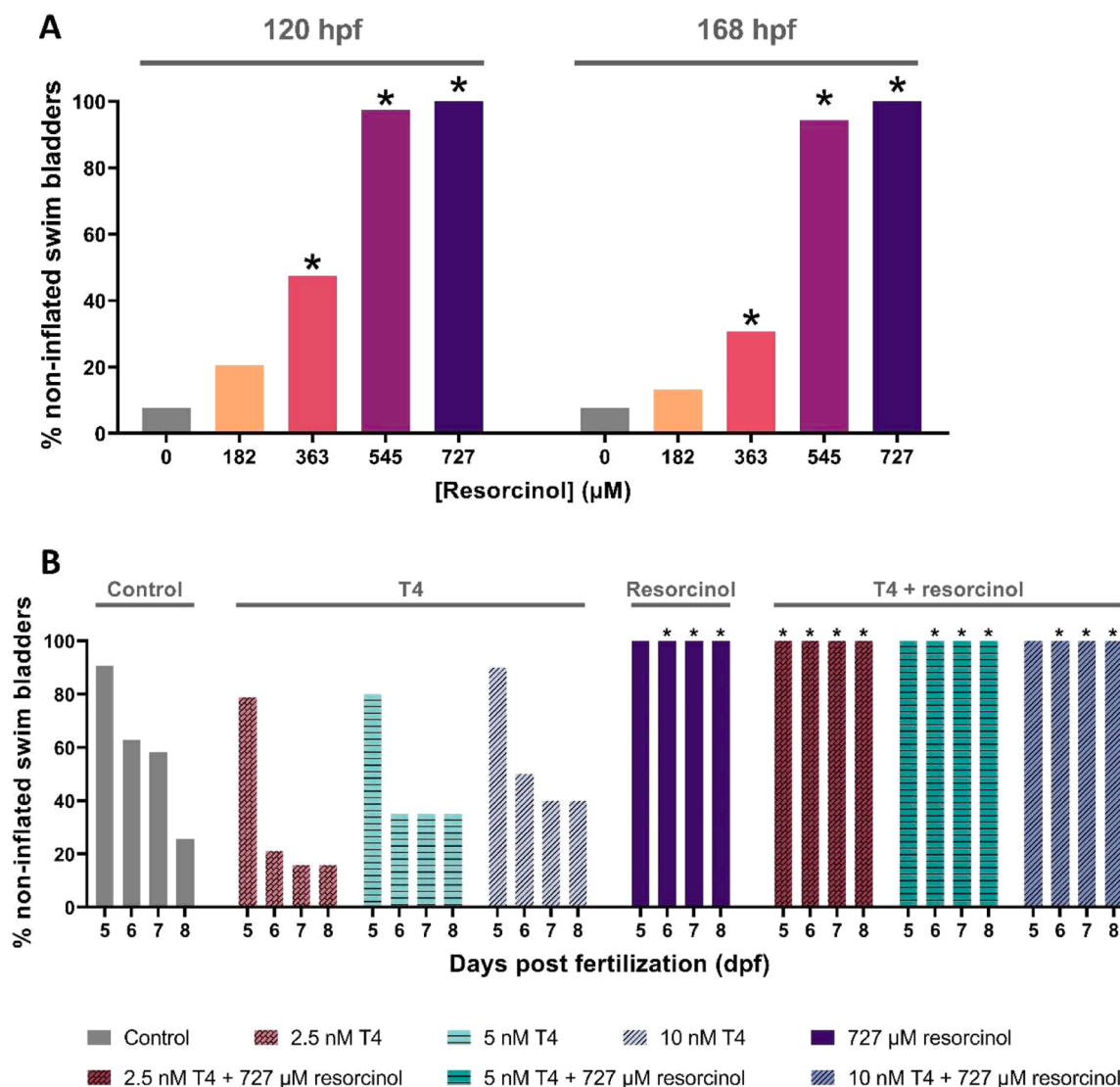


Fig. 2. : Effect of resorcinol exposure on swim bladder inflation. Percentage of non-inflated swim bladders (A) following resorcinol exposure at both 5 and 7 dpf at 28°C (experiment 5). Significant differences from the control (0 µM) are indicated with an asterisk. Effect of T4 supplementation on swim bladder inflation (B) from 5 up to 8 dpf at 26°C (experiment 6). Zebrafish eleutheroembryos were exposed to either T4, T4 in combination with 727 µM resorcinol or solely 727 µM resorcinol. Asterisks indicate significant difference in swim bladder inflation at a given day between either the control and 727 µM resorcinol exposed embryos or between 2.5, 5 or 10 nM T4 and 2.5 nM T4 + resorcinol, 5 nM T4 + resorcinol or 10 nM T4 + resorcinol exposed embryos, respectively.

45–90°) with increasing resorcinol concentration was observed (Fig. 3E). This increase in small turning angles resulted in erratic swimming behavior, that was visually confirmed. As THSD can affect neurodevelopment (Crofton and Zoeller, 2005; Li et al., 2021; Zhu et al., 2018), it is possible that the effects observed in the path-angle analysis are the result of THSD-induced (developmental) neurotoxicity. An unpublished study showed increased locomotor activity on post-natal day 60 in Sprague-Dawley male rats (ECHA, 2020), indicating that resorcinol might indeed be able to interfere with neurodevelopment. Furthermore, one human clinical case reported symptoms of neurological confusion and disorientation almost immediately after application of a resorcinol-containing lotion (Bontemps et al., 1995). This hypothesis does, however, not explain why such an effect was not observed in fish that were exposed to resorcinol but had normally inflated swim bladders. Also, it should be noted that a potential neurotoxic effect may also have been caused by a (yet unknown) mechanism unrelated to THSD.

3.4. Effects of resorcinol on eye and retinal layer size were not observed

No effects were observed on the photoreceptor layer thickness, inner plexiform layer thickness or on eye diameter (Figure S11). While eye size is easily observable, it does not provide the most sensitive read-out of impaired eye development. A *tpo*^{-/-} mutant zebrafish did not show a significant decrease in eye diameter at 5 dpf either (Fang et al., 2022), but since no histopathology was performed, no conclusion can be drawn regarding the effects of TPO knockout on retinal layer structure. TPO inhibition has been linked to altered retinal layer structure observed by histopathology in a recently published AOP, with some remaining uncertainties in the exact timing at which the effect is caused as well as the potential contribution of other (THSD-unrelated) mechanisms (Gözl et al., 2022). The TPO inhibitor 6-propyl-2-thiouracil (PTU) was shown to decrease retinal pigment epithelium thickness in 5 dpf zebrafish (Baumann et al., 2016). Another TPO inhibitor, methimazole, was shown to decrease the ganglion cell layer thickness in 3 dpf zebrafish (Reider and Connaughton, 2014) and the retinal pigment epithelium and outer nuclear layer thickness in 5 dpf zebrafish (Chang et al., 2023).

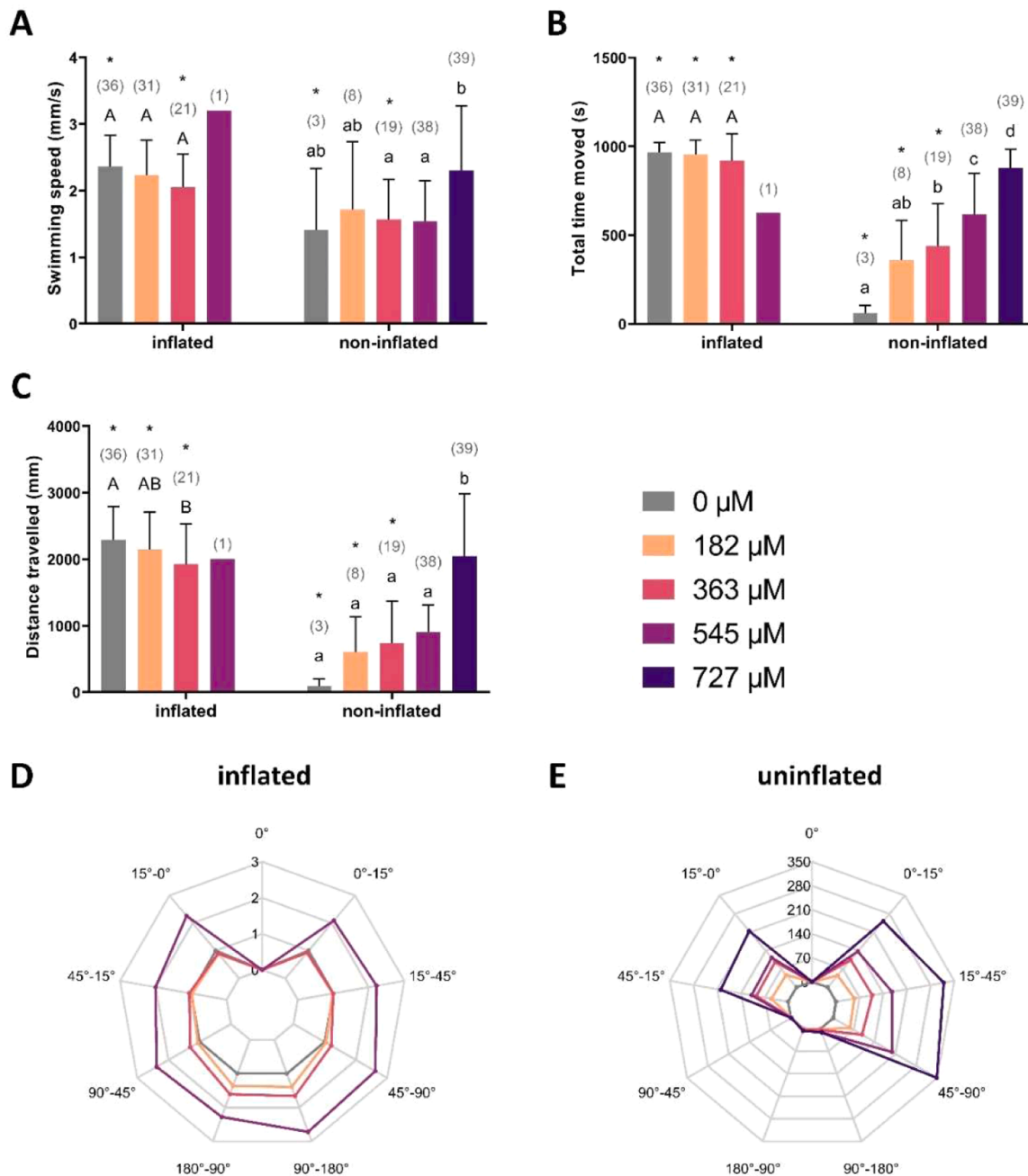


Fig. 3. : Effect of resorcinol exposure on swimming activity at 5 dpf Effects of resorcinol on swimming speed (A), total time moved (B) and the total distance travelled (C). Error bars show standard deviation and sample sizes are given in parentheses. Different letters indicate significant differences. Capital and lowercase letters indicate the significant differences within inflated or uninflated swim bladders, respectively. Significant differences within a concentration (inflated vs non-inflated) are indicated with asterisks. Effect on turning angles in eleutheroembryos with inflated (D) and uninflated swim bladders (E) illustrated using radar plots. Each corner of the radar plot represents a range of different turning angles. The y-axis is represented by the concentric circles showing the average turning angle normalized to both the sum of the swimming distance and the controls.

Since these layers differ from the ones studied in the current research, effects of resorcinol exposure on eye development may have been overlooked. AC50 (half maximal activity concentration) values for TPO inhibition of resorcinol are about 10-fold lower than those of PTU (Paul Friedman et al., 2016) and effects on zebrafish retinal layer morphology were reported at 587 μM (100 mg/L) PTU (Baumann et al., 2016), a concentration that is only two-fold higher than the highest concentration tested in the current study. Therefore, effects on the eyes were expected at the concentrations investigated in the present study. Effects of resorcinol exposure may, however, occur at higher exposure concentrations and/or when additional, more sensitive, endpoints would be

used. For example, Morash et al. (2023) found that expression of transcripts involved in phototransduction and eye structure were altered after exposure of zebrafish from 3 to 5 dpf to resorcinol concentrations similar to those used in our work. Such transcriptional changes have been previously reported in response to THSD (Baumann et al., 2019; Houbrechts et al., 2016). Overall, our study is not conclusive as to whether resorcinol has an impact on eye development.

Table 1

Characterization of the effects of resorcinol on important molecular initiating events involved in thyroid hormone disruption by *in vitro* battery of bioassays. The detailed dose-response relationships are shown in Supporting Information Fig. S12. ^a Data from Stinckens et al. (2018), ^b IC50 value is an estimate based on extrapolation.

Endpoint	Effect	Mean EC50/IC50 ±SD (µM)	Max tested concentration (µM)	Material source
Inhibition of TYR-IOD (ICP-MS)	Very strong	0.074 ± 0.024	3	rat thyroid
TPO inhibition AUR	Very strong	0.77 ± 0.27	5000	rat thyroid
TR antagonism	Strong	23.5 ± 6.6	100	human TR in PZ-TR cell line
TTR-binding inhibition	Strong	136.1 ± 31.3	2000	human TTR
AhR agonism	Weak	2883 ± 866	10000	human AhR in AZ-AhR cell line
DIO1 inhibition ^a	Weak	5921	5096	porcine liver
DIO1 inhibition	Weak	5669 ± 1599	10000	human DIO1 in HepG2 cell line
DIO2 inhibition ^a	Weak	10292 ^b	5013	porcine liver
NIS inhibition	Weak	3604 ± 2765	10000	human NIS transfected in HEK293T cell line
TR agonism	No	-	100	human TR in PZ-TR cell line

3.5. Resorcinol also functions as a TR antagonist and TTR-binding inhibitor

In vitro assays were performed to determine potential additional THSD targets of resorcinol (Table 1, Figure S12). Resorcinol is mainly known as a TPO inhibitor, with varying AC50 values of 0.025 µM (Paul Friedman et al., 2016) and 253 µM (Paul et al., 2014) reported previously. The authors hypothesized that this difference was due to differences in test chemical quality or purity (Paul Friedman et al., 2016). In our study, the TPO inhibitory potential of resorcinol was assessed using two different assays: the TPO-AUR assay focused on the inhibition of the peroxidation step, and the assay targeting inhibition of TPO-mediated tyrosine iodination (TYR-IOD). Measured IC50 values for these two assays were 0.77 ± 0.27 and 0.074 ± 0.024 µM, respectively, similar to what was found by Paul Friedman et al. (2016) and indicating very potent inhibition. This is in line with IC50 values reported for methimazole (0.025–0.06 µM) and propyl-2-thiouracil (0.12–0.23 µM), two other well-known TPO inhibitors (Paul Friedman et al., 2016; Paul et al., 2014). In the present study, our *in vitro* assays further identified resorcinol as TR antagonist and as a TTR-binding inhibitor (Table 1). Quantitative structure-activity relationship (QSAR) modelling has previously predicted binding activity of resorcinol to human TRs, albeit at high concentrations (2861–160000 µM) (Tukes, 2017). It should be noted that the IC50 values for TR antagonism and TTR-binding inhibition are considerably higher than those for TPO inhibition, and TPO inhibition is likely to be the predominant mechanism *in vivo*, with the important caveat that TPO inhibition is only a biologically relevant mechanism after the thyroid gland has become active during embryonic development (around 3 dpf). Furthermore, resorcinol was also identified as a weak DIO1 inhibitor. This is in accordance with our previous study in which we reported a weak DIO1 and DIO2 inhibition potential and concluded that resorcinol is unlikely to act as an *in vivo* DIO1/2 inhibitor (Stinckens et al., 2018). Resorcinol also inhibited NIS activity at high concentrations, making it unlikely that NIS inhibition would occur *in vivo* following resorcinol exposure. The same was observed for the AhR

activation assay where activation was only observed at concentrations close to cytotoxicity. Based on the strong conservation of TH system targets across vertebrates, as shown through sequence alignment analyses as well as by empirical evidence (Haigis et al., 2023; LaLone et al., 2018), in the present study, results from these mammalian *in vitro* assays were used to provide supportive data regarding potential underlying mechanisms of *in vivo* effects in fish in the context of THSD.

3.6. Resorcinol exposure affects transcript levels of key thyroid-related genes *in vivo*

To further assess the effect of resorcinol exposure on the TH system *in vivo*, a whole-body gene transcript analysis was performed for several important TH system-related genes (Fig. 4). Significantly higher *tpo* mRNA levels were observed following resorcinol exposure (Fig. 4A). This increase might be caused by a compensation mechanism counteracting the reduced TPO activity and reduced T4 levels by generating new *tpo* transcripts. Indeed, at least in mammals *tpo* transcription is known to be mediated by thyroid-stimulating hormone (TSH) (Godlewska and Banga, 2019), an important player in the feedback mechanism in the HPT-axis. Since the zebrafish thyroid does not become functional until 3 dpf, this compensatory response probably originated between 3 and 5 dpf, similar to what was observed by Baumann et al. (2016) after exposure to PTU treatment. Such a response was not observed for the genes involved in the other MIEs identified by the *in vitro* assays: TTR-binding inhibition and TR antagonism. *Ttr* transcript levels were significantly decreased following resorcinol exposure (Fig. 4B). Other zebrafish embryo studies have found both a decrease in whole-body T4 levels and in *ttr* mRNA levels (Chen et al., 2012; Fu et al., 2020). However, since there is currently no knowledge about the presence of a thyroid hormone response element in the *ttr* gene, it is difficult to conclude whether the decrease in transcript levels is directly caused by the change in TH levels. For the TRs, *thraa* mRNA levels were decreased following resorcinol exposure, while *thrb* levels were unchanged. There are indications that TRs might be able to autoregulate in goldfish, *Xenopus* and mice (Nelson et al., 2011; Sadow et al., 2003; Shi et al., 1998; Tata, 1994). This means that activation of the receptor leads to transcription of its own gene. Decreased TH levels could therefore lead to reduced TR transcript levels. The decrease in *thraa* mRNA levels could, therefore, be a direct response to the decrease in TH levels. Gene transcript levels for *dio1*, were only significantly altered for 363 µM exposed eleutheroembryos with inflated swim bladders (Fig. 4E). The human DIO1 gene contains a TRE (Zhang et al., 1998) and evidence in mice has shown that TRβ is important in the regulation of DIO1 (Ammann et al., 2001). Although no significant effects were observed on *thrb* transcription, transcript levels for *dio1* seem to follow the same pattern as those of *thrb*. A significant increase in *dio2* transcript levels was observed for 363 µM exposed eleutheroembryos. DIO2 expression is negatively regulated by THs (Burmeister et al., 1997). This could explain the increase in transcript levels at 363 µM, but not the lack of effect at 727 µM. Gene transcript levels for *dio3a* and *dio3b*, the inactivating deiodinases, on the other hand, were significantly decreased (Fig. 4G,H), which is consistent with what is expected in a hypothyroid state (Johnson and Lema, 2011) as DIO3 transcription is positively regulated by T3 (Bianco et al., 2002).

Transcript levels of resorcinol-exposed eleutheroembryos with an inflated swim bladder differed significantly from those with uninflated swim bladders within the same treatment for several of the tested genes (*thrb*, *dio1*, *dio3b*, Fig. 4D,E,H). Each of these transcriptional expression changes could play a role in compensatory responses suggesting that at 363 µM some eleutheroembryos were more capable than others of coping with the stressor, resulting in normally inflated swim bladders.

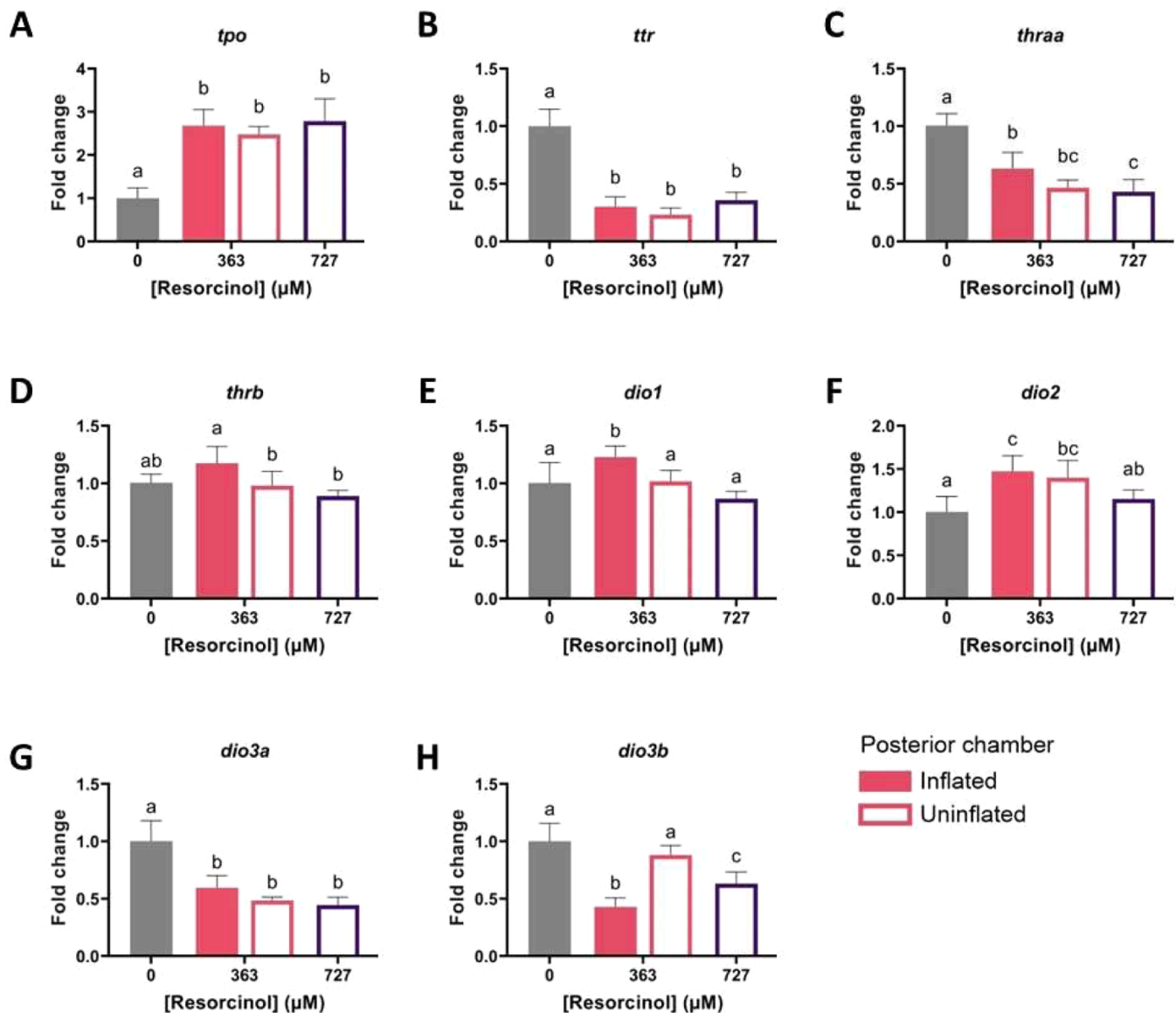


Fig. 4. : Effect of resorcinol exposure on gene transcription of key genes of the thyroid hormone system. Relative gene transcript levels (relative to the control (0 μM)) of *tpo* (A), *ttr* (B), *thraa* (C), *thrb* (D), *dio1* (E), *dio2* (F), *dio3a* (G) and *dio3b* (H) following resorcinol exposure at 120 hpf. Significant differences are indicated with different letters. Inflation status of the posterior chamber of the swim bladder is indicated by either a filled bar (inflated) or an unfilled bar (uninflated). Five replicate samples were used per condition and each sample consisted of ten eleutheroembryos.

3.7. Adding TR antagonism and TTR-binding inhibition to the fish THSD AOP network

Although there is currently limited evidence, the two additional THSD mechanisms that were identified by the *in vitro* assays (TTR-binding inhibition and TR antagonism) could plausibly be linked to impaired swim bladder inflation. In *thraa* and *thrb* morphants, swim bladders were uninflated at 5 dpf (Marelli et al., 2016), confirming that reduced TR activity leads to reduced swim bladder inflation. As TRs have a higher affinity for T3 than for T4 (Marchand et al., 2001; Sandler et al., 2004), this could explain why T4 supplementation did not rescue the effect on the swim bladder (Fig. 2B). TTR is a TH-binding transport molecule in blood plasma and chemical-induced displacement of TH from TTR increases TH availability for liver clearance. In contrast to mammals, fish TTR has a higher affinity for T3 than for T4 (Eales, 2019; Eneqvist et al., 2004; Li et al., 2023). As there is no thyroid binding globulin (TBG) found in fish, albumin is likely the main T4-binding protein (Deal and Volkoff, 2020). Since TTR probably mainly

transports T3, binding of resorcinol to TTR would mostly displace T3, resulting in a decrease in T3 levels as a result of liver clearance. Liver development is only complete at 5 dpf (Lu et al., 2011) and the precise timepoint at which liver clearance becomes physiologically relevant in the zebrafish embryo is still uncertain. However, after 3 dpf a clear decrease in T3 levels can be observed in control eleutheroembryos (Van Dingenen et al., 2023), indicating that the liver is already active at this point. It should be noted that although there is some level of conservation in TTR between humans and fish, the percent of similarity between humans and zebrafish is only 57.96% (Haigis et al., 2023b). Therefore, the *in vitro* assay for human TTR-binding inhibition that was used in our study might not be as predictable for fish.

The relevance of TTR-binding inhibition and TR antagonism as MIEs in the overall THSD AOP network is further supported by a recent taxonomic domain of applicability analysis of the network linking several THSD-related MIEs to multiple adverse outcomes across different vertebrate taxa (Haigis et al., 2023b). Out of the 13 MIEs described in this network, only eight of them (TR antagonism, dual

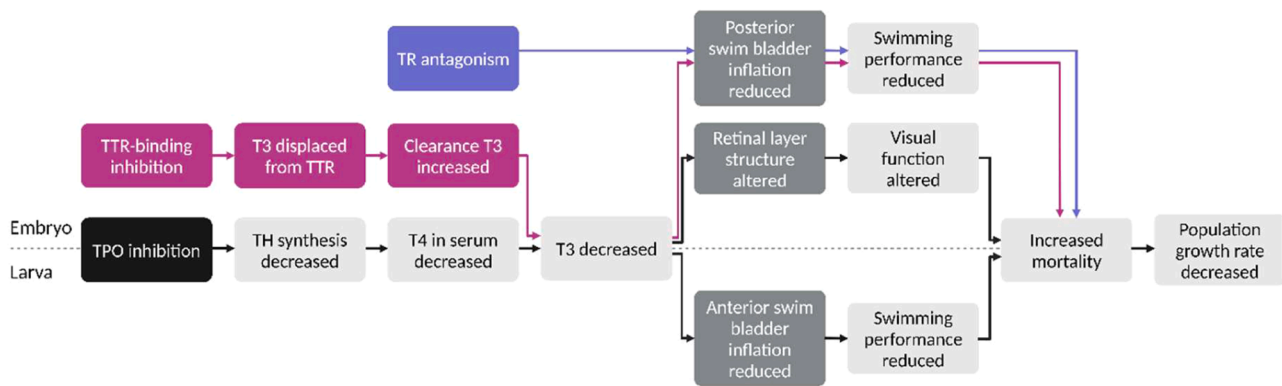


Fig. 5. : Hypothesis of how transthyretin (TTR)-binding inhibition and thyroid hormone receptor (TR) antagonism could be added into the existing fish adverse outcome pathway (AOP) network. The existing AOPs linking thyroperoxidase (TPO) inhibition to impaired anterior swim bladder inflation and altered retinal structure, AOPs 159 and 363 (<https://aopwiki.org/>), respectively, are indicated in grey/black. The two new candidate molecular initiating events (MIEs) that were identified in the current study as well as additional key events (KEs) related to these candidate MIEs are indicated in color (purple: TTR-binding inhibition, blue: TR antagonism). The dotted line indicates all the mechanisms that are important during embryonic development (above the line) and during larval development (below the line). Created with biorender.com.

oxidase (DUOX) inhibition, TPO inhibition, NIS inhibition, TTR-binding inhibition, DIO1–3 inhibition) were reported to be applicable to fish based on available evidence. As the development of the posterior swim bladder takes place before the thyroid follicles become active at around 3 dpf, only mechanisms that do not target endogenous TH synthesis are expected to have an effect (Van Dingenen et al., 2023). This means that DUOX inhibition, TPO inhibition, and NIS inhibition are not expected to result in impaired posterior swim bladder inflation. Apart from DIO inhibition which was previously shown to lead to impaired posterior SB inflation (Cavallin et al., 2017; Stinckens et al., 2018), only two mechanisms remain that could potentially interfere with development of the posterior swim bladder: TR antagonism and TTR-binding inhibition. The fact that resorcinol is only a weak DIO inhibitor (as demonstrated in the current study and (Stinckens et al., 2018)) further strengthens the plausibility that these two mechanisms are indeed involved in the effect on posterior swim bladder inflation. The two mechanisms are, therefore, two candidate MIEs that could be added to the fish swim bladder AOP network in the future (illustrated in Fig. 5). It should be noted that additional research is required in order to establish a key event relationship between these two candidate MIEs and the existing key events (KEs) in the swim bladder AOPs.

4. Conclusion

We confirmed that resorcinol is a TPO inhibitor both *in vitro*, as well as most likely *in vivo* as whole-body T4 levels in 5-day old zebrafish eleutheroembryos were significantly decreased following resorcinol exposure. In contrast to our hypothesis, swim bladder inflation was significantly impaired at 5 dpf. However, this effect was not caused by TPO inhibition since T4 supplementation did not rescue the effect on the swim bladder. Eleutheroembryos with uninflated swim bladders showed erratic swimming behavior suggesting that resorcinol could cause developmental neurotoxicity, either through THSD or via a different mechanism. No effects were observed on eye morphology.

The *in vitro* assays identified two new possible mechanisms through which resorcinol could interfere with the TH system and impair posterior chamber inflation: TTR-binding inhibition and TR antagonism (albeit at concentrations considerably higher than those that inhibit TPO). As both of these mechanisms can be plausibly linked to impaired swim bladder inflation based on available biological data, they represent two candidate MIEs for future addition to the existing AOP network. Although these are plausible THSD-related mechanisms that can explain the effect of resorcinol on swim bladder inflation, it should be noted that the effect could also be due to a THSD-unrelated mechanism. Overall,

the current study provides more insight into the THSD potential of resorcinol both *in vitro* and in an ecotoxicologically relevant *in vivo* context. The relevance of the fact that resorcinol is a TTR-binding inhibitor and TR antagonist should be further investigated in relation to human health relevance as this information could affect the European regulation of resorcinol under CLP.

CRediT authorship contribution statement

Emma Andersen: Writing – review & editing, Investigation, Formal analysis. **Dries Knapen:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Sina Volz:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Monica Christiansen:** Writing – review & editing, Investigation, Formal analysis. **Jirí Novák:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Daniel L. Villeneuve:** Writing – review & editing, Supervision, Resources. **Lucia Vergauwen:** Writing – review & editing, Project administration, Conceptualization. **Klára Hilscherová:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Imke Van Dingenen:** Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Henrik Holbech:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Ann-Cathrin Haigis:** Writing – review & editing, Investigation. **Emma Stacy:** Writing – review & editing, Investigation, Formal analysis. **Brett Reginald Blackwell:** Writing – review & editing, Supervision, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data is available in Supplementary File 2.

Acknowledgments

The authors would like to thank Kato Huyghe for performing the qPCR analyses, Bente Frost Holbech for performing the analytical measurements, Puja Kumari and Runze Liu for *in vitro* bioanalysis and Jan Kuta for ICP-MS/MS analysis. This project has received funding

from the University of Antwerp Research Fund (project 44602), the European Union's Horizon 2020 research and innovation programme under grant agreement No. 825753 (ERGO) and the European Union's Horizon 2020 research and innovation program under grant agreement No 857560. Authors thank the RECETOX Research Infrastructure (No LM2023069) financed by the Czech Ministry of Education, Youth and Sports for supportive background. The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the European Union or the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2024.116995](https://doi.org/10.1016/j.ecoenv.2024.116995).

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