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Research Article

Thyroid Disrupting Chemicals in Mixture Perturb Thymocyte Differentiation in *Xenopus laevis* Tadpoles

Connor C. McGuire,[*,†] B. Paige Lawrence,[*,†] and Jacques Robert[*,†,1]

[*]Department of Microbiology & Immunology and [†]Department of Environmental Medicine, University of Rochester Medical Center, Rochester, New York 1462

[1]To whom correspondence should be addressed at Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642. Fax: (585)-473-9573. E-mail: Jacques_Robert@urmc.rochester.edu.

ABSTRACT

Endocrine disrupting chemicals (EDCs) can perturb the hypothalamic-pituitary-thyroid axis affecting human and wildlife health. Thyroid hormones (TH) are crucial regulators of metabolism, growth, and differentiation. The perinatal stage is most reliant on TH, thus vulnerable to TH disrupting chemicals. Dysregulation of TH signaling during perinatal development can weaken T cell function in maturity, raising the question of whether TH disrupting chemicals can perturb thymocyte development. Using *Xenopus laevis* tadpoles as model, we determined TH disrupting effects and thymocyte alterations following exposure to a mixture of common waterborne TH disrupting chemicals at concentrations similar to those found in contaminated water. This mixture included naphthalene, ethylene glycol, ethoxylated nonylphenol, and octylphenol, which have documented TH disrupting activity. Besides hypertrophy-like pathology in the thyroid gland and delayed metamorphosis, exposure to the mixture antagonized TH receptor-induced transcription of the Krüppel-like factor 9 transcription factor and significantly raised thyroid-stimulating hormone gene expression in the brain, two genes that modulate thymocyte differentiation. Importantly, exposure to this mixture reduced the number of *Xenopus* immature cortical thymocyte-specific-antigen (CTX+) and mature CD8+ thymocytes, whereas co-exposure with exogenous TH (T3) abolished the effect. When each chemical of the mixture was individually tested, only ethylene glycol induced significant antagonist effects on brain, thymic gene expression, and CD8+ thymocytes. These results suggest that EDCs in mixture are more potent than each chemical alone to perturb thymocyte development through TH-dependent pathway, and provide a starting point to research TH influence on thymocyte development.

Key words: water pollutants; immune toxicant; amphibians; developmental immunology; T cell development; mixtures.

Thyroid hormones (TH) are critical drivers of post-embryonic development in all vertebrates, and are required for metamorphosis in amphibians (Buchholz, 2017; Miyata and Ose, 2012). The thyroid hormone axis (TH axis) consists of the thyroid gland, and neuro-endocrine regulatory organs, particularly the hypothalamus and pituitary gland in the brain. Disorders in thyroid function have varied causes, ranging from dietary iodine deficiency, autoimmune diseases, and exposure to environmental pollutants (Carvalho and Dupuy, 2017). Very little is known about how TH affects the immune system, particularly during development. The TH receptor (TR)-regulated transcription factor Krüppel-like factor 9 (*klf9*) may play an important role in thymic lymphopoiesis, as evidenced by the significant decrease in thymus size and thymocyte numbers resulting from RNAi silencing of *Klf9* in zebrafish (Zhang *et al.*, 2017). Additional evidence that TH influences the developing immune system comes from mouse models of congenital hypothyroidism, which have demonstrated exacerbated autoimmune symptoms when the synthesis of TH is impaired during *in utero* development (Albornoz *et al.*, 2013). Also, in amphibians, expression of major histocompatibility complex (MHC) class II receptors on adult T lymphocytes is dependent on adequate TH signaling during development (Rollins-Smith and Blair, 1990). All of this information prompts further investigation of the association between TH and immune system development.

The post-embryonic stage of development is a critical window for the maturation of T cells in the thymus of all jawed vertebrates (Famili *et al.*, 2017). This is the earliest point in time point in which the thyroid gland begins to secrete TH, and lymphoid progenitor cells have migrated from the liver to the thymus to begin their thymic education (Manley and Capecchi, 1998). The thymus is a specialized compartment where immature lymphoid progenitor cells develop into CD8 and CD4 lineage T cells, which then exit the thymus and surveil the periphery for invading microorganisms and tumor cells. Thymocytes undergo positive and negative selection by binding to MHC molecules on thymic epithelial cells (TEC), which ensures that all mature T cells express functional T cell receptors (TCR) that can bind to host MHC-peptide with strong enough affinity for TCR signaling, without potential autoimmune responses should TCR-MHC affinity be too strong (Geenen, 2017). Data are lacking on how endocrine disrupting chemicals (EDCs) may affect the thymus, particularly for EDCs that exert at least some of their pathophysiological effects through TH axis pathways.

Modulation of the TH axis by environmental chemical exposures is a growing concern. Indeed, some EDCs have been shown to block binding of TH to the TR, and may perturb the molecules required for TH synthesis in the thyroid gland (Calsolaro *et al.*, 2017; Capen, 1994). Previous research demonstrated immune system disruption, notably involving CD8+ T cells, from both acute and long-term exposure to water containing a model mixture of 23 EDCs, that include chemicals which may act on the TH axis (Robert *et al.*, 2018, 2019). However, it is not clear by which mechanism EDCs alter the development of the immune system. In addition, the respective contributions of the different EDCs in mixtures have remained elusive. Therefore, to investigate the mechanisms of immune system disruption by the EDCs that act on the TH axis, we focused on 4 chemicals from this previously studied complex mixture that are known or suspected to act, at least in part, via effects on the TH axis. These 4 chemicals include the surfactants octylphenol and nonylphenol ethoxylates, which have been previously shown to induce thyroid pathology in rats and amphibians (Wang *et al.*, 2019; Xie *et al.*, 2019); the insecticide naphthalene, which has been shown to decrease circulating TH levels in fish (Yarahmadi *et al.*, 2016); and the anti-freeze ethylene glycol, which exhibits *in vitro* thyroid receptor antagonist effects (Kassotis *et al.*, 2015). A mixture of these 4 chemicals provides an environmentally relevant tool to examine TH disruption from the combination of thyroid synthesis inhibition and thyroid receptor antagonism. Furthermore, a 4-chemical mixture allows for ease of single chemical exposures to analyze the relative contributions of individual mixture components, which is less feasible for mixtures with a larger number of components.

To determine an association between TH disruption and adaptive immune cell development, we leveraged the strengths of the amphibian *Xenopus laevis*, an experimental organism with a fully annotated genome and extensive characterization of their immune systems (Robert and Ohta, 2009). All anuran amphibians undergo a TH-dependent metamorphosis in which tadpoles mature into adult frogs, and it is already known that T cell development in the thymus is completely remodeled due to apoptosis and replacement of all thymocytes at the height of metamorphosis (Rollins-Smith *et al.*, 1992; Rollins-Smith and Blair, 1990). As such, *X. laevis* tadpoles provide an ideal experimental organism to investigate the effects of TH disruption on intra-thymic T cell development. The objectives of this study were to investigate the effects of TH disruption on *X. laevis* tadpole thymocyte development by comparing a mixture of TH disrupters to their individual components.

Table 1. TH Disrupting Chemicals Used in Mixture and Environmental Reference Ranges

Chemical	Reference Range	Reference
Napthalene	0.74–1.0 μg/l	Preuss *et al.* (2003)
Ethylene glycol	2.0–.8 μg/l	Tran *et al.* (2014)
Ethoxylated nonylphenol	0.17–3.41 μg/l	Jie *et al.* (2017)
Ethoxylated octylphenol	6.9–474.2 ng/l	Yang *et al.* (2013)

MATERIALS AND METHODS

Animal husbandry and exposure paradigm. All animals were obtained from the *X. laevis* Research Resource for Immunology at the University of Rochester (https://www.urmc.rochester.edu/microbiology-immunology/research/xenopus-laevis.aspx). All animal experiments were carefully handled with the prior approval and under the University of Rochester Committee on Animal Resources regulations (approval number 100577/2003-151). For treatment, pre-metamorphic outbred *X. laevis* tadpoles (Niewkoop and Faber stage 51-52, see Nieuwkoop and Faber, 1994) were treated with water containing DMSO vehicle control (0.01% DMSO), an equimass mixture of 4 chemicals (Table 1) at 0.1, 1, or 10 μg/l, and/or 1.25 nM triiodothyronine (T3) for either 6 days or 3 weeks. To produce the 10 μg/l equimass dose of the mixture, the relative weights of all chemicals were equal to each other, for a total of 40 μg/l chemical substances. The chemicals used in these experiments were DMSO (CAS 67-68-5; 99.9% purity, VWR, Radnor, Philadelphia), ethylene glycol (CAS 107-21-1, anhydrous, liquid, 99.8% purity, Sigma Aldrich, St Louis, Missouri), Napthalene (CAS 91-20-3, crystals, 99% purity, Sigma Aldrich, St Louis, Missouri), ethoxylated nonylphenol (CAS, 84133-50-6, 70% purity in H_20, Sigma Aldrich, St Louis, Missouri), ethoxylated octylphenol (CAS 9002-93-1, liquid, 99% purity, Sigma Aldrich, St Louis, Missouri), and Tri-iodo-thryonine (T3) (CAS 6893-02-3, powder, 95% purity, Sigma Aldrich, St Louis, Missouri). The 1.25 nM concentration of T3 was chosen based on prior optimization reported in literature, to produce efficient systemic TH receptor activation without causing overt mortality (Yao *et al.*, 2017). All chemical stocks were prepared in the DMSO vehicle in order to ensure solubility in water. Up to 20 tadpoles per tank were maintained in 4 l tanks of the spiked water and fed with algae daily. Solutions were refreshed by a complete water change every 7 days in the 3-week duration experiments.

Real-time qPCR. Thymus and brain from 6 tadpoles per group were harvested and total RNA was extracted from the tadpole's tissues using TRIzol reagent, following the manufacturer's protocol (Invitrogen, Waltham, Massachusetts). A total of 500 ng of RNA from each sample was used to synthesize complementary DNA (cDNA) by the Moloney Monkey Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, Waltham, Massachusetts) with oligo(dT) primers (Invitrogen, Waltham, Massachusetts). For reverse transcription (RT)-PCR, 125 ng of cDNA was used to determine the expression levels of genes of interest by ΔΔCT value using an ABI 7300 Real-Time PCR System and PerfeCTa SYBR Green FastMix (Thermo Fisher Scientific, Waltham, Massachusetts) following the manufacturer's protocol. Primers used are indicated in Table 2. Relative gene expression levels were assessed using the by ΔΔCT method (Livak and Schmittgen, 2001). Briefly, expression levels

Table 2. qPCR Primer Sequences

Gene Name (Abbreviation)	Forward Sequence
Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*)	F: 5′—ACCCCTTCATCGACTTGGAC—3′ R: 5′—GGAGCCAGACAGTTTGTAGTG—3′
Krüppel-like factor 9 (*klf9*)	F: 5′—GTGGCCACTTGATTTCCCCT—3′ R: 5′—AAAGACACAAAACAGCGGCG—3′
Thyroid receptor β (*trβ*)	F: 5′—CTCATAGAAGAAAACAGAGAAAARAGA—3′ R: 5′—GAAGGCTTCTAAGTCCACTTTTCC
Deiodinase 3 (*dio3*)	F: 5′—GATGCTGTGGCTGCTGGAT—3′ R: 5′—ATTCGGTTGGAGTCGGACAC—3′
Thyroid-stimulating hormone β (*tshβ*)	F: 5′—ACTGCATGACAAAGGATCCAAA—3′ R: 5′—CAGGGTAGGAAAAGAGCGGG—3′
Thryoid-releasing hormone (*trh*)	F: 5′—ACGTGAAGATCTCAAGAAGGT T—3′ R: 5′—CACCAGAACTGTGTGGCAAG—3′
Corticotropin-releasing hormone (*crh*)	F: 5′—CCCAGCCCTTTTTACTGCGA—3′ R: 5′—AGATGCCTCAGGAAACGAGC—3′

Note: F: forward primers; R: reverse primers.

were normalized to an endogenous housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), then further normalized against the control group. GAPDH is a useful housekeeping gene due to its consistent and high expression levels between the same tissues of different individuals (Barber *et al.*, 2005), which did not significantly differ between groups in our experiments. All the primers listed in Table 2 were validated prior to use by gradient PCR as described in prior literature (Navarro *et al.*, 2015).

Histology. Tadpoles (3 per group) were humanely sacrificed, and fixed with paraformaldehyde (2%) and 1% glacial acetic acid overnight. Glacial acetic acid was necessary for a softer fixation for the tadpole eye, which is in-line with the thyroid gland on a transverse section. Trimmings of the tadpole head were prepared for histology through paraffin embedding and sectioning by microtome. Sections (10 μm) were evaluated for presence of thyroid gland, and stained with hematoxylin and eosin. Thyroid glands were imaged using a Nikon Eclipse E200 microscope. Epithelial cell height and follicle thickness were determined by converting number of pixels to μm using ImageJ software (NIH, Schneider *et al.*, 2012). Epithelial cell height was defined as the median height of each epithelial cell in a gland in a given section, whereas follicle thickness was the distance between the outer and inner follicle walls, which can often consist of multiple epithelial cells stacked on top of each other (Grim *et al.*, 2009; Opitz *et al.*, 2006).

Flow cytometry. Flow cytometry was conducted with freshly isolated tadpole thymocytes. Each of the 4–5 biological replicates consisted of pooled thymocytes from 3 tadpoles from the same tank. To isolate thymocytes, the whole thymus was disrupted using a 100-μm pore size mesh and rinsed with amphibian phosphate buffered saline (APBS), a more dilute form of PBS suited to the lower tonicity required for amphibian cells. Cells were then suspended in APBS containing 1% BSA and 0.05% sodium azide, and labeled with monoclonal antibodies. All antibodies were produced in-house by the URMC *X. laevis* Research Resource for Immunology. Each monoclonal antibody (Abs) was generated using hybridomas cultures, and has been tested and titrated for use in flow cytometry using *X. laevis* splenocytes (1:100 dilution). Dual staining of monoclonal Abs was enabled by biotin conjugation of the second Ab used in each cellular stain (Even-Desrumeaux and Chames, 2012). Monoclonal Abs were then labeled with secondary Abs (Goat anti-mouse FITC [catalog: A32723] and APC conjugated with streptavidin [catalog: SA1005], both from Thermo Fisher Scientific, Waltham, Massachusetts) at a 1:100 dilution. Thymocytes were evaluated using an Accuri C6-plus flow cytometer (BD Biosciences). Fifty thousand events per sample were collected to ensure adequate number of thymocytes were analyzed relative to other cell types such as thymic epithelial cells or red blood cells. To direct the identification of distinct thymocyte populations, gating controls consisting of unstained samples, secondary antibody only controls, and single stained controls. Flow cytometry data were analyzed using FCS Express (Version 7, BD Biosciences).

Statistical analysis. For all experiments, statistical analyses were performed in Graphpad Prism 7 according to the experimental design of each experiment. For experiments with two groups a Student's *t* test was used, whereas 1-way ANOVA and Tukey's post hoc test was used for experiments with more than 2 groups. Data on metamorphic timing were treated as a survival analysis, and a log-rank test was used.

RESULTS

Exposure to Thyroid Disrupter Mixture Antagonizes TR Activation In Vivo

To determine whether the mixture of chemicals had *in vivo* antagonist effects for TR signaling, we exposed tadpoles to exogenous T3, or an equimass mixture of the four chemicals, or both T3 and the mixture together. Prior literature has demonstrated *in vitro* antagonist effects from these chemicals using a reporter cell line for the human TR, but *in vivo* antagonist effects have not been examined (Kassotis *et al.*, 2015). After 6 days of exposure, we collected tissues and performed qPCR to examine the level of expression of 3 TR-controlled genes: *klf9*, thyroid receptor β (*trβ*), and deiodinase 3 (*dio3*) (Figure 1). In tadpoles exposed to T3, *klf9* gene expression was markedly increased not only in the brain, as expected, but also in the thymus, when compared with tadpoles exposed to the DMSO vehicle control (Figure 2). *Klf9* is a key transcription factor induced by TR activation and plays a role in autoinduction of the TR (Hu *et al.*, 2016). Exposure to the thyroid disrupter mixture alone did not alter *klf9* gene expression in either the brain or thymus. Co-exposure of T3 and the mixture significantly reduced the increase in klf9 gene expression elicited by T3 exposure alone in both the brain and

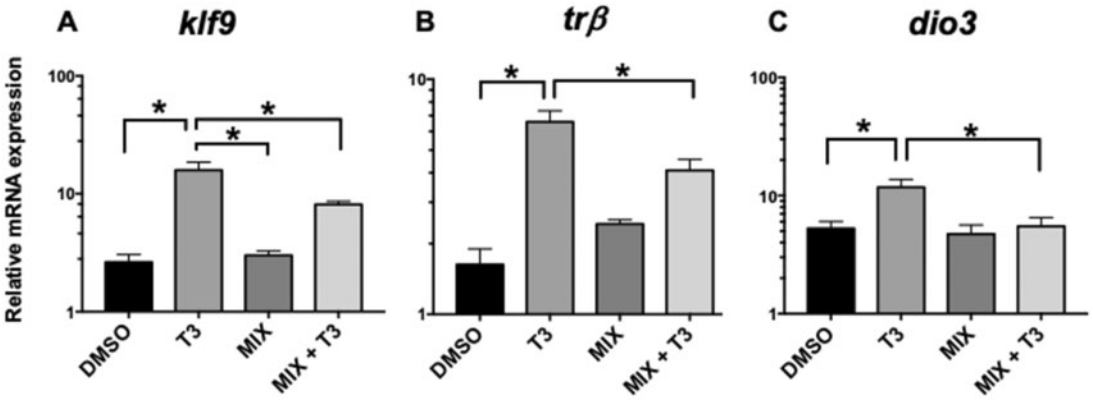

Figure 1. Changes in relative gene expression by qPCR of (A) Kruppel-like factor 9 (*klf9*), (B) thyroid hormone (TH) receptor beta (*trβ*), and (C) deiodinase 3 (*dio3*) in tadpole whole brain tissue following exposure to DMSO control, 1.25 nM T3, 10 μg/l mixture (MIX), or 10 μg/l mixture and 1.25 nM T3 for 6 days. Data presented include mean and SEM with $N = 6$ tadpoles in each group. Statistical significance is denoted by * where $p < .05$ using 1-way ANOVA and Tukey's post hoc test.

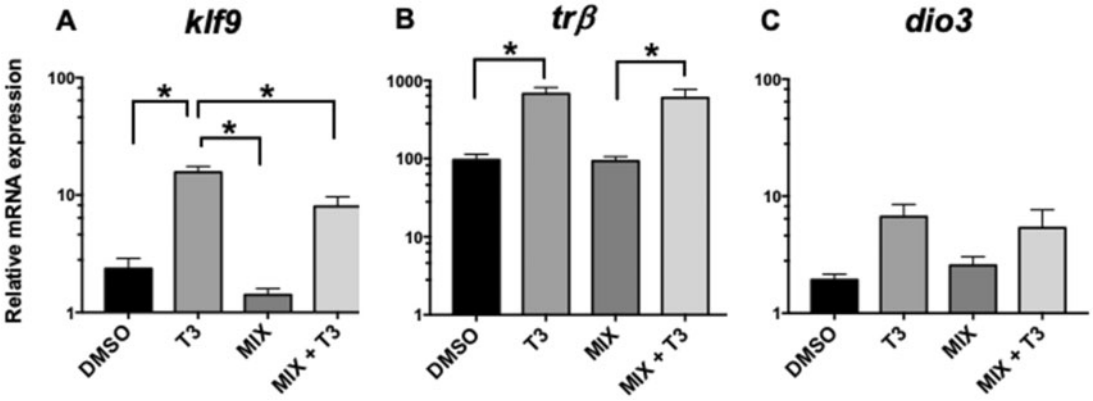

Figure 2. Changes in relative gene expression by qPCR of (A) *klf9*, (B) TH receptor beta (*trβ*), and (C) deiodinase 3 (*dio3*) in tadpole whole thymus tissue following exposure to DMSO control, 10 μg/l mixture (MIX), with or without 1.25 nM T3 for 6 days. Data presented include mean and SEM with $N = 6$. Statistical significance is denoted by * where $p < .05$ using 1-way ANOVA and Tukey's post hoc test.

thymus. These data suggest that exposure to the mixture antagonizes TR activation, reducing downstream TR-mediated gene expression. Co-exposure of the mixture and T3 also attenuated the effect of T3 on *trβ* and *dio3* gene expression in the brain, but not the thymus, suggesting that there may be aspects of antagonism of the TH axis that are tissue specific. Given that antagonist activity was detectable in both thymus and brain tissues, there is a potential for whole body antagonism of the TR.

Exposure to Thyroid Disrupter Mixture Induces TSH Release and Causes Pathological Changes to the Thyroid Gland

To investigate whether exposure to this mixture of 4 chemicals had detrimental effects on TH synthesis, we monitored changes in the relative expression of genes involved in the hypothalamic-pituitary-thyroid axis's negative feedback response on whole brain tissue taken from tadpoles. Exposure to the chemical mixture alone significantly induced thyroid-stimulating hormone β (*tshβ*) gene expression, with an average fold change increase of 1.9 (Figure 3A). In contrast, treatment with T3 significantly reduced TSHβ expression. Exposure to T3 combined with the mixture did not significantly alter *tshβ* expression (Figure 3A). The chemical mixture did not significantly change expression of thyroid-releasing hormone (TRH) or corticotrophin-releasing hormone (CRH) (Figs. 3B and 3C). Given that TRH and CRH are known to be hypothalamic in origin while TSHβ is expressed by the pituitary gland, it is possible that the pituitary gland rather than the hypothalamus is affected by exposure to this chemical mixture.

We then conducted a histopathological evaluation of the thyroid glands from tadpoles that had been exposed to the mixture of 4 chemicals for 2 weeks. Thyroid tissues from mixture exposed tadpoles displayed signs of moderate follicular cell hyperplasia, marked by increased number and clumping of follicular cells and a loss of their normal cuboidal morphology (Figs. 4A and 4B). Quantification of metrics associated with morphological observations revealed a significant increase in average follicular epithelial cell height (Figs. 4C and 4D), which is a known phenotype of chemically induced hypothyroidism (Grim *et al.*, 2009). Some instances of the colloid spaces suggested thickened follicular walls. Quantification supported that the average follicle thickness was about 40% greater in thyroids glands from mixture exposed tadpoles, but the difference was not statistically significant (Figs. 4C and 4E).

Exposure to the TH Disrupter Mixture Affects Metamorphosis

To determine if transient exposure to the mixture during development results in long-term changes to TH activity in tadpoles later on during development, the timing of metamorphosis, a

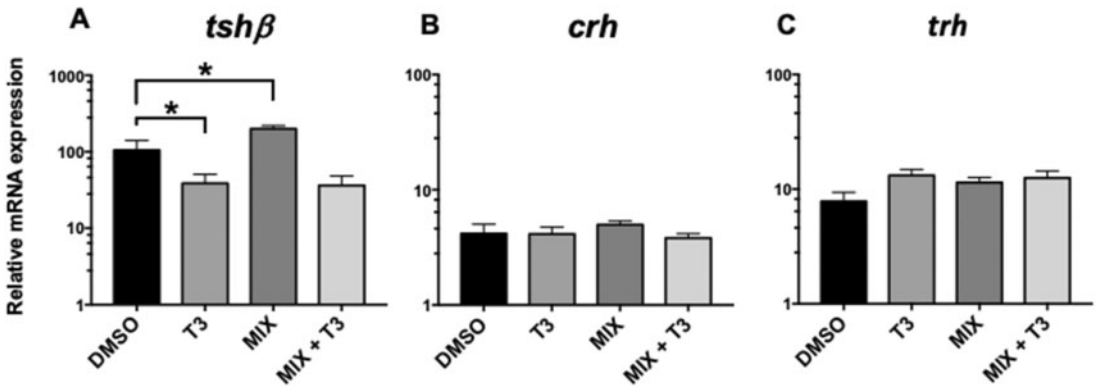

Figure 3. Changes in relative gene expression by qPCR of (A) thyroid-stimulating hormone-β (*tshβ*), (B) corticotrophin-releasing hormone (*crh*), and (C) thyroid-releasing hormone (*trh*), in tadpole whole brain tissue following exposure to DMSO control, 10 μg/l mixture (MIX), with or without 1.25 nM T3 for 6 days. Data presented include mean and SEM with $N = 6$. Statistical significance is denoted by * where $p < .05$ using 1-way ANOVA and Tukey's post hoc test.

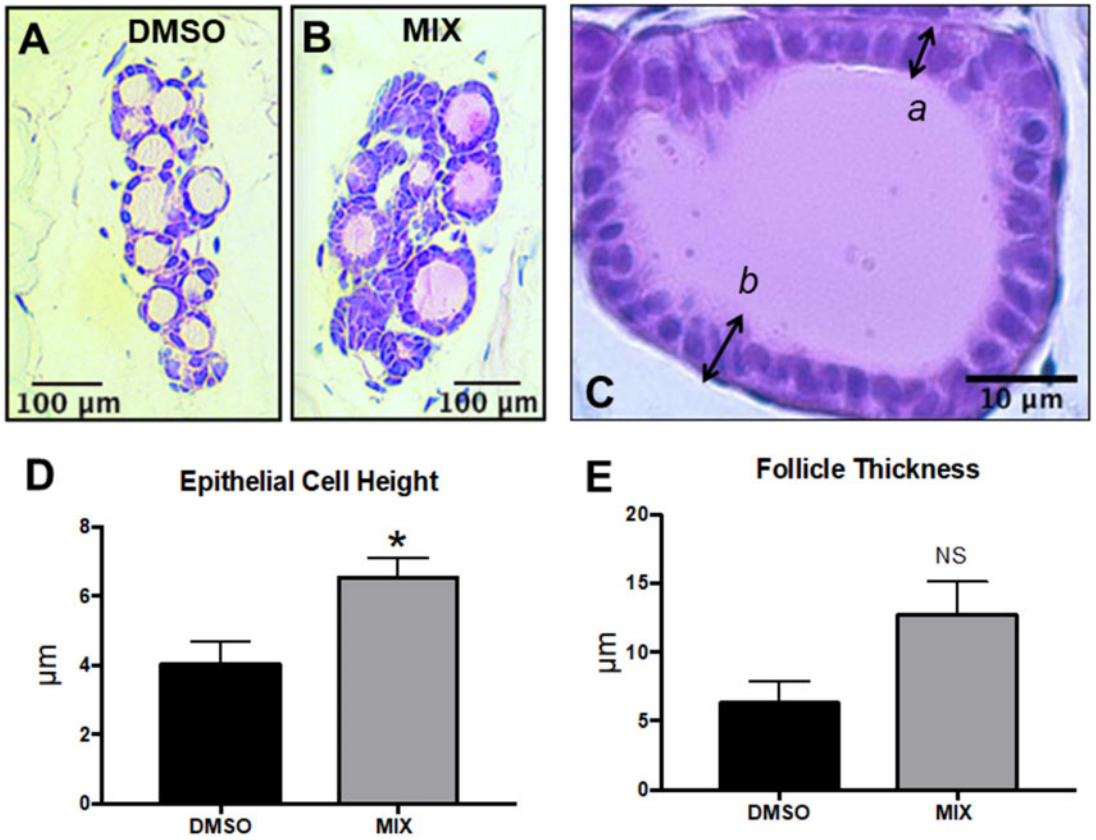

Figure 4. Tadpole thyroid gland histopathology following 2-week-long exposure to DMSO control or 10 μg/l mixture (MIX). (A) Representative thyroid gland cross section from a DMSO control exposed tadpole. (B) Representative thyroid gland cross section from a 10 μg/l mixture exposed tadpole. (C) Measurement of (a) epithelial cell height and (b) follicle thickness indicated on a representative control tadpole thyroid follicle. (D) Comparison of thyroid gland average epithelial cell height following mixture exposure. (E) Comparison of thyroid gland average follicle thickness following MIX exposure. Data presented include mean and SEM with $N = 3$, statistical significance is determined via Student's *t* test where * denotes $p < .05$. Bar: 10 μm.

key TH-dependent event, was measured. Tadpoles were exposed to the mixture for 3 weeks. Following exposure, tadpoles were raised in clean water and monitored daily to record the date that each tadpole completed metamorphosis, which is defined as full regression of tail or NF stage 66 (Nieuwkoop and Faber, 1994) (Figure 5). Tadpoles that were exposed to mixture-spiked water completed metamorphosis significantly later than control tadpoles. Specifically, the median completion of metamorphosis increased from 21 weeks in the DMSO control-treated tadpoles to 26 weeks at 0.1 μg/l and 23 weeks for tadpoles dosed with 1 μg/l of the mixture. These results suggest a long-term alteration of the TH axis that has implications for amphibian metamorphosis and development.

Exposure to the TH Disrupter Mixture Alters Cellular Composition in the Thymus

Changes in thymus cellularity were assessed to determine whether exposure to the mixture perturbed thymocyte

development. Tadpoles were exposed to the mixture, with or without T3, or to the DMSO control for 1 week, and then thymocytes were collected for evaluation by flow cytometry. Using a combination of *X. leavis*-specific monoclonal antibodies directed against CD8, CTX (cortical immature thymocyte marker, Robert and Cohen, 1999) and CD5 (a pan T cell marker), we evaluated the frequency of the following populations of thymocytes: CD8 positive T cells (CD8+/CD5+), CD8 negative T cells (CD8−/CD5+, putative CD4-like), more immature CD8+/CTX+ (putative double positive [DP]-like), and CD8−/CTX+ (putative double negative-like) cells. The gating strategy is depicted in Figures 6A–C (Robert *et al.*, 2019, 2001; Robert and Cohen, 1999). There were no statistically significant differences in the total number of thymocytes recovered from thymuses among these groups of tadpoles (Table 2). But there were 0.6-fold fewer of the more mature CD8+/CD5+ thymocyte subset in tadpoles treated with the mixture and T3 alone, compared with vehicle-treated tadpoles (Figure 6D). Furthermore, the combination of the chemical mixture and T3 abrogated this decrease, suggesting that TR activation by exogenous T3 can rescue or ameliorate the combined disrupting activity of the mixture. Similar to mature CD8+/CD5+ thymocytes, treatment with the mixture and T3 alone decreased the more immature CD8+/CTX+ thymocytes (Figure 6E). However, the loss of CD8+/CTX+ thymocytes induced by the mixture was only partially restored to DMSO control level by co-administration of T3. Thus, both cell types (immature CD8+/CTX+ and more mature CD8+/CD5+) displayed a reduction in cell number following mixture exposure that was all or partially reversed by co-exposure with T3, which suggests that the mixture affects the thymus in a TH-dependent manner.

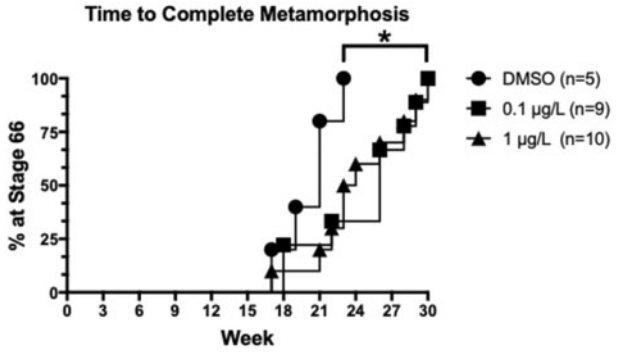

Figure 5. Time in weeks required to complete metamorphosis in tadpoles following 3-week-long exposure to 0.1 and 1 µg/l of the mixture and switch to clean water. Metamorphosis completion was defined as the amount of time needed to reach Niewkoop and Faber (NF) stage 66 (eg, tail fully regressed). * denotes statistical significance ($p < .05$) using log-rank test.

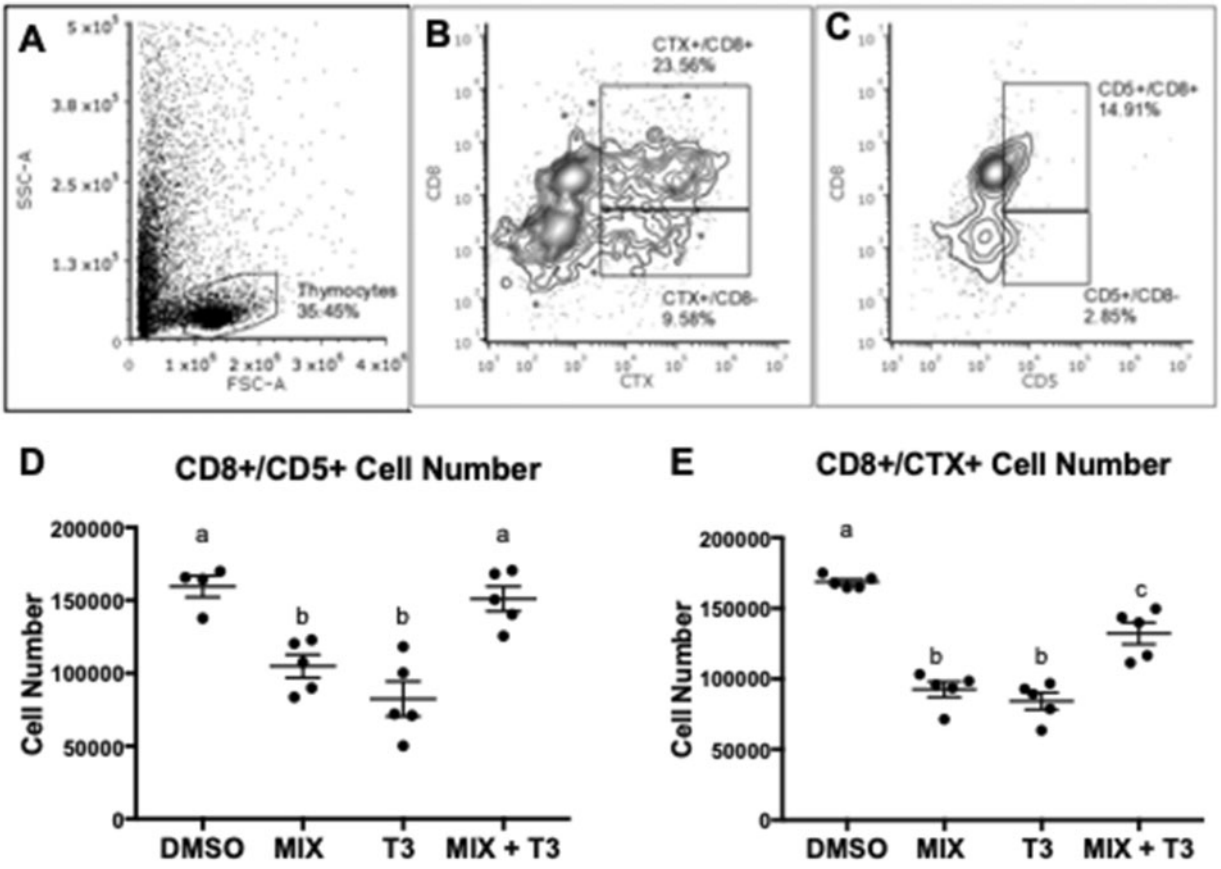

Figure 6. Flow cytometry of tadpole thymocytes following 1-week-long exposure to DMSO control, 10 µg/l of the mixture, with or without 1.25 nM T3 for 6 days. (A) Representative gating strategy to identify thymocyte populations. (B) Representative gating of DP (CD8+/CTX+) DN (CD8-/CTX+) and CD8 SP (CD8+/CD5+) and CD4-like (CD8−/CD5+) thymocyte populations. (C) Thymocyte cell number for mature CD8+/CD5+ and (D) immature CD8+/CTX+ thymocyte cell populations. Data presented include mean and SEM with $N = 5$. Statistical significance is denoted by differences in letters (a, b, c, and d) where means that differ significantly ($p < .05$ using 1-way ANOVA and Tukey's post hoc test) have different letters from one another, whereas means that do not significantly differ have the same letter.

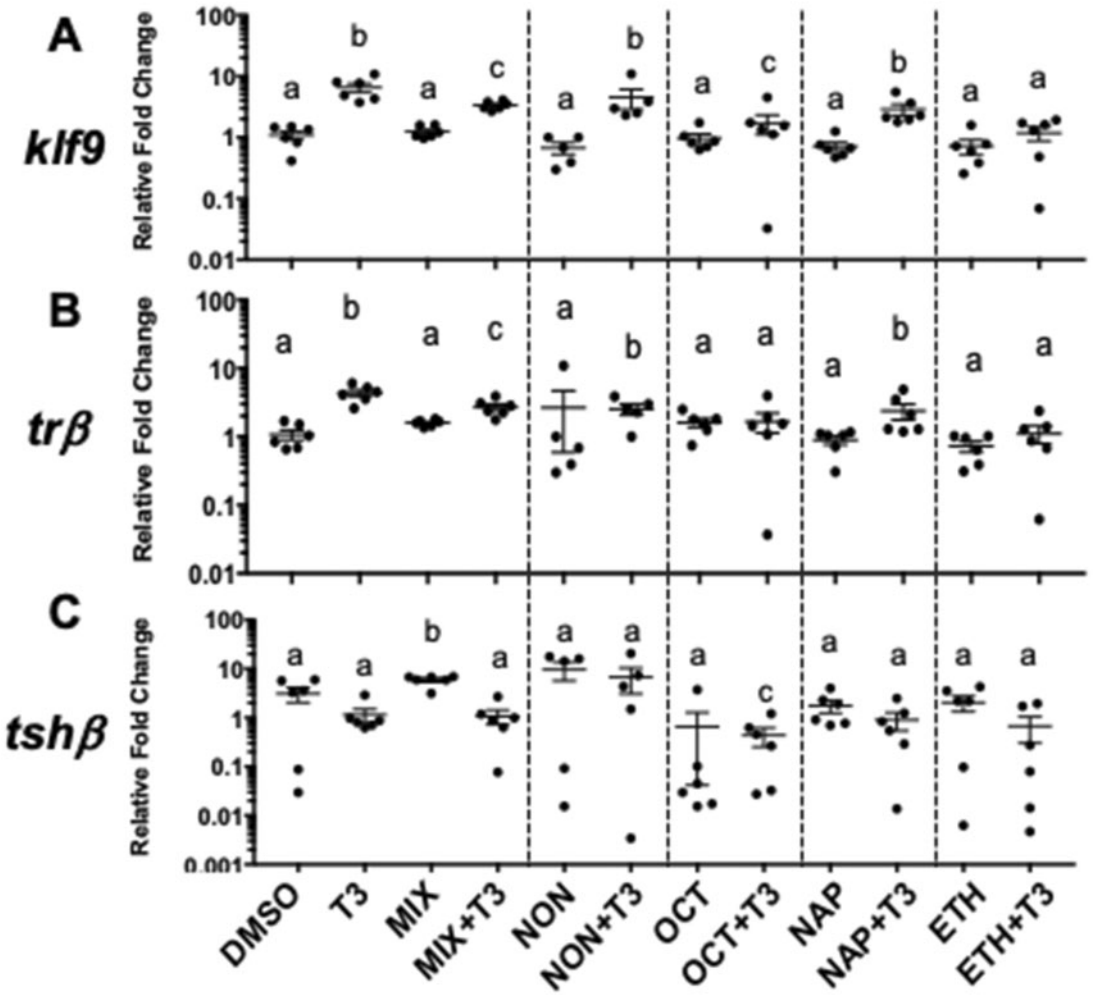

Figure 7. Changes in relative gene expression by qPCR of (A) *klf9*, (B) TH receptor beta (*trβ*), and (C) *tshβ* in tadpole whole brain tissue following 6-day exposure to the individual chemicals of the mixture by qPCR. Chemicals included are the mixture (MIX; data from Figure 1), nonylphenol ethoxylate (NON), octylphenol ethoxylate (OCT), naphthalene (NAP), and ethylene glycol (ETH). Data presented include mean and SEM with $N = 6$. Statistical significance is denoted by differences in letters (a, b, c, and d) where means that differ significantly ($p < .05$ using 1-way ANOVA and Tukey's post hoc test) have different letters from one another, whereas means that do not significantly differ have the same letter.

Exposure to Individual Chemicals Partially Recapitulates the Combined Effects of the Mixture

We next examined the respective TH disruption activity of each of the 4 chemicals alone compared with their combined effect *in vivo* when used in mixture. Tadpoles were exposed to each of the 4 chemicals independently at the same 10 μg/l dose, with or without T3. Gene expression was monitored in the brain and thymus. In the brain, naphthalene and nonylphenol ethoxylate did not cause any detectable modification to *klf9*, *trβ*, or *tsfβ* gene expression, whereas octylphenol ethoxylate and ethylene glycol antagonized *klf9* and *trβ* gene expression levels, by preventing the full induction of these genes by T3 (Figure 7). In the thymus, exposure to naphthalene and ethylene glycol demonstrated antagonist effects on *klf9*, whereas nonylphenol ethoxylate and octylphenol ethoxylate did not (Figure 8). There were no single chemical antagonist effects in the thymus for the TRβ. Furthermore, none of the chemicals had any effect on *tshβ* gene expression in the brain, suggesting that the individual chemicals at these doses caused no overt changes to TH axis negative feedback. The reason for the relatively high individual variability of brain *tshβ* expression is unclear. A similar high variability has reported in human populations that seems to be in part due to circadian rhythms in clinical studies (Ehrenkranz *et al.*, 2015).

Because ethylene glycol was the only chemical with significant individual effects on TR-dependent gene expression in both the brain and thymus, we further determined by flow cytometry whether exposure to this chemical was sufficient to alter thymocytes. Ethylene glycol exposure reduced the total number of thymocytes, whereas total thymocyte numbers were not significantly lowered by the full mixture (Table 3). Ethylene glycol also decreased the number of CD8+/CD5+ thymocytes, but co-exposure to ethylene glycol with T3 did not reverse the effect. CD8+/CTX+ thymocytes were also reduced, albeit the reduction from ethylene glycol was not statistically significant (Figure 9, Table 3). These results suggest that any TH protective effects observed when tadpoles were co-exposed to the mixture and T3 were not due to ethylene glycol alone. Therefore, we conclude that the individual components of the mixture alone

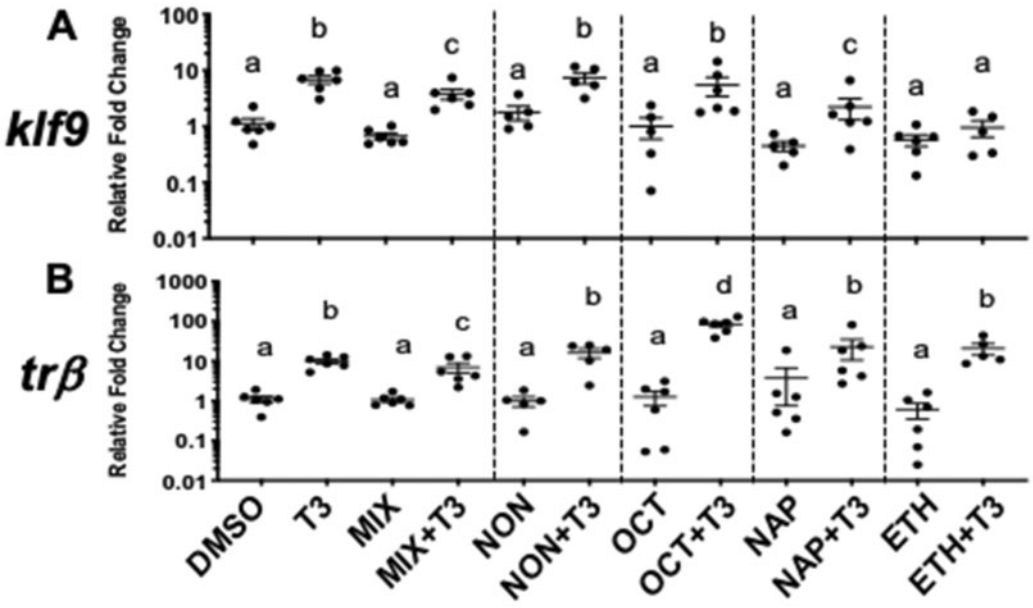

Figure 8. Changes in relative gene expression by qPCR of (A) *klf9* and (B) TH receptor beta (*trβ*) in tadpole whole thymus tissue following 6-day exposure to the individual chemicals of the mixture by qPCR. Chemicals included are the mixture (MIX; data from Figure 2), nonylphenol ethoxylate (NON), octylphenol ethoxylate (OCT), naphthalene (NAP), and ethylene glycol (ETH). Data presented include mean and SEM with $N = 6$. Statistical significance is denoted by differences in letters (a, b, c, and d) where means that differ significantly ($p < .05$ using 1-way ANOVA and Tukey's post hoc test) have different letters from one another, whereas means that do not significantly differ have the same letter.

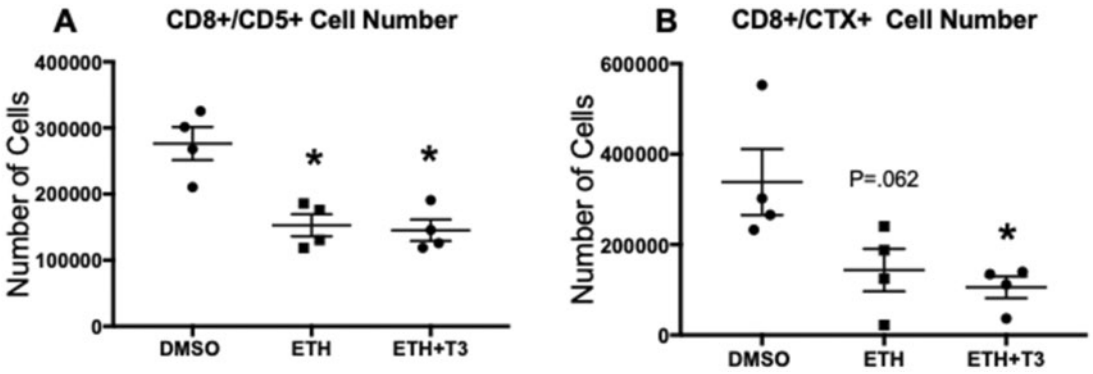

Figure 9. Cell Numbers for (A) mature CD8+/CD5+ and (B) immature CD8+/CTX+ tadpole thymocytes determined by flow cytometry following 1-week-long exposure to ethylene glycol (ETH) with or without T3. Data presented include mean and SEM with $N = 4$. Statistical significance is denoted by * where $p < .05$ using 1-way ANOVA and Tukey's post hoc test.

only recapitulate some, but not all phenotypes observed in tadpoles exposed to the full mixture.

DISCUSSION

This study provides new evidence that early life exposure to a mixture of common TH disrupting water contaminants is associated with delayed amphibian metamorphosis and alterations to thymocyte differentiation. Specifically, we showed that environmentally relevant doses of specific mixture of four EDCs can disrupt the TH axis through 2 different mechanisms: (1) attenuation of T3-induced gene expression; (2) hypothalamic-pituitary regulation of thyroid gland structure and function. Importantly, the effects of TH disruption from this environmentally relevant mixture can be reversed by co-exposure with exogenous hormone. Furthermore, we demonstrated that each component of the mixture tested individually can only incompletely recapitulate the effects induced by the mixture, including T3 antagonism.

The co-exposure with exogenous T3 showed that the chemical mixture can act as antagonist to TR-mediated gene expression in both the brain and thymus, although the thymus

Table 3. Total Number of Thymocytes

Mixture Experiment	Average Thymocyte Number	Ethylene Glycol Experiment	Average Thymocyte Number
DMSO	214500	DMSO	695000
Mixture	169600	Ethylene glycol	378000***
T3	140800	Ethylene glycol +T3	358000***
Mixture + T3	300000		

***Note:* denotes statistically significant difference relative to DMSO control via 1-way ANOVA and Tukey's post-test.

appears to be less sensitive to these effects. Considerable prior evidence has demonstrated that the brain is a sensitive target of TH disrupting chemicals, and that the tadpole brain serves as a reliable model to predict how these EDCs may perturb the development of the mammalian brain (Fini *et al.*, 2017; Fini and Demeneix, 2019). Similarly, thymocyte differentiation is fundamentally conserved between amphibian and mammals (Robert and Cohen, 1999; Robert *et al.*, 2001; Rollins-Smith *et al.*, 1992). Thus, our data imply that TH disrupting chemicals may affect the thymus in similar ways across all jawed vertebrates, including mammals and humans. Of the 3 differentially expressed genes examined, the chemical mixture antagonized *klf9* gene expression in thymus. The function of *klf9* during thymocyte differentiation is still unclear. It is a TR-controlled transcription factor that is a known regulator of lymphoid development in mammals, and a component of the TR's autoinduction loop (Zhang *et al.*, 2017; Hu *et al.*, 2016). Our findings in *X. laevis* suggest that a relatively short exposure to this mixture of suspected thyroid disruptors can alter TH-mediated *klf9* regulation and affect thymocyte differentiation. Consistent with this, RNAi silencing of *klf9* RNAi in zebrafish reduced the number of thymocytes (Zhang *et al.*, 2017). These findings provide evolutionary support for a critical role of this transcription factor in T cell development.

Another interesting finding was that TSH gene expression in the tadpole brain was increased following exposure to the mixture, whereas CRH gene expression remained unchanged. In amphibians, CRH rather than TRH regulates release of TSHβ from the pituitary gland (Denver, 1993). This suggests that overall effects of the mixture on the TH axis may be subtle, and act only on parts of this pathway. However, this partial activity is still sufficient to perturb thyroid gland morphology. The histopathological effects induced by the mixture on the thyroid gland are consistent with pathology found in early-stage cases of hypothyroidism in mammals (Degitz *et al.*, 2005), which suggests that TH levels within tadpoles exposed to the mixture are lower than in control-exposed tadpoles. Yet, exposure to the individual chemical constituents of the mixture did not alter *tsh* gene expression; therefore, it is likely that all 4 chemicals are required to significantly alter regulation of TH synthesis. This is consistent with documented evidence in previous studies that have compared the toxicity of mixtures and single chemicals. For example, multiple studies have revealed that mixtures of EDCs are able to "produce something from nothing," in which biological effects observed after exposure to a mixture are not observed in when same doses of the components of a mixture are used one at a time (Silva *et al.*, 2002; Versieren *et al.*, 2016). In our study, we note that some other effects of the mixture, such as the effects on *klf9* gene expression or thymocyte number can be replicated by at least ethylene glycol alone, yet the effect on *tsh* gene regulation requires all 4 chemicals together.

The metamorphic delay observed for animals exposed to the mixture substantiates a physiological perturbation of the thyroid gland. Effect of multiple EDCs on metamorphosis is well documented (reviewed in Thambirajah *et al.*, 2019). By extension to other anuran species, metamorphic delay is likely to have consequences for amphibian wildlife in areas contaminated by thyroid disrupters, because taking longer time to finish development may reduce the number of animals that survive predation and other conditions to reach adulthood. Although the 4 chemicals in the mixture may either act as TR antagonists and TH synthesis inhibitors separately, it is also possible that some chemicals may be acting on the TH axis through both mechanisms. Amphibian metamorphosis is also known to be regulated by glucocorticoid signaling in combination with TH signaling, and both pathways share neuro-endocrine regulation through hypothalamic stimulation of the pituitary gland with CRH (Kulkarni and Buchholz, 2014). Due to the complexity of adrenocorticotropic hormone (ACTH) synthesis in the pituitary gland through processing and post-translational modification of pre-pro-opiomelanocortin (pre-POMC), we could not determine whether ACTH producing cells were affected in a way similar to the TSH producing cells.

Fewer studies have integrated the role of TH on thymocyte development. Of relevance to these results, TSH-receptor activation within mouse thymocytes has been shown to reduce the fidelity of positive selection, so that fewer DP cells die from neglect if their TCR: MHC-peptide affinity is too low (Wu *et al.*, 2017). In *X. laevis* tadpoles, exposure to this mixture of 4 chemicals resulted in hypothyroid effects, along with a decreased number of mature CD5+/CD8+ thymocytes. This implies that fewer thymocytes survive positive selection, which is contradictory to previous evidence in mammals where elevated TSH allows more thymocytes to survive positive selection (Wu *et al.*, 2017). The administration of exogenous T3 also decreased CD5+/CD8+ and immature CD8+/CTX+ thymocytes, which may be due to induction of early metamorphic changes, as thymocytes are lost by apoptosis during metamorphosis in *X. laevis* (Robert and Ohta, 2009; Rollins-Smith *et al.*, 1997). It will be interesting, in future studies, to determine whether T3 or exposure to TH disrupting EDCs affects thymocyte apoptosis, which is influenced by paracrine release of other hormones, such as glucocorticoids (Ashwell *et al.*, 1996). The loss of mature CD5+/CD8+ and immature CD8+/CTX+ thymocytes from either the mixture or T3 alone was prevented when tadpoles were co-exposed to both the mixture and T3. Whether this is due to an alleviation of disrupted TH synthesis or competition for the TR binding site is unclear. Gene expression data provide a possible explanation in that the increase in TSH gene expression in the brain caused by the mixture was also abrogated in tadpoles treated simultaneously with T3. This suggests that competition for TR binding sites in the hypothalamus may be one potential mechanism by which the co-exposure of T3 and mixture results in a reduced loss of thymocytes compared with either exposure alone. Collectively, these results suggest that the effects of this EDC mixture on thymocytes are dependent on a TH-related pathway, as it has previously been shown that exogenous TH supplementation can rescue immune effects of TH deficiency in murine models (Albornoz *et al.*, 2013).

Among the effects observed using a single chemical of the mixture, it is noteworthy to mention the alteration in thymocyte composition induced by exposure to ethylene glycol, the only chemical showing antagonistic effects on *klf9* gene expression in both the tadpole brain and thymus. There is very little established literature on ethylene glycol's potential thyroid toxicity. This includes an *in vitro* antagonist assay screen data (Kassotis *et al.*, 2015), which is confirmed by our *in vivo* data. Our results suggest that subtle changes to TR signaling are sufficient to drive the loss of CD8+/CD5+ and CD8+/CTX+ thymocytes. As TSH expression in tadpole brains was unaltered by exposure to ethylene glycol alone, it is likely that changes in thymocytes composition were independent of changes in neuro-endocrine regulation of the thyroid gland.

The overall findings of this study provide new evidence about the ways in which disruption of the TH axis by exogenous chemicals can alter thymocyte development. These results show that subtle effects on TH regulation, such as through TR antagonism, may be more impactful on thymocyte

development even if they do not trigger substantive shifts in TH synthesis. Furthermore, our study significantly contributes to understanding of the toxicity of mixtures by showing that the individual components of an EDC mixture only partially recapitulate the effects of that mixture. As such some combinations of chemicals can induce stronger pathophysiological outcomes than are observed when chemicals are evaluated one at a time.

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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