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Investigation of NH3 as a selective thyroid hormone receptor modulator in larval zebrafish (*Danio rerio*)

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ABSTRACT

Thyroid hormones (THs) are essential for normal vertebrate development and diverse environmental chemicals are hypothesized to cause developmental toxicity by disrupting TH-mediated signaling. The larval zebrafish (*Danio rerio*) is an emerging *in vivo* model of developmental TH disruption; however, the effects of TR antagonism have not yet been studied in zebrafish. NH3, generally considered a potent and specific thyroid hormone receptor (TR) antagonist, has been used in rodents and *Xenopus laevis* to characterize phenotypes of TR antagonism. The objective of this study is to determine the effects of NH3 on endpoints previously determined to be TH-sensitive in larval zebrafish, specifically teratology and mortality, photomotor behavior, and mRNA expression of TH signaling genes. Zebrafish embryos were exposed to NH3 *via* static waterborne exposure at concentrations ranging from 0.001 to 10 μM beginning at 6 h post-fertilization (hpf) through 5 days post fertilization (dpf). Significant mortality and teratogenesis was observed at 3, 4, and 5 dpf in zebrafish exposed to NH3 at 10 μM. At concentrations that did not cause significant mortality, NH3 did not exert a consistent antagonistic effect on photomotor behavior assays or mRNA expression when administered alone or in the presence of exogenous T4. Rather, depending on the NH3 concentration and larval age NH3 decreased or increased swimming triggered by transition from light to dark. Similarly, inconsistent antagonistic and agonistic effects on mRNA expression of TH signaling genes were noted following treatment with NH3 alone. NH3 did inhibit T4 (30 nM)-induced gene expression; however, this was only consistently observed at a concentration of NH3 (10 μM) that also caused significant mortality. Collectively, these results suggest that NH3 does not act solely as a TR antagonist in larval zebrafish, but instead exhibits complex modulatory effects on TR activity. These data support the hypothesis that NH3 is a selective thyroid hormone receptor modulator. Further studies of NH3 interactions with the zebrafish thyroid hormone receptor are required to characterize the activity of NH3 in target tissues of the larval zebrafish at the molecular level, highlighting the importance of characterizing NH3 effects in specific models of TH-disruption to better interpret its actions in mechanistic screens of environmental chemicals for TH action.

1. Introduction

In vertebrates, tight regulation of thyroid hormone (TH) signaling is critical for proper development of the somatic and central nervous system systems. Disruption of TH signaling during critical developmental windows is, therefore, widely postulated as a mechanism by which environmental chemicals may adversely impact development, particularly neurodevelopment (Gore et al., 2015; Miller et al., 2009). One mechanism by which chemicals can interfere with TH signaling is *via* direct agonistic or antagonistic interactions with nuclear TH receptors (TRs) [reviewed in (Boas et al., 2012; Gore et al., 2015)]. While phenotypes of TR agonism can be identified using THs as pharmacologic probes, characterizing the phenotypes of TR antagonism has been challenging because of the lack of TR-specific antagonists. NH3 (Fig. 1) is one of the more potent and selective TR antagonists developed to date (Grover et al., 2007; Schapira et al., 2003; Singh et al., 2016). NH3 binds directly to both TRα and TRβ and inhibits T3-induced transcription *in vitro* (Lim et al., 2002). NH3 has been used to characterize phenotypes of

Abbreviations: Dio, deiodinase; Dpf, days post-fertilization; FW, fish water; Hpf, hours post-fertilization; NCOA, nuclear coactivator; NCOR, nuclear corepressor; T3, triiodothyronine; T4, thyroxine; TH, thyroid hormone; TR, thyroid hormone receptor; TSH, thyroid stimulating hormone.

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TR antagonism in rats (Grover et al., 2007), *Xenopus laevis* (Bronchain et al., 2017; Fini et al., 2007, 2012; Mengeling et al., 2017), TR reporter cell lines (Mengeling et al., 2017), and cultured human and rat neural progenitor cells (NPCs) (Dach et al., 2017; Fritsche et al., 2005). In these models of TH-mediated development and TH disruption, NH3 has proven to be a useful tool in establishing phenotypes of TR-antagonism; however, NH3 has consistently been shown to have mixed antagonistic and agonistic activity that varies across NH3 concentrations and models, making it essential to characterize the activity of NH3 within individual models (Mengeling et al., 2017; Nguyen et al., 2002).

Zebrafish (*Danio rerio*) larvae are increasingly being leveraged as an *in vivo* model of developmental toxicology (Dach et al., 2019; Noyes et al., 2015; Tal et al., 2020); however, the effects of NH3, or any other direct TR antagonist, on TH-sensitive endpoints in larval zebrafish have not been thoroughly characterized. Terrien et al. (2011) demonstrated that NH3 decreased TRIAC-induced fluorescence in a transient transgenic zebrafish expressing GFP under the control of a TH/bZIP promoter from *Xenopus laevis*. Yet, the utility of using NH3 as a reference compound for TR antagonism in zebrafish developmental assays routinely used for screening chemical compounds is unknown. Recently, we identified multiple endpoints in larval zebrafish that are sensitive to experimental manipulation of TH signaling, including mortality and teratogenesis, photomotor behavior, and transcriptional regulation of TH signaling genes (Walter et al., 2019b, c). The goal of this study was to evaluate the effects of NH3 on these TH-sensitive endpoints in larval zebrafish to determine the feasibility of using NH3 as a reference compound in screens of environmental chemicals for TH disruption in larval zebrafish.

2. Materials and methods

2.1. Zebrafish husbandry

Zebrafish work was approved and performed in accordance with the University of California Davis Institutional Animal Care and Use Committee (IACUC) protocols 17645 and 19391. Adult wildtype zebrafish (5D) were originally obtained from the Sinnhuber Aquatic Research Laboratory (SARL) and subsequent generations were raised at UC Davis. Water in zebrafish tanks was maintained at 28.5 ± 0.5 °C, pH 7.2 ± 0.4, and conductivity of 700 ± 100 μS. Adult zebrafish 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). Groups of 8–10 adult zebrafish were spawned naturally to obtain fertilized embryos. Embryos were staged as previously described (Kimmel et al., 1995) and kept in an incubator at 28.5 °C in fish water (FW) from the adult zebrafish husbandry racks (pH of 7.2 ± 0.4, and conductivity of 700 ± 100 μS) until 4 h post fertilization (hpf) at which time experimental manipulations were initiated.

2.2. Chemicals

The TR antagonist NH3 was synthesized in-house and its identity and purity (98 %) confirmed by ^{1}H and ^{13}C-NMR as previously described (Singh et al., 2016). Aliquots of 10 mM NH3 stock solution in DMSO were stored at −20 °C. The thyroid hormone L-thyroxine (T4, >98 %; Sigma T2376) was purchased from Sigma-Aldrich (St. Louis, MO, USA). T4 was reconstituted by first solubilizing 1 mg in 1 mL NaOH (1 N), per the manufacturer's recommendation, and then adding this solution to 49 mL deionized water. Aliquots of 25.7 μM T4 stock solutions were stored at −80 °C. Stock aliquots of T4 and NH3 were diluted to yield final concentrations at the time of exposure. An equivalent concentration of DMSO was added to controls and samples treated with T4 in the absence of NH3 so that all wells across all groups had a final DMSO concentration of 0.1 %.

2.3. T4 and NH3 exposures

Zebrafish embryos used for teratological and behavioral assessments 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 transferred to 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 NH3 and T4 exposures, 100 μL of 2X NH3 with or without T4 in EM or DMSO control solution was added to each well. Final DMSO concentrations in all wells was 0.1 %. The use of NaOH to solubilize T4 did not alter the pH of the final treatment solutions as confirmed by testing the pH of the final treatment solution, which was 7.3. Plates were kept in an incubator at 28.5 °C with 14 h light (~300 lx):10 h dark cycles. Evaporation was minimized by covering all plates with Parafilm M (Bemis NA, Neenah, WI). Zebrafish embryos were exposed continuously

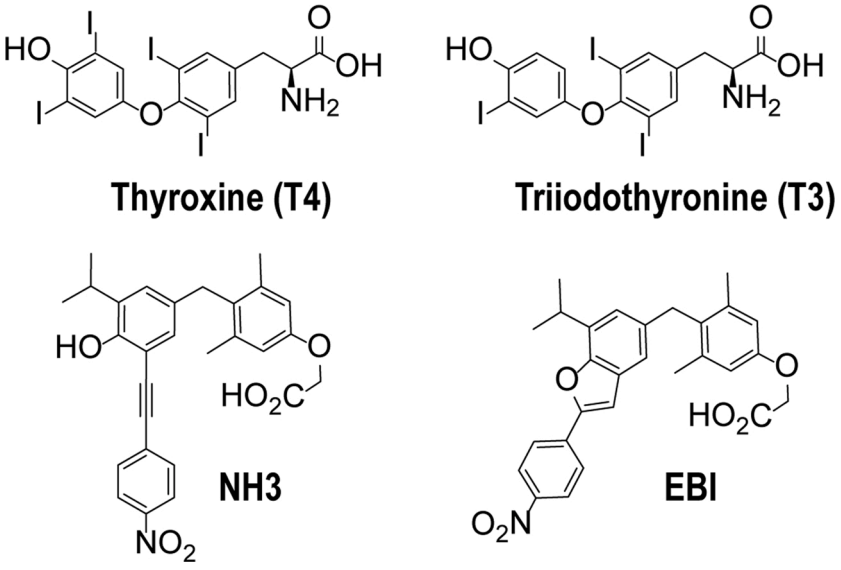

Fig. 1. Structures of thyroxine (T4), triiodothyronine (T3), TR antagonist NH3, and the benzofuran NH3 metabolite EBI, which acts as a TR agonist.

from 6 hpf through 5 days post fertilization (dpf) *via* static waterborne exposure, and teratogenesis and photomotor behavior was assessed at 3, 4, and 5 dpf (Fig. 2A). All experimental conditions were tested 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 (exact sample sizes for each endpoint are provided in the Supplemental material).

Zebrafish embryos used for quantitative real-time RT-PCR (qRT-PCR) were placed in 6-well plates (BD Falcon, Corning) with 20 embryos per well in 2 mL of EM. At 6 hpf, 1 mL of 3X NH3 with or without T4 in EM, or DMSO control, was added to each well. Plates were covered with Parafilm M to minimize evaporation and kept in an incubator at 28.5 °C with 14 h light (~300 lx):10 h dark cycles. Zebrafish embryos were exposed continuously from 6 hpf through 5 dpf *via* static waterborne exposure (Fig. 2A). At 5 dpf, fish were removed from treatment solution, washed in FW, and placed in 2 mL Eppendorf tubes with 1 mL RNAlater (Thermo Fisher Scientific, Waltham, MA, USA). Tubes were left at 4 °C for 24 h and then stored at −20 °C until RNA extraction. Three biological replicates of 20 pooled embryos each were collected for each TH treatment combination using embryos spawned from different adult pairs for each replicate.

2.4. Assessment of teratogenesis and larval photomotor behavior testing

Teratology and mortality were assessed at 24 hpf and at 3, 4, and 5 dpf as previously described (Walter et al., 2019b). Briefly, deaths at 24 hpf (<10 %) were attributed to pronase treatment during dechorionation and were not included in subsequent mortality totals. Larvae were evaluated at 3, 4, and 5 dpf for mortality (absence of heartbeat) and gross developmental malformations. Scored malformations included yolk sac edema, pericardial edema, body axis curvature, altered trunk length, abnormalities of the caudal fin, pectoral fin, somites, and eyes, craniofacial malformation, and gross abnormalities in brain, notochord, and circulatory system (Truong et al., 2011; Walter et al., 2019c). The percent of viable, malformed, and dead larvae at each time point were calculated across all replicates and significant differences in the incidence of these outcomes between groups was determined by chi-squared analysis followed by Fisher's exact test. Sample sizes for all treatment groups are listed in Table S1 in the Supplemental material.

Larval photomotor behavior was assessed at 3, 4, and 5 dpf using a DanioVision system (Noldus, Leesburg, VA, USA) as previously described (Walter et al., 2019b). We previously showed that repeated testing of larval zebrafish over 3 days does not influence photomotor response at later time points (Dach et al., 2019). 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 35 min test paradigm consisted of an initial 5 min light period (~1900 lx) to allow for acclimation, followed by a 5 min light period (~1900 lx) to record baseline swimming, a 5 min dark period to stimulate increased swimming activity, a 5 min light period to induce freezing behavior, and a final 15 min dark period to observe increased swimming activity and acclimation to dark conditions (Fig. 2B). Dead or malformed larvae were excluded from the behavior analysis. For each test period, Light 1 (5–10 min), Dark 1 (11–15 min), Light 2 (16–20 min), and Dark 2 (21–35 min), the area under the curve (AUC) of the distance swam by each fish was computed using the trapezoid method as previously described (Walter et al., 2019b). The AUC for each 1 min bin was calculated and 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 controls. Data are reported as the AUC, as a percent of control, focusing on the comparison during the dark periods. Sample sizes for all treatments are listed in Supplemental Tables 2 and 3.

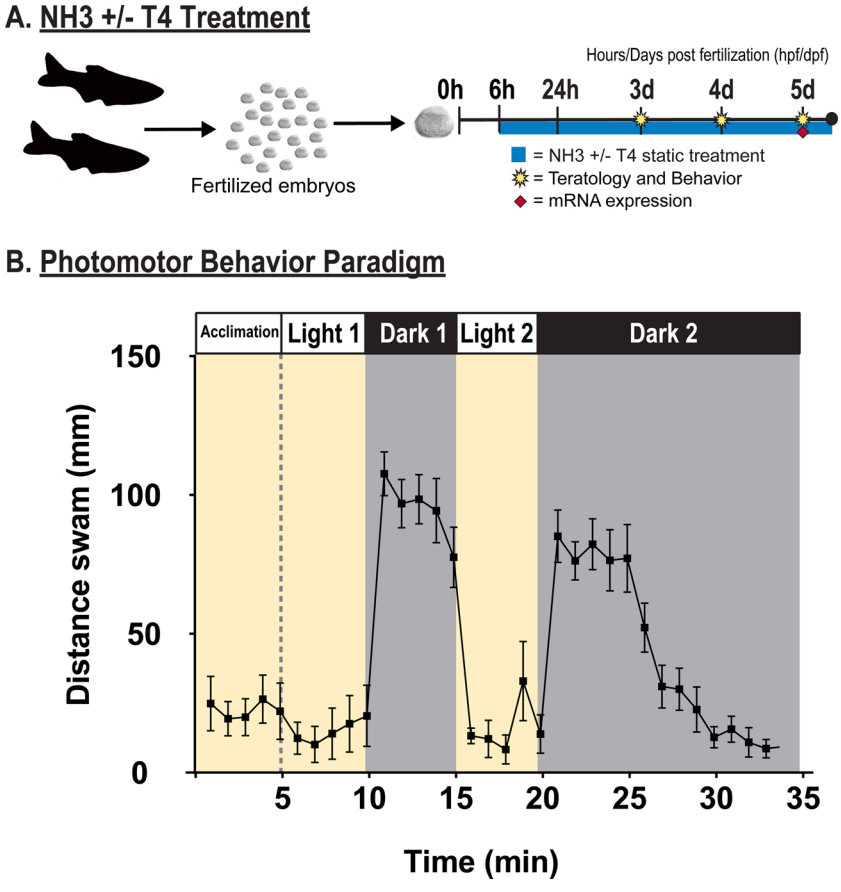

Fig. 2. Overview of experimental paradigm. (A) Fertilized zebrafish embryos were treated with NH3, T4, or a combination beginning at 6 hpf. Teratology was assessed and photomotor behavior quantified at 3, 4, and 5 dpf and the mRNA expression of TH-signaling genes was measured by qRT-PCR at 5 dpf. (B) Schematic illustrating the photomotor behavior assay and the typical response of larval zebrafish to alternating light and dark. The initial 5 min was an acclimation period, 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). Statistical comparison of the natural log of the area under the curve (lnAUC) was performed using a mixed effects model. Adapted from (Walter et al., 2019b).

2.5. Quantitative real time RT-PCR (qRT-PCR) and expression analysis of TH-signaling genes

RNA extraction, cDNA synthesis, and qRT-PCR were performed as previously described (Walter et al., 2019a, c). Expression of a select group of genes either previously shown to be influenced by TH exposure or suspected of being TH-responsive were analyzed by qRT-PCR. The Ct values for each target gene were normalized to the geometric mean of the Ct values for two reference genes, *β-actin* and *elf1a,* within the same sample (McCurley and Callard, 2008). Amplification efficiency (AE) adjusted delta-delta Ct values (ΔΔCt) were determined using the following equation as previously described (Walter et al., 2019a; Yuan et al., 2008): $\Delta\Delta Ct = [(Ct_{tgt\_trt} \times AE_{tgt\_trt}) - (Ct_{ref\_trt} \times AE_{ref\_trt})] - [(Ct_{tgt\_ctrl} \times AE_{tgt\_ctrl}) - (Ct_{ref\_ctrl} \times AE_{ref\_ctrl})]$ where tgt is the target gene, ref is the reference gene, trt is the treatment, and ctrl is the control. Comparisons of mRNA levels across treatment conditions were evaluated as ΔΔCt and compared to DMSO controls using one-way ANOVA with Bonferroni's multiple comparison test. $P < 0.05$ was considered significant.

3. Results

3.1. Teratogenic and lethal effects of developmental exposure to NH3

NH3 has previously been reported to antagonize the TR at low concentrations, but exhibit mild agonistic effects at high concentrations (Lim et al., 2002). Therefore, we evaluated a wide range of NH3 concentrations for teratogenic and lethal effects. Zebrafish larvae were exposed to NH3 at 0.001–10 μM beginning at 6 hpf and continuing until 5 dpf. A significant increase in the incidence of teratogenesis and mortality was observed only in larvae exposed to NH3 at 10 μM. Adverse effects of 10 μM NH3 were observed at 3, 4, and 5 dpf with the incidence of teratogenesis and mortality increasing with increased exposure time (Fig. 3).

3.2. Effects of developmental NH3 exposure on photomotor behavior

The photomotor behavior test is based on stereotypic changes in locomotor activity of larval zebrafish in response to sudden changes in light conditions (Emran et al., 2008). An abrupt change from dark to light causes larval zebrafish to decrease or stop swimming, whereas a sudden change from light to dark triggers increased locomotion (MacPhail et al., 2009) (Fig. 2B). Photomotor behavior was tested in zebrafish larvae at 3, 4, and 5 dpf following exposure to NH3 beginning at 6 hpf (Fig. 4). At 3 dpf, exposure to NH3 at 0.01 μM significantly decreased locomotor activity during Dark 2. Higher NH3 concentrations elicited a non-significant but concentration-dependent trend of increased locomotor activity during the Dark 1 and Dark 2, which peaked at 1 μM then returned to baseline at 3 μM, and decreased at 10 μM (Fig. 4A). While the effect of 10 μM NH3 was significant during Dark 1, none of the other changes reached statistical significance due to high variability in swimming distance. At 4 dpf, the two highest concentrations of NH3, 3 and 10 μM, significantly increased larval swimming activity during both the Dark 1 and Dark 2 periods (Fig. 4B). However, at 5 dpf there were no significant changes in photomotor behavior at any concentration of NH3 Fig. 4C).

We previously reported that developmental exposure to exogenous T4 increases locomotor activity in the photomotor behavioral assay (Walter et al., 2019b). To determine whether NH3 inhibited this effect of T4, zebrafish were exposed to T4 (10 nM) alone or in combination with NH3 (1, 3, or 10 μM), and photomotor behavior was evaluated at 3, 4, and 5 dpf (Fig. 5). At 3 dpf, T4 significantly increased locomotor activity during both Dark 1 and Dark 2, which was blocked by NH3 at all three concentrations tested (Fig. 5A). At 4 dpf, T4 significantly increased locomotor activity during the Dark 2 period. Addition of NH3 at 1 or 3 μM did not significantly decrease the effects of T4 on locomotor activity (Fig. 5B). At 5 dpf, T4 significantly increased locomotor activity during both Dark 1 and Dark 2 periods. The addition of NH3 at 3 μM reduced swimming activity to a level similar to controls; however, NH3 concentrations of 1 and 10 μM had no effect on T4-induced locomotor activity (Fig. 5C).

3.3. Effects of NH3 on transcription of TH signaling genes

The mRNA expression of TH signaling genes in larval zebrafish at 5 dpf was measured by qRT-PCR following exposure to NH3 (0.01–10 μM) (Fig. 6). The genes investigated included TH transporters *mct8*, *oatp1c1*, *lat1*, *lat2*, TH deiodinases *dio2* and *dio3-b*, TH receptors *trα-a* and *trβ*, and suspected TH-responsive genes *mbp-a*, *bdnf*, *klf9*, and *mag*. Only two genes showed significant changes in mRNA levels following exposure to NH3. Expression of *mct8* was increased (Fig. 6A) while the expression of *dio3-b* was decreased (Fig. 6F) relative to DMSO control by NH3 at 0.01 and 0.1 μM, respectively. The other NH3 concentrations that were tested had no significant effect on transcript levels of this panel of genes.

To determine whether NH3 can inhibit TH-induced mRNA upregulation, four genes previously shown to be TH-responsive in larval zebrafish (Walter et al., 2019c) were evaluated by qRT-PCR following treatment with T4 (30 nM) alone or in combination with NH3 at 1 or 10 μM. Genes investigated include *mct8*, *dio3-b*, *trα-a*, and *trβ* (Fig. 7). T4 significantly increased transcription of all 4 of these genes. Co-exposure to NH3 at 1 or 10 μM blocked T4-induced transcription of *mct8* (Fig. 7A), *trα-a* (Fig. 7C), and *trβ* (Fig. 7D). For *dio3-b,* co-exposure to NH3 attenuated T4-induced mRNA expression but did not reduce expression to control levels (Fig. 7B).

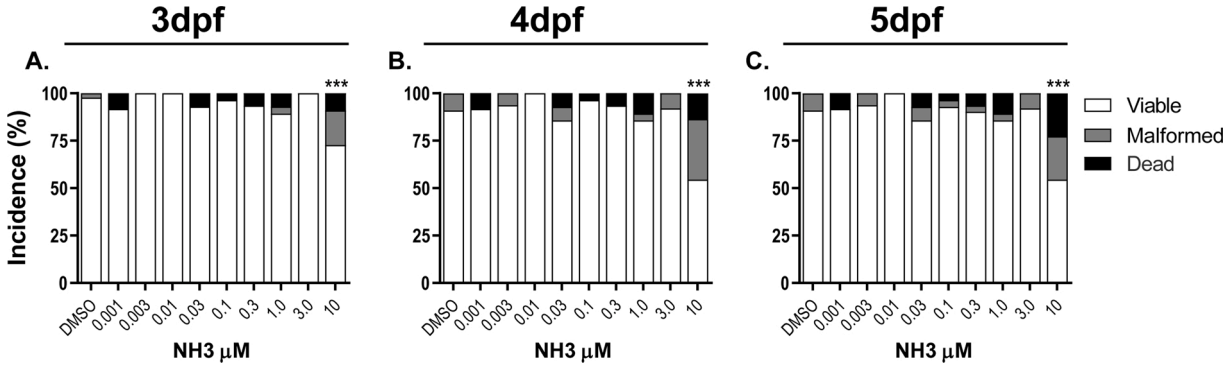

Fig. 3. Effects of NH3 on the viability and incidence of teratological outcomes in larval zebrafish. Data are presented as the percentage of viable, malformed, and dead embryos observed following exposure to exogenous NH3 from 6 hpf through 5 dpf. Significantly different from DMSO controls at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ 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.

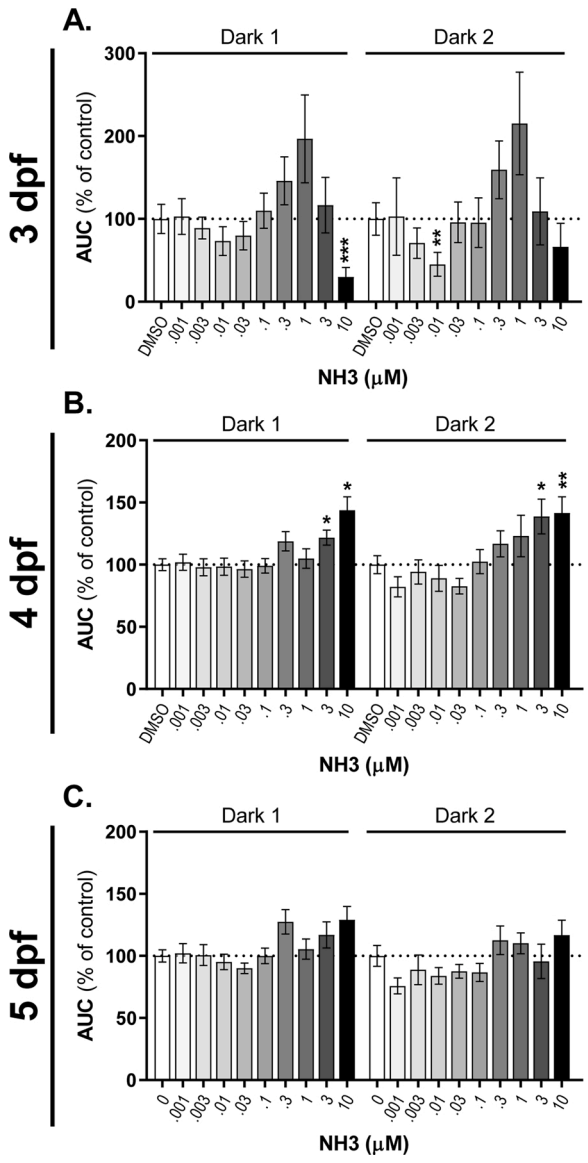

Fig. 4. Photomotor behavior of zebrafish larvae exposed to NH3. Zebrafish larvae were exposed to varying concentrations of NH3 beginning at 6 hpf and locomotor behavior in response to changes in light were assessed in the same fish on three consecutive days (3, 4, and 5 dpf). Only live larvae with normal 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 first 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. Significantly different from DMSO controls at *p < 0.05 and **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 results of the photomotor behavior test are also presented as the average distance swam during each 1 min bin over the course of the behavior test for groups with significant differences in swimming activity in Fig S1 of the Supplemental material.

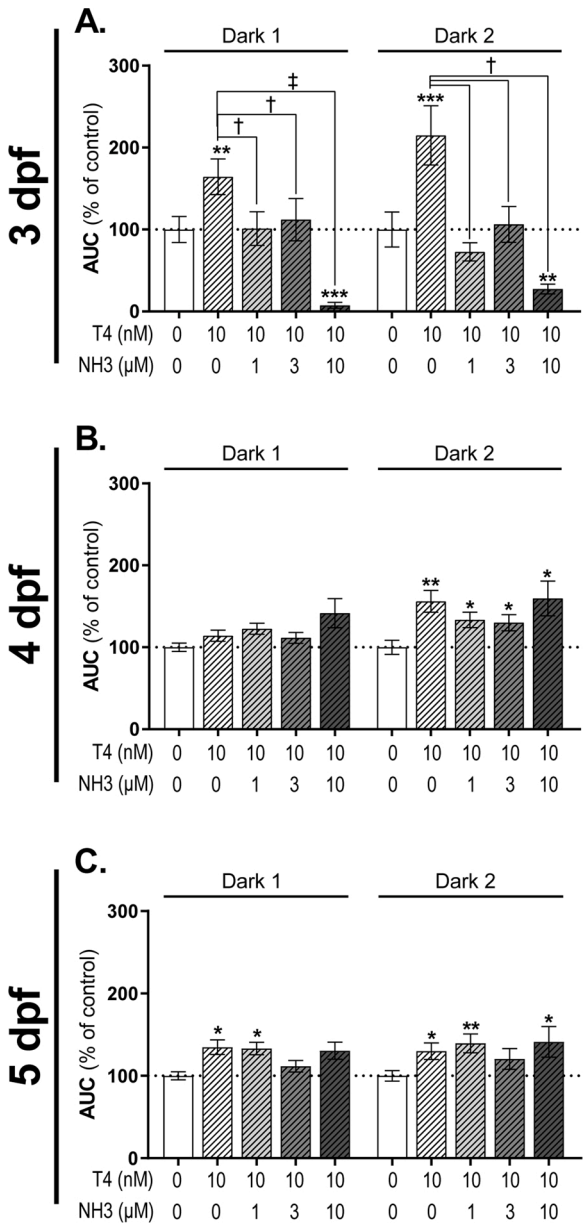

Fig. 5. Photomotor behavior of zebrafish larvae exposed to exogenous T4 and NH3. The locomotor behavior of zebrafish larvae in response to light changes was assessed at 3, 4, and 5 dpf following treatment with T4 alone or in combination with NH3. Data are presented as the mean ± SE of the percent change in the area under the curve (AUC) from DMSO control during the two dark periods of the photomotor behavior test. Sample size for each group is listed in Table S3 in the Supplemental material. Only live larvae with normal morphology were included in behavior analyses. *Significantly different from DMSO control at *p < 0.05, **p < 0.01; †significant differences between larvae treated with both T4 and NH3 relative to those treated with T4 alone 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 results of the photomotor behavior test are also presented as the average distance swam during each 1 min bin over the course of the behavior test for groups with significant differences in swimming activity in Fig S1 of the Supplemental material.

4. Discussion

Zebrafish larvae are a promising *in vivo* model for studying developmental toxicity associated with TH active chemicals (Haggard et al., 2018; Noyes et al., 2015; Walter et al., 2019b, c). We previously demonstrated that experimentally-induced developmental hyper- and hypothyroidism in larval zebrafish caused opposing effects on teratogenesis, mortality, photomotor behavior, and transcription of TH signaling molecules and TH-responsive genes (Walter et al., 2019b, c). In these previous experiments, developmental hyperthyroidism was modeled by exposure of developing wildtype zebrafish to exogenous T4 or T3, while developmental hypothyroidism was simulated by exposure to 6-propyl-2-thiouracil (PTU) or morpholino knockdown of TH transporter *mct8* in developing zebrafish spawned from adult females whose

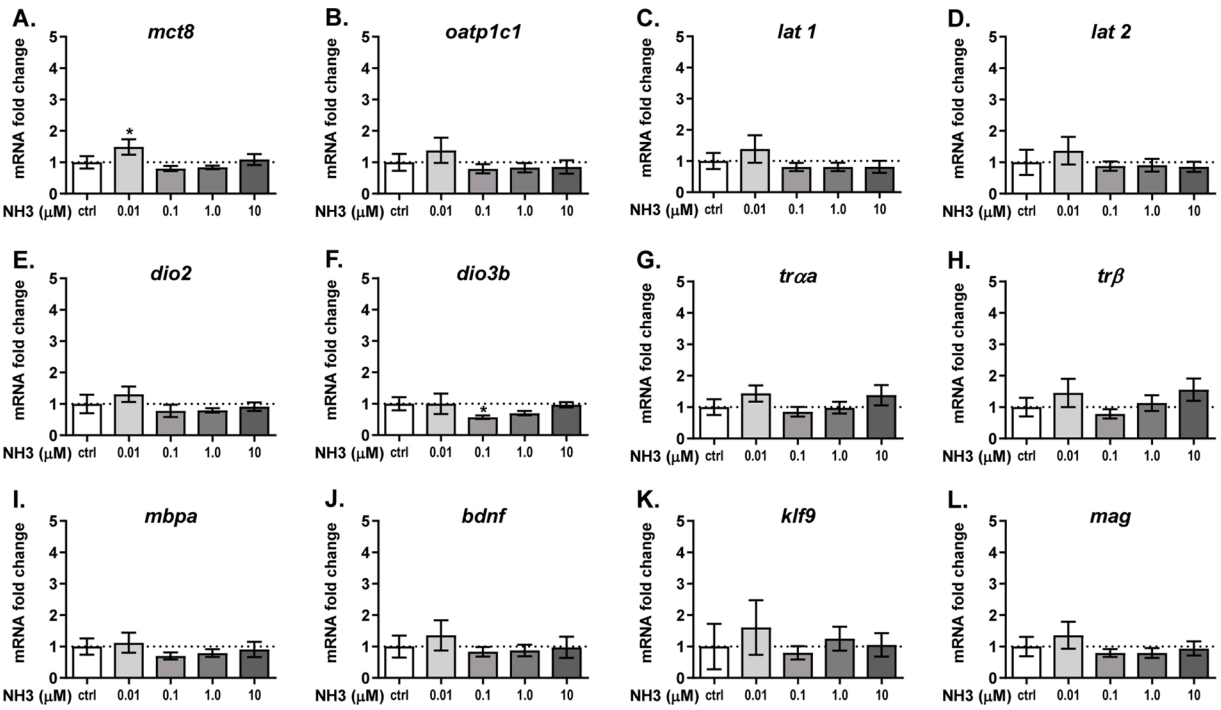

Fig. 6. NH3 effects on mRNA levels of TH-signaling genes. Transcript levels following treatment with NH3 were determined by quantitative real time PCR (qRT-PCR) in pooled samples of zebrafish collected at 5 dpf. Expression of each gene is presented as the relative expression measured relative to DMSO controls ($2^{-\Delta\Delta Ct}$), as determined using the Pfaffl delta-delta Ct method. All samples were normalized to the reference genes *β-actin* and *elf1a*. Data are shown as the means + SE (n = 3 replicates of 30 pooled embryos per group). *Indicates a significant difference at $p < 0.05$ from DMSO control samples as determined by ANOVA with Bonferroni multiple comparison test.

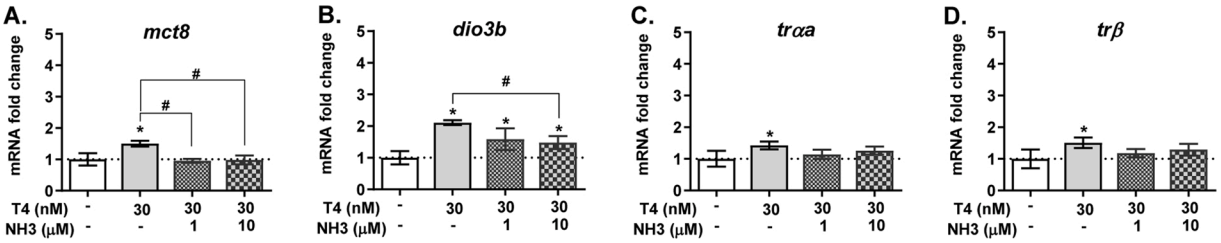

Fig. 7. Effects of NH3 on T4-induced changes in mRNA expression. Transcript levels following treatment with T4 in the absence or presence of NH3 were determined by quantitative real-time PCR (qRT-PCR) in pooled samples of zebrafish collected at 5 dpf. Expression of each gene is presented as the relative expression measured relative to DMSO controls ($2^{-\Delta\Delta Ct}$), as determined using the Pfaffl delta-delta Ct method. All samples were normalized to the reference genes *β-actin* and *elf1a*. Data are shown as the means + SE (n = 3 replicates of 30 pooled embryos per group). *Indicates a significant difference at $p < 0.05$ from DMSO control; #significant differences between larvae treated with both T4 and NH3 relative to those treated with T4 alone at # $p < 0.05$ as determined by ANOVA with Bonferroni multiple comparison test.

thyroid follicles had been ablated to prevent deposition of maternal TH in the yolk sac of embryonic fish. The current study reports novel data using these endpoints previously shown to be sensitive to TH modulation to assess the utility of NH3 as a reference compound of TH disruption in larval zebrafish. The major findings of this study are: (1) NH3 modulates TH-dependent endpoints in larval zebrafish; however, it does not consistently phenocopy developmental hypothyroidism or hyperthyroidism; and (2) NH3 inhibits the effects of supraphysiological levels of T4 on photomotor behavior and gene expression in an inconsistent manner and predominantly at concentrations that also cause significant morbidity and mortality. These data highlight the complexity of studying TH-mediated developmental endpoints and TH disruption *in vivo*, and suggest that NH3 does not act solely in an antagonistic or agonistic fashion in regards to photomotor behavior responses or mRNA expression in larval zebrafish, but instead exhibits mixed agonistic/antagonistic activity. Therefore, further investigation into the molecular action of NH3 at the zebrafish TR and the factors that influence its modulatory activity is needed to interpret effects of NH3 when used as a reference compound for TR modulation in a zebrafish model of TH disruption.

Previous *in vivo* studies similarly demonstrated mixed antagonist and agonist activity of NH3; however, contrary to our findings, TR antagonism was observed at the majority of concentrations tested and weak TR agonism only at higher NH3 concentrations (Grover et al., 2007; Lim et al., 2002; Mengeling et al., 2017). The initial characterization of NH3 in *Xenopus laevis* showed that NH3 arrested TH-dependent spontaneous metamorphosis and inhibited TH-induced early metamorphosis in a concentration-dependent manner. However, at higher concentrations (2 μM), NH3 exhibited partial agonist activity in a tail resorption assay (Lim et al., 2002), although the combined treatment of T3 + 2 μM NH3 did not have an additive effect in this assay. In rats, lower doses of NH3 exerted antagonistic effects on three TH-responsive endpoints: decreasing heart rate and increasing plasma cholesterol and circulating TSH (Grover et al., 2007). However, at higher doses, NH3 had significant

agonistic effects on each of these endpoints. In addition, NH3 inhibited the effects of exogenous T3 on cholesterol, TSH, and heart rate (Grover et al., 2007). One previously proposed hypothesis for the agonistic activity of NH3 was the presence of a co-purifying EBI compound (Fig. 1) that has TR-agonistic activity (Singh et al., 2016). To test this hypothesis, a revised protocol for synthesizing NH3 was developed to eliminate the co-purifying EBI compound (Singh et al., 2016). NH3 synthesized using this modified protocol retained weak, but not significant, TR agonist activity in both a TRE-luc reporter assay and cellular proliferation assay (Mengeling et al., 2017). Currently, there are two prevailing hypotheses to explain the observed agonistic effects of high concentrations of NH3 on TH-dependent endpoints. First, is that NH3 may be converted or metabolized to a compound with agonistic activity, including EBI, which can form in basic solutions (Singh et al., 2016). Second, agonism of NH3 at high concentrations reflects dual biological activities, where in addition to the antagonistic effects induced by prevention of NCOA binding, NH3 also causes de-repression by preventing the binding of NCORs (Mengeling et al., 2017; Nguyen et al., 2002).

Using the larval zebrafish photomotor behavior assay, we previously observed that exposure to exogenous T4 or T3 increased locomotor activity triggered by sudden changes from light to dark conditions, and this effect exhibited a non-monotonic inverted-U shaped concentration-response relationship; in contrast, developmental hypothyroidism decreased locomotor activity (Walter et al., 2019b). Based on these prior observations, we hypothesized that if NH3 acted purely as a TR antagonist, larval zebrafish exposed to NH3 would exhibit decreased photomotor activity during dark periods. While our results do point to some NH3-mediated modulation of TH action, neither a consistent antagonistic nor agonistic effect was seen. We observed that exposure to NH3 alone significantly decreased locomotor activity only at 3 dpf and only in larvae exposed to NH3 at 0.l or 10 μM. At other concentrations, NH3 had no significant effect on photomotor behavior in 3 dpf zebrafish, although there was a concentration-dependent trend towards increased locomotor activity that peaked at 1 μM. The decreased swimming at the low end of the NH3 concentration range may reflect TR antagonism, whereas the decreased locomotor activity at 10 μM may be due to TR antagonism or subtle adverse effects on larvae viability. While the latter is supported by observations of decreased viability in 3, 4, and 5 dpf larvae exposed to NH3 at 10 μM, it is not consistent with the observation that at 4 dpf, NH3 at 3 and 10 μM significantly increased locomotor activity. These observations suggest that at higher concentrations and in the presence of physiologic levels of THs, NH3 may exert agonistic effects on the TH-mediated pathways that influence larval photomotor swimming activity.

In consideration of the variable effects of NH3 treatment on larval zebrafish, it is important to note that TR antagonism may not truly phenocopy hypothyroidism. Previous studies have demonstrated that neither TRα nor TRβ knockout mice phenocopy hypothyroidism (Flamant and Samarut, 2003). This is believed to be due to the repression of TH-responsive genes secondary to unliganded TRs bound to NCORs at TH response elements (TREs) on DNA (Flamant and Samarut, 2003). Therefore, the dynamics of NCORs and NCOAs in association with TRs and NH3 treatment may have a significant impact on the *in vivo* effects of NH3. Previous *in vivo* studies have demonstrated that NH3 exhibits mixed antagonist and agonist activity, with antagonism exhibited lower range of concentrations, and mild agonism seen at higher concentrations (Grover et al., 2007; Lim et al., 2002; Mengeling et al., 2017). This phenomenon has previously been attributed to NH3-stimulated release of NCORs in addition to its inhibition of NCOA recruitment (Lim et al., 2002; Nguyen et al., 2002; Webb et al., 2002). Thus, it has been hypothesized that the *in vivo* effects of NH-3 are likely dependent on both the endogenous level of THs present and the relative importance of NCOA binding and NCOR release within target cells (Grover et al., 2007; Webb et al., 2002).

An additional consideration is that the previously observed effects of developmental hyper- and hypothyroidism on larval zebrafish photomotor behavior are not solely mediated by nuclear TRs. In the initial characterization of larval photomotor behavior, compared to T3, treatment with exogenous T4 elicited a more robust increase in larval swimming activity as evidenced by increased sensitivity of larvae to T4 *vs*. T3 and T4 increased swimming activity at 3, 4, and 5 dpf, whereas T3 increased swimming activity only at 3 and 4 dpf (Walter et al., 2019b). Measurements of T4, T3, rT3, and T2 over the five days of exposure to T4 did not show significant increases in tissue concentrations of T3, suggesting that T4 exposure did truly generate a stronger effect in the photomotor behavior assay. The authors of the previous work suggested two primary hypotheses for the stronger action of T4 compared to T3, including: (1) the influence of THs on larval photomotor behavior may be partially or completely mediated by non-genomic TH signaling pathways; and/or (2) T4 has stronger genomic TR activity than traditionally believed. The traditional view of T4 as a prohormone that must be converted to T3 for genomic TR activity has recently been challenged in studies showing that the relative abundance of corepressors and coactivators expressed in target cells can influence the direct action of T4 on TRs (Schroeder et al., 2014). In addition, biological activity of T4 has previously been observed in tissues with low expression of dio1 and dio2, supporting a direct action of T4 in TR-mediated signaling (Maher et al., 2016). We observed that at supraphysiological T4 levels, NH3 can act as a TR antagonist. This observation suggests that either T4-induced increases in photomotor behavior require activation of nuclear TRs or that NH3 has effects on non-genomic TH signaling pathways in addition to its action on nuclear TRs. Previous studies have demonstrated that NH3 action relies on its ability to prevent co-activator recruitment to nuclear TRs (Nguyen et al., 2002); however, the action of NH3 on non-genomic TR-mediated signaling pathways has not been thoroughly investigated. Therefore, it is plausible the NH3 may have activities in addition to its previously described activity involving nuclear TRs.

An interesting pattern observed across photomotor studies conducted in the absence and presence of T4 was that NH3 had minimal impact on photomotor behavior in 5 dpf larvae. One potential explanation of the differential responses to NH3 over time is that the compound becomes less bioactive with time. While we do not have data regarding the stability of NH3 in our zebrafish model, two observations suggest that increasing NH3 degradation with time is not a major reason for the decrease in NH3 potency with increasing larval age. First, the teratogenic and lethal effects of NH3 increased over time with the most potent effects observed at 5 dpf. Second, NH3 significantly inhibited T4-induced transcription, which was measured at 5 dpf. It is possible that the effects of NH3 observed at 5 dpf are manifestations of NH3 activity at earlier developmental time points. Future investigation of variably timed exposure to NH3 and measurement of NH3 in solution across the 5 days treatment window would help to clarify the time-dependent effects of NH3.

While NH3 significantly inhibited T4-induced transcription, in the absence of exogenous TH, NH3 had minimal effect on the transcription of TH signaling molecules or TH-responsive genes. We previously demonstrated that exposure to exogenous T4 or T3 significantly increased transcript levels of *mct8*, *dio3-b*, *trα-a*, *trβ*, and *mbp-a,* and significantly decreased the mRNA expression of *oatp1c1* in 5 dpf zebrafish larvae (Walter et al., 2019c). While NH3 significantly decreased *dio3-b* mRNA expression, consistent with TR antagonism, it significantly increased *mct8* expression, consistent with TR agonism. Given that these effects were only observed at 0.01 and 0.1 μM NH3, respectively, it is difficult to interpret their biological significance. Overall, the qRT-PCR data do not point to a clear and consistent mechanistic explanation of the observed impacts of NH3 on mRNA expression of TH responsive genes. It is plausible, given the observed mixed agonist/antagonist action of NH3 in previous studies that the expectation that NH3 would show consistent influences on TH-mediated mRNA expression is too simplistic. Selective estrogen receptor modulators (SERMs), such as tamoxifen, have previously been demonstrated to have varying agonistic and antagonistic activity in different target

organs, which is hypothesized to be the result of selective recruitment of coactivators and corepressors in target cells (Feng and O'Malley, 2014). Therefore, one possible explanation for the inconsistent effects of NH3 on mRNA expression of TH-responsive genes is that varying effects of coregulators in the target cells where genes such as *mct8* and *dio3b* are predominantly expressed determine the modulatory effects of NH3. Therefore, a more accurate description of the action of NH3 may be as a selective thyroid hormone receptor modulator (STRM).

In addition, it is important to note that the effects of THs and NH3 on expression of TH-responsive genes such as *mct8* vary between species, target tissue, and target gene. For example, in our previous studies both T4 and T3 treatment increased *mct8* mRNA expression; in contrast, T3 and T2 exposure repressed *mct8* expression in organotypic cerebellum cultures from Tilapia but had no effect *in vivo* (Hernández-Linares et al., 2019). In addition, a previous study in Xenopus demonstrated NH3 phenocopied T3 in inducing expression of *klf9* and *thrb* in head and brain tissue; however, NH3 had opposite effects of T3 on expression of *dio1* and *dio3* in Xenopus heads (Fini et al., 2012). This further highlights the complexity of characterizing patterns of TH regulation, and thus the effects of TH-disruption, between species and across target organs and genes.

We observed mixed antagonist and agonist activity of NH3 in larval zebrafish. However, in contrast to prior studies (Grover et al., 2007; Lim et al., 2002), we saw minimal antagonistic action of NH3 when administered to larval zebrafish in the absence of exogenous THs and the concentrations at which agonistic effects were seen are of questionable biological significance. There are multiple caveats that must be considered regarding the observed agonistic action of NH3 at high concentrations. Agonistic effects were only observed at very high concentrations > 1 μM. Considering that the *in vitro* IC_{50} of NH3 in a GH3. TRE-luc reporter assay is 55.2 nM (Singh et al., 2016), the high concentrations at which NH3 exerted agonistic action in zebrafish may represent non-specific effects instead of direct TR agonism. The uptake of NH3 in larvae has yet to be characterized due to logistic issues in resolving NH3 *vs*. EBI in complex biological matrices; thus, it is difficult to determine the target concentrations responsible for this observed agonism and to assess whether agonistic effects are due to off-target effects. Another caveat is that the TR agonist EBI can be formed spontaneously from NH3 in basic solutions (Singh et al., 2016). While all treatments were conducted in pH buffered EM, it is still possible that some EBI may form in solution over the course of the 5 day exposure. This is less likely considering that the agonistic action was most prominent at 4 dpf and diminished at 5 dpf. Another caveat is that we did not determine whether NH3 blocked the effects of T3.

The discrepancies between studies of NH3 highlight key considerations for the use of NH3 as a reference compound for studying TH-mediated development and TH-disruption by environmental chemicals. First, despite evidence indicating that TH-signaling pathways are highly conserved across vertebrate species (Blanton and Specker, 2007; Porazzi et al., 2009), NH3 does not consistently act as an antagonist across species. Instead, NH3 demonstrated both antagonist and agonistic action, which varied across developmental time, endpoint assessed, concentration of NH3, and addition of exogenous T4. While NH3 may have utility as a selective TR modulator (STRM); its utility as a reference compound for TH disruption in the *in vivo* zebrafish model must be further investigated. Therefore, it is critical that its effects be characterized within each model prior to its use as an assay control or mechanistic tool for studying TH-disruption. Second, the effects of NH3 may be dependent on the level of endogenous THs present in the target system, and the developmental stage at the time of outcome assessment. This may reflect the varying importance of NCORs and NCOAs within different target cells and across developmental time points and should be further investigated on a molecular level (Astapova and Hollenberg, 2013; Moore et al., 2004). Third, since NH3 is not commercially available and has historically required a challenging 13-step synthesis in which an agonistic side-product is produced, its synthesis remains a significant source of variability that may influence results and further limits the utility of NH3 in zebrafish. An alternative synthesis published by Placzek and Scanlan (2015) may improve availability of this compound and facilitate further study into the complex mechanisms of action of NH-3.

In summary, our results suggest that the effects of NH3 are species-specific, highlighting the importance of characterizing NH3 effects in specific models of TH-disruption to better interpret its use as a reference compound in screens or mechanistic studies of environmental chemicals for TH-disrupting activity. In addition, the modulatory capability of NH3 may vary by target cell and endpoint. For zebrafish specifically, NH3 does exhibit TR modulatory activity; however, the inconsistent effects of NH3 *in vivo* may limit its reliability as a reference compound of TH disruption, at least until further molecular characterization can be conducted to elucidate the specific action of NH3 on TH-mediated signaling pathways in larval zebrafish. It is, however, important to consider that environmental chemicals with TH-disrupting capacity may behave similarly to NH3 instead of acting as a true antagonist or agonist. Further investigation is necessary to determine the molecular activity of NH3 on nuclear and non-genomic TH-mediated signaling pathways and how modulation of these pathways influences *in vivo* developmental endpoints of TH disruption.

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CRediT authorship contribution statement

K.M. Walter: Conceptualization, Methodology, Data Curation, Investigation, Writing-Original Draft, Visualization. **L. Singh:** Methodology, Investigation, Editing. **V. Singh:** Methodology, Investigation, Writing-Original Draft, Editing. **P. Lein:** Conceptualization, Writing-Review & Editing, Supervision, Project Administration, Funding Acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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