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Full Length Article

Changes in thyroid hormone activity disrupt photomotor behavior of larval zebrafish

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ABSTRACT

High throughput *in vitro*, *in silico,* and computational approaches have identified numerous environmental chemicals that interfere with thyroid hormone (TH) activity, and it is posited that human exposures to such chemicals are a contributing factor to neurodevelopmental disorders. However, whether hits in screens of TH activity are predictive of developmental neurotoxicity (DNT) has yet to be systematically addressed. The zebrafish has been proposed as a second tier model for assessing the *in vivo* DNT potential of TH active chemicals. As an initial evaluation of the feasibility of this proposal, we determined whether an endpoint often used to assess DNT in larval zebrafish, specifically photomotor behavior, is altered by experimentally induced hyper- and hypothyroidism. Developmental hyperthyroidism was simulated by static waterborne exposure of zebrafish to varying concentrations (3–300 nM) of thyroxine (T4) or triiodothyronine (T3) beginning at 6 h post-fertilization (hpf) and continuing through 5 days post-fertilization (dpf). Teratogenic effects and lethality were observed at 4 and 5 dpf in fish exposed to T4 or T3 at concentrations $\geq$ 30 nM. However, as early as 3 dpf, T4 ($\geq$ 3 nM) and T3 ($\geq$ 10 nM) significantly increased swimming activity triggered by sudden changes from light to dark, particularly during the second dark period (Dark 2). Conversely, developmental hypothyroidism, which was induced by treatment with 6-propyl-2-thiouracil (PTU), morpholino knockdown of the TH transporter *mct8*, or ablation of thyroid follicles in adult females prior to spawning, generally decreased swimming activity during dark periods, although effects did vary across test days. All effects of developmental hypothyroidism on photomotor behavior occurred independent of teratogenic effects and were most robust during Dark 2. Treatment with the T4 analog, Tetrac, restored photomotor response in *mct8* morphants to control levels. Collectively, these findings suggest that while the sensitivity of photomotor behavior in larval zebrafish to detect TH disruption is influenced by test parameters, this test can distinguish between TH promoting and TH blocking activity and may be useful for assessing the DNT potential of TH-active chemicals.

1. Introduction

Thyroid hormone (TH) disruption is widely posited to be a mechanism of developmental neurotoxicity (DNT) (reviewed in Gore et al., 2015; Miller et al., 2009). The scientific premise underlying this hypothesis is that TH signaling is essential for proper neurodevelopment and nervous system function (reviewed in Horn and Heuer, 2010; Zoeller and Rovet, 2004). Marked TH deficiency during gestation and early development results in moderate to severe mental retardation (DeLong et al., 1985), while more moderate reductions in circulating THs have been linked to cognitive deficits in humans (Haddow, 2005; Kooistra et al., 2006) and neurodevelopmental and behavioral impairments in rodents (Gilbert et al., 2016; Sharlin et al., 2010). In further support of the TH disruption hypothesis of DNT, environmental chemicals linked to adverse neurodevelopmental outcomes, such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), perchlorate, phthalates, and bisphenol A (BPA), have been shown to disrupt TH activity *via* effects on the hypothalamic-pituitary-thyroid (HPT) axis, or interference with delivery of TH to cellular targets and/or intracellular TH-signaling (reviewed in Boas et al., 2012; Gore et al.,

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2015). However, to date, experimental evidence causally linking TH disrupting effects to DNT is lacking for most environmental chemicals (Lein, 2015).

Developing zebrafish have been proposed as a promising *in vivo* model for investigating the DNT potential of TH active chemicals (Haggard et al., 2018; Walter et al., 2019). Inherent advantages of using zebrafish for this purpose include they are easy to breed, have a short developmental time, are relatively inexpensive to maintain, and are genetically tractable (Lein et al., 2005). Importantly, both the homeostatic regulation of circulating TH levels by the HPT axis, and the molecular signaling components of the TH system, are highly conserved in vertebrates, including zebrafish (Blanton and Specker, 2007; Porazzi et al., 2009; Walter et al., 2019). Additionally, the fundamental processes of neurodevelopment are the same in zebrafish and humans, and zebrafish express orthologues of human genes known to be important in normal and atypical neurodevelopment (Gilbert and Barresi, 2016; reviewed in Lein et al., 2005).

Zebrafish have been used to investigate chemical-induced TH disruption, with most of these previous reports quantifying effects of chemical exposures on TH concentrations and transcription of genes in the HPT axis (Chen et al., 2012; Liang et al., 2015; Shi et al., 2009; Tu et al., 2016; Wang et al., 2013; Yu et al., 2010). However, these outcomes are not necessarily predictive of changes at the cellular level in target tissue (Zoeller et al., 2000). A more recent study identified several morphologic abnormalities associated with developmental exposure to the T4 and T3 analogs, Tetrac and Triac, respectively (Haggard et al., 2018). While these findings are an important advance, this study did not examine DNT specifically, nor did it assess the effects of developmental hypothyroidism on phenotypic outcomes in developing zebrafish. The latter is an important data gap given that both increased and decreased T4 and T3 levels have been reported in developing zebrafish following chemical exposures (Chen et al., 2012; Huang et al., 2016; Liang et al., 2015; Liu et al., 2011; Shi et al., 2009; Tang et al., 2015; Tu et al., 2016; Wang et al., 2013; Yang et al., 2016; Yu et al., 2010) and that a number of TH disrupting chemicals, including many linked to DNT, such as pesticides, PCBs, PBDEs, perchlorate, bisphenol-A, phthalates, and perfluorinated chemicals, are associated with decreased serum TH levels in humans (reviewed in Boas et al., 2012).

The goal of this study was to generate proof-of-concept data that developmental hypo- or hyperthyroidism causes deficits in photomotor behavior, a widely used endpoint of nervous system function in high throughput zebrafish screens for DNT (Nishimura et al., 2015; Padilla et al., 2011).

2. Materials and methods

2.1. Chemicals

The thyroid hormones L-thyroxine (T4, > 98%; Sigma T2376), triiodothyronine (T3, > 95%; Sigma 2877), as well as 6-propyl-2-thiouracil (PTU, > 98%; Sigma 82460), and 3,3′,5,5′-tetraiodothyroacetic acid (Tetrac, > 98%; Sigma T3787) were purchased from Sigma-Aldrich (St. Louis, MO, USA). T4 and T3 were reconstituted by solubilizing 1 mg in 1 ml NaOH (1 N) and then adding this basic solution to 49 ml deionized water. Aliquots of T4 and T3 were stored as stock solutions of 25.7 and 30.7 μM, respectively, at −80 °C. PTU was reconstituted by solubilizing 50 mg in 1 ml NaOH (1 N) and then adding to 4 ml deionized water to make a 72.5 mM stock (1.0%) that was stored at −20 °C. Tetrac was reconstituted by solubilizing 10 mg into 1.337 ml DMSO to make a 10 mM stock and stored at −20 °C. Stock aliquots of T4, T3, PTU, and Tetrac were diluted at the time of exposure to yield final concentrations. Addition of these stock solutions was confirmed to not change the pH of the final treatment solutions. Analytical standards used for LC/MS/MS determination of thyroid hormones in zebrafish embryos were described previously (Chen et al., 2018b).

2.2. Zebrafish husbandry

All zebrafish work was performed in accordance with protocols approved by the University of California Davis Institutional Animal Care and Use Committee (IACUC). Adult wildtype zebrafish (5D) were originally obtained from the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University (Corvallis, OR) and subsequent generations were raised at UC Davis. Embryos from the Tg(*tg:nVenus-2a-nfnB*)$^{wp.rt8}$ line were obtained from David Parichy (University of Washington-Seattle). All zebrafish were raised under standard laboratory conditions with a 14 h light (~850 lx): 10 h dark photoperiod (Harper and Lawrence, 2016). Water was maintained at 28.5 ± 0.5 °C, pH 7.2 ± 0.4, and conductivity of 700 ± 100 μS. Adult fish were fed twice daily with a combination of live *Artemia nauplii* (INVE Aquaculture, Inc., Salt Lake City, UT, USA) and a mixture of commercial flake foods, including Zeigler Zebrafish Granule (Ziegler Bros, Inc. Gardners, PA, USA), Spirulina flake (Zeigler Bros, Inc.), Cyclopeeze (Argent Aquaculture, Redmond, WA, USA), and Golden Pearl (Brine Shrimp Direct, Ogden, UT, USA). Dietary iodine and selenium content can affect TH synthesis and metabolism (Gore et al., 2015; Triggiani et al., 2009); however, the dietary iodine and selenium concentrations were not available for the majority of the dietary components fed to adult zebrafish. We minimized this potential source of variability by using embryos from three different spawning groups for experimental replicates. Embryos were obtained by natural spawning of adult zebrafish in groups of 8–10 fish. Embryos were collected and staged following fertilization (Kimmel et al., 1995) and kept in an incubator at 28.5 °C in fish water (FW) until 4 h post fertilization (hpf) at which time experimental manipulations were initiated. FW consisted of filtered water removed directly from the adult zebrafish husbandry racks, maintained at a temperature of 28.5 ± 0.5 °C, pH of 7.2 ± 0.4 and conductivity of 700 ± 100 μS with the addition of Instant Ocean aquarium salt.

2.3. Exposures

For teratology and behavior assessments, zebrafish embryos were enzymatically dechorionated at 4 hpf using 50 μl of 41 mg/ml pronase (protease from Streptomyces griseus; Sigma: P5147) in 25 ml FW for 6 min as previously described (Truong et al., 2011). At 5–6 hpf, dechorionated embryos were placed in polystyrene 96-well plates (BD Falcon, Corning, Lowell, MA, USA) containing 100 μl of embryo media (EM: 15 mM NaCl, 0.5 mM KCl, 1.0 mM $MgSO_4$, 150 μM KH_2PO_4, 50 μM Na_2HPO_4, 1.0 mM $CaCl_2$, 0.7 mM $NaHCO_3$) (Westerfield, 2000). For chemical exposures (T4, T3, PTU, or Tetrac), 100 μl of a 2x concentration of the selected compound or control solution was added to each well. Final exposure concentrations for T4 and T3 were 3, 10, 30, 100, and 300 nM; this range was chosen to encompass and exceed previously reported reference intervals for circulating T4 and T3 in human pediatric patients and measurement in zebrafish larvae tissue (Chen et al., 2018a; Zurakowski et al., 1999). Final exposure concentrations for PTU were 0.001% (0.073 mM) and 0.01% (.73 mM) and were chosen based on previous research documenting that these levels reduce T4 immunoreactivity in developing zebrafish with minimal teratogenic effects (Elsalini and Rohr, 2003). EM was used as the control solution for T4, T3, and PTU exposures. The final concentration of Tetrac used was 3 nM, based on a previous report that this concentration rescued phenotypes associated with *mct8* deficiency in mice (Horn et al., 2013). Control solution for exposures with Tetrac contained 0.1% DMSO. All plates were covered with Parafilm M (Bemis NA, Neenah, WI) to minimize evaporation and kept in an incubator at 28.5 °C with 14 h light (~300 lx):10 h dark cycles. Fish were exposed continuously from 6 hpf through 5 dpf *via* static waterborne exposure. Treatment paradigms are shown in Fig. 1. All experimental conditions were tested

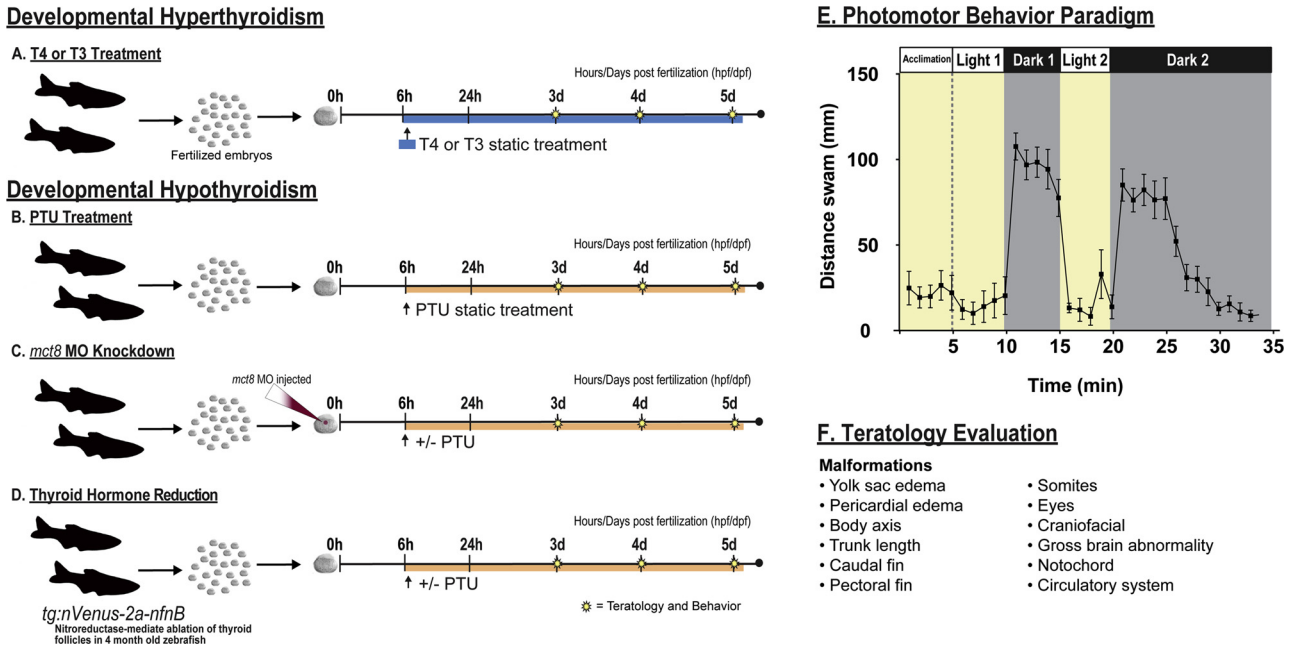

Fig. 1. Overview of experimental paradigms. **(A)** Developmental hyperthyroidism was simulated by exposing zebrafish to exogenous T4 or T3 at 3, 10, 30, 100, or 300 nM. **(B–D)** Three methods were employed to simulate developmental hypothyroidism: **(B)** treatment with the thyroperoxidase inhibitor propylthiouracil (PTU) at 0.001 or 0.01%; **(C)** knockdown of the TH transporter *mct8* by injection of a splice blocking morpholino at the 1–2 cell stage; and **(D)** reduction of maternally-derived thyroid hormone in spawned eggs using nitroreductase-mediated ablation of thyroid follicles in adult females of the Tg(*tg:nVenus-2a-nfnB*)$^{wp.rt8}$ transgenic line. **(E)** Schematic illustrating the typical response of larval zebrafish in the photomotor behavior test conducted at 3, 4, and 5 dpf prior to assessment of fish for teratological outcomes. Fish were allowed to acclimate in the light for 5 min, then locomotor behavior was automatically tracked during sequential exposures to 5 min of light (Light 1), 5 min of dark (Dark 1), 5 min of light (Light 2), and 15 min of dark (Dark 2). The natural log of the area under the curve (lnAUC) was generated for statistical comparison of photomotor behavior between experimental groups using a mixed effects model. **(F)** Endpoints examined in the teratology evaluation at 3, 4 and 5 dpf.

in two or more independent experiments conducted on independent days using larvae from different spawns. For each experiment, 16 larvae were tested per experimental condition, thus each experimental condition had a minimum sample size of 32 larvae. Each experiment was conducted in a 96-well plate with all treatment conditions represented. Treatment groups were randomized by column and differed between all plates.

2.4. Monocarboxyl transporter 8 (mct8) morpholino (MO) knockdown

Knockdown of the TH transporter *mct8* was performed as previously described (Walter et al., 2019) using the fluorescein-tagged splice blocking morpholino-modified antisense oligonucleotides (MO) *mct8*(E2I2) MO (5′-ataaaatcatgtatttacgtggcga-3′) and the Gene Tools standard control MO (5′-ctcttacctcagttacaatttata-3′), also tagged with fluorescein (Gene Tools, Philomath, OR, USA). The *mct8*(E2I2) MO interferes with the splicing of the second exon/intron by introducing a premature stop codon and has been validated to decrease *mct8* expression during zebrafish development (Vatine et al., 2013). Zebrafish embryos at the 1–2 cell stage were injected with 2–5 nL of a solution comprised of 0.5 µl stock morpholino (1.5 mM), 0.5 µl phenol red (Sigma Aldrich), and 4 µl Ultrapure distilled water (Invitrogen, Carlsbad, CA, USA) for a total injection amount of 0.3 - 0.75 pmol of morpholino per embryo. MO solutions were injected into the embryo yolk with a fine-tipped needle, and consistent injection volumes were achieved using a pressure microinjector (Picospritzer II, General Valve Corp., USA) and micromanipulator (Narishige, Tokyo, Japan) connected to a compressed nitrogen air source as previously described (Rosen et al., 2009; Xu et al., 2008). Phenol red was added to verify injection location and volume. At 6 hpf, injected embryos were placed in polystyrene 96-well plates in EM in the absence or presence of 0.01% PTU w/v (0.73 mM). Embryos that did not express fluorescein at 24 hpf were not included in behavior analyses. The extent of knockdown of *mct8* mRNA was confirmed in a subset of fish expressing fluorescein at 24 hpf and 5 dpf by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) as previously published previously (Walter et al., 2019).

2.5. Nitroreductase-mediated conditional thyroid ablation in adult female zebrafish

Zebrafish embryos develop externally and receive maternal T4 and T3 through the yolk, and we previously established that 24 hpf zebrafish contain approximately 0.1 pg T4/embryo and 0.37 pg T3/embryo (Walter et al., 2019). To decrease the maternal TH contribution, thyroid follicles in adult female fish from the transgenic Tg(*tg:nVenus-2a-nfnB*)$^{wp.rt8}$ zebrafish line (McMenamin et al., 2014) were conditionally ablated at 4 months of age using nitroreductase-mediated cell ablation (Curado et al., 2008). These fish express the bacterial gene nitroreductase in thyroid follicles under control of the promoter for thyroglobulin, a protein that is specifically expressed in thyroid follicles. When fish are treated with metronidazole, a cytotoxic metabolite is formed by cells that express nitroreductase, resulting in ablation of thyroid follicular cells. Thyroid ablations were performed on 4-month old female Tg(*tg:nVenus-2a-nfnB*)$^{wp.rt8}$ zebrafish by incubating zebrafish in 10 mM metronidazole (Mtz; Sigma M1547) with 1% DMSO and 0.0004% clove oil (Sigma C8392) for 5 h. A parallel control group was incubated in 1% DMSO and 0.0004% clove oil for 5 h in the absence of metronidazole. Clove oil acts as a mild sedative and was added to decrease stress (Rohner et al., 2011). During this treatment, zebrafish were protected from light to minimize visual stimulation and prevent metronidazole degradation. Loss of nVenus, indicating ablation of thyroid follicles, was confirmed 3 d post-treatment by fluorescence microscopy. Thyroid ablated females and control females with intact thyroids were spawned with age-matched males with intact thyroids at 15 weeks following thyroid ablation, and embryos from these spawns

are referred to as THKO embryos. From each spawn of THKO embryos, samples of 150 embryos were collected at 24 hpf and pooled for TH measurement by LC–MS/MS (Chen et al., 2018b; Walter et al., 2019). LC–MS/MS measurements of T4, T3, and rT3 in embryos following T4, T3, and PTU exposure were previously reported (Walter et al., 2019). The limits of detection for T4, T3, and rT3 using this method were 0.5 pg, 0.6 pg, and 0.5 pg, respectively (Chen et al., 2018a). The THKO embryos not used for LC–MS/MS measurements were placed in 96 well plates for teratology and behavior assessment at 3, 4, and 5 dpf. A subset of embryos from thyroid ablated females were additionally exposed to 0.01% PTU (0.73 mM) beginning at 6 hpf.

2.6. Assessment of teratogenesis

Teratology and mortality were assessed at 24 hpf and at 3, 4, and 5 dpf. Deaths at 24 hpf (< 10%) were attributed to pronase treatment during dechorionation and were not included in mortality totals reported at 3, 4, and 5 dpf. Larvae were evaluated at 3, 4, and 5 dpf for survival and the presence or absence of the following gross developmental malformations: yolk sac edema, pericardial edema, body axis curvature, altered trunk length, abnormalities of the caudal fin, pectoral fin, somite, and eye, craniofacial malformation, and gross abnormalities in brain, notochord, and circulatory system as described previously (Truong et al., 2011). Varying degrees of decreased pigmentation were observed in many fish exposed to exogenous T4 or T3, which appeared to be otherwise morphologically normal. Thus, in the interest of evaluating behavioral changes in fish with mild TH disruption, fish with minor variations in pigmentation but no structural abnormalities were not classified as malformed. No variations in pigmentation were observed in fish maintained under conditions of developmental hypothyroidism. Mortality was defined as the absence of a visible heartbeat. The percent of viable, malformed, and dead larvae at each time point were calculated across all replicates (n = 3–5), and significant between-group differences in the incidence of these outcomes was determined by chi-squared analysis followed by Fisher's exact test. Sample sizes for all groups are listed in Table S1 in the Supplemental material.

2.7. Larval photomotor behavior testing

To evaluate the effects of TH disruption on an apical endpoint of neurodevelopment, photomotor behavior was assessed at 3, 4, and 5 dpf using a DanioVision system (Noldus, Leesburg, VA, USA). We have previously reported that repeated behavior testing does not influence photomotor response at later time points (Dach et al., 2019). Behavior tests were conducted in the same 96-well plates used to expose zebrafish embryos with temperature maintained at 28.5 ± 0.5 °C using a Noldus temperature control unit. The photomotor behavior test is based on consistently displayed patterns of locomotor activity in response to sudden changes between light and dark conditions. This behavioral test can be used to detect changes in nervous system development or function (Emran et al., 2008). A sudden change from dark to light causes larval zebrafish to decrease or stop swimming, whereas a sudden change from light to dark triggers increased locomotion, which gradually subsides as larvae habituate to dark conditions (MacPhail et al., 2009). We used a 35 min test paradigm that consisted of a 10 min light period (~1900 lx) to allow for acclimation (5 min) and to record baseline swimming (5 min), followed sequentially by a 5 min dark period (~0 lx) to stimulate increased swimming behavior, a 5 min light period (~1900 lx) to stimulate freezing behavior, and finally a 15 min dark period (~0 lx) to observe increased swimming behavior and acclimation to the dark conditions (Fig. 1E). Swimming behavior was recorded using a GigE camera (Noldus) equipped with an infrared filter to allow accurate recording during both light and dark periods. We confirmed that differences in pigment did not influence behavioral tracking. Movement of individual larvae was tracked by the EthoVisionXT software (Noldus). All dead and/or malformed larvae, as determined by subsequent teratology assessment at each time point, were excluded from the behavior analysis.

The distance swam by each zebrafish larvae during the behavior test was exported from EthoVisionXT in 1 min bins. The first 5 min of each test were considered an acclimation period and data from this period were not included in the statistical analysis. The area under the curve (AUC) of the distance swam by each fish was computed for Light 1 (5–10 min), Dark 1 (11–15 min), Light 2 (16–20 min), and Dark 2 (21–35 min) using the trapezoid method to calculate AUC for each 1 min bin (see Supplemental material for the equation used for these calculations). At 3 dpf, fish swimming less than 15 mm over the course of the behavior test were removed from the statistical analysis to minimize the influence of non-moving fish. Mixed effects regression models, including zebrafish-specific random effects, were used to assess differences between groups defined by varying concentrations of selected chemical treatments and genetic modifications and their respective controls. Separate models were fit for each treatment condition group. Contrasts were specified to compare the area under the curve during each of the lighting conditions between the exposed groups and their respective controls. The mixed effects regression models that we used included all observations across the 3 d for all fish in a given experiment. The animal-specific random effects accounted for the repeated assessments both within a day and across days. Day, itself, was a specific factor included in the models, which enabled a comparison between groups on each day. Exploratory analysis indicated that a natural logarithmic transformation was needed for the area under the curve to stabilize the variance and meet the underlying assumptions of the mixed effects models. Data are reported as the AUC, as a percent of control, focusing on the comparison between treatment groups and their respective controls during the dark periods where behavior changes were observed. All analyses were conducted using SAS 9.4 and an alpha value of 0.05 for statistical significance. Sample sizes for all treatments are listed in Supplemental Tables 2 and 3.

3. Results

3.1. Teratogenic and lethal effects of developmental hyperthyroidism

To assess the impact of developmental hyperthyroidism on zebrafish development, teratogenic outcomes and mortality were quantified in zebrafish larvae exposed to exogenous T4 or T3 (3, 10, 30, 100, or 300 nM) beginning at 6 hpf and continuing until 5 dpf (Fig. 1). In general, affected larvae exhibited multiple developmental abnormalities, with the most common combination of malformations including pectoral fin, body axis, and craniofacial abnormalities. The teratogenic and lethal effects of developmental hyperthyroidism were concentration- and time-dependent. At 3 dpf, T4 and T3 significantly increased the percentage of malformed and dead fish only at the highest concentration tested of 300 nM (Fig. 2A & D). At 4 dpf, the percentage of malformed and dead fish was significantly increased relative to vehicle controls at T4 exposures $\geq$ 100 nM (Fig. 2B) or T3 exposures $\geq$ 30 nM (Fig. 2E), while at 5 dpf, T4 or T3 exposures $\geq$ 30 nM significantly increased teratogenic outcomes and mortality (Fig. 2C & F). The percentage of malformed and dead fish observed at 5 dpf was increased relative to that observed at 4 dpf in the same exposure group. Zebrafish developmentally exposed to T4 or T3 at concentrations of 3 and 10 nM did not exhibit any significant increases in the percentage of malformed and dead fish at 3, 4, or 5 dpf (Fig. 2).

3.2. Effect of developmental hyperthyroidism on photomotor behavior

Photomotor behavior was tested in zebrafish at 3, 4, and 5 dpf following exposure to T4 or T3 at concentrations ranging from 3 to 100 nM. Developmental exposure to either T4 or T3 altered photomotor behavior in a concentration- and time-dependent manner. In general,

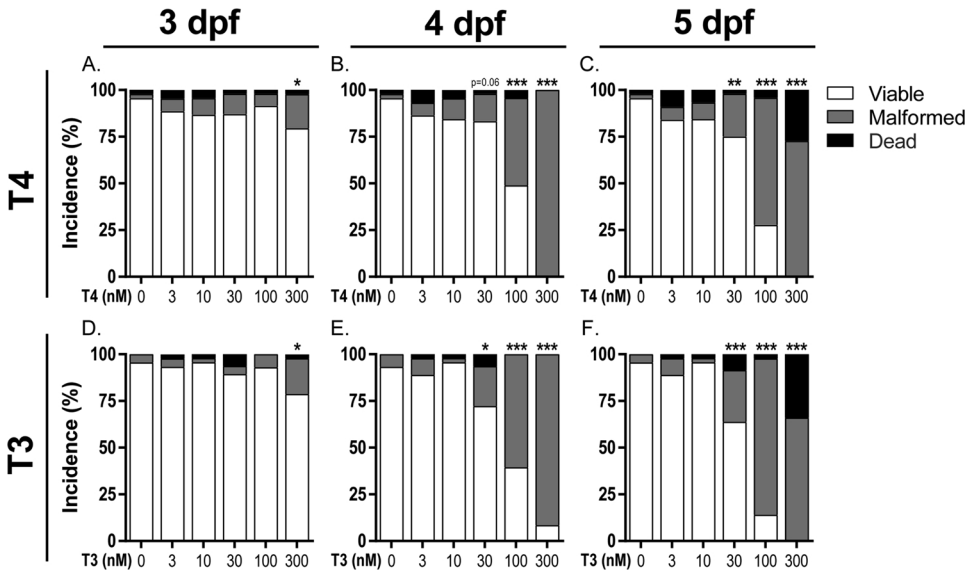

Fig. 2. Concentration-dependent effects of T4 and T3 on the viability and incidence of teratological outcomes in larval zebrafish. Data are presented as the percentage of viable, malformed, and dead embryos observed following exposure to exogenous T4 **(A–C)** or T3 **(D–F)** from 6 hpf through 5 dpf. Significantly different from EM controls at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by chi-squared (X^2) test followed by Fisher's exact test. Sample sizes for each experimental group are listed in Table S1 in the Supplemental material.

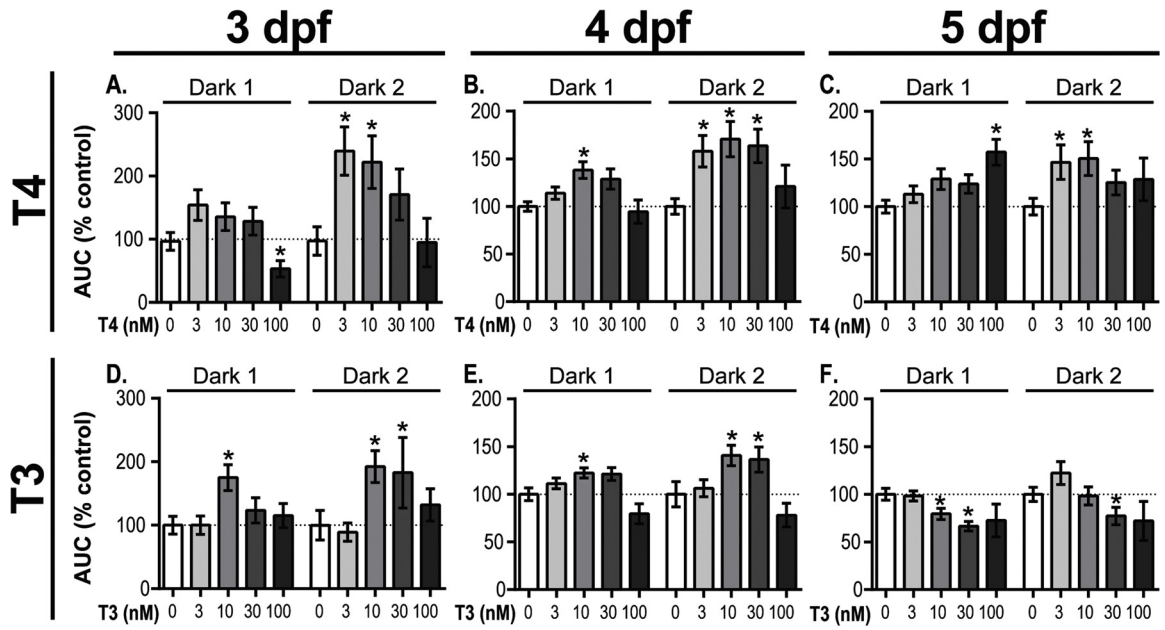

Fig. 3. Photomotor behavior of zebrafish larvae exposed to exogenous T4 or T3. Zebrafish larvae were exposed to varying concentrations of T4 **(A–C)** or T3 **(D–F)** beginning at 6 hpf, and locomotor behavior in response to photoperiod changes was assessed in the same fish on three consecutive days (3, 4, and 5 dpf). Only live larvae with normal morphology were included in behavior analyses. Exposure effects were determined by calculating the percent change in area under the curve (AUC) from respective controls during the two dark periods (Dark 1 and Dark 2). Data are presented as the mean ± SE (sample size for each group is listed in Table S2 in the Supplemental material). The dotted line represents the mean AUC of controls (100%). Significantly different from EM controls at *$p < 0.05$; **$p < 0.01$ as determined using a mixed effects model to compare the natural log of the AUC (lnAUC) to minimize the impact of high outliers. The behavior test results for each group with significant differences in swimming activity are presented as the average distance swam during each 1 min bin over the course of the behavior test in Fig. S1 of the Supplemental material.

T4 increased locomotor activity in response to sudden changes from light to dark and this effect exhibited a non-monotonic concentration-effect relationship (Fig. 3A–C). However, the concentration dependency and the direction of change (decreased *vs*. increased swimming activity) varied across testing days. At 3 dpf, 100 nM T4 decreased locomotor behavior during the Dark 1 period but had no significant effect on locomotor behavior in the Dark 2 period, whereas 3 nM and 10 nM T4 significantly increased swimming activity during Dark 2 (Fig. 3A). At 4 dpf, 10 nM T4 significantly increased locomotor activity during Dark 1, while 3, 10, and 30 nM T4 significantly increased swimming distance during Dark 2 (Fig. 3B). T4 at 100 nM had no effect on swimming activity during either Dark 1 or Dark 2 at 4 dpf. At 5 dpf, 100 nM T4 significantly increased locomotor behavior during Dark 1 whereas 3 and 10 nM T4 significantly increased swimming activity during Dark 2 (Fig. 3C).

Exposure to exogenous T3 caused similar patterns of changes in photomotor behavior at 3 and 4 dpf, eliciting a non-monotonic concentration-dependent increase in swimming activity (Fig. 3D–E). At both 3 dpf (Fig. 3D) and 4 dpf (Fig. 3E), 10 nM T3 increased swimming activity during Dark 1 and Dark 2, while 30 nM T3 increased swimming activity during Dark 2; swimming activity was not altered in fish exposed to 100 nM T3. In contrast to the behavioral response of 5 dpf fish exposed to T4 (Fig. 3C), 5 dpf fish exposed to T3 exhibited decreased swimming activity during dark periods (Fig. 3F). 10 and 30 nM T3

decreased swimming activity during Dark 1, while only 30 nM T3 decreased swimming activity during Dark 1 and Dark 2. During the light periods, no significant differences were observed between TH treatments and control groups, likely due to relatively low swimming activity during these periods.

3.3. Strategies for generating TH-deficient zebrafish

To characterize the teratogenic and lethal effects of developmental hypothyroidism in zebrafish, three mechanistically different approaches were employed to create TH-deficient fish. The goal of these methods was to induce a mild to moderate TH-deficiency, as would likely be triggered by environmental TH disrupting chemicals, rather than a severe TH-deficiency, which is well documented to cause severe developmental abnormalities (DeLong et al., 1985). First, 6-propyl-2-thiouracil (PTU) was used to decrease larval production of T4. PTU inhibits the thyroperoxidase enzyme that catalyzes an essential step in the formation of thyroxine (T4) and triiodothyronine (T3), which is the addition of iodine to tyrosine residues on the hormone precursor thyroglobulin (Engler et al., 1982). PTU can also inhibit iodothyronine deiodinase type 1, which catalyzes the activation of T4 to T3 and the degradation of T4 to rT3 and rT3 to T2. We previously demonstrated that static waterborne exposure to 0.01% PTU beginning at 6 hpf decreased T4 in 72 hpf and 120 hpf fish by greater than 10-fold and 100-fold, respectively, relative to embryo media controls (Walter et al., 2019).

The second strategy for simulating developmental hypothyroidism was to decrease intracellular T4 levels using morpholino knockdown of the TH transporter *mct8* (*mct8*MO) (Zada et al., 2017). In humans, mutations in *mct8* cause Allan-Hernon-Dudley syndrome (AHDS), which manifests clinically as mental retardation and motor deficiencies (Braun and Schweizer, 2018). We previously demonstrated that *mct8* mRNA was significantly decreased in *mct8* morphants by 6.25-fold at 24 hpf and by 1.43-fold at 5 dpf (Walter et al., 2019). Because of the diminishing downregulation of *mct8* mRNA with increasing time post-injection, teratology and photomotor behavior were assessed in *mct8* morphants maintained in the absence and presence of 0.01% PTU to decrease the amount of T4 being produced by larval zebrafish after 72 hpf.

The third strategy to induce developmental TH deficiency was to reduce the maternally-derived TH in zebrafish embryos by conditional ablation of thyroid follicles in adult females. The concentration of T4 in THKO embryos at 24 hpf was not significantly different from that of control embryos (Fig. 4A); however, the concentration of T3 in THKO embryos at 24 hpf was significantly decreased to approximately half the T3 measured in controls (Fig. 4B). THKO and genotype-matched control embryos were raised to 5 dpf in the absence or presence of 0.01% PTU to reduce T4 synthesis by larval zebrafish.

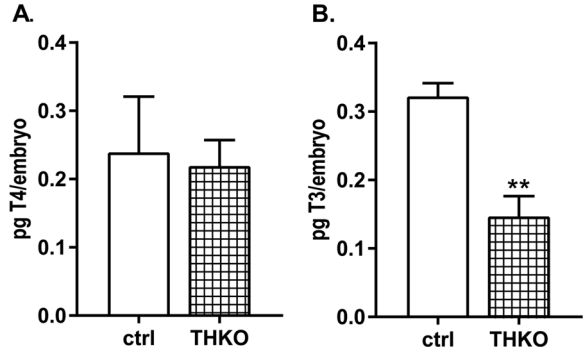

Fig. 4. TH concentrations in embryos spawned from adult female fish with ablated thyroid follicles (THKO). Concentrations of T4 **(A)** and T3 **(B)** were determined by LC–MS/MS in 24 hpf embryos spawned from females with ablated thyroid follicles (THKO) and embryos spawned from the same transgenic line and generation with thyroid follicles still intact (ctrl). Data are presented as the mean TH concentration normalized per embryo (pg TH/embryo) ± SE (n = 3 replicates from 3 independent spawns, each with 150 pooled embryos). *p < 0.05; **p < 0.01 as determined using unpaired student's t-test.

3.4. Teratogenic and lethal effects of developmental hypothyroidism

Teratogenic outcomes and mortality were assessed at 3, 4 and 5 dpf in fish subjected to one of the three methods for inducing TH deficiency (Fig. 1). Compared to controls raised in EM, zebrafish exposed to PTU at 0.001% or 0.01%, from 6 hpf through 5 dpf did not exhibit any significant differences in the percentage of malformed or dead fish at 3, 4, or 5 dpf (Fig. 5 A–C). Similarly, no significant differences in the percentage of malformed or dead fish were observed between *mct8* morphants raised in the absence or presence of 0.01% PTU relative to control morphants (Fig. 5D–F). Teratology and lethality in THKO fish raised in the absence or presence of 0.01% PTU was compared to fish spawned from Tg(*tg:nVenus-2a-nfnB*)$^{wp.rt8}$ females with intact thyroid follicles. At 3 dpf, relative to controls, the percentage of malformed, and dead fish was not significantly different in THKO fish raised in the absence of 0.01% PTU, but it was significantly increased in THKO + 0.01% PTU (Fig. 5G). At 4 dpf (Fig. 5H) and 5 dpf (Fig. 5I), there were no significant differences between groups in the percentage of malformed and dead fish, although the percentage of malformed fish was increased in both the control and THKO + 0.01% PTU groups at these later time points relative to 3 dpf (Fig. 5). The higher than expected incidence of malformations in the control group at 4 dpf and 5 dpf may be due to exposure of control adult females to 1% DMSO, which was used in adult female fish treated with Mtz to ablate thyroid follicles. Alternatively, it could simply reflect a higher background of teratogenic outcomes inherent in this genetic background.

3.5. Effect of TH deficiency on photomotor behavior

In general, developmental TH deficiency caused concentration- and time-dependent decreases in swimming activity during the dark periods, although the timing and extent of this effect varied across the different methods for inducing TH deficiency. Treatment with PTU did not significantly alter swimming activity during Dark 1 or Dark 2 at 3 dpf (Fig. 6A). However, at 4 dpf, swimming activity was significantly decreased during Dark 2, but not Dark 1, in fish exposed to either 0.001 or 0.01% PTU (Fig. 6B). At 5 dpf, only treatment with 0.01% PTU decreased swimming activity, and this was observed during both Dark 1 and Dark 2, although the magnitude of the effect was greater during Dark 2 (Fig. 6C).

Morpholino knockdown of *mct8* also decreased swimming (Fig. 6D–F), and this effect was amplified in *mct8* morphants exposed to 0.01% PTU, which exhibited this behavioral defect earlier than control fish exposed to PTU (Fig. 6A–C). The photomotor response of *mct8* morphants raised in the absence of PTU was not significantly different from control morphants at 3 dpf (Fig. 6D) or 4 dpf (Fig. 6E), but by 5 dpf, their swimming activity was significantly decreased during both Dark 1 and Dark 2 (Fig. 6F). In contrast, mct8 morphants raised in the presence of 0.01% PTU exhibited significantly decreased swimming activity, relative to control morphants at 3, 4, and 5 dpf during both Dark 1 and Dark 2 (Fig. 6D–F).

Fish spawned from adult female Tg(*tg:nVenus-2a-nfnB*)$^{wp.rt8}$ fish with ablated thyroid follicles (THKO) that were raised in the absence of PTU exhibited significantly decreased swimming activity at 3 dpf during Dark 2 compared to control fish derived from adult female Tg (*tg:nVenus-2a-nfnB*)$^{wp.rt8}$ fish with intact thyroid follicles (Fig. 6G). THKO fish raised in the presence of 0.01% PTU, also exhibited significantly reduced swimming activity during both Dark 1 and Dark 2 at 3 dpf, although at this time point, PTU treatment did not amplify the effect. By 4 dpf and 5 dpf, the photomotor response of THKO fish not exposed to PTU was not significantly different from that of control fish;

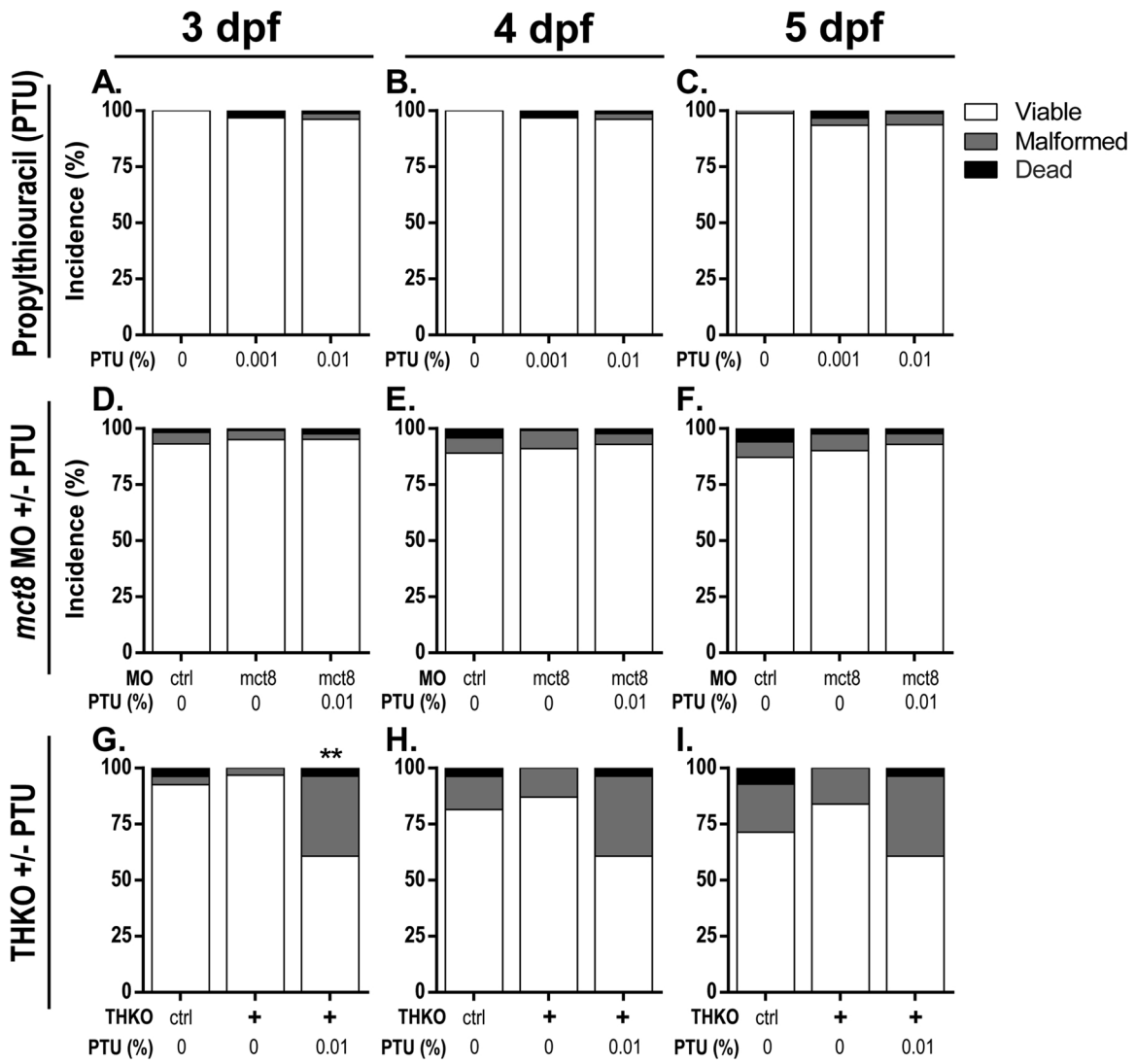

Fig. 5. Effects of experimental hypothyroidism on the viability and incidence of teratological outcomes in larval zebrafish. Hypothyroidism was simulated by treating larval zebrafish with PTU (**A-C**), morpholino knockdown of the TH transporter *mct8* in the absence or presence of 0.01% PTU in the morphants' medium (**D-F**), or reduction of maternally derived T3 *via* nitroreductase-mediated ablation of maternal female thyroid follicles (THKO) with subsequent culture of spawned larvae in the absence or presence of 0.01% PTU (**G-I**). Data are presented as the percentage of viable, malformed, and dead embryos/larvae observed in each experimental group at 3, 4 and 5 dpf. Significantly different from control (ctrl) at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by chi-squared (X^2) test followed by Fisher's exact test. Sample sizes for each group are listed in Table S1 in the Supplemental material.

however, exposure to 0.01% PTU significantly decreased swimming activity of THKO fish during both Dark 1 and Dark 2 (Fig. 6H and I). During the light periods, no significant differences were observed between any of the experimentally-induced hypothyroid conditions and their respective controls, likely due to relatively low swimming activity during these periods.

3.6. Rescue of mct8MO behavioral phenotype with Tetrac

It has been demonstrated previously that treatment with the T4 analog, Tetrac, can rescue phenotypes associated with *mct8* deficiency (Horn et al., 2013). Thus, we used Tetrac (3 nM) to determine whether it would rescue deficiencies in the photomotor response observed in *mct8* morphants raised in 0.01% PTU. All treatment wells contained 0.1% DMSO to match the final concentration of DMSO in treatment wells with Tetrac. Consistent with our earlier observations (above), swimming activity during Dark 2 was significantly reduced at 3, 4, and 5 dpf in *mct8* morphants exposed to 0.01% PTU (Fig. 7). Exposure of control morphants to Tetrac significantly increased swimming activity during Dark 1 at 5 dpf and during Dark 2 at 4 dpf and 5 dpf (Fig. 7). Amongst *mct8* morphants raised in 0.01% PTU, treatment with Tetrac rescued the photomotor response, as evidenced by swimming activity that was not significantly different from that of control morphants (Fig. 7) at all time points in both Dark 1 and Dark 2.

4. Discussion

While TH disruption has been hypothesized as a mechanism contributing to adverse neurodevelopmental outcomes (reviewed in Gore et al., 2015; Miller et al., 2009), the impact of changes in TH activity on DNT endpoints typically evaluated in larval zebrafish has not been previously investigated. Here, we characterized the impact of experimentally induced hyper- and hypothyroidism on teratogenesis and photomotor behavior in larval zebrafish. The major findings of our study are: (1) photomotor responses are more sensitive to TH disruption than teratogenesis at all time points examined (3, 4 and 5 dpf); (2) developmental hyperthyroidism and developmental hypothyroidism exert opposite effects on photomotor responses, with the former

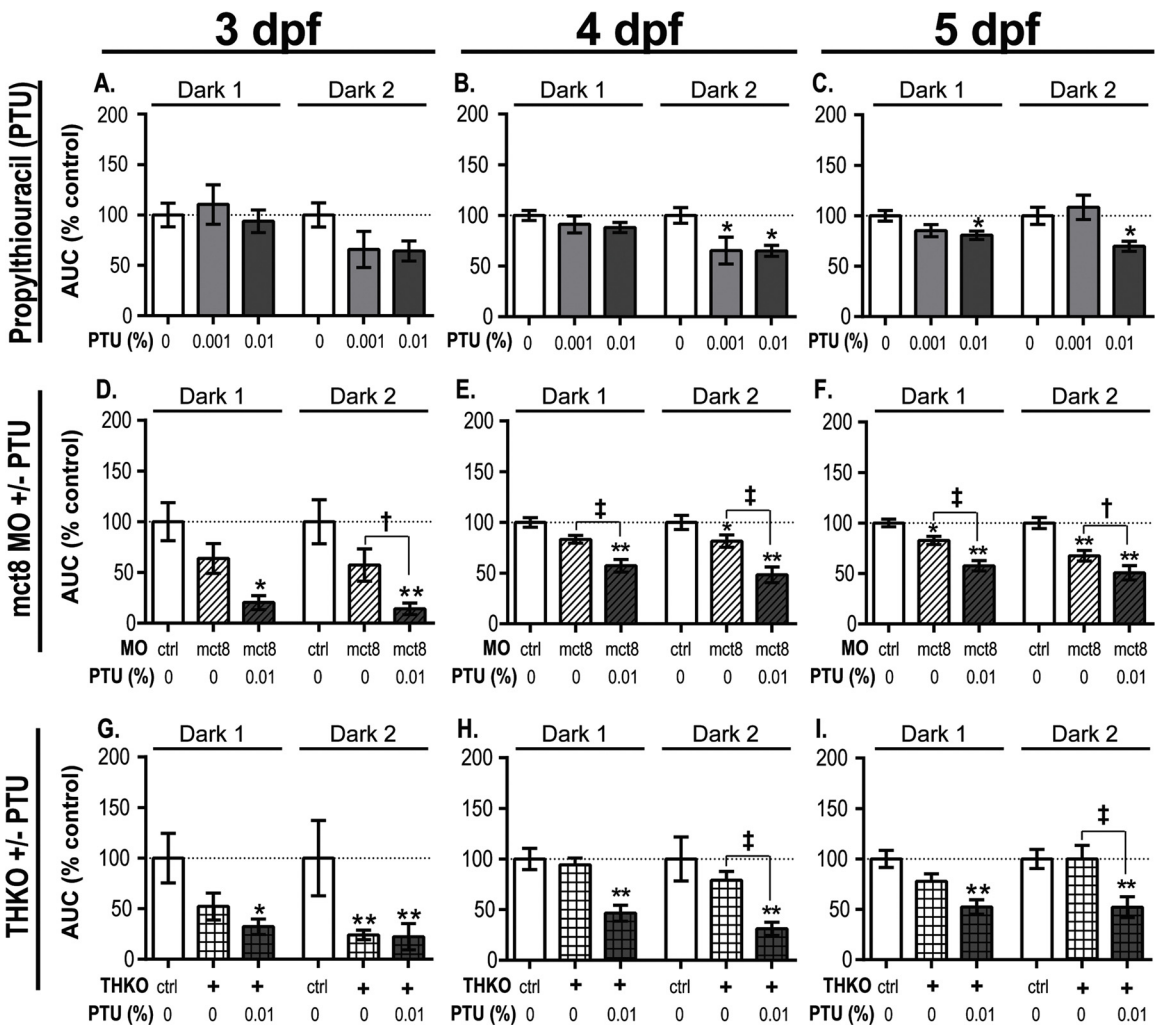

Fig. 6. Developmental TH deficiency alters photomotor behavior of larval zebrafish. The locomotor behavior of zebrafish larvae in response to photoperiod changes was assessed at 3, 4, and 5 dpf following treatment with propylthiouracil (PTU) (**A, B, C**), morpholino (MO) knockdown of *mct8* with subsequent culture of morphants in the absence or presence of 0.01% PTU (**D, E, F**), or in larvae spawned from thyroid ablated females (THKO) and maintained in the absence or presence of 0.01% PTU treatment (**G, H, I**). Data are presented as the mean ± SE of the percent change in the area under the curve (AUC) from respective controls during the two dark periods of the photomotor behavior test (sample size for each group is listed in Table S2 in the Supplemental material). Only live larvae with normal morphology were included in behavior analyses. The dotted line represents the mean AUC of controls (100%). *Significantly different from respective controls at *$p < 0.05$, **$p < 0.01$; †significant differences between *mct8* morphants or larvae from THKO fish grown in the presence of 0.01% PTU relative to their counterparts grown in the absence of PTU at † $p < 0.05$, ‡ $p < 0.01$ as determined using a mixed effects model to compare the natural log of the AUC (lnAUC) to minimize the impact of high outliers. The behavior test results for groups with significant differences in swimming activity are presented as the average distance swam during each 1 min bin over the course of the behavior test in Fig. S2 of the Supplemental material.

increasing and the latter decreasing swimming activity in response to sudden changes from light to dark conditions; and (3) the effects of developmental TH disruption vary as a function of treatment, day of testing, and test period in the photomotor behavioral paradigm.

It has previously been reported that treatment with the T4 and T3 analogs, Tetrac and Triac, respectively, cause morphological abnormalities in larval zebrafish (Haggard et al., 2018). The data reported here confirm and extend these observations by demonstrating that exposure to T4 or T3 causes time- and dose-dependent teratogenesis with the concentrations of T3 or T4 associated with significantly increased incidence of malformations decreasing with increasing age of the larval zebrafish. We also present novel data demonstrating that exposure to T3 or T4 alters photomotor behavior at concentrations lower than those that cause teratogenesis. In general, both T4 and T3 caused hyperactive swimming in response to a sudden change from light to dark with the most pronounced effects observed during the Dark 2 test period. The behavioral effects of both THs exhibited a non-monotonic inverted-U shaped concentration-response relationship, perhaps reflecting subtle adverse effects on viability at the higher concentrations, which were associated with increased teratogenesis. While the biological mechanism(s) responsible for the non-monotonic concentration-response relationship remain to be determined, this is not unprecedented in DNT or in endocrine disruption (Vandenberg et al., 2012; Wayman et al., 2012).

Another somewhat unexpected finding was that photomotor behavior was more sensitive to T4 than to T3, with the lowest effect concentration for T4 being 3 nM compared to 10 nM for T3. T4 is classically viewed as a prohormone that must be converted to T3 by tissue deiodinases 1 or 2 (*dio1/2*) for biological activity (Cheng et al., 2010). Previous LC–MS/MS measurements of TH concentrations in larval zebrafish exposed to exogenous T4 and T3, demonstrate consistently elevated tissue T4 and T3, respectively. However, T4 exposure did not significantly elevate whole body T3 concentrations, although it did significantly increase tissue levels of the metabolites rT3 and T2 (Walter et al., 2019). While these data do not rule out the possibility that there was sufficient conversion of T4 to T3 to trigger biologically relevant T3

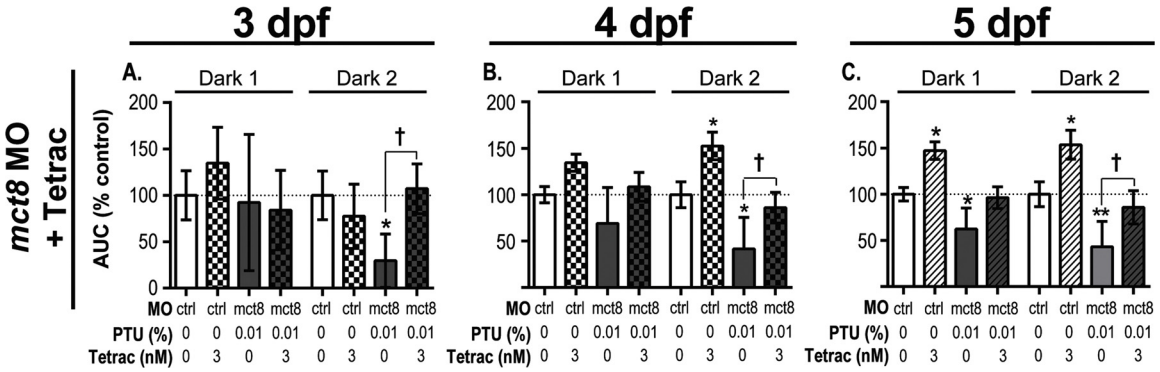

Fig. 7. Tetrac rescues swimming deficits caused by developmental hypothyroidism. The photomotor behavior of zebrafish larvae was assessed at 3, 4, and 5 dpf in fish injected with either control morpholino (ctrl MO) or *mct8* MO, and then grown in the absence or presence of 3 nM Tetrac or 0.01% PTU, singly or in combination. Data are presented as the mean (± SE) percent change in the area under the curve (AUC) from respective controls during the two dark periods of the photomotor behavior test (Dark 1 and Dark 2). Only live larvae with normal morphology were included in behavior analyses. The dotted line represents the mean AUC of controls (100%). *Significantly different from respective controls at *p < 0.05, **p < 0.01; †significant differences between *mct8* morphants grown in the presence of 0.01% PTU relative to morphants grown in the absence of PTU at † p < 0.05 as determined using a mixed effects model to compare the natural log of the AUC (lnAUC) to minimize the impact of high outliers. Sample size for each treatment are listed in Table S3 in the Supplemental material.

signaling in target cells of fish exposed to T4, the absence of a detectable increase in T3 in T4-exposed fish suggest that T4 effects on photomotor behavior are not necessarily mediated by T3. This alternative hypothesis is consistent with recent data challenging the traditional view of T4 as a prohormone and rT3 as an inactive metabolite. For example, biological activity of T4 has been observed in tissues with low expression of *dio1* and *dio2*, suggesting a direct involvement of T4 in TH receptor (TR)-mediated signaling (Maher et al., 2016), and it has been demonstrated that the relative abundance of corepressors and coactivators expressed in target cells may influence the response of TRs directly to T4 (Schroeder et al., 2014). Moreover, both T4 and rT3 can act through non-genomic signaling mechanisms to influence actin polymerization and the migration of neuronal and glial cells (Farwell et al., 2006). Thus, it is plausible that the differing time and concentration dependency of T4 *versus* T3 effects on photomotor behavior are due to direct actions of T4/rT3 mediated by signaling through nuclear TRs and/or non-genomic signaling pathways.

In contrast to experimentally induced hyperthyroidism, experimentally induced TH deficiency caused minimal teratogenesis and induced hypoactivity in response to transitions from light to dark conditions. Similar to developmental hyperthyroidism, effects of TH deficiency on the photomotor response were most robust during the Dark 2 testing period and occurred independent of significant teratogenesis. Different methods were used to generate TH deficiency, and both the timing and magnitude of the hypoactive photomotor response varied between these treatments in a manner consistent with the mechanism of TH disruption. We previously measured baseline thyroid hormone levels across early zebrafish development (up to 5 dpf) and found that at 3 dpf the concentration of T4 increased significantly above levels measured at 24 hpf and continued to rise, indicating the onset of larval T4 production. In addition, mRNA expression of TH signaling molecules were coordinately upregulated at 3 dpf, suggesting an increased susceptibility to interference with TH synthesis or TH signaling pathways may occur after this critical time point (Walter et al., 2019). Zebrafish treated with PTU, which decreases larval production of T4 (Walter et al., 2019), exhibited decreased swimming activity only at 4 and 5 dpf, but not at 3 dpf when maternally derived TH in the yolk is the predominant TH source in larval zebrafish (Walter et al., 2019). In contrast, in zebrafish spawned from adult female Tg (*tg:nVenus-2a-nfnB*)$^{wp.rt8}$ zebrafish with ablated thyroid follicles (THKO), which decreases the amount of maternally derived T3 in the yolk, exhibited significantly decreased swimming activity at 3 dpf only, before larval T4 production predominates. THKO embryos had significantly decreased T3 at 24 hpf, but normal T4 concentrations. THKO larvae treated with 0.01% PTU continued to show decreased swimming activity at 4 and 5 dpf. Together, these results suggest that maternally derived T3 may play a critical role in zebrafish development up to 3 dpf, when larval production of T4 begins. In addition, sufficient larval production and signaling of THs may correct for some of the impacts of early T3 deficiency. These larvae may be most sensitive to TH deficiency after they become reliant on larval-produced TH. We confirmed that hypoactive photomotor behavior was observed in larvae with morpholino knockdown of *mct8*, which would be expected to decrease intracellular TH concentrations in target cells. We also observed that exposure of *mct8* morphants to PTU caused a significantly greater swimming deficit, whereas treatment with the T4 analog Tetrac restored photomotor behavior in *mct8* morphants to control levels. The latter confirms that the effects of morpholino knockdown of *mct8* on photomotor behavior are mediated by TH deficiency. Collectively, these results highlight the ability of the photomotor test of larval zebrafish to detect compounding effects of chemicals targeting multiple mechanisms of TH disruption acting simultaneously, as is likely to occur with chemical mixtures.

We have demonstrated that larval zebrafish photomotor behavior is sensitive to altered TH activity during development, providing proof of concept data to support the use of this assay to assess the DNT potential of TH disrupting chemicals. Thyroid hormones have been shown to influence numerous neurodevelopmental processes at varying developmental time points (reviewed in De Groot et al., 2015; Williams, 2008), including neurogenesis, dendritic and/or axonal growth, synaptogenesis, and myelination (Gothie et al., 2017; Lopez-Espindola et al., 2014; Sharlin et al., 2008; Silva et al., 2017; Wang et al., 2016, 2014). Any one or combination of these processes may be altered by TH disruption, thereby disrupting neurodevelopment and eliciting neurobehavioral deficits. THs also contribute to the development and maintenance of the retina and altered TH signaling has been associated with retinal disease (reviewed in Yang et al., 2018), which presents another potential link between DNT and altered performance in the photomotor behavior assay. The behavioral abnormalities we have observed in this study may be due to many TH-dependent mechanisms of DNT. This is both a strength and weakness of using this behavior platform for chemical screening applications. Initially, this behavior assay may allow us to obtain many positive hits without knowing the specific adverse outcome pathways linking chemical-induced DNT and altered photomotor behavior. Additional mechanistic studies will be needed to confirm that photomotor deficits are mediated by TH-dependent mechanisms and to identify adverse outcome pathways linking TH-disruption to DNT. Additionally, it may be important to include additional

behavior tests probing different neural circuits and behavioral responses such as the 24 hpf PMR assay which measures spontaneous tail contraction stimulated by light through non-ocular photoreceptors located in the hindbrain (Kokel et al., 2013; Noyes et al., 2015a,b). A combination of assays to investigate changes in neurobehavior resulting from chemical exposures will likely be the best approach to capture chemical-induced DNT.

The results from our photomotor behavior assay also highlight important logistical considerations for using the photomotor behavior assay to screen TH disrupting chemicals for DNT. First, the non-monotonic concentration-response relationship observed for the photomotor effects of T4 and T3 indicate that chemicals need to be tested over a large concentration range to fully evaluate their potential for TH disruption (Vandenberg et al., 2012). We observed that the most sensitive and consistent photomotor effects were observed during the second dark period (Dark 2), which is often not included in shorter behavior tests used in high throughput screening. The Dark 2 period in our behavior test was 15 min, compared to the 5 min Dark 1 period. Therefore, the observed differences in sensitivity may be due to either the inclusion of a second dark photoperiod or a prolonged dark photoperiod, which may highlight differences in habituation to dark that result from treatments. Regardless, our data strongly support inclusion of a second dark photoperiod of prolonged duration to significantly increase test sensitivity. In addition, even within treatment groups, variable responses were observed on different testing days (3, 4, or 5 dpf), indicating that neurobehavior tests may need to be conducted at multiple time points to capture the fullest range of possible mechanisms of TH disruption. This will not necessarily increase the number of animals as we have previously demonstrated that repeated testing does not change the outcome at 5 dpf (Dach et al., 2019). Most importantly, photomotor behavior was more sensitive than teratology assessment for all test conditions, which is consistent with previous zebrafish chemical screens (Noyes et al., 2015a,b).

In summary, this study supports the feasibility of using photomotor behavior in larval zebrafish as a second tier test to assess the DNT potential of chemicals identified as TH active in *in vitro*, *in silico*, or computational screens. However, additional mechanistic tests will be needed to confirm that photomotor deficits are mediated by TH-dependent mechanisms and to identify critical exposure windows. This study has identified tools that will be useful for establishing such causal links.

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Conflicts of interest

The authors declare no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.neuro.2019.05.008.

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