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The case for thyroid disruption in early life stage exposures to thiram in zebrafish (*Danio rerio*)

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ABSTRACT

Thiram, a pesticide in the dithiocarbamate chemical family, is widely used to prevent fungal disease in seeds and crops. Its off-site movement to surface waters occurs and may place aquatic organisms at potential harm. Zebrafish embryos were used for investigation of acute (1 h) thiram exposure (0.001–10 μM) at various developmental stages. Survival decreased at 1 μM and 10 μM and hatching was delayed at 0.1 μM and 1 μM. Notochord curvatures were seen at 0.1 and 1 μM thiram when exposure was initiated at 2 and at 10 hpf. Similar notochord curvatures followed exposure to the known TPO inhibitor, methimazole (MMI). Changes were absent in embryos exposed at later stages, i.e., 12 hpf. In embryos exposed to 0.1 or 1 μM at 10 hpf, levels of the thyroid enzyme, Deiodinase 3, increased by 12 hpf. Thyroid peroxide (TPO), important in T4 synthesis, decreased by 48 hpf in embryos exposed to 1 μM at 10 hpf. Thiram toxicity was stage-dependent and early life stage exposure may be responsible for adverse effects seen later. These effects may be due to impacts on the thyroid via regulation of specific thyroid genes including *TPO* and Deiodinase 3.

1. Introduction

Dithiocarbamates have been widely used as pesticides for decades and are considered relatively safe for mammals (Nougadere et al. 2012). Thiram, a representative dithiocarbamate, has seen wide usage as a fungicide on seeds and as an insect repellent, especially in China and India (Mittal et al., 2014). Though it is a fungicide with low toxicity, bioaccumulation has been reported in several studies (Borg and Tjalve, 1988; Mishra et al., 1993; Mittal et al., 2014), raising concerns. Zineb, similar to thiram, has been phased out in several countries due to its strong inhibitory effect on thyroid peroxidase (TPO) (Flippin et al., 2009; Marinovich et al., 1997; Rath et al., 1995); such inhibition can lead to reductions in circulating levels of thyroxine (T4) (Kackar et al., 1997). In a real-world exposure scenario, it is often not a single exposure that occurs but, rather, multiple, inadvertent exposures of short duration of these compounds (Schultz et al., 2003).

The thyroid functions to regulate metabolism, particularly important during development (Polak, 2014). Significant decreases in maternal, fetal or neonatal circulating thyroid hormones (TH) have been associated with neurological deficits and developmental defects. For example, during pregnancy in humans, lower levels of maternal T4 during the first trimester were associated with decreased IQ in children (Gilbert et al., 2012; Lazarus, 2005). Contaminant-induced alterations in TH homeostasis could potentially disrupt fundamental biological processes in aquatic organisms. Both metamorphosis in frogs and in several teleosts are initiated by THs (Kulkarni and Buchholz, 2014;

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Temple et al., 2008; Veldhoen et al., 2014). T4, primarily produced in the thyroid gland is transported to targeted tissues where it is converted by deiodination to active T3 (Sapin and Schlienger, 2003). Xenobiotics can directly alter the circulating levels of THs through changes in iodine uptake, TPO activity, or TH secretion (Simon et al., 2002). In addition, extra-thyroidal mechanisms may affect TH levels by altering binding to hormone transporters, inhibiting deiodination, altering thyroid hormone receptors causing them to be more or less effective, or by a combination of these (McKinney et al., 1985; McKinney and Waller, 1998).

Numerous environmental chemicals including pesticides, polychlorinated dioxins and dibenzofurans, polychlorinated biphenyls, and polybrominated diphenyl esters disrupt TH homeostasis in animal models (Dong et al., 2014; Gabrielsen et al., 2015). Deiodinase plays a vital role in the homeostasis of TH (Johnson and Lema, 2011; Kohrle, 2007). Deiodinase type I (*dio1*) and type II (*dio2*) transform T4 to the active T3, an important hormone for development and reproduction. Deiodinase type III (*dio3*) primarily inactivates thyroid hormones (e.g., converts active T3 to inactive diiodo-L-thyronine (T2)) (Bianco and Kim, 2006; Panicker, 2011). Also, *TPO*, an enzyme normally found in the thyroid gland, plays an important role in the production of thyroid hormones, specifically that of T4 (Bianco and Kim, 2006; Panicker, 2011).

In the USEPA's ToxCast screening of compounds using the zebrafish (*Danio rerio*) model, thiram was identified as one of the most potent compounds to distort the notochord (Reif et al., 2015; Truong et al., 2014; Ellis-Hutchings et al., 2017). The notochord is essential in chordate development and such distortions would likely have consequences for survivors later in life (Stemple et al., 1996). Studies have shown thiram to cause this distortion when zebrafish embryos were exposed at precisely 18 h post-fertilization (hpf), accompanied by an onset of spontaneous rhythmic trunk contractions (Teraoka et al., 2006; Tilton et al., 2006). Additional evidence of thyroid involvement was demonstrated when notochord distortion was observed *in vivo* using *Xenopus tropicalis* embryos exposed to methimazole (MMI) (Tindall et al., 2007), a well-known *TPO* inhibitor. Inhibition of TH by phenylthiourea (PTU) and by MMI also resulted in curvature of the bodies and tails in zebrafish (Elsalini and Rohr, 2003; Komoike et al., 2013). PTU and MMI also caused abnormalities of fetal rats *in vitro* (Stanisstreet et al., 1990). When Ferrand et al. (2003) investigated the effect of dithiocarbamate compounds on *TPO* activity in Chinese hamster ovary (CHO) cells transfected with the human *TPO* gene, results indicated down-regulation of *TPO*. In our previous studies of TH with other compounds (Dong et al., 2014; Dong et al., 2013), similar toxicity was noted. Additionally, THs regulate craniofacial development in zebrafish larvae (Liu and Chan, 2002). For example, adult zebrafish exposed to perchlorate exhibited thyroid disruption and produced offspring with craniofacial changes (Mukhi and Patino, 2006). TH is a very important hormone in zebrafish embryonic development (Brown, 1997); however, no mechanism for TH disruption has been published for thiram in zebrafish, specifically its possible interaction with notochord distortion.

This motivated us to investigate whether thiram might be inhibiting the thyroid, the master gland. If so, exposure might affect the notochord as well as craniofacial development. For the present study we conducted short term (1 h) exposures of embryos to various, environmentally relevant concentrations of thiram, initiated at different stages of development. We examined the role of thiram in the disruption of TH signaling by measuring the gene expression of deiodinase (*dio1*, *dio2*, and *dio3*) and expression and localization of *TPO*. Finally, we repeated the exposure with MMI both with and without T3 to confirm the role of *TPO* and *dio3* in zebrafish notochord curvature.

2. Methods

2.1. Fish husbandry

Adult zebrafish were maintained in a recirculating AHAB system (Aquatic Habitats, Apopka, FL) at 28 °C and under a 14:10 h light:dark cycle. Fish were fed with Ziegler's Adult Zebrafish Complete Diet (Aquatic Habitats) supplemented with *Artemia* nauplii (Dong et al., 2013). Following spawning, embryos were collected and maintained in 30% Danieau medium (2.9 M NaCl, 70 mM KCl, 40 mM $MgSO_4{\cdot}7H_2O$, 60 mM $Ca(NO_3)_2$, 0.5 M HEPES pH 7.2) under the same environmental conditions as above. Broodstock care and reproductive techniques were approved by the Duke University Institutional Animal Care & Use Committee.

2.2. Exposure regime

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Tetramethylthiuram disulfide (CAS No. 137-26-8; a.k.a. thiuram) and methamizole (MMI; CAS No. 60-56-0) were purchased neat (> 99.5% purity) and stock solutions prepared in dimethylsulfoxide (DMSO). Environmentally relevant concentrations of thiram were selected based on reported values in soil samples (0.22–27.04 mg/kg) (Zainol et al., 2010) and in water and palm leaves (0.27–2.52 mg/L and 1.34–12.28 mg/kg, respectively) (Maznah et al., 2018).

For exposure, 30% Danieau medium was used for serial dilution of stock solutions yielding a final concentration of DMSO ≤ 0.1%; controls received the same concentration of DMSO as other treatment groups. To investigate the developmental toxicity of thiram, embryos at 2, 10, 24, or 60 hpf were exposed for 1 h to one of five concentrations (0.001, 0.01, 0.1, 1, or 10 μM), then immediately rinsed with DI water and incubated in 30% Danieau medium. Three replicates, each containing 10–16 embryos, were prepared for each dosing condition in glass scintillation vials (Wheaton, VWR International, Suwanee, GA), resulting in a total 30–48 embryos per treatment group. All embryos were observed daily for development under a stereomicroscope (Nikon SMZ1500, Nikon Instruments Inc., Melville, NY) until 120 hpf, and the presence/absence of phenotypes including mortality, hatching rate, pericardial edema, and curvature of the notochord were recorded. As a comparison for thiram, we also exposed embryos at 10 hpf to MMI in a range of concentrations (12.5, 25, or 50 mM). MMI exposed embryos were observed and imaged every 24 h until 96 hpf. To investigate the role of endogenous active thyroid hormone T3, additional replicates of embryos were co-exposed with it and either thiram or MMI.

2.3. Cartilage and bone staining

At 96 hpf, larvae were euthanized with MS-222 and co-stained for cartilage with Alcian Blue 8GX and bone with Alizarin Red S following published methods (Inohaya and Kudo, 2000). Briefly, larvae were fixed in 4% (w/v) paraformaldehyde (PFA) for 24 h at 4 °C, rinsed twice with PBS, and stained by emersion in 0.1% Alcian Blue 8GX/80% ethanol/20% glacial acetic acid for 16 h. Next, specimens were taken through a series of 1 h rinses with 75 and 50% ethanol/PBS and then incubated in PBS overnight. Afterward, specimens were immersed in 1% KOH/3% H_2O_2 for 20 h, followed by digestion with 0.05% trypsin/30% sodium carbonate tetraborate for 1 h. Specimens were then stained in Alizarin Red S solution at room temperature until bones were noticeably red, washed two times in 0.5% aqueous KOH, and then placed in 80% glycerol solution. Finally, all specimens were observed and photographed (Nikon DXM1200 digital camera; Nikon NIS-Elements 3.20.01; Nikon Instruments Inc.).

Another subset of embryos was exposed to 0.2 μM thiram for 1 h starting at 10 hpf, cultured under clean conditions, euthanized at 33 days post fertilization (dpf), stained according to the above description, and then directly observed and imaged.

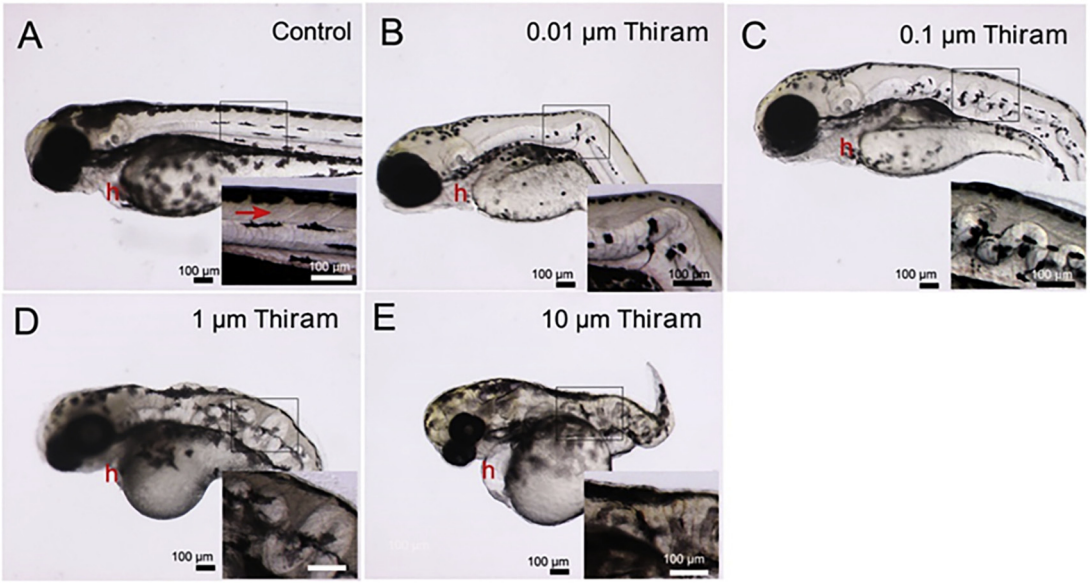

Fig. 1. Examples of pericardial edema and notochord curvatures in 96 hpf zebrafish larvae exposed to various concentrations of thiram at 10 hpf for 1 h. A) control; B) 0.01 μM thiram; C) 0.1 μM thiram; D) 1 μM thiram; and E) 10 μM thiram. Pericardial edema is evident in the region of the heart (h) in highest concentration of thiram (E), and undulation of the notochord evident in all exposures (B-E) compared to control (red arrow in inset of panel A indicates straight notochord). Scale bars are 100 μm.

An assessment of jaw morphometry was conducted by measuring the lengths of Meckel's and ceratohyal cartilage complexes in 10 larvae per treatment according to Mukhi and Patino (2006). These authors provide a method that utilizes how the U- or V-shaped nature of these complexes forms an isosceles triangle. Stained individuals were imaged in a dorsal orientation and the sides (s) and bases (b) were digitally measured (Fig. S3). These measurements were used in the Pythagorean formula, $l = \sqrt{(s^2 - b^2/4)}$ to calculate length (l), or forward protrusion, of the lower jaw.

2.4. Whole mount in situ hybridization

Upon reaching desired life stages, embryos were fixed in 4% (w/v) PFA in PBS (pH 7.4) overnight. Embryos were dechorionated using watchmaker's forceps and then stored in methanol at −20 °C until further processing. Whole mount *in situ* hybridization (WISH) was conducted using our previously reported methods (Dong et al., 2013; Fang et al., 2013). In brief, embryos were hybridized overnight at 64 °C with an antisense probe of 528 base pairs for zebrafish thyroid oxidase (*TPO*). *TPO* probe was cloned with the following primers: forward primer, 5′-TCTCCAGATCAGCAGAAATC-3′; reverse primer, 5′- AAGCA GGGATCTGCACTGAC-3′. Next, embryos were washed with 2 × SSC (300 mM NaCl, 30 mM sodium citrate pH 7.0) and then 0.2 × SSC for 30 min, twice respectively. Following hybridization, embryos were blocked using a 2% blocking reagent (Roche, Mannheim, Germany; 10057177103). Embryos were then incubated overnight at 4 °C with 3000 × diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche; 11082736103). Finally, the color reaction was carried out by incubation with BM-purple substrate according to the manufacturer's recommendation (Roche; 11442074001) and individuals were digitally imaged.

2.5. Total RNA extraction, cDNA generation, and TaqMan® gene expression assays

Deiodinase (*dio1*, *dio2*, and *dio3*) and *TPO* expression in zebrafish embryos exposed to thiram at 10 hpf was measured at both 24 and 96 hpf. Total RNA was extracted from 20 pooled embryos with TRIzol® Reagent according to manufacturer's protocol (Grand Island; 12183-555). RNA quantity in each sample was determined by measuring the absorption ratio at 260/280 nm with a NanoDrop ND-100 spectrophotometer (ThermoScientific, Waltham, MA). This purified total RNA was used immediately for cDNA synthesis. A High Capacity cDNA Reverse Transcription Kit (cat #4368814, Applied Biosystems Inc; Foster City, CA), following the reverse transcription protocols of the manufacturer, was used to generate cDNA. The cDNA samples were stored at −20 °C until use. TaqMan® Gene Expression Assays (cat #4331182, Applied Biosystems Inc.) were used to measure gene expression. Reverse transcription reactions were performed after adding 0.2 ng cDNA mixed with TaqMan® Universal PCR Master Mix. Samples were analyzed by PCR using the 7900HT Real-Time PCR System and its Sequence Detection System 2.0 software (Applied Biosystems). Target gene expression data from samples was normalized using housekeeping gene 18S RNA, which compensated for intrinsic variability in the amount of RNA between embryos.

2.6. Statistical analyses

GraphPad Prism software (Version 6, La Jolla, CA) was used for statistical analyses. All expression data were expressed as mean ± SEM. Differences between means ($n \geq 3$) were analyzed using one-way ANOVA with a post-hoc Dunn's test where a p value < 0.05 was considered to be statistically significant. When comparing the endpoints with different exposure times and concentrations, a two-way ANOVA, with concentration and time as independent variables, was conducted.

3. Results

3.1. Survival, hatching, and morphological effects of thiram

After 1 h of exposure at 2, 10, 24, or 60 hpf and incubation for up 120 hpf, the following were recorded: mortality, hatching rate, pericardial edema, and notochord curvature (Figs. 1 and 2). Examples of pericardial edema and notochord curvatures in 96 hpf zebrafish larvae exposed to various concentrations of thiram at 10 hpf for 1 h are shown in Fig. 1. The survival rates of embryos did not differ between different exposure time windows at low concentrations (*i.e.*, 0.001 and 0.01 μM)

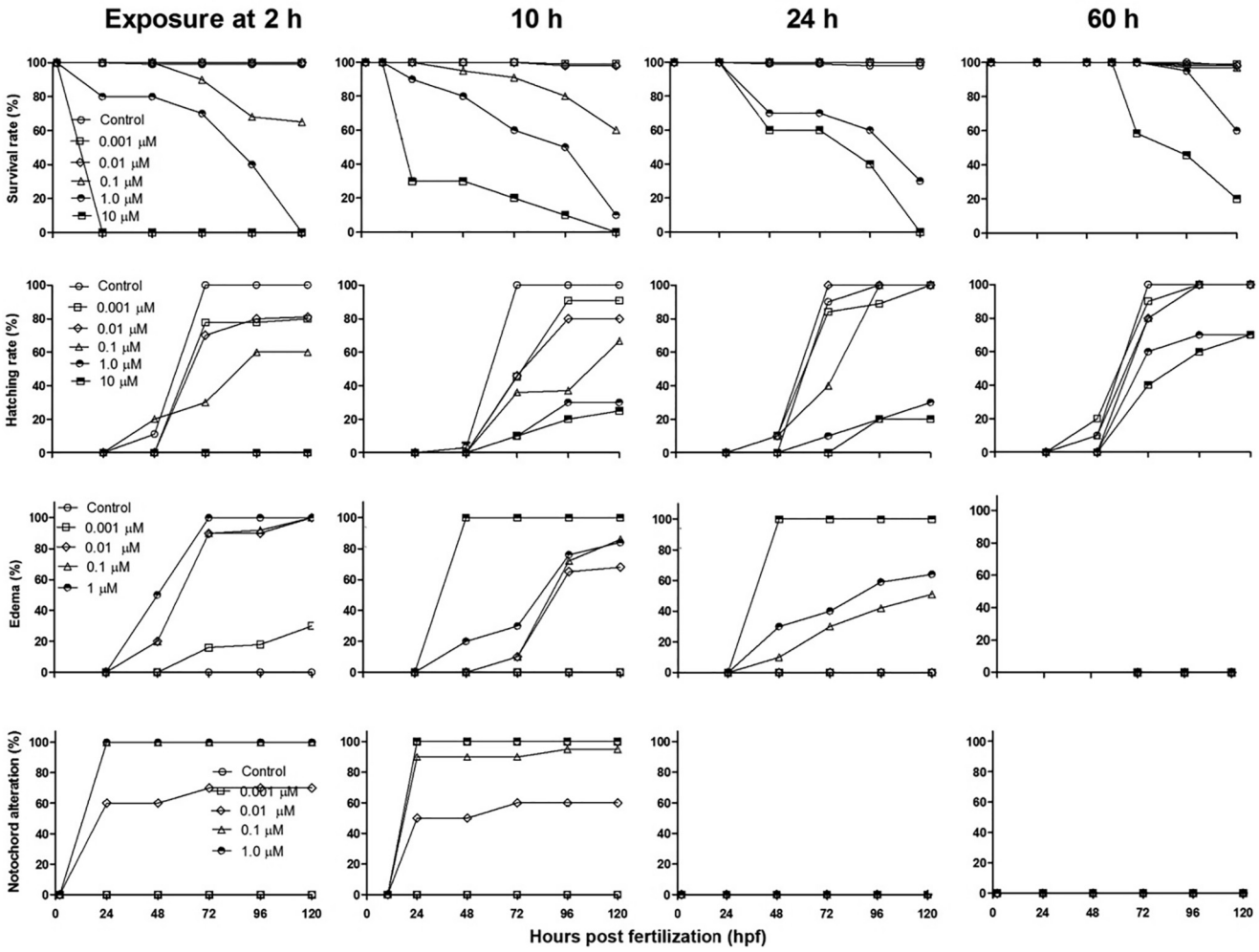

Fig. 2. Survival, hatching, occurrence of pericardial edema, and notochord deformity rates of zebrafish embryos exposed to 0.001, 0.01, 0.1, 1, and 10 μM thiram for 1 h at 2, 10, 24, and 60 hpf. Observations recorded every 24 h until 120 hpf.

(Fig. 2). However, at higher concentrations, age-dependent survival rates were observed. For those embryos exposed at 2 hpf, survival rates decreased with both age and increasing thiram concentration. At 1 μM thiram, the survival rate was less than 10% by 120 hpf, and at 10 μM thiram, 0% survived. Survival rates increased in the embryos dosed in later developmental stages. For example, at 24 hpf exposure, survival remained very low for the two highest concentrations, but individuals survived longer. Survival also increased for embryos exposed to thiram at 0.1 μM, with rates that approached 100%. When embryos aged 60 hpf were exposed to the highest concentration, they showed even greater survival, with the 1 μM group having greater than 50% and the 10 μM group reaching approximately 20% by 120 hpf (Fig. 2).

All embryos hatched in the control group by 72 hpf. Hatching rates were greater than 70% for those embryos exposed at different stages to 0.001 or 0.01 μM thiram. However, a marked reduction of hatching was observed at 72 hpf for the embryos exposed at 2 hpf to 0.1 μM (30% hatched), and no embryos hatched after exposure to 1 μM or at 10 μM concentrations (Fig. 2). For those embryos exposed at 10 hpf, hatching rates decreased in a dose-dependent manner. For those exposed at 24 hpf, hatching rates at 72 hpf were 40.5%, 10.2%, and 0% for 0.1, 1 and 10 μM, respectively. By 120 hpf, only 1 and 10 μM had hatching rates below 40%. At both the 72- and 120 hpf time points, hatching rate increased for those embryos exposed at 60 hpf, with hatching above 50% by 120 hpf (Fig. 2).

Thiram exposure produced pericardial edema (Figs. 1 and 2). All embryos exposed to 10 μM thiram at either 10- or 24 hpf developed pericardial edema by 48 hpf. In the embryos exposed at 2 hpf, only the lowest concentration showed edema below 100% by 120 hpf. At 120 hpf in the 10 hpf initiation group, all treatment groups had a greater than 65% incidence of edema. This decreased in the 24 hpf groups, with only 0.1, 1, and 10 μM treatments showing edema (51.2, 64.1, and 100%, respectively). Interestingly, no edema was observed when exposure was initiated at 60hpf. Time at initiation of exposure proved critical for pericardial edema in later life stages (Figs. 1 and 2).

Notochord curvature proved a prominent phenotype with onset at 24 hpf. Degree of curvature was strongly correlated with the exposure concentration (Fig. 3). At 0.1 and 1 μM thiram, 100% of embryos exposed at 2 hpf and 10 hpf showed completely distorted notochords resembling a tangled string (Fig. 3). Embryos exposed at 2 hpf to 0.01, 0.1, or 1.0 μM concentrations showed notochord curvature, with the latter two concentrations having an incidence of 100% by 120 hpf. This trend was reflected in embryos exposed to thiram at 10 hpf. However, no notochord deformities were observed in those embryos with onset of exposure at 24 hpf or 60 hpf. As with pericardial edema, notochordal defects reflected importance of developmental stage at onset of exposure if notochord changes were to be seen in later stages (Figs. 1–3).

Craniofacial alterations differed depending on the cartilage complex. Meckel's cartilage complex had no statistical differences between treatment groups. Conversely, the ceratohyal cartilage complex had decreased forward protrusion in all but the lowest thiram concentration (Fig. S4).

When embryos were exposed to 0.2 μM thiram at 10 hpf and were

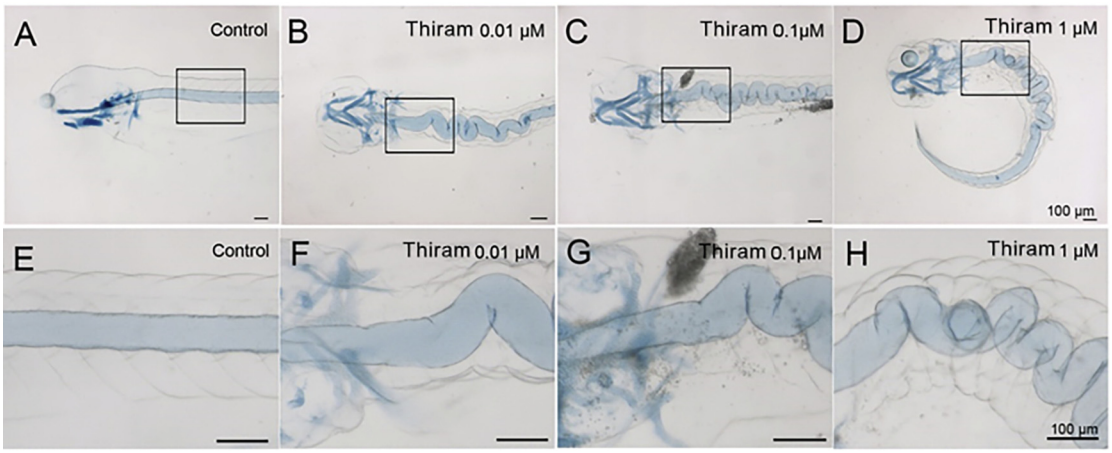

Fig. 3. Alcian blue staining illustrating notochord curvature of zebrafish larvae at 10 dpf that had been exposed to thiram at 10 hpf for 1 h. The bottom row (E-H) is magnified views of boxes drawn in the top row (A-D). A, E) controls; B, F) 0.01 μM thiram; C, G) 0.1 μM thiram; D, H) 1 μM thiram. Scale bars are 100 μm.

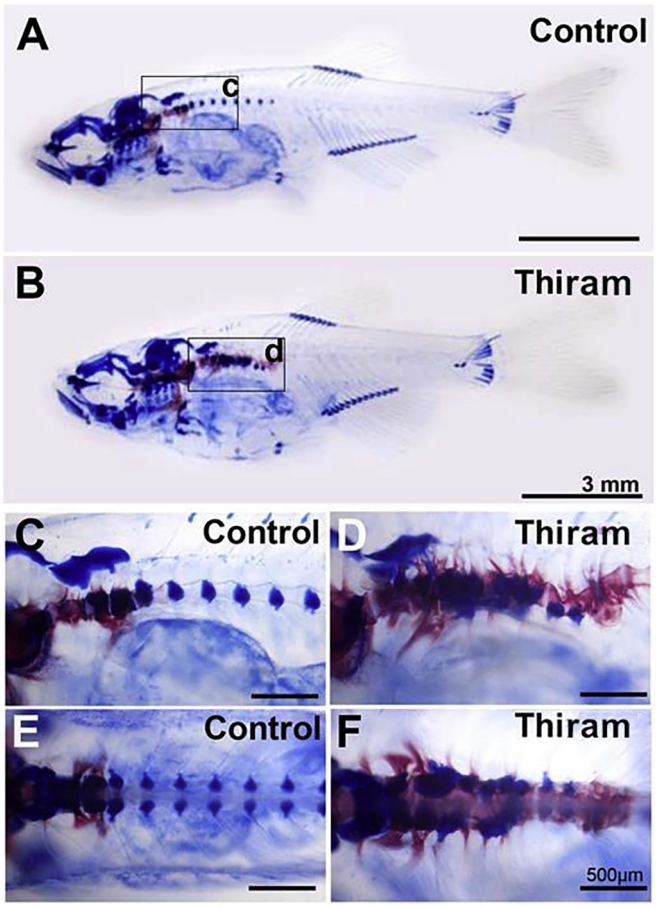

Fig. 4. Skeletal staining showing cartilage (blue) and bone (red) development of 33 dpf zebrafish that were exposed to thiram at 10 hpf for 1 h. A, C, E) control; B, D, F) 20 nM thiram; C, D) lateral magnifications of A and B, respectively; and E, F) dorsal magnifications. Scale bars: 3 mm and 500 μm.

allowed to grow out until they reached 33 dpf, vertebral bone was altered. This included extensive outgrowths of ossified material (Fig. 4). Not only was bone enhanced over normally cartilaginous structures but, when viewed from a lateral position, the spinal column was bent (Fig. 4B–D) and projections of bone were particularly evident in rostral vertebrae, with some of the projections resembling arches (Fig. 4F).

MMI exposure at 2 hpf increased mortality, delayed hatching, and increased occurrences of pericardial edema and notochord curvature by 96 hpf (Figs. S1 and S2). The notochord curvatures observed were similar to those in thiram exposed individuals (Fig. S1). Survival decreased with both age and increasing MMI concentration. In the 25 mM MMI exposure group, the survival rate was 80% by 96 hpf, but with 50 mM MMI, there were no survivors. All embryos hatched in both the control and 12.5 mM MMI exposure groups by 72 hpf. Hatching success was lower in the 25 mM MMI exposure group, with 70% hatched by 96 hpf. No embryos hatched following exposure at 50 mM (Fig. S2). MMI exposure also produced pericardial edema (Fig. S2F). In the 12.5 mM group, 30% of embryos developed edema. In the 25 mM and 50 mM groups, 80% or more had edema by 96 hpf. We also observed a concentration dependent increase in notochord curvatures starting at 24 hpf. The degree of curvature was strongly correlated with exposure concentration (Figs. S1 and S2). When co-exposed with T3, both thiram and MMI had reduced incidence of notochord curvatures in low concentrations (0.01 μM thiram, 12.5 mM MMI; $p < 0.01$) (Fig. S5).

3.2. *dio3* and *TPO* mRNA expression

To quantify the effect of thiram on deiodinase and *TPO* expression, RT-PCR was conducted on those embryos exposed to various concentrations of thiram at 10 hpf and incubated until they reached 12-, 24-, 48- or 96 hpf. As shown in Figs. 5A and S7, thiram induced expression of *dio3* at 12 and 24 hpf in a concentration-dependent manner. Compared with controls at 12 hpf, the expression of *dio3* increased by 1.50 and 1.37 fold for 0.1 and 1 μM concentrations, respectively. At 24 hpf, the expression of *dio3* increased by 1.08, 2.83, 4.14, and 9.38 fold for 0.001, 0.01, 0.1, and 1 μM concentrations, respectively. However, no significant difference was observed in embryos whose exposure was initiated at 96 hpf. Expression of *dio1* and *dio2* was not significantly different at 24 - or 96 hpf (Fig. S7). Thiram did not reduce *TPO* expression at 12-, 24-, or 48 hpf. Significantly decreased expression of *TPO* was observed at 48 hpf for the 1 μM group, a 0.67 fold down-regulation. At 96 hpf in the 0.1 and 1 μM groups, it was down-regulated 0.51 and 0.48 fold, respectively ($p < 0.05$; Fig. 5D).

3.3. *TPO* expression localization using in-situ hybridization

To expand on these results, *TPO* mRNA expression via *in situ* hybridization localized expression of *TPO* in the whole fish. As shown in Fig. 6, at 96 hpf, *TPO* was primarily expressed in the thyroid gland region, ventro-caudal to the mandible. Also, the amount of expression of *TPO* with 0.1- and 1 μM thiram exposure was apparently reduced, consistent with RT-PCR results. However, we did not see expression in embryos at 24–48 hpf using *in situ* hybridization. Therefore, the high concentrations of thiram could inhibit *TPO* expression and/or *TPO*

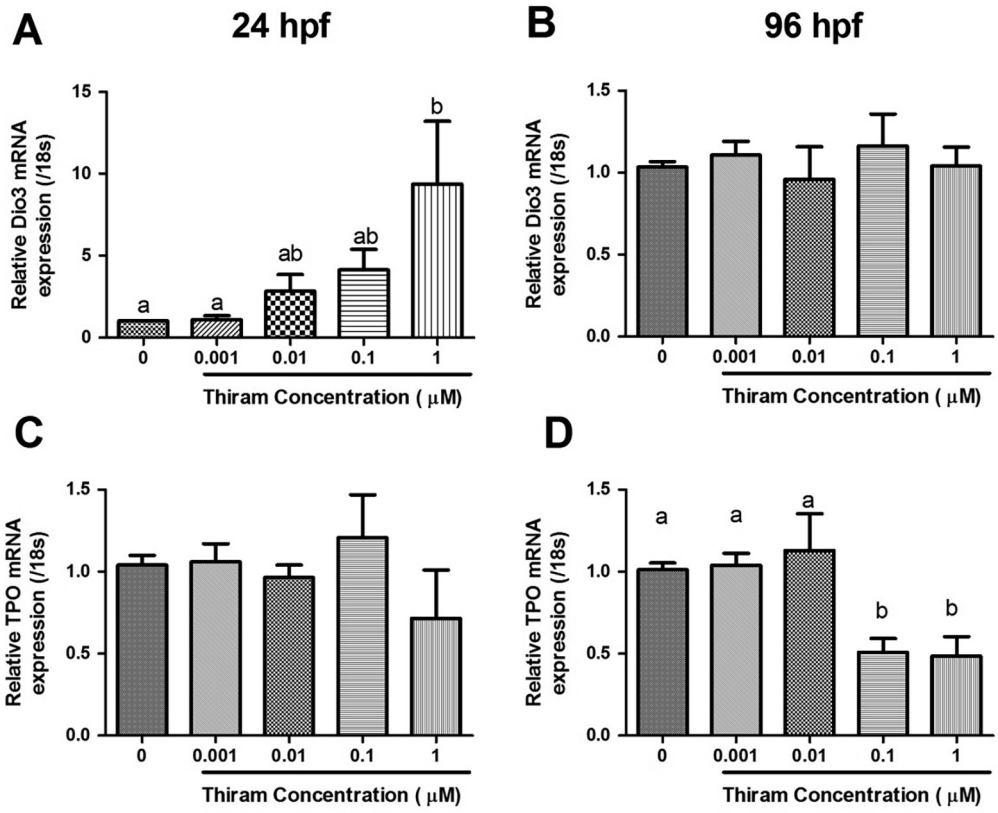

Fig. 5. *dio3* and *TPO* gene expression of zebrafish embryos exposed to thiram at 10 hpf for 1 h. Embryos were collected at 24 hpf or 96 hpf and RT-PCR was conducted. A, B) *dio3* mRNA expression at 24 and 96 hpf, respectively; and C, D) *TPO* mRNA expression at 24 and 96 hpf, respectively. Bars represent means ± SEM. Different letters indicate significant differences ($p < 0.05$).

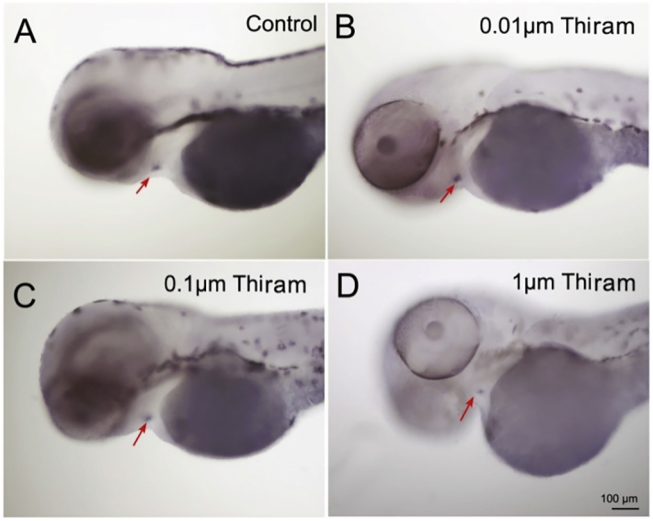

Fig. 6. Whole mount *in situ* hybridization for *TPO* gene expression in 48 hpf zebrafish exposed to thiram at 10 hpf for 1 h. A) control; B) 0.01 μM thiram; C) 0.1 μM thiram; and D) 1 μM thiram. Red arrow indicates *TPO* expression ventral to the mandible, in the region of the thyroid. Scale bars are 100 μm.

expression may be age-dependent during development, which is anatomically consistent with thyroid (Fig. 6).

4. Discussion

Short duration exposures (1 h) of zebrafish embryos to thiram at different stages of development resulted in changes in mortality, altered hatching, and deformity rates. The most severe forms were only seen with exposures initiated at the earliest stages of development. The developmental toxicity that followed exposure at 2 hpf proved similar to our previous observations using continuous exposure regimes until the phenotype was observed (Teraoka et al., 2006; Tilton et al., 2008; van Boxtel et al., 2010a). The fact that these short duration exposures to thiram showed a similar toxicity to a more prolonged exposure suggests that timing is important and that there are "windows of sensitivity." Additionally, gene expression of deiodinase and *TPO* suggested that thiram disrupted TH signaling.

Both concentration and age at initiation of exposure proved important in resultant toxicity. The two highest concentrations of thiram (1 and 10 μM) decreased embryo survival after 24 h exposure, and less than 20% of embryos hatched by 72 hpf with exposure from 2 to 24 hpf. However, if embryos were allowed to age to 60 hpf, these endpoints were lessened and incidence of alterations were likewise decreased. Age at initiation of exposure influenced occurrence of morphological changes, specifically pericardial edema and notochord curvature. Particularly striking in our study was the prevalence of edema (100%) with high thiram concentrations when individuals were exposed before 60 hpf. While edema is not a specific marker of thiram, it is an indicator of developmental toxicity and has been shown to be induced by multiple agents in early life stages (Asharani et al., 2008; Hamm and Hinton, 2000; Muscatello et al., 2006).

Notochord curvature appears to be a more specific marker for thiram-induced developmental toxicity. Our literature review revealed only a few instances where this defect occurred with other agents (Asharani et al., 2008), primarily disulfiram and other dithiocarbamates (Haendel et al., 2004; Strecker et al., 2013; Tilton et al., 2006). Several possible mechanisms for such toxicity have been proposed. van Boxtel et al. (2010b) analyzed dithiocarbamate in embryonic zebrafish and found eight down-regulated genes within the transforming growth factor beta-1 (TGF-beta1) signaling pathway. These included an essential transcription factor for zebrafish craniofacial development, the SRY-box-containing gene 9a (sox9a) (van Boxtel et al., 2010a,b). This reduced expression in sox9a affected bone development in the ceratobranchial arches of zebrafish. Teraoka et al. (2006) found that curvature of the notochord in zebrafish was not due to genes related

specifically to notochord development. Rather, their finding of alteration at 18 hpf following thiram exposure was well after gene expression for the notochord had occurred. Accordingly, they regarded distortion of the notochord to be related to the onset of spontaneous and rhythmic trunk contractions (Teraoka et al., 2006). When tricaine, alpha-bungarotoxin, or a paralytic mutant *sofa potato* were used to abolish spontaneous trunk movements during thiram exposure, the wavy notochord was replaced with normal morphology (Teraoka et al., 2006). Bohnsack and Kahana (2013) connected alterations in TH signaling to disruptions in craniofacial and ocular morphogenesis, specifically neural crest migration. While the exact mechanism(s) explaining notochord changes after thiram remain unclear, the central nervous system, endocrine regulation, and/or tetanic contractions of skeletal muscle are likely involved.

When we extended our observations to a juvenile life stage (33 dpf), we found alterations in cartilage and bone. Couch et al. (1977) analyzed the scoliosis caused by low concentrations of the pesticide Kepone (decachlorooctahydro-a,e,4metheno- 2H-cyclobuta[c,d]pentalen-2-one) in young adult sheepshead minnows (*Cyprinodon variegatus*), noting skeletal muscle alterations, breaking of the centra of vertebrae accompanied by osteoblastic repair, and tetanic muscular contractions. Those authors hypothesized that these changes may be the result of neurological- and/or neuromuscular dysfunction or changes in systemic calcium metabolism. It is unclear at this point if thiram induces similar disruptions in notochord formation early in life and how this might translate into the bone alterations we observed in juveniles. When compared with the dithiocarbamate-induced craniofacial abnormalities observed in other studies (van Boxtel et al., 2010b), effects on cartilage and its transformation to bone need further attention. Given the timing of this transition, the juvenile life stage, in particular, warrants further investigation.

We hypothesized a thyroid-related mechanism and used mRNA expressions of deiodinase and *TPO* to test this. Our results showed that thiram increased *dio3* mRNA expression at 12 hpf and 24 hpf and decreased *TPO* mRNA expression at 48 hpf and 96 hpf. *In situ* hybridization was used to localize the expression of *TPO*, pinpointing the region of the thyroid gland. Both *dio3* and *TPO* influence thyroid hormone homeostasis. *dio*3 deactivates T3 in peripheral tissues by converting them to the inactive form, T2 (Eales et al., 1993; Garcia et al., 2004). *TPO* affects production of T4 by liberating iodine for binding to tyrosine residues in thyroglobulin (Deme et al., 1978; Fragu, 1981). Since thyroid hormones play a vital role in early development and metamorphosis, delayed hatching and development may be modulated by decreased T3. TH level has been shown to be associated with the frequency and severity of acute transverse myelitis (Weng et al., 2017), an inflammatory condition of the spinal cord, and ectopic interplay *in vivo* in human neural tube defects (Li et al., 2015). In the present study, *TPO* inhibitor, MMI, induced similar notochord morphology at 24 hpf, suggesting that *TPO* inhibition might play a partial role in the observed thiram toxicity.

Thyroid gland toxicity of thiram or similar compounds has been reported in several previous studies. Flippin et al. (2009) performed oral administration of thiram to Long-Evans rats aged 23 days and showed a 45% decrease in circulating T4 in the serum. Furthermore, their chronic exposure led to a compensatory goiter. When rats were exposed to dithiocarbamates, T3 and T4 were reduced in the serum level of TSH, and led to thyroid disease and hyperplasia (Kurttio et al., 1986). *In vivo* experiments with ethylenebisdithiocarbamates and ethylene thiourea (ETU; a metabolite of thiram) revealed thyroid toxicity (Marinovich et al., 1997). *TPO* was thought to be the molecular target of these compounds as it liberates iodine for the addition onto tyrosine residues in thyroglobulin for the production of T4. That said, though several *in vivo* studies have shown that the embryonic toxicity of thiram might be closely related with its disruption effect on TH, the specific mechanism(s) have not been determined. Zineb, similar to thiram, inhibits *TPO* activity, reducing the level of circulating T4 (Kackar et al., 1997). Bhaskar and Mohanty (2014) found that mancozeb, another dithiocarbamate, competitively bound THR inactivating T3. The present study demonstrated that embryonic toxicity of thiram may be similarly correlated with the homeostasis of thyroid hormones, specifically the reduction of T4 (*i.e.*, precursor of T3) and conversion of T3 to inactive T2. Further investigation of this hypothesis is warranted.

Endogenous thyroid hormone (THs), thyroxin (T4) and triiodothyronine (T3) production begins shortly before hatching in zebrafish (Opitz et al., 2011; Ji et al., 2012). THs are important in early development where they affect cell proliferation along the dorsal and ventral edge of the body, relative to the change of body size and metamorphosis in both fish and amphibians (Xu et al. 2016). We found TPO mRNA expression at 12, 24, 48 and 96 hpf, but thiram only decreased *TPO* mRNA expression at the latter two timepoints. In addition, this expression was localized to the region of the thyroid gland, ventral-caudal to the mandible. We compared these results to markers of gene expression for thyroid primordium (Porazzi et al. 2009), both of which are localized to the same region. The thiram-induced notochord curvatures we observed may be relative to TH and not only a TPO relative effect. It is possible that the delayed effect of thiram-induced change may indicate a blockage of maternal THs and enzymes that may have led to deficiencies later in development.

Mukhi and Patino (2006) found impaired thyroid endocrine systems in adult female zebrafish exposed to perchlorate that suppressed fecundity. The larvae of those females also exhibited hypothyroidism as well as craniofacial alterations. Liu and Chan (2002) observed that zebrafish embryos treated with MMI had reduced head depths and shorter lower jaws. A review of maternal transfer of THs in fish by Ruuskanen and Hsu (2018) regarded regulation of embryo TH levels occurs but can vary according to several factors (e.g., deiodinase expression). An absence of iodide impairs TH production (Pesce and Kopp, 2014). Marinovich et al. (1997) transfected CHO cells with human *TPO* and exposed them to zineb. They found that zineb inhibited the iodinating activity as well as the oxidative activity of *TPO*. The authors suggested that zineb blocks *TPO*-catalyzed reactions but does not inactivate the enzyme itself, a contrast to the inhibition seen with MMI. In our study, early exposure of thiram induced hypothyroidism in the embryos, likely via down-regulation of *TPO* and up-regulation of *dio3*. It is possible that *TPO* inhibition described by Marinovich et al. (1997) may be mechanistically similar to what we observed.

The increased expression of *dio3* (0.1 and 1 μM thiram) and decreased expression of *TPO* mRNA at 12 and 48 hpf supports our hypothesis that thyroid hormones are causally related to the notochord curvatures we observed in larvae treated with either thiram or MMI. It has been shown that type 2 iodothyronine deiodinase (D2) is present in early embryonic stages where it plays a pivotal role in producing T3 (Porazzi et al., 2009). It is possible that this up-regulation of *dio3* reduced maternally inherited- or embryo synthesized active T3 into inactive T2. We found that the co-exposure of thiram or MMI with T3 resulted in fewer notochord curvatures at low concentrations. Together, these results suggest that thiram and MMI may increase thyroid hormone inactivation or inhibit its synthesis, at least partially.

In conclusion, this study revealed windows of sensitivity for embryonic toxicity of thiram. Short duration exposure, as little as 1 h, during these windows resulted in adverse outcomes, including decreased survival, delayed hatching, pericardial edema, and notochord curvature. There is evidence to suggest that thiram disrupts thyroid function, thereby leading to such changes. Early life exposure also resulted in skeletal defects later in life, likely a result of this thyroid disruption. Alterations at any life stage would be expected to inhibit the organism's ability to grow, avoid predation, and capture food (Stout et al., 2002; Trust et al., 2000). However, there is a need to develop a better, more complete picture of this mechanism in order to detect unwanted genetic and ecological effects of this and related pesticides.

Conflict of interest statement

The authors have no competing interests, financial or otherwise.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.11.003.

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