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Published in:
Chemosphere

DOI:
[10.1016/j.chemosphere.2023.140776](https://doi.org/10.1016/j.chemosphere.2023.140776)

Publication date:
2024

Document version:
Final published version

Document license:
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Citation for pulished version (APA):

Volz, S. N., Poulsen, R., Hansen, M., & Holbech, H. (2024). Bisphenol A alters retinal morphology, visually guided behavior, and thyroid hormone levels in zebrafish larvae. *Chemosphere*, 348, Article 140776. <https://doi.org/10.1016/j.chemosphere.2023.140776>

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Bisphenol A alters retinal morphology, visually guided behavior, and thyroid hormone levels in zebrafish larvae

Sina N. Volz^{a,*}, Rikke Poulsen^b, Martin Hansen^b, Henrik Holbech^a

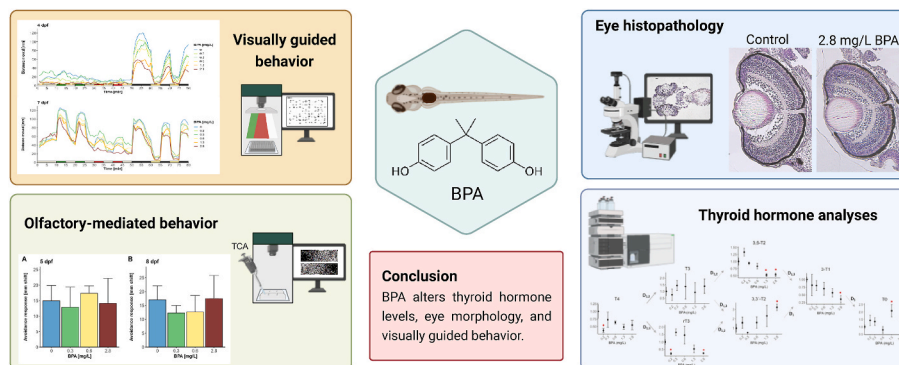
^a Department of Biology, University of Southern Denmark, Campusvej 55, 5230, Odense M, Denmark

^b Department of Environmental Science, University of Aarhus, Frederiksborgvej 399, 4000, Roskilde, Denmark

HIGHLIGHTS

- BPA alters retinal layering in zebrafish larvae.
- Zebrafish larvae exposed to BPA show reduced swimming activity and lack responsiveness to red light.
- BPA alters whole-body levels of several thyroid hormones.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Alvine C.Mehinto

Keywords:

Endocrine disruption
Bisphenols
Thyroid hormone system
Zebrafish
Eye development
Behavior

ABSTRACT

Bisphenols are industrial chemicals that are produced in large quantities and have been detected in all parts of the environment as well as in a multitude of different organisms including humans and fish. Several bisphenols, such as bisphenol A (BPA) and bisphenol F, have been shown to disrupt endocrine systems thereby affecting development and reproduction. While numerous studies investigated the effect of bisphenols on estrogen signaling, their impact on the thyroid hormone system (THS), which is vital for neurodevelopment including sensory development, has been explored to a lesser extent. The present work selected BPA as a representative for structurally similar bisphenols and assessed its impact on the THS as well as sensory development and function in zebrafish. To this end, zebrafish were exposed to BPA until up to 8 days post fertilization (dpf) and thyroid hormone levels, eye morphology, and sensory-mediated behaviors were analyzed. Zebrafish larvae exposed to BPA showed altered retinal layering, decreased motility across varying light conditions, and a loss of responsiveness to red light. Furthermore, whole-body levels of the thyroid hormones thyroxine (T4) and 3,5-diiodothyronine (3,5-T2) were significantly decreased in 5 dpf zebrafish. Taken together, BPA disrupted THS homeostasis and compromised visual development and function, which is pivotal for the survival of fish larvae. This work underlines the necessity for ongoing research on BPA and its numerous substitutes, particularly concerning their

* Corresponding author.

E-mail addresses: volz@biology.sdu.dk (S.N. Volz), rikkepoulsen@envs.au.dk (R. Poulsen), martin.hansen@envs.au.dk (M. Hansen), hol@biology.sdu.dk (H. Holbech).

<https://doi.org/10.1016/j.chemosphere.2023.140776>

Received 1 September 2023; Received in revised form 12 November 2023; Accepted 19 November 2023

Available online 22 November 2023

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effects on the THS and neurodevelopment, to ensure a high level of protection for the environment and human health.

1. Introduction

Bisphenols are industrial chemicals commonly used in the production of epoxy resins and polycarbonate plastics. Due to their high production volume and widespread utilization in consumer products, bisphenols are ubiquitously present in the environment and have been detected in waterways (Petrie et al., 2019; Radwan et al., 2020; Idowu et al., 2022) and numerous organisms, including humans (Calafat et al., 2005; Vandenberg et al., 2007; Corrales et al., 2015). Bisphenol A (BPA) is the most well-known compound of this group of chemicals. In the European Union, BPA is recognized as a substance of very high concern (SVHC) due to its endocrine-disrupting properties including its estrogenic activity, interaction with the thyroid hormone system (THS), and resulting adverse effects on fetal development and reproduction (Rochester, 2013; Beausoleil et al., 2018). Despite the increasing restrictions on BPA within the European Union (European Commission, 2018), the chemical is still registered as imported or manufactured above one million tons per year (ECHA, 2022). A recent re-evaluation of the risk posed by BPA to public health conducted by the European Food Safety Authority (EFSA) resulted in the drastic reduction of the tolerable daily intake rate from 4 μg to 0.2 ng BPA/kg bw per day (EFSA CEP Panel, 2023). This adjustment underscores the substantial health concern associated with BPA. Moreover, there has been rapid growth in the availability of BPA alternatives on the market. Bisphenol S, for instance, is now registered in the EU as imported or produced above 10,000 tons per year (ECHA, 2023). Understanding the potential risks and benefits of these alternatives is crucial for making informed decisions about their usage, yet research on these compounds remains scarce. Existing published data predominantly suggest comparable modes of action and potencies across BPA substitutes (Cano-Nicolau et al., 2016; Qiu et al., 2016; Usman and Ahmad, 2016; Lee et al., 2019; Faheem and Bhandari, 2021), emphasizing the value of continued research on BPA as a representative of structurally related bisphenols. While BPA's estrogenic effects have been extensively studied (Rochester, 2013; Yuan et al., 2023), its impact on other endocrine pathways, including the THS, has received less attention. However, QSAR data as well as *in vitro* and *in vivo* data on different vertebrate species suggest that BPA is also modulating the THS (DTU, 2023; Heimeier and Shi, 2010; Yang and Chan, 2015).

The THS is highly susceptible to the influence of environmental contaminants. Various compounds have been demonstrated to interfere with thyroid hormone signaling through diverse mechanisms, leading to adverse effects on for instance (neuronal) development, metabolism, or cardiovascular function. (Howdeshell, 2002; Murthy and Murthy, 2012; Ghassabian and Trasande, 2018; Köhrle and Frädrieh, 2021). On this account, significant efforts have been made in recent years to investigate sensitive endpoints and develop suitable test systems for the assessment of THS disruption in different vertebrate classes (Holbech et al., 2020; Knäpen et al., 2020). Given the aim to reduce chemical testing on mammals while ensuring a robust level of protection for higher vertebrates including humans, lower vertebrate models such as zebrafish are increasingly being used in (eco)toxicology, pharmacology, and medical science (Bambino and Chu, 2017; Cassar et al., 2020). Since endocrine systems are highly conserved across vertebrate classes (Pickford, 2010; Holzer et al., 2017; McArdle et al., 2020), research conducted on zebrafish can inform about possible effects on higher vertebrates including humans. Within only a few days, the zebrafish thyroid gland develops in the form of thyroid follicles that are loosely distributed along the ventral aorta and start producing thyroxine (T4) at 3 days post fertilization (dpf) (Porazzi et al., 2009; Marelli and Persani, 2017). Until this time point, zebrafish embryos rely on maternally transferred thyroid

hormones (Campinho et al., 2014). At about 5 dpf, the thyroid follicles are fully developed (Opitz et al., 2013). Hence, zebrafish larvae offer a unique opportunity to study the development and chemical disruption of THS-dependent processes in a time frame of a few days.

Neurodevelopment, including sensory organ development, is strongly dependent on the THS. During zebrafish eye development, the expression of thyroid hormone receptor beta 2 (Tr β 2) is vital for the differentiation of red cones (Suzuki et al., 2013; Deveau et al., 2020). Furthermore, treatment of zebrafish embryos with thyroid hormones causes a shift in spectral sensitivity towards longer wavelengths (Mackin et al., 2019). THS-disrupting chemicals such as propylthiouracil (PTU) and tetrabromobisphenol A (TBBPA) have been demonstrated to reduce relative eye diameter and retinal pigment epithelium height in 5 dpf zebrafish and alter the expression of genes involved in photo-transduction (Baumann et al., 2016, 2019). Zebrafish eyes resemble human eyes on morphological, physiological, and gene expression levels (Bibliowicz et al., 2011; Gestri et al., 2012) and retinal signal transduction is highly conserved in vertebrates (Cohen et al., 2022). At 3 dpf, the general structure of the zebrafish retina is developed (Schmitt and Dowling, 1999); the signal transmission from photoreceptors to downstream neurons is fully functional at 5 dpf (Biehlmaier et al., 2003). Behavioral changes in response to varying light conditions can be assessed from 4 dpf onwards (Legradi et al., 2015). Apart from the visual system, evidence suggests that the olfactory system can also be affected by disruption of the THS. Severe hypothyroidism in humans has been found to correlate with a decline in olfactory acuity (Świdziński et al., 2016) and olfactory bulb size (Inal et al., 2022). Studies in mice revealed that exposure to PTU resulted in a loss of olfactory function, which could be prevented by simultaneous administration of T4 (Mackay-Sim and Beard, 1987). Bisphenol F was demonstrated to impair olfactory-mediated behavior in response to social cues (Vancamp et al., 2023). Since the overall structure and function of the olfactory system are conserved across vertebrates (Byrd and Brunjes, 1995; Hildebrand and Shepherd, 1997), zebrafish is a suitable model to investigate THS-dependent development and function of the olfactory system.

The present study aims to further the understanding of the effects of BPA on sensory system development and function in relation to thyroid hormone signaling. We hypothesize that BPA, in addition to its effects on the sex hormone system, alters THS homeostasis thereby disrupting sensory development and impairing related behaviors. To test this hypothesis, zebrafish embryos were exposed to BPA to up to 8 dpf and thyroid hormone levels, eye morphology as well as visually and olfactory-mediated behaviors were assessed (Figure 1).

2. Material and methods

2.1. Zebrafish husbandry and breeding

Wild-type zebrafish (Westaquaarium strain) were kept in a circulating husbandry system (Zebcare, The Netherlands) at 26 ± 1 °C and a 14:10 h light:dark cycle. Water was prepared by the addition of instant ocean sea salt and sodium bicarbonate (Sigma-Aldrich) to reverse osmosis water. Conductivity was 560 ± 5 μS and pH was 7.5 ± 0.1 . Zebrafish were fed *ad libitum* with SDS 400 (Special Diets Services, Brogaarden, Denmark) in the mornings and artemia spec. nauplii in the afternoons. The evening prior to egg collection, breeding groups of 6–9 zebrafish with a female-to-male ratio of 2:1 were transferred in 7 L glass tanks that contained a custom-made stainless-steel slope breeding inlet. Eggs were collected the following morning within 1 h of spawning.

2.2. Exposure

BPA (CAS: 80-05-7, purity $\geq 99\%$, Sigma-Aldrich, Germany) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany) and treatment solutions were prepared by addition of BPA stock solution (50 mg/mL) to reconstituted water (ISO 7346-3, Table S1). Exposure concentrations were selected based on the results of a range findings test, ensuring that they are within the sublethal range when exposing from 1 to 192 h post fertilization. The 192h-EC₅₀ of 4 mg/L BPA was used as the highest exposure concentration. In accordance with OECD test guideline 236, a total of five concentrations with a constant spacing factor of two were selected. The lowest exposure concentration was expected to show no observable effect. Nominal exposure concentrations were 0, 0.25, 0.5, 1, 2, and 4 mg/L BPA. These exposure concentrations significantly exceed the typical levels of BPA detected in the environment. Yet they are well-suited for performing a hazard-based assessment of THS disruption as well as the impairment of sensory development and function in zebrafish larvae. DMSO concentration was 0.01% (v/v) in all treatment solutions as well as the solvent control. There were three replicate dishes per treatment.

Three hundred fertilized eggs in the 4-64-cell stage (1–2 h post fertilization) were selected per treatment using a dissecting microscope and randomly distributed into three 300 mL replicate glass dishes with the respective treatment solution. Dishes were covered with parafilm® and incubated in a climate chamber at 26 ± 1 °C and under a 14:10 h light:dark cycle. Treatment solutions were exchanged daily. Mortality remained below 5% in all dishes and did not differ among treatments.

2.3. Actual BPA exposure levels

Water samples were taken at the beginning of the exposure, after 24 h just before the first water exchange, and at 7 dpf after exchanging the solutions. BPA concentrations were quantified using an LC-MS/MS triple quadrupole system (Agilent 6495 & 1260 Infinity HPLC). Detailed information on the method can be found in the supplementary material (Table S2). Measured concentrations differed more than 20% from nominal concentrations (Table S3). Mean measured concentrations (0.2, 0.3, 0.6, 1.3, and 2.8 mg/L BPA) are used in the results and discussion sections.

2.4. Visually guided behavior

One day prior to the behavioral assessment, 96-well plates were pre-incubated with 300 μ L/well of the treatment solutions. The next morning (at 5 and 7 dpf), plates were emptied, and larvae were transferred in 300 μ L of the respective treatment solution to the wells. Subsequently, inflation of the posterior chamber (PC) of the swim bladder was assessed under a microscope. Larvae were then left to acclimate for a minimum of 30 min before the plates were transferred into the DanioVision Observation Chamber connected to the EthoVision XT 15 software (Noldus, The Netherlands). In the behavioral trials, zebrafish larvae were exposed to a sequence of varying light conditions. After 10 min acclimation in a low-intensity white light (5%) followed two cycles of alternation between green light and 5% white light and two cycles of alternation between red light and 5% white light (5 min each). The second part of the trial consisted of 10 min acclimation in the dark followed by two cycles of alternation between 5 min high-intensity white light (100%) and 5 min darkness. (Fig. S1).

Larvae were recorded and tracked at a rate of 30 frames/second. The mean distance moved per minute was calculated and data were exported to Excel files for further analysis. In line with Leuthold et al. (2019), only the data from the second period of each light condition was used for statistical comparisons to minimize data variation.

2.5. Histopathology

Zebrafish larvae analyzed in the behavioral study were euthanized in 500 mg/L buffered MS-222, fixed in formalin, and stored in 70% ethanol at 4 °C. Next, larvae were pre-embedded in molds prepared from 1% agarose (UltraPure™, Invitrogen) and images were taken using a digital camera mounted on a dissecting microscope. A scale was included to measure the total length with a precision of 0.1 mm. Afterward, the agarose mold containing the larvae was dehydrated in an ascending series of ethanol (70, 96, and 99%), incubated in Tissue Clear (Sakura Finetek Europe B.V.), and embedded in paraffin. Coronal sections (3 μ m) were prepared employing a rotating microtome (Thermo Scientific, Microm HM 355S), transferred on glass slides, and stained with the hematoxylin-eosin stain in an automatic slide stainer (Sakura Tissue-Tek DRSTM, Sakura Finetek Europe HQ, the Netherlands). Analysis was performed using a Nikon Eclipse Ti microscope (Nikon Europe B.V., the Netherlands) equipped with a DFK 33UX250 camera (The Imaging Source Europe GmbH, Germany). Pictures from sections showing the optic nerve entering the retina were taken using the NIS Elements AR software version 4.60.00 (Nikon Europe B.V). Eye diameter and thicknesses of selected retinal layers were measured with Image J 1.52n (Schneider et al., 2012). The eye diameter was measured at the widest part of the eye. For measurements of retinal layer thicknesses, the retina was divided into quarters, and thicknesses of the retinal pigment epithelium, photoreceptor layer, and inner plexiform layer were measured in each of the quarters to the nearest 0.1 μ m.

2.6. Olfactory avoidance response

At 5 and 8 dpf, the response of zebrafish larvae to an aversive olfactory cue was assessed using a similar setup and method as previously described (Heffern et al., 2018; Shamchuk et al., 2018). Stock solutions of 0.1 M taurocholic acid (TCA, CAS: 345909-26-4, Sigma-Aldrich) were prepared on the morning of the behavioral trial. Acrylic troughs (120 x 40 x 20 mm) with three equally sized chambers (left, middle, right) separated by acrylic sliders and filled with 14.85 mL of reconstituted water were used to assess the avoidance response. Ten larvae were transferred into the middle chamber and two troughs with larvae were placed in the water bath (26 °C) of the DanioVision Observation Chamber. Larvae were left to acclimate for 5 min. In the following, 150 μ L of 0.1 M TCA solution was randomly added to either the left or right chamber. After 5 min, the sliders were removed, and the larvae were filmed for 10 min. Afterward, larvae were euthanized by rapid cooling in ice-cold water and washed twice. Next, they were transferred into cryotubes, snap-frozen in liquid nitrogen, and stored at -80 °C until the thyroid hormone analyses were performed. Videos of the larvae were processed and analyzed employing Image J 1.52n (Schneider et al., 2012) following the protocol of Wyeth et al. (2011).

2.7. Thyroid hormone measurements

Samples were extracted for targeted thyroid hormone determination as previously described (Pannetier et al., 2023). Three replicates were analyzed per treatment and twenty larvae from the same dish were pooled to obtain a sufficient amount of tissue. The analysis was performed on an Agilent 6495c triple-quadrupole system with a hyphenated Agilent 1290 Infinity II ultra-high performance liquid chromatography (UHPLC) system (binary pump, degasser, and auto-sampler; Agilent Technologies, Santa Clara, CA USA) as previously described (Pannetier et al., 2023). Targeted analytes were thyroxine (T4), 3,3',5-triiodothyronine (T3), 3,3',5'-triiodothyronine (rT3), 3,5-diiodothyronine (3,5-T2), 3,3'-diiodothyronine (3,3'-T2), 3-iodothyronine (T1), thyronine (T0), 3-iodothyronamine (T1Am), 3-iodothyroacetic acid (T1Ac), 3,5-diiodothyroacetic acid (Diac), triiodothyroacetic acid (TriaC), and tetraiodothyroacetic acid (TetraC). Neat standard ten-point equimolar calibration curves (0.04–50.0 pmol/mL thyroid

hormone, $n = 2$) were prepared in 5% methanol and all vials contained a fixed amount IS and ICS (15.2 pmol/mL). Data analysis was conducted in MassHunter version 10.1 (Agilent Technologies, Santa Clara, CA USA). Detectable hormones in our samples were T4, T3, rT3, 3,3'-T2, 3,5-T2, 3-T1, and T0. To allow for statistical comparisons, thyroid hormone levels below the limit of detection (LoD) were replaced by $\frac{1}{2}$ LoD. Data between the LoD and limit of quantification (LoQ) remained as they were.

2.8. Statistical analysis

All statistical analyses were performed in R Version 4.1.2 (R Core Team, 2021) combined with R Studio Version 1.3.959 (RStudio Team, 2020). For histopathology and visually guided behavior, mean values were calculated for each replicate. In the case of the olfactory-mediated behavior and thyroid hormone analyses, ten and twenty individuals, respectively, were pooled. This resulted in a sample size of three for each endpoint and treatment. Data were tested for normality using the Shapiro-Wilk test; Levene's test (car package) (Fox and Sanford, 2019) was performed to assess for homogeneity of variances. Differences between the blank control and solvent control were analyzed using a Welch two-sample *t*-test. Since controls did not significantly differ for any of the endpoints, control data were pooled. If the data conformed with the parametric assumption, a univariate analysis of variances (ANOVA) followed by a Dunnett's test (multcomp package) (Hothorn et al., 2008) was conducted. In the case of non-parametric data, a Kruskal-Wallis test followed by a Dunn's test was employed. For trend analyses, the Jonckheere-Terpstra test was performed. Graphs were generated using the ggplot2 (Wickham, 2016) and cowplot packages (Wilke, 2019).

3. Results

3.1. Histopathology

BPA exposure altered eye morphology in 4 dpf zebrafish (Fig. 2). Eye diameter was significantly reduced at 2.8 mg/L BPA (Dunnett's test, $p = 0.02$). Thicknesses of the retinal pigment epithelium and the inner plexiform layer were significantly reduced at the low (Dunnett's test, $p = 0.03$ and $p = 0.02$, respectively) and high BPA concentrations (Dunnett's test, $p = 0.01$ and $p = 0.008$ respectively). For the photoreceptor

layer, a decreasing trend was observed, which was, however, not statistically significant (Jonckheere-Terpstra test, $p = 0.07$). The total length of larvae remained unchanged (Fig. S2).

3.2. Posterior chamber inflation and sensory-guided behaviors

Impaired inflation of the posterior chamber (PC) of the swim bladder was observed in all treatments, except at 0.3 mg/L BPA (Fig. S3, Dunnett's test, $p < 0.001$ for 0.2, 0.6, and 1.3 mg/L and $p = 0.001$ for 2.8 mg/L BPA). This effect was no longer present at 7 dpf.

Following 4 days of exposure to BPA, zebrafish showed a decreased distance moved during the dark phase which was statistically significant at 0.6–2.8 mg/L (Fig. 3A, Dunnett's test, $p < 0.05$). Zebrafish larvae did not respond to green or red light (Fig. S4) In the white phase, the distance moved by larvae exposed to BPA was not significantly different from the one in the controls.

At 7 dpf, the distance moved in the dark phase was decreased at 0.2 (Fig. 3B, Dunnett's test, $p = 0.02$) and 2.8 mg/L BPA (Dunnett's test, $p = 0.04$). No statistically significant differences were detected in red or white light ($p = 0.08$ and $p = 0.14$, respectively). In the green phase, a reduced distance moved was observed at 2.8 mg/L BPA (Dunnett's test, $p = 0.03$).

As a measure of the response to the change in light condition, the difference between the distance moved in the first minute of the stimulus phases (green, red, darkness) and the last minute of the preceding white light phases was assessed. The response to red light, but not to green light or darkness was significantly impaired at 2.8 mg/L BPA (Fig. 3C, Dunnett's test, $p = 0.04$).

No significant alteration or trend was observed for the olfactory avoidance response of 5 and 8 dpf zebrafish larvae exposed to BPA (Fig. S5).

3.3. Thyroid hormone analyses

In 5 dpf larvae (Fig. 4) T4, T3, rT3, 3,3'-T2, 3,5-T2, 3-T1, and T0 were detected. The level of T4 was significantly decreased at 0.2 mg/L BPA. At higher exposure concentrations, T4 levels were below those in the control, but the decline was not statistically significant. No significant changes or trends were observed for the active thyroid hormone triiodothyronine (T3). The inactive thyroid hormone reverse T3 (rT3) showed a non-monotonic concentration-response curve and was

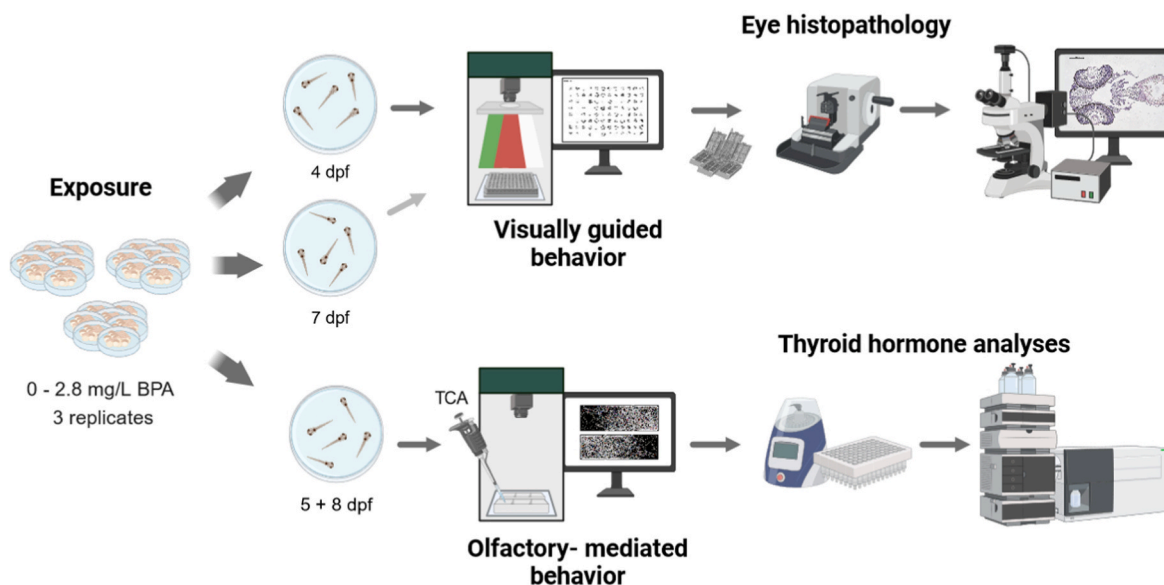


Fig. 1. Study design. Following exposure to BPA, 4–8 dpf zebrafish were assessed in behavioral experiments measuring visually or olfactory guided responses. Subsequently, larvae were euthanized and either processed for eye histopathology or thyroid hormone analyses. Created with BioRender.com.

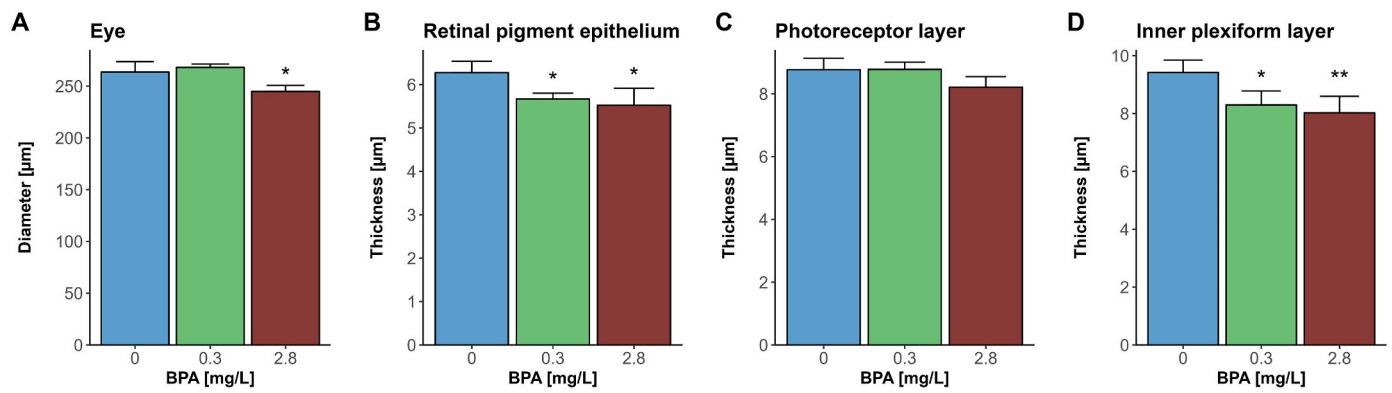


Fig. 2. Eye histopathology in 4 dpf zebrafish following exposure to BPA. Eye diameter (A), as well as thicknesses of the retinal pigment epithelium (B), photoreceptor layer (C), and inner plexiform layer (D) were measured. Data are presented as mean + standard deviation. $n = 3$ replicates (8–24 individuals measured per replicate). Asterisks indicate a statistically significant difference between the treatment groups and the control. * $p < 0.05$, ** $p < 0.01$.

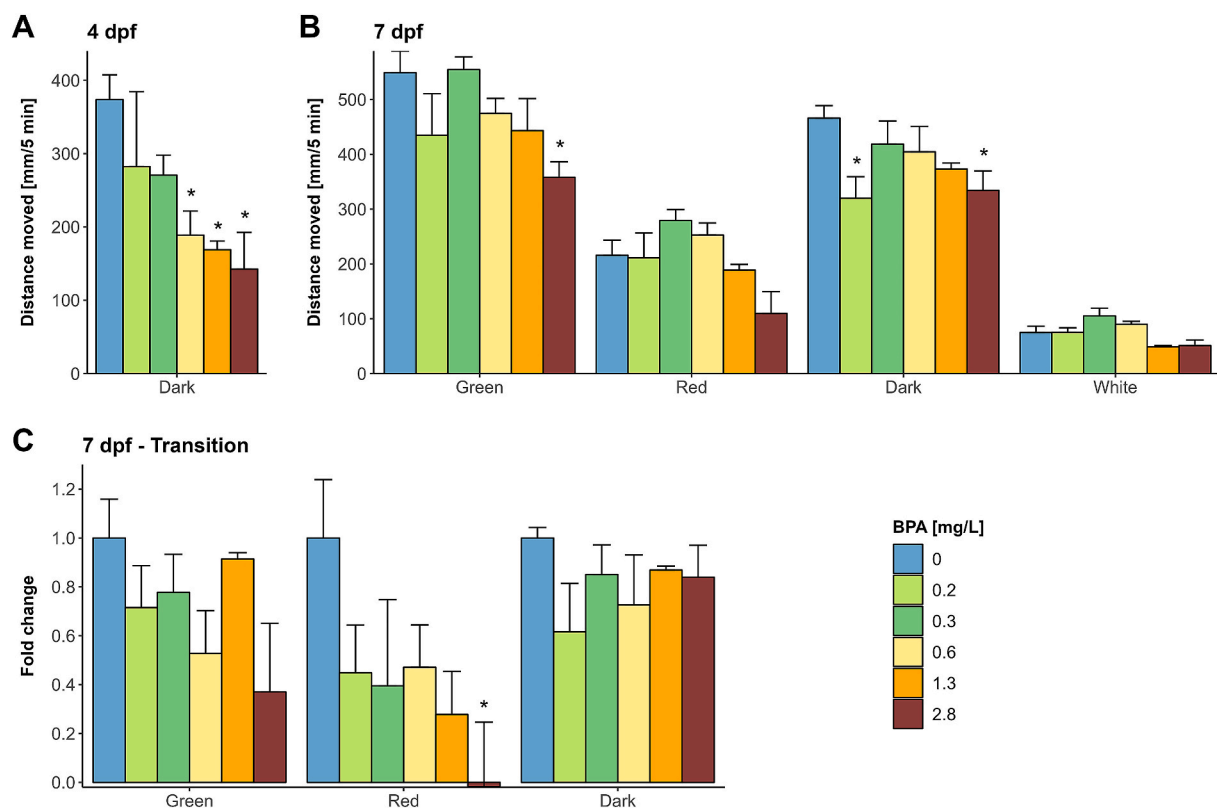


Fig. 3. Visually guided behavior of zebrafish larvae exposed to BPA in response to different light conditions (green, red, dark, white). A: Distance moved of 4 dpf zebrafish in the dark. B: Distance moved of 7 dpf zebrafish in green light, red light, white light, and in the dark. C: Transition response of 7 dpf zebrafish to the change from white light to green light, red light or dark, respectively, presented as fold change to control. $n = 3$ replicates with 12 individuals per treatment group. Results are displayed as mean + standard error. Asterisks indicate a statistically significant difference between the treatment groups and the control. * $p < 0.05$.

significantly decreased at 0.2 and 2.8 mg/L BPA. 3,5-T2 and 3,3'-T2 presented mirrored dose-response relationships. While a decline was observed for 3,5-T2 with statistically significant differences at 1.3 and 2.8 mg/L BPA, 3,3'-T2 levels increased by a factor of three. The level of 3-T1 decreased monotonically and was statistically significant from the control at 2.8 mg/L BPA. T0 showed a non-monotonic concentration-response relationship with a peak at 1.3 mg/L BPA.

At 8 dpf, no significant differences were detected between the treatments and the control, yet trends similar to those at 5 dpf were observed (Fig. S6). Baseline levels of thyroid hormones in control animals did not significantly change between the two time points, except for the levels of rT3, 3,3'-T2, and T1, which were significantly lower at 8

dpf. There were however more values close to the limit of detection at 8 dpf resulting in higher data variation compared to at 5 dpf.

4. Discussion

4.1. BPA exposure alters eye development

Only little is known about how BPA affects vertebrate eye development and published findings on this endpoint are inconsistent. In the present study, BPA exposure decreased eye diameter and altered retinal structure in 4 dpf zebrafish larvae. Previous publications reported increased (Huang et al., 2020), decreased (Martínez et al., 2018), and

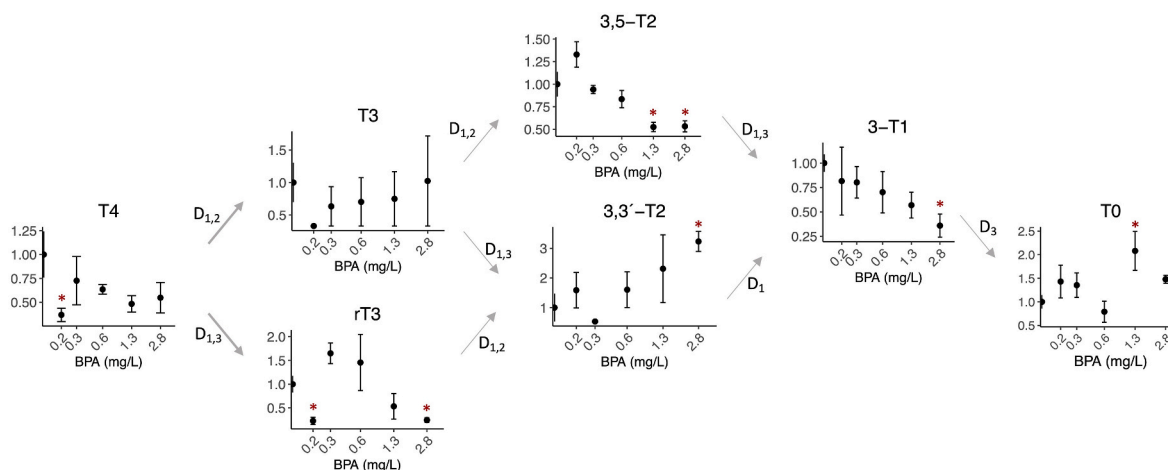


Fig. 4. Relative thyroid hormone levels in 5 dpf larvae shown in the form of the deiodination pathway going from T4 to T0. The deiodinase 1–3 (D1–3) enzymes responsible for the conversion are indicated. Data are presented relative to the controls with the control mean set to 1. The mean \pm SEM has been plotted as a function of the average of actual water concentrations. $n = 3$ (20 larvae pooled per replicate). The control means in fmol/individual were T4: 20 ± 5 ; T3: 1.2 ± 0.4 ; rT3: 13 ± 2 ; 3,3'-T2: 0.6 ± 0.3 ; 3,5-T2: 2.2 ± 0.3 ; T1: 53 ± 5 ; T0: 1.4 ± 0.2 .

unchanged (Lee et al., 2019) eye size in 5 dpf zebrafish larvae at concentrations similar to those tested in this study. To the best of our knowledge, no published data are available on the effects of BPA on retinal layering in fish. Research conducted in rats demonstrated that BPA reduced the thickness of the inner plexiform layer and the outer nuclear layer (Picard et al., 2021). The BPA substitute bisphenol S decreased the thickness of the inner plexiform layer and ganglion cell layer and increased gene expression of opsins related to the perception of red, green, and dim light (Liu et al., 2018). Proper development and function of the visual system depend on both estrogen and thyroid hormone signaling (reviewed in Cohen et al., 2022). A potential explanation for the observed changes in eye development could be a reduction in thyroid hormone levels. In fact, T4 levels exhibited a significant decrease at 0.2 mg/L and displayed a declining trend at higher concentrations. Stinckens et al. (2018) further reported that BPA inhibits thyroid peroxidase (TPO), a crucial enzyme involved in thyroid hormone synthesis, with an IC50 of 13.16 μ M (corresponds to 3 mg/L). This mode of action was also predicted for BPA by the Leadscope QSAR model (DTU, 2023). In addition, it is important to note that the concentration of T4 and T3 in target tissues can significantly differ from whole-body levels owing to localized deiodination processes. Pannetier et al. (2023) analyzed T3 and T4 levels in the eyes of 5 dpf zebrafish larvae following exposure to PTU and TBBPA and found local alterations of thyroid hormone levels which were not observed in the whole body. Deiodinases (Dio) 1 and 2 are responsible for the conversion of T4 to T3, whereas Dio1 and Dio3 catalyze the conversion of T4 to the inactive rT3. Dio2 and dio3 expression was observed in the retina of 2 and 5 dpf zebrafish, respectively (Thisse et al., 2003; Heijlen et al., 2013; Guo et al., 2014). Combined knockdown of Dio1 and Dio2 as well as knockdown of Dio3 have been linked to effects on phototransduction and eye development (Bagci et al., 2015; Houbrechts et al., 2016). In male rats, BPA inhibited DIO1 (da Silva et al., 2019); further, it reduced the expression of *dio1* and *dio3* in a zebrafish liver cell line (Yang and Chan, 2015). To further elucidate the involvement of THS disruption in BPA-induced impairment of eye development, localized analysis of thyroid hormone levels as well as expression of *dio2*, *dio3*, and thyroid hormone receptors could be performed. Rescue experiments employing supplementation of T4, T3, and 3,5-T2 could provide additional mechanistic information. Although the focus of the present study was on sensory development and its association with the THS, it is important to acknowledge that the observed effects may also be influenced by the disruption of estrogen signaling. Aromatase (CYP19), an enzyme responsible for estrogen synthesis, as well as estrogen receptors, are

found in all retinal layers across vertebrate taxa (Ogueta et al., 1999; Cascio et al., 2007; Cohen et al., 2022). Short exposure of zebrafish embryos to 4-hydroxy androstenedione, an inhibitor of aromatase, resulted in long-term effects on their optomotor response (Gould et al., 2017). In addition, previous research by Cascio et al. (2015) supports the notion that changes in estrogen levels in humans are implicated in retinal disease. Thus, the observed BPA-induced disruption of eye development may be attributed to impairment of the THS, estrogenic activity, or a combination of both. Based on the finding that BPA but not E2 influenced the expression of genes associated with phototransduction (Martínez et al., 2018) – a mechanism known to be affected by other THS-disrupting chemicals (Baumann et al., 2019) – it is likely that BPA-induced effects on the visual system are at least partially attributed to alterations in the THS.

4.2. BPA impairs visually but not olfactory-mediated behavior

Both 4 and 7 dpf zebrafish showed a robust response in the light/dark transition part of the behavior trial. The effect of BPA on locomotion in the dark phase was more pronounced at 4 compared to 7 dpf. Swim bladder inflation is a potential factor that can influence swimming behavior. In the present study, posterior chamber inflation was delayed by BPA leading to the increased occurrence of uninflated posterior chambers in all treatments except at 0.3 mg/L in 4 dpf (but not 7 dpf) larvae. Yet, locomotion was only reduced in a concentration-dependent manner at 0.6–2.8 mg/L and individuals exposed to 0.2 mg/L presented normal swimming behavior despite strongly impaired posterior chamber inflation. Previously published work on BPA-induced effects on locomotion in 4–5 dpf zebrafish larvae demonstrated decreased swimming activity in the darkness in similar concentration ranges as in the present study (Wang et al., 2013; Kim et al., 2020). In addition, Wang et al. (2013) discovered that the formation of reactive oxygen species (ROS), DNA damage, and cell death in the trunk musculature correlated with the observed effects on swimming activity. Both ROS formation and disruption of muscle development and function have previously been associated with altered thyroid hormone signaling (Villanueva et al., 2013; Bagci et al., 2015). While BPA-induced formation of ROS and impaired muscle development and function appear to be plausible causes for the decrease in swimming activity observed in the present study, further investigations are needed to conclude the mechanism(s) underlying this effect.

Responses to red and green lights were only observed at 7 dpf. This aligns with previous studies showing that the optomotor response, a

behavior depending on the input from red and green cones, can be reliably elicited from 7 dpf (Orger and Baier, 2005; Portugues and Engert, 2009). Crowley-Perry et al. (2021) further showed that transient exposure of 3 and 7 dpf zebrafish to 23 µg/L BPA increased the positive optomotor response measured one and two weeks post exposure. In addition to causing long-lasting effects on visually guided behavior, this may indicate that BPA alters the function of red and/or green cones. In the present study, larvae exposed to 2.8 mg/L BPA showed decreased swimming activity in green light. The transition response, however, was only inhibited when switching to red light, suggesting an impairment of the perception of long wavelengths. Notably, the retina of zebrafish expresses thyroid hormone receptor genes, including *thrb* (Volkov et al., 2020). During cone development, the expression of *trβ2*, a splice variant of *thrb*, is crucial for the differentiation of cones expressing the red-sensitive opsin *opn1lws1* (Suzuki et al., 2013; Mackin et al., 2019). Consistent with this, knockout of *trβ2* resulted in a loss of the optomotor response of zebrafish larvae to a red stimulus, while the response to a black stimulus remained unaffected (Deveau et al., 2020). BPA's structural similarity to T3 allows it to bind to thyroid hormone receptors, in particular to TRβ, and function as an antagonist in rats (Moriyama et al., 2002; Zoeller et al., 2005). In *Xenopus* tadpoles, exposure to 10 µM BPA (corresponds to approx. 2.3 mg/L) inhibits TRβ-dependent transcription (Heimeier et al., 2009). Taken together, the antagonism of TRβ2 by BPA could explain the impaired response to red light observed in the present study.

Olfactory-mediated behavior of 5 and 8 dpf zebrafish larvae was not significantly altered by BPA. To the best of our knowledge, this is the first study to assess the effect of BPA on olfactory-mediated behavior in zebrafish larvae. In rats, BPA increased the avoidance response to predator odor (Fujimoto and Aou, 2018) and impaired scent discrimination and memory in a preliminary study performed by Vetleson-Trutza et al. (2022).

4.3. BPA exposure alters levels of thyroid hormones and their metabolites

BPA induced alterations in the levels of six out of seven analyzed thyroid hormones or metabolites in 5 dpf zebrafish. Levels of T3, which is recognized as the bioactive thyroid hormone, remained unchanged compared to the control group. T4 levels significantly declined at 0.2 mg/L. Only a limited number of studies have assessed thyroid hormone levels in zebrafish following BPA exposure and reported a slight increase of T3 at 0.4 mg/L but not at 2 mg/L (Lee et al., 2019) as well as unchanged T4 levels (Pelayo et al., 2012; Lee et al., 2019). Exposure of brown trout to BPA for two or eight weeks did not affect T3 or T4 levels (Frenzilli et al., 2021). Yet, it is important to mention that plasma or whole-body levels of thyroid hormone might be unaltered due to compensatory mechanisms, even if peripheral thyroid hormone metabolism is disrupted, potentially resulting in modified local T3 levels. (Coimbra et al., 2005). Decreased T4 levels may suggest an impairment of thyroid hormone synthesis, for instance via inhibition of Tpo or the Sodium/Iodide Symporter (Nis). *In vitro* studies in thyroid follicular FRTL-5 cells and rat thyroid microsomes showed that BPA inhibited iodide transport by NIS but not the activity of TPO (Wu et al., 2016). By contrast, studies conducted in zebrafish did not find significant changes in the expression of *nis* and *tpo* (Chan and Chan, 2012; Gentilcore et al., 2013; Lee et al., 2019).

Levels of 3,5-T2 were significantly reduced in a concentration-dependent manner. In the last decade, a growing body of evidence has indicated that 3,5-T2 is a potent bioactive thyroid hormone in teleost fish (Navarrete-Ramírez et al., 2014; Olvera et al., 2017; Hernández-Linares et al., 2019; Lazcano et al., 2019). Studies conducted in tilapia have demonstrated that 3,5-T2 specifically binds to a TRβ1 isoform referred to as long TRβ1, which is characterized by an insert in the ligand binding domain (Mendoza et al., 2013; Navarrete-Ramírez et al., 2014) and is also present in zebrafish (Marelli et al., 2016). Navarrete-Ramírez et al. (2014) further demonstrated that both 3,5-T2 and T3 promote

growth in tilapia and that their actions are mediated via distinct TRβ1 isoforms. In the cerebellum of tilapia, 3,5-T2, and T3 altered the expression of oligodendrocyte marker genes including *olig2*, *sox10*, and *mbp*, and mitigated the disruption of myelination induced by methimazole. We believe that reduced 3,5-T2 levels may potentially disrupt myelination processes in the zebrafish thereby affecting locomotion and behavior. To test this hypothesis, zebrafish could be co-exposed to a THS-disruptor, for instance methimazole, and 3,5-T2, followed by the assessment of effects on myelination and behavior.

In this study, no significant effect of BPA on thyroid hormone levels was detected at 8 dpf, however, trends were similar to those at 5 dpf. Only very few studies have assessed changes in thyroid hormone levels during zebrafish development and solely for T3 and T4. Control levels vary considerably between these studies (Liu et al., 2011b; Chang et al., 2012). The lack of effects of BPA after the additional exposure time may therefore be due to the decreased analytical sensitivity at the slightly lower thyroid hormone levels at 8 dpf. Another possible explanation could be the adaptation of the hypothalamus-pituitary-thyroid axis. Adjustment of endocrine axes in response to long-term exposure to endocrine-disrupting chemicals has been observed in several studies (Dang et al., 2015; Ramhøj et al., 2020; Pannetier et al., 2023). However, without measurement of for instance thyroid stimulating hormone or the expression of relevant genes, we are not able to conclude whether or not that was the case in this work.

4.4. Crosstalk between the estrogen and thyroid hormone system

The effects observed on eye development are likely attributed to multiple modes of action of BPA. In a recent review, Cohen et al. (2022) assessed the influence of estrogen and thyroid signaling on the visual system of zebrafish and concluded that these endocrine pathways likely exert synergistic effects on its development and function. It is well-established that the THS can influence other endocrine pathways and vice versa, including the estrogen signaling pathway (Thambirajah et al., 2022). Yet only a few studies investigated crosstalk between endocrine systems and our understanding of the mechanisms underlying these interactions remains limited. Liu et al. (2011a,b) investigated the effects of prochloraz on crosstalk between endocrine axes. They observed a decrease in T4 plasma levels and a reduction in the expression of *tshb* in the brain of female zebrafish that positively correlated with estradiol plasma levels. In addition, endocrine-disrupting chemicals with known estrogenic activity have been shown to alter thyroid hormone signaling (Sheikh, 2020). In the current and numerous previously published studies (for example Angle et al., 2013; Vandenberg, 2014; Risalde et al., 2021), BPA presented a non-monotonic concentration-response curve. In this work, rT3 levels at 5 dpf and swimming activity in the dark at 7 dpf exhibited a U-shaped concentration-response curve, with strong effects at the lowest and highest concentration and no effects at intermediate concentrations. Non-monotonic dynamics can have different causes, among others cytotoxicity, receptor competition, and negative feedback loops (reviewed in Vandenberg, 2014). However, they can also be the result of crosstalk between signaling pathways (van Wijk et al., 2015). While exploring interactions between endocrine systems was beyond the scope of the current study, future research should focus on how bisphenols impact the crosstalk between the THS and other endocrine systems. Understanding the effects of bisphenols on these interactions is essential for a comprehensive assessment of the potential hazards to human health and the environment associated with exposure to these compounds.

4.5. Limitations of the study

The present study assessed olfactory-mediated avoidance behavior by analyzing the combined response of ten larvae to an olfactory cue in three replicates. This resulted in high data variation. Hence, the observed lack of a significant alteration of the avoidance behavior may

also be attributed to limitations in the experimental design. Future studies could increase the number of replicates by assessing the behavior of single or fewer individuals in several arenas simultaneously. For the thyroid hormone analyses, twenty larvae were pooled per replicate and measured whole-body levels were close to the limit of quantification and/or exhibited high variability for some thyroid hormones. Consequently, an increase in the number of both individuals pooled and replicates would be preferable in future studies to improve robustness, minimize variability, and enhance statistical power.

5. Conclusion

In conclusion, our findings show that BPA alters thyroid hormone levels and disrupts eye development and visually guided behavior in developing zebrafish. Given that fish larvae strongly rely on visual perception for crucial activities such as foraging and predator avoidance, this study emphasizes the need to evaluate BPA and its substitutes regarding their impact on the THS and associated sensory processes. Currently available OECD fish test guidelines, however, do not encompass endpoints directly linked to THS homeostasis and neurodevelopment. Eye histopathology, visually guided behavior, swim bladder inflation, and thyroid hormone analyses are promising endpoints that could be integrated into existing fish OECD test guidelines covering developmental stages, such as OECD test guideline 210 and 236. While the current study analyzed whole-body levels of thyroid hormones and their metabolites to demonstrate a general effect on the THS, localized measurements in specific body segments or organs may provide more pertinent insights into the disruption of distinct developmental processes. Further research should therefore aim to elucidate the significance of chemically induced changes in both whole-body thyroid hormone levels as well as local thyroid hormone levels in relation to sensory development and function. Such insights are vital for enhancing our understanding of the multifaceted impact of altered thyroid hormone signaling on neurodevelopment and sensory processes and for the development of THS disruption assessment strategies that safeguard human health and the environment.

Credit author statement

Sina Volz: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Validation, Visualization, Writing – original draft Rikke Poulsen: Methodology, Investigation, Formal analysis, Data curation, Validation, Visualization, Writing – original draft Martin Hansen: Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition Henrik Holbech: Conceptualization, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition

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 will be made available on request.

Acknowledgments

The authors would like to thank Bente Frost Holbech for performing the analytical measurements. We would like to acknowledge Annette Duus and Karin Lund Kinnberg for their support with the histology sample preparations. We are grateful to the Aquatic Ecology and Toxicology group (Heidelberg University, Germany) for providing us with the zebrafish breeding groups used in this study. This project has

received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No. 825753 (ERGO). The view expressed in this paper solely reflects the authors' view; the European Union cannot be held responsible for any use that may be made of the information contained therein. The graphical abstract was created with BioRender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2023.140776>.

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