

## The generation gap in endocrine disruption: Can the integrated fish endocrine disruptor test (iFEDT) bridge the gap by assessing intergenerational effects of thyroid hormone system disruption?

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### ARTICLE INFO

#### Keywords:

Endocrine disruption  
Maternal effects  
Intergenerational effects  
Swim bladder inflation  
Photomotor response  
Test guideline  
Zebrafish

### ABSTRACT

Thyroid hormones (THs) act early in ontogenesis, even prior to the differentiation of thyrocytes. Maternal transfer of THs is therefore known to play an essential role in early development. Current OECD test guidelines for the assessment of TH system disruption (THSD) do not address inter- or transgenerational effects. The integrated fish endocrine disruptor test (iFEDT), a test combining parental and developmental exposure of filial fish, may fill this gap. We tested the ability of the iFEDT to detect intergenerational effects in zebrafish (*Danio rerio*): Parental fish were exposed to propylthiouracil (PTU), an inhibitor of TH synthesis, or not exposed. The offspring was submitted to a crossed experimental design to obtain four exposure scenarios: (1) no exposure at all, (2) parental exposure only, (3) embryonic exposure only, and (4) combined parental and embryonic exposure. Swim bladder inflation, visual motor response (VMR) and gene expression of the progeny were analysed. Parental, but not embryonic PTU exposure reduced the size of the swim bladder of 5 d old embryos, indicating the existence of intergenerational effects. The VMR test produced opposite responses in 4.5 d old embryos exposed to PTU vs. embryos derived from exposed parents. Embryonic exposure, but not parental exposure increased gene expression of thyroperoxidase, the target of PTU, most likely due to a compensatory mechanism. The gene expression of *pde-6 h* (phosphodiesterase) was reduced by embryonic, but not parental exposure, suggesting downregulation of phototransduction pathways. Hence, adverse effects on swim bladder inflation appear more sensitive to parental than embryonic exposure and the iFEDT represents an improvement in the testing strategy for THSD.

### 1. Introduction

Maternal thyroid hormones (THs) control morphological, motor, and neurocognitive development in mammals (Andersen et al., 2018; Haddow et al., 1999; Moog et al., 2017; Salazar et al., 2021). Likewise, in oviparous taxa such as birds (Darras, 2019; Groothuis et al., 2019) and fish (Power et al., 2001; Deal and Volkoff, 2020), maternal transfer

of THs is known to affect the development of the progeny. Despite the importance of maternal THs for embryonic development, no OECD test guideline (TG) covers intergenerational (F0 to F1) or transgenerational (F0 to F2 or further) effects of thyroid hormone system disruption (THSD) in non-target organisms, a fundamental gap in the assessment of endocrine disruption (ED). The present study aims at evaluating potential intergenerational effects resulting from suppression of TH

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<https://doi.org/10.1016/j.aquatox.2024.106969>

Received 12 December 2023; Received in revised form 3 May 2024; Accepted 20 May 2024

Available online 21 May 2024

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synthesis, and simultaneously tests the ability of the iFEDT (integrated Fish Endocrine Disruptor Test (Pannetier et al., 2023a; Gözl et al., 2023) to detect such effects.

Both the revised OECD conceptual framework for ED (OECD, 2018) and the European ED regulatory approach (ECHA et al., 2018) are currently based on TGs that mainly use (1) fish as models for investigating potential endocrine activity and adversity of chemicals on the sex steroid hormone system and (2) amphibians for activity and adversity on the TH system (THS). The available TGs addressing effects on the sex steroid hormone system cover different life-stages: either adult fish (TG 229 (OECD, 2012) and TG 230 (OECD, 2009)) or developing fish (TG 234 (OECD, 2011)), or target-specific endocrine mechanisms in embryos, e.g. TG 250 (EASZY (OECD, 2021)) and TG 251 (RADAR (OECD, 2022)). Only higher tier tests such as TG 240 (OECD, 2023) cover exposure effects on 2 generations and, thus, maternal effects, but do not include THS-diagnosing endpoints. TGs specifically addressing THS activity and adversity, which use amphibians exclusively in one-generation approaches, completely disregard inter- or trans-generational effects. In an ecotoxicological context, the absence of a regulatory tool allowing to test maternal effects of TH transfer is a major gap in the regulatory approach, since effects on earliest development stages of organisms may entail severe adverse apical effects (McCullum et al., 2011; Spaan et al., 2019) potentially relevant at the population level.

The iFEDT is a recently proposed test protocol (Pannetier et al., 2023a; Gözl et al., 2023) that aims at evaluating several endocrine modalities over two generations of fish, involving the continuous exposure of both parental and filial fish. The test has been conceptualized as the merge between the FSTRA (Fish Short-Term Reproduction Assay; TG 229 (OECD, 2012)) and the FSST (Fish Sexual Development Test; TG 234 (OECD, 2011)) with additional implementation of THS-sensitive endpoints (Gözl et al., 2023). The iFEDT uses the zebrafish (*Danio rerio*) as a test organism but can easily be adapted to other fish species. Combining all relevant life-stages (adult, embryo, larva, juvenile) in a single 2-generation test protocol, the iFEDT covers intergenerational effects and addresses all endocrine modalities listed in current EU regulations (EATS, i.e., estrogens, androgens, THs and steroidogenesis).

In fish, THs play a fundamental role in metabolism and somatic growth, in neural development including central nervous system and sensory organ development (Cohen et al., 2022), and in morphogenesis, comprising cranial, fin and swim bladder development (Deal and Volkoff, 2020). In recent years, multiple adverse outcome pathways (AOPs) describing the effects of THs on the development of the swim bladder of zebrafish have been developed (e.g. AOPs #155 – 159 (Knapen et al., 2020)) and the inflation of the posterior chamber, which occurs in 4 to 6 d old embryos, has been recently shown to depend on maternally transferred THs (Van Dingenen et al., 2023). In zebrafish, the onset of the endogenous THs production occurs at approx. 3 d after fertilisation and the embryos are able to sustain TH production from 4 d onwards (Brown, 1997; Elsalini and Rohr, 2003; Porazzi et al., 2009). Yet, early developing organs may require THs for their development and depend, therefore, on the THs of maternal origin present in the yolk.

Van Dingenen et al. showed that development of the posterior chamber is not affected by inhibition of embryonic TH synthesis, and that exposure of zebrafish embryos to a deiodinase inhibitor before the onset of endogenous TH synthesis disturbed embryonic Wnt and hedgehog signalling, and impaired swim bladder inflation (Van Dingenen et al., 2023). In other words, the activation of THs not produced by the embryo is required for correct swim bladder development. The swim bladder is essential for the maintenance of buoyancy, and impaired development of this organ reduces swimming efficiency leading to increased energy consumption (Schwebel et al., 2018; Woolley and Qin, 2010), reduces feeding efficiency (Czesny et al., 2005) and most likely affects predator avoidance abilities. Zebrafish embryos that fail to inflate their posterior swim bladder have exceptionally high mortality rates

between days 7 and 10 after fertilisation (Goolish and Okutake, 1999). Due to the central role of THs in the control of swim bladder development, the failure of (and possibly even mere delay in) swim bladder inflation represents an important adverse endpoint for the identification of THSD. Based on the pleiotropic effects of THs on swim bladder development, metabolism as well as neural and cognitive development, disruption of the THS will likely also manifest in behavioural changes. The light-dark visual motor test, a behavioural assay which evaluates the photomotor response, was initially designed to test anxiety-like behaviour in fish but has also been applied as a screening tool for disruption of visual abilities, e.g., in the detection of different light intensities and in THSD research (Spaan et al., 2019; Walter et al., 2019).

Across different vertebrate classes, THs and TH receptors have been shown to affect the development of the visual system, in particular the organization of the retina as well as the differentiation of photoreceptors and their patterning (Cohen et al., 2022; Eldred et al., 2018; Mackin et al., 2019; Ng et al., 2001; Suzuki et al., 2013), and may, therefore, be disrupted by THSD chemicals, potentially affecting vision and colour perception. Exposure of fish to various THSD chemicals resulted in changes of the retinal structure (Havis et al., 2006; Molla et al., 2019; Trimarchi et al., 2008), for example the structure of the retinal pigment epithelium (RPE), for which an AOP has recently been developed (AOP #363 (Gözl et al., 2022)) and which has been shown to affect vision (Baumann et al., 2016). If vision allows the perception of the abrupt light changes generated by the light-dark visual motor test, a stress response is triggered, which can be measured as increased locomotion (Lee et al., 2019). For zebrafish embryos, which prefer light environments, the transition from light to dark is considered a stressful stimulus, and the embryos are typically more active during the dark phases of the test (Lee et al., 2019). As such, the photomotor response is also modulated by the “stress” axis, the hypothalamus-pituitary-adrenal (HPA) axis, or in fish, -interrenal (HPI) axis. In non-mammalian vertebrates, the hypothalamus-pituitary-thyroid (HPT) axis and the HPA/HPI axes show crosstalk, namely at the level of the pituitary, where corticotropin-releasing hormone (CRH) elicits the production of the thyroid stimulating hormone (TSH) and subsequently the production of THs (De Groef et al., 2006; Okada et al., 2007). Conversely, in mammals, THs induce CRH expression and activate the HPA axis (Moog et al., 2017). THSD may therefore alter photomotor response through any combination of these mechanisms: vision, interference with the HPI axis, and/or swimming ability (under influence of swim bladder). In order to explore the physiological mechanisms underlying the morphological and behavioural endpoints being tested, we quantified the expression of selected genes related to or hypothesised to be affected by the activity of the HPT axis (Table 1).

In the present study, the iFEDT protocol served as a basis to test the

**Table 1**

List of HPT-, visual system-, and stress-related genes selected for analysis.

Gene name	Gene	Function
<i>tpo</i>	Thyroperoxidase	Synthesis of THs
<i>trb</i>	Thyroid hormone receptor TRβ	Receptor for THs
<i>crf</i>	Corticotropin-releasing factor	Control of corticotropin release in the HPI (“stress”) axis
<i>pde-6h</i>	cGMP-specific phosphodiesterase 6 h phosphodiesterase	Enzyme essential in initial triggering of the neural signal; involved in phototransduction
<i>opn1mw1</i> and <i>opn1lw2</i>	Opsin 1 medium-wave-sensitive 1 and opsin 1 long-wave-sensitive 2	Visual pigments coding for RH2-1, and LWS2 visual pigments in phototransduction (absorbing at 467 and 548 nm, respectively)
<i>spb</i>	Surfactant protein b	Surfactant protein hypothesised to be involved in swim bladder development and inflation (Zheng et al., 2011)
<i>18s</i>	18S	Ribosomal RNA, reference gene

occurrence of intergenerational effects of THSD and the suitability of the iFEDT to detect effects of potentially disrupted maternal TH transfer during early development. While exposure to a TH synthesis inhibitor will not affect TH levels of embryos before the onset of TH synthesis, at ca. 3 d (Brown, 1997; Elsalini and Rohr, 2003; Porazzi et al., 2009), exposure of parental fish to inhibitors of the TH synthesis may well affect the transfer of THs to the yolk. Therefore, we tested exposure to propylthiouracil (PTU), an inhibitor of TH synthesis. PTU is a goitrogenic, antithyroid pharmaceutical used to treat hyperthyroidism as in Grave's disease. It strongly inhibits the synthesis of T4 and T3 by blocking thyroid peroxidase (TPO), thereby lowering TH levels, which has also been shown in zebrafish in our previous study (Pannetier et al., 2023b). In mammals, PTU is known to also inhibit deiodinase 1 (DIO1), in fish, however, this remains a matter of controversy (Stinckens et al., 2020). PTU was tested in different combinations of parental and/or embryonic exposure. As endpoints, we tested the impact of PTU exposure on (1) posterior swim bladder inflation, (2) visual motor response (VMR) and (3) expression of genes involved in mechanisms potentially underlying the effects observed in up to 5 d old zebrafish embryos.

## 2. Methods

### 2.1. Test chemical

Propylthiouracil (PTU, 6-propyl-2-thiouracil, CAS no: 51-52-5) was purchased from Sigma-Aldrich (Deisenhofen, Germany). Stock solutions of 1055 mg/L PTU were prepared daily and continuously stirred for 24 h to guarantee complete dissolution before application to the flow-through system to obtain a final test concentration of 78 mg/L in the exposure tanks. The final PTU exposure concentration of 78 mg/L was selected as a level for which only ED-specific effects, but no unspecific symptoms of toxicity, including mortality, had been reported in literature. Samples for analytical chemical verification of actual PTU concentrations were taken at weekly intervals (for details (Pannetier et al., 2023a)).

### 2.2. Animal breeding and maintenance

Fish maintenance and breeding were licensed by regional animal welfare authorities under reference 35-9185.64/BH Braunbeck, and animal experiments were authorized under license no. 35-9185.81/G-263/19. Zebrafish (*Danio rerio*) of the Westaquarium strain from the stocks of the Aquatic Ecology and Toxicology Group at the Centre for Organismal Studies, University of Heidelberg, were used as test organisms. Sexually mature fish at an age between 6 and 12 mo were exposed at  $26 \pm 1$  °C and used for breeding in 12 L tanks, housing 5 females and 5 males in a flow-through system (exchange rate 1.25 L/h; photoperiod of 14 h light/10 h dark). Breeding groups were randomly distributed across experimental tanks and fed ad libitum three times a day: at least once with fresh *Artemia* sp. nauplii (Great Salt Lake *Artemia* Cysts, Sanders, Ogden, USA) and with dry flake food (TetraMin™, Tetra, Melle, Germany) the remaining times.

### 2.3. The integrated fish endocrine disruptor test (iFEDT)

The experiments presented in this report are part of a larger study conducted by Pannetier et al. (2023a) and Gözl et al. (2023) evaluating the feasibility of the iFEDT protocol. The iFEDT combines a 21-d period of parental exposure (F0) according to OECD TG 229 with the subsequent exposure of the progeny (F1) to the same test concentrations for 63 d, according to OECD TG 234 (Pannetier et al., 2023a; Gözl et al., 2023).

In a pre-exposure phase of at least 14 d, breeding groups of the parental generation (5 females and 5 males per replicate) were allowed to acclimate to the test conditions. A minimum of 10 eggs/female/day produced during the pre-exposure phase was used as a quality standard

qualifying the start of the exposure phase.

During the parental exposure phase, egg production was monitored daily and, at the end of this phase eggs were collected for exposure of the progeny and the parental generation (F0) was sacrificed for assessment of: TH levels, vitellogenin levels, and histopathology of gonads and thyroid follicles (Pannetier et al., 2023a; Gözl et al., 2023). The progeny (F1) was then exposed to the same concentrations as their parents, until fish reach the late juvenile stage. In F1 embryos, juvenile and sexually differentiated fish, the following endpoints for the different endocrine modalities were assessed: swim bladder inflation, in 5 d old; body size and weight, eye histopathology, at 5 and 60 d old; as well as histopathology of gonads, and of thyroid follicles, TH levels, sex ratio, vitellogenin levels and gonad maturity index, in 60 d old. For further details of the protocol and results, see (Pannetier et al., 2023a; Gözl et al., 2023).

### 2.4. Experimental design: the iFEDT adapted to crossed design

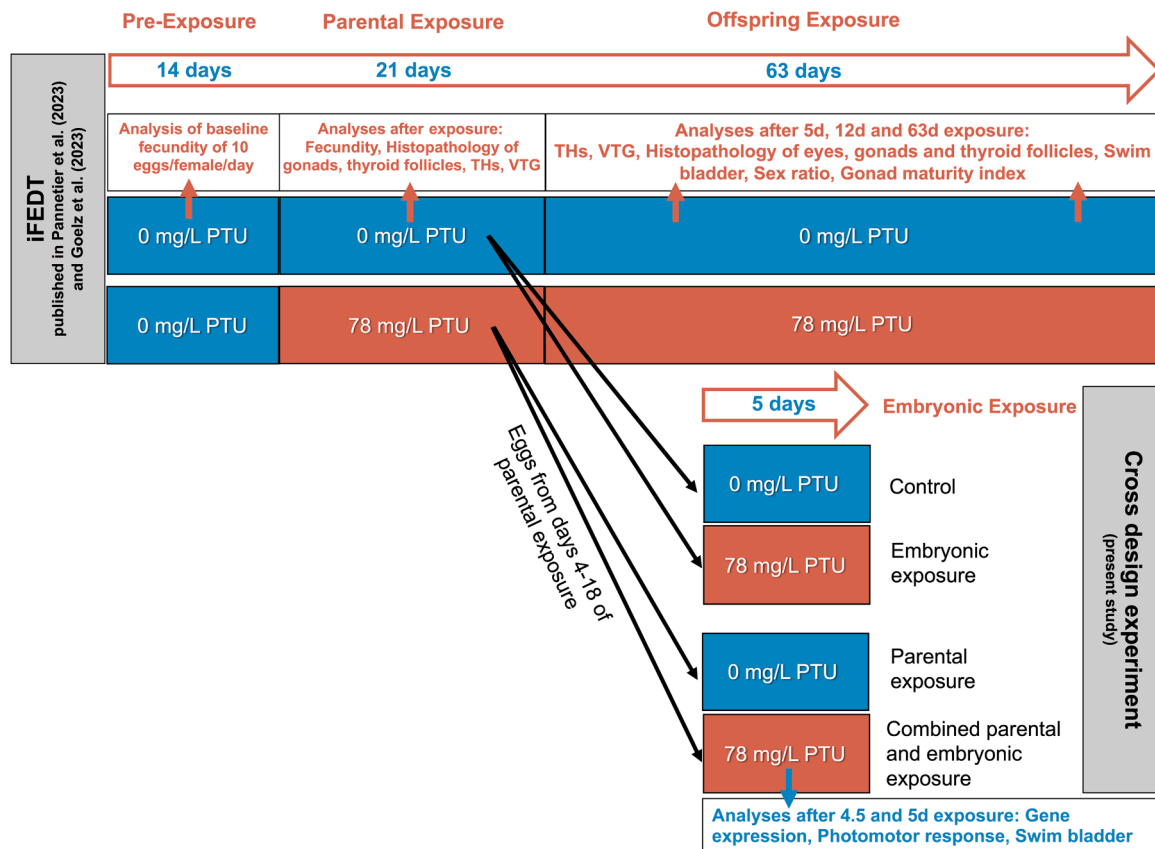
The experiment of the present study followed the iFEDT protocol until the end of the parental exposure (F0) phase, with the following modifications (Fig. 1):

- (1) Instead of a full dose-response series of concentrations, only one concentration was tested (nominal concentration: 78 mg PTU/L; measured concentration 71.3 mg PTU/L, see Pannetier et al., 2023a) against a water control (0 mg PTU/L).
- (2) Eggs were collected throughout the parental exposure phase (days 4 to 18) instead of only at the end of the parental exposure phase (day 21).
- (3) Instead of consistently exposing F1 fish to the same concentration as their parents, the eggs were distributed to different treatments in order to identify the independent contributions of parental and embryonic exposures in a cross design.
- (4) Endpoints were evaluated only in 4.5 to 5 d old embryos: area of swim bladder (similarly to the iFEDT), photomotor response and gene expression (endpoints not evaluated in the iFEDT)

Only fertilized, healthy eggs were selected for exposure of F1, which started at ca. 2.5 h post fertilisation. Eggs were reared in replicate glass dishes in groups of approx. 50 individuals in 200 ml of PTU solution or reconstituted water (International Standards, 1996; OECD, 2013) under semi-static conditions in a temperature-controlled chamber at  $26 \pm 1$  °C under a 14/10 h light/dark cycle. 50 - 60 % of the test solutions were renewed daily, and development was monitored. No malformations or mortality of  $\geq 10$  % of embryos were observed.

- (1) Eggs collected from unexposed parents (0 mg PTU/L) were divided into two groups: (a) "Control" with no parental and no embryonic exposure to PTU (5 replicates) and (b) "Embryonic exposure" in which eggs were exposed to 78 mg PTU/L (4 replicates).
- (2) Eggs collected from exposed parents (78 mg PTU/L) were divided into: (c) "Parental exposure" group, with eggs not exposed to PTU (6 replicates) and (d) "Combined parental and embryonic exposure" group, eggs exposed to the same PTU concentration as the parents (5 replicates).

Eggs were exposed to PTU in a randomized way and according to availability. Since not all parental groups ( $n = 4$  per treatment) produced eggs every day, this resulted in variable numbers of replicates in the filial exposure groups. From each replicate, 50 % of the individuals were used as a pooled sample for analysis of gene expression (at day 4.5), 50 % were used for behaviour testing (at day 4.5) and photographic documentation for measurement of the area of the swim bladder (at day 5). Genes were selected for analysis to represent: synthesis of THs, HPI axis, visual transduction and opsins, and (hypothetically) inflation of the swim bladder (Tables 1 and SM 1 [Supplementary Materials]).



**Fig. 1.** Exposure design of iFEDT experiment and cross design experiment: Zebrafish (*Danio rerio*) parents and offspring were exposed to PTU (iFEDT, results published in Pannetier et al. 2023a and Goelz et al. 2023). For the present study, eggs from the non-exposed parental generation (0 mg PTU/L) were exposed to either 0 or 78 mg PTU /L. Likewise, eggs from the parental generation exposed to 78 mg PTU/L were exposed to either 0 or 78 mg PTU /L. Between 4 and 18 days of exposure of the parental generation, eggs were collected and directly exposed for 4.5 d for evaluation of photomotor response and gene expression, or for 5 d for measurement of area of swim bladder.

Endpoints recorded in parental fish were reported previously (Pannetier et al., 2023a; Gözl et al., 2023).

### 2.5. Behavioural observations

The behavioural response of 4.5 d old embryos was tested in the light-dark locomotion test (also termed light-dark transition test or visual motor response, VMR). The test consists of a sequence of transitions between dark and light in which the locomotion behaviour of the fish is quantitatively recorded, usually as distance moved (Basnet et al., 2019; Legradi et al., 2015). Embryos were tested in sterilized and pyrogen-free tissue culture 96-well plates (TPP 96F, Trasadingen, Switzerland). Plates were pre-exposed to the corresponding test solutions for 24 h. The light-dark locomotion test was conducted in the DanioVision® observation chamber (Noldus, Wageningen, The Netherlands) at 26 °C. Embryos were allowed to acclimate to the DanioVision® observation chamber for 2 h under low intensity white light (525 nm, 2.3 lux). For assessment of normal mobility, the swimming behaviour of the embryos was analysed for 15 min under the same light conditions (525 nm, 2.3 lux) as in the acclimation period.

Tests for PTU exposures started at 7 p.m. with a 10 min dark phase (0 lux) followed by 10 min low intensity green light phase (525 nm, 2.3 lux). These alternating phases were repeated for a total of 10 cycles. Green light was chosen, because a previous study had shown that genes encoding green photoreceptors (*ops1mw*) were strongly dysregulated in zebrafish embryos after PTU exposure, suggesting that the visual response to green light might be altered (Baumann et al., 2019). Based on embryo availability (breeding success of the parents decreased over time under PTU exposure), a total of 11 tests were run within 14 days.

The sequence of tests was randomized with respect to exposure scenarios.

In each test, 22 embryos were analysed, and exposure treatment was randomized by testing days. Embryos, which did not move during the test periods, did not react to touch or showed scoliosis or other morphological malformations or edema, were excluded from the analysis. The test was recorded on video and analysed with Ethovision XT® (v. 11.5; Noldus). In cases focal embryos were not detected automatically by the software for more than 1 % of the observation time, the corresponding 1-min interval was discarded. Behavioural analyses were carried out for the parameters (1) total distance moved per minute and (2) total distance moved by embryos during the dark or during the green light phases respectively.

### 2.6. Assessment of posterior swim bladder size

Following the behavioural tests, embryos were photographed for biometric measurements. After anaesthesia with buffered MS-222 (tricaine methane sulfonate, 400 mg/L), embryos were positioned in sagittal plane on a microscope slide following the procedure described by Stinckens et al. (Stinckens et al., 2020). The size of the posterior swim bladder of the embryos was measured via microphotography at 4 × magnification on an inverted microscope under diascopic illumination (Nikon ECLIPSE Ts2; Nikon, Düsseldorf, Germany; or Olympus CK40, Olympus, Hamburg, Germany). The standard length (SL) of embryos and area of posterior swim bladder were calculated with the NIS Elements D software (v. 5.10; Nikon Europe, Amstelveen, The Netherlands). After photographic documentation, anaesthetised embryos were euthanized in an overdose of MS-222.

The effect of PTU on swim bladder inflation was evaluated according to Hagenaaers et al. (2014) with adaptations: From the control group, the relationship between the SL and surface of the posterior swim bladder was estimated as a linear regression ( $\text{Swim bladder surface}_{\text{expected}} = a + b \times \text{standard length}_{\text{observed}}$ ). In contrast to Hagenaaers et al. (2014), who used the ratio observed /expected swim bladder surface, the difference between the observed surface of the posterior swim bladder and its expected surface as a function of SL was used to test the effect of experimental treatments.

### 2.7. Gene expression analysis

After anaesthesia with MS-222 (400 mg/L), 4.5 d old embryos were frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  until further analysis. Samples of approx. 30 Embryos per tube were homogenised in a TissueLyser II (Qiagen, Hilden, Germany) and frozen in 1 ml TRI Reagent® RNA isolation reagent (Sigma-Aldrich, T9424) and RNA was isolated according to the manufacturer's instructions. RNA concentration and quality were measured in a NanoVue 4282 v.1.7.3 spectrophotometer (General Electrics Healthcare, Lindesnes, Norway). For the synthesis of cDNA, the ReadyScript cDNA synthesis Mix (Sigma-Aldrich) was used according to the manufacturer's instructions. Real time RT-qPCR (StepOne® system; AB Applied Biosystems, Life Technologies, Darmstadt, Germany) with LUNA Universal qPCR Master Mix (New England Biolabs Frankfurt, Germany) was conducted to evaluate the expression of the genes listed in Table 1. Primers were obtained from Sigma-Aldrich; sequences are listed in Table SM 1. Each sample was analysed in triplicate.

Gene expression was calculated by the comparative CT method ( $2^{-\Delta\Delta CT}$ ) as described by Schmittgen and Livak (Schmittgen and Livak, 2008). 18S rRNA was chosen as reference gene due to its constant expression across treatments (Table SM 2) measured as  $\log_2 2^{-CT}$ . Normalized expression of target genes,  $2^{-\Delta CT}$  was compared in different treatments. To produce the heatmap,  $\log_2 2^{-\Delta\Delta CT}$  values were calculated, in which the control treatment was used as calibrator, i.e., the heatmap represents the  $\log_2$  of the fold-change of normalized expression of the target gene in different treatments in comparison to the same gene in the control. The heatmap was produced in R using the ggplot2 package (Wickham, 2016).

### 2.8. Statistical analyses

Data were analysed in R v. 3.6.1 (Core Team, 2012). All tests were two-tailed, and the significance level was set at 0.05 throughout. Data were logarithm- or square root-transformed when necessary to improve model validity.

In order to evaluate the effects of parental and embryonic exposure on behaviour and on swim bladder inflation, linear mixed models (LMM) were applied using the lme4 package (Bates et al., 2015), and the fixed effects were tested by Kenward-Roger F-statistics. Parental and embryonic exposure were considered fixed factors, whereas experimental day was considered a random factor, controlling for the non-independency of embryos tested simultaneously on the same day. This correction for the lack of independence of embryos according experimental day was necessary, since: (1) embryos were raised simultaneously; (2) in behavioural observations, embryos were also observed in groups (in 96-well plates) simultaneously; and (3), if PTU accumulated in the exposed parental generation from day 4 to day 18, the effect of parental exposure might have varied with experimental day as well.

Gene expression data were analysed by ANCOVA considering experimental days as a covariate, using the R package car (Fox and Weisberg, 2019). *Post-hoc* multiple comparisons Tukey tests were computed with the multcomp R package (Hothorn et al., 2008).

## 3. Results

### 3.1. Summary of iFEDT study results

This work is part of an extensive study evaluating the feasibility of the iFEDT presented by Pannetier et al. (2023a) and Gözl et al. (2023) and demonstrates that PTU exposure to 78 mg/L (measured average concentration 71.3 mg/L) caused reproductive impairment of parental fish but had no effect on gonad histopathology and vitellogenin levels of the exposed parents on day 21 of exposure. TH level measurements of the parental generation showed no significant changes, most likely due to technical problems outlined in Gözl et al. (2023). This was surprising, since strong proliferation of thyroid follicles was observed in both the parental generation and the offspring at 78 mg/L PTU exposure, confirming the expected impact of PTU on TH synthesis. Impaired TH synthesis in PTU-exposed zebrafish embryos was confirmed in one of our previous studies using different analytical methods (Pannetier et al., 2023b). In the F1 generation of the iFEDT study (Pannetier et al., 2023a; Gözl et al., 2023), growth was reduced, and eye development was impaired, as revealed by histopathological changes in retinal layers at all sampling time points.

### 3.2. Swim bladder inflation

Parental exposure to PTU significantly reduced the surface of the posterior swim bladder in 5 d old offspring. In contrast, exclusive embryonic PTU exposure did not affect the surface of the swim bladder at the concentration tested, and no interaction between parental and embryonic exposure was detected (Fig. 2; Table 2).

### 3.3. Behavioural observations

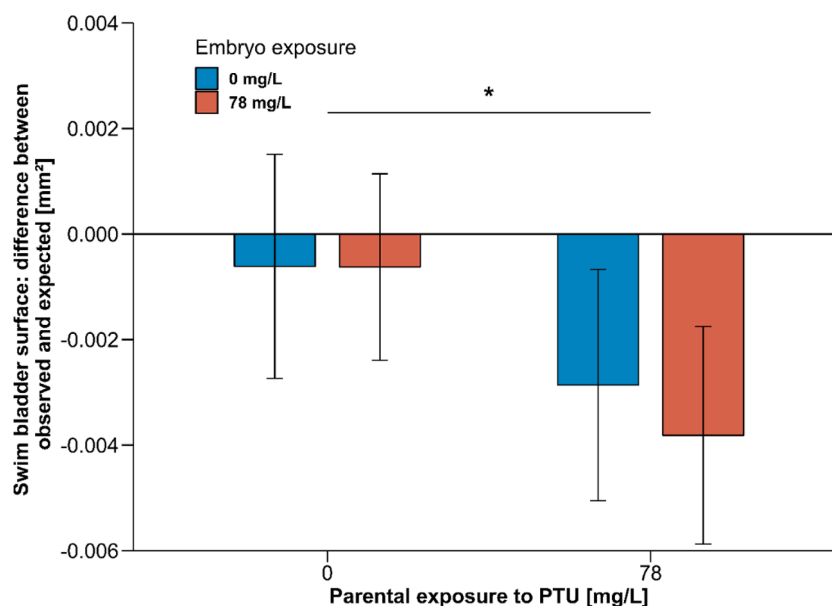
The movement of control and PTU-exposed embryos followed the typical pattern of the light-dark locomotion test, with embryos swimming longer distances during the dark phases. However, mobility during the dark phases gradually declined as the test progressed. This decline tended to be attenuated in embryos exposed to PTU and accentuated in embryos whose parents were exposed to PTU as the test progressed (Fig. 3).

During the dark phases of the light-dark locomotion test, embryonic exposure to 78 mg/L PTU increased the distance moved by embryos. In contrast, parental exposure to 78 mg/L PTU decreased the distance moved by the embryos, but no interaction between parental and embryonic PTU exposure was detected (Fig. 4A and Table 2). The effect of the combined exposure of embryos and their parents was not significant, in that the behaviour of these embryos was similar to that of the control group. The distance moved by exposed embryos from non-exposed parents in comparison to the control group was marginally non-significant (*post-hoc* Tukey comparison:  $z = 2.48$ ,  $p = 0.06$ ). Non-exposed embryos from exposed parents moved significantly less than all other experimental groups (Fig. 4A).

During the green light phases, no significant differences between treatments were detected. Furthermore, PTU exposure of either parents or embryos did not have a significant effect on the embryos' reaction to the transition from dark to green light phases, as measured by the total distance moved during the green light phase, and no interaction of embryo and parental exposure was detected either (Fig. 4B and Table 2).

### 3.4. Gene expression

PTU increased the expression of the thyroperoxidase gene (*tpo*) in 4.5 d old embryos only when the embryos themselves were exposed. No effect of parental exposure could be detected. Likewise, there was no interaction between parental and embryonic exposure and no effect of experimental day (Figs. 5A, SM 1 and Table 3). *Post-hoc* analysis revealed that embryonic exposure alone upregulated *tpo* expression in



**Fig. 2.** Residual effect (difference between observed surface and expected surface according to standard length) of parental and/or embryonic exposure of zebrafish (*Danio rerio*) to propylthiouracil (PTU) on the surface of the posterior swim bladder (mean  $\pm$  95 % confidence interval) of 5 d old zebrafish embryos. Control: 4 replicates,  $N = 81$ ; embryonic exposure: 5 replicates,  $N = 117$ ; parental exposure: 3 replicates,  $N = 76$ ; combined parental and embryonic exposure: 4 replicates,  $N = 86$ . \* Significant main effect of “parental exposure” ( $p < 0.05$ ).

**Table 2**

Statistical results of the light-dark locomotion test. Dark and light phases were analysed independently by linear mixed models (lme4 package) with parental and embryonic exposure as fixed effects tested by Kenward-Roger F-statistics, and experimental day as random factor.

Effects	Dark Phase (squared root transformed data)	Light Phase (log-transformed data)	Swim bladder surface (difference between observed and expected by body length)
Fixed Parental exposure	$F_{1,26.6} = 6.83^*$	$F_{1,20.3} = 2.57$	$F_{1,39.1} = 4.32^*$
Embryonic exposure	$F_{1,27.6} = 17.41^{***}$	$F_{1,21.1} = 2.00$	$F_{1,50.2} = 0.08$
Interaction	$F_{1,27.6} = 2.58$	$F_{1,19.9} = 0.27$	$F_{1,55.5} = 0.38$

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

comparison to the control group, and PTU-exposed embryos from exposed parents showed higher levels of *tpo* expression than non-exposed embryos derived from exposed parents (Figs 5A and SM 1).

The expression of the phosphodiesterase gene (*pde6h*) was, on the contrary, reduced in embryos exposed to PTU, but not in embryos derived from exposed parents, and no interaction was detected (Figs. 5B, SM 1, Tables 3). No *post-hoc* pairwise comparison proved significant, but embryonic exposure alone was only marginally non-significantly reduced (*post-hoc* Tukey comparisons: control vs. embryonic exposure alone  $t = -2.76$ ,  $p = 0.05$ ).

Neither embryo, nor parental exposure significantly affected the expression of the thyroid receptor beta (*trb*), the corticotropin releasing factor (*crf*), the L-cone pigment LWS1-2 (*opn1lw2*) and the M-cone pigment RH2-1 (*opn1mw1*) genes at 4.5 d (Fig. SM 1, Table 3).

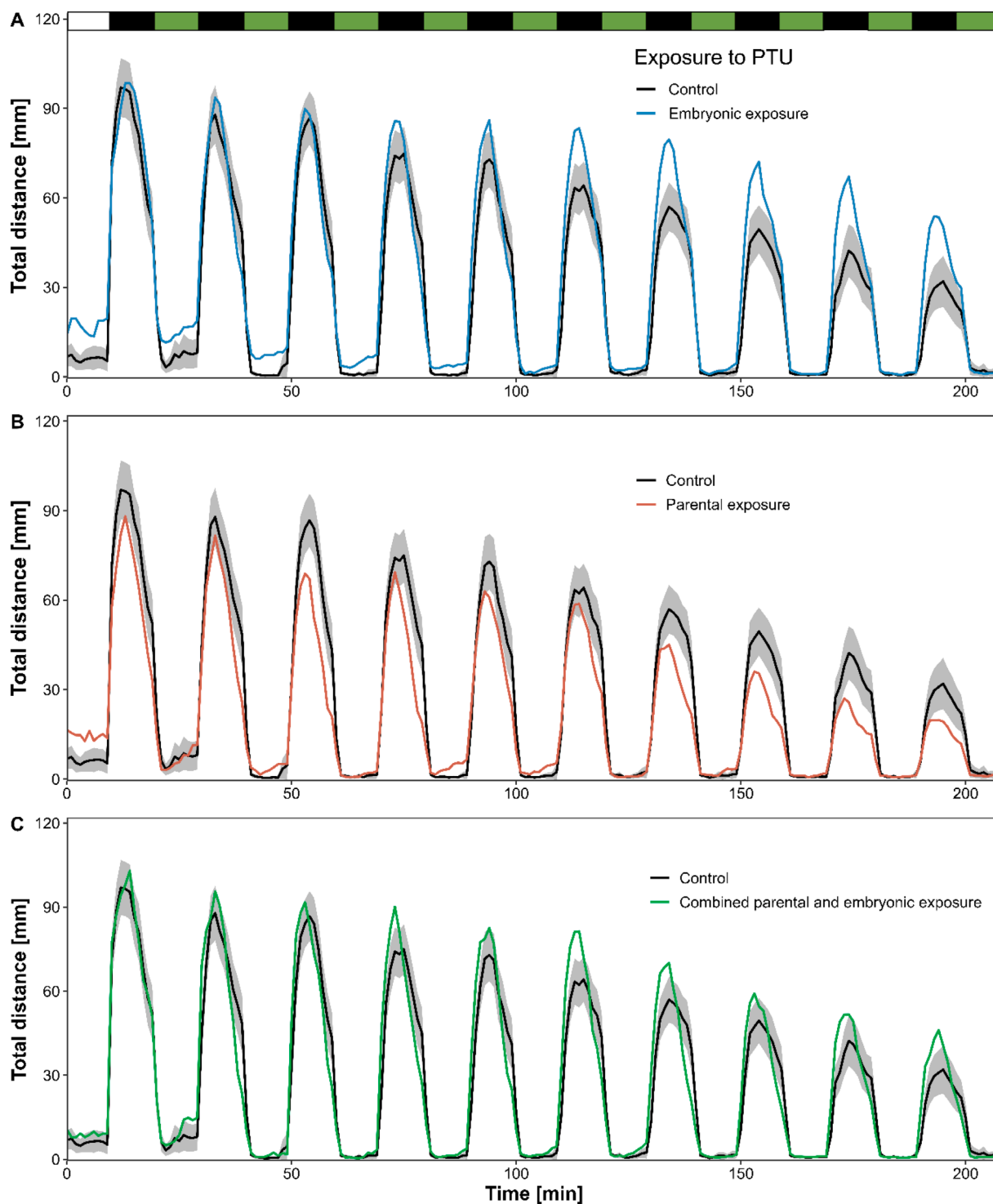
## 4. Discussion

### 4.1. Swim bladder inflation and related gene expression

In the present study, the development of the posterior swim bladder in embryos was not affected by PTU but was reduced by exposure of their parents, suggesting that the swim bladder development is more

sensitive to PTU by its potential effects on TH synthesis of the parents than a direct effect of exposure of the embryos. The hypothesised mechanism of maternal TH transfer to the eggs could not be demonstrated in the present experiment, as it was not possible to quantify the TH content in eggs, and since no statistically significant decrease in TH could be detected in the exposed parents due to technical problems (Pannetier et al., 2023a; Götz et al., 2023). Yet, impaired TH synthesis in PTU-exposed zebrafish embryos was confirmed in one of our previous studies using different analytical methods (Pannetier et al., 2023b). The results are nevertheless in line with a recent study by Van Dingenen et al. (2023), who described reduced swim bladder inflation in zebrafish embryos exposed to a deiodinase inhibitor, iopanoic acid, before the onset of endogenous TH production of the embryos. These results indicate a suppression of the activation of T4 (present in the embryo, but not produced by it) to T3 by iopanoic acid and, hence, that T4 is present in the egg from other sources than endogenous. Further support to the hypothesis of maternal transfer of THs comes from observations by Brown et al. (1988), who provided evidence for maternal transfer of THs by injecting female striped bass (*Morone saxatilis*) with T3, resulting in elevated T3 concentrations in the eggs, a higher percentage of larvae with an inflated swim bladder and lower long-term mortality.

Embryonic exposure to PTU and other TPO inhibitors (e.g. methimazole and 2-mercaptobenzothiazole) affected the development and inflation of the anterior swim bladder at a later age (Stinckens et al., 2020; Stinckens et al., 2016). However, only concentrations of PTU significantly higher (259 mg/L) than those used in the present study (78 mg/L) led to the decrease in the number of 5 d old embryos with inflated posterior swim bladder, whereas other inhibitors of TH synthesis such as methimazole and 2-mercaptobenzothiazole did not affect posterior swim bladder inflation on day 5 (Stinckens et al., 2016; Stinckens et al., 2018). PTU has been hypothesised to additionally affect posterior swim bladder inflation in exposed embryos as a consequence of deiodinase 1 (DIO 1) inhibition (Stinckens et al., 2020), a mechanism not yet elucidated in fish. Knocking down or inhibiting deiodinase enzymes does affect the development of the posterior chamber (Van Dingenen et al., 2023; Bagci et al., 2015; Heijlen et al., 2014; Houbrechts et al., 2016) by preventing the conversion of T4 (whether endogenous and/or of maternal origin) to T3, the most active TH form. If PTU does indeed

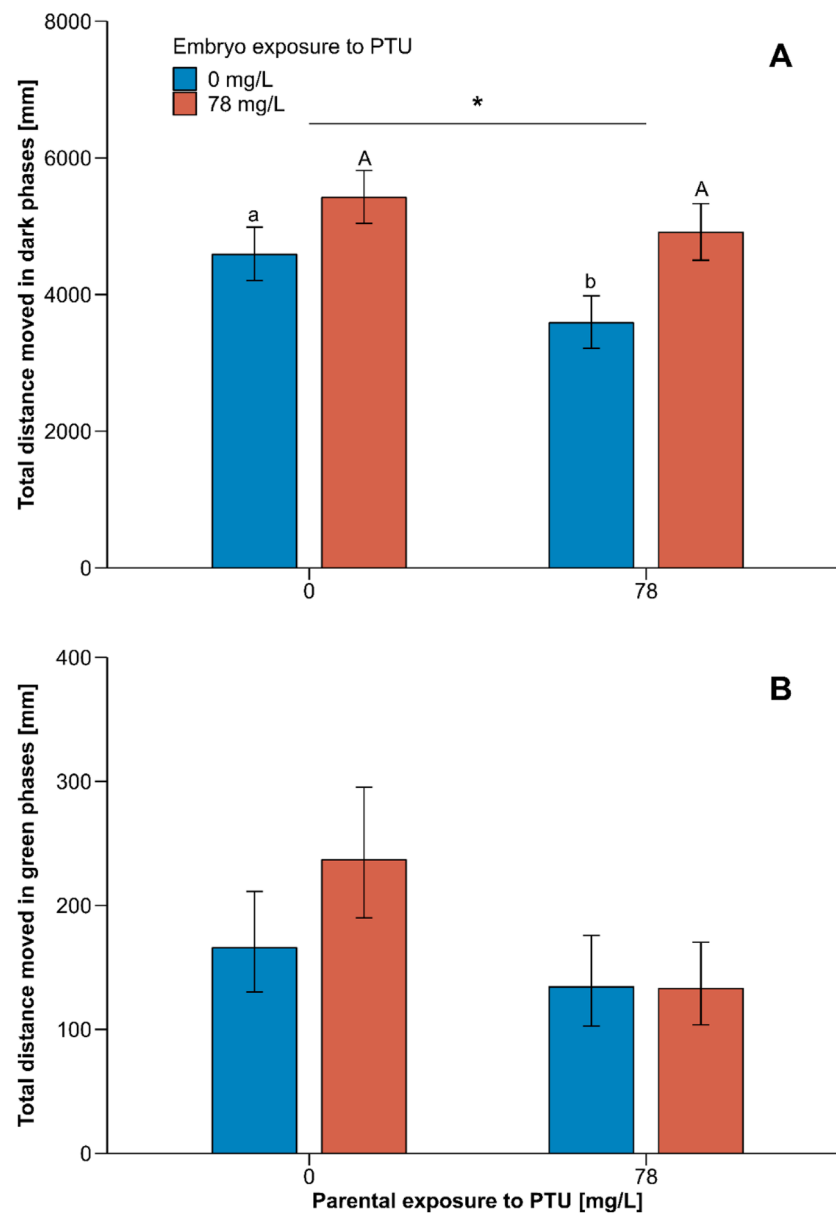


**Fig. 3.** Distance moved per minute by 4.5 d old zebrafish (*Danio rerio*) embryos during the green light-dark locomotion test, after parental exposure to propylthiouracil, PTU (A: blue line, 4 replicates,  $N = 88$ ), after embryonic exposure (B: red line, 6 replicates,  $N = 131$ ) or after combined exposure of parents and embryos (C: green line, 5 replicates,  $N = 104$ ) in comparison to the control (black lines in A-C, 5 replicates,  $N = 109$ ). Areas shaded in grey represent 95% confidence intervals of the control group, and the bar on top of the figure represents the green colour light or the darkness of each of the 10 min phases of the test.

inhibit DIO1 in fish, the concentration that embryos of the present study were exposure to, has not been sufficient to affect posterior swim bladder development; and would not have been an explanation for reduced swim bladder in embryos of exposed parents unless a mechanism of TH transfer to the embryos was involved. Whether inhibition of TH synthesis in parents alone or additional inhibition of TH activation through DIO 1 in the embryo are responsible for the reduction of swim

bladder inflation observed in the present study, remains unclear. In either case, the effect of parental exposure to PTU is definitely stronger than that of embryo exposure, which shows the presence of intergenerational effects and indicates the involvement of maternally transferred THs in the development of the swim bladder. Moreover, it underlines the need to test for inter- and transgenerational effects of THSD.

The hypothesis that surfactant protein-b might be involved in the



**Fig. 4.** Total distance moved (mean  $\pm$  95 % confidence interval) by 4.5 d old zebrafish (*Danio rerio*) embryos in the green light-dark locomotion test, in response to exposure of parents and/or embryos to PTU during the a) dark phases ( $10 \times 10$  min.) or b) green light phases ( $10 \times 10$  min.). Replicates and individual observations: control: 5 replicates,  $N = 109$ ; embryonic exposure: 6 replicates,  $N = 131$ ; parental exposure: 4 replicates,  $N = 88$ ; combined parental and embryonic exposure: 5 replicates,  $N = 104$ . Different letters: significant differences between groups by *Post-hoc* Tukey test; capital and lowercase letters: differences in the main effect of embryonic exposure. \* Statistically significant effect of main effect parental exposure ( $p < 0.05$ ) by LMM and Kenward-Roger F-statistics (see Table 2).

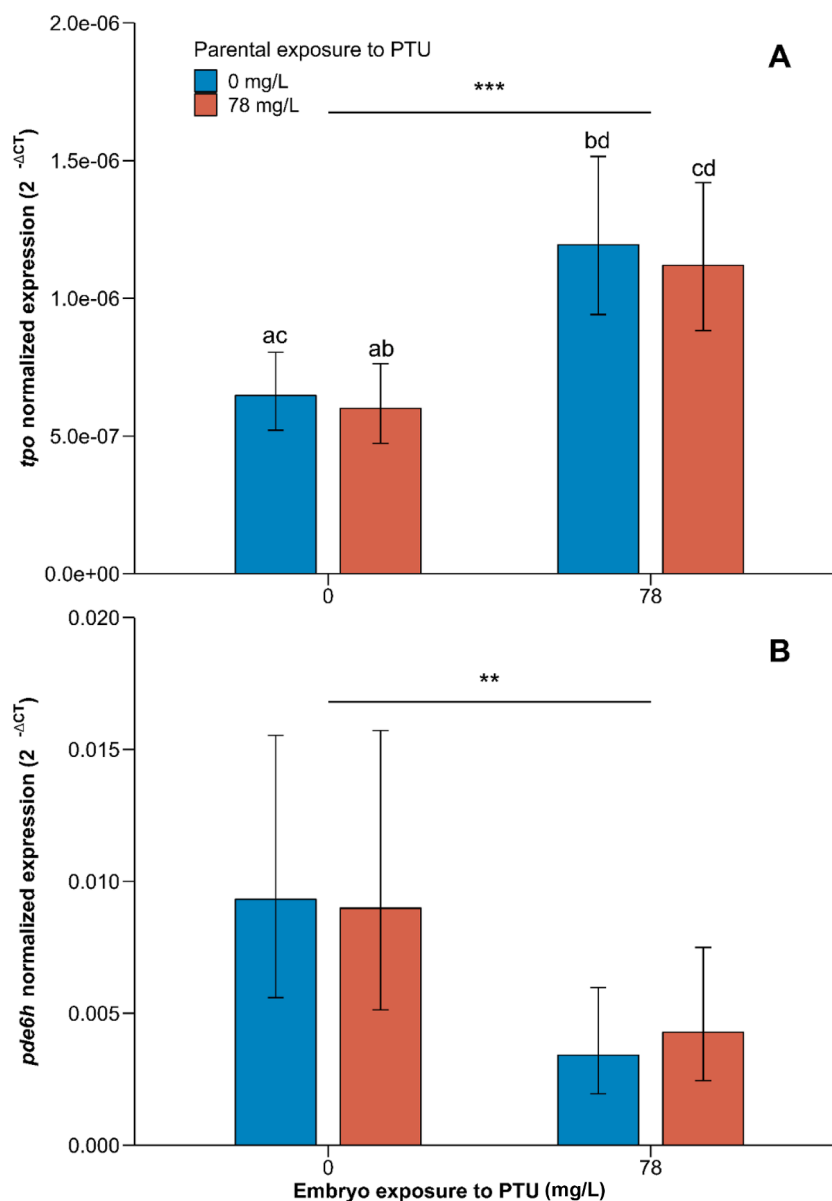
impaired development and inflation of the posterior swim bladder was not supported by the present results: Gene expression of *spb* was not altered by any of the treatments tested, although a recent study showed that Wnt and hedgehog signalling, which are essential for swim bladder formation, can be disrupted by DIO inhibition in zebrafish embryos (Van Dingenen et al., 2023).

#### 4.2. Behavioural changes and related gene expression

Embryos of all treatments showed increased swimming activity, i.e. moved longer distances, during the dark phase than under green light. This pattern is consistent with the typical behaviour of zebrafish embryos in the light-dark locomotion test (Lee et al., 2019), indicating that all embryos retained light detection ability at least to some extent. The distance moved during the green light phases of the test following PTU treatment did not differ from controls, which, however, does not support

our initial hypothesis that the visual response to green light might be altered. Green light was chosen, because a previous study had shown that genes encoding green photoreceptors (*ops1mw*) were strongly dysregulated in zebrafish embryos after PTU exposure (Baumann et al., 2019).

If compared to controls, the distance moved throughout the dark phases of the test was reduced in non-exposed embryos whose parents had been exposed to PTU and increased in exposed embryos derived from non-exposed parents. In contrast, previous studies applying different behavioural assays reported reduced swimming activity in 5 d old embryos exposed to higher concentrations of PTU (111 mg/L) under light and in the absence of stimuli (Stinckens et al., 2020) or under alternating light and dark regimes. The increase in activity observed in the present study under visual and assumed stressful stimuli (Lee et al., 2019) suggests a specific reaction to the transition from green light to dark phases.



**Fig. 5.** Expression ( $2^{-\Delta CT}$ ) of A) thyroid peroxidase (*tpo*) and B) phosphodiesterase (*pde6h*) genes (mean  $\pm$  95 % confidence interval) by 4.5 d old zebrafish (*Danio rerio*) embryos in response to exposure of parents and/or embryos to propylthiouracil PTU. Gene expression normalised to the reference gene 18S rRNA. Control: 6 replicates; embryonic exposure: 5 replicates; parental exposure: 5 replicates; combined parental and embryonic exposure: 5 replicates. Each replicate is a sample from ca 30 pooled embryos. Different letters: significant differences between groups by *Post-hoc* Tukey test; capital and lowercase letters: differences in the main effect of parental exposure. Statistically significant effects of embryonic exposure: \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  (ANCOVA; see Table 3).

The physiological mechanisms responsible for the observed photomotor response could not be identified conclusively: reduced swim bladder inflation could not completely explain the observed behavioural pattern. Parental exposure reduced inflation of the posterior swim bladder and the distance moved by the embryos, in agreement with AOPs in the dark phases of the light-dark locomotion test but embryonic exposure alone increased the photomotor response but had no effect on swim bladder development. Thus, swim bladder inflation was likely not the reason, or at least not the exclusive reason for the observed changes in behaviour.

Alternative mechanisms leading to changes in photomotor behaviour include compromised visual function. Given the crucial role of THs in eye development of fish (Cohen et al., 2022), disruption of eye development in PTU-exposed embryos was expected (Pannetier et al., 2023a; Baumann et al., 2016; Baumann et al., 2019). Against expectations, however, the reduction observed in the expression of *pde6h* suggesting

down-regulation of the phototransduction pathway described after embryonic exposure to 350 mg/L PTU by Baumann et al. (2019) did not reduce, but increase the photomotor response (Baumann et al., 2016; Lee et al., 2019).

A specific sensitivity of vision to a wavelength of 525 nm might also modify the photomotor response. Reduced light perception in this section of the spectrum, as expected by reduction of sensitive L-cones (in particular cones with LWS2 opsins absorbing at 548 nm) should decrease the photomotor response by reducing the contrast between light and dark phases. Differentiation of L cones is dependent on THs and starts very early in zebrafish development before day 4 (Suzuki et al., 2013). Parental exposure, if reducing THs of maternal origin in the embryo, could be expected to reduce the number of L cones and subsequently inhibit photomotor response. On the other hand, THs promote the expression of LWS1 opsins in detriment of LWS2 opsins in L cones. Reduction of THs after day 4, as would be achieved by embryonic

**Table 3**

Results of the bifactorial ANCOVA on gene expression, with factors parental exposure and embryonic exposure, their interaction, and the interaction between test day and parental exposure as covariates.

Gene <sup>1</sup>	Factors / covariate (F <sub>df groups, dfn</sub> )			
	Parental exposure (F <sub>1,15</sub> )	Embryonic exposure (F <sub>1,15</sub> )	Interaction parental and embryonic exposure (F <sub>1,15</sub> )	Interaction parental exposure and experimental day (F <sub>2,15</sub> )
Thyroperoxidase ( <i>tpo</i> )	0.14	<b>28.73***</b>	0.01	0.76
Phosphodiesterase 6 h cGMP-specific ( <i>pde-6 h</i> )	0.87	<b>11.22**</b>	0.18	0.49
Thyroid receptor TR $\beta$ ( <i>trb</i> )	0.16	0.50	2.19	0.47
Surfactant protein b ( <i>sp-b</i> )	<b>3.18<sup>#</sup></b>	0.21	1.96	1.99
Opsin 1 medium-wave-sensitive 1 ( <i>opn1mw1</i> );	0.16	0.15	1.45	0.39
Opsin 1 long-wave-sensitive 2 ( <i>opn1lw2</i> )	1.07	0.36	0.90	0.82
Corticotropin releasing factor ( <i>crf</i> )	0.74	1.02	1.45	0.81

<sup>#</sup> $p < 0.1$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , <sup>1</sup>Gene expression normalized values  $2^{-\Delta\Delta CT}$  were log transformed; df: degrees of freedom.

exposure, would no longer affect L cones differentiation, but rather reduce LWS1 opsin expression in favour of LWS2, increasing light sensitivity at 548 nm, the peak of absorption of LWS2 opsins. An increase in sensitivity to light with a wavelength closed to the one tested might be expected to increase the photomotor response.

Although this would be consistent with the behavioural pattern observed, the lack of expression changes of the LWS2-coding gene (*opn1lw2*) does not corroborate such a hypothesis. Likewise, the unchanged expression of the gene coding for RH2-1 (*opn1mw1*) provides no further clue to the mechanisms underlying the photomotor response. This is in contrast to a previous study (Baumann et al., 2019), which reported upregulation of *opn1mw1* in zebrafish embryos exposed to 350 mg/L PTU, which is, however, a higher concentration than the one used in the present study (78 mg/L).

Interference of THs with the HPI axis may also alter photomotor response indirectly by changing the stress response of fish. As the light to dark transition induces stress in zebrafish embryos, an altered response could be expected, if the HPI axis were affected by PTU exposure. The CRF (Corticotropin Releasing Factor) is a fulcral interception point between the HPI and the HPT axes. In fish, the effect of THs on the HPI axis is not completely understood and may be species-, life-stage- and physiological state-dependent (Paul et al., 2022; Peter and Peter, 2009). Administration of PTU to adult freshwater tilapia (*Oreochromis mossambicus*) did not affect cortisol levels, but subsequent administration of T4 during hypothyroid states increased plasma cortisol levels (Peter and Peter, 2009). PTU administration to 5 d old zebrafish embryos resulted in activation of *cyp 11b* and *crhpb* genes, the first gene coding for cytochrome P450 11B involved in the synthesis of corticosteroids, and the latter gene coding for the CRF binding protein (Liu et al., 2013). The present results, in which embryos exposed to PTU moved longer distances in response to the light to dark transition, suggest an induction of stress responses and probably increased cortisol levels, though apparently not through CRF modulation.

Larvae exposed to methimazole, another TH synthesis inhibitor, also showed increased anxiety behaviour at day 10 using a different test paradigm (Reider and Connaughton, 2015). Walter et al. (2019)

analysed in detail the effect of maternal TH transfer on embryo behaviour, using the same behaviour paradigm, the light-dark locomotion test, but using a shorter test, designed with different durations of dark and light phases. In striking contrast to the results of the present study, Walter et al. (2019) showed that exposure of embryos to PTU reduced swimming activity during the dark phases. However, in agreement with the present results, lowering maternal TH transfer to eggs by ablation of the thyroid follicles in mothers decreased activity of offspring in the dark phases. These contradicting observations might be caused by the use of different light wavelengths, and differences in the number of light-dark cycles and their durations might also lead to diverging results. The results of the present study indicate an increase in the difference between treatment and controls with test progression. Hence, tests with the same approach, but different numbers of light cycles may produce different results, supporting the idea of behaviour as a highly plastic endpoint and highlighting the need for a high degree of standardisation of behaviour tests.

## 5. Conclusions

The present study clearly demonstrates the potential of the iFEDT approach to elucidate intergenerational effects of THSDs. Given the delay in endogenous TH production by the embryos and the known transfer of maternal hormones into eggs, the test may be particularly helpful in the identification of adverse effects in early stages of development caused by inhibition of TH synthesis in the parental generation, which the current repertoire of ecotoxicological OECD TGs may fail to detect. Impairment of swim bladder inflation is an important population-relevant adverse outcome; it is not affected by embryonic exposure alone but affected by both parental exposure alone and combined exposure of parents and their embryos. Therefore, two-generation tests like the iFEDT can identify this effect more reliably than the presently available OECD TGs. The iFEDT protocol can detect different morphological and behavioural responses in the progeny of zebrafish specific to the type of exposure to PTU, namely parental, embryonic or combined parental and embryonic exposure. The mechanisms determining the behavioural differences are not yet understood, probably due to the pleiotropic effect of THs affecting a variety of physiological pathways and interactions between HPT and HPI axes, which render the effects on apical endpoints difficult to predict. Due to its sensitivity, photomotor behaviour of zebrafish embryos is a promising instrument to identify THSD and/or developmental neurotoxicity – an endpoint likely of population relevance. However, the test design clearly influences the results observed; therefore, standardisation of test designs is essential. Until effective methods are available to explain the mechanisms underlying patterns of photomotor response, behaviour tests can at least be used to monitor potential adversity of THSDs or other environmental pollutants.

## CRedit authorship contribution statement

**Teresa Fagundes:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pauline Pannetier:** Writing – review & editing, Visualization, Methodology, Investigation. **Lisa Götz:** Writing – review & editing, Visualization, Methodology, Investigation. **Laura Behnstedt:** Writing – review & editing, Visualization, Methodology, Investigation. **Jane Morthorst:** Writing – review & editing, Validation, Conceptualization. **Lucia Vergauwen:** Writing – review & editing, Validation, Resources, Funding acquisition, Conceptualization. **Dries Knapen:** Writing – review & editing, Validation, Resources, Funding acquisition, Conceptualization. **Henrik Holbech:** Validation, Resources, Funding acquisition, Formal analysis, Conceptualization. **Thomas Braunbeck:** Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Lisa Baumann:** Writing – original draft, Validation, Supervision, Project administration,

Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

### Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

### Data availability

Data will be made available on request.

### Acknowledgments

We acknowledge the advice and support of the steering group, the scientific expert group and the scientific advisors of the iFEDT project, namely: Gerald Ankley, Maria Arena, Mara Ceridono, Zhichao Dang, John Green, Tom Hutchinson, Aude Kienzler, Marc Leonard, Peter Matthiessen, Sharon Munn, Francesca Pellizzato, Laia Quiros, Maristella Rubbiani, Helmut Segner, Georg Streck, Charles Tyler, and Jordane Wodli. We thank the team of the Aquatic Ecology and Toxicology section (University of Heidelberg) for practical support with the extensive experimental work, namely: Lukas Frese, Maximilian Rinderknecht and Paula Weddeling.

### Funding

This work was funded by the EU Tender project “Development of a study protocol for regulatory testing to identify endocrine disrupting substances in biotic systems” under contract no. 070203/2018/794670/ETU/ENV.B.2. We also acknowledge funding from the European Union's Horizon 2020 research and innovation program under grant agreement no. 825753 (ERGO). This output reflects only the authors' views, and the European Union cannot be held responsible for any use that may be made of the information contained therein. Author LB received additional funding from the “Olympia Morata Program” of the Office of Equal Opportunities, University of Heidelberg.

### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.aquatox.2024.106969](https://doi.org/10.1016/j.aquatox.2024.106969).

### References

- Andersen, S.L., Andersen, S., Vestergaard, P., Olsen, J., 2018. Maternal thyroid function in early pregnancy and child neurodevelopmental disorders: a Danish nationwide case-cohort study. *Thyroid Off. J. Am. Thyroid Assoc.* 28, 537–546. <https://doi.org/10.1089/thy.2017.0425>.
- Bagci, E., Heijlen, M., Vergauwen, L., et al., 2015. Deiodinase knockdown during early zebrafish development affects growth, development, energy metabolism, motility and phototransduction. *PLoS One* 10, e0123285. <https://doi.org/10.1371/journal.pone.0123285>.
- Basnet, R.M., Zizioli, D., Taweedet, S., et al., 2019. Zebrafish larvae as a behavioural model in neuropharmacology. *Biomedicines* 7, 1–16. <https://doi.org/10.3390/biomedicines7010023>.
- Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. <https://doi.org/10.18637/jss.v067.i01>.
- Baumann, L., Ros, A., Rehberger, K., et al., 2016. Thyroid disruption in zebrafish (*Danio rerio*) larvae: different molecular response patterns lead to impaired eye development and visual functions. *Aquat. Toxicol.* 172, 44–55. <https://doi.org/10.1016/j.aquatox.2015.12.015>.
- Baumann, L., Segner, H., Ros, A., et al., 2019. Thyroid hormone disruptors interfere with molecular pathways of eye development and function in zebrafish. *Int. J. Mol. Sci.* 20 (1543), 1–17. <https://doi.org/10.3390/ijms20071543>.
- Brown, C.L., Doroshov, S.I., Nunez, J.M., et al., 1988. Maternal triiodothyronine injections cause increases in swim bladder inflation and survival rates in larval striped bass, *Morone saxatilis*. *J. Exp. Zool.* 248, 168–176.
- Brown, D.D., 1997. The role of thyroid hormone in zebrafish and axolotl development. *Proc. Natl. Acad. Sci. U.S.A.* 94, 13011–13016. <https://doi.org/10.1073/pnas.94.24.13011>.
- Cohen, A., Popowitz, J., Delbridge-Perry, M., et al., 2022. The role of oestrogen and thyroid hormones in zebrafish visual system function. *Front. Pharmacol.* 13, 837687. <https://doi.org/10.3389/fphar.2022.837687>.
- Core Team, R., 2012. R: A language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- Czesny, S.J., Graeb, B.D.S., Dettmers, J.M., 2005. Ecological consequences of swim bladder non-inflation for larval yellow perch. *Trans. Am. Fish. Soc.* 134, 1011–1020. <https://doi.org/10.1577/T04-016.1>.
- Darras, V.M., 2019. The role of maternal thyroid hormones in avian embryonic development. *Front. Endocrinol.* 10, 66. <https://doi.org/10.3389/fendo.2019.00066>.
- Deal, C.K., Volkoff, H., 2020. The role of the thyroid axis in fish. *Front. Endocrinol.* 11, 596585. <https://doi.org/10.3389/fendo.2020.596585>.
- De Groef, B., Van der Geyten, S., Darras, V.M., Kühn, E.R., 2006. Role of corticotropin-releasing hormone as a thyrotropin-releasing factor in non-mammalian vertebrates. *Gen. Comp. Endocrinol.* 146, 62–68. <https://doi.org/10.1016/j.ygcen.2005.10.014>.
- ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the Joint Research Centre (JRC), Andersson N., Arena M., et al., 2018. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. *EFSA Journal* 2018;16(6): 5311, 135 pp. <https://doi.org/10.2903/j.efsa.2018.5311>. ECHA-18-G-01-EN.
- Eldred, K.C., Hadyniak, S.E., Hussey, K.A., et al., 2018. Thyroid hormone signalling specifies cone subtypes in human retinal organoids. *Science* 362, eaau6348. <https://doi.org/10.1126/science.aau6348>.
- Elsalini, O.A., Rohr, K.B., 2003. Phenylthiourea disrupts thyroid function in developing zebrafish. *Dev. Genes. Evol.* 212, 593–598. <https://doi.org/10.1007/s00427-002-0279-3>.
- Fox, J., Weisberg, S., 2019. An R companion to applied regression, 3rd ed. SAGE, Los Angeles.
- Gölz, L., Baumann, L., Pannetier, P., et al., 2022. AOP report: thyroperoxidase inhibition leading to altered visual function in fish via altered retinal layer structure. *Environ. Toxicol. Chem.* 41, 2632–2648. <https://doi.org/10.1002/etc.5452>.
- Gölz, L., Pannetier, P., Fagundes, T., et al., 2023. Development of the integrated fish endocrine disruptor test – part B: implementation of thyroid-related endpoints. *Integr. Environ. Assess. Manage.* <https://doi.org/10.1002/ieam.4828>.
- Goolish, E.M., Okutake, K., 1999. Lack of gas bladder inflation by the larvae of zebrafish in the absence of an air-water interface. *J. Fish. Biol.* 55, 1054–1063.
- Groothuis, T.G.G., Hsu, B.-Y., Kumar, N., Tschirren, B., 2019. Revisiting mechanisms and functions of prenatal hormone-mediated maternal effects using avian species as a model. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 374, 20180115. <https://doi.org/10.1098/rstb.2018.0115>.
- Haddow, J.E., Palomaki, G.E., Allan, W.C., et al., 1999. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *N. Engl. J. Med.* 341, 549–555. <https://doi.org/10.1056/NEJM199908193410801>.
- Hagenaars, A., Stinckens, E., Vergauwen, L., et al., 2014. PFOS affects posterior swim bladder chamber inflation and swimming performance of zebrafish larvae. *Aquat. Toxicol.* 157, 225–235. <https://doi.org/10.1016/j.aquatox.2014.10.017>.
- Havis, E., Anselme, I., Schneider-Maunoury, S., 2006. Whole embryo chromatin immunoprecipitation protocol for the in vivo study of zebrafish development. *Biotechniques* 40 (34), 36. <https://doi.org/10.2144/000112098>, 38 passim.
- Heijlen, M., Houbrechts, A.M., Bagci, E., et al., 2014. Knockdown of type 3 iodothyronine deiodinase severely perturbs both embryonic and early larval development in zebrafish. *Endocrinology* 155, 1547–1559. <https://doi.org/10.1210/en.2013-1660>.
- Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. *Biom. J. Biom. Z.* 50, 346–363. <https://doi.org/10.1002/bimj.200810425>.
- Houbrechts, A.M., Vergauwen, L., Bagci, E., et al., 2016. Deiodinase knockdown affects zebrafish eye development at the level of gene expression, morphology and function. *Mol. Cell Endocrinol.* 424, 81–93. <https://doi.org/10.1016/j.mce.2016.01.018>.
- International Standards, 1996. ISO 7346 Water Quality – Determination of the Acute Lethal Toxicity of Substances to a Freshwater Fish [*Brachiodanio Rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]. International Organization for Standardization.
- Knapen, D., Stinckens, E., Cavallin, J.E., et al., 2020. Toward an AOP network-based tiered testing strategy for the assessment of thyroid hormone disruption. *Environ. Sci. Technol.* 54, 8491–8499.
- Lee, H.B., Schwab, T.L., Sigafos, A.N., et al., 2019. Novel zebrafish behavioural assay to identify modifiers of the rapid, nongenomic stress response. *Genes. Brain Behav.* 18, e12549. <https://doi.org/10.1111/gbb.12549>.
- Legradi, J., el Abdellaoui, N., van Pomeran, M., Legler, J., 2015. Comparability of behavioural assays using zebrafish larvae to assess neurotoxicity. *Environ. Sci. Pollut. Res. Int.* 22, 16277–16289. <https://doi.org/10.1007/s11356-014-3805-8>.
- Liu, C., Yu, H., Zhang, X., 2013. Zebrafish embryos/larvae for rapid determination of effects on hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-interrenal (HPI) axis: mRNA expression. *Chemosphere* 93, 2327–2332. <https://doi.org/10.1016/j.chemosphere.2013.08.026>.
- Mackin, R.D., Frey, R.A., Gutierrez, C., et al., 2019. Endocrine regulation of multichromatic colour vision. *Proc. Natl. Acad. Sci. U.S.A.* 116, 16882–16891. <https://doi.org/10.1073/pnas.1904783116>.
- McCullum, C.W., Ducharme, N.A., Bondesson, M., Gustafsson, J.-A., 2011. Developmental toxicity screening in zebrafish. *Birth Defects Res. C Embryo Today* 93, 67–114. <https://doi.org/10.1002/bdrc.20210>.
- Molla, M.H.R., Hasan, M.T., Jang, W.J., et al., 2019. Thyroid hormone-induced swim bladder and eye maturation are transduced by IGF-1 in zebrafish embryos. *Aquac. Res.* 50 (11), 3462–3470. <https://doi.org/10.1111/are.14305>.
- Moog, N.K., Entringer, S., Heim, C., et al., 2017. Influence of maternal thyroid hormones during gestation on foetal brain development. *Neuroscience* 342, 68–100. <https://doi.org/10.1016/j.neuroscience.2015.09.070>.

- Ng, L., Hurley, J.B., Dierks, B., et al., 2001. A thyroid hormone receptor that is required for the development of green cone photoreceptors. *Nat. Genet.* 27, 94–98. <https://doi.org/10.1038/83829>.
- OECD, 2009. Test No. 230: 21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. <https://doi.org/10.1787/9789264076228-en>.
- OECD, 2011. Test No. 234: Fish Sexual Development Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. <https://doi.org/10.1787/9789264122369-en>.
- OECD, 2012. Test No. 229: Fish Short Term Reproduction Assay, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. <https://doi.org/10.1787/9789264185265-en>.
- OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. <https://doi.org/10.1787/9789264203709-en>.
- OECD, 2018. Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, OECD Series on Testing and Assessment, No. 150. OECD Publishing, Paris. <https://doi.org/10.1787/9789264304741-en>.
- OECD, 2021. Test No. 250 EASZY Assay: Detection of Endocrine Active substances, Acting Through Oestrogen receptors, Using Transgenic tg(cyp19a1b:GFP) Zebrafish Embryos. OECD.
- OECD, 2022. Test No. 251: Rapid Androgen Disruption Activity Reporter (RADAR) Assay, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. <https://doi.org/10.1787/da264d82-en>.
- OECD, 2023. Test No. 240: Medaka Extended One Generation Reproduction Test (MEOGRT), OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. <https://doi.org/10.1787/9789264242258-en>.
- Okada, R., Miller, M.F., Yamamoto, K., et al., 2007. Involvement of the corticotropin-releasing factor (CRF) type 2 receptor in CRF-induced thyrotropin release by the amphibian pituitary gland. *Gen. Comp. Endocrinol.* 150, 437–444. <https://doi.org/10.1016/j.ygcen.2006.11.002>.
- Pannetier, P., Gözl, L., Pissarreira Mendes Fagundes, M.T., et al., 2023a. Development of the integrated fish endocrine disruptor test (iFEDT) – part A: merging of existing fish test guidelines. *Integr. Environ. Assess. Manage.* <https://doi.org/10.1002/ieam.4819>.
- Pannetier, P., Poulsen, R., Gözl, L., et al., 2023b. Reversibility of thyroid hormone system-disrupting effects on eye and thyroid follicle development in zebrafish (*Danio rerio*) embryos. *Environ. Toxicol. Chem.* 42 (6), 1276–1292. <https://doi.org/10.1002/etc.5608>.
- Paul, B., Sterner, Z.R., Buchholz, D.R., et al., 2022. Thyroid and corticosteroid signalling in amphibian metamorphosis. *Cells* 11, 1595. <https://doi.org/10.3390/cells11101595>.
- Peter, M.S., Peter, V.S., 2009. Action of thyroid inhibitor propyl thiouracil on thyroid and interrenal axes in the freshwater tilapia *Oreochromis mossambicus* Peters. *J. Endocrinol. Reprod.* 37–44.
- Porazzi, P., Calebiro, D., Benato, F., et al., 2009. Thyroid gland development and function in the zebrafish model. *Mol. Cell Endocrinol.* 312, 14–23. <https://doi.org/10.1006/j.mce.2009.05.011>.
- Power, D.M., Llewellyn, L., Faustino, M., et al., 2001. Thyroid hormones in growth and development of fish. *Comp. Biochem. Physiol.* 130C, 447–459. [https://doi.org/10.1016/s1532-0456\(01\)00271-x](https://doi.org/10.1016/s1532-0456(01)00271-x).
- Reider, M., Connaughton, V.P., 2015. Developmental exposure to methimazole increases anxiety behaviour in zebrafish. *Behav. Neurosci.* 129, 634–642. <https://doi.org/10.1037/bne0000087>.
- Salazar, P., Villaseca, P., Cisternas, P., Inestrosa, N.C., 2021. Neurodevelopmental impact of the offspring by thyroid hormone system-disrupting environmental chemicals during pregnancy. *Environ. Res.* 200, 111345. <https://doi.org/10.1016/j.envres.2021.111345>.
- Schmittgen, T.D., Livak, K.J., 2008. Analysing real-time PCR data by the comparative C (T) method. *Nat. Protoc.* 3, 1101–1108. <https://doi.org/10.1038/nprot.2008.73>.
- Schweibel, L.N., Stuart, K., Lowery, M.S., Wegner, N.C., 2018. Swim bladder inflation failure affects energy allocation, growth, and feed conversion of California Yellowtail (*Seriola dorsalis*) in aquaculture. *Aquaculture* 497, 117–124. <https://doi.org/10.1016/j.aquaculture.2018.07.050>.
- Spaan, K., Haigis, A.-C., Weiss, J., Legradi, J., 2019. Effects of 25 thyroid hormone disruptors on zebrafish embryos: a literature review of potential biomarkers. *Sci. Total Environ.* 656, 1238–1249. <https://doi.org/10.1016/j.scitotenv.2018.11.071>.
- Stinckens, E., Vergauwen, L., Ankley, G.T., et al., 2018. An AOP-based alternative testing strategy to predict the impact of thyroid hormone disruption on swim bladder inflation in zebrafish. *Aquat. Toxicol.* 200, 1–12. <https://doi.org/10.1016/j.aquatox.2018.04.009>.
- Stinckens, E., Vergauwen, L., Blackwell, B.R., et al., 2020. The effect of thyroperoxidase and deiodinase inhibition on anterior swim bladder inflation in the zebrafish. *Environ. Sci. Technol.* 54, 6213–6223. <https://doi.org/10.1021/acs.est.9b07204>.
- Stinckens, E., Vergauwen, L., Schroeder, A.L., et al., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part II: zebrafish. *Aquat. Toxicol.* 173, 204–217. <https://doi.org/10.1016/j.aquatox.2015.12.023>.
- Suzuki, S.C., Bleckert, A., Williams, P.R., et al., 2013. Cone photoreceptor types in zebrafish are generated by symmetric terminal divisions of dedicated precursors. *Proc. Natl. Acad. Sci. U.S.A.* 110, 15109–15114. <https://doi.org/10.1073/pnas.1303551110>.
- Trimarchi, J.M., Stadler, M.B., Cepko, C.L., 2008. Individual retinal progenitor cells display extensive heterogeneity of gene expression. *PLoS One* 3, e1588. <https://doi.org/10.1371/journal.pone.0001588>.
- Van Dingenen, I., Vergauwen, L., Haigis, A.-C., et al., 2023. Deiodinase inhibition impairs the formation of the three posterior swim bladder tissue layers during early embryonic development in zebrafish. *Aquat. Toxicol.* 261, 106632. <https://doi.org/10.1016/j.aquatox.2023.106632>.
- Walter, K.M., Miller, G.W., Chen, X., et al., 2019. Changes in thyroid hormone activity disrupt photomotor behavior of larval zebrafish. *Neurotoxicology* 74, 47–57. <https://doi.org/10.1016/j.neuro.2019.05.008>.
- Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York. <https://doi.org/10.1007/978-0-387-98141-3>.
- Woolley, L.D., Qin, J.G., 2010. Swimbladder inflation and its implication to the culture of marine finfish larvae. *Rev. Aquac.* 2, 181–190. <https://doi.org/10.1111/j.1753-5131.2010.01035.x>.
- Zheng, W., Wang, Z., Collins, J.E., et al., 2011. Comparative transcriptome analyses indicate molecular homology of zebrafish swim bladder and mammalian lung. *PLoS One* 6, e24019. <https://doi.org/10.1371/journal.pone.0024019>.