1 **Exposure to the persistent organic pollutant 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD,**
2 **dioxin) disrupts development of the zebrafish inner ear**

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8 **Key Words**

9 zebrafish, dioxin, TCDD, ear, otic vesicle, semicircular canal

10 **Abstract**

11 Dioxins are a class of highly toxic and persistent environmental pollutants that have been shown
12 through epidemiological and laboratory-based studies to act as developmental teratogens.
13 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent dioxin congener, has a high affinity
14 for the aryl hydrocarbon receptor (AHR), a ligand activated transcription factor. TCDD-induced
15 AHR activation during development impairs nervous system, cardiac, and craniofacial
16 development. Despite the robust phenotypes previously reported, the characterization of
17 developmental malformations and our understanding of the molecular targets mediating TCDD-
18 induced developmental toxicity remains limited. In zebrafish, TCDD-induced craniofacial
19 malformations are produced, in part, by the downregulation of *SRY-box transcription factor 9b*
20 (*sox9b*), a member of the SoxE gene family. *sox9b*, along with fellow SoxE gene family members
21 *sox9a* and *sox10*, have important functions in the development of the otic placode, the otic vesicle,
22 and, ultimately, the inner ear. Given that *sox9b* in a known target of TCDD and that transcriptional
23 interactions exist among SoxE genes, we asked whether TCDD exposure impaired the
24 development of the zebrafish auditory system, specifically the otic vesicle, which gives rise to the
25 sensory components of the inner ear. Using immunohistochemistry, *in vivo* confocal imaging, and
26 time-lapse microscopy, we assessed the impact of TCDD exposure on zebrafish otic vesicle
27 development. We found exposure resulted in structural deficits, including incomplete pillar fusion
28 and altered pillar topography, leading to defective semicircular canal development. The observed
29 structural deficits were accompanied by reduced collagen type II expression in the ear. Together,
30 our findings reveal the otic vesicle as a novel target of TCDD-induced toxicity, suggest that the
31 function of multiple SoxE genes may be affected by TCDD exposure, and provide insight into how
32 environmental contaminants contribute to congenital malformations.

33 **Highlights**

34 1. The zebrafish ear is necessary to detect changes in motion, sound, and gravity.
35 2. Embryos exposed to TCDD lack structural components of the developing ear.
36 3. TCDD exposure impairs formation of the fusion plate and alters pillar topography.
37 4. The semicircular canals of the ear are required to detect changes in movement.
38 5. Following TCDD exposure embryos fail to establish semicircular canals.

1 Introduction

Exposure to environmental contamination is a persistent, global issue that shapes the health of aquatic species and humans alike. The complexity of exposures is increasing due to the generation of new contaminants produced from continuous industrial growth and inadequate environmental regulations, as well as the mobilization of legacy contamination by extreme weather events (Briggs, 2003). Aquatic environments are highly susceptible to the deleterious effects of toxic environmental contaminants with toxicant exposures in fish populations having the potential to produce morphological and behavioral changes that lead to decreased reproduction, health, and survival (Bashir et al., 2020; Melvin & Wilson, 2013)

Dioxins are a class of structurally related, widespread, toxic, and highly persistent contaminants released as byproducts of industrial practices such as fossil fuel combustion and medical waste incineration (Dopico & Gómez, 2015; Patrizi & de Cumis, 2018; Rysavy, Maaetoft-udsen, & Turner, 2013; White & Birnbaum, 2009). Dioxins are chemically stable teratogens that bio-persist and bio-accumulate in the food chain (Manzetti & Spoel, 2014). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is the most potent and toxic dioxin congener (Manzetti & Spoel, 2014) with its toxicity mediated primarily through activation of the aryl hydrocarbon receptor (AHR), a ligand activated transcription factor (Carney, Prasch, Heideman, & Peterson, 2006; Kim et al., 2013; Mimura & Fujii-Kuriyama, 2003; Patrizi & de Cumis, 2018; Xu et al., 2015). Additional environmental pollutants, such as dioxin-like polychlorinated biphenyls (DL-PCBs) and polycyclic aromatic hydrocarbons (PAHs), also induce developmental toxicity through activation of AHR (Mimura & Fujii-Kuriyama, 2003; Sugden, Leonardo-Mendonça, Acuña-Castroviejo, & Siekmann, 2017).

In this study, we utilized the zebrafish (*Danio rerio*) model to identify and characterize novel targets of embryonic TCDD-induced developmental toxicity. Zebrafish are an excellent model for understanding the impact of environmental contamination on embryonic development, as they develop *ex vivo* and are optically transparent during early stages; thus, allowing for the visualization of morphological development in real time (Dai et al., 2014; Scholz et al., 2008; Yang et al., 2009). The rich repertoire of transgenic tools available in the zebrafish provide toxicologists with an opportunity to examine the adverse effects of contaminant exposure with a high degree of resolution. Previous research using zebrafish has shown that embryonic TCDD exposure disrupts the development of multiple organ systems including the nervous and cardiovascular systems and produces craniofacial malformations (Burns, Peterson, & Heideman, 2015; Hofsteen, Plavicki, Johnson, Peterson, & Heideman, 2013; Plavicki, Hofsteen, Peterson, & Heideman, 2013; Xiong, Peterson, & Heideman, 2008, and reviewed in Carney et al., 2006). Previous work demonstrated that inhibition of *sox9b*, a member of the SoxE gene family, is a critical molecular mediator of multiple TCDD-induced phenotypic endpoints. Given the critical role of multiple SoxE genes in teleost and mammalian ear development, we asked whether TCDD exposure affected the development of the auditory and vestibular systems, which are inherently intertwined(Barrionuevo et al., 2008; Liu et al., 2003; Park, Byung-Yong, 2010; Saint-Germain, Lee, Zhang, Sargent, & Saint-Jeannet, 2004; Szeto et al., 2022; Yan et al., 2005).

In zebrafish the otic vesicle, also referred to as the ear, is required to sense changes in sound, gravity, and motion (Abbas & Whitfield, 2010; Baxendale & Whitfield, 2016; Dutton et al., 2009; Geng et al., 2013; Whitfield, 2002; Whitfield, Riley, Chiang, & Phillips, 2002). Fish depend on sensory cues to acquire nutrients, escape from prey, and survive in changing currents. Therefore, proper ear development is critical for fish health and fitness. The zebrafish ear is composed of three semicircular canals, three cristae, two otoliths, and two macula (Bever & Fekete, 2002; Liu et al., 2003; Whitfield, 2020; Whitfield et al., 2002). Together, the dorsolateral septum, anterior

pillar, ventral pillar, and posterior pillar establish three semicircular canals required to detect changes in angular acceleration and rotational movement (Baxendale & Whitfield, 2016). The cristae, otoliths, and macula house sensory hair cells, which are necessary for vestibular function. Failure to establish semicircular canals or to develop the hair cells of the ear results in sensory perception defects.

To examine how early embryonic exposure to TCDD affects otic development, we exposed zebrafish embryos carrying a transgenic reporter for *sox10*, an early neural crest marker required for development of the otic vesicle epithelium as well as the peripheral nervous system, melanocytes, heart, and jaw (Dutton et al., 2009). Embryos were exposed to a 1-hour waterborne exposure of 10 parts-per-billion (ppb) TCDD at 4 hpf, an exposure paradigm that results in an internal body burden of 14.7 ±2.04 pg/embryo at 48 hpf and 9.78 ±1.31 pg/embryo at 72 hpf respectively (Kossack, Manz, Martin, Pennell, & Plavicki, 2023). Using confocal microscopy of fixed and live samples, we characterized the effects of exposure on otic vesicle morphology, quantifying fusion plate formation and pillar topography. We determined that TCDD exposure significantly disrupted the structural development of the ear, resulting in the failure of semicircular canals to form. In addition to the significant morphological malformations observed, we found TCDD exposure reduces collagen type II expression in the developing otic vesicle. Our data demonstrate that the developing otic vesicle is a target of TCDD toxicity and, given our prior work demonstrating TCDD exposure disrupts olfactory system development, lead us to conclude that embryonic TCDD exposure impairs development of multiple sensory modalities.

2 Methods

2.1 Zebrafish Husbandry and Transgenic Lines

All experiments in this study were conducted utilizing zebrafish from the Plavicki Lab Zebrafish Facility. All procedures were covered by protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Brown University in adherence with the National Institute of Health's "Guide for the Care and Use of Laboratory Animals." Transgenic zebrafish lines were kept in an aquatic housing system (Aquaneering Inc., San Diego, CA) with automatic pH and conductivity stabilization, centralized filtration, reverse osmosis (RO) water purification, temperature maintenance (28.5 ± 2°C), and ultraviolet (UV) irradiation for pathogen control. Fish were reared and maintained with a 14 hour:10 hour light:dark cycle, according to Westerfield (2000). The Plavicki Lab Zebrafish Facility undergoes routine monitoring for disease including the semiannual quantified Polymerase Chain Reaction (qPCR) panels to detect common fish pathogens.

Adult zebrafish were spawned in 1.7L slope tanks (Techniplast, USA). Fish were placed in tanks 15-18 hours prior to breeding with a transparent divider that separated males and females. The following day the divider was removed within 2-3 hours of the initiation of the light cycle. Embryos were collected over the course of 1 hour and subsequently reared in fresh egg water (60 mg/l Instant Ocean Sea Salts; Aquarium Systems, Mentor, OH) in 100 mm non-treated culture petri dishes (CytoOne, Cat. No. CC7672-3394). Embryonic and larval zebrafish were maintained in temperature regulated incubators with a 14 hour:10 hour light:dark cycle (Powers Scientific Inc., Pipersville, PA). At 24 hpf, embryos were manually dechorionated using Dumont #5XL forceps (Cat. No. 11253-10, Fine Science Tools, Foster City, CA) and moved to fresh egg water containing 0.003 % 1-phenyl-2-thiourea (PTU, Sigma) to prevent pigment deposition. The following transgenic lines were used in this study: *Tg(Sox10:RFP)*(Kirby et al., 2006) and *TgBAC(cyp1a:NLS:EGFP)* (Kim et al., 2013).

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2.2 TCDD exposure

Embryonic TCDD and dimethyl sulfoxide (DMSO) control exposures were performed in accordance with Plavicki et al., (2013) (Plavicki et al., 2013). Within 1 hour of adults spawning, embryos were collected and maintained in a temperature-controlled incubator for future assessment. At 3 hpf fertilized embryos were evaluated and selected based on healthy developmental quality. Selected embryos were exposed at 4 hpf to either a 0.1% DMSO vehicle control or to a 10 ppb TCDD dosing solution for 1 hour while gently rocking. Twenty embryos were exposed per 2 mL of dosing solution in 2 mL glass amber vials (Cat. No. 5182-0558, Agilent, Santa Clara, CA). Control and dosing solutions were made by adding solution constituents to amber vials already containing 20 embryos and gently rocking. For TCDD exposures, a 10 ppb TCDD (ED-901-B, Cerriliant, St. Louis, MO) dosing solution was prepared by adding 2 mL of egg water and 2 μL of pre-prepared TCDD stock (10ng/mL). Vehicle controls were exposed to a 0.1% DMSO control dosing solution, which is matched to the DMSO concentration in the TCDD dosing solution. Amber vials and caps were wrapped with Parafilm® to limit evaporation.

2.3 Immunohistochemistry

Zebrafish embryos and larvae were collected at 72 hpf and fixed in groups of 10-15 fish per 1.5 mL Eppendorf® Safe-Lock microcentrifuge tube (Millipore Sigma, Cat. No. T9661-500EA) in 4% paraformaldehyde (PFA, Sigma-Aldrich, Cat. No. P6148) overnight at 4°C while gently rocking. Samples were washed 5 times with PBS-T (phosphate buffered solution + 0.6% Triton-X 100) post-fixation. Samples were permeabilized with acetone for 5 minutes at -20°C. Post-permeabilization, samples were placed in blocking solution (PBS-T + 4% bovine serum albumin, BSA) overnight while gently rocking at 4°C. After blocking, samples were incubated with 1° antibodies at 4°C for 1 day. Samples next underwent 5 quick washes with PBS-T and then washed overnight in fresh PBS-T at 4°C. Once PBS-T wash solution was removed, the 2° antibody and Hoechst stain (1:10,000) were added to the samples and left to incubate for approximately 18 hours at 4°C. 2° antibody and Hoechst were removed by 5 quick washes performed with PBS-T followed by overnight wash at 4°C in PBS-T. PBS-T solution was fully removed from the Eppendorf tubes using a Samco™ Fine Tip Transfer Pipette (Millipore Sigma, Cat. No. Z350605-500EA) and VECTASHIELD Mounting Media (Vector Labs, Cat. No. H-1000) was added for clearing and storage. Samples were stored for a minimum of 24 hours in VECTASHIELD prior to imaging. The 1° antibody used was Collagen Type II (II-II6B3) (1:100, mouse monoclonal [IgG1]). II-II6B3 was deposited to the Developmental Studies Hybridoma Bank (DSHB) by Linsenmayer, T.F. (DSHB Hybridoma Product II-II6B3). The 2° antibody used was Alexa Fluor® 488 Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody (1:100,ThermoFisher Scientific, Cat. A21121)

2.4 Microscopy and Image Analysis

Live embryos and larvae were anesthetized with 0.02% tricaine (MS-222) for embedding and subsequent imaging. Both live and fixed embryos were mounted in 2% low-melting agarose (Fisher Scientific, bp1360-100) in 35 mm glass bottom microwell dishes (MatTek, Part No. P35G-1.5-14-C). Confocal imaging was performed using a Zeiss LSM 880 confocal with Airyscan Fast. Z-stacks spanned approximately 110 μm with images collected at 0.69 μm intervals. Images were analyzed and prepared for publication using Adobe Photoshop, Adobe Illustrator, Zeiss Zen Black, and Zeiss Zen Blue software. Graphs were generated for data visualization utilizing GraphPad Prism and R studio.

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2.5 Statistical Analysis

Chi-squared and Fishers exact test were used to assess differences between categorical data collected from the control and TCDD-exposed groups. Significant differences between groups were determined using Chi-squared and Fishers exact test with a 95% confidence interval in GraphPad Prism. Significance is denoted as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

3 Results

3.1 Cyp1a, a biomarker of AHR activation, is induced in the developing otic vesicle following TCDD exposure

TCDD-induced AHR activation induces expression of cytochrome P450 1 A (*cyp1a*), a xenobiotic metabolizing enzyme and a biomarker of AHR activation (Carney et al., 2006; Kim et al., 2013; Mimura & Fujii-Kuriyama, 2003; Xu et al., 2015). Correspondingly, *cyp1a* mRNA or protein expression can be used to identify cellular targets of TCDD-induced developmental toxicity. To determine if the developing otic vesicle was a target of TCDD toxicity, we exposed transgenic zebrafish embryos carrying a green fluorescent protein reporter in the start codon of Cyp1a (*Tg(cyp1a:nls-egfp)*) to 10 ppb TCDD at 4 hpf and used confocal microscopy to visualize Cyp1a expression at 48 and 72 hpf. Previous work has shown that during early stages of development there is endogenous expression of Cyp1a in the ear (Kim et al., 2013). Therefore, we sought to determine if TCDD exposure led to an expansion of the Cyp1a expression domain in the developing otic vesicle. Our findings show that exposure to TCDD results in robust expansion of the Cyp1a expression domain in the ear at both 48 and 72 hpf (Fig. 2 & Fig. 3, Supp. Fig 1).

By 48 hpf, the otic vesicle has developed Sox10+ otic epithelium throughout the ear. At this developmental stage, the three cristae have been established; however, the pillar structures are not yet present (Fig. 1 A-D). Our data showed that by 48 hpf control embryos had Cyp1a expression in the dorsal most portion of the ear, which we will refer to as the apex of the ear, and within a small subset of cells within in the anterior crista. (Fig. 2 A-C, G-I; Supp. Fig. 1). In contrast, in TCDD-exposed embryos, Cyp1a was expressed throughout the anterior crista, ventral crista, and posterior crista at 48 hpf, suggesting the cristae are an early target of TCDD-induced toxicity (Fig. 2 D-F, J-L; Supp. Fig 1).

By 72 hpf, the ear has developed four additional structures: the anterior pillar, ventral pillar, posterior pillar, and dorsolateral septum, which together establish the semicircular canals required for vestibular function (Fig. 1 E-H). At 72 hpf, only the apex and the dorsolateral septum show expression of Cyp1a in control larvae (Fig. 3 A-C, G-I; Supp. Fig. 1). In contrast, the Cyp1a expression domain in TCDD exposed larvae at 72 hpf encompassed the apex, anterior crista, ventral crista, posterior crista, and the present anterior pillar, ventral pillar, posterior pillar, and dorsolateral septum (Fig. 3 D-F, J-L; Supp. Fig. 1). Thus, suggesting that the otic vesicle cristae and the pillar structures that make up the semicircular canals continue to be targets of TCDD-induced toxicity.

The three cristae house sensory hair cells that aid in, but are not required for, accurate tethering of the otoliths (Whitfield, 2020; Whitfield et al., 2002). Since TCDD induced Cyp1a expression in the cristae, we evaluated ears for the presence of otoliths and found that the otoliths were present

214   in all 48 hpf control and TCDD exposed embryos (n=11/group with 3 replicates); as well as in all
215   72 hpf control and TCDD exposed embryos (n=8-9/group with 3 replicates).

216   **3.2 Ears of TCDD exposed embryos display structural deficits**

217   Proper establishment of the semicircular canals requires the development and subsequent fusion
218   of the anterior pillar, ventral pillar, posterior pillar, and dorsolateral septum. Given that the
219   expanded pattern of Cyp1a expression at 72 hpf encompassed the developing pillars, we sought
220   to determine if TCDD exposure disrupts development of the semicircular canals. Semicircular
221   canals form between 60 hpf to 90 hpf (Baxendale & Whitfield, 2016). Therefore, to determine if
222   TCDD exposure disrupted development of the semicircular canals, we characterized pillar fusion
223   at 72 hpf and 96 hpf. We began by quantifying the presence of structural components of the ear
224   and saw that by 72 hpf, 95% of controls had all of the semicircular structural components present.
225   Meanwhile, only 4.17% of TCDD exposed embryos showed the presence of all structures (Fig.
226   4A). 75% of TCDD embryos lacked the ventral pillar, 20.83% the anterior pillar, and 8.33% the
227   posterior pillar (Fig. 4B). Only 5% of control embryos were missing the ventral pillar, which likely
228   reflected variability in developmental timing of ventral pillar development (Fig. 4B). In contrast,
229   TCDD exposed fish showed a range of deficits with 70.83% of embryos lacking at least two
230   structural components and 25% lacking one structure alone (Fig. 4A). Coincidence of a structural
231   absence varied in severity with 8.33% of TCDD exposed fish having no structural components
232   present and 12.50% showing coincident deficits in anterior pillar, ventral pillar, and dorsolateral
233   septum establishment (Fig. 4A). The most common coincident phenotype following TCDD
234   exposure was absence of both the ventral pillar and dorsolateral septum, 50% of embryos
235   presented this defect (Fig. 4A). The dorsolateral septum was the most frequently absent structure
236   with 91.67% of TCDD exposed embryos showing a deficit (Fig. 4B).

237   The rates of coincident phenotypes varied at 96 hpf compared to those at 72 hpf. At 96 hpf the
238   percentage of fish with all structures present increased to 100% in the control group and 15% in
239   the TCDD exposed group, compared to 95% and 4.17% respectively at 72 hpf. Additionally, at
240   96 hpf, only 60% of TCDD exposed embryos, compared to 70.83% at 72 hpf, had at least two
241   coincident phenotypes. However, while only 8.33% of TCDD exposed larvae showed absence of
242   all structural components at 72 hpf, this increased to 35% at 96 hpf (Supp. Fig. 2). Overall, these
243   data indicate that TCDD exposure leads to significant structural deficits with ranging degrees of
244   severity at both 72 hpf and 96 hpf.

245   **3.3 Embryos dosed with TCDD fail to establish a fusion plate**

246   In addition to pillar development, complete pillar fusion is required for establishment of
247   semicircular canals. For pillar fusion to occur the anterior pillar, ventral pillar, and posterior pillar
248   must be present and connect at the fusion plate in the center of the ear (Whitfield et al., 2002).
249   To capture whether complete fusion occurred, we scored larvae for complete and incomplete
250   pillar fusion at both 72 hpf and 96 hpf. Ears were classified as having an incomplete fusion
251   phenotype if the anterior pillar, ventral pillar, and posterior pillar did not form a fusion plate.
252   Therefore, embryos that lacked a pillar or had pillars present that did not fuse were categorized
253   as having incomplete fusion. In controls, 30% of larvae showed an incomplete fusion of the ventral
254   pillar at 72 hpf (Fig. 5). This was likely due to variability in development, since at 96 hpf 0% of
255   controls were scored as having incomplete fusion phenotypes. In contrast, 100% TCDD exposed
256   embryos showed a defect in fusion at both 72 hpf and 96 hpf (Fig. 5 and Supp. Fig. 3).

257 Incomplete fusion phenotypes can occur even if all pillars are present. A pillar was categorized
258 as having incomplete fusion if it was absent or if it did not fuse with at least one of the other pillars.
259 At 72 hpf, 100% of TCDD exposed embryos displayed incomplete fusion of the ventral pillar; 75%
260 of embryos lacked a ventral pillar all together, while 25% had a ventral pillar present that did not
261 link at the fusion plate. Likewise, 87.5% of larvae previously dosed with TCDD showed incomplete
262 fusion of the anterior pillar by 72 hpf; 20.83% of larvae showed absence of the anterior pillar,
263 whereas 66.67% had an existing anterior pillar that did not reach the fusion plate. Finally, 83.33%
264 of TCDD exposed larvae demonstrated an incomplete posterior pillar fusion phenotype; 8.33% of
265 larvae were missing the posterior pillar, whereas 75% lacked adequate formation of the fusion
266 plate formation (Fig. 5). Although we have delineated fusion defects per pillar by categorizing
267 which pillars did not fuse at the plate, it is important to note that presence of all three pillars is
268 required to form the fusion plate. Therefore, after exposure to TCDD 100% embryos at 72 hpf fail
269 to establish the fusion plate. We were able to capture these defects in establishment and fusion
270 of pillars occurring between 52-72 hpf though time-lapse confocal microscopy (Supp. Movie 1).

271 Lastly, to determine if the pillars in TCDD exposed fish fuse at a later time point, we assessed
272 fusion plate establishment at 96 hpf. Consistent with data at 72 hpf, 100% of TCDD exposed
273 larvae had incomplete pillar fusion at 96 hpf. 85% showed defects in the anterior pillar's ability to
274 reach and fuse with the fusion plate, 95% showed defect at the ventral pillar, and 95% showed
275 defect at the posterior pillar (Supp. Fig. 3). Together, these data indicate that embryonic exposure
276 to TCDD impairs establishment of the semicircular canals by altering both establishment and
277 fusion of pillar structures.

278 **3.4 TCDD exposure changes topography of ear structures**

279 Through the process of quantifying semicircular canal establishment, we noted all pillars in control
280 larvae develop in a stereotyped position relative to the adjacent crista. In control fish, the anterior
281 pillar buds above the anterior crista, the ventral pillar buds on top of or slightly to the right of the
282 ventral crista, and the posterior pillar buds above the posterior crista. In contrast, the topography
283 of pillars in TCDD exposed fish was inconsistent; therefore, we quantified how many fish showed
284 a topographical displacement of pillar development. Pillars that were categorized as displaced
285 were present and budding, but in an incorrect position relative to the pillar-crista relationship
286 observed in all control larvae. 100% of the pillars in control larvae develop in the same
287 topographical relation to the cristae. At 72 hpf, 59.09% TCDD exposed fish showed displacement
288 of the posterior pillar and 9.09% showed a coincident displacement of both the anterior and
289 posterior pillars (Fig. 6A). Specifically, the posterior pillar developed under the posterior crista,
290 while the anterior pillar developed on top of or under the anterior crista (Fig. 6B-G). Meanwhile,
291 at 96 hpf, 83.33% of TCDD exposed embryos had one pillar displaced, but no coincident
292 displacements (Supp. Fig. 4). This data indicates that TCDD exposure altered the patterning and
293 overall structural development of the otic vesicle.

294 **3.5 The collagen type II expression in the ear is reduced following TCDD exposure**

295 Collagen type II (Col2a1) is expressed early on in the developing otic vesicle epithelium, pillar
296 structures, and in the anterior macula (Chung et al., n.d.; Dale & Topczewski, 2011; Williams,
297 2022; Yan et al., 2005). Col2a1 plays an important role in maintaining structural integrity in
298 developing skeletal structures. Given that *Sox9*, a target of AHR signaling in mammals, is a direct
299 regulator of Col2a1 (Bell et al., 1997) and that *sox9b*, one of 2 orthologues of *Sox9*, is known to
300 be repressed in multiple zebrafish tissues following TCDD exposure, we asked if expression of
301 Col2a1 in the ear was affected by embryonic TCDD exposure. By 72 hpf, all control embryos

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302 showed expression of Col2a1 in the otic epithelium and present pillar structures. Meanwhile, 90%
303 of TCDD exposed fish had reduced Col2a1 expression in the ear (Fig. 7).

304 **4 Discussion**

305 **4.1 TCDD exposure disrupts sensory system development**

306 Organisms rely on their sensory modalities for the acquisition of nutrients, mating, and evasion of
307 predators. Therefore, the proper structural development of sensory organs as well as appropriate
308 neural innervation of these organs is essential for survival and fitness. Zebrafish possess all of
309 the primary sensory modalities: hearing, balance, taste, touch, smell, and vision (Moorman,
310 2001). Research in fish and mammals has demonstrated that TCDD exposure at different life
311 stages results in neurodevelopmental phenotypes in the CNS including impaired neurogenesis
312 and disrupted development of the olfactory bulb, cerebellum, optic tectum, habenula, and
313 hippocampus (Collins et al., 2008; Hill, Howard, Strahle, & Cossins, 2003; Iida, Kim, Murakami,
314 Shima, & Iwata, 2013; Kimura et al., 2016; Williamson, Gasiewicz, & Opanashuk, 2005).
315 However, considerably less is known about how TCDD exposure effects sensory system
316 development. In this study, we identify the developing zebrafish ear as a novel target of TCDD-
317 induced toxicity, revealing that TCDD exposure impairs development, fusion, and topography of
318 essential ear structures. The ear is required for sensing changes in sound and, in conjunction with
319 the lateral line, detecting changes in gravity, movement, and rotation. TCDD-induced disruption
320 of ear development, therefore, has the potential to impair a number of ecologically relevant
321 behaviors. While we did not observe gross malformations in the neural innervation of the ear,
322 more nuanced studies using genetically encoded calcium indicators and/or electrophysiology
323 could reveal neurofunctional changes in the vestibuloacoustic nerve that innervates the hair cells
324 and/or the octavolateralis efferent system that carries information from the auditory vestibular
325 system to the periphery (Beiriger, Narayan, Singh, & Prince, 2021; Blaxter, 1987). Previous work
326 examining the impact of TCDD exposure on peripheral nervous system development in the red
327 seabream found multiple cranial nerves to be disrupted by TCDD exposure; however, these
328 studies did not specific examine the effects of TCDD exposure on the vestibuloacoustic nerve
329 (Iida et al., 2013).

330 Previous work conducted in our lab showed that early TCDD exposure disrupts development of
331 the olfactory organs and olfactory bulb, the structures required to detecting and coding olfactory
332 cues (Martin et al., 2022). Additionally, while it is beyond the scope of this paper, we note that
333 TCDD-exposed embryos and larvae examined in this study had altered eye morphology,
334 indicating that the eye is also a target of TCDD-induced toxicity (Supp. Fig. 5). Consistent with
335 our observation that TCDD-induced dysregulation of AHR signaling alters eye development, the
336 invertebrate homologs of *AHR*, *spineless*, is necessary for retinal development (Wernet et al.,
337 2006). Together, these studies suggest TCDD toxicity impairs development of multiple sensory
338 systems.

339 **4.2 Understanding the molecular mechanisms that mediate TCDD-induced otic**
340 **phenotypes**

341 Our study characterized the effects of TCDD toxicity on otic vesicle development. To our
342 knowledge, no previous studies have described the ototoxic effects of TCDD in zebrafish or other
343 aquatic animals. Zebrafish mutant analysis studies have revealed key signaling pathways and
344 transcriptional regulators of otic vesicle development and semicircular canal establishment
345 (Whitfield et al., 2002). These mutants highlight potential targets of interest in the quest to uncover
346 the molecular mechanisms of TCDD-induced toxicity. Mutants with defects in semicircular canal

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347 establishment, similar to our TCDD exposed embryos, include the *dog-earred*, *valentino,*
348 *lauscher,* and *colourless* mutant. The *dog-eared* mutant, which disrupts function of *eyes absent-*
349 *1* (*eya1*), has an altered semicircular canal phenotype with defects in maculae and cristae
350 establishment (Kozlowski, Whitfield, Hukriede, Lam, & Weinberg, 2005). The *valentino* mutant
351 displays varying degrees of severity in semicircular canal establishment with some embryos
352 showing partial pillar fusion, which was also observed following TCDD-exposure (Moens, Cordes,
353 Giorgianni, Barsh, & Kimmel, 1998). The *lauscher* mutation caused by a disruption to *gpr124,* an
354 adhesion class G protein-coupled receptor gene, results in defective pillar budding and fusion
355 (Geng et al., 2013). A large deletion mutant b971, which encompasses *sox9b*, produces smaller
356 and misshapen ears and *sox9a* and *sox9b* double mutants lack ears altogether(Yan et al., 2005).
357 Finally, the *colourless* mutation caused by loss of *sox10* results in small ears, abnormal sensory
358 patches, underdeveloped semicircular canals (Dutton et al., 2009; Lopes et al., 2001). In our
359 study, we found that TCDD exposure targets Sox10+ epithelial structures in the ear and that
360 TCDD induced disruption of these structures results in defects in sensory canal establishment.
361 TCDD exposure has also been shown to reduce the number of Sox10+ oligodendrocytes in the
362 developing brain (Martin et al., 2022). Given that in certain developmental contexts *sox10* is
363 downstream of *sox9b*, a known target of the AHR signaling pathway, it could be that TCDD-
364 induced AHR activation indirectly reduces Sox10 expression by impairing Sox9b expression (Yan
365 et al., 2005). Alternatively, as we discuss below, multiple SoxE genes could be direct targets of
366 TCDD-induced toxicity.

4.3 SoxE genes as targets of TCDD-induced toxicity

368 SoxE proteins are a family of transcription factors grouped together by sequence identity in their
369 high-mobility group (HMG) domains (Chiang et al., 2001). The SoxE family in zebrafish is
370 composed of the transcription factors Sox8, Sox9a, Sox9b, and Sox10, which are critical for
371 embryonic development of the otic vesicle, jaw, heart, fin, gonads, CNS, and PNS (Hofsteen et
372 al., 2013; She & Yang, 2017; Stolt & Wegner, 2010; Yan et al., 2005). Previous work has shown
373 that TCDD exposure reduces expression of Sox9b in the developing jaw, heart, and fin
374 (Andreasen, Mathew, & Tanguay, 2006; Burns et al., 2015; Carney et al., 2006; Plavicki et al.,
375 2013; Xiong et al., 2008). Zebrafish exposed to TCDD at 96 hpf show reduced expression of
376 *sox9b* in the jaw at 4 and 12 hours post-exposure (Xiong et al., 2008). In the developing heart,
377 loss of Sox9b can account for a significant subset of the cardiac phenotypes produced by
378 embryonic TCDD exposure (Hofsteen et al., 2013). However, it remained unknown whether other
379 SoxE genes such as *sox9a*, *sox10*, and *sox8* are TCDD targets.

380 The otic placode and the developing otic vesicle express Sox9a, Sox9b and Sox10 (Chiang et al.,
381 2001; Dutton et al., 2009) *sox10* expression initially overlaps with *sox9a* and *sox9b*, but eventually
382 expression of *sox10* is maintained in all otic epithelial cells while *sox9a* and *sox9b* expression
383 becomes restricted to specific domains (Dutton et al., 2009). Previous studies have shown that
384 loss of Sox10 results in impaired in otic placode establishment and, ultimately, in structural and
385 sensory deficiencies including defects in the semicircular canals (Dutton et al., 2009). Our data
386 indicate that TCDD targets Sox10+ otic epithelium and that TCDD toxicity results in structural
387 defects.

388 Future work should be conducted to determine if other SoxE genes are targets of TCDD
389 independent of Sox9b and to understand the downstream targets of Sox10 in the ear. Potential
390 targets include those found in loss of *sox10* function studies. For instance, a loss of function *sox10*
391 mutation in zebrafish results in altered expression of signaling molecules *fibroblast growth factor*
392 *8a* (*fgf8*) and *bone morphogenetic protein 4* (*bmp4*) in the ear (Dutton et al., 2009). Fgf8 is
393 required to induce the otic vesicle placode and to form the otic epithelium (Liu et al., 2003).

10

394 Correspondingly, in mice, loss of Bmp4 impaired cristae and semicircular canal establishment
395 (Chang et al., 2008) and in zebrafish, excess Bmp4 treatment resulted in ectopic or absent pillar
396 formation, suggesting Bmp4 is involved in semicircular canal establishment. Although previous
397 studies have not investigated the effects of TCDD exposure on expression of Bmp4 and Fgf8
398 specifically in the ear, previous research found that following TCDD exposure Bmp4 expression
399 was expanded in the heart and, based on whole embryo qPCR data, is globally upregulated in
400 embryos and larvae (Mehta, Peterson, & Heideman, 2008; Yue, Martin, Martin, Taylor, & Plavicki,
401 2021). TCDD was shown not affect Fgf8 expression following fin amputation (Andreasen,
402 Mathew, Löhr, Hasson, & Tanguay, 2007); however, it is not clear whether Fgf8 expression is
403 altered in other tissues in response to TCDD exposure. Expression analysis of both Fgf8 and
404 Bmp4 in the ear following TCDD exposure would help to further elucidate the molecular
405 mechanisms mediating the phenotypes described in this paper.

4.4 TCDD induced dysregulation of collagen deposition

407 Our studies revealed that TCDD exposure reduces expression of Col2a1, a critical collagen
408 subtype found in many structures including the inner ear. Mutations in COL2A1 can lead to
409 hearing loss in humans (Acke, Dhooge, Malfait, & De Leenheer, 2012). Zebrafish have two
410 *Col2a1* homologues, *col2a1a* and *col2a1b*, with distinct and overlapping expression domains
411 (Dale & Topczewski, 2011). The Col2a1 antibody utilized in this study broadly marks collagen
412 type II and it is therefore not possible for us to distinguish if there are differential effects on the
413 homologues following TCDD exposure. Notwithstanding, we were able to capture a noteworthy
414 reduction of Col2a1 in the otic vesicles suggesting that one or both collagen type II homologs are
415 affected by TCDD exposure. TCDD exposure has also been shown to impair osteogenesis and
416 affect type II collagen expression in medaka; thus indicating that TCDD exposure affects collagen
417 deposition in multiple fish species (Dong, Hinton, & Kullman, 2012; Watson et al., 2017).

418 In the mouse, *Col2a1* is known to be a direct target of Sox9 (Bell et al., 1997). The observed
419 reduction in Col2a1 is likely the result of inhibited Sox9b expression. The previous studies have
420 shown AHR activation represses *sox9b* function through the expression of a long noncoding RNA,
421 *sox9b long intergenic noncoding RNA* (*slincR*) (*slincR*)(Garcia et al., 2018). It is not clear if
422 repression of Sox9b is the primary mediator of the observed otic vesicle phenotypes or, if so, if
423 Sox9b repression is occurring through the same mechanism. Sox9 is also known to be a target
424 of retinoic acid signaling (Dale & Topczewski, 2011; Williams, 2022). A previous study found that
425 retinoic acid signaling suppressed SOX9, the human orthologue of zebrafish Sox9b, in
426 chondrocytes and reduced *Col2a1 e*xpression (He, Brysk, Tyring, Ohkubo, & Brysk, 2001).
427 Conversely, overexpression of SOX9 reversed retinoic acid induced suppression of *Col2a1* (He
428 et al., 2001)*.* Similar regulatory interactions between Sox9 and retinoic acid have been observed
429 in the testis (Bowles et al., 2018). These studies indicate that retinoic acid is an important regulator
430 of *Sox9* and *Col2a1* expression*.*

431 Retinoic acid signaling has also been implicated in zebrafish ear development, specifically
432 regulating the development of the semicircular canals and otoliths. Disruption of this pathway
433 results in a circling behavior previously associated with inner ear malformations (Mackowetzky et
434 al., 2022). A number of lines of evidence suggest an interaction between TCDD exposure and
435 retinoic acid signaling in the generation of adverse outcome (Fiorella, P. D., Olson, J. R., & Napoli,
436 1995; Herlin et al., 2021; Jacobs et al., 2011; Murphy, Villano, Dorn, & White, 2004; Schmidt et
437 al., 2003; Weston, W. M., Nugent, P., & Greene, 1995). Consequently, the retinoic acid pathway
438 presents exciting prospects for future studies of TCDD-induced disruption of otic vesicle
439 development.

11

5 Conclusions

This study reveals the otic vesicle as a novel target of embryonic TCDD toxicity. Through this work, we characterized the auditory-vestibular structures and developmental processes in otic vesicle formation affected by early TCDD exposure. Specifically, our data demonstrates that embryonic exposure to TCDD leads to defects in pillar establishment, pillar fusion, and topography, ultimately leading to impaired development of the ear's semicircular canals required for detecting angular rotation. Furthermore, we found TCDD significantly inhibited expression of collagen type II, a collagen subtype with important functions in mammalian ear development. Future studies will help determine the molecular mechanisms that produce otic phenotypes following TCDD exposure.

6 Conflict of Interest

The authors declare no conflicts of interest. This study was completed without any financial affiliations that could be interpreted as a potential conflict of interest.

7 Author Contributions
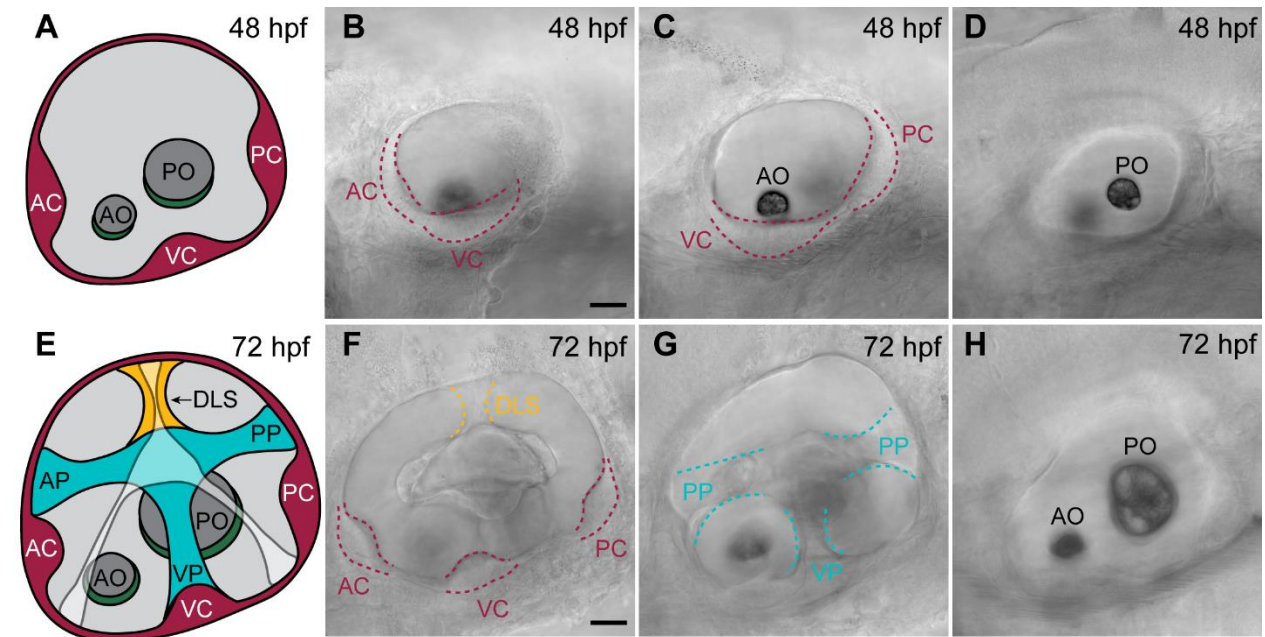
8 Acknowledgements

468 **Figures**

470 **Figure 1. Anatomy of the wild-type zebrafish ear.** (A, E) Schematics of wild-type zebrafish ear
471 at 48 hpf (A) and 72 hpf (E). (B-D) Representative brightfield images of live wild-type ears showing
472 the developing cristae and otoliths at 48 hpf. (F-H) Representative brightfield images of live wild-
473 type ears capturing the cristae, otoliths, pillars, and dorsolateral septum at 72 hpf. Abbreviations:
474 anterior crista (AC), ventral crista (VC), posterior crista (PC), anterior pillar (AP), ventral pillar
475 (VP), posterior pillar (PP), dorsolateral septum (DLS), anterior otolith (AO), posterior otolith (PO).
476 All images are 40x. Scale bar in B & F is 25µm.
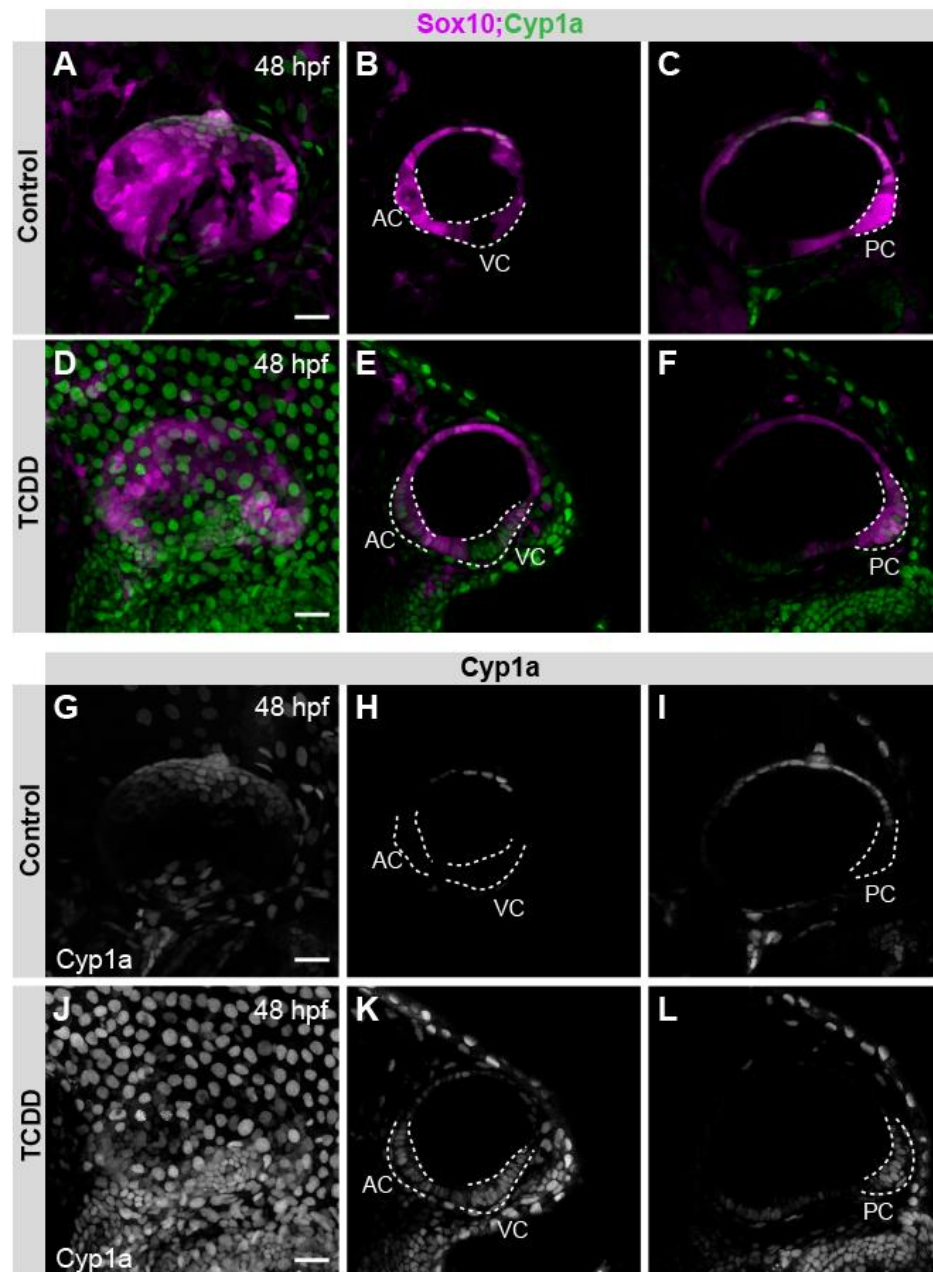
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Figure 2. TCDD exposure expands the Cyp1a expression domain in the developing embryonic ear. (A-F) Confocal micrographs of control (A-C) and TCDD-exposed (D-F) embryos carrying reporters for *sox10* (magenta) and *cyp1a* (green) [*Tg(sox10:RFP)*; *TgBAC(cyp1a:NLS:EGFP)*]. *sox10* expression encompasses the otic epithelium and *cyp1a* acts as a biomarker of AHR activation. (H-I, K-L) Representative slices taken from confocal z-series capturing *cyp1a* induction (white) in control (G-I) and TCDD-exposed (J-L) embryos. Maximum intensity projections of confocal z-series are shown in A, D, G, & J. Abbreviations: anterior crista (AC), ventral crista (VC), and posterior crista (PC). Scale bar in A, D, G, & J = 25µm. n=8-10/group with 3 a minimum of replicates.
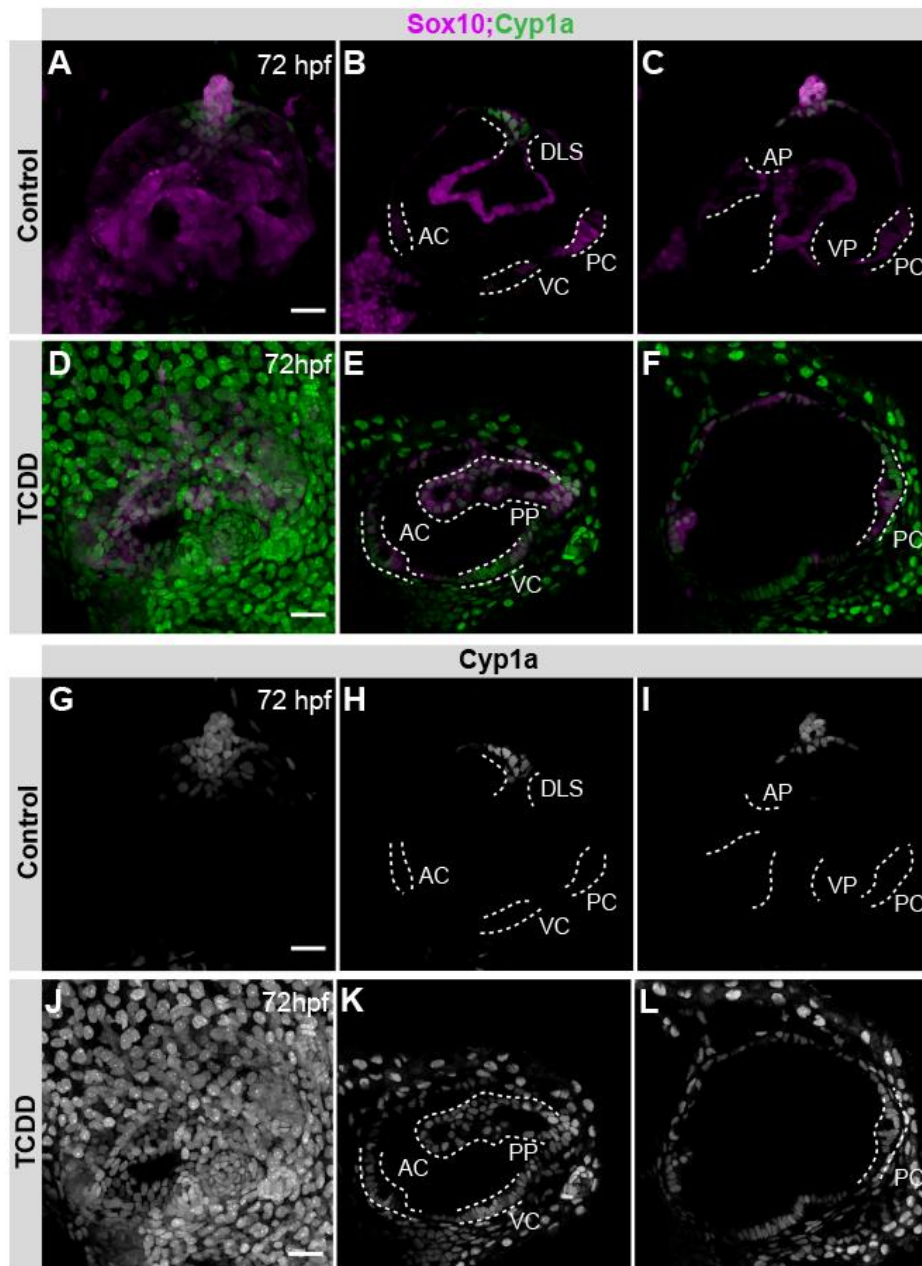
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Figure 3. TCDD-induced expansion of Cyp1a expression is maintained in the larval ear.
(A-F) Confocal micrographs of control (A-C) and TCDD-exposed (D-F) larvae carrying reporters for *sox10* (magenta) and *cyp1a* (green) [*Tg(sox10:RFP)*; *TgBAC(cyp1a:NLS:EGFP)*]. (G-L) Cyp1a transgenic reporter in black and white in controls (G-I) and TCDD exposed (J-L) fish. Maximum intensity projections of confocal z-series are shown in A, D, G, & J. Abbreviations: anterior crista (AC), ventral crista (VC), posterior crista (PC), anterior pillar (AP), ventral pillar (VP), posterior pillar (PP), dorsolateral septum (DLS). Scale bar in A, D, G & J is 25µm. n=10-13/group with a minimum of 3 replicates.
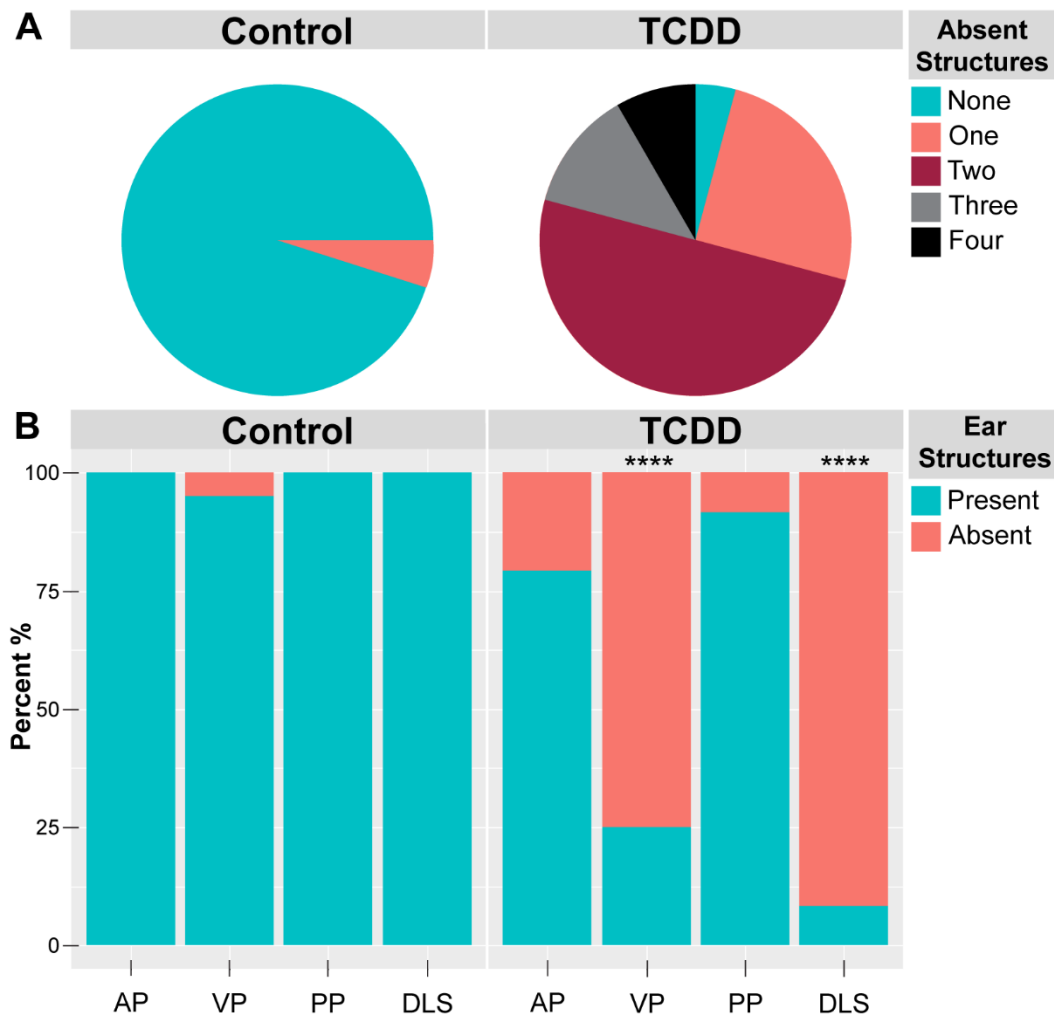
15

Figure 4. Quantification of structural deficits induced by TCDD exposure. (A) Percentage of coincident absent structures in control and TCDD exposed larval at 72 hpf. (B) Percentage of specific ear structure absence. Abbreviations: anterior pillar (AP), ventral pillar (VP), posterior pillar (PP), dorsolateral septum (DLS). n=20-24/group with a minimum of 3 replicates. ****p < 0.0001.
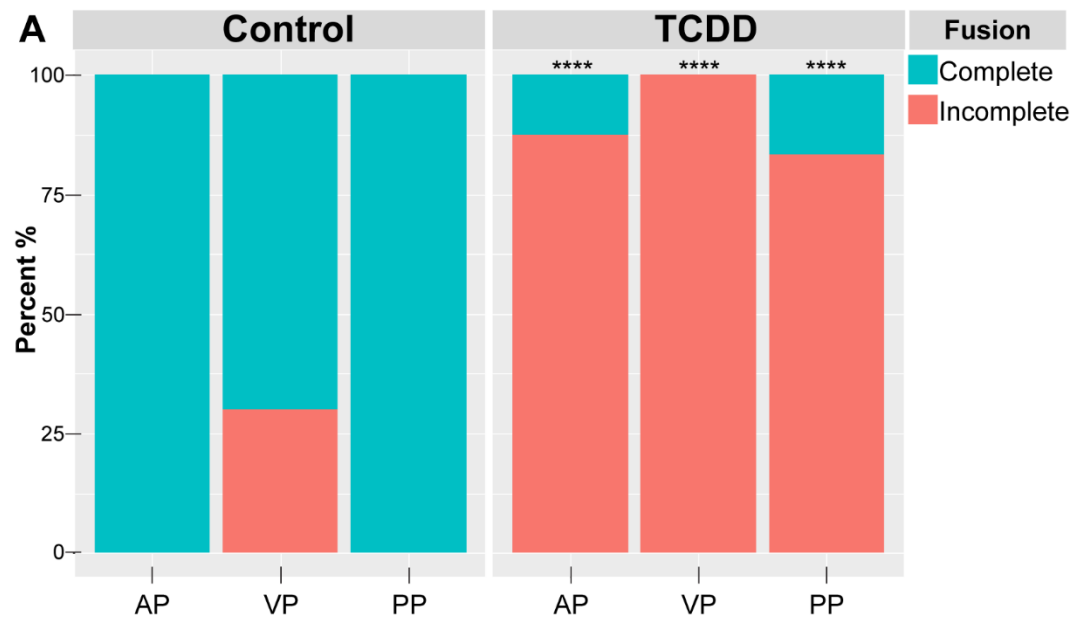
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Figure 5. Quantification of incomplete pillar fusion. Percent of structures that failed to establish a connection at fusion plate in control and TCDD exposed embryos at 72hpf (A). Abbreviations: anterior pillar (AP), ventral pillar (VP), posterior pillar (PP). n=20-24/group with a minimum of 3 replicates. ****$p < 0.0001$.
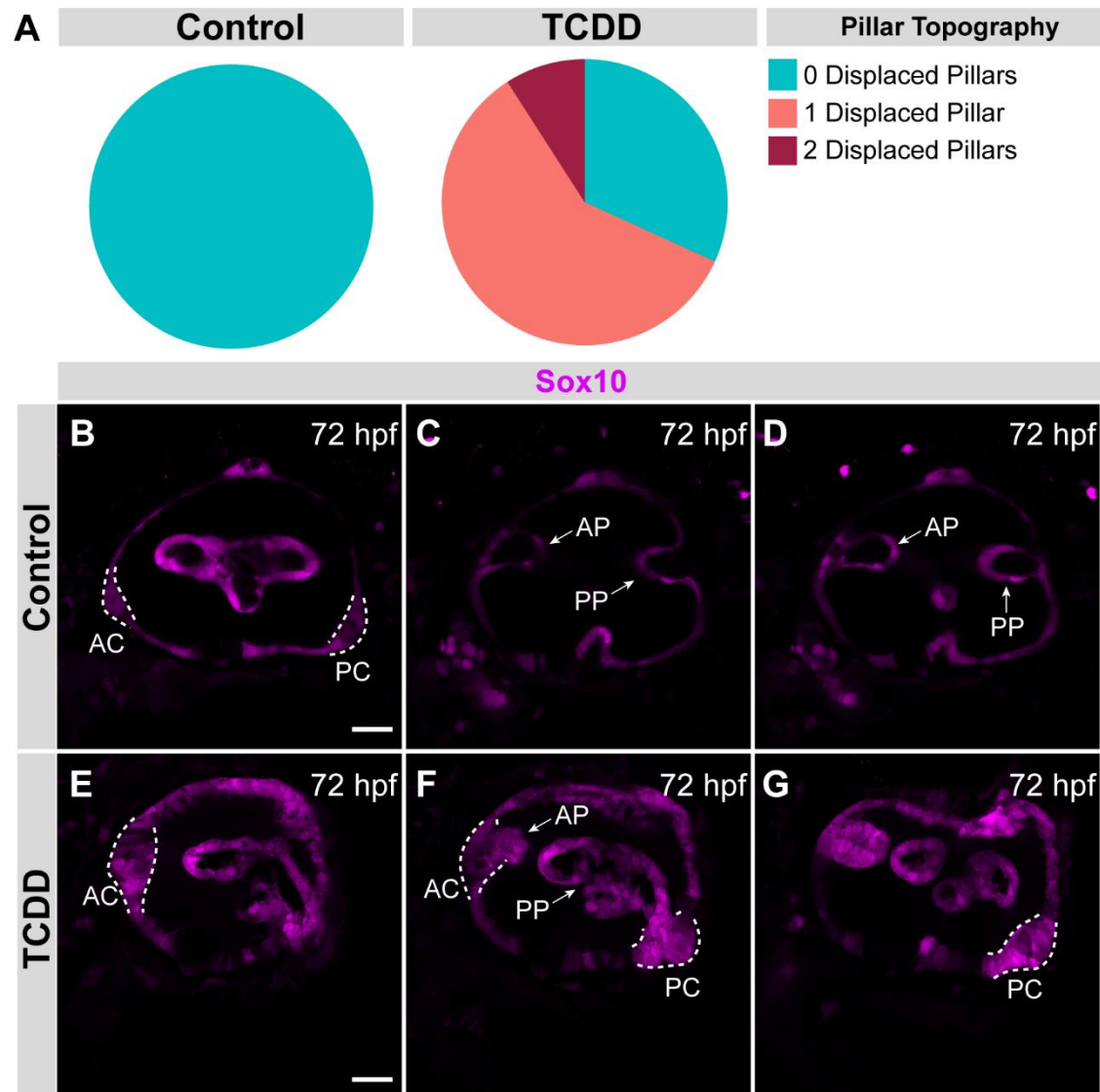
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Figure 6. Pillar topography defect quantification and phenotypes. Percent of control and TCDD exposed embryos with zero, one, or two displaced pillars at 72 hpf (A). Confocal slices capturing the fluorescent reporter *Tg(Sox10:RFP),* in magenta, in the developing otic epithelium at 72hpf with a 40x magnification (B-G). Control ear displaying typical pillar topography (B-D). TCDD exposed ear exhibiting displacement of both the anterior and posterior pillar (E-G). Abbreviations: anterior pillar (AP), ventral pillar (VP), posterior pillar (PP), dorsolateral septum (DLS), anterior crista (AC), posterior crista (PC). 25µm scale bar for reference (B,E). n=20-24/group with a minimum of 3 replicates.
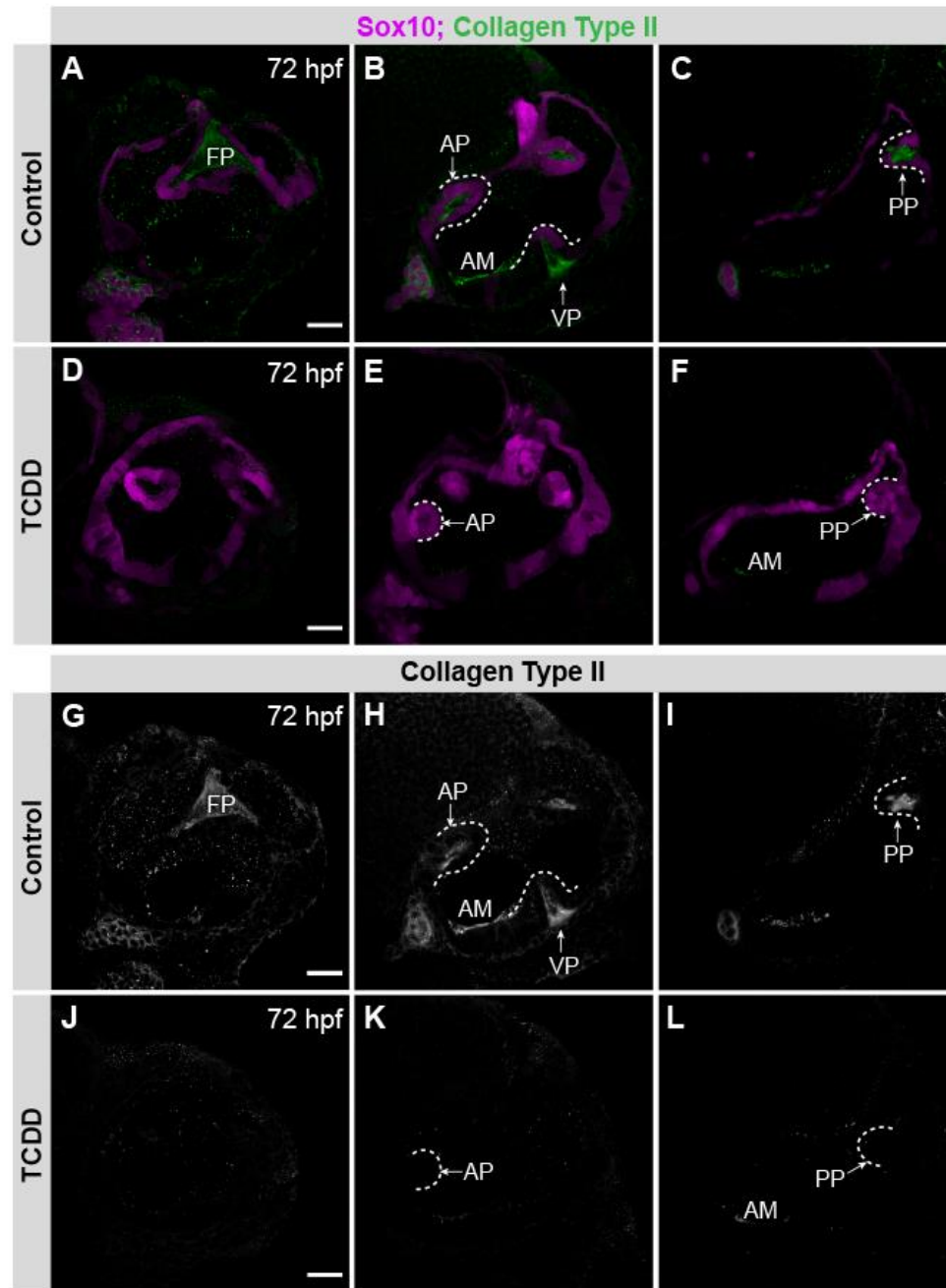
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Figure 7. Loss of collagen type II expression in the ear following TCDD exposure. (A-L) 40x confocal micrographs highlighting different portions of the developing ear at 72 hpf. Control (A-C) and TCDD exposed embryos (D-F) expressing the fluorescent reporter *Tg(sox10:RFP)* (magenta) and immunostained for collagen type II (green). (G-L) Black and white images of collagen type II in control (D-F) and TCDD exposed (J-L) larval ears. Abbreviations: fusion plate (FP), anterior macula (AM), anterior pillar (AP), ventral pillar (VP), posterior pillar (PP). Scale bar in A,D,G,J = 25μm.n=11-8/group with 3 replicates.

9 References

Abbas, L., & Whitfield, T. T. (2010). The zebrafish inner ear. *Fish Physiology*, *29*(C), 123–171. https://doi.org/10.1016/S1546-5098(10)02904-3

Acke, F. R. E., Dhooge, I. J. M., Malfait, F., & De Leenheer, E. M. R. (2012). Hearing impairment in Stickler syndrome: A systematic review. *Orphanet Journal of Rare Diseases*, *7*(1), 1. https://doi.org/10.1186/1750-1172-7-84

Andreasen, E. A., Mathew, L. K., Löhr, C. V., Hasson, R., & Tanguay, R. L. (2007). Aryl hydrocarbon receptor activation impairs extracellular matrix remodeling during zebra fish fin regeneration. *Toxicological Sciences*, *95*(1), 215–226. https://doi.org/10.1093/toxsci/kfl119

Andreasen, E. A., Mathew, L. K., & Tanguay, R. L. (2006). Regenerative growth is impacted by TCDD: Gene expression analysis reveals extracellular matrix modulation. *Toxicological Sciences*, *92*(1), 254–269. https://doi.org/10.1093/toxsci/kfj118

Barrionuevo, F., Naumann, A., Bagheri-Fam, S., Speth, V., Taketo, M. M., Scherer, G., & Neubüser, A. (2008). Sox9 is required for invagination of the otic placode in mice. *Developmental Biology*, *317*(1), 213–224. https://doi.org/10.1016/j.ydbio.2008.02.011

Bashir, I., Lone, F. A., Bhat, R. A., Mir, S. A., Dar, Z. A., & Dar, S. A. (2020). *Concerns and Threats of Contamination on Aquatic Ecosystems*.

Baxendale, S., & Whitfield, T. T. (2016). Methods to study the development, anatomy, and function of the zebrafish inner ear across the life course. In *Methods in Cell Biology* (Vol. 134). https://doi.org/10.1016/bs.mcb.2016.02.007

Beiriger, A., Narayan, S., Singh, N., & Prince, V. (2021). Development and migration of the zebrafish rhombencephalic octavolateral efferent neurons. *Journal of Comparative Neurology*, *529*(7), 1293–1307. https://doi.org/10.1002/cne.25021

Bell, D. M., Leung, K. K. H., Wheatley, S. C., Ling Jim Ng, Zhou, S., Kam Wing Ling, … Cheah, K. S. E. (1997). SOX9 directly regulates the type-II collagen gene. *Nature Genetics*, *16*(2), 174–178. https://doi.org/10.1038/ng0697-174

Bever, M. M., & Fekete, D. M. (2002). Atlas of the developing inner ear in zebrafish. *Developmental Dynamics*, *223*(4), 536–543. https://doi.org/10.1002/dvdy.10062

Blaxter, J. H. S. (1987). Structure and Development of the Lateral Line. *Biological Reviews*, *62*(4), 471–514. https://doi.org/10.1111/j.1469-185x.1987.tb01638.x

Bowles, J., Feng, C. W., Ineson, J., Miles, K., Spiller, C. M., Harley, V. R., … Koopman, P. (2018). Retinoic Acid Antagonizes Testis Development in Mice. *Cell Reports*, *24*(5), 1330–1341. https://doi.org/10.1016/j.celrep.2018.06.111

Briggs, D. (2003). Environmental pollution and the global burden of disease. *British Medical Bulletin*, *68*, 1–24. https://doi.org/10.1093/bmb/ldg019

Burns, F. R., Peterson, R. E., & Heideman, W. (2015). Dioxin disrupts cranial cartilage and dermal bone development in zebrafish larvae. *Aquatic Toxicology*, *164*, 52–60. https://doi.org/10.1016/j.aquatox.2015.04.005

Carney, S. A., Prasch, A. L., Heideman, W., & Peterson, R. E. (2006). Understanding dioxin developmental toxicity using the zebrafish model. *Birth Defects Research Part A - Clinical and Molecular Teratology*, *76*(1), 7–18. https://doi.org/10.1002/bdra.20216

Chang, W., Lin, Z., Kulessa, H., Hebert, J., Hogan, B. L. M., & Wu, D. K. (2008). Bmp4 is essential for the formation of the vestibular apparatus that detects angular head movements. *PLoS Genetics*, *4*(4). https://doi.org/10.1371/journal.pgen.1000050

Chiang, E. F. L., Pai, C. I., Wyatt, M., Yan, Y. L., Postlethwait, J., & Chung, B. chu. (2001). Two sox9 genes on duplicated zebrafish chromosomes: Expression of similar transcription activators in distinct sites. *Developmental Biology*, *231*(1), 149–163. https://doi.org/10.1006/dbio.2000.0129

Chung, B., Development, J. H., Yan, Y., Miller, C. T., Nissen, R. M., Singer, A., … Westerfield,

574   M. (n.d.). *A zebrafish sox9 gene required for cartilage morphogenesis DEVELOPMENT*
575   *AND DISEASE A zebrafish sox9 gene required for cartilage morphogenesis.* 5065–5079.
576 Collins, L. L., Williamson, M. A., Thompson, B. D., Dever, D. P., Gasiewicz, T. A., & Opanashuk,
577   L. A. (2008). 2,3,7,8-Tetracholorodibenzo-p-dioxin exposure disrupts granule neuron
578   precursor maturation in the developing mouse cerebellum. *Toxicological Sciences*, *103*(1),
579   125–136. https://doi.org/10.1093/toxsci/kfn017
580 Dai, Y. J., Jia, Y. F., Chen, N., Bian, W. P., Li, Q. K., Ma, Y. B., … Pei, D. S. (2014). Zebrafish
581   as a model system to study toxicology. *Environmental Toxicology and Chemistry*, *33*(1),
582   11–17. https://doi.org/10.1002/etc.2406
583 Dale, R. M., & Topczewski, J. (2011). Identification of an evolutionarily conserved regulatory
584   element of the zebrafish col2a1a gene. *Developmental Biology*, *357*(2), 518–531.
585   https://doi.org/10.1016/j.ydbio.2011.06.020
586 Dong, W., Hinton, D. E., & Kullman, S. W. (2012). TCDD disrupts hypural skeletogenesis during
587   medaka embryonic development. *Toxicological Sciences, 125*(1), 91–104.
588   https://doi.org/10.1093/toxsci/kfr284
589 Dopico, M., & Gómez, A. (2015). Review of the current state and main sources of dioxins
590   around the world. *Journal of the Air and Waste Management Association*, *65*(9), 1033–
591   1049. https://doi.org/10.1080/10962247.2015.1058869
592 Dutton, K., Abbas, L., Spencer, J., Brannon, C., Mowbray, C., Nikaido, M., … Whitfield, T. T.
593   (2009). A zebrafish model for Waardenburg syndrome type IV reveals diverse roles for
594   Sox10 in the otic vesicle. *DMM Disease Models and Mechanisms*, *2*(1–2), 68–83.
595   https://doi.org/10.1242/dmm.001164
596 Fiorella, P. D., Olson, J. R., & Napoli, J. L. (1995). *2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin*
597   *induces diverse retinoic acid metabolites in multiple tissues of the Sprague-Dawley rat.* (pp.
598   134(2), 222-228.). pp. 134(2), 222-228.
599   https://doi.org/https://doi.org/10.1006/taap.1995.1187
600 Garcia, G. R., Shankar, P., Dunham, C. L., Garcia, A., La Du, J. K., Truong, L., … Tanguay, R.
601   L. (2018). Signaling events downstream of AHR activation that contribute to toxic
602   responses: The functional role of an ahr-dependent long noncoding RNA (slincR) using the
603   Zebrafish Model. *Environmental Health Perspectives*, *126*(11).
604   https://doi.org/10.1289/EHP3281
605 Geng, F. S., Abbas, L., Baxendale, S., Holdsworth, C. J., George Swanson, A., Slanchev, K., …
606   Whitfield, T. T. (2013). Semicircular canal morphogenesis in the zebrafish inner ear
607   requires the function of gpr126 (lauscher), an adhesion class G protein-coupled receptor
608   gene. *Development (Cambridge)*, *140*(21), 4362–4374. https://doi.org/10.1242/dev.098061
609 He, N., Brysk, H., Tyring, S. K., Ohkubo, I., & Brysk, M. M. (2001). Transcriptional suppression
610   of Sox9 expression in chondrocytes by retinoic acid. *Journal of Cellular Biochemistry*,
611   *81*(SUPPL. 36), 71–78. https://doi.org/10.1002/jcb.1077
612 Herlin, M., Sánchez-Pérez, I., Esteban, J., Korkalainen, M., Barber, X., Finnilä, M. A. J., …
613   Håkansson, H. (2021). Bone toxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin
614   (TCDD) and the retinoid system: A causality analysis anchored in osteoblast gene
615   expression and mouse data. *Reproductive Toxicology*, *105*(August), 25–43.
616   https://doi.org/10.1016/j.reprotox.2021.07.013
617 Hill, A., Howard, C. V., Strahle, U., & Cossins, A. (2003). Neurodevelopmental defects in
618   zebrafish (Danio rerio) at environmetally relevant dioxin (TCDD) concentrations.
619   *Toxicological Sciences*, *76*(2), 392–399. https://doi.org/10.1093/toxsci/kfg241
620 Hofsteen, P., Plavicki, J., Johnson, S. D., Peterson, R. E., & Heideman, W. (2013). Sox9b is
621   required for epicardium formation and plays a role in TCDD-induced heart malformation in
622   zebrafish. *Molecular Pharmacology*, *84*(3), 353–360.
623   https://doi.org/10.1124/mol.113.086413
624 Iida, M., Kim, E. Y., Murakami, Y., Shima, Y., & Iwata, H. (2013). Toxic effects of 2,3,7,8-

tetrachlorodibenzo-p-dioxin on the peripheral nervous system of developing red seabream (Pagrus major). *Aquatic Toxicology*, *128–129*, 193–202. https://doi.org/10.1016/j.aquatox.2012.12.009

Jacobs, H., Dennefeld, C., Féret, B., Viluksela, M., Håkansson, H., Mark, M., & Ghyselinck, N. B. (2011). Retinoic acid drives aryl hydrocarbon receptor expression and is instrumental to dioxin-induced toxicity during palate development. *Environmental Health Perspectives*, *119*(11), 1590–1595. https://doi.org/10.1289/ehp.1003075

Kim, K. H., Park, H. J., Kim, J. H., Kim, S., Williams, D. R., Kim, M. K., … Choi, S. Y. (2013). Cyp1a reporter zebrafish reveals target tissues for dioxin. *Aquatic Toxicology*, *134–135*, 57–65. https://doi.org/10.1016/j.aquatox.2013.03.010

Kimura, E., Endo, T., Yoshioka, W., Ding, Y., Ujita, W., Kakeyama, M., & Tohyama, C. (2016). In utero and lactational dioxin exposure induces Sema3b and Sema3g gene expression in the developing mouse brain. *Biochemical and Biophysical Research Communications*, *476*(2), 108–113. https://doi.org/10.1016/j.bbrc.2016.05.048

Kirby, B. B., Takada, N., Latimer, A. J., Shin, J., Carney, T. J., Kelsh, R. N., & Appel, B. (2006). In vivo time-lapse imaging shows dynamic oligodendrocyte progenitor behavior during zebrafish development. *Nature Neuroscience*, *9*(12), 1506–1511. https://doi.org/10.1038/nn1803

Kossack, M. E., Manz, K. E., Martin, N. R., Pennell, K. D., & Plavicki, J. (2023). Environmentally relevant uptake, elimination, and metabolic changes following early embryonic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish. *Chemosphere*, *310*(October 2022), 136723. https://doi.org/10.1016/j.chemosphere.2022.136723

Kozlowski, D. J., Whitfield, T. T., Hukriede, N. A., Lam, W. K., & Weinberg, E. S. (2005). The zebrafish dog-eared mutation disrupts eya1, a gene required for cell survival and differentiation in the inner ear and lateral line. *Developmental Biology*, *277*(1), 27–41. https://doi.org/10.1016/j.ydbio.2004.08.033

Liu, D., Chu, H., Maves, L., Yan, Y. L., Morcos, P. A., Postlethwait, J. H., & Westerfield, M. (2003). Fgf3 and Fgf8 dependent and independent transcription factors are required for otic placode specification. *Development*, *130*(10), 2213–2224. https://doi.org/10.1242/dev.00445

Lopes, S. S., Elworthy, S., Haffter, P., Rauch, J., Dutton, K. A., Pauliny, A., … Kelsh, R. N. (2001). Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development*, *128*(21), 4113–4125. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed&cmd=Retrieve&dopt=AbstractPlus&list_uids=11684650

Mackowetzky, K., Dicipulo, R., Fox, S. C., Philibert, D. A., Todesco, H., Doshi, J. D., … Waskiewicz, A. J. (2022). Retinoic acid signaling regulates late stages of semicircular canal morphogenesis and otolith maintenance in the zebrafish inner ear. *Developmental Dynamics*, (September 2021), 1798–1815. https://doi.org/10.1002/dvdy.510

Manzetti, S., & Spoel, D. Van Der. (2014). *Chemical Properties, Environmental Fate, and Degradation of Seven Classes of Pollutants*.

Martin, N. R., Kossack, M. E., Tian, L., Camarillo, M. A., Cintrón-rivera, L. G., Gawdzik, J. C., … Nwagugo, F. O. (2022). *Proper modulation of AHR signaling is necessary for establishing neural connectivity and oligodendrocyte precursor cell development in the embryonic zebrafish brain*. (November), 1–18. https://doi.org/10.3389/fnmol.2022.1032302

Mehta, V., Peterson, R. E., & Heideman, W. (2008). 2,3,7,8-Tetrachlorodibenzo-p-dioxin Exposure Prevents Cardiac Valve Formation in Developing Zebrafish. *Toxicological Sciences*, *104*(2), 303–311. https://doi.org/10.1093/toxsci/kfn095

Melvin, S. D., & Wilson, S. P. (2013). The utility of behavioral studies for aquatic toxicology testing: A meta-analysis. *Chemosphere*, *93*(10), 2217–2223. https://doi.org/10.1016/j.chemosphere.2013.07.036

676   Mimura, J., & Fujii-Kuriyama, Y. (2003). Functional role of AhR in the expression of toxic effects
677   by TCDD. *Biochimica et Biophysica Acta - General Subjects*, *1619*(3), 263–268.
678   https://doi.org/10.1016/S0304-4165(02)00485-3
679   Moens, C. B., Cordes, S. P., Giorgianni, M. W., Barsh, G. S., & Kimmel, C. B. (1998).
680   Equivalence in the genetic control of hindbrain segmentation in fish and mouse.
681   *Development*, *125*(3), 381–391. https://doi.org/10.1242/dev.125.3.381
682   Moorman, S. J. (2001). Development of sensory systems in zebrafish (Danio rerio). *ILAR*
683   *Journal*, *42*(4), 292–298. https://doi.org/10.1093/ilar.42.4.292
684   Murphy, K. A., Villano, C. M., Dorn, R., & White, L. A. (2004). Interaction between the aryl
685   hydrocarbon receptor and retinoic acid pathways increases matrix metalloproteinase-1
686   expression in keratinocytes. *Journal of Biological Chemistry*, *279*(24), 25284–25293.
687   https://doi.org/10.1074/jbc.M402168200
688   Park, Byung-Yong, and J. S. (2010). *Long-term consequences of Sox9 depletion on inner ear*
689   *development* (pp. 1102-1112.). pp. 1102-1112. Developmental Dynamics.
690   Patrizi, B., & de Cumis, M. S. (2018). TCDD toxicity mediated by epigenetic mechanisms.
691   *International Journal of Molecular Sciences*, *19*(12), 1–15.
692   https://doi.org/10.3390/ijms19124101
693   Plavicki, J., Hofsteen, P., Peterson, R. E., & Heideman, W. (2013). Dioxin inhibits zebrafish
694   epicardium and proepicardium development. *Toxicological Sciences*, *131*(2), 558–567.
695   https://doi.org/10.1093/toxsci/kfs301
696   Rysavy, N. M., Maaetoft-udsen, K., & Turner, H. (2013). *Dioxins : diagnostic and prognostic*
697   *challenges arising from complex mechanisms*. (August 2011), 1–8.
698   https://doi.org/10.1002/jat.2759
699   Saint-Germain, N., Lee, Y. H., Zhang, Y., Sargent, T. D., & Saint-Jeannet, J. P. (2004).
700   Specification of the otic placode depends on Sox9 function in Xenopus. *Development*,
701   *131*(8), 1755–1763. https://doi.org/10.1242/dev.01066
702   Schmidt, C. K., Hoegberg, P., Fletcher, N., Nilsson, C. B., Trossvik, C., Håkansson, H., & Nau,
703   H. (2003). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the endogenous metabolism
704   of all-trans-retinoic acid in the rat. *Archives of Toxicology*, *77*(7), 371–383.
705   https://doi.org/10.1007/s00204-003-0457-8
706   Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., & Voelker, D. (2008). The
707   zebrafish embryo model in environmental risk assessment - Applications beyond acute
708   toxicity testing. *Environmental Science and Pollution Research*, *15*(5), 394–404.
709   https://doi.org/10.1007/s11356-008-0018-z
710   She, Z. Y., & Yang, W. X. (2017). Sry and SoxE genes: How they participate in mammalian sex
711   determination and gonadal development? *Seminars in Cell and Developmental Biology*, *63*,
712   13–22. https://doi.org/10.1016/j.semcdb.2016.07.032
713   Stolt, C. C., & Wegner, M. (2010). SoxE function in vertebrate nervous system development.
714   *International Journal of Biochemistry and Cell Biology*, *42*(3), 437–440.
715   https://doi.org/10.1016/j.biocel.2009.07.014
716   Sugden, W. W., Leonardo-Mendonça, R. C., Acuña-Castroviejo, D., & Siekmann, A. F. (2017).
717   Genetic dissection of endothelial transcriptional activity of zebrafish aryl hydrocarbon
718   receptors (AHRs). *PLoS ONE*, *12*(8), 1–22. https://doi.org/10.1371/journal.pone.0183433
719   Szeto, I. Y. Y., Chu, D. K. H., Chen, P., Chu, K. C., Au, T. Y. K., Leung, K. K. H., … Cheah, K.
720   S. E. (2022). SOX9 and SOX10 control fluid homeostasis in the inner ear for hearing
721   through independent and cooperative mechanisms. *Proceedings of the National Academy*
722   *of Sciences of the United States of America*, *119*(46), 1–12.
723   https://doi.org/10.1073/pnas.2122121119
724   Watson, A. L. T. D., Planchart, A., Mattingly, C. J., Winkler, C., Reif, D. M., & Kullman, S. W.
725   (2017). Embryonic exposure to TCDD impacts osteogenesis of the axial skeleton in
726   Japanese medaka, Oryzias latipes. *Toxicological Sciences*, *155*(2), 485–496.

727   https://doi.org/10.1093/toxsci/kfw229

728 Wernet, M. F., Mazzoni, E. O., Çelik, A., Duncan, D. M., Duncan, I., & Desplan, C. (2006).
729   Stochastic spineless expression creates the retinal mosaic for colour vision. *Nature*,
730   *440*(7081), 174–180. https://doi.org/10.1038/nature04615

731 Weston, W. M., Nugent, P., & Greene, R. M. (1995). *Inhibition of retinoic-acid-induced gene*
732   *expression by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin.* (pp. 207(2), 690–694). pp. 207(2),
733   690–694. https://doi.org/https://doi.org/10.1006/bbrc.1995.1242

734 White, S. S., & Birnbaum, L. S. (2009). *An Overview of the Effects of Dioxins and Dioxin- Like*
735   *Compounds on Vertebrates , as Documented in Human and Ecological Epidemiology An*
736   *Overview of the Effects of Dioxins and Dioxin-Like Compounds on Vertebrates , as*
737   *Documented in Human and Ecological Epi. 0501.*
738   https://doi.org/10.1080/10590500903310047

739 Whitfield, T. T. (2002). Zebrafish as a model for hearing and deafness. *Journal of Neurobiology*,
740   *53*(2), 157–171. https://doi.org/10.1002/neu.10123

741 Whitfield, T. T. (2020). Cilia in the developing zebrafish ear. *Philosophical Transactions of the*
742   *Royal Society B: Biological Sciences*, *375*(1792), 0–3.
743   https://doi.org/10.1098/rstb.2019.0163

744 Whitfield, T. T., Riley, B. B., Chiang, M. Y., & Phillips, B. (2002). Development of the zebrafish
745   inner ear. *Developmental Dynamics*, *223*(4), 427–458. https://doi.org/10.1002/dvdy.10073

746 Williams, A. L. (2022). *Zebrafish Model of Stickler Syndrome Suggests a Role for Col2a1a in the*
747   *Neural Crest during Early Eye Development.*

748 Williamson, M. A., Gasiewicz, T. A., & Opanashuk, L. A. (2005). Aryl hydrocarbon receptor
749   expression and activity in cerebellar granule neuroblasts: Implications for development and
750   dioxin neurotoxicity. *Toxicological Sciences*, *83*(2), 340–348.
751   https://doi.org/10.1093/toxsci/kfi031

752 Xiong, K. M., Peterson, R. E., & Heideman, W. (2008). Aryl hydrocarbon receptor-mediated
753   down-regulation of Sox9b causes jaw malformation in zebrafish embryos. *Molecular*
754   *Pharmacology*, *74*(6), 1544–1553. https://doi.org/10.1124/mol.108.050435

755 Xu, H., Li, C., Li, Y., Ng, G. H. B., Liu, C., Zhang, X., & Gong, Z. (2015). Generation of
756   Tg(cyp1a:gfp) Transgenic Zebrafish for Development of a Convenient and Sensitive In Vivo
757   Assay for Aryl Hydrocarbon Receptor Activity. *Marine Biotechnology*, *17*(6), 831–840.
758   https://doi.org/10.1007/s10126-015-9669-1

759 Yan, Y. L., Willoughby, J., Liu, D., Crump, J. G., Wilson, C., Miller, C. T., … Postlethwait, J. H.
760   (2005). A pair of Sox: Distinct and overlapping functions of zebrafish sox9 co-orthologs in
761   craniofacial and pectoral fin development. *Development*, *132*(5), 1069–1083.
762   https://doi.org/10.1242/dev.01674

763 Yang, L., Ho, N. Y., Alshut, R., Legradi, J., Weiss, C., Reischl, M., … Strähle, U. (2009).
764   Zebrafish embryos as models for embryotoxic and teratological effects of chemicals.
765   *Reproductive Toxicology*, *28*(2), 245–253. https://doi.org/10.1016/j.reprotox.2009.04.013

766 Yue, M. S., Martin, S. E., Martin, N. R., Taylor, M. R., & Plavicki, J. S. (2021). 2,3,7,8-
767   Tetrachlorodibenzo-p-dioxin exposure disrupts development of the visceral and ocular
768   vasculature. *Aquatic Toxicology*, *234*(November 2020), 105786.
769   https://doi.org/10.1016/j.aquatox.2021.105786

770