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Development of a Zebrafish Embryo-Based Test System for Thyroid Hormone System Disruption: 3Rs in Ecotoxicological Research

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Abstract: There is increasing concern regarding pollutants disrupting the vertebrate thyroid hormone (TH) system, which is crucial for development. Thus, identification of TH system–disrupting chemicals (THSDCs) is an important requirement in the Organisation for Economic Co-operation and Development (OECD) testing framework. The current OECD approach uses different model organisms for different endocrine modalities, leading to a high number of animal tests. Alternative models compatible with the 3Rs (replacement, reduction, refinement) principle are required. Zebrafish embryos, not protected by current European Union animal welfare legislation, represent a promising model. Studies show that zebrafish swim bladder inflation and eye development are affected by THSDCs, and the respective adverse outcome pathways (AOPs) have been established. The present study compared effects of four THSDCs with distinct molecular modes of action: Propylthiouracil (PTU), potassium perchlorate, iopanoic acid, and the TH triiodothyronine (T3) were tested with a protocol based on the OECD fish embryo toxicity test (FET). Effects were analyzed according to the AOP concept from molecular over morphological to behavioral levels: Analysis of thyroid- and eye-related gene expression revealed significant effects after PTU and T3 exposure. All substances caused changes in thyroid follicle morphology of a transgenic zebrafish line expressing fluorescence in thyrocytes. Impaired eye development and swimming activity were observed in all treatments, supporting the hypothesis that THSDCs cause adverse population-relevant changes. Findings thus confirm that the FET can be amended by TH system–related endpoints into an integrated protocol comprising molecular, morphological, and behavioral endpoints for environmental risk assessment of potential endocrine disruptors, which is compatible with the 3Rs principle. *Environ Toxicol Chem* 2024;00:1–18.
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INTRODUCTION

Endocrine-disrupting chemicals (EDCs) have become a major focus of ecotoxicological research and regulation in European chemical legislation (Slama & Demeneix, 2019). Numerous research projects, including the EURION cluster (https://eurion-cluster.eu/), are actively working on enhancing the identification of EDCs for their potential impact on both the environment and human health. As part of the EURION cluster, the research project Endocrine Guideline Optimization (ERGO; Holbech et al., 2020) is dedicated to assessing chemicals that disrupt the thyroid hormone (TH) system (THSDCs) and aims to improve endocrine testing strategies by extrapolating data between different vertebrate classes and by building a cross-species adverse outcome pathway (AOP) network for THSDCs (Haigis et al., 2023).

In chemical testing, Organisation for Economic Co-operation and Development (OECD) test guidelines are key for the

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standardization of testing methodologies to evaluate the safety of chemicals for the environment and human health. In the context of endocrine-disrupting effects, for example, those related to estrogen, androgen, thyroid, and steroidogenesis (collectively referred to as "EATS" modalities), several test guidelines have been developed to cover the effects of EDCs on organisms like fish and amphibians (European Chemicals Agency [ECHA] et al., 2018). However, currently existing test guidelines only address specific aspects of EDC effects in certain species, life stages, and mechanisms of action.

Although fish are *the* established vertebrate model for aquatic ecosystems and are, therefore, anchored in multiple national and international test guidelines, there are currently no established test guidelines for evaluating the impact of EDCs on the thyroid modality in fish (Dang et al., 2021). So far, in an environmental context, research into the potential disruption of the TH system is conducted in amphibians because they show the most conspicuous metamorphosis of tadpoles into adult amphibians. Consequently, in a regulatory context, it is common to conduct multiple tests to assess the potential impact of environmental EDCs reliably: Amphibians are used to detect THSDCs, while fish are used to detect disruption of the steroid hormone system.

The integration of TH system–related endpoints into existing fish test guidelines would thus significantly reduce extra amphibian testing, except for cases where it is explicitly required to protect wild amphibians. In this context, the use of nonprotected early embryonic life stages of fish (European Commission, 2010; Strähle et al., 2012; Tindall et al., 2023) seems especially relevant because the reduction in the use of protected juvenile or adult life stages, which has already been initiated for other pathways, for example, estrogen-related pathways (Christophe et al., 2019), also improves the compatibility of endocrine testing with the 3Rs (replacement, reduction, refinement) principles of Russell & Burch (1959), which has been gaining increasing importance in regulatory and scientific research (Maestri, 2021; Schiffelers et al., 2014).

To date, three OECD test guidelines (231, 241, and 248) have been established for thyroid modality testing in amphibians; however, in contrast to corresponding mammalian tests, none of these include risk assessment and extrapolation to humans (ECHA et al., 2018). In contrast, per the European Union (EU) regulation on industrial chemicals known as Registration, Evaluation, Authorization, and Restrictions of Chemicals (REACH; European Commission, 2006), fish are commonly used as the primary vertebrate group for the assessment of aquatic toxicity. In fish, most recent investigations focusing on the TH system and its potential disruption have employed zebrafish (*Danio rerio*) as a nonmammalian model (Couderq et al., 2020): Among 117 nonmammalian ecotoxicity studies conducted over the last 5 years, 81 used fish (77% zebrafish), 30 used amphibians, and six used birds (online literature search on Pubmed, Sciencedirect/Scopus, Web of Science, and ResearchGate, publications until September 2023). Zebrafish is widely used as a model species in scientific research (Laale, 1977; MacRae & Peterson, 2015; Spitsbergen & Kent, 2003) and one of the most popular model organisms in (eco)toxicology for its ease of maintenance, cost-effectiveness, rapid sexual maturation within months, and continuous production of numerous offspring (Westerfield et al., 1997).

Thus, it seems not only cost-, labor-, and resource-efficient but also logical to incorporate TH system–related endpoints into existing fish test guidelines, especially during developmental periods when THs play a critical role (Power et al., 2001). Several established fish OECD test guidelines appear suitable for the integration of TH system–sensitive endpoints: For example, test guideline 210, the Fish, Early-Life Stage Test (OECD, 2013a); test guideline 240, the Medaka One Generation Reproduction Test (OECD, 2023); or test guideline 236, the Fish Embryo Toxicity (FET) test (OECD, 2013b) could be adapted to include such endpoints. Recently, a two-generation test combining test guidelines 229 and 234 that includes TH system–related endpoints has been developed (Gölz et al., 2023; Pannetier, Poulsen, et al., 2023).

The present study explored the suitability of a modified FET protocol with zebrafish embryos for integration of TH system–relevant endpoints at different biological levels. Given that zebrafish embryos develop within a fully transparent chorion, which does not represent a major obstacle to the uptake and bioaccumulation of chemicals except for very large molecules >4000 Da (Henn & Braunbeck, 2011; Kais et al., 2013; Pelka et al., 2017), allowing continuous observation of tissues and organs also before hatching (Kimmel et al., 1995), exposure can be initiated immediately after fertilization. As holds for all vertebrates, early development of zebrafish is at least partially regulated by THs (Power et al., 2001), making it an interesting model for assessing effects by THSDCs. During early stages of zebrafish development, THs are maternally supplied and stored in the yolk sac until embryonic TH synthesis becomes active at 72 h of development (Porazzi et al., 2009). Thyroid hormones are critical in regulating fish development from fertilization throughout the embryonic, larval, and juvenile stages to adulthood (Parichy et al., 2009; Power et al., 2001). They are regulators central to multiple morphological and physiological changes during embryonic and larval development (Campinho, 2019; Evans & Fernald, 1990); in particular, THs are key players in the development of the central nervous system (Gothié et al., 2020) and sensory organs (Besson et al., 2020).

Previous research has identified multiple relevant endpoints sensitive to the effects of THSDCs in fish (Dang et al., 2021), including the expression of specific genes (Baumann et al., 2019; Reinwald et al., 2021), TH levels (Pannetier, Poulsen, et al., 2023), thyroid follicle morphology in embryos (Fetter et al., 2015; Jaka et al., 2023; Kraft et al., 2023) and older life stages (Gölz et al., 2023; Schmidt & Braunbeck, 2011), eye development (Baumann et al., 2016; Gölz et al., 2022; Pannetier, Poulsen, et al., 2023), swim bladder inflation (Stinckens et al., 2018), as well as changes in behavior (reviewed by Spaan et al., 2019). All of these endpoints have been demonstrated to be responsive to various modes of action of THSDCs and principally hold promise for use in the regulatory assessment of potential THSDCs. Consequently, AOPs (Ankley et al., 2010) linking disruption of the TH system to swim bladder inflation and eye development in zebrafish have been

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established, which can support and improve our understanding of the impact of THSDCs on crucial biological processes (see AOPs 155–159 and 363–365 in the AOPWiki: https://aopwiki.org/).

Molecular endpoints such as changes in gene expression and TH levels provide information about the endocrine activity of potential THSDCs. However, to assess the impact of THSDCs at the population level, there is a need for endpoints that inform about endocrine adversity (ECHA et al., 2018). Swim bladder inflation, eye development, and swimming behavior are such population-relevant endpoints that might play an important role in decision-making within a regulatory context.

The present study proposes an experimental protocol incorporating various parameters specific to endocrine activity and adversity in embryo-larval stages of zebrafish, following the AOP concept. Four well-characterized THSDCs representing different modes of action were used as model substances: Propylthiouracil (PTU), a pharmaceutical, acts as an inhibitor of thyroperoxidase (TPO), thereby reducing TH levels. Potassium perchlorate (PCL), an environmental pollutant, inhibits iodine uptake into thyrocytes, resulting in decreased TH levels through a different mechanism. Iopanoic acid (IOP), a commonly used radiocontrast medium, hinders the conversion of thryroxine (T4) to triiodothyronine (T3) by deiodinases, thus lowering their activity. The active TH form, T3, activates TH receptors and associated processes.

To assess the impact of the selected THSDCs at the molecular level, gene expression analyses (quantitative real-time polymerase chain reaction [qPCR]) of thyroid- and eye-related genes were performed. In addition, changes in the morphology of thyroid follicles were monitored by use of the transgenic zebrafish line Tg(*tg:MA-mCherry*)ulb1 (Opitz et al., 2012), which expresses a thyroglobulin-bound fluorescent protein in thyrocytes, a method compatible with conventional histopathological analyses typically performed in older life stages (Schmidt & Braunbeck, 2011). Moreover, eye histopathology was employed as an adverse endpoint to investigate the effects of THSDCs on eye development (Gölz et al., 2022). Finally, behavior analyses were performed to evaluate the effects of THSDC exposure on the overall fitness of the embryos, particularly their swimming activity, and as a proxy of potential developmental neurotoxicity caused by disruption of the TH system.

The present study thus introduces a novel strategy for the assessment of EDCs in fish representing the first Level 4 test guideline covering endocrine adversity, which, however, is regarded as an alternative method ("in vitro") and, thereby, contributes substantially to the 3Rs principle.

MATERIALS AND METHODS

Chemicals

The chemicals used in this proof-of-concept study were selected as model compounds with well-investigated effects on the TH system of different organisms at sublethal concentrations. Exposure concentrations do not necessarily represent environmentally relevant concentrations. Unless stated otherwise, all chemicals were purchased at the highest purity available (>98%) from Sigma-Aldrich (Deisenhofen, Germany). Stock solutions of PTU (Chemical Abstracts Service [CAS] no. 51-52-5), PCL (CAS no. 7778-74-7), IOP (CAS no. 96-83-3), and T3 (CAS no. 6893-02-3) were prepared 24 h before usage. Because of the low water solubility of T3 and IOP, dimethyl sulfoxide (DMSO) was used as a solvent. The two components were stirred in DMSO for at least 10 min at room temperature. To obtain the final concentrations, the IOP and T3 stock solutions were diluted with artificial water according to OECD test guideline 236 for each experiment with a final concentration of 0.02% DMSO.

Zebrafish husbandry and breeding

Zebrafish rearing, breeding, and exposure were conducted following the specifications outlined in OECD test guideline 236 (FET test) with slight modifications: The exposure duration was extended to 5 days postfertilization (dpf) to include eye development as an endpoint and to analyze more complex behavior. Zebrafish eggs used in the exposure experiments were obtained from different parental zebrafish lines, depending on the specific endpoints assessed. The reasons behind using different zebrafish lines are of a practical nature, depending on the availability of lines in different laboratories and some of the experiments being part of different projects. For gene expression analysis, wildtype zebrafish (*D. rerio*, Westaquarium strain) were utilized, while the transgenic line Tg (*tg:MA-mCherry*)ulb1 (Zebrafish Information Network [ZFIN] code: https://zfin.org/ZDB-ALT-130213-1; Opitz et al., 2012) was used for the analysis of thyroid follicle morphology and eye histopathology. Both zebrafish lines were maintained under standard rearing conditions (Lammer et al., 2009) at the facilities of the Aquatic Ecology and Toxicology Group, Center of Organismal Studies, University of Heidelberg (licensed by local authorities: 35-9185.64/BH Braunbeck). Behavioral analyses were performed at the MARBEC Palavas Experimental Marine Platform (licensed under D34121926) using the cyp19a1b:GFP zebrafish line (ZFIN code: http://zfin.org/ZDB-ALT-110126-5).

Exposure experiments

Freshly fertilized zebrafish eggs were collected in the morning after the onset of light and carefully assessed for quality: Only eggs of the highest quality, characterized by uniform cell division, transparent yolk, and a uniformly shaped chorion between the 4- and 64-cell stages, were used for experiments. Exposure concentrations for PTU, PCL, IOP, and T3 were determined based on range-finding tests, which helped to establish the threshold to unspecific toxicity (Table 1). Final exposure levels were set at concentrations below 10% effect concentration values to avoid any interference with malformations or even mortality (cf. Wheeler et al., 2013).

For all analyses except behavior, 24-well plates were preexposed (saturated) for 24 h with 2 ml of the respective test

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TABLE 1: Test substances with median lethal concentration values (based on toxicity testing according to Organisation for Economic Co-operation and Development Test Guideline 236) and final test concentrations used in the modified protocol for investigating the different endpoints in 5 days postfertilization zebrafish (*Danio rerio*) embryos

Substance	CAS no.	LC50	Exposure concentrations	No. of embryos per replicate
Propylthiouracil	51-52-5	635.8 mg/L	0, 100, 150, 200, 250 mg/L	▪ Histopathology: 12–30 individuals; 60–150 follicles ▪ qPCR: three replicates of 24 pooled individuals each ▪ Behavior: 24 individuals
Potassium perchlorate	77778-74-7	>1000 mg/L	0, 0.1, 0.5, 1.0, 1.5 mg/L	▪ Histopathology: 12–30 individuals; 60–150 follicles ▪ qPCR: three replicates of 24 pooled individuals each ▪ Behavior: 24 individuals
Iopanoic acid	96-83-3	3.5 mg/L	0, 0.5, 1.0, 1.5, 2.0 mg/L (in 0.02% DMSO)	▪ Histopathology: 12–30 individuals; 60–150 follicles ▪ qPCR: three replicates of 24 pooled individuals each ▪ Behavior: 24 individuals
Triiodothyronine	6893-02-3	5.6 mg/L	0, 0.65, 6.5, 65 µg/L (in 0.01% DMSO)	▪ Histopathology: 12–30 individuals; 60–150 follicles ▪ qPCR: three replicates of 24 pooled individuals each ▪ Behavior: 24 individuals

CAS = Chemical Abstracts Service; LC50 = median lethal concentration; qPCR = quantitative real-time polymerase chain reaction; DMSO = dimethyl sulfoxide.

solutions or control medium. For exposure, each well was used to hold a single egg. Plates were incubated with a 14:10-h day: night cycle at a temperature of 26.0 ± 1.0 °C. For behavioral analyses, batches of 25 fish embryos per treatment were exposed at 27.0 ± 1.0 °C in glass crystallizers containing 25 ml of prewarmed exposure solution. Exposure solutions were renewed daily, and embryos were closely monitored for any symptom of malformation or toxicity.

All experiments were carried out in triplicate on 5-dpf embryos. For each endpoint, details on specific exposure concentrations and the number of embryos used per replicate can be found in Table 1.

qPCR of thyroid- and eye-related genes in zebrafish embryos

The qPCR analysis was conducted on every THSDC treatment at the highest concentration except for PTU, where the second-highest concentration was selected because PTU had already shown strong effects in eye-related endpoints. Wild-type embryos (5 dpf) were anesthetized using an ice-cold solution of 400 mg/L buffered tricaine mesylate (MS-222). Pooled groups of 12 embryos were transferred into 2-ml Eppendorf tubes, and the exposure solutions were removed. Subsequently, the reaction tubes were rapidly frozen in liquid nitrogen and then stored at −80 °C until further analysis. For RNA isolation, TRI Reagent® RNA Isolation Reagent (Sigma Aldrich; product no. T9424) was used, following the manufacturer's protocol. Concentration and purity of the isolated RNA were assessed using a Nanodrop NanoVue 4282 spectrophotometer (GE Healthcare, Chicago, IL). The isolated RNA was processed further to synthesize complementary DNA (cDNA) using ReadyScript cDNA synthesis mix (Sigma-Aldrich; product no. RDRT). The qPCR was performed on an AB Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Darmstadt, Germany), utilizing the StepOne® real-time PCR system (ThermoFisher Life Technologies, Darmstadt, Germany). The qPCRs were conducted using Luna Universal qPCR Master Mix (New England BioLabs, Ipswich, MA) following the manufacturer's protocol, with the respective primers (for details see Supporting Information, Table S1) and the cDNA. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, as described by Schmittgen and Livak (2008). The 18 S ribosomal RNA gene was employed as a reference gene because its expression remained stable across treatments, as determined by $\log_2 2^{-CT}$. The normalized expression of the target genes, $2^{-\Delta\Delta CT}$, was then compared across the different treatments.

Anatomy and morphology of thyroid follicles

For morphological analyses, 5-dpf embryos of the thyroid transgenic zebrafish Tg(*tg:MA-mCherry*)ulb1 line were used as previously described by Kraft et al. (2023). Embryos were sedated using a 0.016% MS-222 solution and placed on glass microscopy slides covered with 3% methylcellulose for in vivo imaging of the thyroid follicles on an inverted epifluorescence microscope equipped with a camera (Nikon Eclipse Ti-S and DS-Fi3; Nikon, Düsseldorf, Germany) using a ×20 magnification lens and the software NIS-Elements (Ver. 4.60). The head region of each embryo was first put in focus in brightfield for orientation, and thyroid follicles were imaged using the

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tetramethylrhodamine-isothiocyanate-epifluorescence filters. Two Z-stacks consisting of nine images with 2.5-µm spacing were captured: one using brightfield, one using the epifluorescence filters.

In total, 15 embryos per treatment were analyzed. Analysis of the Z-stacks was automated using a custom-made macro using the software FIJI (Schindelin et al., 2012; for macro code, see Supporting Information, Macro S2). During the analysis, images were converted to gray scale, and the overall fluorescence (the product of follicle area by their mean gray value) of the thyroid follicles was calculated (for details, see Supporting Information, Figure S1). Following imaging, embryos were returned to artificial water to remove remnants of methylcellulose and euthanized by an overdose of ice-cold MS-222.

Eye histopathology and morphometry of retinal layers

After live imaging of the thyroid follicles, 5-dpf zebrafish embryos (Tg(*tg:MA-mCherry*)ulb1 line) were euthanized and fixed overnight for histological procedures in cold modified Davidson's fixative (Braunbeck et al., 2010). Prior to standard processing for histopathological analyses, groups of 12 embryos were embedded in agarose blocks following the method described by Sabaliauskas et al. (2006) and modified by Kraft et al. (2023). The agarose blocks underwent standard dehydration and paraffin embedding for 48 h using an automated tissue processor (TP1020; Leica, Nussloch, Germany). The paraffin-embedded blocks were sectioned at 4 to 5 µm using a rotary microtome (Microm HM 355 S; ThermoFisher, Wiesbaden, Germany). The sections were mounted on adhesion slides (SuperFrost® Plus; Menzel, Thermo-Fischer) and stained with hematoxylin and eosin G using an automated stainer (Cellstain®15; Tharmac, Wiesbaden, Germany).

Histopathological analyses of the eyes were conducted following Kraft et al. (2023) as well as Pannetier, Poulsen, et al. (2023): Coronal sections at the level of the optic nerve (Figure 1) were used to record histopathological alterations in the eye and to measure the diameter of the eye and the thickness of the retinal pigment epithelium (RPE), the photoreceptor layer (PRL), and the inner plexiform layer (IPL) by means of Fiji software at eight different locations per layer (Figure 1). Especially in embryos exposed to the highest concentrations of IOP, the IPL displayed conspicuous deformations such as gaps and "dents" (Supporting Information, Figure S7). To characterize such malformations, the severity of deformations and dents was graded on a scale ranging from 1 (control) to 5 (severe change); the mean severity grade of a treatment was calculated across the three replicates.

In addition, a semiquantitative analysis was performed to evaluate the pigmentation intensity and detachment of the RPE on a scale from 1 to 4, with 1 representing almost no detachment and normal pigmentation, while 4 indicated significant detachment and conspicuously low pigmentation.

Analysis of behavior in zebrafish exposed to THSDCs

The swimming activity of 5-dpf zebrafish (cyp19a1b:GFP line) embryos was evaluated in the photomotor response assay using the DanioVision™ system (Noldus, Wageningen, The Netherlands) between 1:00 and 5:00 p.m. (MacPhail et al., 2009). At least 2 h before testing, zebrafish embryos were carefully transferred from the glass crystallizers into 24-well plates (TPP, Trasadingen, Switzerland; one larva per well) and kept for acclimation in an illuminated incubator at 28 °C.

For analysis of swimming behavior, the well plates with the embryos were transferred to the DanioVision system and acclimated to darkness for 10 min. Then, swimming activity was recorded during a first 5-min period of light (LON1), then during 5 min of darkness (LOFF) and again during another period of light (LON2). The swimming tracks of the embryos

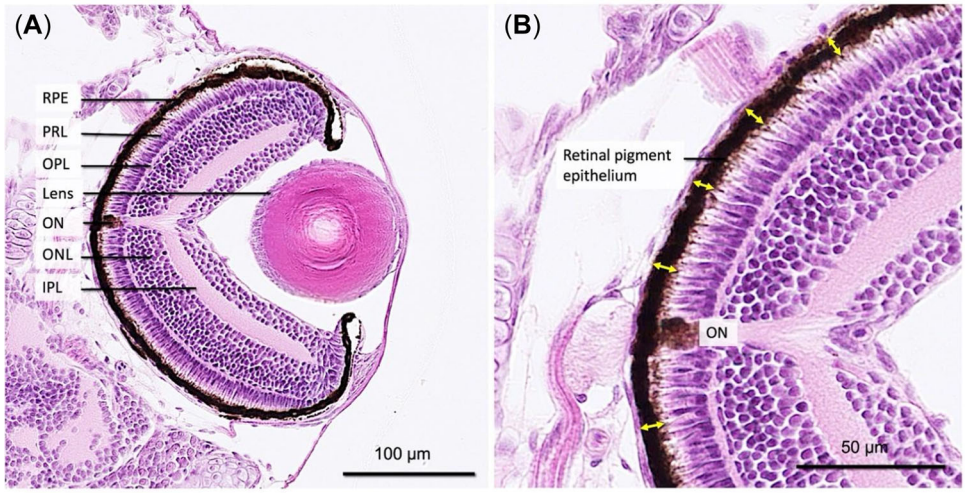

FIGURE 1: Histological sections of the eye of a 5–days postfertilization control zebrafish (*Danio rerio*) embryo stained with hematoxylin and eosin reveal a clear stratification of the retina (**A**). For morphometric analyses, five out of eight measurements of the RPE evenly distributed over the retina were used (**B**, yellow arrows). RPE = retinal pigment epithelium; PRL = photoreceptor layer; OPL = outer plexiform layer; ON = optic nerve; ONL = outer nuclear layer; IPL = inner plexiform layer.

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were automatically recorded and analyzed using the Noldus Ethovision™ computer software. The locomotion data of interest (distance traveled [centimeters], meandering of path [degrees per centimeter], and time spent in the well center [seconds]) were automatically extracted and used for statistical analyses. At the end of the experiment, larvae were euthanized in a saturated solution of benzocaine (CAS no. 94-09-7; Merck).

Statistical analyses

Statistical data analysis was conducted using Prism 9 (Ver. 9.1.2 (226), 2021; GraphPad Software; Statcon, Witzenhausen, Germany) for qPCR, eye histology, and thyroid follicle data. Behavioral data were analyzed using R (Ver. 4.2.2; R Core Team, 2021).

For the analysis of qPCR data, data normal distribution was ensured, and an unpaired *t* test was performed to compare exposed embryos to the corresponding controls.

For the analysis of eye histopathology, data from the three independent replicates were pooled if there were no statistically significant differences between the negative controls. Before the individual experiments for IOP and T3 were pooled, the negative and solvent controls were compared with a *t* test. If no statistically significant differences were found, these groups were pooled, resulting in a single control per substance. Data distribution was checked using the D'Agostino-Pearson test. Potential statistical outliers were identified and removed using the ROUT method (robust regression and outlier removal; Motulsky & Brown, 2006) with Q = 1%. When comparing control and exposure groups, a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used. If a data set contained data which did not follow a Gaussian distribution, a Kruskal-Wallis test followed by Dunn's multiple comparisons test was used instead.

During the analysis of the thyroid follicles, data for the fluorescence of the thyroid follicle analysis were found to follow a log-normal distribution and were, therefore, log-transformed (Base 2) before further analyses were performed. Statistical outliers were identified, followed by a normalization against the respective control group for each substance, resulting in relative fluorescence values. One-way ANOVA followed by Dunnett's multiple comparisons test or a Kruskal-Wallis test followed by Dunn's multiple comparisons test was used depending on the normality of data distribution.

Data from behavior analyses were analyzed using Kruskal-Wallis tests followed by Dunn's multiple comparisons for each of the three light periods (LON1, LOFF, LON2).

To avoid giving biased weight to one variable compared to another one, data summarized in Table 2 were used as "+1" for an increase of one variable compared to its respective control, "–1" for a decrease, and "–" otherwise. Then Euclidean distances and tree topology were obtained using the joining clustering method with complete linkage (Statistica Ver. 13, 2015; Tibco, Palo Alto, CA).

For all experiments, results were deemed statistically significant for (adjusted) $p < 0.05$.

RESULTS

Molecular changes in genes associated with eye and thyroid functions in zebrafish embryos

Expression patterns of genes associated with eye and thyroid functions were altered after treatment with all THSDCs in wild-type zebrafish embryos. Particularly strong (and statistically significant) effects were observed following exposure to PTU and T3 (Figure 2).

Exposure to 200 mg/L PTU lowered the relative expression of eye-related genes after 5 d of exposure. Most prominently, PTU exposure reduced phosphodiesterase (*pde6h*) and retinal pigment epithelium (*rpe65a*) gene expression levels to ~25% (Figure 2). Expression of thyroid receptors alpha and beta (*trα*, *trß*) and deiodinase type 3 (*dio3*) was significantly reduced, while expression of *dio2* increased on exposure to PTU.

Embryos treated with T3 showed a trend for up-regulation of nearly all eye- and thyroid-related gene transcripts. Statistically significant changes were observed for the thyroid-related genes *dio2* and thyroid-stimulating hormone (*tsh*) and the eye-related gene *pde6a*.

In contrast, exposure of zebrafish embryos to 1.5 mg/L PCL or 1.5 mg/L IOP for 5 days did not produce any statistically significant changes in the expression of thyroid- or eye-related genes. However, a slight trend of overexpression was detectable for the *dio2* ($p = 0.2$) and *dio3* ($p = 0.3$) genes following PCL exposure.

Thyroid follicle analysis

Following exposure to PTU, analysis of fluorescent images of the thyroid follicles in 5-dpf zebrafish embryos (Tg(*tg:MA-mCherry*)ulb1 line) revealed a consistent increase in both their quantity and size, indicating substantial developmental changes along the dorsoventral and anteroposterior axes (Figure 3). This effect could be quantified as a relative change in fluorescence, which demonstrated a statistically significant increase across all tested PTU concentrations (Figure 4).

Exposure to increasing concentrations of PCL induced a trend toward an increase in thyroid follicle fluorescence with a statistically significant increase at the highest concentration of 1.5 mg/L PCL. In contrast, IOP exposure produced only minor changes except for a transient yet significant decrease at the lowest exposure concentration (Figure 4).

The hormone T3 significantly decreased thyroid follicle fluorescence at 6.5 µg/L, whereas the slight decrease observed at 65 µg/L was statistically not significant.

Histopathological analysis of retinal layers

After 5-day exposure of (Tg(*tg:MA-mCherry*)ulb1 line) zebrafish embryos, PTU caused multiple effects in the architecture of the retina (Figure 5; for summary, see Table 2). The most prominent alterations were observed in the RPE: Following exposure to 200 and 250 mg/L PTU, the thickness of the RPE declined from 3.6 µm to 2.8 and 2.3 µm, respectively

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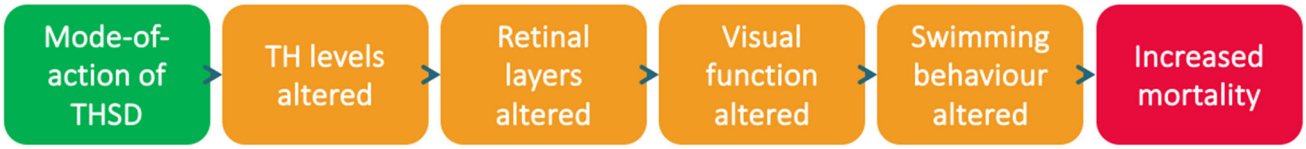
TABLE 2: Summary of effects observed in the modified fish embryo toxicity tests (Organisation for Economic Co-operation and Development Test Guideline 236) for zebrafish (*Danio rerio*) embryos exposed to propylthiouracil, potassium perchlorate, iopanoic acid, and triiodothyronine at concentrations below the 10% lethal concentration



	qPCR of genes related to		Thyroid follicles	Histological analysis of retinal layers			Behavioral analysis		
	Thyroid	Eye	Integrated density	RPE	PRL	IPL	Distance	Meander	Thigmotaxis
PTU	↓	↓	↑	↓	–	–	↓	↓	↓↑
PCL	–	–	↑	↓	↑	↑	–	–	↓
IOP	–	–	↓	↓	↓	↑	↓	↓	↑
T3	↑	↑	↓	↓	↓	–	↓	↑	↓

The test design followed the adverse outcome pathway concept from molecular events to adverse outcomes at higher levels and covered the following modes of action: thyroperoxidase inhibition (PTU), competitive inhibition of iodine uptake (PCL), inhibition of deiodinases (IOP), and supplementation of thyroid hormone (T3). Arrows indicate a significant increase or decrease of the analyzed endpoint in any exposure concentration relative to controls;–indicates no effect. Hierarchical clustering (right end of table) by joining clustering method integrates effects and gathers PTU and PCL into one group and IOP and T3 into another one.
qPCR = quantitative real-time polymerase chain reaction; RPE = retinal pigment epithelium; PRL = photoreceptor layer; IPL = inner plexiform layer; PTU = propylthiouracil; PCL = potassium perchlorate; IOP = iopanoic acid; T3 = triiodothyronine.

(Supporting Information, Figure S2). Likewise, a significant loss of pigmentation was evident after exposure to ≥200 mg/L PTU, which was accompanied by a strong trend toward detachment of the RPE at 250 mg/L. In contrast, histopathological analysis did not reveal any significant decrease in the thickness of either the PRL or the IPL.

Exposure to ≥0.1 mg/L PCL induced significant effects in all of the retinal layers investigated (for summary, see Table 2):

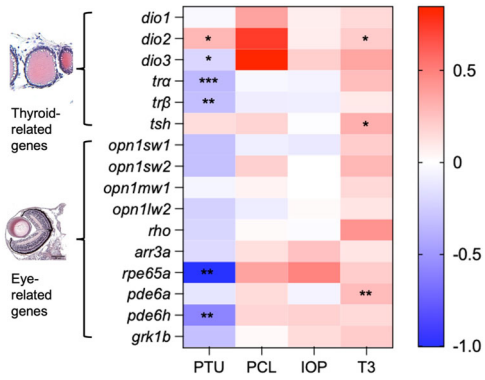


FIGURE 2: Heat map of log2-transformed fold-changes of gene expression in 5–days postfertilization zebrafish (*Danio rerio*) embryos exposed to 200 mg/L propylthiouracil, 2.0 mg/L perchlorate, 2.0 mg/L iopanoic acid (0.02% dimethyl sulfoxide [DMSO]) or 65 µg/L triiodothyronine (0.01% DMSO). Data are given for $n = 3$ replicates of 24 embryos each ($N = 72$). Statistically significant differences from controls: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$ (unpaired *t* test). Thyroid-related genes: *dio1–3* = deiodinase types 1–3; *trα/trβ* = thyroid receptors alpha/beta; *tsh* = thyroid-stimulating hormone. Eye-related genes: *opn1sw1/2* = opsin 1 short-wave-sensitive 1/2; *opn1mw1* = opsin 1 medium-wave-sensitive 1; *opn1lw2* = opsin 1 long-wave-sensitive 2; *rho* = rhodopsin; *arr3a* = arrestin 3a; *rpe65a* = retinal pigment epithelium-specific 65-kDa; *pde6a/6h* = protein phosphodiesterase 6a/6h; *grk1b* = G protein–coupled receptor kinase 1b. PTU = propylthiouracil; PCL = perchlorate; IOP = iopanoic acid; T3 = triiodothyronine.

The thickness of the RPE was reduced in embryos exposed to concentrations from 0.1 to 1.5 mg/L PCL, whereas the thickness of the PRL and the IPL was increased at 1.5 mg/L and 1.0 as well as 1.5 mg/L PCL, respectively (Supporting Information, Figure S3). No change was seen for pigmentation.

Exposure to IOP at concentrations of 0.5, 1.0, 1.5, and 2.0 mg/L had an impact on various retinal layers (Table 2; Supporting Information, Figure S4): The height of both the RPE and the IPL was significantly reduced in embryos exposed to concentrations ranging from 0.5 to 2.0 mg/L and 2 mg/L IOP, respectively. Because the thickness of the PRL showed particularly high variance, the inner segment of this layer was measured to calculate the ratio to the PRL: In fact, even though there was no decrease in the overall PRL thickness, the relative size of the inner segment of the photoreceptors decreased significantly (Supporting Information, Figure S4). The semiquantitative severity grading of the malformations revealed structural defects of the IPL (Figure 6): After exposure to 1.5 and 2.0 mg/L IOP, severe malformations such as dents and gaps could be seen, leading to a mean severity index of >1.5 (Figure 6). Again, no effect on relative pigmentation was observed.

Finally, exposure to T3 induced a significant decrease of RPE and PRL thickness in 5-dpf zebrafish embryos (Supporting Information, Figure S5). In contrast, no effects were seen in the IPL. Exposure to T3 did not induce any effect on relative pigmentation.

Behavioral analysis

At 100 and 200 mg/L, PTU induced a significant decrease in distance traveled by the embryos (cyp19a1b:gfp line) during LON2 and LON1/LON2, respectively (Figure 7); the same trend could be revealed for LOFF for these two concentrations and

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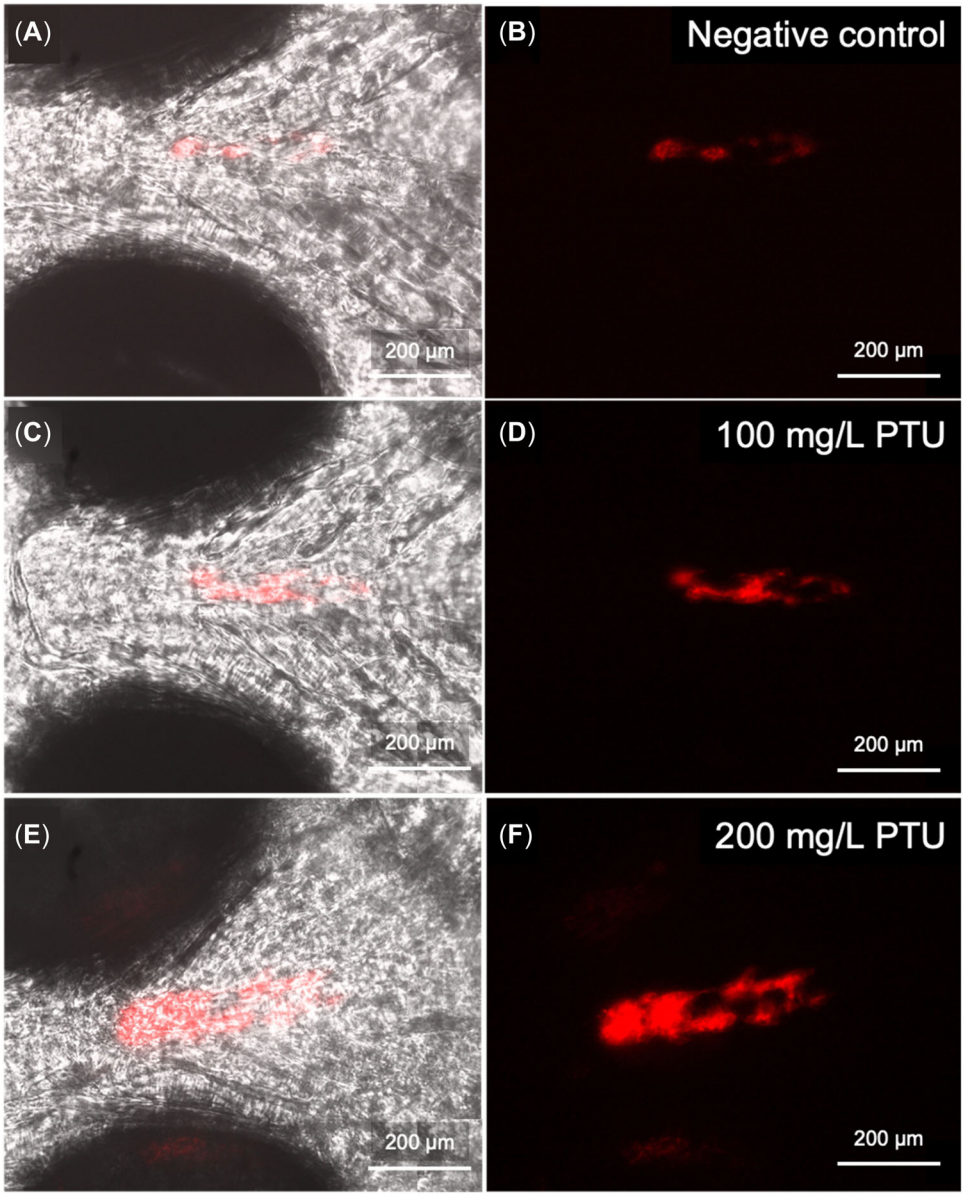

FIGURE 3: Head region of 5–days postfertilization transgenic (Tg(*tg:MA-mCherry*)ulb1) control zebrafish (*Danio rerio*) embryos (**A**, **B**) as well as embryos exposed to 100 mg/L (**C**, **D**) or 200 mg/L propylthiouracil (PTU; **E**, **F**). Left column: Overlays of brightfield and tetramethylrhodamine-isothiocyanate (TRITC) channels identifying the position of thyroid follicles in the embryo head region. Right column: Red color representation of the gray-scale TRITC channel. Both size and intensity of the fluorescence signal clearly increased following PTU treatment, if compared to negative controls.

for 50 mg/L during LON periods. Path sinuosity was affected in a light- and concentration-dependent manner: 200 mg/L PTU did not affect path meandering during LON, although it induced a strong increase (+150%) in meandering during LOFF ($p = 0.086$). In contrast, 50 mg/L ($p = 0.060$–0.090) and 100 mg/L PTU ($p \leq 0.001$) led to a decrease in path sinuosity during both LON periods (Figure 7). Thigmotaxis showed a significant increase after exposure to 100 (LON) and 200 (LON, LOFF) mg/L PTU, whereas 50 mg/L led to a significant decrease during LOFF (Supporting Information, Figure S6).

Effects following exposure to 1 and 1.5 mg/L PCL were restricted to an increase in thigmotaxis during LOFF (Supporting Information, Figure S6).

Exposure to IOP led to a pronounced decrease in distance traveled during LON1 (1 and 1.5 mg/L), LOFF (0.5 and 1.5 mg/L), and LON2 (1 and 1.5 mg/L; Figure 7), which was accompanied by a significant decrease in path sinuosity at 1 and 1.5 mg/L IOP. Thigmotactic behavior was significantly reduced following exposure to 0.5 mg/L (LON1, LOFF) and 1 mg/L (LOFF; Supporting Information, Figure S6).

Exposure of zebrafish embryos to 65 µg/L T3 produced a significant decrease in distance traveled and an increase in path sinuosity during LOFF (Figure 7), associated with an increase in thigmotactic behavior restricted to light periods (significant in LON1; Supporting Information, Figure S6). An increase in path sinuosity was also observed after exposure to

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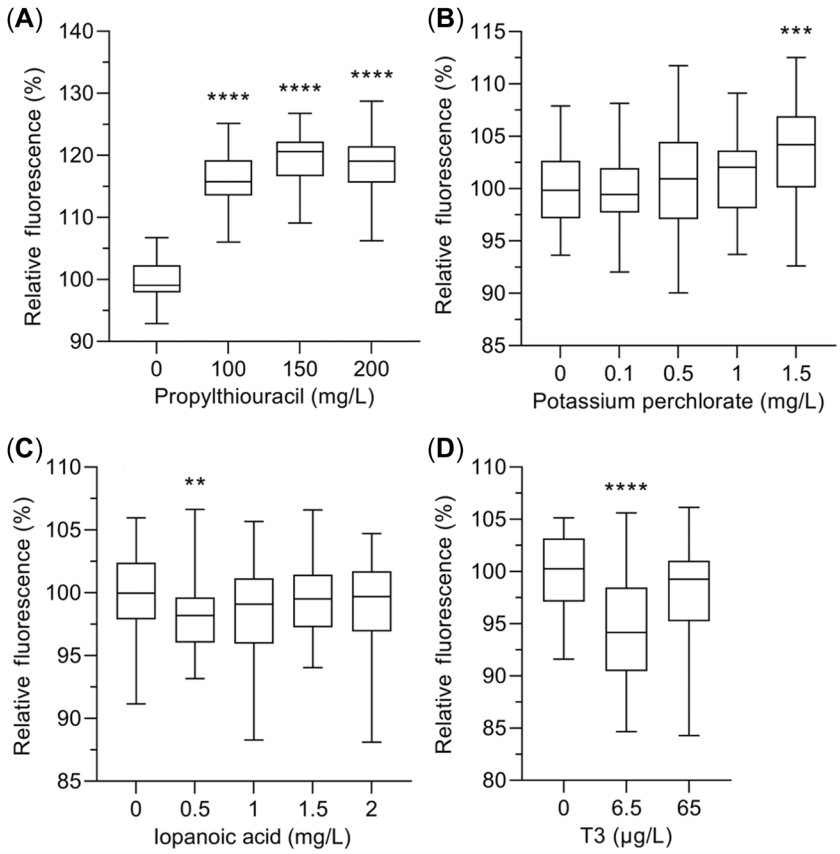

FIGURE 4: Relative fluorescence (log-fold-change) of thyroid follicle fluorescence in 5–days postfertilization transgenic (Tg(*tg:MA-mCherry*)ulb1) zebrafish (*Danio rerio*) embryos following exposure to propylthiouracil (**A**), perchlorate (**B**), iopanoic acid (**C**), or triiodothyronine (**D**) compared to control. Data are given for $n = 3$ replicates of 15 embryos each. Whiskers indicate minimum and maximum values. Statistically significant differences from controls (0): $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$ (one-way analysis of variance, Dunn's multiple comparisons test). T3 = triiodothyronine.

6.5 µg/L T3 during both LON and LOFF periods (Figure 7), and a decrease in thigmotaxis was evident after exposure to 0.65 µg/L T3 during LOFF (Supporting Information, Figure S6).

DISCUSSION

The present study clearly documents the suitability of zebrafish embryos for the assessment of various TH system–relevant endpoints in a regulatory context by using a modified FET protocol according to OECD test guideline 236. For all of the four THSDCs selected for different modes of action, effects relevant in an AOP context could be recorded from the molecular, over the morphological, to the potentially population-relevant behavioral level (for summary, see Table 2). In fact, all endpoints were significantly affected by at least one of the THSDC treatments. The study thus indicates that the approach to implement novel thyroid-related endpoints into a FET-based exposure scenario, which is compatible with the 3Rs approach, might be an interesting component of a future OECD testing framework for THSD assessment in fish. Based on an extended data set on effects for additional THSDCs in a fingerprint-like fashion, hierarchical clustering might help to group THSDCs according to their modes of action by comparing with reference molecules. In the present study, hierarchical clustering resulted in grouping of PTU and PCL into one group and IOP and T3 in another one, meaning that they show similar effects. In a regulatory context, this could be useful to propose modes of action for unknown compounds.

Expression of eye- and thyroid-related genes

Based on existing knowledge about the impact of PTU, PCL, IOP, and T3 on the TH system (Jaka et al., 2023; Opitz et al., 2009; Schmidt et al., 2017) and eye development (Baumann et al., 2016, 2019; Bhumika et al., 2015; Gölz et al., 2022; Havis et al., 2006), changes in the expression of TH system–related genes as well as eye-related genes were selected as a potential source of molecular endpoints, which might support the mechanistic interpretation of effects by the different THSDCs. The results demonstrate that PTU lowered the expression of at least three eye-related genes after 5 days of exposure and thus confirm conclusions from a previous complete transcriptomic analysis, which described a >90% downregulation of phototransduction- and eye-related pathways (Baumann et al., 2019). The downregulation of *pde6h* seems especially relevant because *pde6h* and *pde6a* are known regulators of visual signal transduction (Cote, 2004). The decreased expression of *rpe65a*, which is important for cone and rod chromophore synthesis (Kiser, 2022), further corresponds to the morphological observation of a reduced

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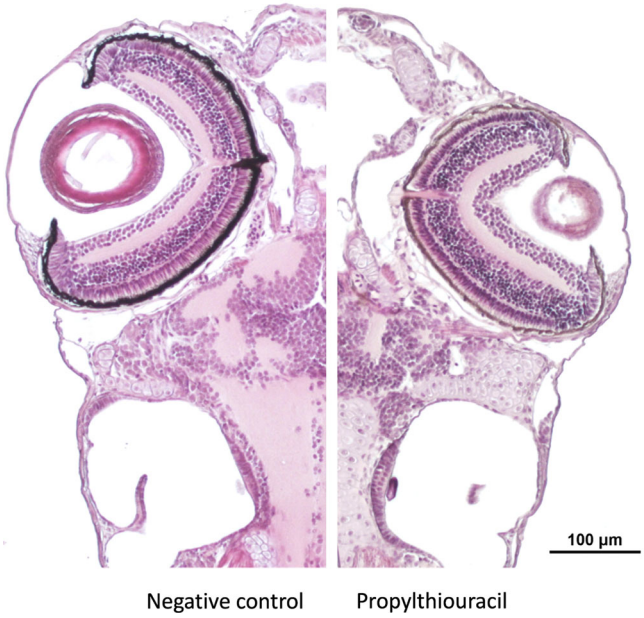

FIGURE 5: Effects of 200 mg/L propylthiouracil treatment on eye development in a 5–days postfertilization zebrafish (*Danio rerio*) embryo. Histopathological sections of 2.5 µm thickness stained with hematoxylin and eosin. Loss of pigmentation of the retinal pigment epithelium (RPE) in combination with a reduction of the thickness of the RPE can be seen in the right histological section as a typical effect of propylthiouracil on the retinal structure.

RPE cell height. The findings thus support the hypothesis that—through TPO inhibition—TH system disruption leads to a downregulation of various eye-related pathways, as postulated in the recently published AOP (Gölz et al., 2022).

In contrast to PTU, PCL and IOP failed to induce significant changes in the expression of thyroid- or eye-related genes in zebrafish embryos. Only a minor trend toward an upregulation of *dio2* and *dio3* after PCL exposure could be observed. This lack of response could be due to the low number of samples ($n = 3$), resulting in low statistical power of the test. In fact, this observation would be consistent with the assumption that PCL competitively inhibits iodide uptake by the sodium/iodide symporter in the thyroid follicles, thereby lowering the synthesis of THs (Schmidt et al., 2012). This could lead to a compensatory upregulation of DIO enzyme expression. Compared to PTU, IOP is a relatively weak TPO inhibitor, but it still inhibits all three *dio* genes (Paul et al., 2014; Renko et al., 2012; Stinckens et al., 2016). The most important consequence is thought to be suppression of the conversion of T4 to T3 by *dio1* and *dio2*, which is consistent with the observation that IOP exposure lowered T3 levels but did not affect T4 levels in juvenile (32-day) zebrafish exposed to 1 mg/L IOP (Stinckens et al., 2020). As holds true for PCL, there remains a fundamental knowledge gap on IOP-related effects on eye-related genes.

Embryos treated with T3 showed a clear trend towards upregulation of almost all eye- and thyroid-related gene transcripts, which was statistically significant for the thyroid-related genes *dio2* and *tsh* and the eye-related gene *pde6a*. This upregulation was likely a compensatory reaction to lowered T4 levels caused by T3 supplementation, as shown by Wang et al. (2013). Given the high standard deviation of expression data, however, some uncertainties remain; and more research is needed to confirm the upregulation of thyroid- and eye-related genes. At least for *pde6a*, the present study confirms an impact of T3 exposure on eye-related genes.

Yet, although the genes selected did show some changes in expression after treatment with the different THSDCs, we do not recommend the inclusion of qPCR into a modified test guideline 236 protocol because a very high number of embryos (i.e., animals) is needed to arrive at a satisfactory statistical robustness. Whereas thyroid follicles, eye histopathology, and behavior can be assessed in the same individuals, qPCR analyses require additional animals, which is not compatible with the 3Rs principle. In fact, the set of genes selected represents only a snapshot of the complex TH and eye systems, and we cannot exclude that effects would be stronger for other genes. Given, however, that at least one mechanistic (molecular) endpoint should be included into the modified test guideline 236 protocol to confirm specificity for the TH system, the measurement of TH levels appears more promising, even though this also requires additional animals (Pannetier, Poulsen, et al., 2023). Previous results indicate that liquid chromatography coupled with tandem mass spectrometry would be the preferred method to precisely analyze low concentrations of THs in zebrafish embryos (Gölz et al., 2023; Pannetier, Poulsen, et al., 2023).

Thyroid follicle analysis in transgenic zebrafish embryos

There are different methods for the morphometric analysis of thyroid follicles (Grim et al., 2009; Mohorea et al., 2023; Opitz et al., 2006, 2009). Given that direct measurement on histological sections is a very time-consuming procedure, attempts have been made to quantitatively analyze the fluorescence intensity in whole mounts of transgenic zebrafish expressing thyroid-related signals (Kraft et al., 2023; Opitz et al., 2012; Pannetier, Gölz, et al., 2023). For the present study, different methods for analysis of the transgenic fluorescence signal were compared, and an optimized macro (Supporting Information, Macro S2) was developed on the basis of the relative fluorescence values, which, if compared to our previous method (Kraft et al., 2023), may be less sensitive but is superior in terms of standardization and reproducibility. Yet, for optimization of statistical robustness of future experiments, a slight increase of the number of embryos is recommended to facilitate the identification of even more subtle effects on thyroid follicle morphology and activity.

In at least one exposure concentration, significant changes of thyroid size and/or activity could be documented for all compounds tested, confirming an impact on the TH system and corroborating the conclusion by Grim et al. (2009) that direct morphological analysis of the thyroid follicular epithelium is probably the most reasonable and straightforward approach for the detection of specific THSDC effects. A considerable number

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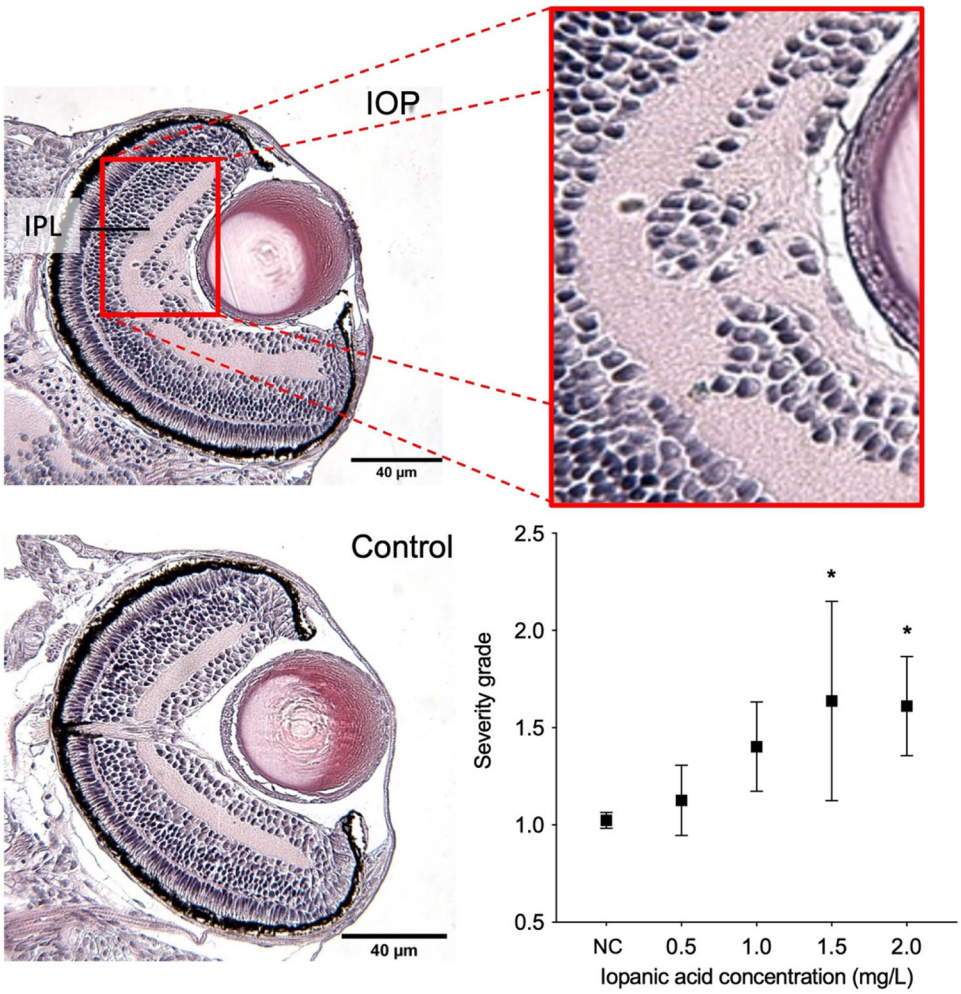

FIGURE 6: Malformations of the inner plexiform layer after exposure of 5–days postfertilization zebrafish (*Danio rerio*) embryos to iopanoic acid (IOP). A severity grading shows a significant increase in the number of malformations (gaps and dents) at concentrations of 1.5 and 2.0 mg/L IOP. Histopathological sections of 2.5 µm thickness stained with hematoxylin and eosin. Statistically significant deviation from negative controls: $*p < 0.05$ (one-way analysis of variance, Dunn's multiple comparisons test). IPL = inner plexiform layer; NC = negative control.

of studies have documented histopathology of thyroid follicles to be a sensitive method to reveal effects not only in different developmental stages of THSDC-exposed fish (Gölz et al., 2023; Pinto et al., 2013; Schmidt & Braunbeck, 2011; Sharma et al., 2016; Sharma & Patiño, 2013; Van Der Ven et al., 2006) but also in amphibians (Dang, 2019). The use of immunostaining or transgenic lines (Jaka et al., 2023; Kraft et al., 2023; Raldúa & Babin, 2009; Rehberger et al., 2018) also proved a highly effective method to profit from the advantages of such morphological analyses.

Propylthiouracil inhibits the enzyme TPO and, consequently, the iodination of thyroglobulin, resulting in impaired synthesis of THs (Elsalini & Rohr, 2003). Thus, thyroid follicles proliferate to compensate for lowered TH levels (present study; Gölz et al., 2023; Jaka et al., 2023). Schmidt and Braunbeck (2011) made similar findings after histological analyses of thyroid follicles in juvenile zebrafish exposed to PTU. Similar effects could be observed after PCL exposure, which significantly increased size and fluorescent intensity of thyroid follicles (Jaka et al., 2023), reflecting the expected compensatory reaction to sodium/iodide symporter (NIS) inhibition.

Iopanoic acid interferes with deiodinases, which convert inactive T4 to active T3 by deiodination in peripheral tissues (primarily the liver). There is only one study on the effects of IOP on zebrafish: Exposure of up to 32-h-old zebrafish embryos to IOP in a fish early life–stage test (OECD test guideline 210) resulted in decreased whole-body T3, which is in line with observations in the present study that IOP induces a decrease of size and fluorescence intensity of the thyroid follicles. Likewise, fathead minnow (*Pimephales promelas*) embryos exposed to IOP from Day 6 to Day 21 showed a decrease in whole-body T3 concentrations and an increase in whole-body T4 concentrations (Cavallin et al., 2017).

Exposure to T3 resulted in the expected decrease in thyroid follicle size and fluorescence intensity due to decreased TH synthesis following excess exogenous TH administration (feedback mechanism; Trubiroha et al., 2018). Thyroid atrophy as the morphological counterpart of decreased TH synthesis has also been reported in *Xenopus* tadpoles exposed to T4 in the amphibian metamorphosis assay (AMA; Coady et al., 2010).

The present study thus underlines the suitability of the transgenic zebrafish line Tg(*tg:MA-mCherry*)ulb1 as a

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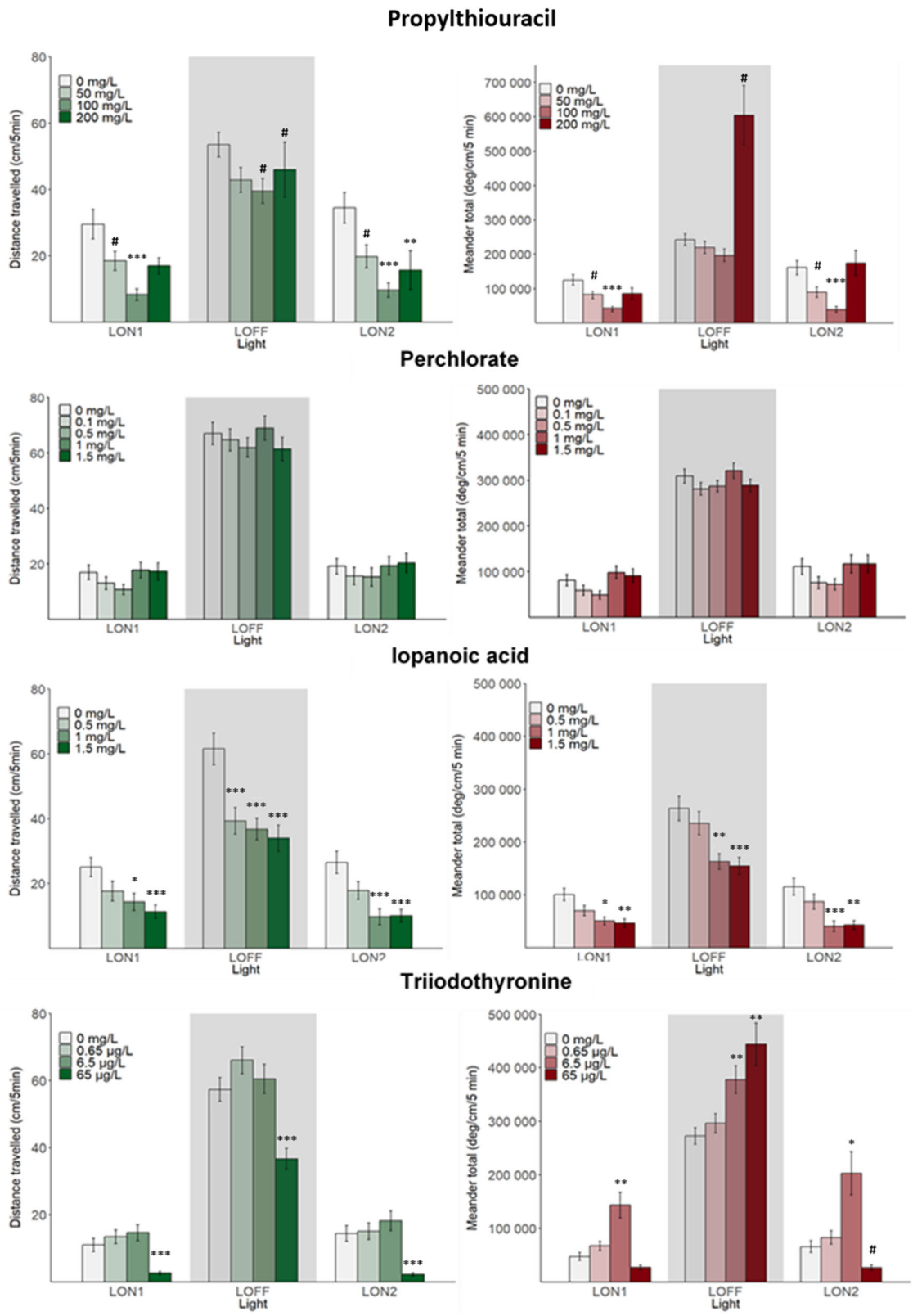

FIGURE 7: Modification of distance traveled (left) and meandering (right) in 5–days postfertilization zebrafish (*Danio rerio*) embryos after exposure to propylthiouracil, perchlorate, iopanoic acid, and triiodothyronine with lights on and lights off. Statistically significant differences from controls: $^{\#}p \leq 0.1$; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ (Kruskal-Wallis test, Dunn's multiple comparisons test). LON = lights on; LOFF = lights off.

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promising tool for the screening of THSDCs with various modes of action (Jaka et al., 2023; Pannetier, Gölz, et al., 2023). Alterations in thyroid follicular morphology can, therefore, be recommended as an endpoint for endocrine activity in prospective AOPs for TH system disruption in fish based on OECD test guideline 236. Again, measurement of TH levels is recommended as an important mechanistic complement to morphological endpoints to strengthen the key event relationships in the AOP-based approach.

Eye histopathology in zebrafish embryos

Histopathological analyses range among the most reliable, sensitive, and comprehensive endpoints for the qualitative and quantitative determination of morphological changes and play an important role in bridging subcellular (e.g., molecular) and apical (i.e., population-relevant) endpoints (Wolf et al., 2015). In fact, all THSDCs tested produced changes in the organization of the retina of zebrafish embryos, including cell size, shape, height and structure, organization, and height of photoreceptors and pigmentation (Allison et al., 2006; Baumann et al., 2016; Gamborino et al., 2001; Houbrechts et al., 2016; Kraft et al., 2023; Pannetier, Poulsen, et al., 2023; Vancamp et al., 2019; Viets et al., 2016). The present study confirmed RPE cell height to be the most sensitive and easy-to-assess endpoint for the demonstration of disruption of eye development in zebrafish embryos; however, given the continuous gradient from control to pathological conditions, quantification of the structural changes in retinal layers appears indispensable.

Propylthiouracil-induced changes in RPE height and pigmentation intensity as well as the structure of IPL, PRL, and RPE basically confirm observations in our previous studies (Baumann et al., 2016; Pannetier, Poulsen, et al., 2023). Similar changes are characteristic of IOP and PCL exposure, although the present study is the first to report on PCL-specific alterations of the RPE. Despite different modes of action, both IOP and PCL thus have adverse effects on retinal development, possibly via restricted availability of T3. In addition to quantitative changes, IOP exposure produced conspicuous, specific pathological alterations of the IPL in the form of dents and gaps, which may suggest deficits in signal transmission of visual signals.

In contrast, although external administration of T3 only affected the structure of the PRL, not the development of IPL or RPE, the observations unequivocally demonstrate that T3 exposure has an impact on phototransduction pathways and eye development. In fact, previous studies described comparable effects of T3 and concluded that THs are regulators of cone development in the retina (Fischer et al., 2012; Gamborino et al., 2001).

In summary, the findings on eye development and structure demonstrate that the retinal layers, namely RPE, PRL, and IPL, provide an important source of valuable and sensitive endpoints for the detection of adverse THSDC-induced effects when assessed collectively and in combination with mechanistic thyroid-sensitive endpoints. The relationship of these endpoints has recently been described in an AOP for TPO inhibition (Gölz et al., 2023; https://aopwiki.org/aops/363). Based on molecular and morphological observations, exposure to all THSDCs tested clearly affected crucial components of the visual system. Therefore, PTU, PCL, IOP, and T3 can be assumed to have significant effects on vision in zebrafish, which might well interfere with the overall performance of the fish under environmental conditions. Eye histopathology thus most likely represents a population-relevant endpoint that can easily be implemented into test protocols with nonprotected stages of zebrafish, for example, in OECD test guideline 236.

Locomotor behavior

Monitoring of behavior has the advantage of improving the functional relevance of risk assessment with endpoints known to be relevant to individuals and populations (Clotfelter et al., 2004; Saaristo et al., 2018). Besides, the automatic, noninvasive tracking of organisms using designed high-throughput platforms combined with the use of early life stages leads to the generation of robust and reliable data sets (Ågerstrand et al., 2020).

Effects of several THSDCs on behavioral endpoints have been identified previously (reviewed in Spaan et al., 2019), although specific mechanisms remain unknown. Mechanistic studies established a link between constitutive TH inhibition (e.g., knockout of *mct8* transporters, knockout of *dio2*) and decreased swimming activity in zebrafish embryos (De Vrieze et al., 2014; Houbrechts et al., 2016; Walter et al., 2019; Zada et al., 2014). This effect could be rescued by the addition of a T4 analogue, thus proving the direct influence of TH on activity reduction (De Vrieze et al., 2014; Walter et al., 2019).

Whereas exposure to chemicals inducing a decrease in TH levels seems to reduce swimming activity in a similar manner (Chae et al., 2023; Walter et al., 2019), stimulation of the TH system by THSDCs seems to produce a more complex picture (Walter et al., 2019; Zhu et al., 2021). In particular, results from exposures made in the present study show that simulation of hyperthyroidism by T3 and induction of TH depletion by PTU, PCL, or IOP overall lead to a decrease in swimming activity, thereby making it impossible to distinguish behavioral effects between hypo- and hyperthyroidism contexts. Notably, the present study documents exogenous exposure to T3 leading to decreased swimming activity, which is in line with previous results obtained in 5-dpf zebrafish (Walter et al., 2019), while studies with earlier stages reported hyperactive embryos. However, minor differences in terms of sensitivity between studies may directly reflect differences in sensitivity of fish lines and minor modifications of the experimental design (Fraser et al., 2017).

As discussed above, TH signaling is highly important for the development of the visual system and may directly translate into altered performance in the photomotor response assay. Nonetheless, THs are also involved in numerous neurodevelopmental and neuromuscular processes and can thus result in behavioral changes. For example, deficiency in mobility and decreased psychomotor reaction to sensory stimuli are linked to muscle hypotonia and are hallmarks of the Allan-Herndon-Dudley

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syndrome associated with a dysregulation of the TH system (De Vrieze et al., 2014; Zada et al., 2014). Moreover, swim bladder inflation is known to be regulated by THs (Van Dingenen et al., 2023). The present study revealed a notable lack of swim bladder inflation (≥90%) on exposure to the highest concentration of T3, which is logically linked to a strong decrease in swimming activity. However, this observation alone cannot explain the effects observed for other tested THSDCs, which did not have such an impact.

Besides such potential confounding factors, the present study was successful in demonstrating that behavioral responses, that is, the combination of effects on distance traveled, thigmotactic behavior, and path sinuosity, were highly specific for the test compound and could not generally be linked to TH system activation or inhibition. Behavior is an integrative indicator that increases the sensitivity of ecotoxicological studies but can, however, not easily be linked to specific mechanisms without assessment of additional specific endpoints such as eye malformation or lack of swim bladder inflation. Taken together, it is likely that the behavioral changes observed are due to various TH-dependent mechanisms and/or other TH-independent pathways that may concomitantly be affected (Fraser et al., 2017; Spaan et al., 2019).

CONCLUSIONS

The present study confirms evidence that zebrafish embryos are sensitive to THSDC treatment and represent a promising model for the assessment of TH system–related effects at different levels of biological organization. Previous work had already indicated that zebrafish embryos are useful for assessment of THSDC-induced changes at the transcription (Baumann et al., 2019) and the hormonal (Pannetier, Poulsen, et al., 2023) levels. At the morphological level, eye development, an ecologically highly important developmental process regulated by THs, was shown to be disrupted by different THSDCs (Baumann et al., 2019; Kraft et al., 2023; Pannetier, Poulsen, et al., 2023). A link to behavioral defects has also been highlighted (Baumann et al., 2016; Spaan et al., 2019; Walter et al., 2019) but not for different modes of THSDCs. The present study is the first to connect these endpoints in an AOP-based approach that directly links the different key events together in one testing protocol. The differences in responsiveness to different modes of action of TH system disruption underline the need for such a comprehensive approach in which single endpoints are not interpreted in an isolated manner and cannot be weighed as more or less important. The causal link between endocrine activity and adversity can only be made in an AOP-based approach.

Moreover, the present study was able to close some research gaps: While transcriptional analyses still suffer from statistical robustness (number of animals), the present approach provides a testing protocol that successfully covers both mechanistic and population-relevant endpoints for the assessment of TH system disruption in fish embryos, which is particularly relevant because, by definition, for the assessment of endocrine disruption the causal link between endocrine activity and adversity must be demonstrated (ECHA et al., 2018). In a regulatory context, this is of particular importance because the use of zebrafish embryos has, so far, not been established for the detection of endocrine adversity.

The test protocol of the present study not only covers different population-relevant endpoints but also contributes to the reduction and refinement of animal experimentation for testing of endocrine disruption because zebrafish embryos are not regarded as protected. The current testing framework for THSDC assessment in nontarget organisms only consists of Level 3 tests with amphibians (*Xenopus* eleutheroembryonic thyroid assay, AMA) for endocrine activity and one Level 4 test with amphibians (larval amphibian growth and development assay) for endocrine adversity (ECHA & EFSA, 2018). So far, assessment of endocrine adversity based on fish tests is generally not established in any Level 4 test. Within the EU Horizon 2020 project ERGO (Holbech et al., 2020), TH system disruption endpoints are being implemented into existing OECD test guidelines; based on the data provided by the present study, the FET (OECD test guideline 236) has a high potential to be used in future EDC testing as a test procedure compatible with the 3Rs principle.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5878.

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Writing—review & editing. **Xavier Cousin**: Conceptualization; Formal analysis; Resources; Supervision; Visualization; Writing—original draft; Writing—review & editing. **Thomas Braunbeck**: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Validation; Visualization; Writing—original draft; Writing—review & editing. **Lisa Annie Baumann**: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Writing—original draft; Writing–review & editing.

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