THE ROLE OF THYROID HORMONES IN VERTEBRATE DEVELOPMENT

EDITED BY: Marco António Campinho and Laurent M. Sachs
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THE ROLE OF THYROID HORMONES IN VERTEBRATE DEVELOPMENT

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Table of Contents

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EDITORIAL
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Editorial: The Role of Thyroid Hormones in Vertebrate Development

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Keywords: thyroid hormones, development, vertebrates, teleosts, anurans, birds, mammals

Editorial on the Research Topic

The Role of Thyroid Hormones in Vertebrate Development

In vertebrates, thyroid hormones (TH) have long been recognized as essential factors in vertebrate natal and postnatal development. Since TH role was first suggested in the Eighteenth century when a relationship between goiter and cretinism was found, TH was shown to play key roles in development of the nervous system in vertebrates, controlling diverse processes such as neurogenesis, cell migration, apoptosis, differentiation, and maturation. Furthermore, the role of TH is more diversified. TH act on several other key events during development including morphogenesis and metabolism. Notwithstanding, research into the role of TH in vertebrate development is still lagging. This is mainly due to the relatively easy by which thyroid/TH linked pathologies could be treated "simply" by TH supplementation and also the pleiotropic nature of TH action that makes it difficult to identify and study discrete roles of TH in various aspect of development. However, for the patient, the therapeutic interventions are not always satisfactory, with the conservation of adverse effects on the quality of life. In this special issue of Frontiers in Endocrinology, we aim to address state of the art on TH role in vertebrate development, with a comparative perspective from teleosts to humans to highlight the huge conservation of the TH signaling pathway in vertebrates. The biology of TH is complex with highly regulated production of the TH in the gland, entry, activation, and binding of the TH to cognate receptors in target cells. Actually, new scientific advances and technologies are now providing tools to dissect the developmental role of TH on vertebrate development, both embryonic and post-natal. Given that TH constitute integrative physiological signals in vertebrates a new scenario is emerging whereas TH seem to be important in the development and maturation of most organ systems in vertebrates during their different life transitions.

The manuscripts published highlight the evolutionary conserved role of TH on vertebrate natal and post-natal neurodevelopment from fishes, anurans, birds to humans. First, Rabah et al. review into the role of blood and cerebrospinal fluid TH transporter proteins in vertebrate development with special incidence in transthyretin (TTR) in humans and associated pathologies. The original localization of TTR in the cerebrospinal fluid open new perspectives on the biological understanding of TH function and TH availability in brain formation. In the same register, Stepien and Huttner review the current knowledge about TH delivery (transport), conversions (metabolism), and function in the developing mammalian brain, including neurogenesis and brain maturation. They also discuss their potential role of TH in vertebrate brain evolution and offer future directions for research.

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Next, thanks to the contribution of non-mammalian models to dissect the developmental role of TH on vertebrate development, both embryonic and post-natal. The post-natal role of TH in vertebrate development is long known from anuran studies. Amphibian metamorphosis is a valuable model to study postembryonic development in vertebrates because this developmental process is independent of maternal influence and avoids the difficulty to manipulate the uterus-enclosed embryos and neonates. Interestingly, TH is not the only endocrine signal involved in frog metamorphosis. As reported by Sachs and Buchholz, if TH is probably the most important hormone, glucocorticoids (GCs) can modulate the rate of developmental progress induced by TH and may also have direct actions required for completion of metamorphosis independent of their effects on TH signaling. Here, they provide a new review and analysis of the crosstalk between the two endocrine pathways that are strongly conserved during vertebrate evolution. Next, coming back on the effect of TH on brain development, Wen et al. were able using an inactivating mutation in the gene that encodes TH receptor alpha (TRα) to provide evidences that TRα is required for TH-dependent neural cell proliferation during tadpole metamorphosis, a suggested for mammals. Indeed, TRα accounts for 95% of the gene regulation responses to TH. Another pertinent use of amphibian model is the understanding of TH action on the most notable phenomena in developmental biology resulting in morphological changes: the limb development and growth along with tail resorption. These two phenomena are linked to locomotive switch. Yaoita highlights how this switch require elaborate regulation with differential TH sensitivity of the tail and hindlimbs. He also reviews the mechanism leading to tail resorption that occurs through two mechanisms, suicide and murder.

Moreover, today the access to transcriptome analysis in several Anuran species strongly improve capacity to distinguish between species specific transcriptional program and shared program. Wang et al. findings suggest that tail resorption in tow *Microhyla fissipes* and *Xenopus laevis* shares many programs. These results help to reveal important mechanistic insights governing mammalian postembryonic developments, with strong impact to understand the life transition from an aquatic environment to an air breathing environment. Another example comes from an Anuran with a different life cycle than the classical model, the Xenopus. Indeed, although frogs present indirect development with larval phase and metamorphosis other frogs have direct development and bypass tadpole stage. It is remarkable that the developmental action of TH is highly conserved. Laslo et al. show that, in comparison to biphasic anuran species, in the direct-developing frog *Eleutherodactylus coqui*, TH also promotes limb development and the same TH-signaling genes found in Xenopus are involved. Remarkably, limb development occurs before thyroid gland development highlighting the critical role of maternal TH in these anurans, critical role that has also been reported in mammals. Amphibian clade provides another group of model organism in biomedical research. Urodela, such as *Ambystoma mexicanum* (Axolotl) has amazing ability to regenerate after injury and ability to retain juvenile morphology into the adult phase of life. The axolotl does not typically undergo a metamorphosis, but TH can induce transformation. Crowner et al. discuss paedomorphic development and metamorphosis of axolotl. This anuran constitutes a formidable example of how different regulation of TH signaling during metamorphosis can give rise to singular developmental outcomes in vertebrates. With the recent completion of the axolotl genome assembly and established methods to manipulate gene functions, the axolotl is poised to provide new insights about paedomorphosis and the role of thyroid hormone in development and evolution. Using the same model and a combination of developmental endocrinology, functional genomics, and network biology to compare the transcriptional response of tailfin to TH, in the post-hatching paedormorphic axolotl and *Xenopus* tadpoles, Kerdivel et al. first show that axolotl tailfin undergoes a TH-dependent transcriptional response at post embryonic transition, despite the lack of visible anatomical changes and second propose that a subnetwork of cellular sensors and regulators, display opposite transcriptional programs conducting alternative tailfin fate (maintenance vs. resorption) post-hatching.

Today, amphibians are not the only non-mammalian models to dissect the developmental role of TH on vertebrate development. In most teleosts, metamorphosis encompasses a dramatic post-natal developmental process where the free-swimming larvae become a fully formed juvenile fish (Campinho). In the flatfishes, the morphological changes are more dramatic with the migration of one eye to the opposite side of the head while simultaneously the symmetric pelagic larva develops into an asymmetric benthic juvenile. Thus, flatfish metamorphosis is a remarkable example of the capacity of TH-signaling in shaping adaptation and evolution. Only recently, the powerful Zebrafish model has taken attention to learn more about TH function during development. Here, Lazcano et al. generated crispants for all the TR to show that TH signaling participates in early zebrafish development and to identify TR isoform-specific mediated regulation of early gene expression. Birds also provided their contribution thus highlighting its position as a formidable vertebrate model to study maternal thyroid function (Darras). The data presently available clearly indicate that maternal derived THs play an important role in avian embryonic neurodevelopment linked to post hatch fitness. As a whole, this works highlights the conserved role of maternal thyroid hormones in vertebrate neurodevelopment.

There is no doubt that fine-tuning of TH signaling is essential for proper vertebrate development. Unfortunately, aquatic and terrestrial environments are increasingly contaminated by anthropogenic sources that have the potential to disrupt endocrine function, including TH action. Again, anuran postembryonic metamorphosis provides a powerful model to serve as a sensitive test for the detection and mechanistic elucidation of TH disrupting activities of chemical contaminants and their complex mixtures. As reviewed by Thambirajah et al., sensitive assays are indispensable for the timely detection of TH disruption. Thus, providing a comprehensive understanding of how TH signaling is disrupted with building

of adverse outcome pathway is required to isolate molecular dysfunction that precedes adverse effects and complement morphological, behavioral, and histological assessments. In the same context, Leemans et al. focuses on plant protection products, because some pesticides and biocides were recognized as potential TH signaling disrupting chemicals particularly during pre- and perinatal development, two vulnerable periods of exposure. Finally, both reviews highlight the absence from regulatory directives of thyroid-specific endpoints for neurodevelopmental effects.

With this Research Topic, we provide a developmental as well as evolutionary picture to how TH action become so important in vertebrate development. There is no doubt that, actual research on TH role in invertebrates, vertebrate such as amphioxus and lamprey, fishes, amphibians, turtles/reptiles, birds and mammals will provide pertinent results to fully understand the evolution of TH signaling pathway in order to better characterize and treat TH associated pathologies or anthropomorphic disruption of TH signaling.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the manuscript.

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ORIGINAL RESEARCH
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Gene Expression Program Underlying Tail Resorption During Thyroid Hormone-Dependent Metamorphosis of the Ornamented Pygmy Frog *Microhyla fissipes*

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Thyroid hormone (T3) is essential for vertebrate development, especially during the so-called postembryonic development, a period around birth in mammals when plasma T3 level peaks and many organs mature into their adult form. Compared to embryogenesis, postembryonic development is poorly studied in mammals largely because of the difficulty to manipulate the uterus-enclosed embryos and neonates. Amphibian metamorphosis is independent of maternal influence and can be easily manipulated for molecular and genetic studies, making it a valuable model to study postembryonic development in vertebrates. Studies on amphibian metamorphosis have been largely focused on the two highly related species *Xenopus laevis* and *Xenopus tropicalis*. However, adult *X. laevis* and *X. tropicalis* animals remain aquatic. This makes important to study metamorphosis in a species in which postmetamorphic frogs live on land. In this regard, the anuran *Microhyla fissipes* represents an alternative model for developmental and genetic studies. Here we have made use of the advances in sequencing technologies to investigate the gene expression profiles underlying the tail resorption program during metamorphosis in *M. fissipes*. We first used single molecule real-time sequencing to obtain 67, 939 expressed transcripts in *M. fissipes*. We next identified 4,555 differentially expressed transcripts during tail resorption by using Illumina sequencing on RNA samples from tails at different metamorphic stages. Bioinformatics analyses revealed that 11 up-regulated KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and 88 Gene Ontology (GO) terms as well as 21 down-regulated KEGG pathways and 499 GO terms were associated with tail resorption. Our findings suggest that tail resorption in *M. fissipes* and *X. laevis* shares many programs. Future investigations on function and regulation of these genes and pathways should help to

reveal the mechanisms governing amphibian tail resorption and adaptive evolution from aquatic to terrestrial life. Furthermore, analysis of the *M. fissipes* model, especially, on the changes in other organs associated with the transition from aquatic to terrestrial living, should help to reveal important mechanistic insights governing mammalian postembryonic developments.

Keywords: SMRT sequencing, RNA-Seq, metamorphosis, *Xenopus*, thyroid hormone receptor, tail resorption, *Microhyla fissipes* (Neobatrachia)

INTRODUCTION

Thyroid hormone (T3) plays a critical role during vertebrate development. In mammals, the most important period of T3 action is the so-called postembryonic development, which is about 4 months before to several months after birth for human when plasma T3 level peaks (1, 2). Many important developmental changes take place during this period. Among them include brain development, organ maturation, the changes from fetal to adult hemoglobin, etc. Compared to embryogenesis, postembryonic development is poorly studied in mammals largely because of the difficulty to manipulate the uterus-enclosed embryos and neonates. On the other hand, defects during postembryonic development can lead to life-long diseases/abnormalities. Thus, alternative non-mammalian models are needed to understand this developmental period, especially the role of T3. Among vertebrates, frog metamorphosis bears the strong similarities with postembryonic development in mammals (1, 2). Like mammalian postembryonic development, frog metamorphosis is also characterized by a peak level of plasma T3. Furthermore, T3 plays a necessary and sufficient role for the transformation of a tadpole to a frog (3–6). Unlike mammalian development, frog metamorphosis is independent of maternal influence and can be easily manipulated by controlling the availability of T3 to the tadpoles. Furthermore, most, if not all, individual organs are genetically pre-determined to undergo organ-autonomous changes in response to T3, making it possible to induce metamorphosis in organ- and primary cell cultures with T3 treatment (1, 2, 7). These make frog metamorphosis a highly valuable model to study postembryonic development in vertebrates.

The most widely and best-studied frog models for metamorphosis are *X. laevis* and *X. tropicalis*, especially after the advent of transgenic and gene-editing technologies (8–11). Studies on the *Xenopus* models have yielded important insights on the roles of T3 and its two receptors, TRα and TRβ during development, and identified many T3-reponse genes and gene regulation profiles underlying metamorphosis in a number of organs and tissues (3, 4, 7, 12–17). On the other hand, it remains to be investigated if the findings from the *Xenopus* models apply to other frogs. This had been difficult due to the lack of genome sequence information for other frog species. Although a genome-wide transcriptome analysis for the developing tadpoles of the northern leopard frog (Lithobates pipiens) was reported but the study did not analyze changes during metamorphosis (18). Thus, it is important to study the gene expression program during metamorphosis in other frog species, especially considering that adult *X. laevis* and *X. tropicalis* remain aquatic.

Microhyla fissipes offers a number of advantages as an alternative model for developmental and genetic studies. *M. fissipes* is a typical anuran from the family of Microhylidae belonging to the Neobatrachia while *Xenopus* is a representative of Mesobatrachia (19). Studies on *M. fissipes* will thus allow a comparison between two different genuses to reveal adaptive mechanisms from aquatic to terrestrial life (19). Compared to the two *Xenopus* species, *M. fissipes* animals are much smaller in body size and have a shorter developmental time through metamorphosis (20, 21). *M. fissipes* is a diploid anuran and has large egg size (0.8–1.0 mm) (19), making it easy to adapt gene-editing tools for knockout studies of gene function during development. Most importantly, *M. fissipes* metamorphosis changes an aquatic tadpole to a terrestrial frog, better resembling postembryonic development in mammals. Thus, while many metamorphic changes, such as tail resorption and limb development, are expected to be conserved between *Xenopus* and *M. fissipes,* others that are critical for terrestrial living, such as the transformation of the lung and skin, may diverge.

To investigate whether we can use global gene expression analysis to study *M. fissipes*, we have made use of the advances in sequencing technologies to investigate the gene regulation profiles underlying the tail resorption program during metamorphosis in *M. fissipes.* Tail was chosen for this study in part due to easiness to characterize the changes at morphological and cellular level and in part due to available information from earlier studies on *Xenopus*. We have used Illumina RNA sequencing (RNA-Seq) combined with single molecule real-time (SMRT) sequencing for transcriptome assembly on RNA isolated from *M. fissipes* tails at different developmental stages. This allowed us to obtain 50,577 expressed transcripts and identified 4,555 differentially expressed transcripts (DETs) during tail resorption. We further analyzed the enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among the DETs. Our finding revealed similar gene regulation programs underlying tail resorption in both *M. fissipes* and *Xenopus.* Future studies on *M. fissipes,* especially on the metamorphosis of tissues critical for terrestrial living, should help to reveal the mechanisms governing the adaptive evolution from aquatic to terrestrial life.

MATERIALS AND METHODS

Experimental Animals

Microhyla fissipes breeding adults were collected from Shuangliu, Chengdu, China (30.5825°N, 103.8438°E). All animal care and treatments were done as approved by Animal Care Committee, Chengdu Institute of Biology (Permit Number: 20150121003). Fertilized eggs were obtained from one pair of frogs, incubated in glass petri dishes (diameter, 160 mm) with dechlorinated tap water. After hatching, every 60 tadpoles was transferred to a plastic container ($420 \times 300 \times 230$ mm^3) with 80 mm depth dechlorinated tap water at 25 ± 0.6 °C, and tadpoles were fed with spirulina powder once daily. All animals were maintained under the 12: 12 h light: dark cycle. Developmental stages were determined as described (21).

Tissue Collection

Six tadpoles at stage 39 (S39), S40, S41, and S43 each were randomly collected and anesthetized in 0.01% MS222 for tail morphological measurement. Three animals at each stage were used for hematoxylin-eosin (HE) staining and the rest were used for terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling (TUNEL) analysis. For gene expression analysis, three biological replicates, each containing one animal tail, were used for RNA-seq at each developmental stage. To maximize the discovery of *M. fissipes* gene pools, heart, liver, spleen, lung, kidney, skin, ovary, testis from adult *M. fissipes*, tails (at S38, S40, S41, S43) and dorsal muscle (at S36, S40, S43, S45) were dissected for SMRT sequencing. All dissected tissue samples were immediately frozen in liquid nitrogen and stored at −80°C.

Morphological Measurements and Histological Analysis

Length Measurement

Tail length (TL, from posterior edge of vent to the end of tail tip) and snout-vent length (SVL) were measured with a stereo microscope (JSZ8T, Jiang Nan Yong Xin, Nanjing, China) and Mshot Image Analysis system (Mc50-N) as described (21). To reduce allometric bias, tail length at each stage were presented as a ratio of TL/SVL.

HE Staining

After length measurement, tails were dissected and fixed in 4% paraformaldehyde for 24 h, and then embedded in paraffin. Next, tails were cross-sectioned at 5 μm with a Rotary Microtome (Leica, RM 2016, Germany), and tissue sections were stained with HE.

TUNEL Assay

Apoptotic cells in the tail during metamorphosis were detected by using a TUNEL detection kit (Roche, 11684817910) according to manufacturer's instruction.

Illumina RNA Sequencing (RNA-Seq)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and DNase I (Sigma, St. Louis, MO, USA) was used to remove DNA. RNA concentration was measured using Qubit 2.0 Fluorometer (Life Technologies, CA, USA) and RNA purity was checked by using Nanodrop 2000 Spectrophotometer (IMPLEN, CA, USA). Additionally, the integrity of RNA (RIN) was determined using Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) with RIN>8.0. Briefly, mRNA was purified from total RNA by using poly-T oligo-attached magnetic beads and chemically fragmented. First strand cDNA was synthesized by using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H–). Second strand cDNA synthesis was subsequently performed by using DNA Polymerase I and RNase H. Twelve cDNA libraries were generated by using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) following the standard protocols. The libraries were sequenced on the Illumina X-ten platform to obtain 150 nt paired-end reads at NovoGene (Beijing, China). All the raw data from Illumina sequencing were deposited in the NCBI Short Read Archive (SRA) database with the accession number PRJNA504611.

SMRT Sequencing

Tissue samples were homogenized and total RNA was isolated. The concentration and quality of RNA were assessed as above for RNA-Seq. The first full length strand cDNA was synthesized from 3 μg total RNA by using the SMARTer PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA). After PCR cycle optimization, the double-strand cDNA was generated with 11 PCR cycles, and the PCR products were separated with agarose gel-based size selection into cDNA fractions of length 1–2, 2–3, and 3–6 kb. Then, large-scale PCR was utilized to amplify the cDNAs. In the end, three SMRTbell template libraries were generated from the amplified cDNA by using Pacific Biosciences template preparation kit (Menlo Park, CA, USA) as per the standard protocol. Then SMRT sequencing was performed on a PacBio RS II platform at NovoGene (Beijing, China). Full-length transcripts were obtained by using Pacific Biosciences' SMRT Analysis Server 2.0 (22). Finally, full-length transcripts were corrected with Proovread Software (23) by using Illumina Hiseq reads. The proofread-corrected sequences after removing the redundant sequences with using CD-HIT-EST (24) were used as the reference sequences for further analyses. All the raw data from Illumina sequencing were deposited in the NCBI Short Read Archive (SRA) database with the accession number PRJNA504611.

Functional Annotation of Transcripts

The final corrected clean reads above were searched against the databases NR, Swiss-Prot and KOG by using diamond software version 0.8.22 with an E-value threshold of 1.0E-5 and KOG with an E-value threshold of 1.0E-3 to predict the gene identities. NT annotation was done with NCBI blast 2.2.28$^+$ software version 2.2.28$^+$ with an E-value threshold of 1.0E-5. Hmmer 3.0 package hmmscan was used for PFAM annotation with an E-value threshold of 0.01. GO annotations were determined based on the best BLASTX hit from the NR database using the Blast2GO software version 2.5 (*E*-value = 1.0E−6). KEGG pathway analyses were performed using the KEGG Automatic Annotation Server (KAAS, *E*-value = 1.0E−10). Clean reads were mapped to the full length reference transcripts by using

Bowtie, and then gene expression level was calculated with RSEM (RNA-Seq by expectation-maximization) (25).

DET Analysis

To compare the transcript expression levels among the four stages, the reads of each transcript were normalized to yield the number of sequenced fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) for the four developmental stages. Pairwise comparisons of transcript expression levels among the four stages were carried out by using the DESeq R package (1.10.1) to identify DETs. Transcripts were considered as DETs at q value < 0.05 after adjustment for the false discovery rate (FDR). For heatmap analysis, $\log_{10}^{(FPKM+1)}$ values were used for each tested transcript. Gene expression profile and log2 (ratios) values were used for K-means clustering.

GO and KEGG Enrichment Analysis of DETs

DETs were subjected to GO enrichment analysis by using the GOseqR package based on the Wallenius non-central hypergeometric distribution (26). KEGG pathway enrichment analysis of the DETs was done with the KOBAS software (27).

RESULTS

Microhyla fissipes as a Model for Studying Tail Resorption

Studies on *X. laevis* indicate that tail resorption takes place mainly after stage 62 (S62) when rapid reduction in tail length occurs (28). This process involves apoptosis in essentially all tail tissues, especially, the epidermis, muscle, and notochord (29–31). To determine if *M. fissipes* undergoes a similar tail resorption process, we first compared the morphology of *M. fissipes* tadpoles at different stages of metamorphosis to that of *X. laevis* tadpoles (28). As shown in **Figure 1**, *M. fissipes* tadpoles at S39 resemble *X. laevis* tadpoles at S56, considered to be the early phase of metamorphosis (with stage 54/55 typically considered the onset of metamorphosis in *X. laevis*) (28). Under comparable rearing temperatures, *M. fissipes* tadpoles at S39 complete metamorphosis (reaching S45) in about 10 days, about ½ of the time that it takes for *X. laevis* tadpoles at S56 to reach the end of metamorphosis when tail resorption is complete (**Figure 1**). In particular, tail resorption, as defined from the onset of tail length reduction to complete resorption, requires only 3 days for *M. fissipes* tadpoles (from S42–S45) but 10 days for *X. laevis* tadpoles (from S61–S66) (**Figure 1**), indicating that *M. fissipes* tadpoles undergo a faster T3-dependent metamorphosis.

We next isolated tails from animals at 4 different metamorphic stages, S39, S40, S41, and S43 for further analysis. *M. fissipes* S39 is equivalent to *X. laevis* S56 (**Figure 1**). The only noticeable metamorphic change that takes place at this stage is the hindlimb development (28). For this reason, *X. laevis* at S56 is often referred to as a premetamorphic stage and for simplicity, we will refer *M. fissipes* tadpoles at S39 as premetamorphic animals as well. S40–S43 are metamorphic climax stages, although tail length reduction occurs only after S41. When tail sections at these different metamorphic stages were analyzed, it was clear that significant muscle atrophy was observed only at S43 (**Figure 2A**). In addition, TUNEL assay showed that while some apoptotic cells were observed by S40/41, drastic increase in apoptotic cells were found at S43 (**Figure 2B**), consistent with rapid tail length reduction. These findings indicate that *M. fissipes* undergoes a similar tail resorption program as that during *X. laevis* tail metamorphosis (29–31).

Transcript Assembly and Identification of DETs During Tail Resorption

To facilitate the genome-wide analysis of the gene expression program underlying tail resorption, we first carried out SMRT sequencing of different *M. fissipes* organs at different stages. From 108,614,218 nucleotide sequences thus obtained, a total of 67,939 transcripts were assembled. The lengths of these assembled transcripts ranged from 103 to 8,847 bp with an average length of 1,599 bp and N50 of 1,956 bp (**Table S1**). Annotations through NR, NT, KEGG, Swiss-Prot, PFAM, GO and KOG databases showed that 52,881 transcripts (78% of the 67,939 transcripts) were annotated in at least one of the databases (**Table S1**).

To determine the gene expression program underlying tail resorption, we carried out Illumina RNA-Seq on the tail at the four metamorphic stages (S39, S40, S41, S43). The gene expression level was determined from the sequence data by using RSEM (25). 50,577 expressed transcripts with FPKM >0.3 were thus identified in the tail among these four metamorphic stages and the vast majority of transcripts (33,264 transcripts) were commonly expressed at all four stages (**Figure 3**).

Next, pair-wise comparisons of gene expression among the four stages were carried out, leading to the identification of 4,555 DETs in the tail during natural metamorphosis (**Figure 4**, **Table S2**). Among the 4 developmental stages, most DETs were found when comparing gene expression at S43 to any one of the other three stages, while relatively few DETs were found when the comparison was done among S39, S40, S41 (**Figure 4**). Consistently, Venn diagrams of the DETs between S39 and any one of the other three stages (**Figure 5A**) or the DETs between consecutive two stages (**Figure 5B**) showed very few commonly regulated genes. In contrast, Venn diagram of the DETs between S43 and any one of the other three stages showed that most of the DETs were common (**Figure 5C**). Finally, a heatmap of all 4,555 DETs showed that the vast of majority of the genes fell into two categories: highest and lowest expression at S43, respectively, but with similar expression levels among S39, S40, and S41 (**Figure S1**). These findings indicate that the tail at S43 has a very different gene expression program compared to those at the other three stages, while the tail gene expression programs at S39, S40, and S41 were very similar to each other, consistent with the lack of significant changes in the tail from S39 to S41 but drastic tail resorption by S43 (**Figure 1**).

Identification of the Gene Regulation Programs Underlying Tail Resorption During Natural Metamorphosis

To identify the critical gene regulation programs governing tail resorption, we further analyzed the 4,555 DETs in the tail

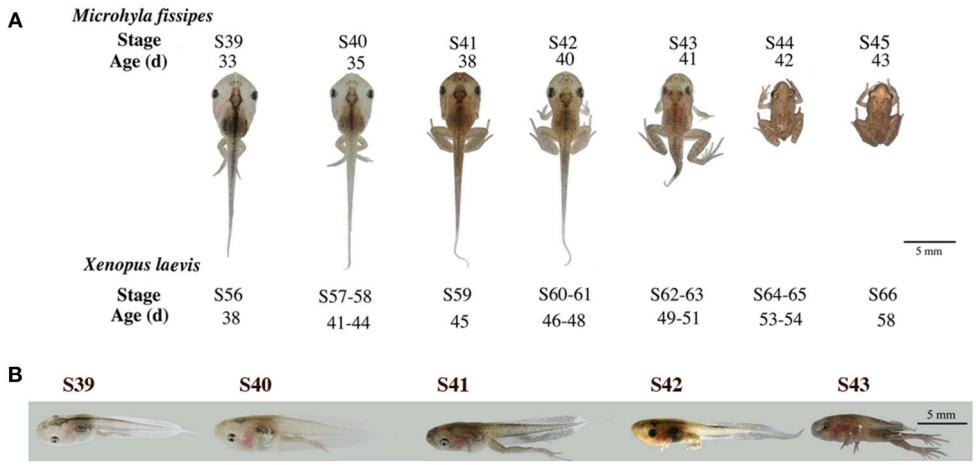

FIGURE 1 | Morphological changes associated with tail resorption during *M. fissipes* metamorphosis. **(A)** A representative *M. fissipes* animal at indicated developmental stages from S39 (around the onset of metamorphosis) to S45 (end of metamorphosis) is shown together with typical age (in days, d) of the animal when reared at 22.9–25.4°C. Shown at the bottom are the corresponding stages of *X. laevis* animals and their ages when reared at 22–24°C (28), scale bar: 5 cm. **(B)** Lateral views of the tail at indicated developmental stages.

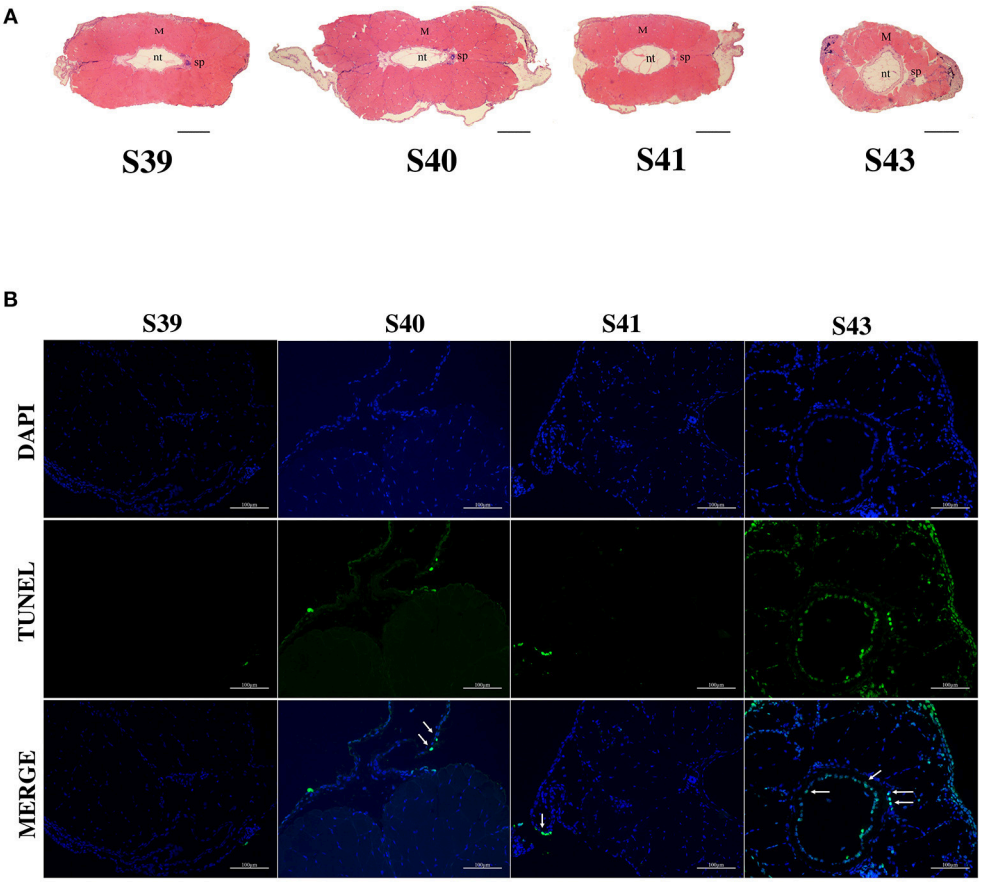

FIGURE 2 | Extensive apoptosis in the epidermis and notochord is associated with tail resorption during natural metamorphosis. **(A)** H&E staining of tail cross-sections at indicated stages. Magnification, 40×, scale bar: 200 μm, M, muscle; nt, notochord; sp, spinal cord. **(B)** TUNEL labeling reveals apoptotic cells mainly in the epidermis and notochord at S43 when tail length reduces rapidly (see **A**). Tail sections at indicated stages were co-stained with TUNEL labeling (green) for apoptotic cells (indicated by white arrows) and DAPI (blue) for DNA. Magnification, 200×, scale bars: 100 μm.

during natural metamorphosis. First, K-means clustering was performed to divide the DETs into the 26 possible expression patterns (clusters) (**Figure 6**). In agreement with the findings above, most of the DETs fell into two groups of clusters: one group of clusters (the up-clusters #5, 8, 13, 21, 23, 26) in which the DETs were gradually upregulated by S43 and another group of clusters (the down-clusters #3, 9, 10, 12, 20, 22, 25) in which the expression of the DETs changed little from S39 to S41 but were downregulated significantly between S41 and S43. Since metamorphosis begins around S39 and drastic tail resorption starts between S41 and S43, we focused on these two groups of clusters. Analyses of gene functional categories and biological pathways with GO and KEGG, respectively, on the 1,030 genes in the up-clusters revealed no pathways or GO terms was significantly enriched in the DETs based on the more stringent criterion of q<0.05. On the other hand, a number of significantly enriched KEGG pathways and GO terms were found based on a value of $p < 0.01$ (**Tables S3, S4, Figure S2**). Most significant among them was the proteolysis GO term with 60 DETs (**Figure S2**), essentially all of which were upregulated by S43 but changed little from S39 to S41 (**Figure S3**, **Table S5**). The upregulation of this GO term is consistent with the rapid protein degradation associated with tail resorption after S41.

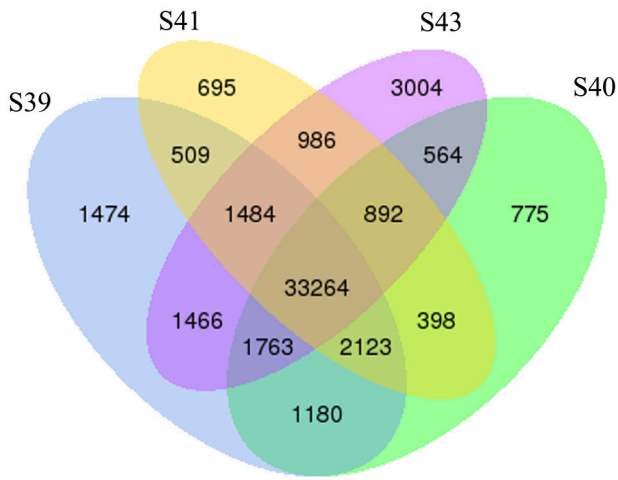

FIGURE 3 | Venn diagram analyses reveal stage-specific of gene expression. Venn diagrams showing the number of expressed transcripts (FPKM > 0.3) at different stages in the tail during natural metamorphosis. Note that total 50,577 transcripts were expressed in which 33,264 transcripts were expression at all four stages and that each stage had some uniquely expressed transcripts, with S43 having the most. FPKM, fragments per kilobase of exon model per million mapped reads.

Similar analyses on the 1,596 genes in the down-clusters revealed the enrichment of 21 KEGG pathways (**Figure 7A**, **Table S6**) and 499 GO terms (**Figure 7B**, **Table S7**) even based on the more stringent statistical criterion of $q < 0.05$. Many of the most significantly enriched KEGG pathways were related to metabolism, consistent with the tissue resorption process as the cells undergo apoptosis and reduce their own metabolism. The top 30 most significantly enriched GO terms were related to three higher level GO terms "Biological Process" (BP), "Cellular Component" (CC), and "Molecular Function" (MF) (**Figure 7B**), and were strongly associated with the protein import into nucleus, intermediate filament, myosin complex, motor activity and nucleotide binding (**Figure 7B**, boxed terms). When we carried out the analysis with directed acyclic graph (DAG), we found that the enriched GO terms within BP were all linked to a single node: the GO term

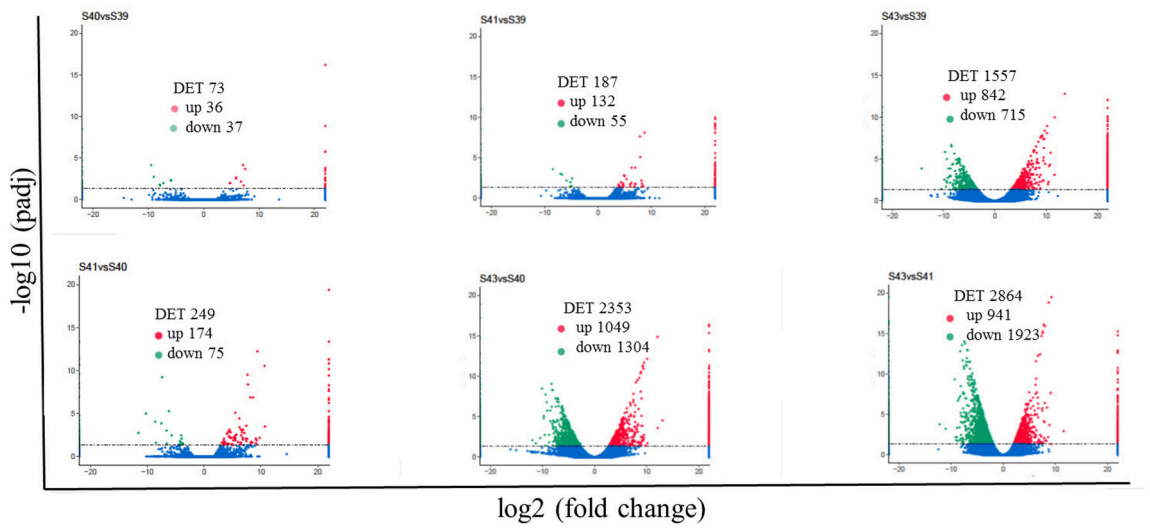

FIGURE 4 | Pair-wise comparisons reveal that S43 tail has the most number of differentially expression transcripts (DETs, $q < 0.05$) during natural metamorphosis. Volcano plot of DETs in the tail for six different comparison groups. X axis represents fold-change (log2FC) of the DETs and the Y axis represents the –log10padj [adjusted *P*-value (*q*-value)] value of the DET, with 0.05 set as the significance cutoff level. Up-regulated DETs are shown in red while down-regulated ones in green; non-significantly DETs are presented in blue.

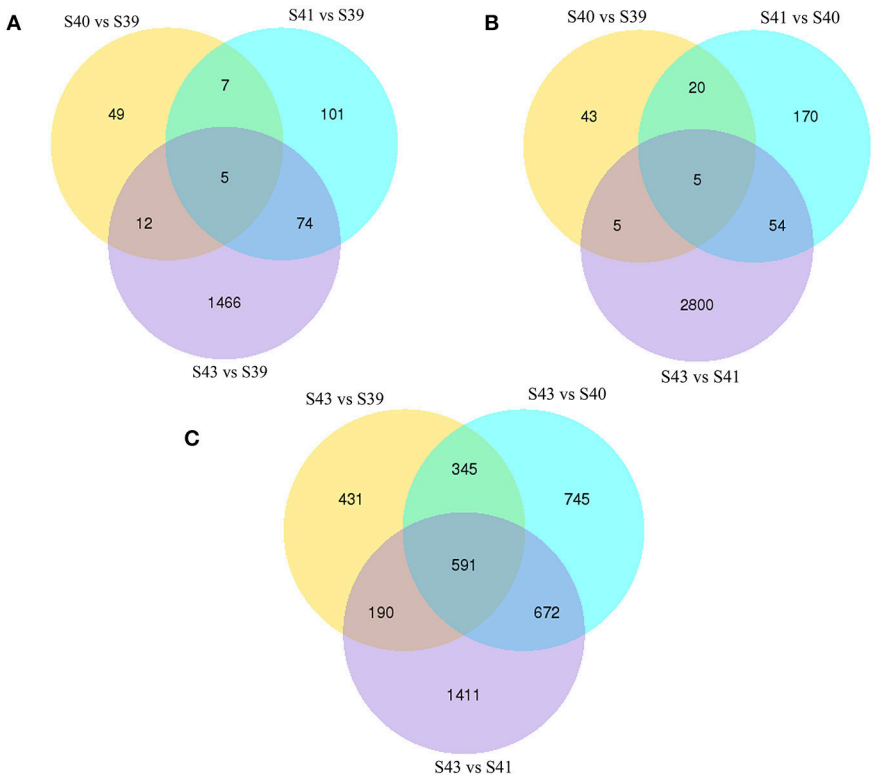

FIGURE 5 | Venn diagrams of differentially regulated transcripts from pair-wise comparisons show transcript expression profiles at S39-S41 are similar but very different from those at S43. **(A)** Venn diagrams of DETs between S39 and the other three stages. Note that there were few DETs between S39 and S40 or S41 but much more between stages S39 and S43. **(B)** Venn diagrams of DETs between two successive stages. Note that there were few DETs between S39 and S40 or between S40 and S41 but much more between S41 and S43, indicating that the gene expression profile changes correlates well with the drastic morphological changes since drastic tail resorption takes place between S41 and S43 (**Figure 1**). **(C)** Venn diagrams of DETs between S43 and the other three stages. Note that there were many DETs between S43 and any other stages, including 591 common DETs between S43 and the other three stages.

"protein import into nucleus" (**Figure 7C**a). On the other hand, the enriched GO terms within CC and MF could be linked to two GO nodes: the GO terms "myosin complex" and "intermediate filament" for CC (**Figure 7C**b), and the GO terms "cytoskeletal components" and "muscle contraction" for MF (**Figure 7C**c), respectively. The enrichment of these GOs among the down-regulated genes is again consistent with the tissue resorption program as the cells undergo apoptosis.

Coordinated Regulation of Gene Categories Related to Tail Resorption During Natural Metamorphosis

The discoveries of many KEGG pathways and GO terms associated with tail resorption above were based on genes that were coordinately regulated in up- or down-clusters. To determine if all DETs in a given KEGG pathway or GO term were coordinately regulated, we compared the gene regulation profiles of all DETs in selected pathways or GO terms that are likely important for tail resorption. It is well-known that matrix metalloproteinases (MMPs) are important for apoptosis and associated ECM degradation. A heatmap of all DETs encoding MMPs showed that all 9 DETs were coordinately upregulated dramatically at S43 (**Figure 8A**). Similarly, over 80% of the DETs belonging to the GO term "muscle function" (**Figure 8B**) or "mitochondrial function" (**Figure 8C**) coordinately down-regulated by S43, consistent with the apoptotic process taking place at S43.

A similar result was obtained on DETs in the KEGG pathways "glycolysis/gluconeogenesis" (**Figure 9A**) or "Alzheimer's disease" (**Figure 9B**). In the former, all enriched DETs were down regulated between S41 and S43 while in the latter, 9 out of 10 DETs were downregulated. Thus, DETs in KEGG pathways or GO terms that are associated with apoptosis and tissue resorption are coordinately regulated during tail resorption.

DISCUSSION

Amphibian metamorphosis has long been used as a model to study postembryonic development in vertebrates, especially the developmental roles of T3. Earlier molecular and genetic studies on amphibian metamorphosis were mostly on *X. laevis* and more recently on *X. tropicalis*, especially with the advent of gene-editing technologies. While there are many

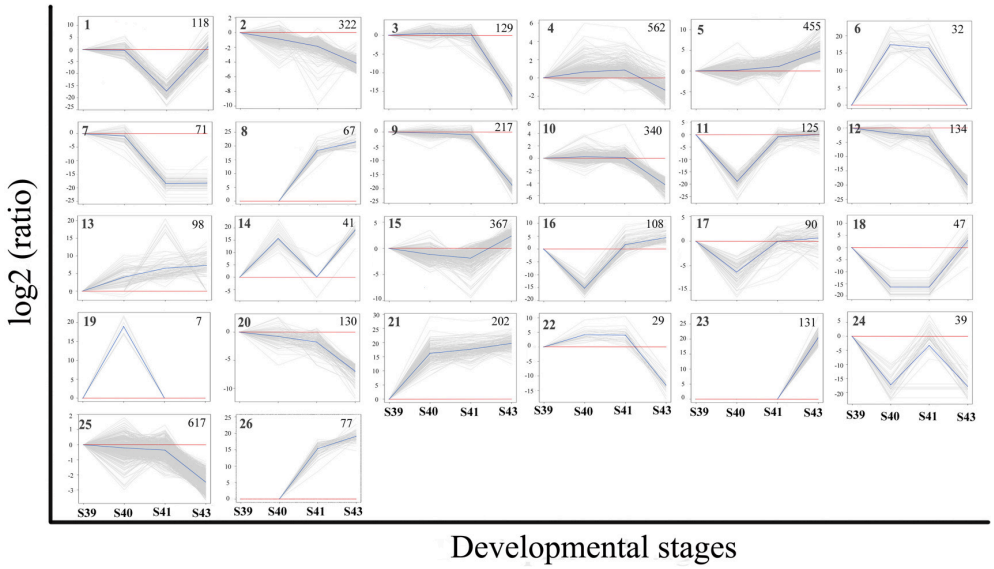

FIGURE 6 | K-means clustering profile of DETs during tail resorption. A total of 4,555 DETs were grouped into twenty-six clusters based on their developmental regulation patterns. (Note that as there are three possible changes in gene expression between any two stages: up, down and unchanged, there are totally 27 possible clusters among 4 stages. One cluster, i.e., the one with no changes in gene expression among 4 stages, is absent among the DETs.) Each gray line represents one transcript and the average relative expression levels of all transcripts are shown as a blue line. The vertical axis represents the expression level and the horizontal axis shows the four developmental stages. The cluster number is shown as a bold number in the upper left corner and the number in the upper-right corner of each cluster indicates the number of transcripts in the cluster.

advantages of using the *Xenopus* models, the aquatic nature of adult *X. laevis* and *X. tropicalis* makes them less than ideal as a model for mammalian postembryonic development. Toward developing the terrestrial diploid anuran *M. fissipes* as an alternative model for developmental and genetic studies, we have here assembled nearly 70,000 expressed transcripts and identified over 4,000 DETs during tail resorption. Our results highlight conserved gene regulation programs for tail resorption during *M. fissipes* and *X. laevis* and suggest that *M. fissipes* may be a valuable model for studying postembryonic development in mammals, especially for organs that are critical for the transition from aquatic to terrestrial living.

Microhy fissipes tadpoles are much smaller in body size and have a shorter developmental time through metamorphosis (**Figure 1**) (20, 21). *M. fissipes* is a diploid anuran and has large egg size (0.8–1.0 mm). These make it possible to adapt transgenic and gene-editing technologies for functional studies *in vivo*. On the other hand, *M. fissipes* genome is not yet sequenced. Using the newest sequencing platforms, we have here assembled nearly 70,000 transcripts and annotated nearly 80% of these transcripts by using various databases. These annotated transcripts should be a valuable resource for studying gene regulation and function during *M. fissipes* development.

We successfully used these annotated transcripts for analyzing RNA-seq data of *M. fissipes* tails at different stages of metamorphosis. In our study, we chose tadpoles at 4 different stages: S39, S40, S41, and S43. S39 is essentially a premetamorphic stage when few T3-depdent metamorphic changes occur. S40 and S41 are early metamorphic climatic stages when extensive metamorphic changes occur, particularly limb development and body/head structure transformations. However, tail remains essentially unchanged up to S41. Finally, S43 is in middle of most dramatic tail resorption with tail length halved (**Figure 1**). Thus, an RNA-seq analysis of these 4 stages should provide a detailed description of the gene expression profiles underlying tail resorption.

In agreement with the morphological changes of tail during these stages, our RNA-seq analyses revealed relatively few DETs among S39, S40, and S41. Most dramatic changes in gene expression profile take place between S41 and S43 when rapid tail resorption occurs, accompanied by increased levels of apoptosis or programmed cell death in the tail. Surprisingly, however, our GO ontology analysis of the upregulated DETs did not identify cell death as an enriched GO term among the DETs. In fact, of all DETs related to cell death, about ½ were upregulated and ½ were down regulated (data not shown) at S43. Thus, while cell death is an essential event during tail resorption, the expression of cell death-related genes was not coordinately regulated. This may reflect the fact that apoptosis often involves post-transcriptional activation of proteins involved in cell death. On the other hand, all DETs encoding MMPs were coordinately upregulated during tail resorption. MMPs are important cell surface or extracellular proteases and have been shown to be important players for development cell death, including amphibian metamorphosis (32, 33). These findings are in agreement with microarray studies of gene regulation during tail resorption in *X. laevis* (31).

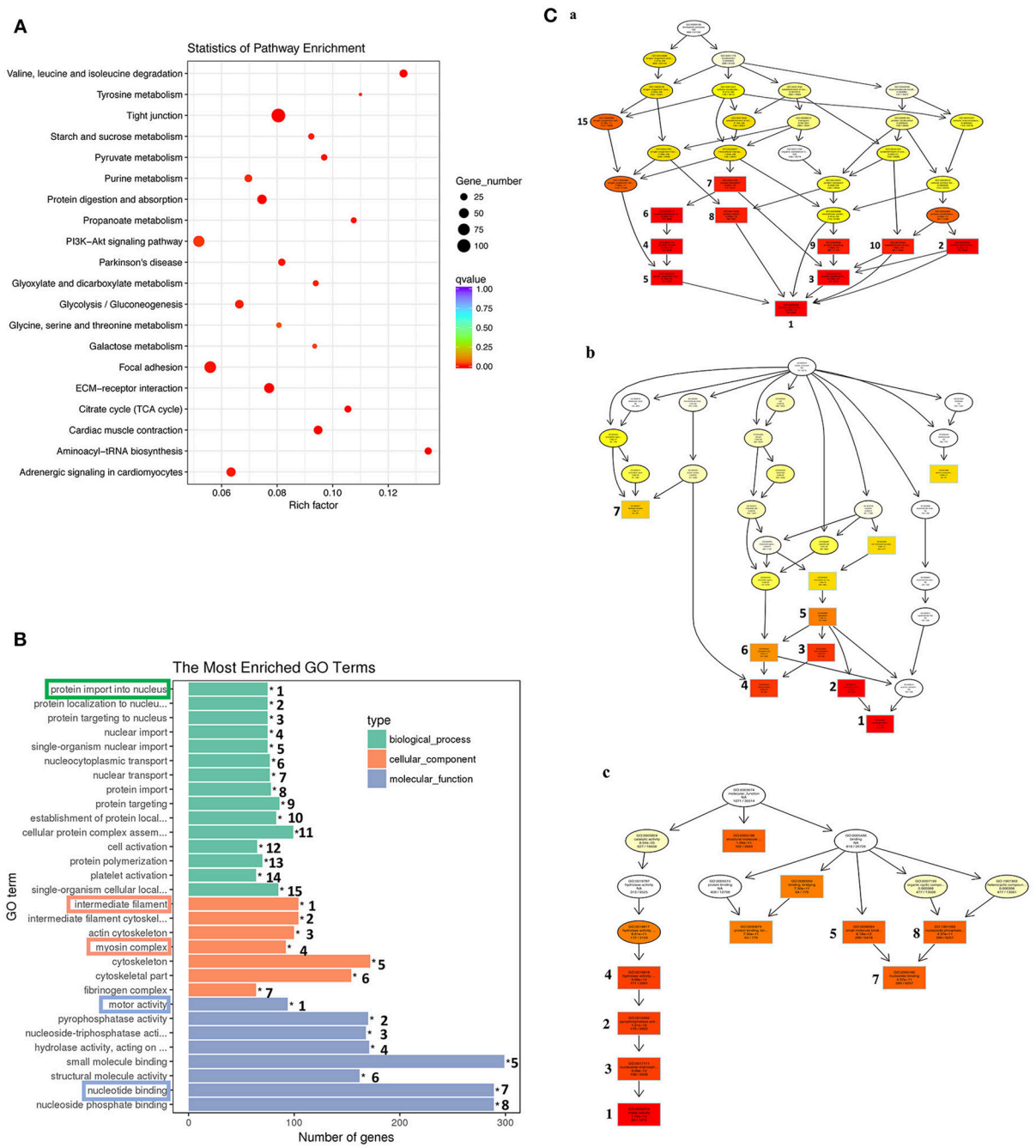

FIGURE 7 | KEGG and GO analyses reveal important down-regulated pathways and GO terms during tail resorption. The 1,596 genes in the down-regulated subcluster-3, 9, 10, 12, 20, 22, 25, in **Figure 6**, were combined and subjected to GO and KEGG enrichment analyses. Twenty-one significantly enriched ($q < 0.05$) KEGG pathways (**Table S6**) and 499 significantly enriched ($q < 0.05$) GO terms (**Table S7**) were obtained. **(A)** Top 20 significantly enriched pathways based on smallest *q*-value (from the top to the bottom). Rich factor: the number of genes enriched relative to the known number of genes in the same pathway. Red color indicates the significant enriched pathways and the size of the circle corresponds to the known number of genes in the pathway. **(B)** Top 30 significantly enriched GO terms. Ranking numbers at right site indicate the significant level of the enriched GO terms; boxed ones on the left represent selected GO terms associated with tail resorption. **(C)** Directed acyclic graph (DAG) displaying the relationship among different GO terms. Each box and circle indicate a GO term and the descriptions inside of which show GO term id, GO description, *q*-value and the enriched DETs numbers comparison with background gene numbers of indicated GO term from top to bottom. Color depth represents the enrichment degree (red is the most enriched). The numbers next to the color boxes correspond to the GO ranking numbers in **B** within the three higher level GO terms as shown here: **(a)** Biological process (shown in green in **B**) with GO term "protein import into nucleus" as the most significantly down-regulated; **(b)**. Cellular component (shown in orange in **B**) with GO terms "myosin complex" and "intermediate filament" as the most significantly down-regulated; and **(c)**. Molecular function (shown in blue in **B**) with the GO term "motor activity" as the most significantly down-regulated.

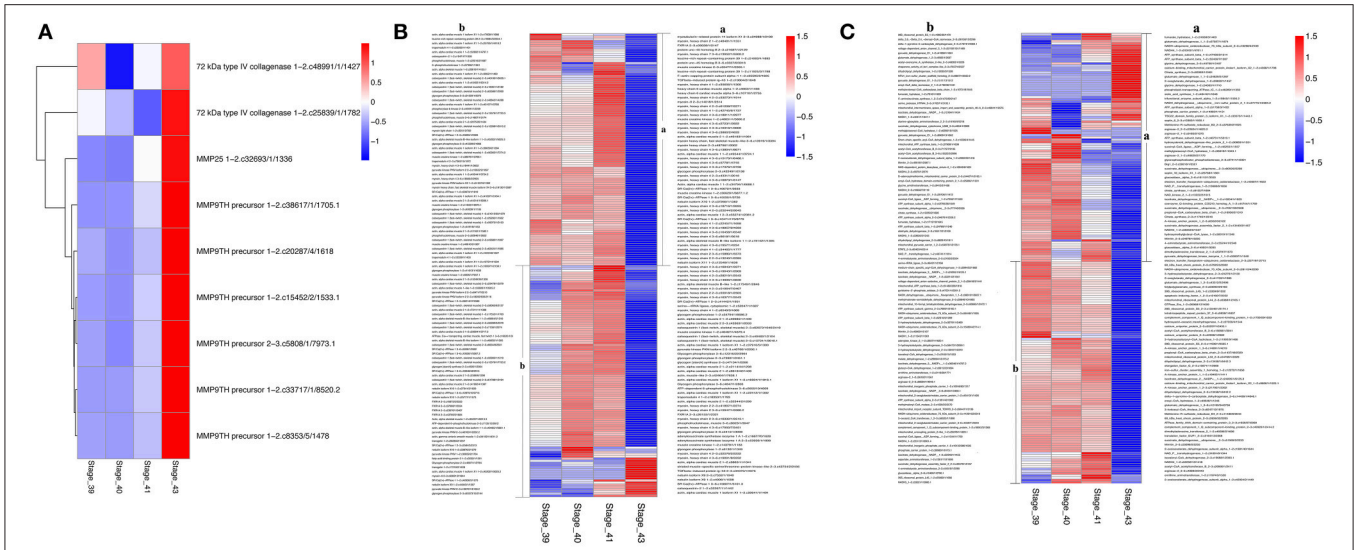

FIGURE 8 | Heatmaps showing coordinated regulation of genes in selected GO terms during tail resorption. The intensity of color indicates relative expression levels. Red to blue corresponds to high to low levels of expression. **(A)** A heatmap of all transcripts encoding matrix metalloproteinases (MMPs) within the 4,555 DETs. Note that all MMP transcripts were highly upregulated at stage 43, although they were from only three different MMP genes. **(B)** A heatmap of all transcripts encoding genes related to muscle function within the 4,555 DETs. Note that nearly all were downregulated at stage 43 when tail resorption takes place. **(C)** A heatmap of all transcripts encoding genes related to mitochondrial function within the 4,555 DETs. Note that nearly all were downregulated at stage 43 when tail resorption takes place.

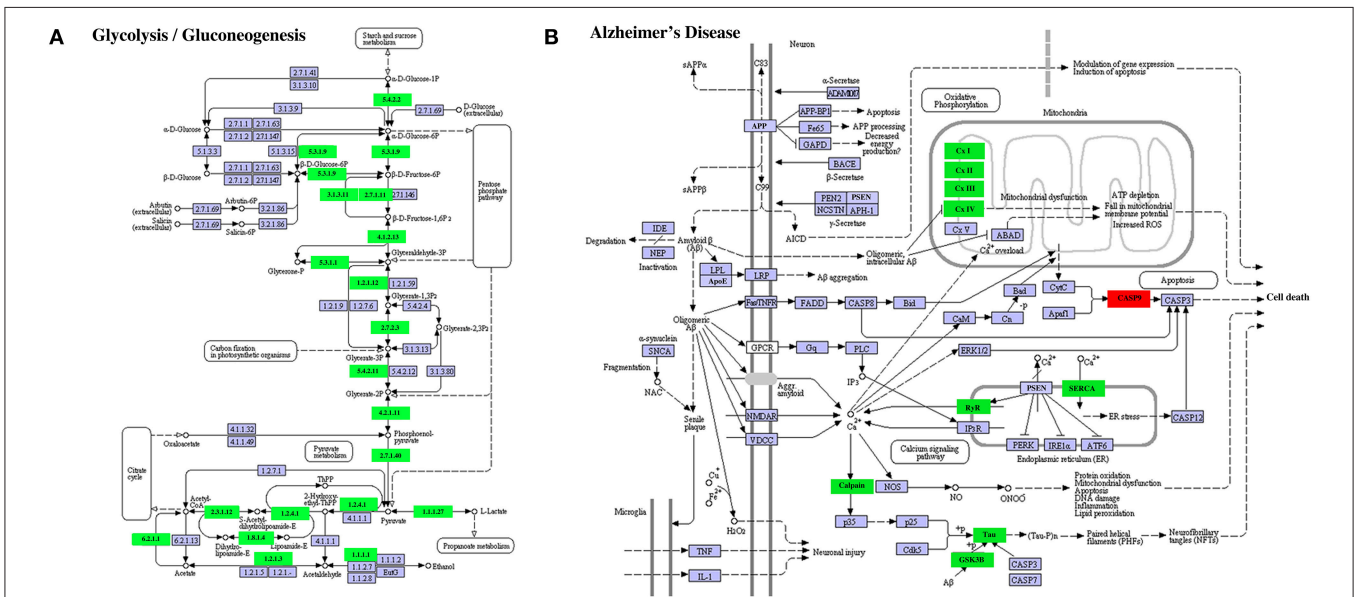

FIGURE 9 | Selected enriched KEGG pathways among DETs between S41 and S43 during tail resorption. **(A)** Glycolysis/Gluconeogenesis pathway. Note that nearly all were downregulated between S41 and S43. A rectangle indicates a gene product (an enzyme). The down-regulated genes are shown in green. The light blue genes have no change in expression level. The numbers in the all boxes correspond to the enzyme commission numbers which are associated with a recommended name for the respective enzyme and clear circles indicate metabolic products. **(B)** Alzheimer's disease pathway. Note that all but one DETs were downregulated. The exception was CASP9, which is important for inducing cell death and not surprisingly upregulated. Green color indicates down-regulated DETs and red represents up-regulated DETs.

The downregulated DETs had many highly enriched KEGG pathways and GO terms. In agreement with a degenerative process for tail resorption, the DETs in the glycolysis/gluconeogenesis pathway were coordinately downregulated by S43 as the tail length shortens rapidly. Interestingly, we also found many DETs in the Alzheimer's disease pathway and Parkinson's disease pathway were coordinately downregulated during tail resorption. This suggests that similarities exist between tail resorption and neuronal degeneration in the development of Alzheimer's disease (**Figure 9B**) and Parkinson's disease (data not shown), and that studying gene function during tail resorption may

provide insights toward understanding these diseases. The GO terms "muscle function" and "mitochondrial function" were two highly enriched terms with some of largest numbers of downregulated DETs. Heatmap analysis of all DETs encoding genes in these two GO terms showed that the vast majorities (>80%) of the DETs in these two GO terms were coordinately downregulated at S43. Many of the DETs were common in these two GO terms. These findings are consistent with the fact that muscle is the predominant tissue in the tail and muscle degeneration is thus one of the critical, major events during tail resorption. As muscle cells undergo apoptosis, genes important for muscle function, including those for mitochondria activities, are expectedly downregulated. Similar findings were also obtained in the microarray studies of tail resorption during *X. laevis* metamorphosis (31), again supporting conserved gene regulation programs governing tail resorption in *X. laevis* and *M. fissipes*.

In summary, we report here the assembly of nearly 70,000 transcripts and annotation of over 50,000 of these transcripts. By using these transcripts and RNA-seq analysis, we have obtained a detailed gene regulation profiles underlying tail resorption during natural metamorphosis in *M. fissipes*. Our findings suggest the existence of conserved gene regulation programs for tail resorption in *X. laevis* and *M. fissipes*. We have also made a novel discovery that the many genes in the Alzheimer's disease pathway and Parkinson's disease pathway were coordinately downregulated during tail resorption, implicating studies on tail resorption in *M. fissipes* may help our understanding on neurodegenerative diseases. Finally, *M. fissipes* undergoes a transition from aquatic to terrestrial living, closely resembling the postembryonic development in mammals. Thus, it is a good alternative model to either *X. laevis* or *X. tropicalis* for studying postembryonic development of organs that are critical for terrestrial living.

ETHICS STATEMENT

The care and treatment of animals used in this study were in accordance with the guidelines of the Animal Care Committee, Chengdu Institute of Biology (2015-AR-JJP-01).

AUTHOR CONTRIBUTIONS

SW, LL, and JJ conceived and designed the experiment. SW and Y-BS analyzed the data and prepared the manuscript. JL, WZ, YT, LF, and LB assisted with the bioinformatics analysis. All authors participated in the manuscript preparation and approve the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00011/full#supplementary-material

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The Role of Maternal Thyroid Hormones in Avian Embryonic Development

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During avian embryonic development, thyroid hormones (THs) coordinate the expression of a multitude of genes thereby ensuring that the correct sequence of cell proliferation, differentiation and maturation is followed in each tissue and organ. Although THs are needed from the start of development, the embryonic thyroid gland only matures around mid-incubation in precocial birds and around hatching in altricial species. Therefore, maternal THs deposited in the egg yolk play an essential role in embryonic development. They are taken up by the embryo throughout its development, from the first day till hatching, and expression of TH regulators such as distributor proteins, transporters, and deiodinases in the yolk sac membrane provide the tools for selective metabolism and transport starting from this level. TH receptors and regulators of local TH availability are expressed in avian embryos in a dynamic and tissue/cell-specific pattern from the first stages studied, as shown in detail in chicken. Maternal hyperthyroidism via TH supplementation as well as injection of THs into the egg yolk increase TH content in embryonic tissues while induction of maternal hypothyroidism by goitrogen treatment results in a decrease. Both increase and decrease of maternal TH availability were shown to alter gene expression in early chicken embryos. Knockdown of the specific TH transporter monocarboxylate transporter 8 at early stages in chicken cerebellum, optic tectum, or retina allowed to reduce local TH availability, interfering with gene expression and confirming that development of the central nervous system (CNS) is highly dependent on maternal THs. While some of the effects on cell proliferation, migration and differentiation seem to be transient, others result in persistent defects in CNS structure. In addition, a number of studies in both precocial and altricial birds showed that injection of THs into the yolk at the start of incubation influences a number of parameters in posthatch performance and fitness. In conclusion, the data presently available clearly indicate that maternal THs play an important role in avian embryonic development, but how exactly their influence on cellular and molecular processes in the embryo is linked to posthatch fitness needs to be further explored.

Keywords: thyroid hormone, development, bird, deiodinase, TH transporter

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INTRODUCTION

One of the major functions of thyroid hormones (THs), and probably also the most ancient one in vertebrate evolution, is coordinating embryonic and early postnatal development. By switching on and off the expression of a multitude of genes, THs ensure that the correct sequence of cell proliferation, differentiation, and maturation is followed in each developing organ/tissue. Many key developmental genes are only or mainly responsive to TH signaling during specific time windows in development. As a result, both untimely or too late expression of these genes may compromise the development and functioning of important organs in a persistent and irreversible way.

Although THs are needed from the start of development, early embryos lack a functional thyroid gland. This structure typically matures half way in embryonic development in precocial species such as chicken and Japanese quail, or only around the time of hatching in altricial species such as ring dove and red-winged blackbird (1–3). Mammalian embryos/fetuses can rely on a continuous supply of maternal THs via the placenta. External development in other vertebrates however precludes this possibility, so they rely on maternal THs deposited in the egg yolk. Early expression of TH distributor proteins, TH transporters, iodothyronine deiodinases, and TH receptors ensures that these hormones can be taken up from the yolk and transported into embryonic tissues where they can be activated and regulate gene transcription.

This review on the role of THs in avian embryonic development focuses on the role of maternal THs during early stages of development and explores the possible consequences for later posthatch life. Most of our current knowledge is derived from chickens where the embryonic thyroid gland is fully functional by mid-incubation (10-day-old embryo, E10) (1). Although the gland may already be able to secrete a small amount of THs a few days earlier, relevant contribution of embryonic THs to circulating levels is thought to start only around E8-E9 and to gradually increase thereafter up till hatching at E20. Fertilized chicken eggs are readily available and can easily be incubated in standard laboratory settings, facilitating experimental manipulation under controlled conditions throughout development. Moreover, the timing of the maturation of the thyroid axis is quite similar to that in the human fetus while maturation occurs much later in the classical rodent models. As a result, chicken is an excellent model system to study the role of THs in prenatal (human) development (4, 5). A limited number of data is also available for other economically relevant galliform species such as turkey and quail. Data on altricial species are scarce, and samples have been collected predominantly from songbird populations in the wild, where experimental manipulation is difficult and often not even allowed. These data are however important; since the thyroid gland of altricial species only matures late in incubation or even posthatch (6), maternal THs are the only source available throughout embryonic development.

MATERNAL TH CONTENT IN AVIAN EGGS AND EMBRYOS

Since THs are lipophilic, only a small amount of maternal TH is found in the egg albumen while the vast majority is deposited in the yolk. Reported average levels for a number of avian species are summarized in **Table 1**. The concentration of 3,5,3′,5′-tetraiodothyronine or thyroxin (T_4) in yolk always exceeds that of 3,5,3′-triiodothyronine (T_3), as found in almost all other oviparous vertebrates investigated so far. Interestingly, reported levels not only vary between species but also within species. In the case of chicken this is not surprising since the different commercial strains have been separated by long term selection. Reported average levels vary 10-fold for T_4 and 3-fold for T_3, showing differences between broilers and layers (10) but also between different layer strains (7, 9, 10). Variation however also occurs between individuals of a given species. In a recent study on songbirds, T_4 levels were found to vary 3- to 4-fold, and T_3 levels 2- to 3-fold, between individuals of the same species (11). Moreover, factors such as laying sequence, temperature and food availability induce changes in maternal TH deposition, with potential consequences for the offspring (14).

The amount of THs deposited in the egg in general varies in parallel with hormone levels in the maternal circulation: induction of maternal hypothyroidism decreases while maternal hyperthyroidism increases yolk TH levels (8, 12). However, females seem to be able to regulate TH deposition to some extent (13, 15), although the mechanisms by which this occurs are not yet understood. It was found in euthyroid Japanese quail that T_4 content in individual eggs of a given hen was relatively constant despite fluctuations in plasma T_4 (15). When quail hens were made hyperthyroid by two different doses of T_4, both T_4 and T_3 content in eggs was significantly increased with both doses although plasma T_3 was only increased following treatment with the higher dose (12). When hens were supplemented with T_3, plasma T_3 was increased and plasma T_4 decreased but TH content in eggs remained stable (12). On the other hand, when chicken hens were made hypothyroid by goitrogen treatment, plasma T_4 and T_3 initially decreased but plasma T_3 returned to normal by 14 weeks of treatment. Nevertheless, both T_4 and T_3 levels in egg yolk remained severely decreased (8).

To perform any function, maternal THs of course must be taken up by the developing embryo. It was found in chicken that significant amounts of TH were already released by area opaca cells before the start of gastrulation and that active TH (T_3) was enriched in the primitive streak and Hensen's node during gastrulation (16). A longitudinal study in chicken by Iwasawa and coworkers from E4 till hatching showed that total yolk weight and total yolk T_4 and T_3 content decreased in an almost linear and parallel way throughout embryonic development (9), suggesting that the yolk transfers THs together with other components to the embryo in a continuous and non-selective way. However, TH measurements in head and trunk in embryos from hypothyroid chicken hens showed that the situation is more complex. Despite severely reduced T_4

TABLE 1 | Maternal TH content deposited in egg yolk of different avian species.

Species	T_4 in ng/g[a]	T_3 in ng/g[a]	T_4/T_3[b]	References
PRECOCIAL BIRDS				
Chicken (*Gallus gallus*)				
- Shaver strain (layer)	3.80 ± 0.81	1.50 ± 0.39	2.13	(7)
- Ross strain (broiler)	14.60 ± 2.24	1.23 ± 0.20	9.93	(8)
- Hy-line strain (layer)	30.4	1.0	25.4	(9)
- White leghorn strain (layer)		1.05 ± 0.36		(10)
- Cornish rocks strain (broiler)		0.46 ± 0.38		(10)
Japanese quail (*Coturnix japonica*)	9.74 ± 2.20 6	3.44 ± 0.88 2.5	2.38 2.01	(11) (12)
ALTRICIAL BIRDS				
Rock pigeon (*Columbia livia*)	3.06 ± 0.99	1.10 ± 0.21	2.33	(13)
Great tit (*Parus major*)	1.15 ± 0.42	0.14 ± 0.07	6.73	(11)
Collared flycatcher (*Ficedula albicollis*)	7.21 ± 0.99	1.97 ± 0.48	3.06	(11)
Pied flycatcher (*Ficedula hypoleuca*)	5.72 ± 1.42	1.86 ± 0.56	2.57	(11)

[a] *Average concentrations ± SD are given when information is available.*
[b] *Ratio calculated on a molar basis.*

and T_3 content in yolk, T_4 levels in head and trunk of E6 embryos were normal and T_3 levels were only reduced in head (8). This indicates that regulatory mechanisms exist, certainly within the embryonic tissues themselves (see next section), but probably also already at the level of the yolk sac membrane. Both transthyretin (TTR) and albumin (ALB) are expressed there and may contribute to TH transfer to the embryonic circulation (9). Next to these TH distributor proteins, yolk sac membrane also expresses the TH transporters monocarboxylate transporter 8 and 10 (MCT8, MCT10) and organic anion transporting protein 1C1 (OATP1C1) as well as all three types of deiodinases (DIO1, DIO2, DIO3) (9). Expression profiles are gene-specific and dynamic throughout embryonic development. The yolk sac membrane may therefore act as a selective barrier, similar to the mammalian placenta where DIOs and TH transporters are also dynamically expressed to regulate TH transfer to the fetus (17, 18).

Direct measurement of T_4 and T_3 in chicken embryonic tissues during the first days of development is difficult due to the limited sensitivity of the currently available methods, but it was shown indirectly by a reporter system that active TH was already present in blastoderm and subsequently also in the primitive streak (16). From E4 onwards, extraction of either whole embryos or brain tissue, followed by sensitive radio-immunoassay, allowed to directly determine the amount of both T_4 and T_3 (7, 19). Uptake of TH from egg yolk into embryonic tissues was additionally confirmed by an increase of T_3 in E3 whole embryo extracts following injection of T_3 into the yolk at E1 (Van Herck & Darras, unpublished results). Similarly, a combined T_4+T_3 injection into the yolk of embryos from different stages (E3-E11) increased levels of both hormones in extracts from brain collected 24 h later (E4-E12) (19). Maternal THs are therefore present in the early chicken embryo and can be active if the mediators of TH action are also available (see next section). Maternal THs were also shown to be present in plasma of altricial species such as ring dove and European starling where sampling was done at later stages of embryonic development but still prior to the presumed timing of thyroid gland maturation (20, 21).

REGULATORS OF TH ACTION AT EARLY STAGES

The majority of TH signaling occurs via binding of T_3 to nuclear thyroid hormone receptors (THRs), so their presence is essential for TH action. Important in the context of development however is the fact that THRs can also function in an unliganded state, in most cases switching from active gene repression to stimulation of gene transcription upon ligand binding (22). Chickens express three isoforms of THRs: THRα, THRβ0, and THRβ2 (23). Messenger RNA for *THRA* is already present at the gastrula stage and *in situ* hybridization (ISH) showed that during neurulation, *THRA* is strongly expressed in the neural tube (16). At later stages and throughout development, this receptor is expressed in a wide array of embryonic tissues (24). Expression of *THRB* starts somewhat later in development and is mainly restricted to brain, eye, lung, kidney and yolk sac (24). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of different brain regions at E4 and E8 revealed a dynamic and region-specific expression pattern for all three *THR* isoforms (19). It was also shown that the *THRA* mRNA present during neurulation was indeed translated to functional receptor protein since injection of high doses of T_3 interfered with normal neural tube morphogenesis (16). Therefore, we can assume that TH signaling in the chicken embryo starts from the first day of development.

As THRα seems to be ubiquitously expressed from early stages (16, 25), the presence of additional regulators is essential to control local T_3 availability and thereby coordinate the switch between unliganded and liganded THR function in a time- and tissue-dependent manner. Iodothyronine deiodinases (DIOs) are intracellular enzymes capable of activating and inactivating THs. DIO1 can stimulate both pathways but with relatively low affinity and is presently thought to be less important in euthyroid conditions (26). The main enzyme for local TH activation (T_4 to T_3 conversion) is DIO2 while DIO3 is the main enzyme for local TH inactivation (conversion of T_4 to reverse T_3 and T_3 to 3,3′-diiodothyronine or 3,3′-T_2) (26, 27). The earliest ISH data are from chicken brain at E3 showing *DIO2* expression in the hypothalamic region and *DIO3* expression in the mesencephalon and the eye (28, 29). Further ISH studies up to E10 showed increasing expression of *DIO2* throughout the brain and adenohypophysis and in the eye and inner ear. *DIO2* was abundantly expressed in endothelial cells of blood vessels throughout the brain, suggesting that in chicken T_4 to T_3 conversion may already occur at least partially just

prior to entry into the brain at the level of the blood-brain-barrier (28). *DIO3* in contrast was highly expressed in the choroid plexus (28, 30), suggesting that the amount of active TH that can reach the cerebrospinal fluid is strictly controlled. Up to E10, *DIO3* mRNA was also found in some sensory brain centers and in the eye, while it was absent from the inner ear (28).

Using the more sensitive qRT-PCR as an alternative approach, mRNA of all three *DIOs* was detected in extracts from whole chicken embryos at E1 (31) and in extracts from brain sampled from E4-E12 (19). Next to the different expression dynamics in telencephalon, diencephalon, mesencephalon and rhombencephalon, the latter study also showed that *DIO2* and *DIO3* mRNA were translated into active enzymes while this was apparently not the case for *DIO1* (19). *DIO2* mRNA and activity were also reported in chicken brain by another research group in a study starting at E7 (32). Information on expression patterns of DIOs in peripheral tissues of chicken embryos during the first half of incubation is surprisingly scarce A relatively old report demonstrated the presence of both outer and inner ring deiodinating activity in liver at E8 (33) and one more recent ISH study showed *DIO1* mRNA at E4 in developing limb bud muscles and *DIO3* mRNA at E3/E4 in mesonephros/somites (29).

Before intracellular (in)activation by DIOs can occur, THs need transporters to facilitate their entry (and exit) through the plasma membrane. Chickens express four different TH transporters: MCT8, MCT10, OATP1C1, and L-type amino acid transporter 1 (LAT1), while LAT2 seems to be absent (34). The characteristics of these four chicken TH transporters are quite similar to those found in humans and zebrafish. MCT8 transports both T_4 and T_3 with high affinity, while MCT10 and OATP1C1 show a preference for T_3 and T_4 transport respectively. LAT1 has a lower affinity but is able to transport T_4 and T_3 next to its preferred iodothyronine, 3,3′-T_2 (34). Messenger RNA for *MCT8* was already detectable by ISH at E1 where expression initially occurred in all three germ layers and subsequently shifted to foregut, head region and neural tube. Expression in the endoderm disappeared at E2 (35). In E3-E4 brain, *OATP1C1* signal was mainly found in the hypothalamic region while *MCT8* staining was more widely spread. Expression of *MCT8* continued to expand throughout the brain at E6-E10 but *OATP1C1* mRNA became more restricted with a strong signal in the adenohypophysis and median eminence (28, 34). *MCT8* but not *OATP1C1* mRNA was also found in the developing eye and inner ear (28).

In relation to the brain barriers, both *MCT8* and *OATP1C1* mRNA were found in the choroid plexus but absent from the endothelial cells at the blood-brain-barrier (28, 34). The only transporter detected by ISH in these cells was *LAT1*. Immunostaining revealed the presence of LAT1 protein both in the luminal and abluminal membrane of the endothelial cells, suggesting that despite its lower affinity, this transporter is an important regulator of T_4 and T_3 entry into the developing chicken brain (5, 34). *LAT1* was also expressed in blood vessels in spinal cord and in some cell types of eye while *MCT10* mRNA was found in eye and to some extent also in the choroid plexus (34). According to the same study, neither of the four transporters was expressed in sufficient amounts in heart, lung, intestine, liver, kidney, and gonads to be detectable by ISH at E10. At that stage positive staining in peripheral tissues was only found in pancreas for *LAT1* and *MCT10* and in feather buds for *LAT1* and *MCT8* (34). qRT-PCR data are only available for *OATP1C1* and *MCT8* expression in brain, showing a strong decrease in *OATP1C1* mRNA in telencephalon and diencephalon from E4 toward E10 while its expression in mesencephalon and rhombencephalon was stable and low. In contrast, *MCT8* expression gradually increased in all brain regions within the same time frame (19).

To have an efficient regulator function, one typically expects the above mentioned players to react to changes in maternal TH availability in the yolk in some sort of feedback system. Injection of THs (T_4+T_3) into the yolk at E3 indeed resulted in clear changes in brain *OATP1C1*, *MCT8*, *DIO2*, *THRA*, and *THRB* expression 24 h later. Surprisingly, this was no longer the case following injection at E7 (19). Although the results at E4 indicate responsiveness of these regulators at early stages, not all changes fit with what one would expect from a typical negative feedback response. Several of them are in line with the normal ontogenetic pattern (19), so they could at least partially be the result of a TH-induced acceleration of development. This shows that although TH regulators respond to TH status at early stages of development, the negative feedback system is still immature, making early embryos extra vulnerable for inadequate maternal TH supply.

In contrast to the substantial amount of data available for chicken (brain), data on expression of TH regulators in early embryos of other precocial avian species are lacking. With regard to altricial species, some information is available for ring dove just before and around the stages where the thyroid gland becomes active (6). *In vitro* testing of hepatic T_4 to T_3 conversion in the perinatal and early posthatch period showed that considerable outer ring deiodinating activity (probably DIO1) was present in embryos shortly before hatching while inner ring deiodinating activity (probably DIO3) may be less important in nestling doves as compared to embryonic quail (6, 20). However, neither DIOs nor TH transporters had been cloned and fully characterized at the time of these studies. Therefore, the only conclusion that can be drawn from them is that altricial embryos also take up maternal THs from the yolk and are capable of TH activation and inactivation.

EFFECTS OF MATERNAL HYPER- AND HYPOTHYROIDISM ON EMBRYONIC DEVELOPMENT

Different methods have been used to alter maternal TH supply to chicken embryos. One way to increase TH availability in the yolk is to supplement laying hens with T_4 and/or T_3. Only a few studies followed this approach. Two papers report on a study with T_4 supplementation in broiler breeder hens. They showed an increase in plasma T_4 but not T_3 of embryos at E18 and at internal pipping (36, 37), but embryos were not studied in more detail. Some information on effects in early development

is available from a study where Japanese quail hens were dosed twice daily with 3x the daily thyroid gland secretion rate of T_4, resulting in an accelerated growth and differentiation of embryonic pelvic cartilage, shown by its increased weight and alkaline phosphatase activity at day 9 of the 16 days incubation period (12).

A more easy alternative to maternal supplementation is to inject T_4 and/or T_3 directly into the yolk of fertilized eggs, allowing to control precisely the administered dose and time of injection. Injecting 1 μg T_4 + 0.5 μg T_3 into the yolk of chicken eggs at different stages (between E3-E11) always resulted in increased levels of both hormones in embryonic tissues 24 h later [(19) and Van Herck and Darras, unpublished results]. As mentioned before, this induced changes in the expression of TH regulator genes in embryonic brain at early stages (19). We took the same approach some years ago, injecting 0.5 μg T_3 at E3 or E7, to analyze the brain transcriptome 48 h later using the chicken 44K microarray from Agilent (38). Statistical analysis revealed 187 differentially expressed genes at E5 and 420 differentially expressed genes at E9 (Van Herck and Darras, unpublished results). Next to gene ontology analysis, gene network analysis was performed with Ingenuity Pathway Analysis using the Genbank identities of the corresponding human proteins. The top gene interaction network identified at E5 (25 genes) was "Developmental disorders, Endocrine system disorders, Neurological disease" and at E9 (26 genes) "Cellular development, Hematological system development and function, Hematopoiesis." Although these results are preliminary and a larger study is needed for full analysis (nowadays rather by RNA sequencing), they clearly prove that increasing "maternal" TH deposit influences biological processes during early brain development.

Injection of THs at the start of incubation has also been done in turkey, another precocial species, resulting in decreased hatchability (39). This differs from two recent studies in altricial species, showing enhanced embryonic development and hatching success in rock pigeon but not in great tit (40, 41). Unfortunately, none of these studies provided data on earlier embryonic stages.

In relation to TH deficiency, injection of goitrogens such as 2-mercapto-1-methylimidazole (MMI), 6-propyl-2-thiouracil (PTU) or ammonium perchlorate (AP) into the egg can block the embryonic thyroid gland and decrease TH availability, but only at stages where the thyroid gland starts contributing to circulating TH levels. Blocking conversion of maternal T_4 into T_3 at earlier stages by injecting PTU or iopanoic acid is also not an option since the high concentrations needed to efficiently block DIO1/DIO2 activity *in vivo* are toxic for the embryo (own observations). Decreasing maternal TH availability throughout development can only be achieved by rendering laying hens hypothyroid, which is typically done by addition of goitrogens to their food or drinking water. Finding the right dose can be a challenge since too mild maternal hypothyroidism does not sufficiently decrease yolk TH content while too severe hypothyroidism results in a reduction or even complete stop of egg laying as found in both quail and chicken [(12, 42) and own unpublished results].

Addition of 0.03% of MMI in drinking water of broiler breeder hens reduced the number of eggs with 70% by week 8. Yolk T_4 and T_3 content of eggs collected between week 10 and 16 were reduced with 70 and 50% respectively. Overall egg quality (egg weight, crude energy content, crude protein content and crude lipid content) was unaffected (8), precluding non-specific effects on embryonic development caused by nutrient deficiency. Morphological scoring of the embryos at E4, E6, and E8 according to the Hamburger and Hamilton stages (43) suggested there was no overall delay in development but strikingly, none of the embryos from the MMI-treated hens hatched, even when incubated up to 24 days (own unpublished results). This latter observation corresponds to what was found for embryos from broiler breeder hens treated with 0.01% PTU in drinking water (36). Also in Japanese quail hens treated with AP, none of the embryos from the high treatment dose (0.4% AP) hatched completely, while embryos from the low dose (0.2% AP) hatched 1 or 2 days late (42). However, we cannot attribute these effects exclusively to a lower maternal TH deposit. Goitrogens are transferred into the egg and taken up by the embryo as shown for instance for MMI (8). One should therefore keep in mind that goitrogens of maternal origin may at least partially inhibit embryonic thyroid gland functioning at later stages. In addition, they can have some direct adverse effects on development, even in early embryos, due to non-TH-related cytotoxicity.

Plasma T_4 and T_3 concentrations at E18 and at internal pipping were found to be lower in embryos from PTU-treated broiler breeder hens (36). E14 embryos from AP-treated Japanese quail hens were reported to have decreased body weight as well as decreased thyroidal T_4 and T_3 content. Expression of *DIO2* in these embryos was increased in liver but not in brain while expression of another TH-responsive gene, *RC3*/neurogranin, in brain was also unaffected (42). As the data in both studies are from rather late stages of embryonic development, both effects on maternal TH deposit and on embryonic thyroid gland activity may contribute to the observed changes. Sampling of brain and peripheral tissues from embryos of MMI-treated broiler breeder hens at E6, E14, and E18 allowed to show that severe maternal hypothyroidism lowered T_4 and/or T_3 levels both prior to and after the start of embryonic thyroid gland functioning. The decrease was in general more pronounced for T_3 than T_4, especially in brain (8). This led us to perform also a prospective microarray analysis on extracts of E4 and E8 brain (telencephalon) of embryos from MMI-treated hens. Again, a larger study with more samples is needed to allow detailed analysis, but we identified many differentially expressed genes, both at E4 and E8. Interestingly, only part of the affected genes were identical at the two stages, hinting at the stage-specific effect of THs on brain development (31).

As adequate lowering of TH availability in the early embryo via maternal hypothyroidism is quite labor intensive, including the maintenance of large stocks of goitrogen-treated laying hens, there is a need for alternative methods applicable directly on normal fertilized eggs. One possibility would be to block TH signaling by exposing the embryo to a specific THR antagonist like NH-3 (44). Such an approach has for instance been used successfully to show the severe impact of blocking maternal TH action on neural crest cell migration in early *Xenopus* embryos (45). However, NH-3 can also have some agonistic activities at higher concentrations (46) so it is important to find the right dose

for *in vivo* treatment of the species studied. Moreover, in contrast to amphibian eggs, which can take up NH-3 continuously from the surrounding water/medium, the barrier of the avian egg shell implies the need for (probably repeated) injection of the compound. Nevertheless, it would be interesting to test the usefulness of NH-3 or other THR modulators in avian eggs in more detail.

EFFECTS OF LOCAL KNOCKDOWN OF MCT8 ON NEURODEVELOPMENT

In recent years our research group opted for an alternative approach, focusing on the TH transporter MCT8. As THs are known to be extremely important for neurodevelopment in all vertebrates and many important steps in chicken central nervous system (CNS) development occur prior to the start of embryonic thyroid gland activity, we chose this target to locally silence *MCT8* gene expression via RNA interference (RNAi) technology. Knockdown of this highly efficient TH transporter that is widely expressed in early chicken CNS (28, 47) allows to reduce cellular uptake of maternal THs and study the consequences for processes such as precursor cell proliferation, migration and differentiation. The MCT8-RNAi vector was generated by cloning synthetic miRNA hairpins within the miRNA operon expression cassette of an pRFPRNAiA vector designed for use in chicken (48, 49) and was transfected into embryonic CNS by electroporation. Changing the timing and site of injection allowed to target specific precursor cell populations that could subsequently be identified by expression of red fluorescent protein (RFP). The fact that knockdown of MCT8 indeed reduced TH signaling in transfected cells (50) and that some of the observed defects could be rescued by supplementation with 3,5,3′-triiodothyroacetic acid (TRIAC), a non-MCT8-dependent TH analog (49), convincingly demonstrated that maternal THs are playing a major role in early CNS development.

Electroporation of the MCT8-RNAi vector into the cerebellar anlage at E3 allowed to knock down MCT8 predominantly in Purkinje cell (PC) precursors. This resulted in a strong decrease in the proportion of LIM homeobox domain transcription factor 1/5 (LHX1/5)-positive cells in the MCT8-RNAi-transfected cell population at E6 compared to similarly treated controls transfected with empty vector (49). This decrease in LHX1/5 protein was accompanied by a decrease in expression of the TH-responsive nuclear receptor retinoic acid receptor-related orphan receptor alpha (*RORα*). As LHX1, LHX5, and RORα are all very important for early PC differentiation and dendritogenesis (51, 52), these observations were in line with the impaired further development of MCT8-RNAi-transfected PCs, showing a significantly smaller and less complex dendritic tree at E18 (49) (**Figure 1C**). Importantly, MCT8 deficiency in PCs also induced non-autonomous effects, since it led to reduced granule cell precursor proliferation as shown by reduced incorporation of the proliferation marker 5-ethynyl-2′-deoxyuridine (EdU) in the external germinal layer at E10, and reduced/delayed migration of differentiating granule cells from the external germinal layer to the internal granular layer observed at E18 (49).

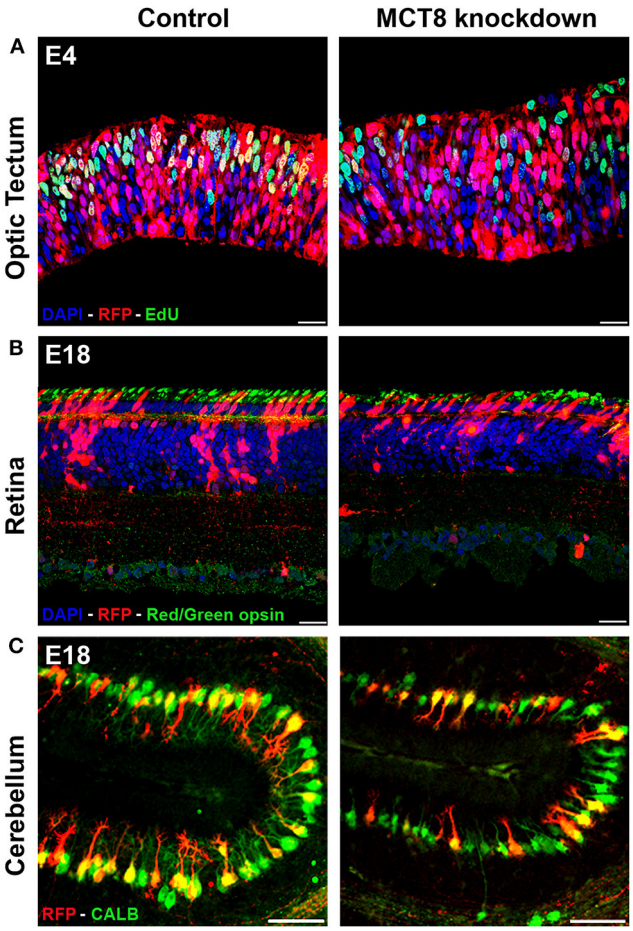

FIGURE 1 | Impact of TH deficiency observed at early and later stages of embryonic chicken CNS development. **(A)** Electroporation of empty vector (control) or MCT8-RNAi vector in the optic tectum at E3 followed by EdU pulse-labeling 1 h before sampling at E4. The strong reduction in the number of proliferating (S phase) transfected cells (yellow) in the knockdown condition illustrates one of the early effects of TH deficiency on CNS development. **(B)** Electroporation of empty vector (control) or MCT8-RNAi vector in the retina at E4 followed by IHC staining for red/green opsin at E18. The lower amount of red/green expressing cones in the mature retina in the knockdown condition at E18 is the combined result of a reduced retinal progenitor cell proliferation and a shift in commitment toward short wavelength sensitive cones at the expense of long/medium wavelength sensitive cones occurring at earlier stages. The picture also shows a reduced thickness of the retina and a disorganization of the sublaminae in the inner plexiform layer in the knockdown condition. **(C)** Electroporation of empty vector (control) or MCT8-RNAi vector in the cerebellar anlage at E3 followed by IHC staining for calbindin (CALB) at E18. The clear reduction in dendritic tree complexity of the Purkinje cells in the knockdown condition may be due to diminished expression of LHX1, LHX5, and RORα, observed at earlier stages. Scale bars represent 20 μm for optic tectum and retina and 100 μm for cerebellum.

Electroporation of the MCT8-RNAi vector in the developing optic tectum at E3 severely disrupted the organization of this layered brain structure. This started with reduced cell proliferation and a premature shift to asymmetric cell divisions in neural progenitors observed at E4, hampering sufficient expansion of the progenitor pool due to precocious neurogenesis (50) (**Figure 1A**). A second problem, shown by EdU birth-dating

experiments between E4 and E12, was impaired migration of both early-born and late-born neuroblasts. This might have been due to the reduced expression of the protein reelin encoded by the TH-responsive gene *RELN* as well as the disorganization of radial glial cell fibers observed at E6 (50). The result of MCT8 deficiency in the optic tectum at E12, a stage where the formation of the different layers in the optic tectum is normally completed (53), was a reduction of optic tectum thickness together with a lower total cell number. This could be linked to the very strong reduction in the multiplication of transfected cells in the MCT8-RNAi condition compared to control-transfected embryos in all different layers of this brain structure (50). In this study too, indications were found for non-autonomous effects, showing a reduction not only in MCT8-RNAi-transfected but also in untransfected GABAergic neurons, a cell type known to depend strongly on TH for its development (50, 54).

Lastly, we also studied the retina, another typically layered structure of the CNS. Knockdown of MCT8 by electroporation at E4 significantly reduced retinal precursor cell proliferation as shown at E6. This resulted in cellular hypoplasia and a thinner retina at E18, where mainly photoreceptors and horizontal cells were lost (55). Although differentiation into retinal ganglion cells and amacrine cells was initially delayed, analysis of the E18 retina showed that the partial loss of some cell types was predominantly due to reduced precursor cell proliferation rather than subsequent differentiation (55). A striking effect of MCT8 deficiency observed at E18, a stage where formation and differentiation of photoreceptors is normally completed (56), was the relative increase in short wavelength-sensitive (UV/blue) cones at the expense of medium/long wavelength-sensitive (red/green) cones (**Figure 1B**), which is in line with results obtained following deficient TH signaling in murine retina (55, 57, 58). As proliferation of immature photoreceptors occurs predominantly around E6-E8 while opsin expression starts around E14 (56), it can be concluded that the reduction in photoreceptors is the result of a local lack of maternal THs while the shift in cone photoreceptor subtype may be the result of reduced local availability of THs of both maternal and embryonic origin.

Taking advantage of the fact that transfected cells expressed RFP, we also performed fluorescence activated cell sorting (FACS) on cell suspensions of pools of E6 retinas transfected with either MCT8-RNAi or empty vector at E4. RNA isolation of the RFP-positive cell fractions was followed by quantification of expression of a small selection of genes by qRT-PCR. Expression of *THRA* and *THRB2* were respectively 4- and 3-fold lower in MCT8-RNAi transfected cells compared to controls, in line with what is expected in TH-deficient cells. In contrast, expression of *OTX2*, encoding a transcription factor stimulating retinal precursor cells to commit to the photoreceptor cell lineage (59), was 4-fold increased (Vancamp, Houbrechts and Darras, unpublished results). This argues against the possibility that the decreased amount of photoreceptors observed at E18 (see above) was due to a decreased commitment of precursor cells to photoreceptors. Unfortunately, the limited amount of material available did not allow a more in depth analysis, but this approach is certainly worthwhile pursuing in the future.

CONSEQUENCES OF VARIATION IN MATERNAL TH AVAILABILITY ON POSTHATCH LIFE

Based on the results from studies on chicken embryos prior to the start of embryonic TH production, we now know for sure that a clear reduction in (local) maternal TH availability has strong detrimental effects on early development, while too high levels also have a negative impact (5, 16). Importantly, the studies with MCT8 knockdown also allowed to identify some of the mechanisms involved. As cell proliferation, migration, and differentiation are restricted in time, differing from one tissue/cell population to another, it is clear that many of these defects cannot be corrected at later stages of development when the lack of maternal TH supply may (or may not) be compensated by increased embryonic TH contribution. For example, PCs are the sole output neurons of the cerebellum and are involved in coordinating movement, posture and balance in real-time, but also in long-term motor learning (60). A disrupted cerebellar circuit due to restricted PC arborization and reduced/impaired synaptogenesis with other cerebellar cell types may therefore cause cerebellar ataxia, balance problems and disturbed locomotion (61). Similarly, the defects observed in the chicken retina and in the optic tectum, where visual input is received, processed, and projected to higher brain areas, are likely

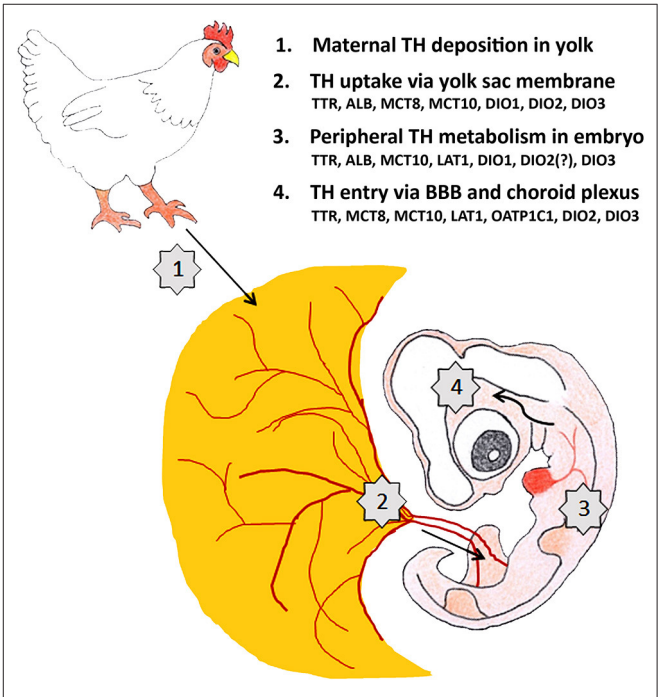

FIGURE 2 | Maternal TH supply to the developing brain of a 4-day-old chicken embryo is regulated at 4 different levels. The factors controlling TH transport and metabolism (TH distributor proteins, TH transporters, deiodinases) shown to be present at E4 at the different levels are mentioned. ALB: albumin, BBB: blood-brain-barrier, DIO1-3: deiodinase 1-3, LAT1: L-type amino acid transporter 1, MCT8-10: monocarboxylate transporter 8-10, OATP1C1: organic anion transporting protein 1C1, TTR: transthyretin.

to have implications for posthatch visual function, including for instance luminance detection and color perception (62). Birds are known to have excellent visual abilities (63) and the connection between the retina and the oculomotor cerebellum via the optic tectum is vital for the control of avian flight (64). The missing link to prove whether the retinal defects observed at E18 have an impact on later life are behavioral studies on posthatch chicks following embryonic MCT8 knockdown. Unfortunately, electroporation in early embryos is a rather invasive technique and so far no hatchlings (control or knockdown) were obtained. Moreover, knockdown by this technique is typically restricted in size, reaching only part of the targeted CNS structure. Knockdown of MCT8 via injection of a viral vector, which is less invasive and allows more widespread knockdown, or the use of CRISPR-Cas9 technology to generate (preferentially conditional) knockout chickens, would provide an important step forward. As mentioned before, injection of a specific THR antagonist would also be an interesting approach, even if repeated injections are be needed to ensure efficient blocking of TH action.

For precocial birds, a literature search only revealed three related papers on the consequences of increased maternal TH availability on posthatch performance of the offspring. They reported that maternal hyperthyroidism via T_4 supplementation in broiler breeder hens induced some changes in intestinal morphology of male chicks but it did not affect the feed:gain ratio nor the carcass weight at slaughter age (6 weeks) (37). The same chicks seemed to have an increased early adaptive immune response (65) and showed a lower incidence of cold-induced ascites, accompanied by lower hematocrit values compared to cold-exposed controls (36). In all three papers the authors stated that the causal mechanisms remained to be elucidated. The study on the effect of maternal hyperthyroidism in Japanese quail mentioned earlier was not continued until posthatch stages (12) and the same was true for the studies on early TH injection in chicken eggs performed in our own laboratory [(19) and Van Herck and Darras, unpublished results].

There are however two recent reports on the posthatch consequences of TH injection into the eggs of great tits and rock pigeons, altricial species in which the thyroid gland is still immature at hatching. In both studies, a combination of T_4+T_3 at slightly elevated physiological doses was injected at the start of incubation. Body weight was decreased in both male and female nestlings in rock pigeon but without concomitant decrease in tarsus length (40). In great tit, both body weight and wing length were decreased in female chicks but increased in male chicks (41). Finally, neither resting metabolic rate nor motor coordination behavior of great tit nestlings seemed to be affected by the treatment nor was the length of the nestling period (41). Since factors such as body weight at fledging are associated with later survival in many birds, both studies indicate that changing maternal TH availability may have an impact on offspring fitness (14). It would have been interesting to have also data on the embryos prior to hatching to find out if any causal links could be found with the changes observed posthatch.

CONCLUSION AND FUTURE PERSPECTIVES

The stock of maternal THs in avian egg yolk is substantial and is used by the embryo until hatching even if the embryonic thyroid gland becomes active. Maternal THs are therefore important throughout embryonic development not only in altricial but also in precocial birds. Multiple control levels collaborate to fine-tune the amount of T_3 that finally reaches the THRs in a given tissue (**Figure 2**). Multiple studies have shown that changes in maternal TH supply have an impact on avian embryonic development while some recent studies point to long lasting effects on posthatch performance and fitness. The challenge for the future is to better understand the link between both observations. From a physiological point of view the focus is on understanding the molecular mechanisms responsible for the observed changes in embryonic development and to link them to changes in posthatch behavior. This can be further investigated in standard laboratory conditions in precocial model species such as chicken and quail, although it is worthwhile to include also comparison with an altricial model species such as laboratory-raised zebra finch. From an ecological point of view it is important to investigate in more detail to what extent environmental factors such as temperature, food, stress, and unfortunately also endocrine disruptors, influence maternal TH deposit in the egg and to find out how this is linked with posthatch fitness of the offspring in changing environmental conditions in both altricial and precocial species.

We should also keep in mind that THs are not the only maternal hormones deposited in avian egg yolk. Other hormones such as sex steroids and corticosteroids are also present and are known to influence embryonic development (66). Moreover, apart from having effects of their own, THs, sex steroids and corticosteroids interact with each other to control development in synergistic as well as antagonistic ways (67, 68). Finally, it is also important to place the results obtained in birds in a broader comparative context. Although it has been debated for quite some time, it is now accepted that THs are needed for early stages of development in all vertebrates. Maternal THs are the only source available for the early embryo, both in mammals and non-mammalian vertebrates, and insights obtained from studies in birds are therefore widely applicable, both in a biological and medical context.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Tail Resorption During Metamorphosis in *Xenopus* Tadpoles

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Tail resorption in anuran tadpoles is one of the most physically and physiologically notable phenomena in developmental biology. A tail that is over twice as long as the tadpole trunk is absorbed within several days, while concurrently the tadpole's locomotive function is continuously managed during the transition of the driving force from the tail to hindlimbs. Elaborate regulation is necessary to accomplish this locomotive switch. Tadpole's hindlimbs must develop from the limb-bud size to the mature size and the nervous system must be arranged to control movement before the tail is degenerated. The order of the development and growth of hindlimbs and the regression of the tail are regulated by the increasing levels of thyroid hormones (THs), the intracellular metabolism of THs, the expression levels of TH receptors, the expression of several effector genes, and other factors that can modulate TH signaling. The tail degeneration that is induced by the TH surge occurs through two mechanisms, direct TH-responsive cell death (suicide) and cell death caused by the degradation of the extracellular matrix and a loss of cellular anchorage (murder). These pathways lead to the collapse of the notochord, the contraction of surviving slow muscles, and, ultimately, the loss of the tail. In this review, I focus on the differential TH sensitivity of the tail and hindlimbs and the mechanism of tail resorption during *Xenopus* metamorphosis.

Keywords: tail resorption, *Xenopus*, metamorphosis, amphibian, thyroid hormone, thyroid hormone receptor, deiodinase, extracellular matrix

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INTRODUCTION

Metamorphosis occurs in most animal phyla and accompanies the concomitant morphological, ecological, and physiological changes. In the case of marine invertebrates, a larva acts mostly as a drifting or free-swimming creature in the ocean and thereby extends its habitat distribution and seeks out the optimal location for its survival, growth, and propagation, whereas the adult becomes a sessile animal or a burrower in the sea bottom after metamorphosis (1). Early Cambrian fossil records show that planktotrophic larvae metamorphosed into filter-feeding sedentary juveniles (2), which demonstrates the ancient origin and importance of metamorphosis in evolution.

An amphibian tadpole undergoes thyroid hormone (TH)-dependent metamorphosis from an aquatic to a terrestrial animal (3). TH-dependent metamorphosis is also reported in sea urchin (4), amphioxus (5), and flounder (6) to alter the lifestyle from that of a planktotrophic or free-swimming larva to that of a sessile or benthic adult. The developmental profile of gene expression in the rodent brain during the first 3 postnatal weeks resembles the corresponding profile in the *Xenopus* brain during the metamorphosis climax, which strongly suggests that the mammalian brain undergoes TH-dependent metamorphosis to adapt to the open-air environment after aquatic (amniotic) life, similar to anuran metamorphosis (7). In amniotes, TH-dependent metamorphosis might

have evolved for adapting to environmental change during the rapid transition from amniotic to terrestrial life (8).

Anuran metamorphosis is characterized by the resorption of larva-specific organs (a tail and gills), the development of adult-specific organs (limbs), and the transformation of organs (brain, liver, intestine, pancreas, skin, etc.) from larval to adult type. Tail resorption is a particularly drastic change that occurs during the climax of anuran metamorphosis (9), and the phenomenon has attracted much scientific attention since the nineteenth century. A historical overview of the anatomical, physiological, and biochemical studies on tail resorption is presented elsewhere (10).

Tadpoles prepare for the smooth locomotive transition from fish-like swimming using a tail to frog-like swimming using hindlimbs by developing hindlimbs and arranging the nervous system to enable their powerful and accurately controlled movement (11), while the tail concurrently works as a constant driving force till the regression starts. This preparation is implemented by regulating blood TH levels. Whereas, hindlimbs can respond to low levels of THs by developing and growing, a tail cannot. Conversely, the tail responds to high levels of THs during the metamorphosis climax and initiates the death of muscle cells and the degeneration of the notochord, which lead to tail resorption. In this review, I introduce the models proposed to explain the differential TH sensitivity of the tail and hindlimbs and the mechanism of tail resorption.

DIFFERENTIAL TH SENSITIVITY OF THE TAIL AND HINDLIMBS

Developmental Regulation of THs and TH Receptors (TRs)

TH binds to a heterodimeric receptor—composed of TR and 9-cis retinoic acid receptor—that inhibits and activates transcription from promoters containing the TH-response element (TRE) in the absence and presence of TH, respectively (12, 13). Vertebrates express two TR subtypes, TRα and TRβ, and hindlimb growth is inhibited by unliganded TRα (14–16) before endogenous TH secretion from the thyroid gland begins, i.e., before Nieuwkoop and Faber (NF) stage 54 of *Xenopus laevis* (17). The plasma level of thyroxine (T4), a low-activity TH precursor, slowly increases from NF stage 54 (0.66 nM) to NF stage 62 (9.7 nM), and this is accompanied by hindlimb growth (**Figure 1A**). Conversely, 3,5,3′-triiodothyronine (T3), a highly active TH derived from T4, surges abruptly at NF stage 58, the beginning of the metamorphosis climax, and peaks at NF stages 61–62 (7.9 nM), when the tail starts shortening. T3 shows 4 to 7-fold-higher binding affinity for TR than T4 (19, 20). The tail starts regressing only after the hindlimbs have grown adequately and move cooperatively to enable swimming, which indicates that hindlimbs can respond to substantially lower levels of THs than the tail.

As THs circulate in the bloodstream throughout the body and all organs and tissues are exposed to the same concentrations of THs, when and how each organ or tissue orchestrates the induced transformation depends on their sensitivity and responsivity to these metamorphosis inducers, and this is expected to be reflected by the developmental expression of the genes involved in TH signaling in the transforming organs. The expression level of *TRα* mRNA in the hindlimbs of *X. laevis* is high at NF stage 54 and decreases up to NF stage 62, whereas *TRβ* mRNA is expressed at very low levels throughout metamorphosis (**Figure 1B**). In contrast, *TRα* mRNA level in the tail rises gradually from NF stage 54 to 62 and then decreases, whereas *TRβ* mRNA is expressed at very low levels and increases starting from NF stage 62 (18, 21–23) (**Figure 1C**). Therefore, the decreasing *TRα* mRNA expression in the hindlimbs and the increasing *TRα* mRNA expression in the tail intersect during development around NF stage 58 when the level of active T3 rises sharply, which suggests that the TH sensitivity of organs depends on the TRα expression level. This is supported by a report that the TH sensitivity of tail tips increases steadily with development from NF stage 38–58 in organ culture, as evidenced by a shortening lag period before the onset of regression and an increased rate of regression (24).

Enhancement of TH Sensitivity by TR Overexpression in the Tail

Somatic gene transfer performed using electroporation enables the introduction of exogenous DNA into many tail muscle cells. Moreover, treatment with an inhibitor of TH synthesis, methimazole, stops tadpole development and hindlimb growth at NF stage 54. If methimazole-treated tadpoles are immersed in low-concentration (0.3–1 nM) T4 and T3 solutions, hindlimb buds can grow within several days in a TH-dose-dependent manner, whereas the tail cannot regress. However, when a *TR*-expression construct is introduced (together with a reporter gene) into the tail muscle cells of methimazole-treated tadpoles, tail cells respond to low levels of THs and die over a time course similar to that of hindlimb growth; therefore, *TR* mRNA overexpression confers responsiveness to low levels of T3 and T4 on tail muscle cells (18). The disappearance of tail muscle cells induced by T4 treatment is delayed compared with the disappearance after T3 treatment, which implies that T4 is converted to the active form, T3, by the induction of type 2 iodothyronine deiodinase (D2). Furthermore, the D2 inhibitor iopanoic acid impairs the death of *TR*-overexpressing tail muscle cells as well as the growth of hindlimbs at a low level of T4 but not T3, which supports a role for D2 in the response to low T4 levels (18).

Developmental Expression of the Gene *D2* and Its Regulation by THs

D2 activity and mRNA levels are the highest in a given tissue at the time of the tissue's major transformation (25). *D2* mRNA is expressed at a high level in growing hindlimbs at NF stage 54 and increases up to NF stage 60, whereas the mRNA is present at a low level in the tail and is abruptly elevated to an extremely high level at NF stage 62, when tail regression starts (18, 26) (**Figures 1B,C**). The *D2* mRNA level in hindlimbs is reduced to one-fourth of the control level after 1 month of methimazole treatment of NF stage 54 tadpoles, which means that only a small fraction of the *D2* mRNA in untreated tadpoles is expressed in hindlimbs

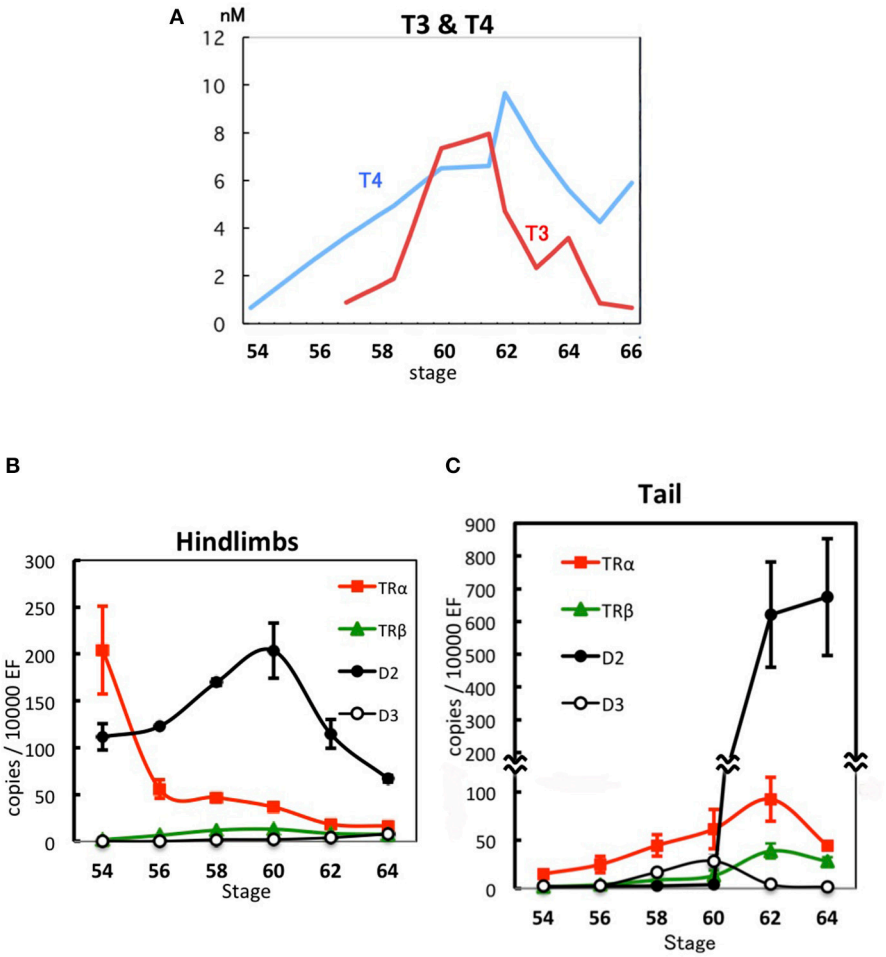

FIGURE 1 | Developmental regulation of THs and gene expression during *X. laevis* metamorphosis. **(A)** TH levels in plasma (17). **(B)** Expression levels of *TRα*, *TRβ*, *D2*, and *D3* mRNAs in hindlimbs (18). **(C)** Expression levels of *TRα*, *TRβ*, *D2*, and *D3* mRNAs in the tadpole tail (18). Data are shown as means ± SE. *EF, elongation factor 1α*.

in the absence of THs and the major fraction is expressed in a TH-dependent manner. Treatment with a low concentration of T4 induces *D2* mRNA in 4 days in the *TRα*-overexpressing tail muscle cells of methimazole-treated tadpoles, and the levels of *D2* mRNA and *TRα* mRNA show a close correlation (18).

D2 mRNA is induced in 8 h by T3 in the tail myoblastic cell line XLT-15, and the induction is only partially abrogated by the protein synthesis inhibitor cycloheximide, which indicates that *D2* is a direct TH-response gene (18). This is supported by the presence of a functional TRE at similar positions within 600 bp of a highly conserved region in *X. laevis* D2.L and D2.S genes and *Xenopus tropicalis* D2 gene; the TREs are located 1–2 kb upstream of the TATA box. The *D2* TREs exhibit lower affinity (i.e., they are weak TREs) for TR *in vitro* and lower homology to the TRE consensus sequence than the TRE of another direct TH-response gene, *TRβ*. Moreover, *D2* mRNA expression is activated 4-fold more by a low level of T3 in *TR*-transfected cultured cells than in vector-transfected cells, whereas the *TRβ* mRNA level shows no difference between *TR*- and vector-transfected cells. A larger amount of the *TR*-expression construct is required for the *D2* TRE to mediate sufficient TH signaling in the oocyte system than for *TRβ* TRE-mediated signaling (18).

Low Sensitivity to T4 in the Tail of Young Tadpoles

Low levels of *TR* mRNAs are produced in the tadpole tail at NF stage 54. In vector-transfected tadpoles treated with methimazole for several days, a small fraction of the transfected tail muscle cells disappears even in the absence of TH, compared with TR-overexpressing muscle cells in the same condition. A similar cell-death tendency of vector-transfected cells is observed in the presence of 0.3–1 nM T4 (18). If a low level of endogenous TR binds to the weak TREs of TH-response genes, including *D2* and several effector genes (27, 28), TR should repress the expression of TH-response genes causing cell death in the absence of T4, as seen in TR-overexpressing muscle cells. The similar death pattern exhibited by vector-transfected tail muscle cells in the presence of 0, 0.3, and 1 nM T4 could be ascribed to the expression

of endogenous TR at a level that is too low to form a stable complex with the weak TREs of TH-response genes (*D2* and other effector genes), which then allows the leaky expression of the genes to induce the death of a small fraction of cells. This view suggests that endogenous unliganded TR cannot completely inhibit the metamorphic change of tail cells. The expression of endogenous TR at insufficient levels to inhibit TH-target genes in young tadpoles may be similar to the inadequate TR levels to elicit the maximal response to TH. The latter is supported by the report that a desert frog, which features the shortest larval period, expresses elevated levels of *TRα* mRNA throughout development and exhibits accelerated expression kinetics of TH-response genes under exogenous TH treatment. Furthermore, overexpression of *TRα* increases the rate of tail muscle cell death in response to TH (29). The death of a small fraction of tail muscle cells should be observed in normal NF stage 54 tadpoles (**Figure 2A**); the dead cells might be replenished through the cell division of myoblasts during development.

In the hindlimbs of *TRα*-knockout tadpoles, TH-response effector genes are no longer repressed by unliganded TR due to the complete loss of TRα and their expression induces precocious development before NF stage 54 (14–16). The hindlimbs show reduced responsiveness to 10 nM T3 at NF stages 53–55 (30), suggesting a low sensitivity to TH. The growth rate of hindlimbs in *TRα*-knockout tadpoles is higher at NF stages 50–54 (in the absence of THs), similar to NF stages 54–56, and lower thereafter than the rates in wild-type and *TRβ*-knockout hindlimbs, which catch up to the *TRα*-knockout hindlimbs in size at NF stage 58 (16). Wild-type and *TRβ*-knockout hindlimbs show no difference in morphology or size, and thus TRβ plays only a minor role in the growth and development of hindlimbs.

When a *TR*-expression construct is introduced into the tail muscle cells of methimazole-treated tadpoles, the overexpressed TR binds to the weak TREs of *D2* and other TH-response effector genes and represses their expression because of the absence of THs (**Figure 2B**). The slow growth of tail muscle cells that is obscured by cell death becomes apparent following the complete inhibition of cell death (18). Given that *TRα* mRNA is abundant in the hindlimbs of wild-type tadpoles before NF stage 54, inhibition of TH-response effector genes by TR in the absence of THs constrains growth and development.

High Sensitivity to T4 in TR-Overexpressing Tail Muscle Cells or Hindlimbs of Young Tadpoles

The organs that express TR abundantly can respond to a low level of T3 as a result of the stable interaction between liganded TR and the weak TREs of effector genes in the absence of *D2* mRNA induction and TH activation; conversely, the organs that express TR at low levels cannot drive the response to a low level of T3, because the high TR expression level is required to increase the occupancy at weak TREs, independent of T3 concentration.

A positive-feedback model involving the upregulation of *D2* and the conversion of T4 to T3 by D2 enzymatic activity in the presence of low levels of T4 has been proposed to explain the organ sensitivity to T4 (18) (**Figure 2C**). Abundant TR forms a stable complex with the weak TREs of TH-response genes, including *D2* and effector genes in the hindlimbs of NF stage 54 tadpoles and TR-overexpressing tail muscle cells of methimazole-treated tadpoles, binds to a low level of T4 (0.3–1 nM) transiently and weakly, and stimulates the *D2* promoter to induce the production of a small amount of the D2 enzyme. Following this subtle induction of D2 production, T4 is converted to T3 by the enzyme, and the generated T3 then binds to the TR recruited on the TREs of *D2* and other effector genes more robustly and efficiently and activates *D2* transcription leading to further conversion of T4. This process might be promoted by the stimulated expression of *TRβ* that has a high affinity TRE, although *TRβ* mRNA is upregulated weakly by 1 nM T4 in 4 days in TR-overexpressing tail muscle cells (18). This positive-feedback loop operating under abundant TR expression amplifies TH signaling through *D2* stimulation and TH activation to enable a response to a low level of T4 earlier than in other organs, and this then establishes the metamorphic changes such as limb growth within several days. During the positive-feedback process, TH-response effector genes harboring weak TREs are also activated to drive the growth in hindlimbs or the cell death in the tail.

Developmental Gene Expression and Function of Type 3 Iodothyronine Deiodinase (D3)

Differential sensitivity of organs and tissues to THs is regulated by multiple molecular mechanisms (31). Whereas D2 converts T4 to T3 and thereby leads to TH activation, D3 inactivates T3 and T4. *D3* mRNA expression level and D3 activity in the tail are low at NF stage 54, increase from NF stage 58 to 60–61, and decrease thereafter (18, 32–34) (**Figure 1C**). D3 activity appears to inhibit the expression of TH-response effector genes before NF stage 61 by reducing the TH concentration in the tail (35). Their prominent and concurrent expression is induced at NF stage 62 after the downregulation of *D3* mRNA (28). The *D3* mRNA level is increased in Meckel's cartilage immediately before the end of its proliferation during the late metamorphosis climax, and, similarly, in late limb development. The *D3* mRNA level typically declines immediately before a tissue's metamorphic change, which enables the tissue to respond to THs, or increases immediately before the completion of a tissue's response to TH. Thus, D3 expression is involved in the elaborate regulation of the TH response of organs (36). Because tail tips can respond to lower levels of THs in organ cultures during developmental progression from NF stage 38–58 (24), D3 might reduce the TH responsivity of the tail at NF stages 59–61 (31).

Developmental Gene Expression and Function of TH Transporters and Cytosolic TH-Binding Proteins

MCT8, MCT10, OATP1c1, and LAT1 are TH transporters, and their mRNAs are upregulated when an organ undergoes TH-dependent metamorphic changes (37). However, *OATP1c1* mRNA is expressed at low levels in hindlimbs and the tail during metamorphosis (38). *LAT1* mRNA is increased at a

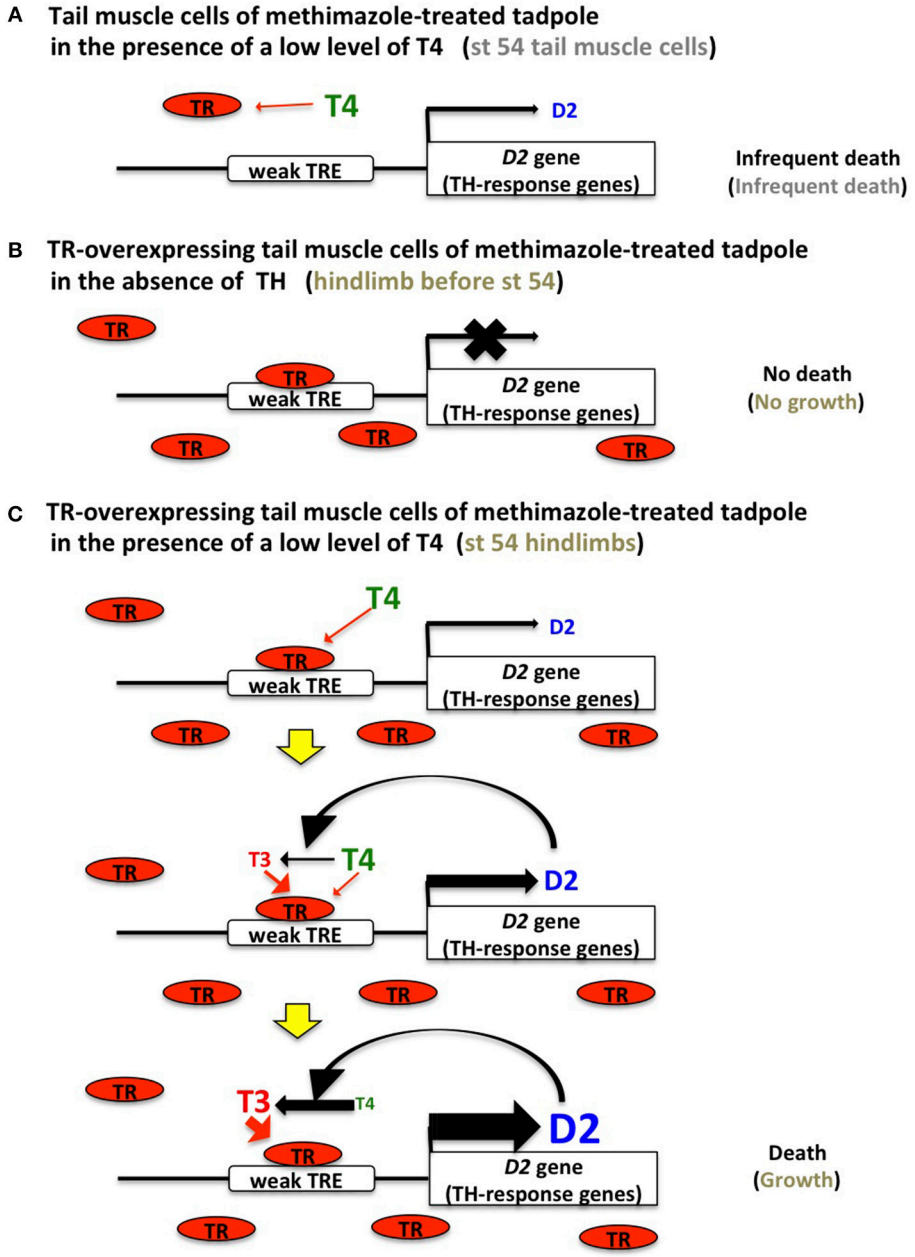

FIGURE 2 | TH sensitivity and response in tail muscle and hindlimb cells. **(A)** TR expression is too low to bind to the low-affinity TREs (weak TRE) of *D2* and TH-response effector genes in tail muscle cells of methimazole-treated tadpoles and stage 54 tadpoles, which results in a leaky expression of these genes and induces cell death infrequently. **(B)** Abundant and unliganded TR binds to TREs in TR-transfected tail muscle cells of methimazole-treated tadpoles and stage 54 tadpole hindlimbs and inhibits cell death and growth, respectively, by repressing the expression of TH-response genes. **(C)** Once a low level of T4 is recruited to the TR on the weak TRE of the *D2* gene, D2 protein is gradually synthesized and converts T4 to T3, which stimulates *D2* expression more efficiently. This positive feedback loop drives the expression of *D2* and TH-response genes and TH activation, leading to cell death in TR-overexpressing tail muscle cells of methimazole-treated tadpoles in the presence of a low T4 level and growth in stage 54 tadpole hindlimbs.

single stage of development in the hindlimbs and tail, NF stage 54 and stage 62, respectively (37). Intriguingly, *MCT8* mRNA expression in hindlimbs is high at NF stage 54, decreases from NF stage 58–60, and then remains at a low level. However, in the tail, the mRNA is expressed at NF stage 54 at a low level and then increases from NF stage 62–64, suggesting the mRNA level reflects the TH sensitivity of each organ. In humans, an *MCT8* mutation causes Allan-Herndon-Dudley syndrome, which is characterized by X-linked mental retardation and markedly elevated serum T3 (39, 40). Skin fibroblasts of patients with the syndrome show decreased T4 and T3 uptake, which indicates that MCT8 is a major TH transporter expressed in

dermal fibroblasts (41). However, *MCT8* overexpression fails to promote TH-induced death of tail muscle cells in response to 2 nM T3, whereas *LAT1* overexpression increases the rate of tail cell death (38).

A previous study analyzed the mRNA expression during metamorphosis of three cytosolic TH-binding proteins, aldehyde dehydrogenase 1, pyruvate kinase subtype M2, and μ-crystallin (CRYM) (38). *CRYM* mRNA expression declines from NF stage 54 to 66 in hindlimbs, but increases from NF stage 63 onward in the tail, thus exhibiting an expression pattern similar to that of *MCT8* mRNA. The affinity constant of T3 binding to CRYM is approximately 2 nM, and T3 concentration in tissues is reduced in *CRYM*-knockout mice without alternations of peripheral T3 action, suggesting that TH is retained inside cells by CRYM. Interestingly, a *CRYM* mutation has been reported in two families with hereditary deafness (42). Lastly, overexpression of *CRYM* or the pyruvate kinase subtype M2 gene, like *LAT1* overexpression, results in enhanced tail muscle cell death in the presence of 2 nM T3, and co-overexpression of *MCT8* and *CRYM* produces a synergistic effect on cell death (38).

MECHANISM OF TAIL RESORPTION

Murder Model

When tailfin explants are incubated in a TH-containing solution, collagenase activity and loss of tissue collagen are induced together with the progression of tailfin resorption (43). The regression program stops in the tail amputated from a tadpole that has been treated with 100 nM T3 for 48 h if a protein synthesis inhibitor is added within 24 h of TH pretreatment, but if the tadpole is treated with TH for >48 h, the regression continues even if TH is removed or the protein synthesis inhibitor is added. These findings suggest that the genes involved in tail regression are activated during the 2 days of TH treatment (44). In this study, roughly 20 TH-upregulated genes were isolated by employing a PCR-based subtractive-hybridization procedure using RNA isolated from the tails of NF stage 54 tadpoles treated with 100 nM T3 for 1–2 days (44). The mRNA levels of these genes increase developmentally in the tail during the normal metamorphosis climax, and the upregulated genes include not only a direct TH-response gene, *TRβ*, but also *collagenase 3* (*MMP13*), *stromelysin-3* (*MMP11*), and *fibroblast activation protein α* (*FAPα*) (33). MMP13 cleaves types I, II, and III collagen and gelatin (45) and belongs to the matrix metalloproteinase (MMP) family of enzymes that degrade extracellular matrix (ECM) proteins between cells (46). FAPα is a homodimeric integral-membrane gelatinase belonging to the serine-protease family. Subsequent work revealed that the regressing tail during the metamorphosis climax expresses several MMP genes, *MMP18* (*collagenase 4*) (47), *MMP2* (*gelatinase A*) (48), *MMP9TH* (27), and *MMP14* (*membrane-type 1 MMP*) (49), which are concomitantly upregulated in response to the TH surge at NF stage 62 when the tail begins to regress (28).

The proteolytic-enzyme mRNAs accumulate at high levels at NF stage 63 in the proliferative fibroblasts of the tail. These cells line and surround the notochord sheath or lie beneath the epidermal lamella and start invading the notochord or their neighboring epidermal collagen lamella, respectively, at late NF stage 63 and early NF stage 64. This fibroblast invasion is suggested to participate in notochord collapse and tail regression. The murder model is proposed based on these observations (31). The fibroblasts around the notochord and under the epidermal lamella start producing ECM-degrading proteases at NF stage 62 in response to the peak level of T3 and migrate to the epidermal lamella, notochord sheath, and the basal lamina between muscle cells. These cells secrete ECM-degrading enzymes that dissolve the basal lamina, and widespread ECM degradation results in the loss of cellular attachment to the ECM, elimination of anchorage, and death of muscle cells.

The tail muscle is mostly composed of fast muscle, whereas the peripheral muscle fibers are slow-muscle fibers. During ECM cleavage and digestion in the tail by MMPs at around NF stage 62, the bulk of fast muscle disappears preferentially. Subsequently, during notochord degeneration and collapse at NF stages 62–65, rapid tail shortening is driven by the contraction of four muscle cords comprising two dorsal and two ventral parallel rows of slow-muscle bundles that run along the tail's length (50).

Suicide Model

Muscle cells die in the resorbing tail during the metamorphosis climax and are fragmented into membrane-bounded muscle pieces, engulfed, and digested by macrophages, which is typical of apoptosis (51). To facilitate analysis of tail resorption, myoblastic cell lines were established from the *X. laevis* NF stage 57 tadpole tail. In these cells, apoptosis occurs in response to physiological TH concentrations, as indicated by positive TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) reaction and internucleosomal DNA cleavage. TH-triggered death of the myoblastic cells is not stimulated by the addition of conditioned medium collected from a cell line incubated with T3 for 2 days, which suggests that cell death is not mediated by a paracrine mechanism, such as a mechanism involving ECM-degrading enzymes that dismantle cell anchorage or secreted factors that induce apoptosis. These findings have engendered the suicide model, which posits that tail muscle cells respond cell-autonomously to TH by undergoing apoptosis, because the myoblastic cell line represents a homogenous population derived from a single cell of tail muscle and includes no fibroblasts (52).

TH induces *caspase 3* mRNA in one of the established cell lines, XLT-15, and the mRNA is also induced in the regressing tail during the metamorphosis climax. Moreover, the apoptosis of XLT-15 cells by TH is blocked by a Caspase 3 and 7 inhibitor (acetyl-Asp-Glu-Val-Asp-aldehyde). These results imply that cell death is triggered by the induction of *caspase 3* mRNA. However, *caspase 3* mRNA is not induced by TH in a subline of XLT-15, XLT-15-11, that also dies in response to TH, which indicates that the induction of *caspase 3* mRNA is not essential for cell death, although Caspase 3 might promote apoptosis and tail resorption. Furthermore, *caspase 1*, *2*, 6, *7*, *8*, *9*, and *10* are not upregulated

by TH in XLT-15-11 cells (53). The induction of these apoptosis-related genes is not necessary for the death of myoblastic cell lines, but the genes might functionally complement each other and coordinate with TH-induced apoptosis-promoting genes that are as yet unidentified. Although the pro-apoptotic genes *bax* and *bid* are suggested to be involved in tail resorption (54, 55), whether they are required for the death of tail cells remains to be determined.

Death Switch From Suicide to Suicide and Murder at the Beginning of Tail Regression

In response to TH, the myoblastic cell line XLT-15 transcribes the mRNAs of *MMP9TH* and *FAPα* (28). *MMP9TH* is one of the duplicated genes of *MMP9*. *MMP9TH* expression is strongly induced by THs, whereas *MMP9* expression is not (27). MMP9TH exhibits gelatin-degrading activity like MMP9. MMP9 cleaves native type IV collagen, which is the major structural component in the basal lamina that underlies all epithelial sheets and tubes and encircles single muscle cells. In NF stage 63 *TRβ*-knockout tadpoles, *MMP9TH* and *FAPα* expression levels are reduced to less than one-tenth in the posterior part of the tail, where almost all muscle flanks and the satellite cells between muscle cells disappear, compared with the corresponding levels in the tail of wild-type and *TRα*-knockout tadpoles at the same stage (16). These observations suggest that muscle cells and/or satellite cells synthesize *MMP9TH* and *FAPα* mRNAs in response to THs and thus dismantle the basal lamina surrounding individual muscle cells.

In a dominant-negative form of TR (DNTR), a mutation in the TH-binding domain compromises the TH-binding ability of the protein. DNTR binds to the TRE in the promoter of TH-response genes and represses their expression by associating with co-repressors, irrespective of the presence of TH (56, 57). Following co-transfection of a *DNTR*-expression construct and a reporter gene into XLT-15 cells, DNTR-overexpressing XLT-15 cells survive for 3 days in the presence of 10 nM T3, whereas half the vector-transfected control cells die. Given that expression of the exogenous reporter gene is detected in only a fraction of the cells, most of the cells respond to T3 and secrete MMP enzymes. Because DNTR overexpression cannot protect even *DNTR*-transfected cells against anchorage dissolution by ECM-degrading enzymes, DNTR inhibition of the death of the cultured cells indicates that DNTR blocks the TH signaling leading to cell-autonomous suicide, which agrees with the result of the experiment conducted using the conditioned medium mentioned above (58). The action of the ECM-degrading enzymes might be compromised by MMP inhibitors contained in the fetal calf serum included in culture medium (59).

A *DNTR*-expression construct has been introduced together with a reporter gene into muscle cells in the tail of live tadpoles to block TH signaling and analyze the effect of DNTR expression on cell death *in vivo*. According to the suicide model, DNTR overexpression represses the suicide process only in *DNTR*-transfected muscle cells and enables their survival in the presence of TH. Conversely, under the murder model, all *DNTR*-transfected cells and non-transfected cells are killed by the translated death-promoting proteins encoded by the TH-response genes that are induced in a majority of non-transfected muscle cells and fibroblasts. The blocking of TH signaling by DNTR overexpression almost completely inhibits muscle cell death until NF stage 61 (i.e., until immediately before tail regression), but does not protect *DNTR*-transfected cells after NF stage 62, when the genes for several ECM-degrading enzymes are upregulated drastically and concurrently and the tail starts regressing (28). Only 10% of *DNTR*-transfected cells survive after the metamorphosis climax, and some of the cells are retained even 3 weeks after metamorphosis, which indicates that DNTR overexpression inhibits cell death partially by attenuating cell death by suicide. These findings show that TH induces tail muscle cells to commit suicide before tail regression and to die through the suicide and murder mechanisms after the regression (58) (**Figure 3**).

Immunological Rejection Model and Its Reexamination

Xenopus laevis juveniles are reported to reject tail skin grafts from syngeneic tadpoles, but trunk skin grafts become increasingly more acceptable with the progression of metamorphosis of the donor tadpoles. This tadpole skin rejection starts with the recognition of the tadpole-specific skin proteins Ouro1 and Ouro2 as non-self-proteins. Precocious tail degeneration is elicited before the climax when *ouro1* and *ouro2* are overexpressed using a heat shock-inducible promoter, whereas tail resorption during spontaneous metamorphosis is delayed in response to antisense *ouro* RNA-induced knockdown of *ouro1* and *ouro2* using the same promoter, which results in the generation of tailed froglets (60). These data suggest that larval organs such as the tail are eliminated during the metamorphosis climax in the tadpole through the recognition of larva-specific proteins as non-self-proteins by the immune system.

The knockouts of *ouro1* and *ouro2* were generated using the genomic-editing technique in *X. tropicalis* to reexamine the immunological rejection model. While *ouro1*-knockout tadpoles express no Ouro1 and express Ouro2 at a very low level, *ouro2* knockouts express no Ouro2, and express Ouro1 at a barely detectable level. However, the *ouro1*- and *ouro2*-knockout tadpoles undergo normal metamorphosis without any morphological differences from wild-type tadpoles, and the knockouts do not retain a tail after metamorphosis (61).

Athymic frogs were created by modifying *Foxn1*, a gene whose mutation in mice results in congenital loss of the thymus and mature T cells, including helper and $CD8^+$ cytotoxic T cells, and in a severe combined immunodeficiency phenotype (62). Similarly, athymic frogs harbor no splenic $CD8^+$ T cells, which are necessary for a cytotoxic reaction, and these frogs can accept major histocompatibility-disparate skin grafts. However, the tadpole tail disappears

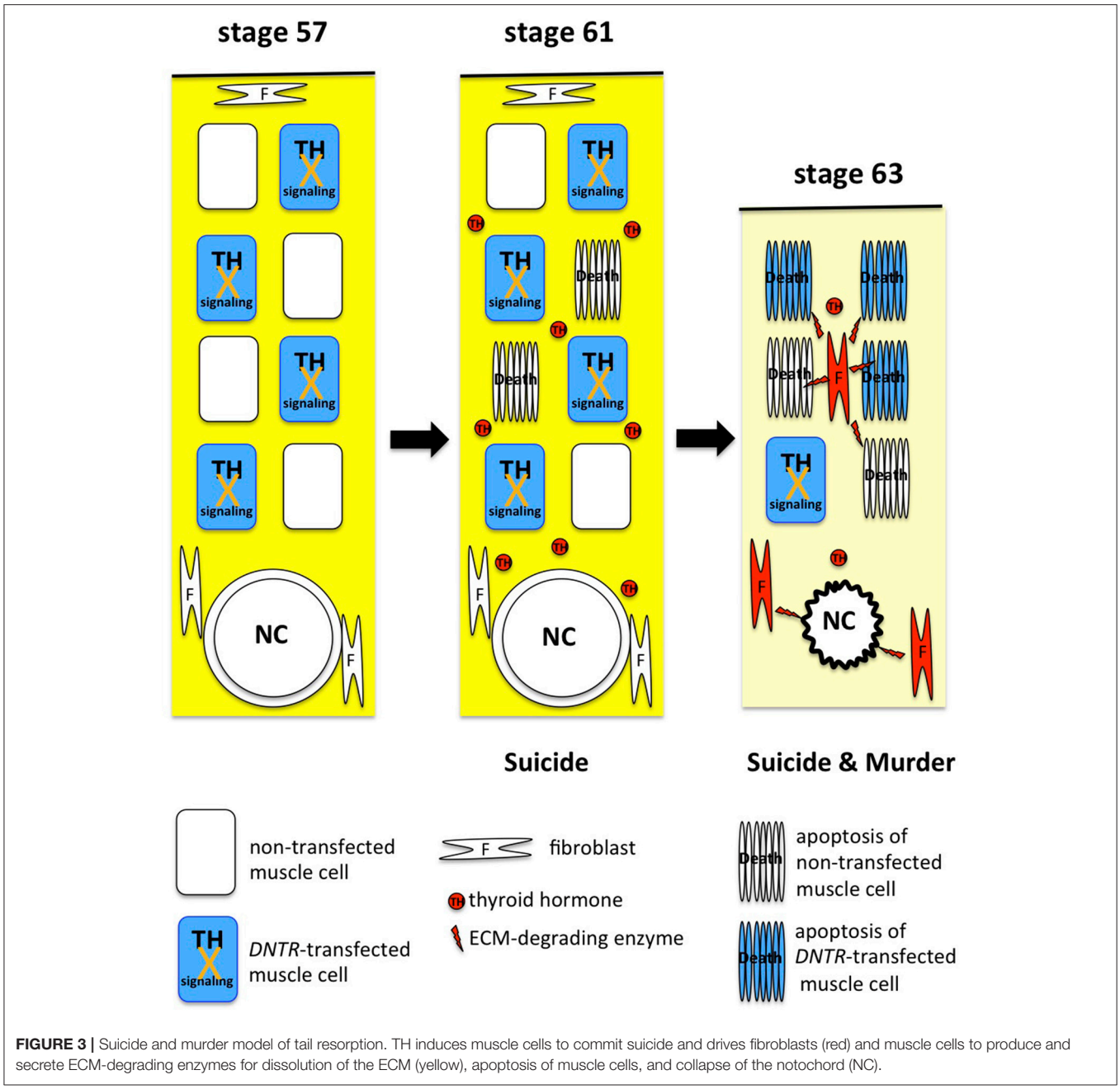

FIGURE 3 | Suicide and murder model of tail resorption. TH induces muscle cells to commit suicide and drives fibroblasts (red) and muscle cells to produce and secrete ECM-degrading enzymes for dissolution of the ECM (yellow), apoptosis of muscle cells, and collapse of the notochord (NC).

normally in these frogs during the metamorphosis climax without delay (61).

Although the skin grafts of tadpoles appear to be rejected reproducibly on the syngeneic frogs of *X. tropicalis* within 2 months as discussed above, the grafts survive for >150 days on syngeneic frogs treated with a TH-synthesis inhibitor (methimazole) for 1 month before the skin transplantation and continuously during the experiment. Because methimazole exhibits no immunosuppressive activity, the result indicates the possibility that the skin grafts become atrophic in response to the THs derived from the recipient frogs (63). This notion is supported by results demonstrating that the serum of wild-type *X. tropicalis* frogs contains 6.3 nM T4, which is comparable to the concentration in the *X. laevis* tadpole featuring a regressing tail during the metamorphosis climax (63). In addition, one of the TH-response genes, *TRβ*, is expressed in the adult brain and liver at a level similar to the late phase of the metamorphosis climax (7). These findings show that the endogenous levels of THs circulating in a frog induce the degeneration of the syngeneic tadpole skin graft.

The results of the analyses on the *ouro*-knockouts, athymic tadpoles, and skin-graft transplantation are incompatible with

the immunological rejection model. I cannot exclude the possibility that this discrepancy is because of the species difference between *X. laevis* and *X. tropicalis*, but it is also likely that the active apoptosis pathway in a regressing tail is impaired by the toxic effect of heat shock, leading to unphysiological results. If heat-shock treatment disrupts the progression of metamorphosis and the cell-death process, the use of other promoters and knockout experiments might be more appropriate for examining how metamorphosis is affected by the overexpression and reduced expression of a gene of interest, respectively.

Tail Resorption in *TR*-Knockout Tadpoles

Wild-type and *TRα*-knockout tadpoles show no differences in developmental tail regression during the metamorphosis climax (14–16) in either histological or quantitative gene-expression analysis (16). However, the precocious development of hindlimbs is observed during premetamorphosis before TH secretion (14–16) and the expression levels of several brain genes are reduced at NF stage 61 in *TRα*-knockout tadpoles (7). The absence of TRα leads to *TRβ* de-repression, accumulation, and subsequent recruitment to the TRE of *TRβ* to repress its expression in the absence of THs (14), but is not enough to bind to the weak TREs. As the T3 level increases, *TRβ* expression is immediately activated by the T3-bound TR recruited on the TRE to compensate for the lack of TRα and to express sufficient levels of TR for binding to the weak TREs of the TH-response effector genes. This augmentation of TH signaling induces cell death and, ultimately, the collapse of the notochord according to the regular timetable.

Tail regression, gill absorption, and olfactory-nerve shortening are markedly delayed in *TRβ*-knockout tadpoles (16). This delay of tail regression is reproduced in *TRβ*-knockdown tadpoles (64). Complete tail loss requires 3–4 weeks after the start of tail regression at NF stage 62 in *TRβ*-knockout tadpoles, compared with 1 week in wild-type and *TRα*-knockout tadpoles, which demonstrates that TRβ plays a dominant role in tail resorption (16). *TRβ*-knockout tadpoles retain a tail after their body undergoes metamorphosis, which make them appear as tailed frogs (16). As *TRβ*-knockout tadpoles complete metamorphosis slowly over time, TRα expression may finally compensate for the loss of TRβ.

Although muscle cells disappear almost completely and the tail is shortened to three-quarters of the trunk length by 5 days after NF stage 62 in *TRβ*-knockout tadpoles, the tail still harbors a healthy notochord, which runs along the tail's length and supports the structure. In contrast, the notochord is dissolved 1 day after NF stage 62 in wild-type and *TRα*-knockout tadpoles featuring a tail of similar size. Interestingly, the anterior part of the tail expresses a higher level of *MMP13* mRNA in NF stage 63 *TRβ*-knockout tadpoles than in wild-type and *TRα*-knockout tadpoles at the same stage, although the expression of all *MMP* mRNAs examined is decreased in the distal part of the tail in *TRβ*-knockout tadpoles. *MMP13* mRNA exhibits very strong expression in the notochord, notochord sheath, and fibroblasts around the notochord during the climax of spontaneous metamorphosis (31). However, the notochord shows no detectable degradation in *TRβ*-knockout tadpoles despite the >2-fold-increased expression of *MMP13* mRNA, whose product exhibits collagenase activity after proteolytic processing. This observation suggests that the activation of MMPs, including MMP13, is attenuated and delayed in the absence of TRβ. To my knowledge, tailed frogs are created in response to treatment with a TH-synthesis inhibitor (50) and overexpression of transgenic *D3* (35), *prolactin* (65), and the gene encoding a dominant-negative form of SRC3 (a member of the steroid receptor coactivator family) (66), all of which inhibit the TH-signaling pathway. Thus, maximal TH signaling might be essential for the collapse of the notochord (50). *TRβ* is a direct TH-response gene that is upregulated, and this culminates in the production of sufficient levels of TR through autoregulation for binding to the weak TREs of effector genes (22). One of these effector genes might encode an activator protein that converts a latent MMP into an active enzyme and thus helps dismantle the notochord. Once the notochord weakens, the tail begins shortening through the contraction of the four cords, as mentioned above (50).

PERSPECTIVES

The TH-dependent anuran metamorphosis might be required for the quick transformation from an aquatic to a terrestrial form that enables anurans to escape from predators and hunt for prey readily and rapidly without interruption. The metamorphic change is repressed by unliganded TRα in the TH-sensitive organs before TH secretion, is induced by a gradual increase of T4 in the organs with abundant expression of TRα for the preparation to adapt to adult life, and occurs in almost all organs at the peak concentration of T3 to eliminate the larval organs and accomplish the transformation by the induction of TRβ. This prompt, coordinated, and systematic remodeling orchestrates appropriate and precise development for survival, which is executed by the differential expression of *TRα* mRNA at high and low levels in the hindlimbs and the tail of young tadpoles, respectively. The 5′-untranslated region of *TRα* mRNA contains several translational repressive elements that control TRα expression at low levels (67). However, the mechanism by which TRα expression is regulated at the transcriptional and translational levels in the hindlimbs and the tail remains to be investigated.

Tadpole tail resorption during metamorphosis has long been studied (10), and the apoptotic pathway involved has been comprehensively characterized at the molecular level (68, 69). However, many of the molecules and genes that play a leading role in the suicide and murder of tail muscle cells and the collapse of the notochord remain unidentified. Although apoptosis-related proteins such as caspases are generally considered to contribute to tail resorption, the specific gene expression necessary for the suicide induction of tail muscle cells during spontaneous metamorphosis is unknown. Several ECM-degrading enzymes can cleave collagens, elastin, and other ECM molecules, but no study thus far has identified an enzyme

essential for the murder of tail muscle cells, and the possibility remains that multiple enzymes complement each other in this process. Although MMP13 has emerged as a favorable candidate for driving notochord collapse, *MMP13* mRNA is accumulated in the anterior part of the *TRβ*-knockout tadpole tail harboring a healthy notochord; this implies that an activator of MMP13 functions as a trigger of notochord dissolution. Addressing these issues in future studies by performing comprehensive RNAseq analyses and using genomic-editing methods to create knockouts of genes of interest will lead to the clarification of the entire mechanism of tail resorption.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Knock-Down of Specific Thyroid Hormone Receptor Isoforms Impairs Body Plan Development in Zebrafish

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The role of thyroid hormones (THs) in development has been extensively studied, however, the specific molecular mechanisms involved are far from being clear. THs act by binding to TH nuclear receptors (TR) that act as ligand-dependent transcription factors to regulate TH-dependent gene expression. Like vertebrates, zebrafish express different isoforms of functional Tr alpha and beta, some of which can bind alternative ligands like 3,5-T2. In this study, we first analyzed the effects of exogenous T3 and 3,5-T2 exposure during embryogenesis. The percentage of affected embryos was similar to those vehicle-injected, suggesting that the early exposure to low TH levels is not sufficient to elicit effects upon the phenotype of the embryo. We then generated crispants for four isoforms of *thr* to learn more about the role of these receptors in early development. We found that crispant larvae from *thraa* and a newly identified *l-thrb*+, but not *thrab* and canonical *thrb1* showed profound deleterious effects upon symmetry and laterality, suggesting early novel roles for these Tr isoforms in the body plan developmental program. Since critical events that determine cell fate start in the late gastrula, we tested if some genes that are expressed during early developmental stages could indeed be TH targets. We identify early development genes, like *sox10* and *eve*, that were specifically over-expressed in *thraa* and *l-thrb*+ crispants, suggesting that these specific *thr* isoforms function as transcription repressors for these genes, while transcription of *zic* and *ets* appear to be *thraa* and *l-thrb*+-mediated, respectively. Overall, present results show that TH signaling participates in early zebrafish development and identify Tr isoform-specific mediated regulation of early gene expression.

Keywords: thyroid hormone receptors, thyroid hormones, CRISPR/Cas9, development, zebrafish

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INTRODUCTION

Thyroid hormones (THs) play important roles in different developmental processes and life transitional events of vertebrates (1–5). The molecular mechanisms that govern these events are complex and not fully described, but there is evidence that several of the TH-regulated effects are exerted through a genomic mechanism mediated by thyroid hormone receptors (TRs) (6), which are members of the nuclear receptor superfamily. In vertebrates, TRs are encoded by two distinct

genes denominated thyroid hormone receptors alpha (*THRA*) and beta (*THRB*), which in turn are transcribed into several TR isoforms with tissue- and species-specific functions (7, 8). Aside from T3 (3,3′,5-triiodo-L-thyronine), T2 (3,5-diiodo-L-thyronine) also functions as an important TR ligand known to bind preferentially to different Tr isoforms, at least in teleosts (9), suggesting that each of these ligands can modulate different transcriptional processes.

In a comparative scenario, zebrafish have become an invaluable tool to start unraveling some of the mechanisms involved in vertebrate developmental processes. For example, during segmentation [10–20 h post fertilization (hpf)] an anteroposterior embryo is well-defined, and somites, tail, and a rudiment of the head and eyes can be observed (10). Some transcription factors that regulate cell fate and differentiation are regulated in a fine way before segmentation starts (5–10 hpf). During gastrulation (5–10 hpf), epiboly, internalization and germinal layer formation, as well as a correct positioning of dorso-ventral, antero-posterior, and left-right axis of embryo occur (11, 12), and several genes involved in cell fate and organogenesis start expression during this stage. Trs are known to function as the TH signal modulators during zebrafish development and in concert their coding mRNA is present in the fertilized egg in high concentrations during the first 6 hpf, after which *thr* mRNA decreases to low or non-detectable levels until 24 hpf (13, 14). The fact that *thr* mRNAs exhibit their highest levels concomitantly with those of intra-ovum THs (15) suggests that both Tr and ligand are from maternal origin and that these can be functional before embryonic transcription of *thr* and the appearance of the thyroid gland for embryonic TH synthesis. Tr manipulation results in stronger effects than those from exogenous TH administration. Indeed, overexpression of Trα has shown dramatic effects upon craniofacial development (16), and recently, human dominant-negative TRs were employed to determine the role of these isoforms during development (14, 17).

The objective of the present study was to further understand the role of THs and their receptors during early development. To that end, we evaluated the effects of exogenous T3 and T2 exposure during embryogenesis as well as disrupted *thr* expression using the CRISPR/Cas9 methodology. Also, we analyzed the expression of some early development genes and identified if they were TH-responsive and direct *thra*- or *thrb*-mediated TH targets.

MATERIALS AND METHODS

Animals

Adult zebrafish (*Danio rerio*) were purchased from a commercial pet store and acclimatized to laboratory conditions: flow-through system with tap water at 28°C and a photoperiod 16:8 (light:dark). Embryos were obtained from natural mating, washed with tap water and cultured with E3 standard medium containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ and methylene blue. Un-injected embryos (UN) were immediately placed into an incubator at 28.5°C. All zebrafish were maintained and handled in accordance with protocols approved by the Ethics for Research Committee of the Instituto de Neurobiología at the Universidad Nacional Autónoma de México (UNAM).

Preparation of sgRNA and mRNA Cas9

The zebrafish *thraa*, *thrab,* and *thrb* gene sequences were obtained from the zebrafish information network (www.zfin.org), and the different isoforms were verified on Ensembl (www.ensembl.org).

A well-established protocol was followed for genome editing (18). The main steps are briefly described: The scoring algorithm designed and tested in zebrafish CRISPRscan [www.crisprscan.org; (19)] was used to design sgRNAs. The selected target regions and selected guides for *thraa* and *thrab* are illustrated in **Supplementary Figure 2** and **Table 1**. In the case of the *thrb* gene, *two* guides were used: sgRNA *l- thrb+*, which only targets the newly identified super long *thrb* (*l- thrb+*, see below), and sgRNA *thrb**, which potentially targets three distinct isoforms (the well-described long (l-)Trβ1 and short (s-)Trβ1 isoforms and the newly described l-Trβ+) (**Supplementary Figure 3**). sgRNA was synthetized by *in vitro* transcription using T7 Quick High Yield RNA Synthesis Kit (New England Biolabs). Cas9 mRNA was also synthetized by *in vitro* transcription using an *Xba*I-linearized pT3TSn-Cas9n plasmid as template through mMESSAGE mMACHINE T3 kit (Life Technologies). sgRNAs and mRNA Cas9 were purified by ethanol precipitation and resuspended in RNAase free water. For the CRISPR/Cas9 microinjection, Cas9 mRNA and sgRNA mix was prepared and zebrafish embryos were injected directly with a final volume of 1 nL equivalent to 100 and 20 pg of Cas9 and sgRNA per embryo, respectively.

Microinjection

Embryos for microinjections were prepared according to Rosen et al. (20). One-cell stage zebrafish embryos were injected directly with a final volume of 1 nL of vehicle or working solutions using 1.5 OD/1.12 ID thinwall capillars (World Precision Instruments) and a Pneumatic PicoPump (PV 820; World Precision Instruments). To calculate intra-ovum TH concentration, an intra-embryonic volume of 170 nL was estimated. Groups of around 50 eggs were injected with the corresponding guide, either TH or vehicle, which consisted in DEPC water for CRISPR/Cas9 experiments and 10^{-7} N NaOH for TH treatments. Three independent experiments were performed per group.

DNA Extraction

A HotSHOT modified protocol (21) was used to extract larval genomic DNA, in which 45 μL of 50 mM NaOH were added to each individual larva, followed by a 30 min incubation at 95°C. The samples were cooled at 4°C, and 5 μL of 1 M Tris-HCl, pH 8.0 were added to neutralize the solution. The samples were centrifuged to pellet debris and 5 μL of the supernatant were used for 50 μL PCR reactions.

Crispant Verification

To verify that the zebrafish larvae contained the desired gene mutations, a fragment of *thraa*, *thrab,* and *thrb* genes that included the CRISPR/Cas9 target sites was amplified. To this end,

TABLE 1 | Primers and templates.

Gene target	Sequence identifiers	Forward primer (5′-3′)	Reverse primer (5′-3′)	Primer position	Length (bp)
REAL TIME PCR PRIMERS					
dio2	NM_212789.4	GCAGCGCATGTTAACCACAG	GTTGTGGGTCTTACCGCTGA	Exon 1–2	160
dio3a	NM_001256003.1	CGCTCGTGTGTCTGCTCATT	CAGAGACTCCCAGCTGAACA	Exon 1	175
thraa	NM_131396.1	ATGGAAAACACAGAGCAGGAG	AGGAACAGAGATGCTCTTGTC	Exon 2–4	132
thrab	ENSDART00000153187.2	GGATGGAAATAAGGTGAATGGAAC	GGTAGTGATATCCGGTAGCTTTG	Exon 3–5	210
s-thrb1	NM_131340.1	AGAAGACTGTATGGGATCGAC	GTCTTCTGGCAGGAATTTGCG	Exon 9–10	134
l-thrb1	ENSDART00000151766.3	AGAAGACTGTATGGGATCGAC	GGCTTGGCTTCCTTCACCC	Exon 7–8	154
mct8	NM_001258230	GTTCGGGAAGATCGGAGACC	AACACGGCACACTGAGGAAT	Exon 21–22	111
ets1	NM_001017558.1	GAGATTTCTGGACCTGGCAC	GAAATATTCGGAGGGATAGCGG	Exon 4–5	145
eve1	NM_131114.1	GGGAACAGCTGACTCGTCTC	TGTCCTTCATTCTCCGGTTC	Exon 2–3	141
fgf1b	NM_001105278	CATGAGACTGGACTATACCTTGC	GTCCTGATATCTCTGCGAACG	Exon 2–3	138
foxd3	NM_131290	GTCCCGTCAAATATCATCTCCG	GCCTATAGTTCGTGCTGTATCG	Exon 2–3	150
msx1a	NM_131273.1	TCACACCCGTTTCACAGAC	CGGCAAACTTCACAAGTCAC	Exon 2–3	147
sox10	NM_131875.1	TCAATATCCGCACCTGCAC	CGCTTATCCGTCTCGTTCAG	Exon 2–3	82
pax7a	ENSDART00000172008.3	TCTGCAAAGTTCCTCCGGATT	CTGCAGTGCACAATGCCAAA	Exon 1	310
zic1	NM_130933.2	CTACACACATCCCAGTTCTCTC	TCTGGTTTTCTGTGGAAGGG	Exon 2–3	143
mbpa	ENSDART00000052556.8	GAGGAGACAAGAAGAGAAAGGG	GAAATGCACGACAGGGTTG	Exon 1–2	83
mpz	NM_194361.2	ACCTGTGATGCCAAGAACC	TTGCCACAACGAGGATCA	Exon 3–4	148
olig2	ENSDART00000060006.5	CGAGTGAACTGGAATAGCCTTAC	GCTCGTGTCAGAGTCCATG	Exon1–2	134
plp	ENSDART00000003514.8	ACACTGTTAACGTCCTGTCAG	CTGGTGCTTTGCATATGTTGG	Exon 4–5	147
lsm12b	NM_213148.1	AGTTGTCCCAAGCCTATGCAATCAG	CCACTCAGGAGGATAAAGACGAGTC	Exon 3–4	300

Gene target	Sequence identifiers	Forward primer (5′-3′)	Reverse primer (5′-3′)
gDNA PRIMERS			
thraa	ENSDARG00000000151	TGTCAGATGGCCAAATGGAGT	CTGGTTGCGGGTGATTTTGT
thrab	ENSDARG00000052654	AGCTCTCGGAGCTGAAAGTG	ACCAGTGTAAGGAATAAAGTTGCT
thrb (l-thrb+)	ENSDARG00000021163	GACATAGCCCATGGTGTAAG	CTTTCTTATGTGGCCCTTGC
thrb (thrb)*	ENSDARG00000021163	GCATGGCTACAGACTGTAAG	GTTGTCAACAGGGAAGAGAC

Templates for *in vitro* transcription of sgRNAs	
thraa	taatacgactcactataGGAGCGGTAATGATAGCCAGgttttagagctagaa
thrab	taatacgactcactataGGGAAAGAACAGCCAGTGTTgttttagagctagaa
l-thrb+	taatacgactcactataGGGTGAGTTATGCACCATGGgttttagagctagaa
*thrb**	taatacgactcactataGGGAGAACCGTGAACGCCGAgttttagagctagaa
Generic for template assembly	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTaacttgctatttctagctctaaaac

a pair of primers for each gene was designed (see **Table 1**). An equal volume of DNA from 8 larvae of the same experimental condition were mixed (un-injected wild type and injected) for PCR amplification using Platinum Taq DNA Polymerase (Invitrogen) and subsequently column-purified (DNA Clean & Concentrator ^{TM}; Zymo Research). Purified PCR amplicons were either directly sequenced or used for subcloning in the TA vector pGEM-T Easy (Promega) to analyze the mutant allele populations from the injected larvae. Isolated colonies resulting from competent bacteria transformations were plasmid extracted and sent to sequencing using the universal primer T7.

Quantitative PCR

The expression of selected genes was quantified in native, TH-treated and crispant larvae. To that end, total RNA was extracted from 0 un-injected zebrafish fertilized eggs and 9 hpf embryo pools (8-16) with Trizol Reagent (Life technologies). RNA was reverse transcribed with RevertAid Reverse Transcriptase (Thermo Scientific) from 1 μg of total RNA and 0.5 μg oligo (dT). Specific oligonucleotides were designed with Real-time PCR tool IDT. PCR products were obtained using a proofreading DNA polymerase for 10′ at 95°C, 10″ at 95°C, 10″ at 61°C and 10″ at 72°C for 40 cycles and were cloned into pJET1.2/blunt vector (Thermo Scientific). Constructs were verified by sequencing, and standard curves that ranged from 10^5 to 10^9 molecules/μL were prepared. In all cases, reactions contained 1 μL of reverse transcribed reaction, 6 μL Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and 250 or 500 nM forward and reverse oligonucleotides in a final volume of 12 μL. A Step One instrument was used for detection and data

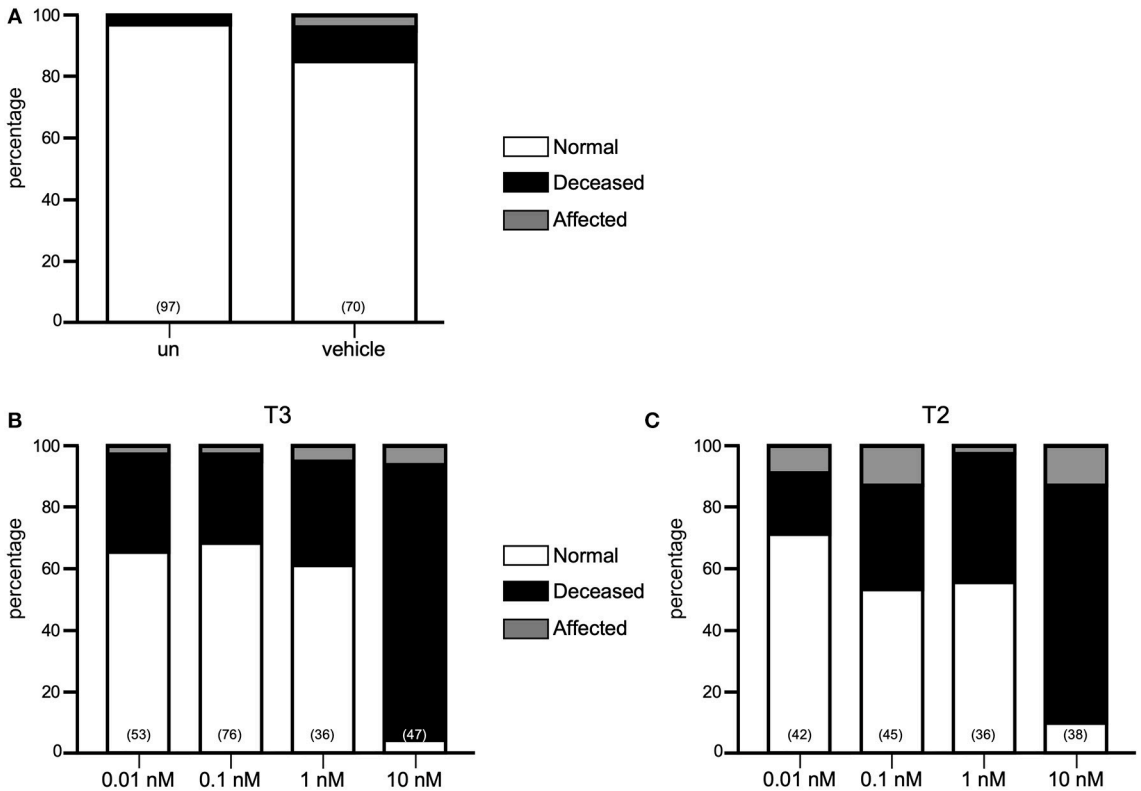

FIGURE 1 | Effect of THs in zebrafish development. **(A)** Percentage of normal, affected and deceased larvae in un-injected (UN), vehicle or TH treatments. Neither T3 **(B)** nor T2 **(C)** had effects upon morphology at 0.1–10 nM but is toxic at 10 nM.

analysis according to the manufacturer's instructions (Applied Biosystems). The absolute mRNA concentration was expressed as molecules per microgram of total mRNA used in RT reaction and obtained by interpolation with the standard curve and normalized with reference gene lsm12b (22) in each experimental sample. Oligonucleotides used for gene quantifications are listed in **Table 1**.

Statistical Analysis

Results were analyzed using ANOVA coupled to a Tukey *post-hoc* test (control vs. treatments) and GraphPad Prism 7. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Effects of Exogenous T3 and T2 Exposure During Embryogenesis

Until now, only immersion administration protocols have been used to deliver exogenous TH into the teleost embryo (23, 24). For this study we microinjected one-cell stage zebrafish embryos with T3 or T2 at concentrations ranging from 0.01, 0.1, 1, and 10 nM and observed for effects on the general body plan or on mortality during the first 48–50 hpf. As depicted in **Figure 1**, 19–41% and 30–33% of mortality was observed after the injection of 0.01, 0.1, and 1 nM of both T3 and T2, respectively, however, mortality increased to 76–89% when embryos were injected with 10 nM of either hormone, clearly showing toxic effects. In all cases, the percentage of affected embryos was similar to those vehicle-injected (below 6% for T3 and 13% for T2), suggesting that the microinjection did not influence development, as well as that the early exposure to the hormone alone was not sufficient to elicit effects at least upon the phenotype of the embryo (**Figure 1**). Since transactivating assays showed that zebrafish TRs were activated with 0.1 nM (**Supplementary Figure 1**), and a slightly lower mortality rate was observed with this concentration compared to 1 and 10 nM, subsequent experiments were performed with 0.1 nM of either T3 or T2.

thraa but Not thrab Impacts Early Zebrafish Development

Two *thra* genes have been described in teleosts, but from these genes, only *thraa* has been more extensively studied. In zebrafish, two *thraa* transcripts have been described, which differ in the presence of an extension of around 12 amino acids in the C-terminal domain (**Figure 2**). In contrast, only one transcript has been described for *thrab*, however, we identified two transcripts in Ensembl that differ by 6 amino acids at the N-terminal of the protein (**Figure 2**). As mentioned above,

FIGURE 2 | Primary sequence of thyroid hormone receptor isoforms from zebrafish analyzed in this study. The alignment depicts the DBD (light blue) and LBD (Blue)which are highly conserved. Isoforms were denoted as short (S) or long (L), according to the respective counterpart isoform. The teleost-specific insert of 9 amino acids from l-Trβ1 and the newly identified 9 plus 20 amino acid sequence from l-Trβ+ are denoted in purple and pink, respectively. We highlight in red the sgRNA targets for each isoform. The alignments were prepared using MEGA 8.

for this study, guides to disrupt *thra* genes were designed to target the two identified isoforms per gene. Designed sgRNA guides were effective to produce a variety of mutations reflected in the electropherograms from crispant DNA with respect to that from wildtype embryos (**Supplementary Figures 2, 3**). Our results show that approximately 35% of the *thraa* crispant larvae presented a clear loss of symmetry and laterality (asymmetric size and position of external morphology mainly of eyes, head, and tail) observed as early as 24 hpf when the effects were severe, or from 3 to 4 days post-fertilization in mildly to moderately affected larvae when the body plan had taken form (**Figure 3A**; **Supplementary Figure 4**). Moreover, this group of crispants exhibited a 30% mortality (**Figure 3B**). In contrast, when *thrab* crispants were analyzed, no effect was observed in larvae (**Figures 4A,B**), and mortality was only 5.5%. Altogether, these results strongly suggest a novel role for *thraa* in the body plan development program, whereas *thrab*, although expressed in early development (13, 25), does not seem to participate in these processes.

Zebrafish L-trb+ Crispants Show Impaired Embryogenesis

As mentioned, several functional teleost-specific *thrb* isoforms have been identified. Of these, the most representative are two gene products that contain or not an insertion of nine amino acids in the ligand binding domain (LBD) of the protein and that have been referred to as long or short thyroid hormone receptor β1 (S- or L-Trβ1). However, some metamorphic species have been shown to express an additional *thrb* isoform that contains 20 amino acids adjacent to the 9-amino acid insert, and that has been denominated L-Trβ1+ (8). Interestingly, we identified (Ensembl) an isoform that had not been previously identified in zebrafish that contains a 111 amino acid N-terminal fragment, similar in length to that of the human Trβ2, but with only 26% of conserved amino acids within this region (**Figure 2**). The low sequence identity in the fragment raises the doubt that the isoform could indeed be a Trβ2. Furthermore, this Trβ isoform contains the 29-amino acid insert, as described for the metamorphic species (L-Trβ1+). Given the ambiguity to clearly identify this isoform in terms of sequence identity, in this study we have denominated it L-Trβ+.

To unravel the putative role of this as well as the other two *trβ1* during early development, crispants for the three isoforms were generated by using the guide *thrb**, as well as crispants that were directly targeted to disrupt *l-trb+*. Surprisingly, only crispants specifically generated to disrupt *l-trb+* presented effects in 30% of larvae, showing a loss of symmetry and laterality in the same manner as *thraa* crispants (**Figures 3A,C**). In contrast, crispants resulting from a target site that disrupts a shared *Trβ1* sequence (**Figure 2**) only showed minor effects as when using the guide that disrupts *l-trb+* alone (**Figures 4A,C**). Although intriguing, these results evidence a clear functional role of the novel *l-trb+* isoform at least during early development. No morphological defects were observed in 60% of crispants generated with sgRNA *thrb**, an observation that could result from the nature of the sgRNA target site, which renders transcripts that still contain the DNA-binding domain (DBD), possibly allowing the truncated protein to bind

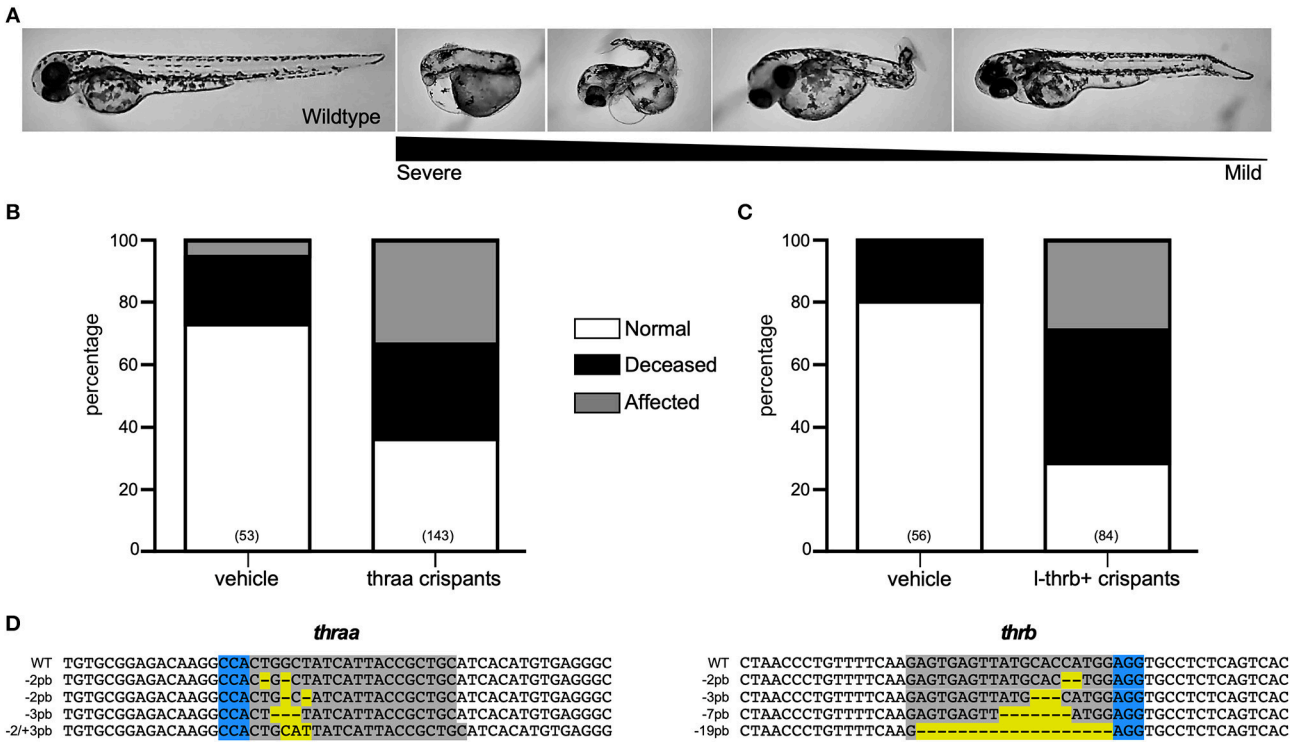

FIGURE 3 | Characterization of *thraa* and *l-thrb+* crispants. **(A)** *thraa* and *l-thrb+* crispants show diverse phenotypes with several degrees of body plan affectations as compared with wild-type at 3 dpf. Various phenotypes were observed due the mosaicism of the mutation. **(B,C)** Represent the percentage of normal, affected and deceased larvae after sgRNA and Cas9 treatment. **(D)** DNA sequence of sgRNA target site for CRISPR/Cas9 is denoted in gray, whereas PAM sequence is denoted in blue. Insertion-deletions (indels) are highlighted in yellow.

to TH-responsive elements in target gene promoters and repress gene transcription. However, the 32% mortality observed in this group could also correspond to the population where *l-trb+* expression was most affected, further suggesting that the canonical *thrb* isoforms do not appear to be involved in early developmental events.

Changes in Development-Related Genes at 9 hpf

Both *thraa* and *l-thrb+* crispants showed a clear loss of symmetry and laterality, visualized at 24 hpf when a well-defined antero-posterior pattern was observed in control larvae. As previously mentioned, critical events that determine cell fate for these developmental stages, like epiboly, internalization and germinal layer formation, start in the gastrula (approximately 5–10 hpf), (10, 26). Thus, we hypothesized that some genes that are expressed during early development could indeed be TH targets, with Tr-specific signaling pathways. To prove our hypothesis, we chose 9 hpf embryos, which were at the onset of segmentation, to analyze the expression of sets of genes known to be part of TH signaling (*dio2, dio3, mct8, thraa, thrab, s-thrb1, l-thrb1*), and genes involved in symmetry and laterality (*eve, fgf, zic, pax 7, msx, foxd3, sox 10, ets*) and in myelination (*mpz, mbp, olig 2, plp1b*) (**Table 1**). Since myelination does not start until 48 hpf in zebrafish (27), the latter set of genes was included as a negative control group. This screen was performed in fertilized un-injected eggs to determine gene expression at time zero and in the 9 hpf vehicle-injected embryos or embryos treated with T3 and T2, as well as in crispants generated for all *thr* isoforms. As 9 hpf is too early to detect body plan malformations, the mRNA pool samples of crispants were heterogeneous since we were unable to distinguish between affected and normal embryos. Nonetheless, we were still able to detect clear changes in mRNA expression in the different experimental groups, with clear statistical significance compared to controls. Exogenous TH exposure influenced the regulation of several genes at this stage of development, showing that the expression of TH-responsive genes is receptive to TH regulation during gastrulation. The fact that T2 had an effect upon gene regulation suggests that in this developmental stage, as in the juvenile and adult stages, T2 is a relevant TR alternative ligand (28, 29) (Lazcano et al. under review)[1].

As observed in **Figures 5–7**, mRNA from *dio2, thraa, s-thrb1, l-thrb1, mbp, ets,* and *fgf* was highly expressed at the stage of one-cell embryo (0 hpf), while mRNA expression of *mct8, mpz, eve, foxd3, msx,* and *zic* was not detected, showing the maternal origin of some transcripts. Indeed, transcripts from maternal origin are present in the oocyte, and zygotic transcription starts around 2 to 3 hpf. Other transcripts (*mct8*, *eve*, *foxd3*, *msx*, and *mpz*) were only detected after 9 hpf, evidencing onset of zygotic

[1]Lazcano I, Hernández-Puga AG, Orozco A. Alternative ligands for thyroid hormone receptors. A molecular perspective. *Mol Cell Endocrinol.*

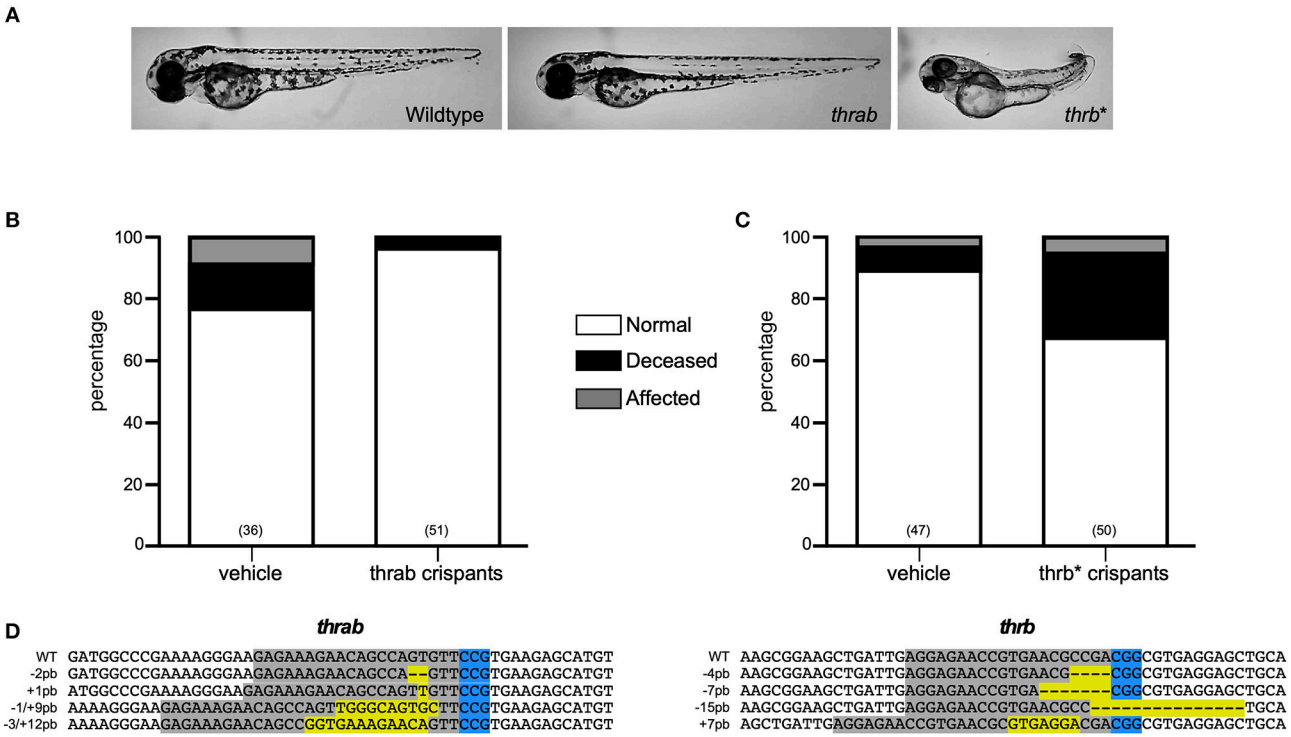

FIGURE 4 | Characterization of *thrab* and *l-thrb** crispants. **(A)** *thrab* crispants do not show effects upon zebrafish morphology, whereas *l-thrb** crispants generated with an sgRNA able to target all *thrb* isoforms showed a low percentage of affected phenotypes characterized by laterality. **(B,C)** Represent the percentage of normal, affected and deceased larvae after sgRNA and Cas9 treatment. **(D)** The DNA sequence of sgRNA target site for CRISPR/Cas9 is denoted in gray, whereas PAM sequence is denoted in blue. Insertion-deletions (indels) are highlighted in yellow.

gene transcription (10). Furthermore, we identified genes that were up- (*thraa, thrab, msx*) or down-regulated (*dio2, eve, pax 7*) by both T3 and T2 at 9 hpf, as compared with vehicle-injected embryos, and genes that were specifically up-regulated by T3 (*sox10*) or by T2 (*mct8, olig2, zic*), as well as genes down-regulated by T2 (*foxd3*). We also identified genes that did not respond to TH treatment (*dio3, mbp, mpz, plp, ets, fgf, pax7*). Thus, out of the three sets of genes, at least those related to TH signaling and early development were indeed TH-responsive. The early exposure of T2 resulted in an up-regulation of *olig2*, a gene associated to myelination, suggesting a direct regulatory effect of this hormone. As an attempt to identify if the TH response was mediated by a specific *thr*, we analyzed the expression of the three sets of genes in *thr* crispants. Only *sox10, eve* and *zic* specifically increased their expression in *thraa* crispants, *sox10, ets* and *eve* in *l-thrb+* crispants and *mbp* in *thrab*, suggesting that for these genes, *thraa, l-thrb+*, and *thrab* respectively, function as repressors of transcription. *thrb** crispants, where all *thrb* isoforms are targeted, exhibited an increased expression of *dio3, eve, pax7,* and *zic*, suggesting that the regulation of *eve* is mediated by *l-thrb+* and that of the other genes by a different *thrb* isoform. In contrast, we observed a more diverse response on gene expression for *thrab* crispants: the expression of *mbp* and *sox10* was discretely increased, while that of *dio2*, *ets*, and *eve* decreased, suggesting that *thrab* could have other roles in later stages of development.

DISCUSSION

This is the first study in which *thr* crispants were analyzed in order to further understand the role of Trs in early development. We found that larvae from *thraa* and *l-thrb+* crispants presented profound deleterious effects upon symmetry and laterality, suggesting early novel roles of these Tr isoforms in the body plan developmental program. We also explored the expression of early development genes known to be involved in symmetry and laterality in *thr* crispants and identified direct *thra-* or *thrb*-mediated TH targets.

Early TR expression has been described before gastrula in teleosts and birds (14, 30) and during the first trimester of gestation in the human fetal brain (31). As in the present study, *THRA* is the most representative TR expressed gene (14, 31), suggesting that TRα-mediated TH signaling regulates early developmental events in all vertebrates. It is thought that THs do not participate in early vertebrate development, only after neural tube closure (32, 33), however, and irrespective of the vertebrate species, the embryo is always exposed to low TH levels from maternal origin, and TR mRNA is present from the onset of development (13, 14). Studies aimed to analyze early ontogenetic TH effects in zebrafish have shown that these hormones accelerate hatching and pigmentation (23). In the present work we did not detect any of these effects with our administration protocol (data not shown), but we did observe

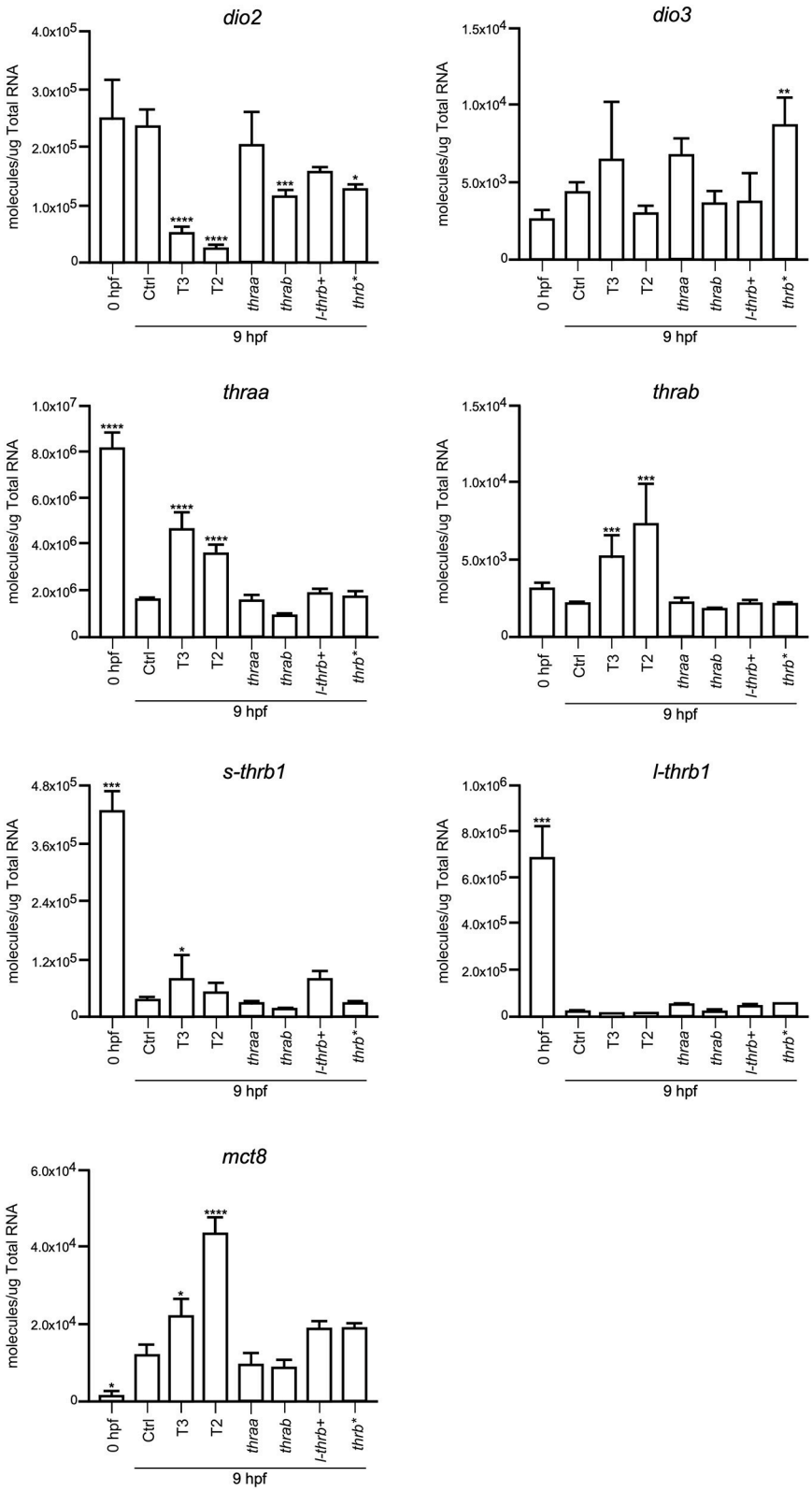

FIGURE 5 | Embryonic mRNA expression of TH-signaling genes *dio2, dio3, thra, l-thrb1, s-thrb1, and mct8*. Total RNA was extracted from 9 hpf zebrafish embryo pools (8–16) for qPCR. The plots show mean values ± SEM results of three independent biological experiments. Statistical analysis was performed using one-way ANOVA coupled with a Tukey's multiple comparisons test with respect to control groups. Significance is indicated $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$.

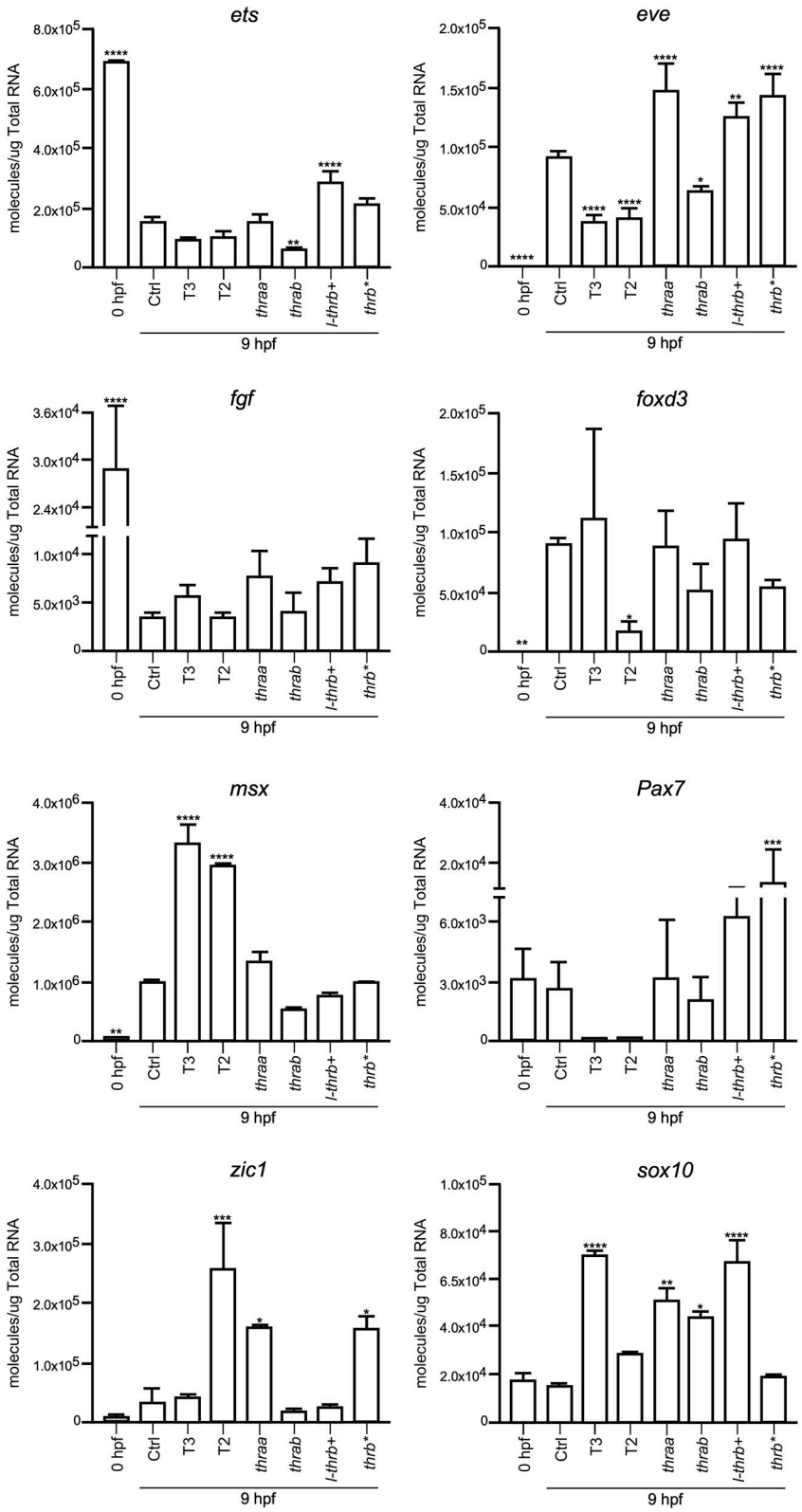

FIGURE 6 | Embryonic mRNA expression of early development genes *eve, fgf, zic, pax7, msx, foxd3, sox10, and ets*. Total RNA was extracted from 9 hpf zebrafish embryo pools (8–16) for qPCR. The plots show mean values ± SEM results of three independent biological experiments. Statistical analysis was performed using one-way ANOVA coupled with a Tukey's multiple comparisons test with respect to control groups. Significance is indicated $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$.

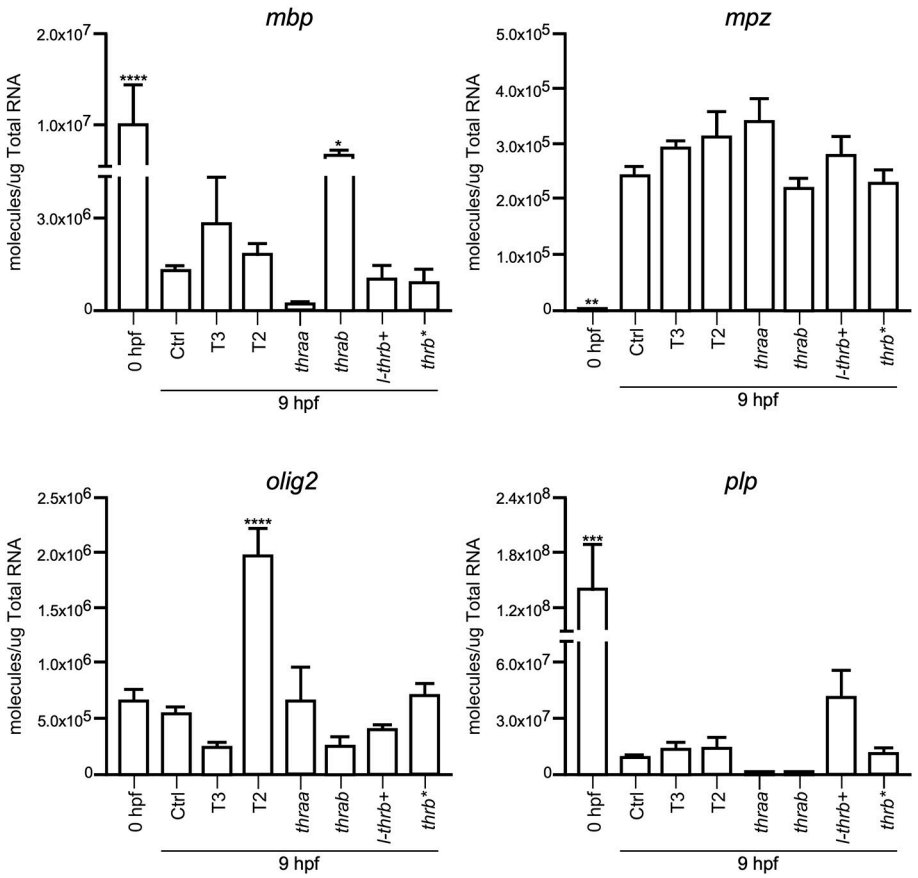

FIGURE 7 | Embryonic mRNA expression of genes involved in myelination (*mpz, mbp, olig2, plp1b*). Total RNA was extracted from 9 hpf zebrafish embryo pools (8–16) for qPCR. The plots show mean values ± SEM results of three independent biological experiments. Statistical analysis was performed using one-way ANOVA coupled with a Tukey's multiple comparisons test with respect to control groups. Significance is indicated $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$.

changes in the transcription of several genes associated with TH signaling and to body symmetry. Indeed, *dio2, thraa, s-thrb1,* and *l-thrb1* were found to be highly expressed in one-cell embryos, emphasizing mRNA of maternal origin and the concomitant early onset of TH signaling. Furthermore, the expression of genes associated with body symmetry like *eve*, *foxd3*, *msx*, and *zic1*, was undetectable at the one-cell stage, but detectable in 9 hpf embryos evidencing the time-specific windows of gene expression during development. The fact that TH regulated some of these genes as early as 9 hpf clearly suggests that maternal THs regulate the transcription of genes involved in their own transport, metabolism and signaling, as well as the onset of cellular and molecular mechanisms involved in body symmetry and laterality. Given these observations, it was puzzling that no clear effects upon larval phenotype were evident after TH treatment. One possible explanation is that the fine down-regulatory effect that THs exert upon *dio2* expression (**Figure 5**) could serve as a protective mechanism from TH excess. Other possible explanations could involve particular TR action mechanisms yet to be elucidated.

The study of TRs and their ligands during development has focused on the central nervous system, where it is known that they are required to perform certain actions mainly related to neurogenesis and myelination (33–36) and other possible roles may have been overlooked. The zebrafish model has recently been used to address some relevant aspects of TR-TH function during development. Since teleosts underwent a specific round of genomic duplication, their genome contains several copies of genes that are absent in other taxa (37). In the case of zebrafish *thr*, the presence of a second copy of the *thraa* gene has been demonstrated and it has been called *thrab* (13, 25). The alignment of the primary sequence of the isoforms resulting from both *thra* genes shows differences only at the N- and C-terminal of the protein (**Figure 2**), while the DBD and the LBD show a high degree of identity, suggesting that all isoforms generated from the *thraa* and *thrab* genes could bind to DNA and ligate THs. In this study, the sgRNA designed to disrupt each gene modifies the open reading frame at the beginning of transcription, prior to the DBD, in both cases affecting the synthesis of all the possible isoforms for *thraa* and *thrab* that we identified by Ensembl. The resultant crispants carry the induced mutation, but the grade of penetrance is undefined because of the nature of the changes in the nucleotide sequence (monoallelic, biallelic in- or out of frame, in different cell types or occur over different time frames). Due to this crispant nature, and as determined by sequencing, we identified a great variety of

mutated alleles in all four target genes analyzed (**Figures 3D**, **4D**), most of them resulting in premature stop codons and frameshifts. This variety of alleles is reflected in the obtained phenotypic diversity (**Supplementary Figure 4**), however, only *thraa* crispants presented severely affected larvae. Indeed, *thraa* crispants showed high mortality, clear malformations, asymmetry and altered laterality, while *thrab* crispants did not. It is possible that *thrab* mutations could affect development in a different way, without evident abnormalities in body plan, whereas *thraa* could regulate the transcription of body plan genes during early development (see below). However, studies to corroborate the biological activity of the receptors encoded by *thrab*, as well as some possible roles during other stages of the zebrafish life cycle must be tested experimentally.

Although only a single copy of the *thrb* gene has been identified with the exception of eels (38, 39) and *Xenopus laevis* (40) which contain two copies of *thrb*, this gene has other characteristics that could confer biological plasticity, like the presence of several isoforms that differ in the N-terminal and/or the LBD (**Figure 2**). Zebrafish, for example, expresses the canonical Trβ1, homologous to the TRβ1 of mammals and other vertebrates, and also an isoform with a 9 amino acid insert in the LBD that has been previously characterized and is able to bind T3 as well as the alternative TR ligand T2 (9). More surprisingly, we also detected a zebrafish isoform that has an up-stream putative alternative start site of transcription different from the other *thrb* isoforms that results in a Tr with an extended N-terminal fragment similar in length but not in sequence identity to that of the human Trβ2, which additionally contains the 9 amino acid insert plus 20 amino acids more located adjacently, probably generated by alternative splicing (**Figure 2**). This isoform, which we denominated *l-trb+*, is the largest *thrb* identified thus far in teleosts and not previously characterized in zebrafish. As in the case of *thraa,* the disruption of *l-thrb+* rendered larvae with deleterious effects upon symmetry and laterality that had not been previously described. In fact, the phenotype of both *thraa* and *l-thrb+* crispants was so strong that no detailed scrutiny was needed to identify affected larvae. Thus, one of the most interesting findings of the present study was the isoform-specific effects that *thr* exerted during development.

In concert with the phenotypic assessments, when gene expression was analyzed in crispant embryos, we identified early development genes, like *sox10* and *eve,* that were specifically over-expressed in *thraa* and *l-thrb+* crispants, suggesting that for these genes, these specific *thr* isoforms function as transcription repressors. This would be in agreement with the prevailing concept that during early vertebrate development, TR act mainly as dominant negatives when unliganded, at least for TH positively regulated genes (30, 31). It is noteworthy that *sox10* and *eve* are determinant for neural crest migration and tail extension (41, 42). Thus, precocious expression of *sox10* and *eve* could explain, at least in part, the dramatic malformations observed in *thraa* and *l-thrb+* crispants. Interestingly, these two genes are regulated by THs in an opposite manner: T3 up-regulates *sox10*, while both, T2 and T3 down-regulate *eve*, showing the dynamic interplay between *thr* and ligands during early gastrulation. It would be very interesting for future experiments to isolate the different obtained alleles and look for differences or redundancy in its phenotype. Other genes that could be involved in these early developmental processes are *zic* and *ets*, which appear to be *thraa-* and *l-thrb+*-mediated, respectively. *zic* is involved in brain and somite development (43), while *ets* is a gene involved in vascularization (44). In contrast, with the exception of *mbp*, the expression of genes involved in the myelination process is not affected in the different crispants. The fact that *mbp* is up-regulated in *thrab* crispants suggest a not myelination-related function of this gene in early development. As previously mentioned, these observations are in concert with the notion that myelination starts around 48 hpf in zebrafish (27). The expression of other analyzed genes known to participate in zebrafish early development was not significantly modified. It is possible that these genes are not TH targets or that they act at different stages of development.

Overall, present results show that TH signaling participates in early zebrafish development. An interesting contribution of this study however is the identification of Tr isoform-specific mediated regulation of early gene expression. Thus, and although at this point, we cannot clearly decipher the respective contribution of each receptor isoform during early development, we did identify at least two genes whose regulation is specifically mediated by Trα and L-Trβ+, showing that the experimental strategies used in the present study will be useful to elucidate TR-specific functions.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

IL and RR-O performed the CRISPR-Cas9 experiments. PV performed the qPCR experiments. AO and IL directly participated in the planning and execution of this study and drafted the manuscript. All authors provided critical comments to the manuscript and revised the text. All authors of this research paper have read and approved the final version submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00156/full#supplementary-material

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ORIGINAL RESEARCH
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Opposite T_3 Response of ACTG1–FOS Subnetwork Differentiate Tailfin Fate in Xenopus Tadpole and Post-hatching Axolotl

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Amphibian post-embryonic development and Thyroid Hormones (TH) signaling are deeply and intimately connected. In anuran amphibians, TH induce the spectacular and complex process known as metamorphosis. In paedomorphic salamanders, at similar development time, raising levels of TH fail to induce proper metamorphosis, as many "larval" tissues (e.g., gills, tailfin) are maintained. Why does the same evolutionary conserved signaling pathway leads to alternative phenotypes? We used a combination of developmental endocrinology, functional genomics and network biology to compare the transcriptional response of tailfin to TH, in the post-hatching paedormorphic Axolotl salamander and *Xenopus* tadpoles. We also provide a technological framework that efficiently reduces large lists of regulated genes down to a few genes of interest, which is well-suited to dissect endocrine regulations. We first show that Axolotl tailfin undergoes a strong and robust TH-dependent transcriptional response at post embryonic transition, despite the lack of visible anatomical changes. We next show that Fos and Actg1, which structure a single and dense subnetwork of cellular sensors and regulators, display opposite regulation between the two species. We finally show that TH treatments and natural variations of TH levels follow similar transcriptional dynamics. We suggest that, at the molecular level, tailfin fate correlates with the alternative transcriptional states of an fos-actg1 sub-network, which also includes transcription factors and regulators of cell fate. We propose that this subnetwork is one of the molecular switches governing the initiation of distinct TH responses, with transcriptional programs conducting alternative tailfin fate (maintenance vs. resorption) 2 weeks post-hatching.

Keywords: Thyroid hormone, Axolotl, network biology, embryonic development, paedomorphosis

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INTRODUCTION

Thyroid Hormones (TH) play central roles in numerous physiological and cellular processes, such as metabolism, cell proliferation, cell death, cell differentiation, and control of homeostasis. It is striking that a single hormone/signaling pathway can mediate such evolutionary conserved but functionally diverse transcriptional responses. TH actions are mediated through binding to specific receptors, the thyroid hormone receptors (THR), that belong to the superfamily of nuclear receptor transcription factors (1). Ligand binding modulates the receptor's biological activity, resulting in the transcriptional regulation of a large set of target genes (2, 3). TH stimulation typically leads to massive changes in the transcriptional state of the cell through both direct and indirect effects (2). To this respect, by offering a highly contrasted biological response, the Xenopus model has been instrumental to decipher the mechanisms of action of TH at physiological, cellular and molecular levels (3–6). The repertoire of TH target genes differs considerably in a cell- and/or tissue-specific manner, as it reflects new differentiation stages, metabolic states or other cell specific programs (7). Nonetheless, a very small number of genes (among which *klf9*) are differentially regulated in almost all tissues, suggesting that they belong to a core set of genes mediating TH response [reviewed in (7)]. Cross species comparison revealed that despite important species-specific TH responsive gene sets, homologous tissues often respond by using a core subset of genes (typically <1% of regulated genes), further strengthening the view of an evolutionary conserved molecular machinery (7). As such, and given its highly contrasted phenotypic changes, post-embryonic development has been a leading model to dissect the molecular, cellular and physiological changes and tissue remodeling initiated by TH signaling (8–10).

Post-embryonic development corresponds to the transition to a phenotypically distinct juvenile. This is an ancestral character shared by all extant tetrapods, and all chordates. Despite similar control by TH, the post-embryonic transition is quite diverse as it coincides with hatching in reptiles and birds (11, 12) and the perinatal period in mammals (10, 13, 14). In some species, this transition is so spectacular that it has been named "metamorphosis," to reflect the profound morphological and ecological differences between a larva and a juvenile. In flatfish, post-embryonic development culminates with the migration through the skull of one eye of the symmetric larvae to the other side of the face (15). In anuran amphibians, this transition corresponds to the transformation of a tadpole into a frog (16). Most of the current knowledge on amphibian metamorphosis results from work on the anuran *Xenopus laevis* and *Xenopus tropicalis,* which have been instrumental in dissecting the anatomical, cellular and molecular processes taking place. In particular, the molecular mechanisms of the TH signaling have been the focus of numerous studies and are now well-described [reviewed in (3) and (17)], although this is still a subject of active research (18).

However, not all amphibians metamorphose and alternative post-embryonic development strategies are common, as seen in the case of paedomorphosis, where sexually mature "adults" of a species retain larval features of their ancestors (e.g., Ambystomatids) (8, 19). This relaxes the dependence on the changes of ecological niche imposed by metamorphosis (19). These alternative developmental strategies are thus ideal "natural experiments" for (1) investigating the link between TH signaling and the control of post-embryonic development, and (2) identifying the changes of transcriptional states driving the adaptive variations of post-embryonic transitions.

The Mexican Axolotl (*Ambystoma mexicanum*) is the textbook example of an amphibian paedomorphic species (19) and is an attractive model to address these issues. Although rare in nature, metamorphosis in sexually mature Axolotl can be induced after long treatments with TH (20). Axolotl expresses functional TH receptors and typical TH response genes are differentially regulated [e.g., collagenase 3 and matrix metallopeptidase 11 (*mmp11*)] upon TH treatment in paedomorphs (21). As soon as 2 weeks post-hatching, the thyroid gland fixes iodine (22) and starts releasing TH, resulting in a peak of 20–40 nM thyroxine (T_4) secretion [(23), **Figure 1**, developmental period later designated as the High TH Period (HTP)], but while limbs develop, larvae fail to metamorphose, as illustrated by other features such as gill growth and tailfin that remain anatomically unaltered (**Figure 1**). This TH peak also correlates with a rapid and transient increase of deiodinase2 (D2, TH activating enzyme) protein content in brain, up to the larvae-paedomorph transition, where closely related *Ambystoma* species undergo metamorphosis (but Axolotl doesn't). From this stage, D2 levels drop and deiodinase 3 levels (D3, TH inactivating enzyme), which were low, start raising (23). In agreement with these observations, early treatment of TH in rearing water prior to the endogenous TH peak does not induce metamorphosis (22), but results in miniature paedomorphs displaying extended growth of limbs and gills, but no tailfin resorption. Although previous report (26) describes accelerated development and early gills regression after intramuscular injection of TH in animals of various age (in the perivitelline space for 3 days post-spawning animals), this certainly does not reflect a physiological response because of the very high doses injected (10 to 60 μg TH per animal) and the fact that most animals died afterward. Therefore, it is not clear whether the lack of metamorphosis results from defect in TH signaling, as hypothesized previously based on experiments carried out on old paedomorphs (27). To date, the molecular determinism controlling paedomorphosis is still unknown and the current perception is that apart from the gonadal development program, paedomorph and larval tissues would be indistinguishable.

In this paper, we hypothesized that Axolotl tailfin almost certainly responds to TH, at the HTP. We ask whether and how Axolotl tailfin responds to TH at HTP. We also compared TH response between the Axolotl and the anuran amphibian *Xenopus tropicalis* (*X. tropicalis*), for which the post-hatching HTP is marked by metamorphosis (limb growth, and gills and tailfin resorption). We found that Axolotl tailfin responds strongly (>400 genes) and quickly (within 24 h) to TH. In the two species, tailfin response to TH mobilizes overlapping networks of biological pathways, despite little overlap between the two sets of differentially expressed (DE) genes. The developmental

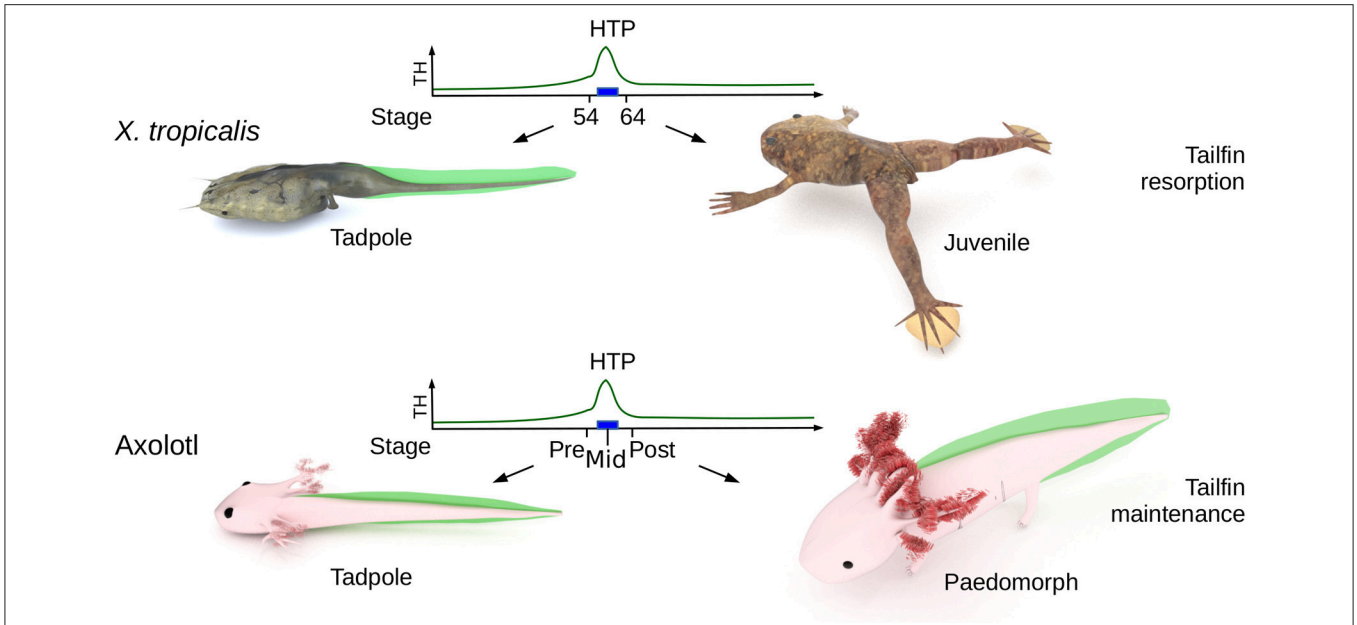

FIGURE 1 | Biological context. The High Thyroid Hormone Period (HTP), that marks the end of tadpole stage (blue rectangle) in *X. tropicalis*, ignites metamorphosis and the resorption of larval tissues (such as tailfin). In Axolotl, the HTP does not induces tailfin resorption. "pre-HTP" animals will refer to class 3 Axolotl [as defined by Rosenkilde et al. (23)], when limb buds start growing (around 2 weeks post-hatching). At this stage, endogenous TH level is low and thyroid gland starts releasing TH. "mid-HTP" animals refer to class 8 Axolotl, with four toes on hind limbs (between 32 and 48 days post-hatching). This stage corresponds to the highest level of endogenous T_4. "post-HTP" animals refer to class 12 Axolotl (around 3 months old), where T_4 endogenous level dropped significantly. *X. tropicalis* tadpoles were staged according to the normal table of *Xenopus laevis* (Daudin) of Nieuwkoop and Faber (24). The TH levels are schematized from data of Leloup and Buscaglia (25) for Xenopus and of Rosenkilde et al. (23) for Axolotl. Digital paintings were carried out with BLENDER v2.8b.

program (maintenance vs. resorption) is mirrored by alternative transcriptional states of a single subnetwork, structured around actg1 and fos. We thus propose that this subnetwork would be a component of a molecular switch involved in tailfin fate at HTP and the commitment into resorption vs. maintenance programs.

MATERIALS AND METHODS

Animal Care and Treatments

All *Ambystoma mexicanum* (Axolotl) animals used in this study were obtained from Maison de l'eau (Villerville, France) and from a generous gift of Yannick Andéol (Sorbonne Université, Paris, France). Embryos were kept in tap dechlorinated water at room temperature and fed daily with artemia. They were staged with respect to the development table proposed by Rosenkilde et al. (23). Experiments have been conducted at class 3 (corresponding to 32 days old post-fertilization, around 2 weeks post-hatching, and prior to the TH peak during post-embryonic development), on class 8 (corresponding to 48 days old post-fertilization, corresponding to 1 month post-hatching, and at TH peak during post-embryonic development) and at class 12–13 (corresponding to 6 months old post-fertilization). We refer to these stages as pre-, mid- and post-HTP, respectively (**Figure 1**). The main active TH, T_3 (T2752, SIGMA, St. Quentin Fallavier, France) was dissolved in 0.1 N NaOH and added to the culture medium to a final concentration of 10 nM. Control treatments correspond to an equivalent amount of 0.1 N NaOH. *X. tropicalis* tadpoles were raised at 26°C and staged according to the normal table of *Xenopus laevis* (Daudin) of Nieuwkoop and Faber (24). For TH treatment, tadpoles at stage NF54 were exposed 24 h to 10 nM T_3. Animals were euthanized after anesthesia (ref: E10505; 0.01% MS222, SIGMA) before dissection. Animal care and experimental work was carried out in accordance with institutional and national guidelines and under permission granted in animal license number 00372.02 (*A. mexicanum*) and 68008 (*X. tropicalis*) delivered by the Cuvier Ethic Committee.

RNA Extraction

For Axolotl, tailfin was collected from 5 individuals (8 to 11 groups per conditions) for pre-HTP (class 3) animals, from 5 individuals (3 groups per conditions) for mid-HTP (class 8) animals, from 3 individuals (5 groups per conditions) for post-HTP (class 12–13) animals and from 5 individuals (8 to 11 groups per conditions) for 6 months old paedomorphs. Tissues were flash frozen, and stored at −80°C before RNA extraction. For *X. tropicalis*, tailfins were collected from 10 tadpoles (3 to 8 groups per treatment conditions), flash frozen, and stored at −80°C. Tissues were lysed in 500 µl of RNAble (GEXEXT00-0U; Eurobio, Les Ulis, France) with one bead (INOX AISI 304 grade 100 AFBMA) using the Tissue Lyser II apparatus (QIAGEN, Courtaboeuf, France) for 1 min at 30 Hz. A total of 100 µL of chloroform was then added to the lysate. After 5 min incubation on ice, they were centrifuged 15 min at 12,000 g, 4°C. RNAs were purified from supernatant with the RNeasy

MinElute Cleanup kit (ref: 74204, QIAGEN) according to the manufacturer's instructions. RNA concentration was measured with nanodrop and RNA quality was assayed using Agilent Bionalyzer with standard procedure. To avoid any potential contamination with genomic DNA, RNA samples were treated with DNAse following the provider instruction (Turbo DNA free; Ambion, Applied Biosystems, Courtaboeuf, France).

Illumina Sequencing

Library preparation and Illumina sequencing were performed at the Paris Genomic Center (France). Messenger (polyA+) RNAs were purified from 1 µg of total RNA using oligo(dT). Libraries were prepared using the strand non-specific RNA-Seq library preparation TruSeq RNA Sample Prep v2 kit (Illumina). Libraries were multiplexed by 2 on a single flow cell lane and

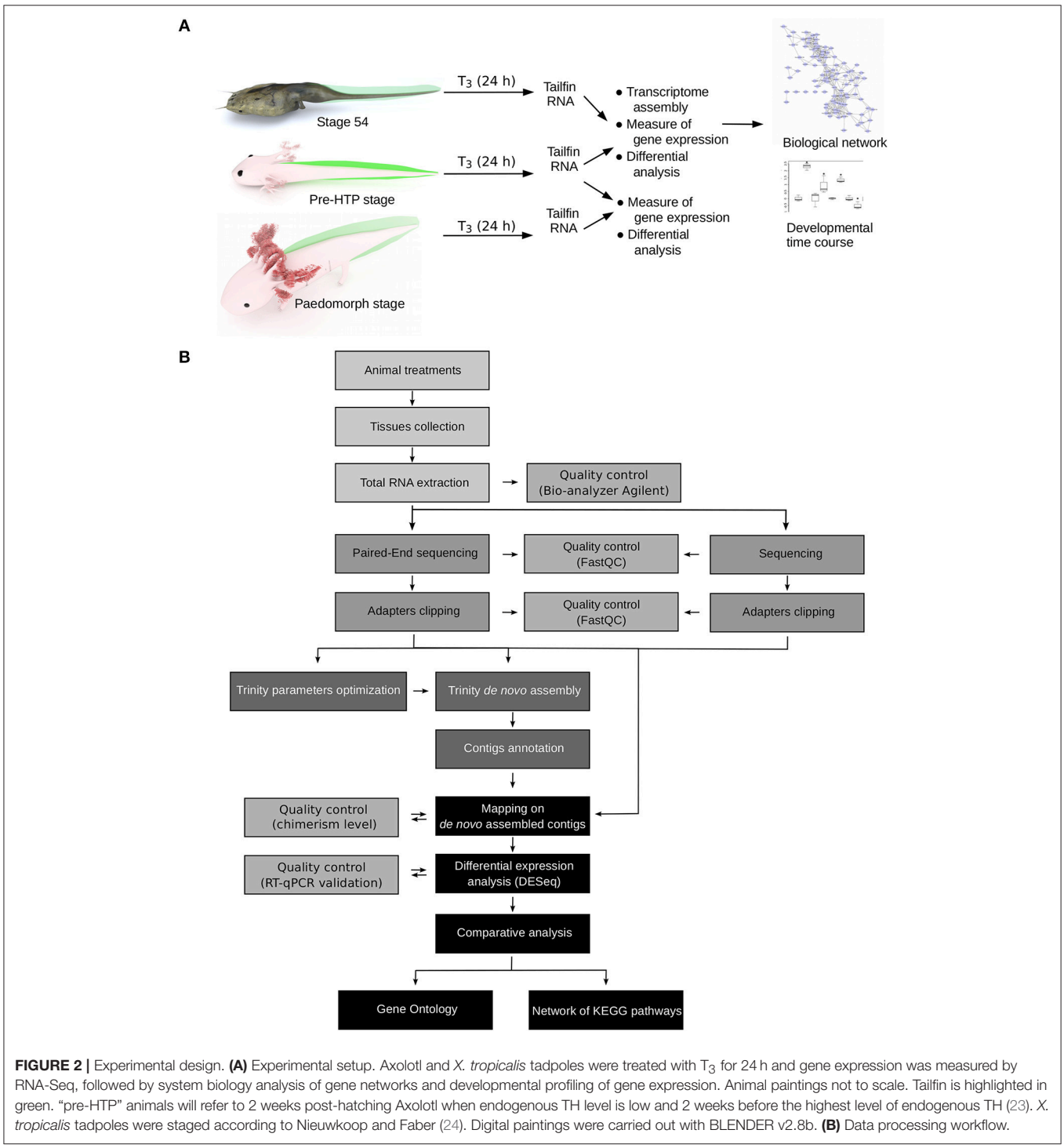

FIGURE 2 | Experimental design. **(A)** Experimental setup. Axolotl and *X. tropicalis* tadpoles were treated with T_3 for 24 h and gene expression was measured by RNA-Seq, followed by system biology analysis of gene networks and developmental profiling of gene expression. Animal paintings not to scale. Tailfin is highlighted in green. "pre-HTP" animals will refer to 2 weeks post-hatching Axolotl when endogenous TH level is low and 2 weeks before the highest level of endogenous TH (23). *X. tropicalis* tadpoles were staged according to Nieuwkoop and Faber (24). Digital paintings were carried out with BLENDER v2.8b. **(B)** Data processing workflow.

subjected to 100 bp paired read sequencing on a HiSeq 1500 device. For the comparison between T_3 response at pre-HTP and 6 months old paedomorphs, libraries were multiplexed on 4 lanes on a NextSeq 5000 apparatus. Reads qualities were assessed with the FASTQC toolkit v0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Axolotl raw data have been deposited on the Short Read Archive under accession numbers SRP067617, SRR810197, and SRR8101977, and X. *tropicalis* under accession numbers PRJNA240154. For the comparison between pre-HTP and 6 months old paedormorphs, samples were prepared following the same protocol, but subject to conventional 75 bp Illumina single end sequencing (TrueSeq), according to the manufacturer recommendations.

ONT Sequencing

RNA samples were sequenced with Oxford Nanopore Technology, following standard procedures on two

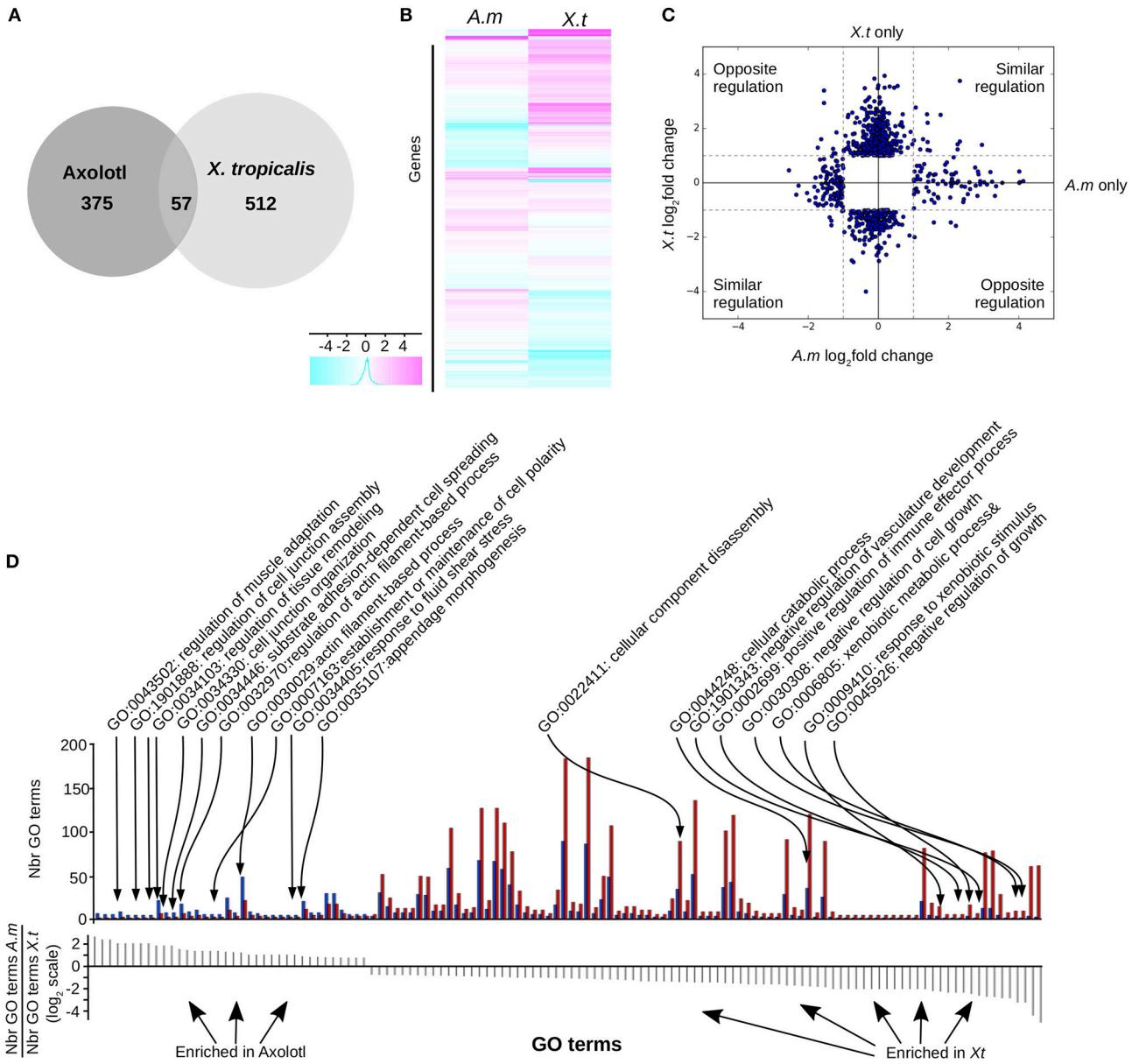

FIGURE 3 | T_3 regulates different gene sets in Axolotl and *X. tropicalis* at the post-embryonic transition. **(A)** Overlap between differentially expressed genes in both species. **(B)** Heatmap of differentially expressed genes in Axolotl (A.m) and *X. tropicalis* (X.t). **(C)** Log2 ratio of TH-induced gene expression changes. **(D)** Gene ontology analysis. Top: Number of genes for each GO term (not shown), on both species. Blue: Number (Nbr) of terms found in Axolotl. Red: Number of terms found in *X. tropicalis*. Bottom: ratio of the number of terms found in both species for each GO term (in the same order as the top panel). Positive and negative values correspond to terms mostly found in Axolotl or *X. tropicalis* gene set, respectively.

1D^2 flow cells, and raw data deposited under the SRA reference PRJNA498010.

Transcripts *de novo* Assembly and Evaluation of the Assembly Procedure

Sequences were trimmed with AlienTrimmer v0.4.0 (28) to remove adaptor contaminants and low-quality sequences (Phred score $\leq$ 26). *De novo* assembly was carried out with Trinity v2.0.6 (29, 30) with $\geq$120 M paired-reads, and contigs shorter than 200 bases were discarded. Assembly with increasing number of paired-reads (5 to 120 M) randomly picked from the initial dataset showed that saturation is reached as early as 50 M reads from tailfin RNA. TRINITY run time options have little effect on assembly statistics. See (31) for a detailed description of the assembly and clustering procedures. The "long" reads produced by ONT sequencing of the same RNA samples were

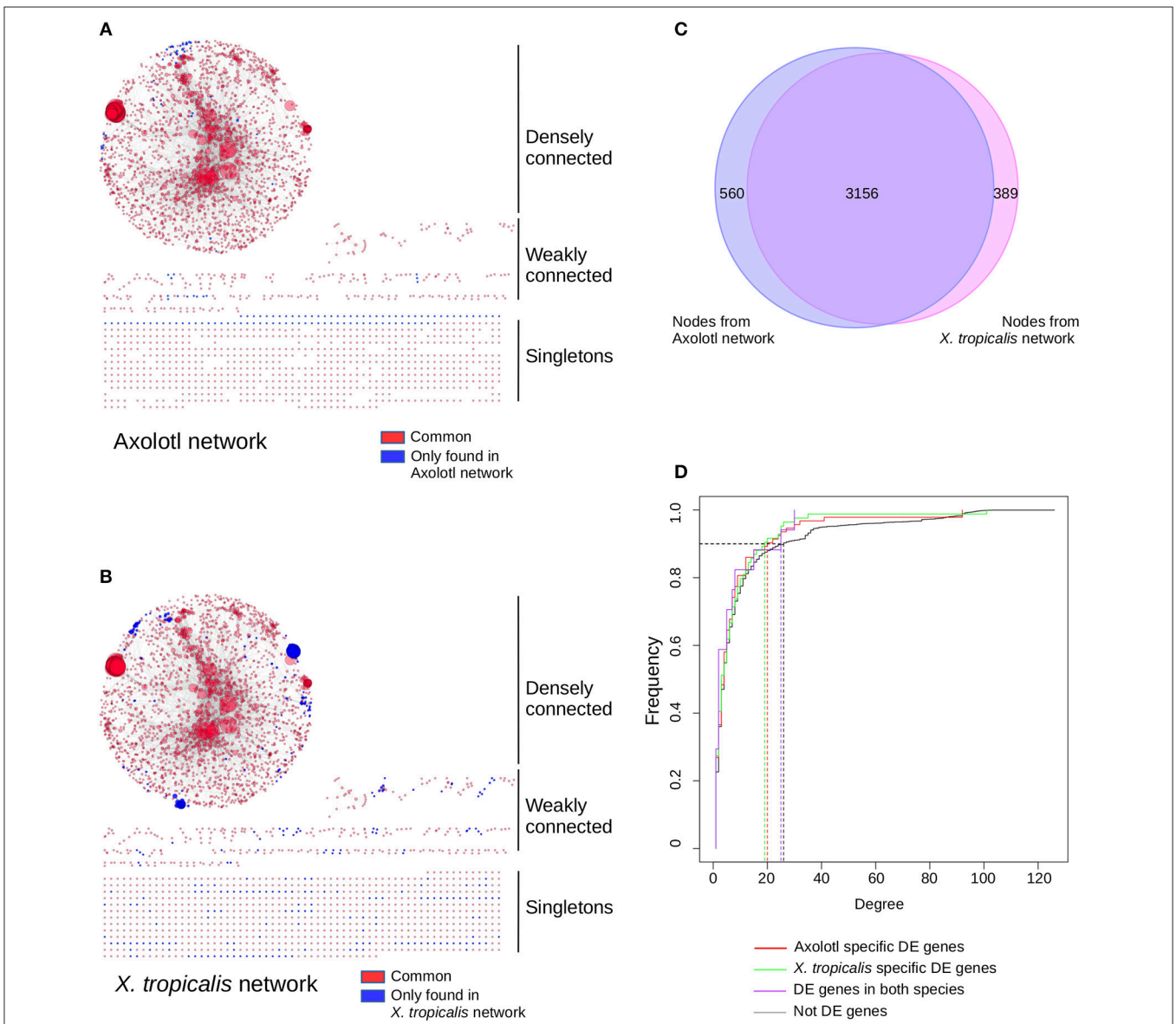

FIGURE 4 | T_3 affect a similar network of pathways in both species, despite regulating different sets of genes. Networks of KEGG pathways affected in Axolotl **(A)** and *X. tropicalis* **(B)**. The reconstructed network for Axolotl is composed of 3,305 nodes and 12,776 edges, with a densely connected component (2,274 nodes and 12,554 edges), some weakly connected genes (273 genes and 222 edges) and a set of singletons (758 nodes). The *X. tropicalis* network is composed of 3,561 nodes and 16,237 edges, with a highly interconnected component (2,443 nodes and 15,950 edges), some weakly connected genes (318 genes and 287 edges) and a set of singletons (800 nodes). Nodes correspond to gene products, linked together by the functional interactions described in the pathways (edges). Individual node size is proportional to the number of nodes connected to it. Large nodes thus correspond to hubs between KEGG pathways. Red: nodes in common to both networks. Blue: nodes only found in one (or the other) network. Layout computed with the prefuse force directed algorithm. **(C)** Overlap between the node (gene product) content of the two networks. **(D)** Cumulative distribution of node connectivity (degree). In both species, T_3 do not target (or avoid) specific network components.

used to assess transcripts chimerism (**Supplementary Figure 1**). Assembly statistics are provided **Supplementary Tables 1–3**.

Annotation of Transcriptomes Assembled *de novo*

Assembled reference sequences were aligned to the non-redundant (nr) protein sequence database (NCBI, release date November 2014) and to all *X. tropicalis* RefSeq protein sequences using BLASTX (32). BLASTX alignments took 720 days (CPU time) on a server with 16 cores and 64 GB of memory. Matches with a bit-score lower than 100 were discarded. Reference sequences were named according to the best reverse BLAST hit (BRBH, highest score). If several reference sequences were assigned to the same protein sequence, only the longest with the best score was kept. Annotation of Axolotl transcripts was carried out with the BLOSUM45 matrix.

Differential Analysis of Gene Expression

For each species, filtered reads were mapped to the corresponding set of reference sequences with Bowtie v0.12.7 (33) and the "-l 32−3 10−5 10 -n 2 -m 1" parameters. Read counts were calculated for each reference sequences matched by at least one read and reference sequences with low read-counts (< 50) were discarded. To further estimate the accuracy of our differential expression analysis procedure in reflecting true biological variations, we compared the *X. tropicalis* gene expression levels to those obtained after conventional RNA-Seq carried out by our group (three biological replicates, single-end reads mapped on the genome sequence, unpublished data). We found that the log ratios from the two approaches correlated very well ($r > 0.8$) (**Supplementary Table 4**), thus illustrating the accuracy of our approach to measure gene expression and report differential levels of RNA species. Raw read counts were subjected to a variance-stabilization transformation as described in Anders and Huber (34) and followed by Principal Component Analysis (PCA), to partition biological and technological variability of the experiments into covariant components. Ideally, one expects the first components to capture most of the biological variability, with little non-biological effect (i.e., technical noise). On the contrary, major components poorly connected to biological variables (treatment, animals) would indicate that the dataset is dominated by noise. We found that the first component (72% of the total variance) corresponds to the species-specific variance, the second and third components (PC2, 17.8% and PC3, 9.9%) correspond to the TH treatment in one or the other species, thus showing a low level of technical noise. Since the lack of replicates did not allow us to run a proper statistical analysis, DE genes were defined as having a fold change (in log2 scale) superior or equal to +/-1, after trimmed mean of M-values (TMM) normalization using DESeq v1.14.0 (34) in blind mode and with fit-only option. Gene Ontology analysis was carried out with goProfiles 1.32.0 (35), which allows one to directly compare the GO terms associated to two DE genes lists for a direct visualization of the biological processes favored in one species vs. the other.

RT-qPCR

Reverse transcription was carried out from 2.5 μg of total RNA, first mixed with dNTP (2 μL, 10 mM, Invitrogen) and random primers (1 μL, 50 μM, Invitrogen) in a final volume of 12 μL (with H_2O DEPC, Ambion), and incubated at 65°C for 5 min. After hybridization, samples were put on ice prior to the addition of 1 μL H_2O DEPC, 1 μL 0.1 M DTT (Invitrogen) and 4 μL 5X first strand buffer (Invitrogen). RNAse inhibitors (1 μL, RNAse out, Invitrogen) and reverse transcriptase (1 μL, SuperScript III, Invitrogen) were added and the reaction was incubated at 25°C for 10 min, followed by an incubation at 42°C for 40 min. Primer express (Applied Biosystems) was used to design primers (see **Supplementary Table 5** for Axolotl and **Supplementary Table 6** for *X. tropicalis*). Primer choice for RT-qPCR validation was optimized by combining coding sequence conservation and the reads distribution along Axolotl reference sequences. BLASTX alignments between Axolotl reference sequences and the corresponding Xenopus analogous coding sequences were visualized by dot-plot (a few illustrative examples are shown in **Supplementary Figure 2**). This allowed us to directly control that evidences for differential expression are unambiguously located in properly assembled transcripts, and are not an assembly artifact resulting in chimeric transcripts. qPCRs were performed on an ABI 7300 (Applied Biosystems) and analyzed with the Prism 7300 system software (Applied Biosystems). *H3f3a* and *rpl8* were selected as control genes for normalization, using Normfinder (36) (data not shown). *H3f3a* was used to compare untreated groups vs. T_3 treated groups and *rpl8* was used to compare developmental stages. Raw data were normalized on the control gene and on the non-treated sample by the 2 CT method. Results are presented as means of Log(2CT) with standard deviation (SD). Statistical analyses, based on 3 to 11 biological replicates, were performed with the Mann and Whitney test ($\alpha = 5\%$).

Network Analysis

The overlap between the sets of DE genes and KEGG pathways (Kyoto Encyclopedia of genes and Genomes database) was carried out with JEPETTO [Java Enrichment of Pathways Extended To Topology (37)], a plugin to Cytoscape v3.2.0 (38). All KEGG pathways containing at least one DE gene were collected and merged to create a global functional interaction network (see main text for description). Cytoscape was used to visualize the network and compute network properties. Hubs are defined as nodes (i.e., gene product) with a degree (a.k.a connectivity) higher than 20. Empirical cumulative degree distributions were computed with the ecdf function of the Hmisc R package. The degree distribution in *X. tropicalis* network exhibit a bimodal degree distribution (not shown). This is due to a complex of 59 DE ribosomal protein coding genes, which are highly interconnected, and as such, are characterized by a high degree. Given that they are also poorly connected to the rest of the network and form a self-contained sub-network, they were excluded from the analysis.

The identification of the dense sub-network of DE genes is based on Z-score statistics, computed by randomly shuffling the gene status (whether they are differentially expressed or not)

and counting the number of DE genes in the neighborhood of actg1 and fos. This process, repeated 1,000 times, estimates the (normal) background distribution of the number of DE genes found by change. The actual number of DE genes observed around actg1 and fos is then compared to the distribution, which is used to derive the z-score (i.e., number of standard deviations from the mean) and *p*-values.

RESULTS

How to explain, in term of transcriptional regulation, that *X. tropicalis* and Axolotl tailfin respond differently to TH at HTP? Our experimental set up and data processing workflow to address this question are described **Figure 2**. Briefly, we used pre-HTP *X. tropicalis* (pre-metamorphic, stage 54) and Axolotl (Class 3 Axolotl as defined by 23), corresponding to 2 weeks post-hatching tadpoles when limb buds start growing, endogenous TH level is low and thyroid gland starts releasing T_4. Animals were treated with 10 nM T_3 for 24 h, tissues were collected and RNAs subjected to paired-end sequencing to measure gene expression. The choice of tissue collection at 24 h is based on the fact that in *X. tropicalis*, expression of typical DE response genes (e.g., *klf9*, *mmp11*, *thbzip*) is strongly induced as soon as 24 h post-treatment. This is also certainly true for Axolotl, where collagenase3 and stromelysin3 expression is also strongly induced of after 48 h of T_3 treatment (21). In fact, a fast transcriptional regulation may be a general feature of T_3 response, since it is also true in mice (39). After differential expression analysis, transcriptomic responses were further characterized by topological analysis of biological networks. We also characterized in Axolotl the transcriptional response naturally occurring at the HTP, or after T_3 treatments at later stages (6 months old). Given that no genome sequence was available for Axolotl at the time of this work, we used RNA-Seq paired-end reads to assemble the transcriptome and generate the repertoire of coding sequences. Importantly, this also helped capture specific transcripts originating from an embryonic tissue. RNA-Seq reads were then mapped to the reference sequences to measure gene expression. In order to circumvent technological biases, and despite the availability of an improved genome sequence and annotation (40), *X. tropicalis* RNAs were subjected to the same procedure (paired-end sequencing followed by transcriptome assembly) to generate an equivalent set of reference sequences. In fact, at the evaluation step of the bioinformatic pipeline, the around 25,000 known *X. tropicalis* coding sequences were used as a gold standard to evaluate the assembly process and set optimal parameters for assembly and clustering (**Supplementary Tables 1**, **2**). Coding sequences were then annotated by comparison to *X. tropicalis* and NCBI's nr databases. Systematic dot-plot comparison of assembled transcripts to homologous sequences together with ONT sequencing of the same RNA samples showed that the chimerism level, a typical artifact of transcriptome assembly, is low (∼2%, **Supplementary Figure 1**) and that the vast majority of transcripts align well to their cognate homolog (see illustrative examples **Supplementary Figure 2**). Overall, we produced a high quality set of 17,990 *X. tropicalis* coding sequences, and 21,141 Axolotl coding sequences, that will be used as a proxy for gene reference sequences and differential analysis of gene expression. This corresponds to a set of 9,006 homologous genes common to the two species.

An Early TH-Dependent Transcriptional Dynamic in Both Axolotl and *X. tropicalis* Tailfin

Measure of gene expression was carried out for each species by mapping the paired-end reads on the gene reference sequences that were assembled (**Supplementary Table 7**), and was followed by differential analysis. Principal Component Analysis (PCA, data not shown) indicates that our data set is dominated by biological signal, with very little experimental noise.

We found (**Figure 3**) 569 (6.3%) differentially expressed (DE) genes in *X. tropicalis*, 303 up- and 266 down-regulated, and 432 (4.8%) in Axolotl, 153 up- and 279 down-regulated (see **Supplementary Tables 8**, **9**, respectively). These genes include known TH responsive genes previously described for both species, such as *klf9* and *mmp11* (21). A number of genes belonging to the TH signaling pathway (*thra*, *dio2,* and *thbzip*) have been filtered out because of low reads count, thus making any differential measure of their expression hazardous. For *thrb*, this agrees well with the work of Safi et al. (21), who also reported a very weak expression level in Axolotl. For Axolotl, we confirmed the differential expression status of 20 up and down regulated genes by RT-qPCR (**Figures 5C**, **6**, **Supplementary Figure 3**). For *X. tropicalis*, the DE gene list is well in line with previously published micro-array experiments (41), and has been validated experimentally (**Supplementary Figure 4**), together with seven additional genes by RT-qPCR (**Figure 5D**). These results unambiguously show that Axolotl tailfin responds to T_3 at the transcriptional level, to an extend somewhat similar to *X. tropicalis*. Only a few genes (57 genes) were T_3 responsive in both species (**Figure 3**, **Supplementary Table 10**), of which 32 exhibited a common regulation (14 up- and 18 down-regulated). This subset of co-regulated genes includes the well-known TH responsive gene *dio3* and is also enriched in transcription factors such as *brca1*, *fosl2*, *klf13*, *klf17*, *klf9*, *nr4a2*, *sox4,* and *znf395*. Except for actg1 and fos, genes with opposite regulation in the two species *(rhof, ankrd1, lamc2, epb41l3, itga11, hhipl2, crispld2, cdh2...*, **Figure 3C**) are exclusively composed of membrane bound or extracellular matrix proteins.

We next carried out Gene Ontology analysis in order to contrast the "biological processes" GO terms found in the gene sets of both species. This analysis revealed significant alternative usage of GO terms (Fischer test, $p<10^{-4}$, see Materials and Methods) between species (**Figure 3D**, **Supplementary Table 11**). In Axolotl, DE genes are involved in various aspects of tissue remodeling (e.g., cell junctions organization, cell adhesion, extracellular matrix (ECM) organization and structure) together with a number of terms related to actin biology. In contrast, in *X. tropicalis*, DE genes are more involved in diverse catabolic processes, disassembly of cellular components, regulation of cell growth, and several components of the immune system, fitting well the known biology of tailfin resorption programs.

TABLE 1 | List of hubs (genes) differentially expressed in either Axolotl or *X. tropicalis*.

Gene name	Axolotl _log2FC	*X. tropicalis* _log2FC	Degree	Neighborhood connectivity
AXOLOTL SPECIFIC HUBS				
AQR	−1.67	0.9	92	85.59
EGFR	−1.77	0.26	92	24.63
ERBB2	−1.71	0.3	41	32.24
PTK2B	−1.33	−0.25	27	43.96
MAP2K1	−1.02	−0.18	24	31.58
HUBS REGULATED IN BOTH SPECIES				
ACTG1	1.42	−1.96	30	12.6
FOS	1.39	−2.26	25	27.08
***X. tropicalis* SPECIFIC HUBS (EXCLUDING RIBOSOMAL PROTEINS)**				
LSM2	0.1	1.25	101	79.47
FAU	0.19	−1.59	72	67.29
PSME3	0.08	1.58	35	34.23
POLR2H	0.21	1.16	26	14.08
IRS2	−0.57	1.13	25	34.04
MCM5	−0.32	−1.44	24	22.67

FC, Fold change.

These results show that as soon as 2 weeks post-hatching, T_3 treatment induces the differential regulation of many genes in Axolotl tailfin, including genes often associated with TH transcriptional responses. Therefore, and this is an important result, the HTP corresponds to a TH sensitive period in Axolotl tailfin, despite the lack of visible anatomical change. The programs induced in Axolotl and *X. tropicalis* differ significantly, except for a core set of genes involved in TH signaling and transcriptional regulation. Likewise, transcriptional responses contrast sharply in term of biological processes (remodeling of the acto-myosin network vs. tail resorption program).

Integrated Functional Relationships Between DE Genes

To get a functional and integrated view of the molecular phenotype described by our dataset, we took advantage of the KEGG pathways (42), a collection of curated functional interactions organized in a number of well-identified pathways. Given that components are often shared between pathways, we undertook to reconstruct a network of all the KEGG pathways having at least one DE gene (i.e., not all genes are DE in the network). In this network, nodes represent gene products and edges represent functional connections (phosphorylates, activates, represses...) between them. The first advantage of this approach is to provide an integrated view of the functional interactions between gene products and thus address how T_3 response is orchestrated between biological pathways, and whether this response is different between the two species. In Axolotl, 116 genes (out of 412 DE genes) could be assigned to 112 KEGG pathways (**Supplementary Table 12**). In accordance with our GO enrichment analysis (and despite the medical orientation of their name), the most represented pathways have strong components related to actin, cytoskeleton and ECM biology: "ECM-receptor interaction," "Hypertrophic cardiomyopathy (HCM)," "Dilated cardiomyopathy," "Focal adhesion." In *X. tropicalis*, 171 (out of 569 DE genes) could be assigned to 136 KEGG pathways (**Supplementary Table 13**), with many metabolism-related pathways. The reconstructed networks are shown **Figure 4**. The Axolotl network has a total of 3,305 nodes and 12,776 edges, together with 758 singletons (unconnected nodes). The *X. tropicalis* network is composed of 3,561 nodes and 16,237 edges, with 800 singletons. From the 57 T_3 responsive genes common to both species, 19 mapped to KEGG pathways and 14 were located in the highly connected components of the two networks. Remarkably, the two networks were highly similar with 99 KEGG pathways and 3,156 nodes in common (95% of nodes from Axolotl network and 89% of *X. tropicalis*, **Figure 4C**), illustrating that despite a limited overlap between DE genes sets, T_3 response affects similar pathways in both species.

The second advantage of a network of pathways is to identify integration points between multiple pathways within a network (i.e., gene products shared between multiple pathways), which ensure communication and signal propagation between subnetworks. They are well-known to have a strong structural role in biological networks, which is (in part) responsible for their robustness, and functionally targeting them has a strong predicted biological impact (43). This is the underlying postulate of our approach: regulating the expression of a gene product shared between multiple pathways will likely have large biological effects since this will simultaneously affect multiple pathways. Here, we addressed how T_3 response preferentially targets (or avoids) the gene products shared between multiple pathways and how this differs between Axolotl and *X. tropicalis* responses. More formally, gene products shared between pathways correspond to highly connected nodes ("hubs") and the question can simply be put as the relationship between biological responses (DE/non- DE) and node connectivity. We first characterized the relationship between the degree (level of connectivity) of DE genes and T_3 response, in both species, in order to characterize the transcriptional dynamic of their network. The connectivity of a node is measured by a simple metric, the degree, which corresponds to the total number of edges (i.e., functional interactions) connected to it. We thus plotted the cumulative degree distribution of DE genes and non-DE genes. This analysis is akin to ROC curve analysis. We found a similar distribution for Axolotl and *X. tropicalis* DE genes, as well as non-DE genes (**Figure 4D**). As a result, in one species vs. the other, the TH response (1) affects nodes with similar connectivity and (2) does not target a specific subset of high or low connectivity. These results clearly show that although the DE gene sets are different in both species, those described in KEGG pathways belong to (almost) identical networks and they have similar transcriptional impact on the network as a whole.

We next focused on hubs (highly connected nodes). Overall, Axolotl and *X. tropicalis* networks contain 309 and 422 hubs, respectively, among which 66 correspond to DE genes in one or the other species. There is a total of five DE hubs specific to Axolotl (*aqr*, *ptk2b*, *erbb2*, *map2k1*, *egfr*), all down-regulated

(**Table 1**). Remarkably, four of them are involved in Pi3k/Akt signaling, suggesting that T_3 treatment strongly impacts this second messenger signal transduction pathway. A total of 57 hubs are only differentially expressed in *X. tropicalis*, corresponding mainly to ribosomal protein coding genes, together with *mcm5*, *psme3*, *polr2h*, *lsm2*, *fau*, and *irs2* (listed in **Table 1**, without 17 the ribosomal protein coding genes) which are involved in metabolism, regulation of gene expression, and DNA repair. Strikingly, only two hubs correspond to DE genes in both species (*actg1* and *fos*), although with opposite regulation (both genes

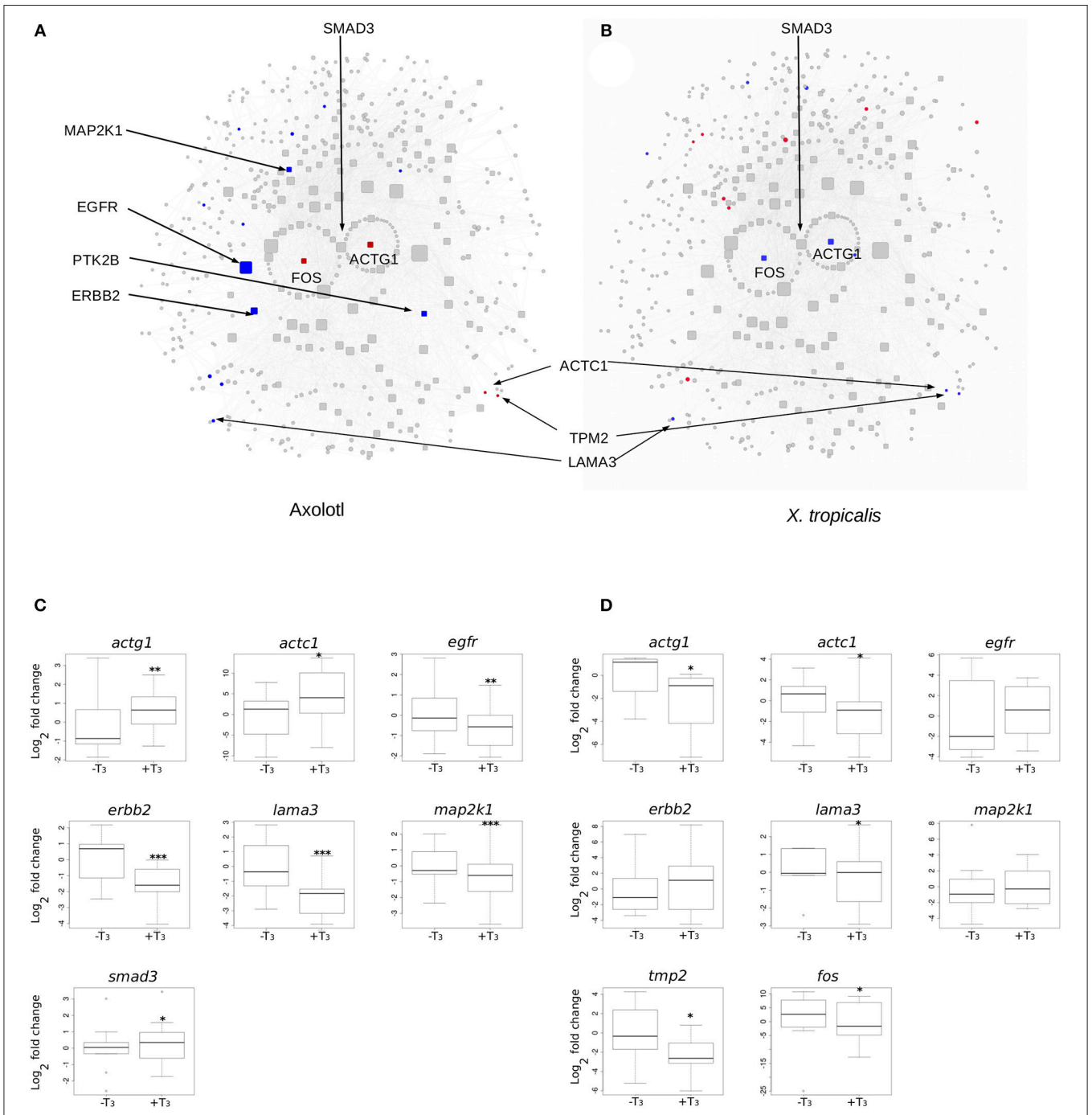

FIGURE 5 | Differential gene expression at the Actg1-Fos subnetwork, in Axolotl and *X. tropicalis*. **(A)** Axolotl subnetwork. **(B)** *X. tropicalis* subnetwork. The subnetworks are composed of the first (laid out in circle) and second neighbors of Actg1 and Fos nodes. Hubs (nodes with degree >20) are shown with rounded squares. Node size is proportional to their degree (connectivity). Colors indicate differentially expressed genes (red: induced, blue: repressed). Homologous nodes are located at the same place in both networks. **(C)** RT-qPCR analysis of DE genes in the Axolotl subnetwork. **(D)** RT-qPCR analysis of DE genes in the *X. tropicalis* subnetwork. Statistical significance (Mann-Whitney test) with $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

up-regulated in Axolotl and down-regulated in *X. tropicalis*) (**Table 1**). These two nodes are very close in the networks and are separated by a single node, *smad3* (**Figures 5A,B** respectively, for Axolotl and Xenopus), for which the differential expression level in Axolotl is well-below our threshold. However, extensive RT-qPCR analysis with a high number of biological replicates confirmed its DE status (**Figures 5C**, **7**). Apart from Smad3, Fos and Actg1 are not directly connected to other DE genes (first neighbors) but their second neighbors are. In fact, these second neighbors are particularly enriched in DE genes (19 for Axolotl, **Figure 5A**, and 13 for *X. tropicalis,* **Figure 5B**). In Axolotl, these DE genes are mainly down-regulated (16/19) and are involved in cell migration and cell apoptosis. In *X. tropicalis*, they do not show any preferred direction of change. They are mainly involved in cell proliferation, cell differentiation, cell survival, and oxidative stress. Three of these DE genes are common between the two species (*actc1*, *tpm2,* and *lama3*). The first two have opposite regulation, where mRNA levels increase in Axolotl and decrease in *X. tropicalis*. In both species, *lama3* mRNA level decreases following T_3 treatment (**Figure 5**). Interestingly, this subnetwork clusters most (four out of five; all except *aqr*) of the hubs that are differentially expressed only in Axolotl (**Figure 5A**). This represents an unusually high concentration of DE hubs around *actg1* and *fos* (z-score $= 2.029$, $p = 0.0424$). The fact that this subnetwork exists in alternative transcriptional states in both species at HTP is an important result and is noteworthy. The species-specific regulation of the components of this subnetwork has been confirmed by RT-qPCR for both Axolotl and *X. tropicalis* (**Figures 5C,D**, respectively). Overall, these results show that although the transcriptional response to TH mobilizes similar networks of biological pathways in both species, their transcriptional changes display sharp contrasts: (1) T_3 affects different network components in both species, and (2) in (and only in) Axolotl, the Fos-Actg1 subnetwork contains most of the hubs that are differentially expressed.

Transcriptional Response to T_3-Treatment Correlates With Development-Dependent Changes of Gene Expression

We next addressed whether T_3-induced gene expression changes recapitulate gene expression changes during normal development in Axolotl. To this end, we carried out quantitative RT-PCR on tailfin mRNA samples extracted from pre-HTP (2 weeks post-hatching when limb buds starts growing, refer to class 3 Axolotl as defined by 21, **Figure 1**), mid-HTP [1 month post-hatching, i.e., class 8 animals as defined by Rosenkilde et al. (23), corresponding to the maximum of the peak, **Figure 1**] and post-HTP animals (3 months old animals, refer to class 12 Axolotl as defined by 21, where TH endogenous level dropped significantly, **Figure 1**). Of the 10 genes tested, five displayed similar transcriptional responses after T_3 treatment and during the course of development (*bcl6*, *cdh2*, *myh7*, *smarcd3*, *tnn*, **Figure 6**). *Junb*, *klf9* and *itga11* are not DE between the two

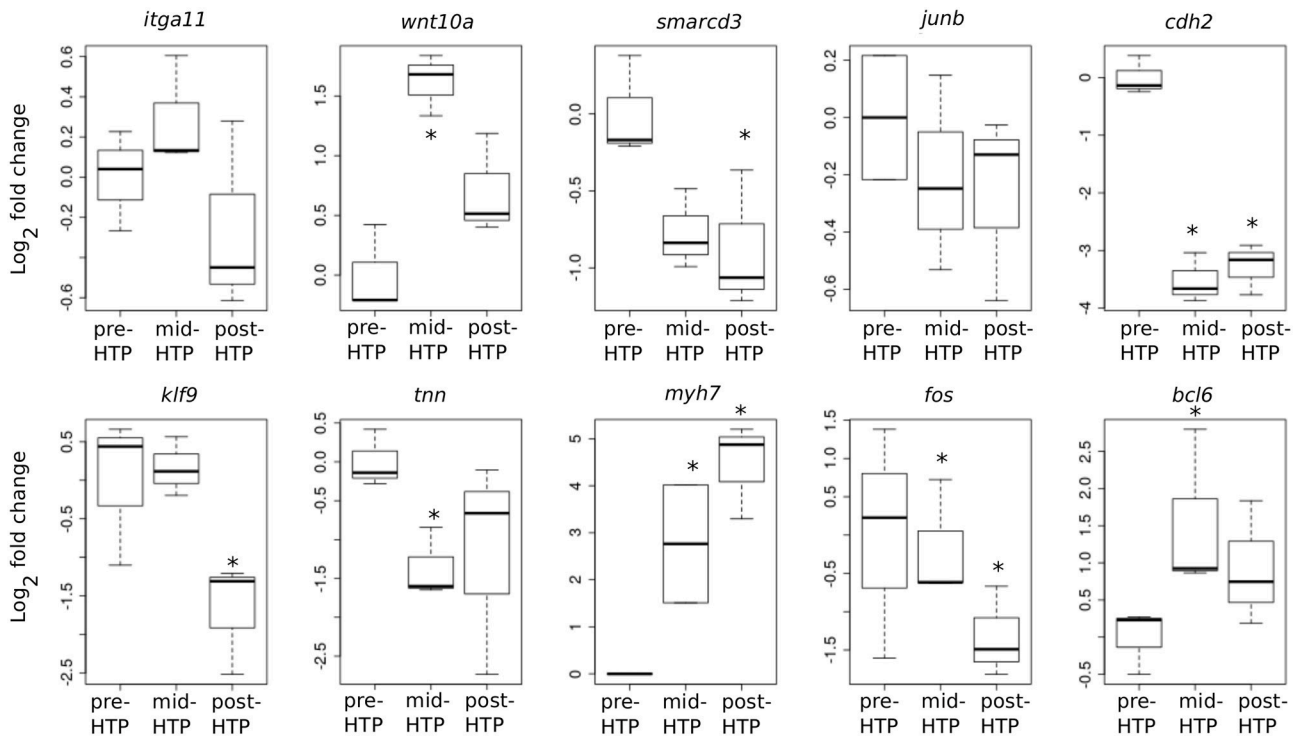

FIGURE 6 | Developmental time course of gene expression. Normalized gene expression changes (log2 Fold Change) before, during or after the endogenous peak of TH, corresponding to pre-, mid-, and post-HTP animals. Statistical significance (Mann-Whitney test) with $^*p \leq 0.05$.

developmental stages, while *junb* and *klf9* were up-regulated and *itga11* down-regulated following T_3 treatment at pre-HTP. Only, *wnt10a* showed an opposite transcriptional response to T_3 vs. developmental changes. We also note that the mRNA levels for *klf9*, a typical T_3-induced gene in tetrapods, decreased during later stages of development (**Figure 6**) as previously shown for *X. tropicalis* (44). This shows that T_3 treatment at pre-HTP induces changes of gene expression that mostly parallel endogenous transcriptional profiles changes through this transition. This also further confirms that Axolotl tailfin responds to endogenous TH as early as 2 weeks post-hatching, despite no apparent anatomical change.

A DISTINCT T_3 TRANSCRIPTIONAL RESPONSES AT HTP AND PAEDOMORPH STAGES

We next addressed whether the ability of Axolotl tailfin to respond to TH changes through development. To this end, we treated pre-HTP tadpoles and adult peadomorphs (6 months old post-hatching) with T_3 for 24 h and measured gene expression changes by RNA-Seq (see Materials and Methods, **Figure 7**). In paedomorphs, we found 109 genes differentially expressed upon T_3 treatment (**Figures 7A–C**, **Supplementary Table 14**), from which only a minority (16/109) was in common with the pre-HTP TH-responsive genes set (*bcl6, klf9, cdh2, chst6, dnajb5, fos, hspb1, klf17, mcoln1, frkb, pla2g7, pprc1, prdm1, sox4, tmprss4, ulk4*). Importantly, none of these DE genes (except *fos*) belong to the *Actg1-Fos* subnetwork, which strongly suggest that the this subnetwork is in an alternative transcriptional state at this stage. This view is also supported by the fact that *actg1* expression is not TH responsive in the paedomorph. Altogether, these results highlights the sharp contrast between TH responses at pre-HTP and paedomroph stages. Gene ontology analysis further confirms this result and shows that biological processes are differentially affected at pre-HTP and paedomorph stages (Fischer test, $p = 0.027900$, see Materials and Methods). At pre-HTP, TH response impacts several developmental processes and actin/muscle biology, which contrasts with paedomorph where TH response focus more on immune system, various differentiation processes and regeneration (**Figure 7D**, **Supplementary Table 15**).

We also carried out additional validation with a set of previously identified T_3 responsive genes, by RT-qPCR, and found good agreement with RNA-Seq data (**Figure 7E**). Interestingly, some genes (*bcl6, fos,* and *klf9*) displayed a similar response between the two stages, whereas for other (*ahr, junb, utx*), T_3 responsiveness reached significance only at the pre-HTP stage, despite a weak, but similar, trend (**Figure 7C**). Of note, *wnt10a* almost reached statistical significance, but failed because of the large biological variability of the samples. The three genes that are members of a core set of genes idiosyncratic of a TH response (7), transcription factors *bcl6, fos,* and *klf9,* display a similar response at 2 weeks post-hatching and in adult. These results strongly suggest that despite its TH responsiveness, tailfin engage specific and distinct responses at different stages. This further strengthens the fact that Axolotl tailfin at HTP undergoes a very specific TH-dependent transcriptional program.

DISCUSSION

TH mediate diverse transcriptional responses in a cell- and/or tissue-specific manner. To dissect the molecular basis underlying the variety of these biological responses, we used the well-known and evolutionary conserved post-embryonic development (frog metamorphosis, perinatal period in mammals...) as a model of TH response (9). Interestingly, Axolotl and *X. tropicalis* tailfins are homologous tissues, but yet respond differently to T_3 at a similar developmental period (limb development at HTP). This transition, quite extreme in anuran amphibians, corresponds to the abrupt and profound change of body shape known as metamorphosis. In contrast, Axolotl shows little or no sign of post-hatching transition, despite a transient surge of TH (23). Since these early works, the apparent lack of transition at this stage in Axolotl has been interpreted as a lack of TH action by some unknown mechanism (45, 46). This point has been questioned later because at this stage, thyroid signaling is already fully operational and T_3 treatment results in accelerated growth of larval features (22). In order to resolve the molecular determinism of this apparent contradiction, we set out to compare the tailfin transcriptional response to T_3 in both species. In this work, we first show that Axolotl tailfin strongly responds to T_3 at a period of high levels of endogenous TH (around 2 weeks post-hatching), despite no visible anatomic change. T_3 responsive gene sets are different between Axolotl and *X. tropicalis* but belong to the same pathways, and mirror phenotypic differences (tailfin resorption vs. maintenance). In term of regulatory mechanism of TH action, we next sought for a possible molecular subnetwork that may act as a switch that controls tailfin developmental fate. By coupling functional genomics to network biology, we could identify an Actg1-Fos subnetwork switching to alternative transcriptional states in both species, and that parallels tailfin fate.

Thyroid Hormones Induce Alternative Molecular Phenotypes in Axolotl and *X. tropicalis*

Despite limited overlap between DE gene sets, a few genes idiosyncratic of TH response (*mmp11*, fos, *klf9*) (7, 47, 48) are also differentially expressed in the 2 weeks post-hatching Axolotl. This important result not only shows that tailfin responds to T_3, but also that the transcriptional program is different from the resorption program induced in *X. tropicalis* tailfin. Tailfin responsiveness to T_3 is further supported by (1) change in gene expression *in vivo* that parallels endogenous variations of TH levels [this work and Rosenkilde et al. (23)], and (2) the fact that despite different DE gene sets (see below), the same molecular pathways are mobilized in both species. In fact, the Axolotl tailfin transcriptional response is not limited to this small subset of genes but includes 432 genes, affecting various cellular processes. While a catabolic response predominates in *X. tropicalis*, Axolotl tailfin seems to respond mainly by engaging a developmental

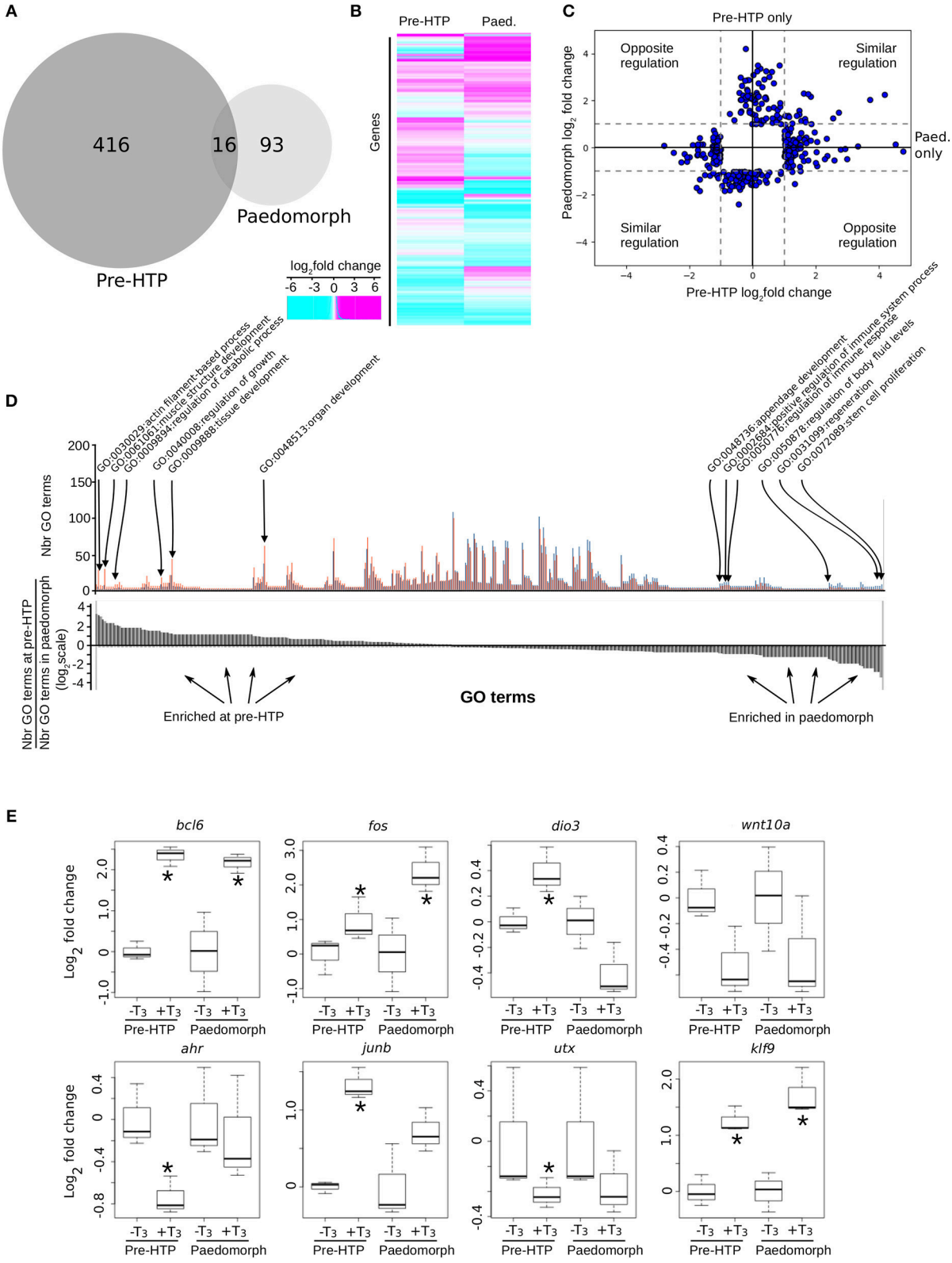

FIGURE 7 | Axolotl tailfin transcriptional responses to T_3 at pre-HTP and paedomorph stages. **(A)** Overlap between T_3-responsive gene sets at pre-HTP and 6 months old paedomorph stages, measured by RNA-Seq. **(B)** Heatmap of differentially expressed genes at pre-HTP and paedomorph stages. **(C)** Expression fold

(Continued)

FIGURE 7 | changes at pre-HTP vs. old paedomorphs. **(D)** Gene ontology analysis. Top: Number of genes for each GO term (not shown), at both stages. Red: Number (Nbr) of terms found at pre-HTP. Blue: Number of terms found in paedomorph. Bottom: ratio of the number of terms found at each stage for each GO term (in the same order as the top panel). Positive and negative values correspond to terms mostly found at pre-HTP or paedomorph gene set, respectively. **(E)** RT-qPCR normalized gene expression changes (log2 Fold Change) after T_3 treatment. Tailfin transcriptional response to T_3 differs between class 3 larvae and 6 months old paedomorphs. Statistical significance (Mann-Whitney test) with $^*p \leq 0.05$.

process. In particular, there is a strong enrichment in terms related to reorganization of the cytoskeleton and the acto-myosin network, suggestive of a transition or a remodeling of the tailfin. This is interesting because this would imply that the tailfin of young (i.e., 2 weeks post-hatching) and older (paedomorph) animals are not equivalent. In term of Axolotl biology, this raises the interesting possibility that paedomorph tailfin may have different mechanical and physical properties, maybe more suited to the increased body size of a fully-grown animal with long-term aquatic life style. This would then make the case for an early TH-sensitive period in *Xenopus* and Axolotl, that would initiate tailfin resorption or remodeling, respectively.

Strikingly, and perhaps not surprisingly, the transcriptional response in the two species was very different, with only 32 genes exhibiting similar regulation. Interestingly, 8 of these 32 genes are known transcription factors that may regulate the expression of large subsets of genes depending on the species-specific and tissue-specific chromatin context or co-regulators expression. A total of 25 genes also show opposite regulation, mainly linked to actin-myosin networks and metabolism, supportive of very different cell fates and organ outcomes. It is noteworthy that the only transcription factor in this list is Fos (see below). These different transcriptional responses (and the resulting phenotypes) in the two species could also reflect differences in the sensitivity or competency of the cells/tissues to respond to the T_3 stimulus. The regulation of THR expression or genes involved in TH availability is quite different between the two species. As expected, we confirmed that the two types of THR (α and β) are weakly expressed and not differentially regulated following T_3 treatment in Axolotl larvae (21). In *X. tropicalis* tadpoles, *thrb* mRNA levels are strongly induced following T_3 treatment; a hallmark of metamorphosis. In contrast, D3 deiodinase, the key enzyme involved in the degradation of biologically active hormone at target tissues (49), shows little species-specific transcriptional responses and its expression is induced in both Axolotl and *X. tropicalis*, following TH treatment (respectively, 4.8 vs. 2.4 fold). Interestingly, older Axolotl animals lose the ability to regulate *dio3* mRNA level in a T_3-dependent manner.

The different transcriptional responses could be mediated by the species-specific differential T_3-regulation of pioneer factors and/or genes involved in histone modification or DNA-methylation. Pioneer factors are proteins contributing to cell type-specific transcriptional competence by binding to and decondensing chromatin (50). Indeed, we observed different T_3 effect for several forkhead box transcription factors, such as Foxp4, Foxa2, and Foxp1 (down-regulated in Axolotl) or Foxo1 (up-regulated in *X. tropicalis*). In addition, the chromatin state landscape could also be quite different between the two species, as suggested by the large set of T_3 regulated chromatin modifying factors: Kdm6b (up), Men1 and Phf8 (down) in Axolotl, Carm1, Dot1L, Ezh2, Kdm6b, and Smarca4 (up) in *X. tropicalis*. The role of DNA methylation and histone methylation in metamorphic processes is currently being actively studied. For example, metamorphosis in lampreys has been associated with DNA methylation (51), and T_3-induced histone modifications have been shown to be part of the mechanism of THR action during amphibian metamorphosis (3).

Actg1 and Fos, Two Hubs With Opposite Regulation in Both Species

As described above, the Axolotl transcriptional response to T_3 is vastly different from that found in *X. tropicalis*. This certainly reflects the opposite tailfin fate (maintenance vs. resorption) characteristic of both species. We identified a small number of biologically relevant genes of interest, through a straightforward network analysis. To this end, we used KEGG pathways as a set of high-quality resources that aggregate a curated knowledge of functional interactions between gene products, organized in independent pathways devoted to specific topics. By building a network of KEGG pathways components, we reconstructed a network of functional interactions which provides us with (1) an integrated view of the global impact of T_3 in the two species, and (2) a data type suitable for formal exploration with the rich framework of network analysis. Our analysis of node topology quickly identified hubs which, by definition, are nodes (gene products) shared by several biological pathways. Affecting their biological activity is likely to have large biological consequences and as such, they are often considered as weakness points in biological networks (43). Remarkably, the two networks (one build for each species) are almost identical despite the very poor overlap between the DE gene lists of *X. tropicalis* and Axolotl, since they both use overlapping sets of KEGG pathways. This nicely illustrates the connections between network evolution, adaptation and biological robustness (52): given a similar network topology, dynamic changes of networks states are flexible enough to accommodate different life history traits. In terms of transcriptional changes in the biological networks, our analysis provides a number of interesting observations, which may help understand the molecular switch that generates alternative functional output and how they translate into opposite organ fates.

The key point is that the Actg1-Fos subnetwork (i.e., a collection of hubs clustered around Fos and Actg1) displays alternative transcriptional states between Axolotl and *X. tropicalis* at pre-HTP, and between pre-HTP and paedomorph stages. By definition, all the components of this subnetwork are functionally connected and collectively contribute to a number of biological pathways, ultimately translating into a coordinated biological process (e.g., tailfin regression or maintenance). We proposed that this sub-network is one of the molecular determinants of the differential response to T_3 in both species and at both stages in Axolotl. In this context, two hubs

are remarkable: *fos* and *actg1*, because they are differentially expressed in both species, but display opposite regulation. The first hub is Fos, which is induced in Axolotl and repressed in *X. tropicalis*. This is a transcription factor that regulates a large subset of genes involved in cell proliferation, differentiation and apoptosis (53). In fact, a recent survey showed that it belongs to a gene set idiosyncratic of TH responses (7). Its ability to control such a large collection of biological processes stems from its mode of action. Fos is a subunit of AP1, which forms homo- or hetero-dimers with other basic region-leucine zipper proteins that all belong to the subfamily of Jun, Fos, Maf, and Atf transcription factors. The opposite effects of AP1 on cell death and survival results from the transcriptional activation of a combination of positive and negative regulators of apoptosis. Interestingly, in Axolotl, *fos* is T_3-responsive at both larval and adult stages whereas *junb* and the other components of the Actg1-Fos subnetwork are not. This leads to the attractive possibility that tailfin fate is governed by the relative ratio of AP1 components regulated in a T_3-dependent manner. In addition, the fact that *fos, junb* and the Actg1-Fos subnetwork display different expression responses between *X. tropicalis* and Axolotl suggests alternative cell fate commitments. Interestingly, the functional connection between Fos and THR is not new, since they are known to share many target genes, on which they act reciprocally to repress the transcription induced by the other (54–56).

The second hub highlighted by our analysis corresponds to *actg1*, which is induced in Axolotl and repressed in *X. tropicalis*. Actg1 is a central element of the acto-myosin network. It is noteworthy that *actg1* null mice are viable during embryonic development, but most die shortly after birth at the post-embryonic transition (57). Actin plays also a key role in apoptosis (58), although its precise mechanism remains elusive. Our network analysis that links functionally Actg1 and Fos by a single node implies that Actg1 may have a nuclear localization. This is now clearly established, and Actg1 is known to participate with transcriptional gene activation (for all three RNA polymerases), editing and nuclear export of mRNAs, DNA repair, chromatin remodeling, development and transcriptional reprogramming (59, 60). In addition, Actg1 and Fos are known to belong to the same synexpression group, since their transcription is controlled by a similar serum response element in their promoter (61).

Gene regulatory networks have a modular structure, with sub-circuits dedicated to specific tasks (62) which can be recruited multiple times in different contexts during the course of evolution (63). The evolution of a regulatory module's output can easily account for the differential recruitment of the Fos-Actg1 subnetwork. Obviously, the step forward would be to address the mechanistic details and decrypt their transcriptional regulatory mechanisms. Unfortunately, our ChIA-PET analysis in *X. tropicalis* shows that neither *fos, actg1* nor the other components of the Actg1-Fos subnetwork belong to the repertoire of genes whose expression is directly controlled by the TH receptor (39 and data not shown), and the transcription factors involved remain to be determined. Thus, even if *fos* and *actg1* are direct TH target genes in Axolotl (which we currently do not know), the comparison between the two species is not trivial.

In this work, we show that despite the lack of visible anatomical changes, Axolotl tailfin responds to known endogenous variations of T_3 levels, at the transition between tadpole and paedomorph stages (2 weeks post-hatching). Compared to the transcriptional response to T_3-treatment at similar developmental period in *X. tropicalis* (metamorphosis), TH signaling acts on different target genes, as illustrated by vastly different DE gene sets. However, the two transcriptional response are mediated by remarkably similar cellular pathways in two species with opposite tailfin fate (maintenance vs. resorption). We also show that tailfin fate correlates with the alternative transcriptional state of a dense subnetwork around fos and actg1. We propose that the transcriptional state of this subnetwork help explains why two similar tissues and at a similar developmental period, respond differently to TH at HTP.

DATA AVAILABILITY

The datasets generated for this study can be found in SRA, under the references SRP067617, SRR810197, SRR8101977 and PRJNA498010.

AUTHOR CONTRIBUTIONS

NB and LMS designed the experiment. GK, NB, and MR carried out the experiments. GK, LMS, and NB processed and analyzed the data. CB and CF sequenced RNA samples. GK, NB, and LMS wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00194/full#supplementary-material

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REVIEW
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Transport, Metabolism, and Function of Thyroid Hormones in the Developing Mammalian Brain

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Ever since the discovery of thyroid hormone deficiency as the primary cause of cretinism in the second half of the 19th century, the crucial role of thyroid hormone (TH) signaling in embryonic brain development has been established. However, the biological understanding of TH function in brain formation is far from complete, despite advances in treating thyroid function deficiency disorders. The pleiotropic nature of TH action makes it difficult to identify and study discrete roles of TH in various aspect of embryogenesis, including neurogenesis and brain maturation. These challenges notwithstanding, enormous progress has been achieved in understanding TH production and its regulation, their conversions and routes of entry into the developing mammalian brain. The endocrine environment has to adjust when an embryo ceases to rely solely on maternal source of hormones as its own thyroid gland develops and starts to produce endogenous TH. A number of mechanisms are in place to secure the proper delivery and action of TH with placenta, blood-brain interface, and choroid plexus as barriers of entry that need to selectively transport and modify these hormones thus controlling their active levels. Additionally, target cells also possess mechanisms to import, modify and bind TH to further fine-tune their action. A complex picture of a tightly regulated network of transport proteins, modifying enzymes, and receptors has emerged from the past studies. TH have been implicated in multiple processes related to brain formation in mammals—neuronal progenitor proliferation, neuronal migration, functional maturation, and survival—with their exact roles changing over developmental time. Given the plethora of effects thyroid hormones exert on various cell types at different developmental periods, the precise spatiotemporal regulation of their action is of crucial importance. In this review we summarize the current knowledge about TH delivery, conversions, and function in the developing mammalian brain. We also discuss their potential role in vertebrate brain evolution and offer future directions for research aimed at elucidating TH signaling in nervous system development.

Keywords: thyroid hormones, neocortex, prenatal development, embryonic brain development, mammalian brain development, mammalian brain evolution, hypothyroidism

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INTRODUCTION

Thyroid hormone (TH) signaling is an ancient regulatory mechanism dating back to early eukaryotes. The use of iodinated amino acids and bona fide THs to control development and trigger major life transitions precedes the ability to produce these molecules internally (1–4). Endogenous TH production within a specialized gland of animals appears in the evolution of basal chordates ∼550 million years ago (1, 2, 4–6). In vertebrates THs are crucial for both development and adult life as they regulate tissue differentiation, maturation and whole body metabolic function (7). They also trigger major life transitions and metamorphosis in multiple chordate species (6, 8).

Although attempts to treat goiter with iodine-rich foods were made already in antiquity (9), the importance of thyroid gland secretions in human health was scientifically recognized only at the end of 19th century. In that time thyroid deficiency was linked to myxedematous cretinism with the first successful treatment by thyroid extract injection published by the end of the century (10, 11). THs were subsequently identified as active components, chemically characterized and synthesized in the early 20th century (12–14). Specific functions of TH signaling in brain development were also recognized with the systematic observations of the neurological cretinism prevalent in regions with iodine deficiency (15, 16). Since then our knowledge about the many roles of THs in the regulation of fetal brain development has grown exponentially. This review focuses on the functions of THs in early development of the mammalian central nervous system (CNS), with an emphasis on cerebral cortex development and evolution. Functions of THs in the postnatal development and brain function, including as regulators of adult neurogenesis, have been reviewed elsewhere (17–20).

PRODUCTION AND METABOLISM OF THs—MATERNAL AND FETAL SOURCES

Mammalian THs are produced in two forms – 3,3′,5-triiodothyronine (T3) and 3′,5′,3,5-tetraiodo-L-thyronine (T4 or thyroxine). T4, the main product of thyroid gland secretion, has a low affinity for nuclear TH receptors (TRs) and therefore is thought to act largely as a prohormone in the classical TH signaling pathway (8). In contrast, biologically active T3 has a high affinity for nuclear TRs (21, 22) and is produced by either the thyroid gland or locally from T4 by target tissues and cells (23–25). Additionally, multiple TH-derivatives arise as products of TH metabolism, some of which have biological activity while others are degradation byproducts and storage forms (26).

There are two main periods in prenatal development of placental mammals with regard to TH production and delivery into the fetal nervous system. In early development an embryo relies solely on the maternal source of THs as its thyroid gland is not yet fully functional. The thyroid gland develops early in pregnancy from an anterior region of the embryonic gut, however, in humans it does not secrete significant TH levels until mid-gestation (27). Therefore the 1st trimester of human pregnancy proceeds with a full dependence on maternal TH secretion, and afterwards fetal TH production raises gradually (28, 29). In agreement with the fetal demand for THs in pregnancy total maternal T4 and T3 levels rise through the 1st trimester and stay elevated for the remainder of pregnancy. In the same time, due to the increased binding to rising levels of maternal serum thyroxine-binding globulin (TBG), free T4 and T3 levels decrease after the initial peak at the onset of pregnancy and remain comparable with non-pregnant women (30). During pregnancy, high total TH levels are needed to meet the rising demands of the fetus as well as the mother (29, 31, 32). In cases of fetal TH production deficiencies caused by events like thyroid gland agenesis, maternal THs are largely able to substitute for fetal TH production (33, 34). Even after the onset of fetal TH production the maternal source of THs seems to be important for proper brain development, as can be deduced from the developmental deficits seen in premature infants (35). Although in the fetus total T4 and T3 concentrations are very low in early pregnancy, free T4 concentrations in the amniotic fluid and fetal serum increase to almost adult levels by mid-gestation, likely due to a low presence of TH binding carrier proteins, and could therefore exert biological function (29, 31). Free T4 is taken up by fetal tissues and gets converted to T3 locally (36).

T3, T4 and some of their metabolites are subject to the activity of three selenocysteine-containing iodothyronine deiodinases (Dio1-3) that produce both active and inactive products, thereby controlling the amount of biologically active THs and targeting their metabolites for further degradation and clearance (37). Type III iodothyronine deiodinase (thyroxine 5-deiodinase, Dio3) robustly catalyzes inner ring deiodination (IRD) of T4 and T3 to rT3 (3,3′,5′-triiodothyronine) and 3,3′-T2 (3,3′-diiodothyronine), respectively (38), resulting in inactivated forms of these hormones that have little affinity for nuclear TRs and undergo rapid removal (39). In contrast, Dio2 (type II iodothyronine deiodinase) primarily activates T4 by converting it to the active receptor-binding T3 form by outer ring deiodination (ORD) (40). Dio1 (type I iodothyronine deiodinase) can catalyze both IRD and ORD, which leads to T4 inactivation or activation, respectively, but with lower activity toward T4 than Dio2 (41). It is mainly expressed postnatally and outside of the placenta or CNS, which make it less important for fetal brain development (42, 43).

In addition, TH modifications, including decarboxylation, deamination, ether-link cleavage, sulfation, and glucuronidation, affect their bioactivity and downstream metabolism (**Figure 1**). Most of them lead to deactivation and eventually degradation of THs (26), however some of the generated compounds, such as rT3 (44, 45), iodothyroacetic acids (tetrac and triac) and thyronamines (46–50), have been shown to convey biological effects in specific contexts. The conversions and main metabolites of THs are shown in **Figure 1**.

Sulfation and glucuronidation of the phenolic 4′-hydroxyl group of THs are considered phase II detoxification reactions as they increase the solubility of the products (51, 52). Sulfation is catalyzed by cytoplasmic sulfotransferases (SULTs) that transfer a sulfate group from the donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to their substrates (53) and is utilized to inactivate THs. T3 sulfate (T3S) does not bind TRs (54)

and Dio1-mediated ORD of T4 sulfate (T4S) is blocked while simultaneously IRD of both T4S and T3S is stimulated (55–58). Normally levels of sulfated THs in circulation and in excretions are low due to fast deiodination and clearance, but high levels of these metabolites are present in fetal circulation, likely due to the absence of Dio1 activity (59–62). Sulfotransferases producing T4S and T3S are present in the placenta, and sulfated TH metabolites can be transferred from the fetus into maternal circulation, potentially playing a role in regulating TH levels (52). Sulfated as well as glucuronidated THs may also serve as a pool of inactive hormones that can be mobilized by bacterial sulfatase or β-glucuronidase activity and reabsorption from the bile in the intestine (63–69) or hydrolysis by tissue sulfatases in the brain, kidneys and liver (70, 71).

TH DELIVERY INTO THE DEVELOPING BRAIN—TRANSPORT ACROSS BIOLOGICAL BARRIERS

TH delivery into the fetal brain requires passage through multiple barriers at the feto-maternal interface and between fetal circulation and the CNS. THs are actively transported across tissue barriers, including placenta, and brain blood barrier (BBB), and into target cells. In circulation free THs are present only in minute amounts and mostly are bound to carrier-proteins. The main TH binding proteins in human plasma are mammalian-specific TBG, albumin and transthyretin (prealbumin, TTR) (72), the latter being also an exclusive TH carrier in the cerebrospinal fluid (CSF), where it makes up to 20% of total protein (73–75). A minor portion of THs is bound to ApoB100 and other lipoproteins (76). Carrier binding determines the amount of free *vs.* total THs in circulation, from which only the free fraction is readily available for uptake by cells, whereas protein bound THs are considered to be biologically inert (77, 78). TH entry and exit from cells are mediated by membrane transporters. A number of proteins capable of TH transport have been identified, including monocarboxylate transporters MCT 8 and 10, organic anion carrier transporter polypeptides (OATPs), Na+/taurocholate co-transporting polypeptide NTCP, and heterodimeric amino acid transporter (HAT) members/L-type aromatic and large branched-chain amino acid transporters LAT1 and 2. They differ in expression pattern and affinity for THs and their metabolites as well as ability to transport other compounds. Multiple TH transporters are expressed already during fetal nervous system development, the most important being MCT8 and OATP1C1 (79–101).

Before the onset of fetal TH production THs enter fetal tissues by passing through the placenta, which serves as an active filter allowing only limited amounts of the active hormone to enter the fetus (31, 34). The main deiodinase expressed in the placenta is Dio3 (102), the ability of which to inactivate THs is thought to protect the developing fetus from toxic levels of the maternal hormones, especially in the brain, which is uniquely vulnerable (103–105). Notably, Dio3 has a preference for T3 as substrate, which contributes to T4 being the main TH passing through the placenta (106). Dio2 is also present in the placenta, albeit at lower levels than Dio3 (107, 108), and is thought to act as a provider of bioactive T3 for local use. Total fetal T4 is kept lower than the adult level for the entire gestation in both human and rodents until birth or at 2 weeks postnatally, respectively (32, 109). An additional mechanism balancing active TH levels involving sulfation was postulated (52), although only low activity toward THs by the placental sulfotransferases was detected (110).

In the 1st trimester of pregnancy most of the THs are thought to be taken up by the fetus from the coelomic and/or amniotic fluid, while from the 2nd trimester onwards direct transfer to the fetal circulation starts to play a more important role (29). Prior to neural tube closure THs can access the developing CNS directly from the amniotic fluid. Afterwards THs get delivered into the brain either through the BBB of the developing vasculature or the choroid plexus (CP) and cerebrospinal fluid (CSF) system. Endothelial cells of both the brain capillaries and the CP express transporters and TH modifying enzymes controlling TH levels entering the brain (111).

CELLULAR SIGNALING OF THs AND ITS FUNCTIONS IN MAMMALIAN FETAL BRAIN DEVELOPMENT

Expression and Signaling Pathways of TH Receptors in the Early Nervous System

THs versatile functions are dependent on cellular responses mediated by their interaction with various receptors expressed in cell- and tissue-specific manner. In target cells THs trigger either genomic responses mediated by DNA-binding nuclear TRs or non-genomic responses by alternative non-nuclear receptors. Genomic effects on gene transcription require members of the nuclear hormone receptor superfamily type II, in mammals encoded by two related genes arising from whole genome duplication in vertebrates: THRA/NR1A1 and THRB/NR1A2, which produce TR α and β, respectively (112–114). Each of these genes can undergo alternative splicing and harbors alternative promoters, resulting in a number of distinct isoforms differing in their ability to bind target DNA sites, ligand binding, and co-factor recruitment (114, 115). The isoforms that possess both DNA and ligand binding capacity and localize to the nucleus are TRα1 and β1-3 (with TRβ3 being rat-specific), and these are the ones that mediate the genomic effects of THs (116, 117). Other isoforms act as dominant-negative regulators or have non-genomic functions (118–120). TRβ1 and 2 possess the same DNA binding domain, but their N-termini differ in the activation domain, which in β2 favors coactivator recruitment (121, 122). TRα1 and TRβ1 differ in DNA-binding affinity and selectivity (123), T3 affinity (124), and the ability to form dimers (125). T3 is the active form of the hormone capable of binding to these receptors as T4 has about 10 times lower affinity for TRs (21, 22). However, direct T4 binding with biologically significant effects has also been shown recently (126, 127).

To affect transcription of target genes TRs bind DNA as either homodimers or heterodimers with retinoid-X-receptors and recognize TH response elements (TREs) in promoter regions of regulated genes (114). TRs lacking bound THs

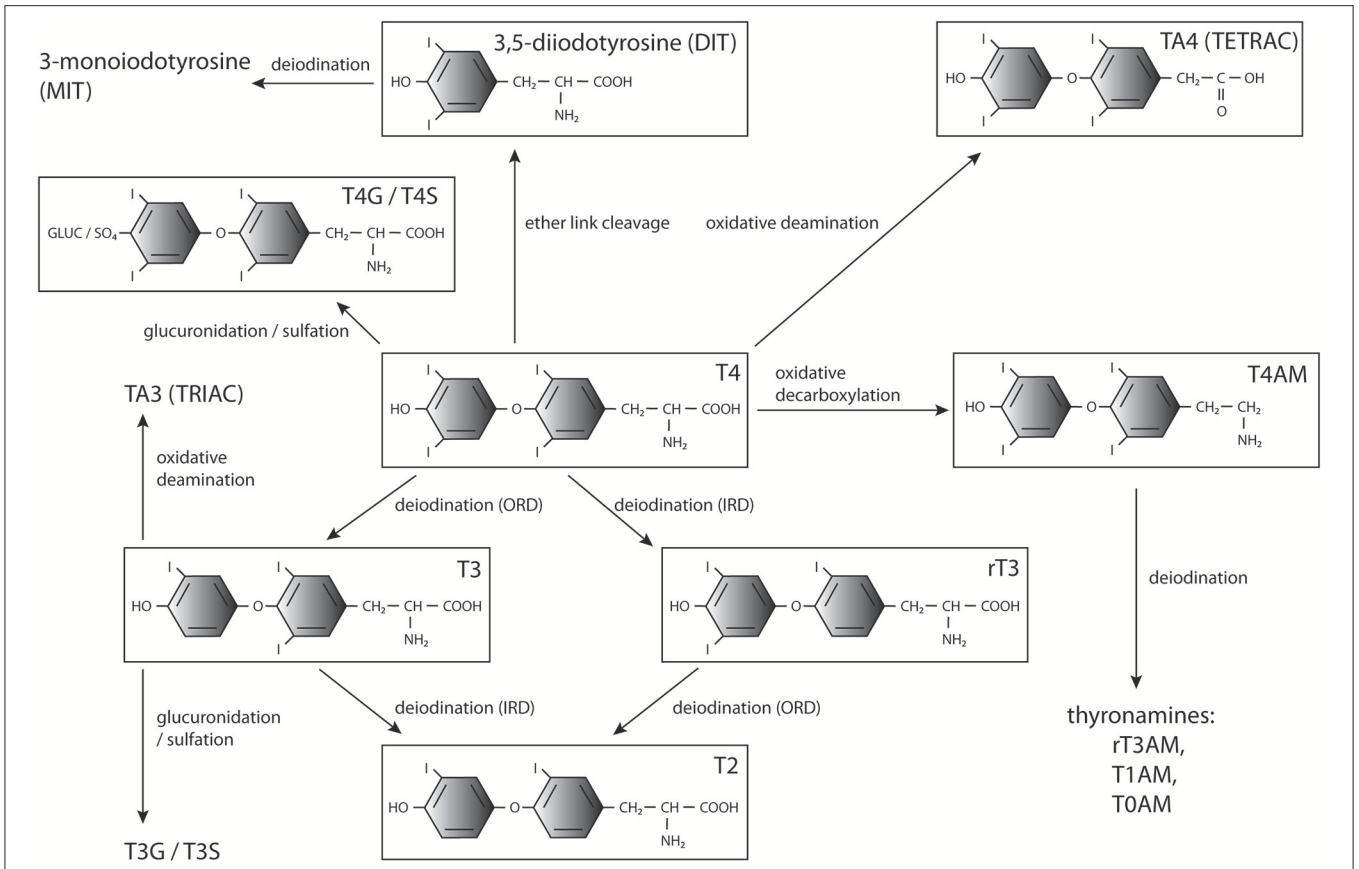

FIGURE 1 | THs and major products of their metabolism. T4, 3′,5′,3,5-tetraiodo-L-thyronine (thyroxine); T3, 3,3′,5-triiodothyronine; T2, 3,3′-diiodothyronine; rT3, 3, 5′,3′,-triiodothyronine; , diiodotyrosine; MIT, monoiodotyrosine; T3G, triiodothyronine glucuronidate; T4G, thyroxine glucuronidate; T3S, triiodothyronine sulfate; T4S, thyroxine sulfate; TRIAC, triiodothyroacetic acid; TETRAC, tetraiodothyroacetic acid; T1AM, 3-iodothyronamine; T0AM, thyronamine.

can bind DNA as aporeceptors, which represses target gene transcription by recruiting corepressor complexes with histone deacetylase activity (128, 129). T3 binding lifts this repression and leads to target gene transcription, which is necessary for normal nervous system development (130–132). While T3/TR interaction results in coactivator recruitment, chromatin restructuring, and transcriptional activation for most targets, some genes can also be repressed by TRs with bound THs (133, 134). Accordingly, a meta-analysis study of genes transcriptionally regulated by THs in the nervous system identified over 700 curated targets, however the extent and mode of their regulation is likely to differ during development and in specific cell types (135). More targeted studies are needed to explain the differential cellular responses to THs in various contexts. The interplay between various TR isoforms, chromatin re-modeling and transcriptional machinery leads to complex tissue and cell-specific responses in various contexts and comprehensive reviews on the mechanistic aspects of the genomic pathway are available (136–138).

Tissues differ in TR isoform expression patterns and cell-specific functions. TR isoforms share many common targets, however, there is marked spatiotemporal variation in the degree and mode of regulation and target overlap. Frequently cells express multiple isoforms with distinct roles arising due to differences in the respective protein levels or intrinsic activity (117). Nuclear TRs are expressed in the developing brain of humans and rodents (22, 139, 140), and T3 binding in the human brain occurs even before fetal thyroid gland maturation (22, 141, 142). TRα1 is the major isoform expressed in neurons from early fetal development in humans and rodents onwards, while TRβ increases perinatally and is more abundant in specific neuronal types such as hippocampal pyramidal and granule cells, paraventricular hypothalamic neurons and cerebellar Purkinje cells (143–145). Interestingly, TRβ1 is also expressed in the germinal zones of cerebral cortex (145). During early postnatal development in rodents TRβ is specifically required for enhancing the expression of the striatum-enriched gene *Rhes* (146). *Rhes* functions in multiple signaling pathways and has been implicated in the regulation of dopamine-mediated synaptic plasticity of striatal neurons, in striatum-related behaviors, and in neurodegeneration in the course of Huntington disease (147). Moreover, TRβ1 and 2 are required for the cochlear and retina development, and TRβ null mice have defects in auditory and visual development (148). TRβ2 also plays a

role in establishment and maintenance of the hypothalamus-pituitary-thyroid gland axis (114). Most neurons express both TRα and TRβ receptors, however, the relative expression levels differ, which can have important functional consequences such as in the hippocampus, where TRα but not β is necessary for proper GABAergic interneuron innervation and behavior (145, 149). The relative abundance of both receptors was also proposed to control proliferation/differentiation balance in the developing brain (145). In addition, certain specific cell types express exclusively either TRα or TRβ form. For instance, parvalbumin (PV) positive cells in the CA1 of the hippocampus express preferentially TRα while the PV^+ interneurons in the somatosensory cortex produce mostly TRβ (149). Also developing cerebellar granule cells express TRα1 but not TRβ while Purkinje cells produce mostly TRβ (144, 145, 150).

TR mutations in both rodents and humans have been linked to a range of behavioral and cognitive phenotypes, including changes in sensory, attention, emotion and memory functions, but their effects are complex and usually more benign than those of hypothyroidism (149, 151–155). Detrimental effects of hypothyroidism are thought to occur largely due to the repressive activity of TRs lacking bound THs, as mice completely lacking both TR receptor types are viable and without major defects (153). Moreover, TRα1 KO rescues the viability of Pax8 KO mice, which present with thyroid agenesis and lethal congenital hypothyroidism during the early postnatal period (156), and partly rescues the Dio3 KO phenotype (157). TRs lacking bound THs are generally implicated in maintaining the proliferative, undifferentiated state of neural progenitors, while T3-bound receptors promote transcription of genes triggering cell differentiation and maturation (129, 158–160).

In addition to the classical pathway mediated by nuclear TRs, a growing list of TH effects have been linked to their non-genomic actions, including regulation of actin polymerization (161), Dio2 activity (162), ion transport (163), Akt/PKB and mTOR pathway activation (164), and fatty acid metabolism (165). Non-genomic effects of THs can also influence cell proliferation and survival (166). Among receptors mediating the non-genomic functions of THs is a cell surface TH receptor, integrin αvβ3 (167, 168), which preferentially binds the T4 pro-hormone to activate the MAPK signaling cascade. This interaction promotes angiogenesis (167) and proliferation in osteoblasts and various cancer cell types (169–171). Signaling through this receptor has also been implicated in neocortical development as T4 binding to integrin αvβ3 upregulates progenitor proliferation in this structure (172). A detailed review of the non-genomic effects of THs in various cell types can be found elsewhere (120).

THs also interact with other signaling pathways during cortical development. In neural development sonic hedgehog (Shh) signaling leads to an increase in Dio3 expression while decreasing Dio2 by ubiquitination (108). In turn both fetal and adult brain T3 upregulated Shh production (134, 173), thus providing a negative feedback loop. TH and Shh pathways interact also in cerebellar development to control granule cell precursor proliferation (174). Brain morphogen retinoic acid (RA) shares common carrier proteins with THs, and their nuclear receptors dimerize. RA can also increase MCT8 expression to increase TH import (175). Another transcription factor, COUP-TF1 (Chicken Ovalbumin Upstream Transcription Factor 1), has been shown to bind to DNA sites overlapping with TREs and to block TR access and activation (176, 177) thereby modulating TH signaling. Genes that show the presence of both TR and COUP-TF1 binding elements include calcium calmodulin-dependent kinase IV (CamKIV) (177, 178), which is important for both GABAergic and glutamatergic neuron production (179, 180). Emx1 and Tbr1 genes are also controlled by both THs and COUP-TF1, with the latter factor modulating the timing and magnitude of the T3 response (180). Similarly, nuclear liver X receptor β interacts with TH signaling in regulating cortical layering, likely by influencing the expression of their common target, the reelin receptor ApoER2 (181).

Developmental Hypothyroidism and Its Impact on Brain Development

The complexity of TH production, delivery, and metabolism contributes to varying clinical presentations of different TH signaling deficiencies during gestation, with the most severe being iodine deficiency which impairs both maternal and fetal TH supply (15, 182). Maternal iodine deficiency or severe hypothyroxinemia alters embryonic brain development even before the fetal thyroid gland becomes functional (183, 184), and leads to profound neurological cretinism with defects in sensory, motor and cognitive functions (15, 28, 185, 186). TH deficiencies, even when limited to the 1st trimester of gestation, are linked to cognitive deficits and neurodevelopmental delay (183, 187, 188). In contrast, fetal TH production defects, such as congenital hypothyroidism caused by thyroid agenesis, can largely be compensated by maternal THs (33, 34, 189), with most deficiencies in development arising postnatally if these defects are not treated (109, 190).

Given the selective placental permeability for T4, even mildly hypothyroid or asymptomatic cases of maternal iodine deficiency, lowering T4 but not T3 levels, can reduce fetal THs enough to cause developmental defects (182, 186, 189). Moreover, maternal T4 but not T3 supplementation protects the brain from hypothyroid injury until birth (34, 189, 191). As in the placenta, the main TH form transported into the CNS is T4, and the majority of the cerebral cortex T3 comes from local tissue production by Dio2 (192, 193), rendering the brain dependent mostly on circulating fetal T4 levels (28). The brain seems to be privileged in taking up T4 from the fetal circulation compared to other tissues, while the opposite is true for T3 (34). TH transporters facilitate entry from the circulation into the developing brain. Postnatally T4 is mainly taken up by astrocytic OATP1C1 and converted to bioactive T3 by the action of Dio2 (94, 194), which is expressed almost exclusively in glial cells (195, 196). Generated bioactive T3 is then provided to neurons, which lack Dio2 activity but express high levels of Dio3, allowing them to deactivate glia-derived excess THs (43, 196, 197). Neurons take up T3 preferentially over T4 via the MCT8 transporter either from astrocytes or directly from the interstitial fluid (198–200).

The Dio2/Dio3 activity balance provides an important mechanism for regulating active T3 levels in the brain to protect against excess THs (201). Both Dio2 and Dio3 activities are present in the fetal brain already from the 1st trimester onwards but show opposing trends with Dio3 being more active early and Dio2 toward the end of gestation (202–204). Dio3 KO in mouse, in contrast to other deiodinases, causes widespread abnormalities in brain and sensory organs, but it is unclear to which degree this phenotype is generated prenatally and arises due to placental or CNS deficiency (104, 105, 205). Similarly, human mutations affecting Dio3 imprinting result in Temple or Kagami-Ogata syndromes that impair brain function; however, whether this phenotype can be fully attributed to the altered dosage from the Dio3 locus is unclear (206). Additional mechanisms controlling active TH levels may also be present as TH sulfotransferases were shown to be expressed and active in the developing human brain (207, 208).

Fetal and perinatal TH deficiency, due to congenital hypothyroidism or iodine deficiency, has a dramatic negative impact on cerebral development, affecting multiple regions including cerebral cortex, hippocampus, amygdala, and basal ganglia as well as motor neurons, cochlea, retina and interregional connectivity (15, 183, 184). Most of the early brain developmental events (proliferation of neural progenitors and neuronal migration in the neocortex, hippocampus, and medial ganglionic eminence) occur before fetal TH production, and thus are predominantly under the control of maternally-derived TH signaling. However, later stage processes (ongoing neurogenesis and migration, axon growth, dendritic arborization, synaptogenesis, and early myelination) occur after the onset of fetal TH production and proceed under the control of both fetal and maternal THs. Further brain developmental events (cortex pyramidal cell, hippocampal granule cell and cerebellar granule and Purkinje cell migration, gliogenesis, and myelination) occur postnatally and are therefore controlled entirely by neonatal THs. TH signaling has an effect on all of these processes (158, 209). The diverse actions of TH in early brain are summarized in **Figure 2**.

Functions of TH Signaling During Development of Mammalian CNS

Human cretinism has been extensively modeled in rodents. In human, cortical neurogenesis occurs between week 5 and 20 of gestation, which is the period when the fetus depends primarily on the maternal source of THs, corresponding roughly to rat E12-18 (27, 209). In cortical development neurons are generated from progenitor cells residing in the subventricular zone and migrate basally along radial glia fibers to form an ordered 6-layered cortical plate, a process controlled largely by pioneer Cajal-Retzius and subplate neurons (210, 211). Perturbations of this migratory process lead to defects in cortical morphology and function (212). Even mild or transient maternal hypothyroxinemia during neurogenesis retards fetal glutamatergic neuron migration along the radial glia scaffold in the rat sensory cortex and hippocampus, without affecting tangentially migrating GABAergic neurons. This deficiency results in reduced neocortical thickness, blurred cortical layering and subcortical band heterotopia, likely responsible for increased seizure susceptibility and altered behavior (184, 190, 213–216). Improper neuronal migration also leads to alterations in callosal connectivity (213, 217). These migration defects can be at least partly attributed to a direct effect of the lack of THs on guiding cues as THs regulate Reelin, Dab1, and Vldlr expression in rat neocortex and cerebellum (218–220). T3 signaling also controls the expression of lipocalin-type prostaglandin D2 in Cajal-Retzius cells and hippocampal neurons during development (221), a protein known to affect glial cell migration (222). Moreover, a large subset of subplate neuron-enriched genes were shown to be under TH regulation (160). Maternal hypothyroidism alters gene expression in the brain by midgestation, and while it can be corrected by T4 application (223), the morphological changes persist if hormones are replaced after the critical window has closed (36).

TH signaling affects not only migration but also enhances progenitor proliferation and cortical neurogenesis, which is regulated by both genomic and non-genomic TH action (172, 180, 224). Hypothyroidism causes cell cycle disruption, increased apoptosis and reduction in both apical and basal progenitor pools and defects in neuronal differentiation, leading to cortical thickness reduction and decreased neuron number, especially in upper cortical layers (224). THs were shown to upregulate genes involved in cell cycle regulation and sustained proliferation in the developing cortex, such as POU2F1/Oct-1 or Nov (178, 223). Signaling through various pathways could have opposing roles in regulating proliferation/differentiation balance as T4 binding to integrin αvβ3 upregulates progenitor proliferation in the developing cortex (172), while T3 regulates gene expression in primary cerebrocortical cells via a nuclear TR-dependent pathway consistent with a role in promoting neuronal differentiation (160). Even mild hypothyroxinemia induces shifts in gene expression in developing hippocampus and neocortex (225). Among TH-regulated targets are genes involved in neuronal specification and function, such as Emx1 (Empty spiracles homolog 1), Tbr1 and neurogranin (180, 226–228), as well as cytoskeleton components and ECM molecules, which impact on both proliferation and neuronal migration (134, 229). T3 also regulates the expression of DNA methyltransferase Dnmt3a in mouse brain, potentially extending the genomic effects of TH action beyond directly regulated genes by affecting global DNA methylation states (230). Seemingly contradictory functions of THs in promoting progenitor proliferation and neuronal differentiation may stem from specific spatiotemporal expression of their transporters, metabolizing enzymes, and effectors that mediate different actions in various cell types in the course of development.

While progenitor proliferation, cortical neurogenesis and early neuronal migration occur largely prenatally, THs have a profound effect also on perinatal CNS developmental events. During that period, the TH deficiency associated with congenital hypothyroidism leads, in both rodent and

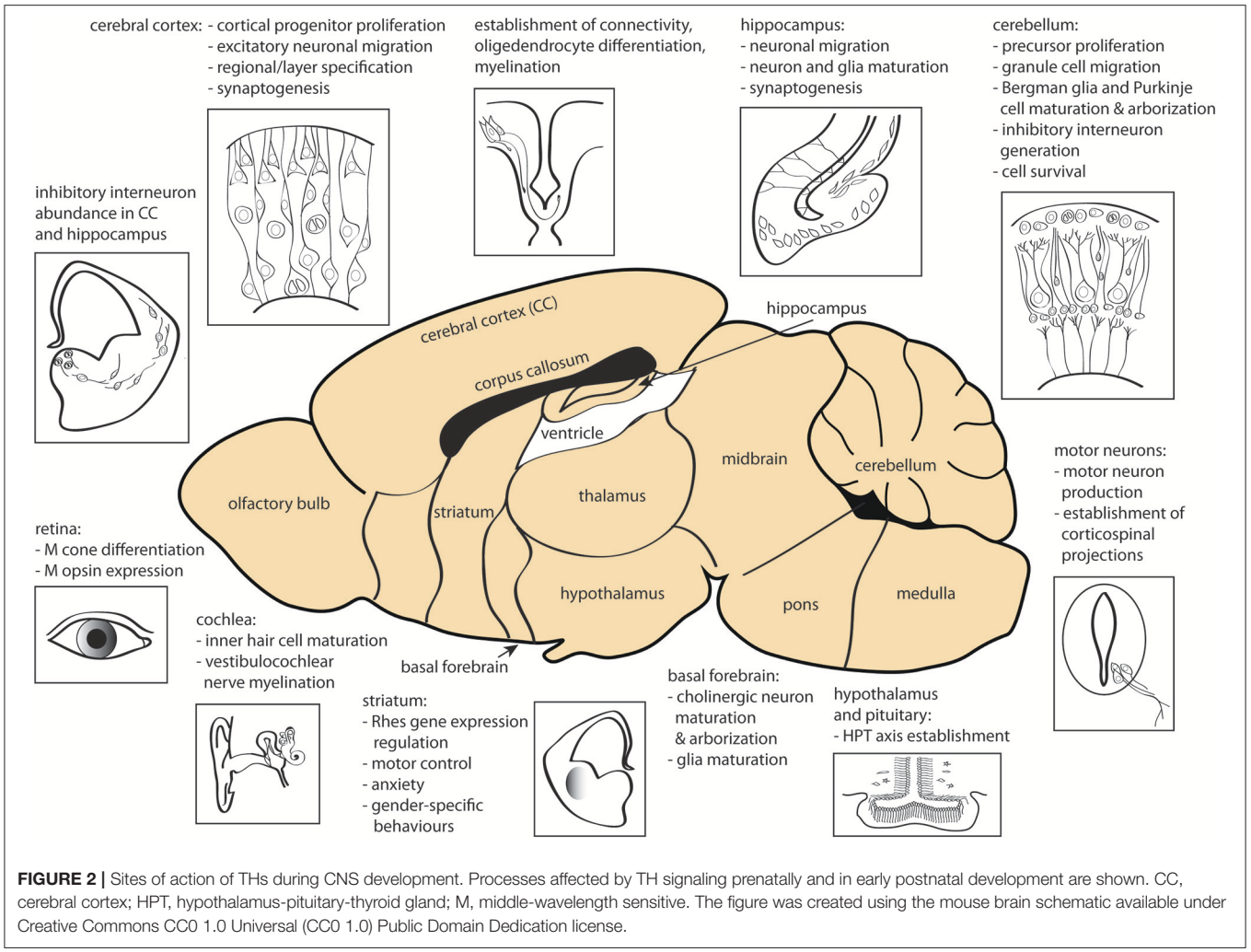

FIGURE 2 | Sites of action of THs during CNS development. Processes affected by TH signaling prenatally and in early postnatal development are shown. CC, cerebral cortex; HPT, hypothalamus-pituitary-thyroid gland; M, middle-wavelength sensitive. The figure was created using the mouse brain schematic available under Creative Commons CC0 1.0 Universal (CC0 1.0) Public Domain Dedication license.

humans, to defects in late neuron migration, cerebellar neuron and glia arborization and maturation (231–233), astrocyte and neuron differentiation in hippocampus (234–236), inhibitory neuron development and function (237, 238), oligodendrocyte differentiation and myelination (129, 239, 240), and synaptogenesis (241, 242). TH signaling also controls spinal motor neuron generation in vertebrates (243) and establishment of corticospinal projections (244).

The impact of perinatal TH deficiency on brain development has been intensely studied in two vital regions associated with hypothyroid injury, especially related to motor function impairment—the striatum and the cerebellum (245). In mammalian cerebellum the final TH-dependent stages of development occur perinatally, when cells from the external germinal layer proliferate and migrate to the inner granular layer forming connections with maturing Purkinje cells (246). TH signaling affects all of these processes. In cerebellum migration of granular cells requires ligand bound TRα, while maturation of Purkinje cells depends on the functions of both TRα and β isoforms. Additionally, TRβ is required for adequate granule cell proliferation (247). Interestingly, the hypothyroid injury on the developing cerebellum can be largely rescued by TRα1 deletion, in agreement with the function of TH in relieving the receptor-mediated transcriptional repression (248).

In the striatum a connection between TH-regulated gene expression and brain-region specific function involves the Ras-like GTP-binding protein Rhes/Rasd1. Despite being expressed in multiple brain regions from midgestation this gene shows a specific striatal upregulation in early postnatal rodent development that is critically dependent on THs (249–251). Developmental Rhes enrichment in this structure is dependent on T3 binding to TRβ isoform (146), however adult expression seems to rely primarily on TRα (252). Interestingly, Rhes functions in G-protein coupled receptor signaling as well as in PI3K/Akt/mTOR pathways (253, 254) to modulate synaptic transmission (255), and Rhes KO animals have deficits in striatum-controlled behaviors (256), providing a potential functional link between hypothyroidism and resulting motor and affect dysfunctions.

THs IN MAMMALIAN BRAIN EVOLUTION

In addition to their relevance regarding neurodevelopmental disorders, THs may have played a crucial role in human brain evolution. Although mostly limited to comparison between human and rodents, a number of important differences in TH signaling have been characterized. Spatiotemporal expression patterns of TH transporters are species-specific and can lead to drastic differences in TH metabolism, evident especially in disease states. Strikingly, the effects of MCT8/SLC16A2 mutations, which in human cause severe brain hypothyroidism with concomitant hyperthyroidism in circulation and peripheral organs, known as Allan-Herndon-Dudley syndrome (AHDS), characterized by severe intellectual and motor disability (257–259), are not fully recapitulated by mice, especially with regard to the neurological phenotype (91, 260–262). In rodents, only MCT8 and OATP1C1 double-inactivation causes cerebral hypothyroidism and associated defects (263). Various explanations, including the presence of compensatory alternative transport or T3 production pathways in rodents (264, 265) or the differential expression of the LAT2 transporter in neurons (91), have been suggested.

A potential evolutionary difference in TH delivery between rodents and human may exist, pertaining to the carrier protein TTR. In human TTR is present in the CSF as early as from the 8th fetal week (75), and in contrast to TBG and albumin there are no known individuals with TTR null mutations, suggesting its vital role in development (72). However, TTR null mice are viable and do not have overt symptoms of hypothyroidism in the CNS (266). Interestingly, TTR evolution in vertebrates, leading to its synthesis in the CP and a shift in specificity from T3 to T4 in the mammalian protein, coincides with the emergence of the cerebral cortex as a novel structure (72). It is tempting to speculate that the evolutionary expansion of the neocortex in the primate lineage may be linked to increased dependence on the function of TTR during development. Subtle differences in serum TTR abundance and posttranslational modifications were detected between human and several other species of great apes, but their functional and evolutionary importance remains to be elucidated (267).

In rodent neocortex development increasing TH-mediated integrin αvβ3 activation promotes basal progenitor proliferation (172). In contrast, blocking integrin αvβ3 has the opposite effect on ferret basal progenitors (268). Increased pool size and proliferative capacity of basal progenitors are thought to have contributed to the evolutionary expansion of the neocortex, especially in the primate lineage (229). Interestingly, a number of human genes implicated in TH metabolism are altered in human basal progenitors compared to mouse (208), which may affect the magnitude and timing of TH action during cortical neurogenesis.

One of the major concepts in human evolution is neoteny, especially in relation to brain development and function (269). Alterations is TH signaling are known to underlie evolutionary heterochrony in various animal species (6), including our closest living relatives, the chimpanzees and bonobos (270). The global TH status in rodents is connected to either accelerated or delayed development in hyperthyroid and hypothyroid pups, respectively (271). Given that in the CNS THs tend to accelerate cell type maturation (272, 273), one could speculate that prolonged or enhanced brain protection from THs and spatiotemporal alterations in metabolic enzyme and effector expression in the primate lineage could have delayed differentiation, contributing to human neoteny. Further studies investigating species-specific differences in TH pathways in brain development, especially including other model species, beyond human and rodent, could help to test this hypothesis.

CONCLUSIONS

TH action with regard to mammalian brain development is highly pleiotropic, and despite many advances the complexity of their delivery, metabolism, and cell-specific responses make it difficult to dissect specific functions in brain regions and cell subtypes in the course of development. With the advent of single-cell transcriptomics and the CRISPR/Cas9 technology, the spatiotemporal dissection of TH signaling in various cell types across the nervous system should become faster and more precise. This is of crucial importance, as in addition to the long-recognized role of TH deficiency in neurodevelopmental defects, undiagnosed developmental hypothyroxinemia may be linked to common neurological disorders such as ataxias and epilepsy (274, 275). Elucidation of the mechanisms underlying these pathologies down to the cellular and subcellular level could aid better diagnostic and therapeutic interventions. Understanding and expanding the existing catalog of the evolutionary differences in TH signaling, which momentarily includes mostly genes linked to human genetic diseases such as AHDS or Kagami-Ogata syndrome, could also contribute to the generation of better disease models. Of note, when reaching conclusions about the role of THs in the human brain from rodent studies, it is important to keep in mind the at times profound phenotypic variation across species and its impact on disease presentation and potential treatments.

AUTHOR CONTRIBUTIONS

BS and WH made substantial contributions to the conception and drafting of the work and revising it critically. WH approved the final version of this manuscript.

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Rediscovering the Axolotl as a Model for Thyroid Hormone Dependent Development

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The Mexican axolotl (*Ambystoma mexicanum*) is an important model organism in biomedical research. Much current attention is focused on the axolotl's amazing ability to regenerate tissues and whole organs after injury. However, not forgotten is the axolotl's equally amazing ability to thwart aspects of tissue maturation and retain juvenile morphology into the adult phase of life. Unlike close tiger salamander relatives that undergo a thyroid hormone regulated metamorphosis, the axolotl does not typically undergo a metamorphosis. Instead, the axolotl exhibits a paedomorphic mode of development that enables a completely aquatic life cycle. The evolution of paedomorphosis allowed axolotls to exploit relatively permanent habitats in Mexico, and preadapted axolotls for domestication and laboratory study. In this perspective, we first introduce the axolotl and the various meanings of paedomorphosis, and then stress the need to move beyond endocrinology-guided approaches to understand the axolotl's hypothyroid state. With the recent completion of the axolotl genome assembly and established methods to manipulate gene functions, the axolotl is poised to provide new insights about paedomorphosis and the role of thyroid hormone in development and evolution.

Keywords: axolotl, paedomorphosis, metamorphosis, thyroid hormone, *ambystoma*

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INTRODUCTION

Mexican axolotls (*Ambystoma mexicanum*) have been studied in laboratories throughout the world for over two-hundred years (1). Beginning in the early nineteenth century, French expeditions to Mexico brought preserved adult specimens back to Paris for examination by curators at the *Jardin des Plantes*. Esteemed zoologist Georges Cuvier originally classified these specimens as larvae of an unknown species (2). It was not until decades later, when living axolotls were brought to Paris and a laboratory population was established by Auguste Duméril, that these presumptive larval forms were found to be reproductively mature and capable of metamorphosis. From the same axolotl spawn, Duméril (3) observed that most sibs reached an adult state and some reproduced while retaining larval characteristics including external gills, while a few individuals metamorphosed into forms typical of terrestrial salamanders (4). The observation of both metamorphic and non-metamorphic forms arising from a single spawn inspired theories and experiments to explain the axolotl's unusual mode of paedomorphic development (5). While much has been learned from studies of the axolotl and other salamanders, the mechanistic basis of paedomorphosis remains largely unknown.

Paedomorphosis

Paedomorphosis is a somewhat confusing term because it has been used to explain variation at evolutionary, ecological, and genetic levels of inquiry. At an evolutionary level, paedomorphosis is used to describe a specific pattern of developmental variation among ancestral and descendant species (6). The ancestral mode of development in salamanders is generally thought to include a single, obligate metamorphosis which partitions the life cycle between an early aquatic phase and a more terrestrial adult phase. Indeed, close tiger salamander (*A. tigrinum*) relatives of the axolotl are known to invariably undergo a metamorphosis (**Figure 1**). In contrast, the axolotl typically does not undergo a metamorphosis; axolotls are paedomorphic because they express ancestral juvenile traits in the adult stage of life. Paedomorphosis thus provides an evolutionary explanation for how new patterns of variation arise among species; the biphasic life cycle of an ancestral species was truncated somehow during evolution to yield a paedomorphic species. Within the lexicon of heterochrony, a theory that associates changes in developmental timing to the origin of new forms, Gould (6) proposed that paedomorphic salamanders arose during evolution as a result of changes in mechanisms that regulate metamorphic timing. While such description is useful for describing evolutionary patterns of developmental variation, what we ultimately seek is proximate-level understanding of timing mechanisms that regulate the expression of paedomorphosis.

In ecological studies, paedomorphosis is used to describe patterns of developmental variation among individuals within species and populations. At this level, paedomorphosis is thought to be an adaptive developmental strategy for exploiting favorable larval habitats for growth and reproduction (8), although non-adaptive variation in the expression of paedomorphosis is known (9). The mechanisms that allow for such plasticity are presumably influenced by an individual's genetic makeup, health status, and environmental cues. For example, studies of salamanders that express paedomorphosis facultatively have shown clear ecological correlates. Paedomorphosis is more frequent in permanent aquatic habitats that do not undergo seasonal drying (10, 11). It has been argued that ecological conditions largely dictate the expression of metamorphosis or paedomorphosis in facultative species to increase fitness-associated traits, such as body size (bigger is generally better) and the probability of earlier and more frequent reproduction (12). It is important to point out that genetics also plays a role. Evidence for a population genetic component of variation has been shown in experiments that altered the heritability of paedomorphosis in *A. talpoideum* over several generations of selection (10). However, such studies do not provide resolution of genetic factors that regulate the expression of paedomorphosis.

Paedomorphosis has also been used to describe developmental variation among siblings within genetic crosses. Taking advantage of the recent evolution of paedomorphosis among tiger salamander complex species, interspecific genetic crosses have been performed in the laboratory to segregate metamorphic and paedomorphic modes of development and map the genomic location of genetic factors (13–18). These crosses have identified major effect quantitative trait loci (QTL) that regulate the timing of metamorphosis and expression of paedomorphosis. For example, second generation backcross individuals of *A. mexicanum* x *A. tigrinum* hybrid crosses that inherit axolotl alleles at the *met1* QTL delay metamorphosis or express paedomorphosis. It is not clear if genetic factors identified from interspecific crosses regulate the expression of metamorphosis and paedomorphosis in natural populations. However, this classical genetic approach presents an unbiased method to identify candidate genes and associated mechanisms that may be operative in natural populations.

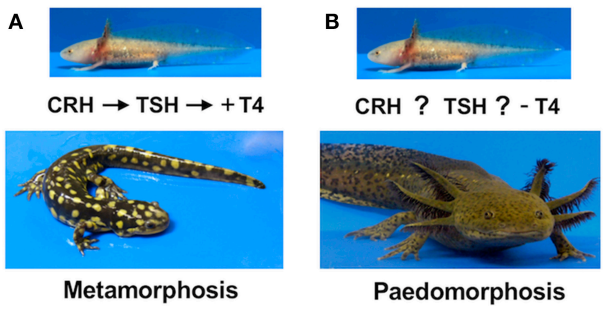

FIGURE 1 | (A) The ancestral salamander mode of development is metamorphosis. Metamorphosis in the tiger salamander is regulated by the hypothalamus-pituitary-thyroid (HPT) axis. At a critical time during larval development, corticotrophin releasing hormone (CRH) from the hypothalamus stimulates thyrotrophic cells in the pituitary to release thyroid stimulating hormone (TSH), which in turn stimulates the thyroid gland to secrete thyroid hormone (TH). Increasing TH (+) triggers metamorphic changes in target cells. **(B)** The derived paedomorphic mode of development. Paedomorphosis in the axolotl results in the retention of ancestral larval characters into the adult phase of life. Although CRH-like and TSH-like activity are present in the axolotl hypothalamus and pituitary respectively, and TSH and TH treatment can induce metamorphosis, the HPT axis is not activated during larval development. Modified from Johnson and Voss (7).

Endocrinology of Paedomorphosis

Over the last century, axolotl paedomorphosis has been the subject of a number of physiological studies. We refer readers to two relatively recent reviews of the literature pertaining to the endocrinology of axolotl paedomorphosis (7, 19). Our goal here is to briefly review salient features of axolotl hypothyroidism to provide context for identifying mechanisms that may regulate metamorphic timing and expression of paedomorphosis.

Thyroid hormones play a central role in regulating amphibian metamorphosis (**Figure 1**). Increasing titers of thyroid hormone during larval development are associated with tissue-specific changes that occur during metamorphosis. Thus, the timing of metamorphosis is potentially associated with a number of mechanisms that regulate TH: (1) the hypothalamus-pituitary-axis that regulates TH synthesis and secretion from the thyroid gland, (2) TH transport and uptake within cells, (3) activation and inactivation of TH within cells, (4) binding of TH to steroid nuclear receptors in the nucleus, and (5) the interaction of TH and TR with protein complexes that regulate transcription. TH levels in the axolotl remain low throughout larval development

and do not increase at the time metamorphosis occurs in related tiger salamanders. However, the axolotl is capable of initiating and completing metamorphosis when thyroid hormone and other endocrine factors of the HPT axis are administered. Collectively, these and other results suggest a defect in the regulation of the HPT axis, perhaps at the level of the hypothalamus or pituitary (19–24). Given the importance of thyroid hormone feedback on HPT axis maturation and control, axolotl paedomorphosis presents a conundrum—are TH levels inherently too low to support normal development and function of the hypothalamus and pituitary, and/or are these glands relatively insensitive to TH feedback?

One mechanism that has been advanced to explain axolotl paedomorphosis is a failure in hypothalamic stimulation of pituitary thyrotropes that secrete thyrotrophin (TSH) to regulate thyroid activity (19). In contrast to mammals, corticotropin (CRH) and not thyrotropin releasing hormone (TRH) mediates release of TSH from the larval amphibian pituitary (25). CRH treatment of metamorphic tiger salamanders decreases the time to metamorphosis (26), consistent with thyrotrophic stimulation, while CRH treatment does not increase circulating T4 levels in the axolotl (24). While this result is consistent with a failure in hypothalamic stimulation of the pituitary, CRHR2 expression in axolotl thyrotropes appears to be normal. This highlights critical knowledge gaps in our understanding of axolotl paedomorphosis. Studies that have interrogated aspects of endocrine regulation have not rigorously controlled axolotl age, body size, or sex. Inducing metamorphosis by TH treatment of adult axolotls ignores early larval developmental windows within which the HPT axis matures and becomes operative. Also, studies have not assessed mechanisms of thyroid hormone regulation within all HPT axis tissues. For example, while it is clear that axolotls have functioning thyroid hormone receptors (27), the expression of TRs in HPT axis tissues has not been assessed. Also, while CRH is clearly essential for releasing TSH during amphibian metamorphosis, the secretion of CRH or CRH-like peptides has not been shown in the axolotl (19). Finally, much of what we know about the endocrinology of amphibian metamorphosis comes from studies of anurans, not from studies of salamanders. These two amphibian groups diverged several hundreds of millions of years ago and salamander families are minimally 150 million years diverged. Thus, it is not clear that the anuran metamorphic knowledge base, or the relatively fewer insights gained among salamander species, can provide a framework to conceptualize the HPT axis in axolotls. While endocrinology-guided approaches have provided important insights, there is need to consider other avenues of reasoning in the study of axolotl paedomorphosis.

Genetics of Paedomorphosis

As was introduced above, genetic studies are beginning to resolve the location of genetic factors within the axolotl genome that regulate metamorphic timing and expression of paedomorphosis. Primarily, axolotls have been crossed to metamorphic tiger salamanders to segregate alleles that affect paedomorph expression and metamorphic timing, however we highlight a study (17) that crossed the axolotl to a paedomorphic relative to identify genetic factors associated with T4 sensitivity. In that study, second generation *A. mexicanum/A. andersoni* paedomorphic hybrids were created and administered 50 mM T4 at the time metamorphosis occurs in metamorphic tiger salamanders. Siblings exhibited tremendous variation in metamorphic timing (160-day range) and some individuals remained paedomorphic after 150 days of continuous T4 treatment. Genetic linkage mapping was then used to identify three moderate effect QTL (*met1*-3) that additively explained variation in metamorphic timing, including *met1* first identified in *A. mexicanum/A. tigrinum* hybrids. This study showed that metamorphic timing is associated with QTL that segregate allelic variation for responsiveness to T4.

At the time these QTL were identified and in lieu of a sequenced axolotl genome, comparative genome mapping was used to identify candidate genes for *met1*-3. The expressed sequence tag (EST) that was used to initially map *met1* showed similarity to *nerve growth factor receptor* (*ngfr*). As more genes were mapped, it became clear that this *ngfr-like* gene corresponded to *nradd* and *met1* located to a genomic region that was uniquely structured during vertebrate evolution. The genes in this region are found on separate chromosomes in all other vertebrates, including the newt (*Notophthalamus viridescens*) which is member of a different salamander family (28). Page et al. (18) speculated that this chromosomal fusion may have brought genes into linkage that are relevant for paedomorph expression in *Ambystoma*, because several of the genes have neurological functions. In addition to *nradd,* which is expressed in the mouse hypothalamus (29), *ccm2, map2k3,* and genes from the Smith-Magenis syndrome region in the human genome associate with *met1. ccm2* is associated with neurovascularization (30) while *map2k3* was recently identified as a superior memory candidate gene in SuperAgers (31). Smith-Magenis syndrome is primarily attributed to deletion polymorphisms of *rai1* (32), a dosage-sensitive transcription factor that regulates multiple functions, including embryonic neurodevelopment, neuronal differentiation, and circadian rhythm. Although *rai* locates outside the *met1* region, it is possible that long range enhancers for *rai1* or other flanking genes may locate within the *met1* region (33). While no candidate genes were identified for *met2, met3* is associated with *pou1f1,* a transcription factor associated with combined pituitary hormone deficiency in humans. This deficiency is associated with incomplete secretion of pituitary hormones involved in regulating growth and development, but not reproduction. This makes *pou1f1* a good candidate for metamorphic regulation because metamorphic and paedomorphic ambystomatids do not show differences in reproductive potential, although paedomorphic species can reproduce multiple times annually while metamorphic species breed once annually (7).

New Axolotl Genome Resources

Five years ago, it was difficult if not impossible to pursue studies of axolotl candidate genes identified by comparative mapping. At that time, the large axolotl genome (32 Gb) had not been sequenced and thus it was difficult to develop molecular probes

and investigate gene functions. However, in just the past couple of years, the axolotl genome has been incrementally sequenced and recently a chromosome-level genome assembly was completed (34). Now it is possible to comprehensively evaluate *met1-3* genomic regions for candidate genes and test gene functions using genome-editing approaches and transgenics. In this section, we review new, critical resources to identify and test candidate genes for future studies of axolotl paedomorphosis.

It is important to note that the method used to assembly the axolotl genome simultaneously increased the resolution of candidate genes within the *met1* genomic region (34). Forty-eight individuals from the meiotic mapping panel that was used to map *met1* (16) were sequenced to 2x depth to identify polymorphisms that in turn were used to order genomic scaffolds into chromosomes by linkage analysis. The 48 individuals that were sequenced were not randomly drawn from the mapping panel. Instead, individuals that exhibited recombination within the *met1* genomic region were purposely chosen for sequencing to more finely resolve the boundaries of recombination that differentiate early (metamorphic) vs. late (paedomorphic) metamorphosing individuals. Inspection of these recombination boundaries against the background of the physically ordered loci yielded a high confidence genomic region of relatively few candidate genes (**Figure 2**).

The ordering of *map2k3-nradd-and setd2* is the same in the genome assembly as it was determined previously by genetic linkage analysis. Page et al. (18) mapped these loci near the maximum inflection point of the *met* 1 LOD peak, implicating them as especially good candidate genes. The new genome assembly revealed new, physically-linked candidates, including *dhrs7b, tmem11*, and *natd1* from the Smith-Magenis syndrome region, and *kcnj12, klhl18, smarcc1,* and three anonymous, predicted genes. Using BLASTn and BLASTx searchers of the anonymous genes against NCBI databases, we discovered the likely identity of all three loci: *camp* (LOC101951429), *ccn1-like* (LOC102363594), and *c16orf89* (LOC102943813). While *camp* is known to be syntenic with *setd2, kif9,* and *khlh18* in the human genome, *ccn1-like* and *c16orf89* are not expected to map to the *met1* region, which only contains loci from human chromosomes 3 and 17. *ccn1* is a member of the CCN gene family, which contains six different members (CCN1-6). CCN family members typically have an N-terminal secretory signal peptide and four structural domains: an insulin-like growth factor binding protein-like domain, a von Willebrand factor type C repeat (VWC) domain, a thrombospondin-homology type 1 repeat (TSP1) domain, and a C-terminal cysteine-knot-containing (CT) domain. Interestingly, the *met1 ccn1-like* gene shows sequence similarity to *ccn1* but has a 30 bp deletion in the hinge region between the VWC and TSP1 domains (**Figure 2**). We determined the location of each presumptive CCN ortholog in the axolotl genome assembly, including a second *ccn1-like* gene that showed higher sequence identity to *ccn1*-vertebrate orthologs. The position of the second *ccn1-like* gene in the axolotl genome assembly suggests it to be the true vertebrate *ccn1* ortholog and thus the *ccn1-like* gene in the *met1* region appears to represent a novel gene. An ancient origin for this novel gene seems likely because it is equally divergent in amino acid similarity from human, *Xenopus tropicalis*, and axolotl *ccn1* orthologs (**Figure 2**). In contrast, the positioning of *c16orf89* in the met genome region likely reflects an intrachromosomal inversion as several genes linked to *c16orf89* in the human genome are found on axolotl chromosome 2, although some distance away.

How to Test the Candidate Genes for Paedomorphosis?

It is important to point out that none of the candidate genes in the *met1* region have been annotated to endocrine gland development or endocrine process gene ontologies (**Table 1**). Two genes (*setd2, smarcc1*) that regulate global patterns of transcription during early neural development in mammals maybe interesting candidates to pursue because brain transcriptional activity is generally lower in larval axolotls than larval tiger salamanders (18, 35). Thyroid hormone and NGF signaling (perhaps via *nradd*) mediate early neuronal development in mammals, and based upon transcriptional data, *c16orf89* is predicted to play a role in thyroid gland development and function. Given the multifunctional roles of CCN family members, *ccn1-like* presents an attractive candidate. However, nothing is known about this genes function and none of the genes discussed above seem to standout above the rest. As a path forward, we believe that all of the genes in the *met1* region can be quickly tested for function using CRISPR-Cas9, which is known to efficiently knock-out genes in the axolotl. For example, CRISPR-Cas9 was used recently to create insertion/deletion polymorphisms in *fat3* to generate offspring that presented limb and kidney defect phenotypes seen in the *short toes* axolotl mutant (36). In a similar vein, CRISPR-Cas9 could be used as a strategy to test candidate genes in the *met1* genomic region using offspring from axolotl x tiger salamander crosses. For example, it is well-established that all axolotl x tiger salamander F1 hybrids undergo metamorphosis. This suggests that dominant tiger salamander alleles at candidate genes are sufficient to induce metamorphosis. Conversely, this suggests that paedomorphosis is associated with the inheritance of recessive alleles that may be partially functional or null in regards to inducing metamorphosis. To test this hypothesis, it would be efficient to knockout candidate genes in F1 hybrids as only the tiger salamander allele would need to be edited. F1 individuals could be assayed for paedomorphosis or delayed metamorphic timing and thus in short order, each of the candidate genes could be tested functionally. We note that it would also be informative to knock out *met1-3* candidate genes in other vertebrates, including frogs which do not present paedomorphosis.

CONCLUSION

Although the Mexican axolotl has been studied for over 150 years, the mechanism associated with its unique paedomorphic mode of development remains unknown. Endocrinology studies have established the importance of thyroid hormone in regulating amphibian metamorphosis. The axolotl does not show an increase in thyroid hormone during early development and thus

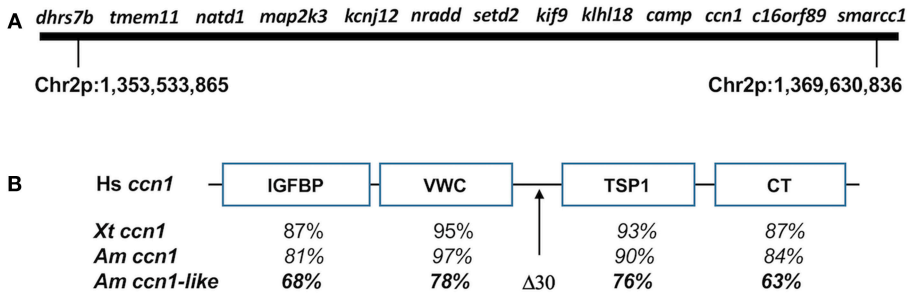

FIGURE 2 | (A) Map of genes from axolotl chromosome 2 (Chr2p) that associate with *met1*. **(B)** General structure of CCN family proteins showing insulin-like growth factor binding protein-like (IGFBP), a von Willebrand factor type C repeat (VWC), thrombospondin-homology type 1 repeat (TSP1), and a C-terminal cysteine-knot-containing (CT) domains. Human (Hs) *ccn1* (GenBank: AAB84227.1) was used as a reference to compare domain-specific amino acid similarity among *Xenopus tropicalis* (Xp) *ccn1* (GenBank: OCA36969.1), *A. mexicanum ccn1* (AMEXTC_0340000257024_cysteine-rich), and *A. mexicanum ccn1-like* (AMEXTC_0340000025590_LOC102363594). The *A. mexicanum ccn1-like* sequence has a unique deletion of 30 amino acids between the VWC and TSP1 domains.

TABLE 1 | Candidate genes from the *met1* region with functional information obtained from the NCBI Gene database.

Gene ID	Gene information
dhrs7b	Short-chain dehydrogenase/reductase family member. Possibly could function in steroid hormone regulation.
tmem11	Mitochondrial inner-membrane protein thought to regulate mitochondrial morphogenesis.
natd1	The function of this gene is unknown but transcripts in mice embryonic stem cells suggest a role in hematopoiesis.
map2k3	MAP kinase-mediated signaling cascade member that activates MAPK14/p38-MAPK in response to mitogens and environmental stress.
kcnj12	Inwardly rectifying K+ channel generally associated with heart function although broadly expressed among other tissues in human.
nradd	Highly similarity to the p75 neurotrophin receptor *ngfr* that functions in neurotrophin signaling in rodents.
setd2	Histone methyltransferase that is specific to lysine-36 of histone H3. Methylation of this residue is associated with active chromatin. Interacts with histone H2A.z to regulate embryonic neurogenesis in mice.
kif9	Kinesin motor protein that functions in the regulation of spindle length and chromosome alignment during mitosis.
klhl18	Associates with *cul3* ubiquitin ligase to regulate cell cycle entry.
camp	An antimicrobial and immune response protein.
ccn1-like	Novel CCN family gene. CCN family proteins regulate many cellular responses that are critical for skeletal, vascular, and neural development.
c16orf89	The function of this gene is unknown but human transcripts are enriched in the thyroid gland.
smarcc1	Important component of the large ATP-dependent chromatin remodeling complex SNF/SWI which functions during brain development to regulate transcription globally.

fails to undergo metamorphosis. While many aspects of the HPT axis seem to be functional in the axolotl, and peripheral tissues are responsive to thyroid hormone treatment, endocrinology-guided studies have not resolved the basis of paedomorphosis. We argue the need to test candidate genes from genetic studies of axolotl paedomorphosis using new genomic resources available to the community. In particular, the new axolotl genome assembly has resolved a short-list of candidate genes for the *met1* genomic region that can be efficiently tested using CRISPR-Cas9 to knock-out gene functions. The recent development of essential genetic and genomic tools for the axolotl brings us closer to identifying mechanisms of paedomorphic development and understanding the role of thyroid hormone in development and evolution.

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REVIEW
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Insufficiency of Thyroid Hormone in Frog Metamorphosis and the Role of Glucocorticoids

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Thyroid hormone (TH) is the most important hormone in frog metamorphosis, a developmental process which will not occur in the absence of TH but can be induced precociously by exogenous TH. However, such treatments including *in-vitro* TH treatments often do not replicate the events of natural metamorphosis in many organs, including lung, brain, blood, intestine, pancreas, tail, and skin. A potential explanation for the discrepancy between natural and TH-induced metamorphosis is the involvement of glucocorticoids (GCs). GCs are not able to advance development by themselves but can modulate the rate of developmental progress induced by TH via increased tissue sensitivity to TH. Global gene expression analyses and endocrine experiments suggest that GCs may also have direct actions required for completion of metamorphosis independent of their effects on TH signaling. Here, we provide a new review and analysis of the requirement and necessity of TH signaling in light of recent insights from gene knockout frogs. We also examine the independent and interactive roles GCs play in regulating morphological and molecular metamorphic events dependent upon TH.

Keywords: thyroid hormone, glucorticoids, metamorphosis, Amphibia Anura, crosstalk

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INTRODUCTION

Vertebrate life history transitions, such as birth or weaning in mammals, smoltification in fish, hatching in birds, and metamorphosis in amphibians, are associated with dramatic morphological and/or physiological changes underlain by striking maxima in several plasma hormone titers (1–6). Chief among the hormones involved are thyroid hormone (TH) and glucocorticoids (GCs), but other hormones with less extensive or recognized roles include prolactin, aldosterone, and insulin (7–11). Lack of GCs is not conducive to neonate survival in mammals (12), and lack of TH signaling precludes developmental progression in tadpoles (13), underscoring the critical importance of hormones during development. Typically, the actions of hormones during life history transitions are studied one hormone at a time, and when studying hormone interaction, the effect of one hormone's ability to affect the tissue sensitivity to other hormones is determined (14–16). However, other modes of hormone interaction are not well-characterized. Here, we focus on the extensively studied roles of TH and GCs in frog metamorphosis to gain insight into how hormones may interact to accomplish developmental changes.

The common thumbnail understanding of hormonal control of frog metamorphosis is that TH signaling is necessary and sufficient for metamorphosis and that GCs increase the rate of transformation via increasing tissue sensitivity to TH (13, 17–20). Similarly, signaling through TH receptors (TRs) is viewed as necessary and sufficient to initiate metamorphic events based

on transgenic overexpression of mutant TRs (21–24). Current understanding of the molecular and developmental roles of TH and TR signaling has been summarized in the dual function model, where TRs act to repress TH-response gene expression in the absence of TH to prevent metamorphic events until TH becomes available in order to signal through TRs to induce TH response gene expression and accomplish metamorphic transformation (25–27). The current review will highlight previous and recent evidence suggesting modifications to this thumbnail sketch, namely that TH is required for complete tissue transformation in wild-type but not mutant animals lacking TRs, that TH signaling is not sufficient to accomplish frog metamorphosis, and that GCs do more than modulate TH tissue sensitivity.

ANALYSIS OF THE REQUIREMENT FOR TH/TR SIGNALING IN FROG METAMORPHOSIS

Early experiments showed that TH is required for metamorphosis (13, 28). Removal of TH via embryonic thyroidectomy or treatment of tadpoles around or before the start of feeding with chemical inhibitors of TH biosynthesis (thiourea, propylthiouracil, potassium permanganate, methimazole) completely inhibits developmental progression beyond the foot paddle stage (**Figure 1A**). The inhibited tadpoles continue to grow at the same or faster rate than control tadpoles but external morphology, internal histology, and biochemistry remain larval with little if any indication of progress toward metamorphosis. Similarly, blockade of TR action by transgenic overexpression of a dominant negative TR inhibits metamorphosis when expressed all over the body and inhibits transformation of specific tissues when overexpressed in those tissues (**Figure 1A**) (21, 24, 29–34). These dominant negative TRs lack the last several C-terminal amino acids such that they cannot bind TH and thus maintain repression of TH response genes even in the presence of TH. Likely exceptions to a requirement for TH signaling to achieve the adult condition include lens crystallin transition, which appears to depend on tadpole size rather than stage (35) and gonad development where gonadal sex differentiation occurs on its own schedule irrespective of somatic developmental progression followed by oocyte and sperm production in the tadpole body in an extended absence of TH (36–40).

TH signaling is indeed required to accomplish metamorphosis, but how much signaling required is not defined. Two non-mutually exclusive models have been proposed to explain how much TH signaling is required, the "stoichiometric" model and the "threshold" model (41). A stoichiometric relationship between TH signaling and metamorphic progression implies that a certain sum total of TH signaling is required, which can be achieved by high levels of signaling for a short duration or lower levels over a longer duration (42). This model stems from the fact that the rate of induced metamorphic development is positively correlated with the concentration of exogenous TH. The stoichiometric model has been mistakenly contrasted with the threshold model of TH in metamorphosis where each developmental stage requires a certain minimum TH concentration in order to be achieved (43). This model stems from the fact that each tissue has its own threshold sensitivity to plasma TH level below which that tissue will not respond. Thus, near the threshold TH sensitivity for a tissue, the tissue transforms slowly with low levels of induced TH response genes, and at TH doses above the threshold, higher peak levels of TH response gene expression and developmental rates are achieved. In agreement with these models, spadefoot toad species with higher rates of metamorphosis have higher peak amounts of TH body content and higher levels of metamorphic gene expression compared to spadefoot species with longer larval periods (44, 45). Also, within a species, tadpoles reared in conditions that accelerate metamorphosis (e.g., low water) exhibit a higher peak in TH body content and TH response gene expression level (46).

Further insights into the role of TH signaling in metamorphosis came from *TRα* or *TRβ* knockout animals. The result that removal of TH and the transgenic expression of dominant negative TRs block metamorphosis has been over-interpreted by virtually every expert in the field to mean that gene induction by TH is required for metamorphosis. Gene induction involves TH binding to TR and recruitment of co-activators that induce gene expression (20, 27). In the absence of TH, TRs recruit co-repressors to actively repress (i.e., "turn-off") genes. In the absence of TRs, such active repression would not occur allowing "leaky" expression of TH response genes, but the level of such expression resulting from lack of repression is usually lower than that induced by TH binding to TR (47, 48). Thus, the blockade of metamorphosis due to lack of TH or overexpression of dominant negative TR has at least two possible interpretations. Either TH induction of genes is indeed required for metamorphosis, or alternatively lack of repression may allow enough expression of TH-regulated genes to enable metamorphosis. Rearing *TRα* knockout animals in methimazole resulted in full development of limbs and skin, suggesting that induction of TH response genes is not required for metamorphosis (**Figure 1A**) (48, 49). Thus, TH signaling is required in wild type animals, but results from *TRα* knockout animals suggest that the observed de-repression of TH response genes rather than TH-mediated induction of metamorphic genes may induce enough gene expression to allow metamorphic completion. It remains to be unequivocally demonstrated that limb and skin and any other organs can undergo full metamorphosis without induction of genes through TR by analyzing *TRα*/*β* double knockouts. The results with *TRα* knockout animals are consistent with the stoichiometric model, where a slight increase in TH response gene expression (de-repressed levels rather than induced levels) is enough to allow limb development to proceed to completion, albeit more slowly than normal. Similarly for *TRβ* knockout animals, tail resorption is delayed, presumably because of the reduced TH signaling from loss of TRβ (50, 51).

The above results reveal consistent relationships between dose of TH, level of gene expression, and rate of developmental change, but the molecular mechanisms that determine how much signaling is required and for how long to achieve full

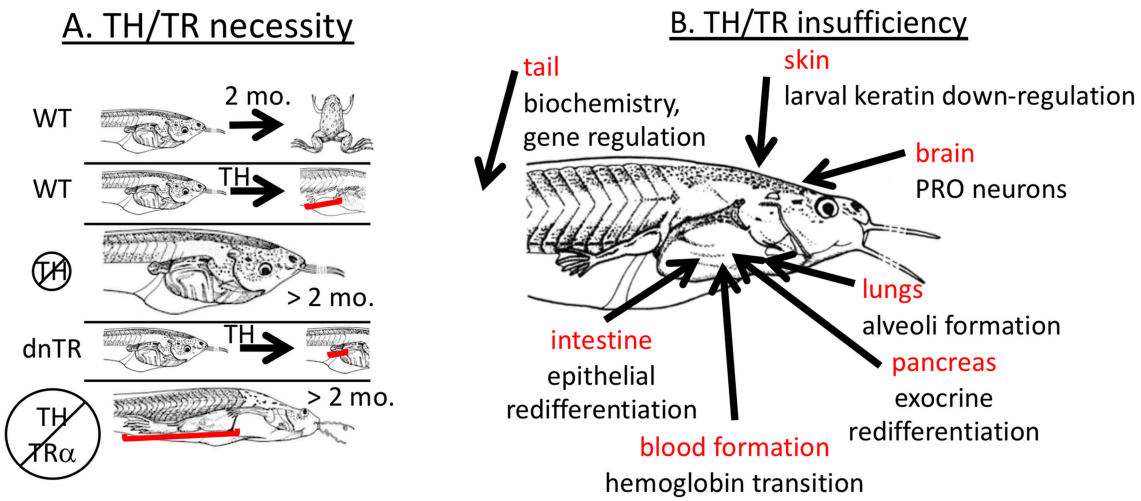

FIGURE 1 | Necessity yet insufficiency of TH signaling in frog metamorphosis. **(A)** TH signaling is necessary. In wild-type (WT) tadpoles, metamorphosis is complete within 2–3 months, and tissue transformation, such as limb development, can be induced prematurely (3–5 days) by exogenous TH. Animals lacking TH are completely inhibited from metamorphic transformation but grow indefinitely in size. Tadpoles overexpressing dominant negative TH receptor (dnTR) do not exhibit limb elongation when treated with exogenous TH showing either that gene induction or at least lack of repression is required. When TH synthesis is blocked in TRα mutant animals (knockout), development of all tadpole tissues is stopped except limbs and skin which predominantly express TRα strongly in non-mutant animals indicating that lack of repression of TH response genes is necessary. Brackets highlight significant effects on limbs. **(B)** TH signaling is not sufficient. Many cases have been identified where exogenous TH is not sufficient to replicate natural metamorphosis. Each indicated tissue has metamorphic events that do not occur properly with just TH signaling. PRO = preoptic recess organ. See text for details.

tissue transformation are little understood. As a start, it has been estimated that two days of TH signaling is enough to achieve full TH signaling required for tissue destructive events but not constructive events (52), but this duration is likely dependent on TH dose, target tissue, and temperature. Removal of thyroidectomized tadpoles from TH treatments led to cessation of developmental progression after 2–3 days, where hind leg growth and tail regression came to a halt (53). More work is required to determine how the amount of TH signaling relates to the expression kinetics of TH response genes in the TH-induced gene regulation cascade that then controls the rate of metamorphosis.

ANALYSIS OF THE SUFFICIENCY OF TH/TR SIGNALING IN FROG METAMORPHOSIS

TH is considered to be sufficient for metamorphosis because addition of TH to premetamorphic tadpoles initiates virtually all known metamorphic changes (13). Continuous treatment of tadpoles with low doses or graded increases in TH dose over time enables animals to survive and complete metamorphosis precociously (53, 54). In addition, signaling through the TR appears to be sufficient to mediate the TH signal for metamorphic tissue transformations, because overexpression of a constitutively active mutant of TRα can initiate all the metamorphic events assessed (22). Addition of any other hormone by itself in the absence of TH, including GCs, aldosterone, and prolactin, has no known developmental effect during the larval period (7, 10, 11). Thus, it is commonly accepted that signaling through TH and TR is sufficient for all metamorphic transformations and that no other hormone is responsible or required. Despite this generalization, natural tissue remodeling is not always replicated by exogenous TH treatment. An obvious example is the jutting lower jaw and subsequent death typically within 7–10 days, prior to completion of metamorphic development in many organs when climax-level doses or higher of exogenous TH are given to young tadpoles (55). Additional discrepancies between natural and TH-induced metamorphosis have been observed in many organs, including lung, brain, blood, intestine, pancreas, tail, and skin (**Figure 1B**).

Lungs

A striking example where TH treatment may not recapitulate the events of natural metamorphosis has been observed in lung transformation (56). In tadpoles, septa buds form and extend into the lumen of the sac-like lung forming numerous, thin-walled alveoli, a process that begins in premetamorphosis. The role of TH in lung development is not well characterized, but expression levels of *TR*α and *TRβ* increase in lung in TH-treated organ culture and reach a peak during natural metamorphosis in *Lithobates catesbeianus* (57). Similarly, exogenous TH induces the TH-response gene *Krüppel-like factor 9* (*klf9*) in organ culture in bullfrog, and reaches a peak at metamorphic climax in *Xenopus laevis*, though not in bullfrog (57, 58). In contrast to the natural septation process, treatment of premetamorphic bullfrog tadpoles with TH appeared to cause an abnormal thickening of the connective tissue in the lung wall and no septation (56). Addition work is required to examine what may

explain this effect of TH and the potential role of GCs in lung morphological development.

Brain

During metamorphosis, many TH-dependent changes occur in the central nervous system, including elaboration of the median eminence where hypothalamic axon terminals release hormones acting on the pituitary causing release of hormones that act to increase TH and GC levels (7, 59). In thyroidectomized or hypophysectomized (removal of pituitary) tadpoles, no monoamine-containing neurons appear in the preoptic recess organ of the hypothalamus, and neither catecholamine terminals nor capillaries appear in the median eminence (60, 61). Treatment with TH induced development of preoptic recess organ catecholamine neurons and capillaries in median eminence in the thyroidectomized bullfrog tadpoles, but surprisingly not in hypophysectomized bullfrog tadpoles even though external morphology was induced in both groups. Importantly, exogenous GCs, specifically corticosterone (CORT), induced the appearance of monoaminergic neurons in the preoptic recess organ in the hypophysectomized larvae but without causing morphological progress. Development of these neurons and capillaries appears to be the only GC-dependent and TH-independent metamorphic events known.

Blood

During the climax of metamorphosis, larval erythrocytes containing larval hemoglobin are replaced by adult erythrocytes containing adult hemoglobin (62). Treatment of bullfrog tadpoles with TH induced minimal hemoglobin transition, and even after 2–4 weeks of treatment only partial adult hemoglobin synthesis and no decrease in larval hemoglobin occurred (63–65). Further, adult erythroblast proliferation was only minimally induced by TH, even though TH induced larval erythroblast apoptosis in the liver. However, erythroblast proliferation was substantially induced by TH plus CORT (though CORT by itself did not affect larval erythrocyte apoptosis) (66). Consistent with these results, inhibition of TH synthesis using propylthiouracil for over one year starting at early limb bud stage produced giant tadpoles of *Xenopus laevis* which exhibited a complete hemoglobin transition from larval to adult in the absence of morphological change (67). Similarly, the axolotl, a facultative neotenic species of *Ambystoma* salamanders, has adult rather than larval hemoglobin in a larval body (68) and the larval to adult hemoglobin transition occurs in thyroidectomized but not hypophysectomized larvae of the salamander *Hynobius* (69). Thus, even though production of adult hemoglobin can be induced by TH to a small extent, TH is not sufficient for the full larval to adult hemoglobin transition and is not necessary for the transition to occur.

Intestine

During metamorphosis, the larval intestinal epithelium undergoes apoptosis, while adult epithelial cells from dedifferentiated larval epithelial cells proliferate, differentiate, and repopulate the intestinal epithelium to accommodate the change in diet from plant material to live prey (70). TH treatment of bullfrog tadpoles reared in thiourea decreased larval brush border hydrolytic enzyme activity, but adult levels of enzyme activity did not come about even after 15 days post treatment (71). TH treatment of small intestine cultured *in vitro* also caused larval cell degeneration (72), but adult epithelium failed to proliferate sufficiently (73), adult-type microvilli did not form (74), and adult-pattern lectin binding failed to occur (72). In addition, the adult epithelium achieved by natural metamorphosis and the epithelium achieved by TH treatment responded to GCs, specifically hydrocortisone, differently (75, 76). In particular, hydrocortisone increased intestinal digestive enzymes after natural metamorphosis but decreased them after TH-induction. However, *in-vitro* TH treatment of small intestine combined with the GC cortisol and/or insulin mimicked complete larval to adult epithelial transition reconstituting a brush border and exhibiting the supranuclear adult lectin binding pattern (72).

Pancreas

During metamorphosis, the pancreas shrinks by 80% due to loss of zymogen granules and exocrine cell apoptosis (31, 77). Also, beta cells of the Islets of Langerhans exhibit a transient decrease in *insulin* mRNA expression though apparently without a decrease in beta cell number as they change from a larval to adult arrangement and cellular histology (32, 78). After climax, rebuilding the adult pancreas involves morphogenesis of the acini and ducts, redifferentiation of exocrine cells, and re-expression of endocrine hormones and begins around tail resorption when TH levels have already returned to baseline (31, 32). TH treatment mimics the morphological (reduction in pancreas mass) and biochemical (increased protein degradation and DNA synthesis) changes associated with remodeling of the larval pancreas that occur before metamorphic climax, but the increase in pancreas size and protein synthesis found in the natural remodeling process after metamorphic climax are not observed even after two weeks of TH treatment (though DNA synthesis does return) (79). Similarly, TH treatment induces the loss of larval alpha-amylase, but the normal replacement by adult alpha amylase does not occur (80). Partial pancreatectomy in premetamorphic tadpoles caused increased islet cell size and changed arrangement in ways reminiscent of metamorphic changes, leading to the view that islet remodeling may not be under TH control (81). However, islet remodeling appears to require TH-dependent remodeling of the exocrine pancreas, even when TH signaling is specifically blocked only in beta cells (32). These results suggest that pancreas resorption is stimulated by TH but that redifferentiation of newly proliferated exocrine cells accompanied by rearrangement of islet cells may not be dependent on TH.

Tail

The sufficiency of TH in tail regression at the end of metamorphosis is not clear. Complete resorption of the tail is not observed upon prolonged treatment with moderate but effective doses of TH in premetamorphic tadpoles (41). However, treatment with a graded series of TH from low to high over successive days to mimic the developmental profile of endogenous plasma TH enables complete metamorphosis

including tail resorption, and TH alone induces nearly complete tail shrinkage in culture devoid of other hormones (37, 53, 82). Even though GCs have no known action to induce tail regression, GCs synergize with TH *in vitro* to accelerate tail shrinkage (14, 83) and inhibition of GC signaling with amphenone B (a corticoid synthesis inhibitor) inhibited TH-induced tail resorption *in vivo* (84). In contrast, cortisol partly inhibited TH-induced reduction in DNA synthesis in tail epidermal cells (85), which is consistent with the observation that GCs by themselves increase tail growth *in vitro* (14, 83). In addition, CORT and TH have synergistic as well as antagonistic interactions at the level of gene expression (see below) (86). At the biochemical level, the role of taurine in tail regression is not known, but the amount of the atypical amino acid taurine in tail increases a few stages before tail regression during spontaneous metamorphosis but not in TH-induced metamorphosis (87). Also, beta-glucuronidase activity levels increase 3-fold more during spontaneous metamorphosis compared to TH-induced metamorphosis (88). On the other hand, cathepsin C increased in tail, gill, liver, lung, and kidney during natural metamorphosis, but TH treatment in premetamorphic bullfrog tadpoles induced cathepsin C activity only in the tail (89).

Skin

Larval skin is glandless with three layers of uncornified epithelial cells containing cytokeratin 8 in the apical cell layer and larval keratin in the suprabasal skein and basal skein cell layers (90–92). Skein cells are strictly larval and have special intermediate filament bundles in them (called Figures of Eberth) (93). Metamorphosis results in the typical vertebrate cornified, stratified skin epithelium, which has a proliferative adult basal layer and expresses several adult keratins. The adult basal layer is derived from a series of three differentiation steps: first, basal skein cells change to adult keratin-positive basal skein cells which then change to larval (or pre-adult) basal cells associated with secondary connective tissue, and then these cells in turn change to adult basal cells (92, 94). In *in-vitro* skin culture, TH plus hydrocortisone but not TH alone reduced larval keratin synthesis in isolated primary epithelial cells after 4 days (95) but longer culture (9 days) with TH alone resulted in production of full adult skin except the secondary connective tissue did not form (96). Also, TH plus hydrocortisone-treated larval epidermal cells produced sheets of cornified cells as seen *in vivo*, while TH by itself induced only scattered single cornified cells (95). In addition, expression of adult keratin in basal skein cells can occur in culture in the absence of TH (96). Thus, the series of differentiation steps of the basal skin cells during skin transformation appear to involve a variety of TH- and GC-dependent and -independent steps.

MODES OF TH/GC INTERACTION

It is not known what explains the numerous cases where exogenous TH does not replicate events of natural metamorphosis. The cause of death after prolonged (7–10 days) TH treatment has been provisionally attributed to simultaneous initiation and highly abnormal rates of tissue transformations (97) or perhaps to thyro-toxicity because exogenous doses in the rearing water can achieve 5–6 times that amount within the tadpole body (98). Within a tissue, discrepancies between natural and induced metamorphosis may be due to incomplete organ competence to respond to TH, inappropriate TH dose, and/or requirement for other hormonal inputs. Future experiments are required to unequivocally establish any of these mechanisms. Here, we examine the possible requirement of GC signaling and interaction with TH for metamorphosis.

The best known role of GCs in metamorphosis is to synergize with TH to accelerate TH-induced metamorphosis in *Xenopus* (14, 17) and in axolotl (99). On the other hand, exogenous CORT by itself does not induce metamorphic development, and in fact inhibits growth and development in premetamorphic tadpoles (100, 101). Surprisingly, even when administered during prometamorphosis when endogenous TH is present, exogenous CORT still inhibits metamorphosis in *Xenopus laevis* (100) but accelerates development by itself in toad species (102, 103). Inhibitory effects of exogenous GCs administered during prometamorphosis may be acting at the level of the hypothalamus resulting in lower plasma TH, but this possibility has not been tested. Also, even though TH-response gene induction may not be required for metamorphosis (see above discussion), it is likely that CORT is required for survival through metamorphosis. In particular, only TH plus ACTH (adrenocorticotropic hormone, the pituitary hormone that stimulates production of GCs), but not TH alone, enabled survival through metamorphosis of hypophysectomized tadpoles (104). In contrast, lack of metamorphosis in hypothalectomized (hypothalamus removed, blocks stimulation of pituitary hormones required for TH and GC production) could not be rescued by treatment with TH and ACTH (13).

It is possible that the explanation for GC acceleration of TH-induced metamorphosis, death from lack of GC signaling, and the observed discrepancies between natural and induced metamorphosis may be related to TH tissue sensitivity. The increased tissue sensitivity induced by GCs (see below) can explain acceleration of TH-induced metamorphosis by GCs, where increased TH sensitivity would increase TH signaling and thus the rate of metamorphic development. Also, death from lack GC signaling and failure of exogenous TH to completely replicate natural development, which presumably includes a lack of a surge in GCs, may be due to lack of GC-induced increase in TH sensitivity, such that insufficient TH sensitivity may disallow development of a critical organ system before TH levels return to baseline or may lead to disruptions of the normal series of asynchronous developmental events (e.g., it would be problematic if leg development were not complete before tail resorption). On the other hand, high exogenous TH doses would presumably negate a need for GC-dependent increases in TH sensitivity. Thus, distinct from altered TH sensitivity, direct and required actions of GCs may explain death from lack of GC signaling and may also explain discrepancies between natural and induced metamorphosis. Examples of direct action of GCs different from just increasing TH sensitivity would be that GC-response genes may integrate with the TH gene regulation

cascade and/or GCs may act on differentiation steps subsequent to differentiation steps accomplished by the TH gene regulation cascade. These modes of GC interaction with TH-dependent development, namely altered TH tissue sensitivity and direct effects of GCs, are discussed in the following sections.

GC REGULATION OF TH SENSITIVITY

A well-researched mechanism to explain synergy between TH and GC signaling is the enhancement of tissue sensitivity to TH by GCs (14). Tissue sensitivity to TH is regulated by GCs through (1) altered deiodinase (TH metabolizing enzymes) expression and/or activity, (2) direct GC regulation of TH-response gene expression, such as the transcription factor *klf9*, and (3) indirect GC induction of TH receptor beta, which is one of the targets of Klf9. Reciprocally, the sensitivity to GCs can be enhanced in some tissues by TH via increased GC receptor expression.

Effect on Deiodinases

Deiodinases are a family of enzymes that catalyze the release of iodine from TH leading to the production of T3 (the most active form of TH) from T4 (often considered a prohormone and binds with lower affinity to TH receptors) and to the degradation of T4 and T3 (105). Such TH activation by deiodinase type II (D2) and TH inactivation by deiodinase type III (D3) presumably allows fine control of intracellular hormone availability. Several studies have shown that the acceleration of TH-induced metamorphosis by GCs is partly due to the increased availability of TH in cells through GC effects on deiodinase expression or activity. First, GCs increased D2 activity in tadpole tissues associated with increased generation of T3 from T4 in *Lithobates catesbeianus* (16) and *Anaxyrus boreas* (106). Also, GCs decreased D3 activity in *Lithobates catesbeianus*, decreasing the degradation of T3 (16). Overall, these two actions of GCs contribute to the global increase in TH availability in metamorphosing tissues. Similarly in the neotenic amphibian, the axolotl (*Ambystoma mexicanum*), treatment with dexamethasone (a synthetic glucocorticoid) increased D2 activity and decreased D3 activity, and such changes were accompanied by an increase in plasma T3 levels (107). Using *Xenopus laevis* prometamorphic tadpoles, tail explant cultures, and frog tissue culture cells (XTC-2 and XL-15) (14) showed that *D2* mRNA levels were induced by GCs (and also by T3) supporting that the synergistic actions of TH and GC in metamorphosis occur at the level of expression of genes for D2, enhancing tissue sensitivity to TH (14). The mechanisms by which GCs regulate *D2* mRNA and enzyme activity levels are not yet defined.

Effect on TRs

The level of *TR* gene expression is another central component of TH sensitivity (108). TH acts by binding to TR that functions as a ligand-activated transcription factor. The number of functional TRs expressed by a cell in large part determines the cell's sensitivity and responsivity to T3 (109, 110). TH itself can induce the expression of *TR* (autoregulation) in tadpoles, thus increasing the sensitivity to TH and driving the transformation process (111). In addition to autoregulation, other stimuli can influence the expression of *TR* (cross-regulation) (112). Such cross-regulation by GCs was first shown in bullfrog tadpole tail fins, where an increase in nuclear binding capacity for T3 was observed (113). A direct measure of *TRβ* mRNA levels showed that CORT by itself upregulated *TRβ* mRNA in the intestine, but not tail or brain, in *Xenopus laevis* (98), but *TRβ* mRNA was synergistically upregulated by T3 plus GCs in tail explants, tail and brain *in vivo*, and tissue culture cells (14, 98). In contrast, T3 treatment or spontaneous metamorphosis lead to an increase in the number of T3 binding sites per nucleus in *Lithobates catesbeianus* red blood cells, but surprisingly this effect was abolished by dexamethasone (glucocorticoid receptor agonist) and sustained by dexamethasone plus RU-486 (glucocorticoid receptor antagonist) (114). Thus, the synergistic actions of TH and GC in metamorphosis involve increased *TRβ* expression, thereby enhancing tissue sensitivity and responsivity to TH, though in a cell/tissue specific manner.

Effect on klf9

TRs directly regulate numerous genes, some of which are transcription factors that in turn regulate the expression of other genes in a gene regulation cascade (20). One such transcription factor induced during metamorphosis is *Krüppel-like factor 9* (*klf9*) (115), which is a member of an evolutionarily conserved class of DNA-binding proteins that influence many aspects of development and physiology (116). Several members of this family were shown to be effectors of nuclear receptor signaling. Specifically, the KLFs can act as accessory factors for nuclear receptor actions, can regulate expression of nuclear receptor coding genes, and can be regulated directly by nuclear receptors. *Klf9* in particular is directly induced by GCs in a protein synthesis independent fashion exclusively via GR in tail, lungs, and brain (58, 117). It was further observed that GCs synergize with TH to superinduce the expression of *klf9* (112). This direct TH and GC regulation of *klf9* is evolutionary conserved as it also occurs in mammals (112). To explain how both TH and GCs synergize to increase *klf9* mRNA expression, a highly conserved 200 bp genomic region of the *Xenopus* and mouse *klf9* genes was identified 5–6 kb upstream of the transcription start site with binding sites for TR and GC receptor (GR) (112, 118). Characterization of this region has shown that TH increased the recruitment of liganded GR to chromatin at the enhancer element and that chromosomal looping allows the interaction of this far upstream enhancer region (5–6 kb) with the *klf9* promoter. This transcriptional mechanism of GC and TH interaction is known for just this gene, *klf9*, but there are likely other synergistic genes when considering that GCs synergize with TH to increase the rate of numerous morphological changes occurring during metamorphosis.

To reveal further how intertwined the relationship is between TH and GC interaction, Klf9 itself is involved in the autoinduction of TRβ by TH (119). Klf9 binds to GC-rich DNA regions present in the proximal promoter of the *Xenopus laevis* *TRβ* gene to enhance its autoinduction by T3. Thus, Klf9 acts as an accessory transcription factor with TRs at TR direct target genes, which increases cellular responsivity to further TH action on developmental gene regulation programs (120). Furthermore,

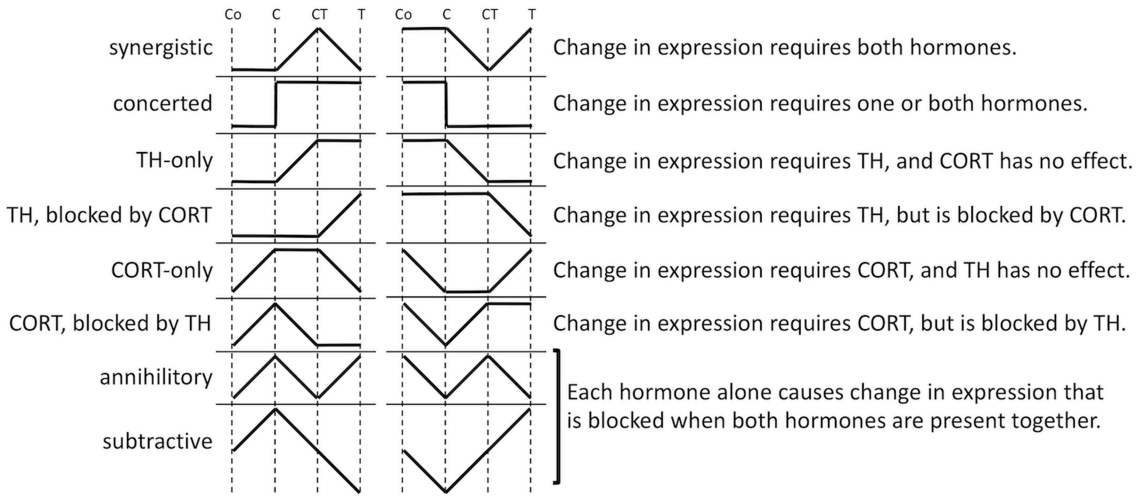

FIGURE 2 | Patterns of gene regulation induced by TH and CORT. Tadpoles were treated with CORT (C), CORT plus TH (CT), or TH (T), or no hormone control (Co), and after 14 h, tails were subjected to microarray analysis. Shown are idealized patterns of changes in gene expression induced by hormone treatments relative to control levels based k-means clustering of significantly regulated genes among treatments (86).

because *klf9* is also a direct GC target gene, GCs not only synergize with TH to induce *klf9* but also thereby increase tissue sensitivity to TH via Klf9 induction of *TRβ*.

Effects of TH on GC Signaling

TH affects GC signaling in at least two ways. First, T4-treatment, but not T3, increased whole body-GC levels in *Anaxyrus boreas* tadpoles, and a corticoid synthesis inhibitor prevented the stimulatory effect of T4 on GC production (106). Second, TH may regulate GR expression, at least in some tissues. During natural metamorphosis with its rising plasma TH titers, *GR* mRNA increases in the brain, lungs, and tail, but not intestine (58, 117). However, T3 treatment increased *GR* expression in the tail and decreased it in the brain (intestine and lungs were not assessed) (14, 98). Mineralocorticoid receptor (MR, the other nuclear receptor for GCs) increased during natural metamorphosis in brain, lungs, and tail and was shown to be inducible in the tail (58, 117). Thus, the synergy of T3 with GC during metamorphosis involves tissue-specific and T3-dependent regulation of *GR* transcripts.

DIVERSITY OF GC AND TH CROSSTALK ON RESPONSE GENE EXPRESSION

As shown above, exogenous TH does not always replicate natural metamorphosis. It is easy to add saturating amounts of TH to rule out insufficient TH signaling as the reason for the discrepancy in induced vs. natural metamorphosis. Also, the lack of metamorphic completion associated with lack of GC signaling (104) suggests that TH is not sufficient and that direct action of GCs not related to TH signaling is required. An important issue, then, is to distinguish between TH and GCs working simultaneously on the same cell vs. independent actions on cells of the same or different stages of differentiation. Also, at the level of gene expression, some changes in gene regulation in the presence of both TH and GCs are inconsistent with the synergistic morphological actions of the two hormones together. High throughput technologies provide a global perspective to help understand the mechanisms of interaction between TH and GCs.

TH and GC Crosstalk: Gene Regulation Profiles

To gain insight into the molecular mechanisms of synergy between TH and GCs, we performed microarray analysis on tail RNA extract from *Xenopus tropicalis* tadpoles treated for 18 h with corticosterone (CORT), T3, CORT plus T3, or vehicle (86). Previously, only one GC response gene was known in tadpoles, i.e., *klf9*, which was also the only known TH/GC synergistic gene. Microarray analysis identified over 5,000 genes whose expression was significantly modified in response to one or more hormone treatments and offered a new opportunity to dissect the interaction between TH and GCs. Cluster analysis led to the identification of numerous patterns of gene regulation (**Figure 2**). The greatest number of these genes was regulated by T3 unaffected by CORT (33%) and by CORT unaffected by T3 (12%). Noteworthy were these so-called "CORT-only" genes because they represent GC response genes not affected by TH signaling. Many genes either required both hormones together (22%) or were regulated by each hormone separately as well as together (16%), which may represent truly synergistic (require both hormones for expression) and/or some form of additive interaction, like *klf9* where both hormones can contribute individually. The remaining genes (17%) represent some form of antagonism on gene expression, predominantly TH gene regulation blocked by CORT and CORT gene regulation blocked

by TH. These antagonistic hormone interactions at the gene expression level contrast sharply with the solely synergistic action of these hormones at the morphological level, i.e., GCs accelerate TH-induced metamorphic change. The tail transcriptome results obtained by DNA arrays showed that the effect of T3 and CORT co-treatment is not simply the addition of T3-regulatd genes plus GC regulated genes. The unexpectedly complex and uncharacterized mechanisms of gene regulation for a large number of genes controlled by TH and GCs represents an open frontier in need of future research to understand how developing organisms interact with the environment to modulate development via altered hormonal input.

TH and GC Crosstalk: Regulated Gene Functions

To expand the understanding of the hormonal cross talk and link clustering with biological functions, gene ontology (GO) analysis was applied to the gene lists (86). The genes significantly regulated by T3 (**Figure 2**, TH-only genes) included GO categories, corresponding to programmed cell death and metallopeptidase activity. It makes sense that an increase in T3 would increase the expression of genes involved in tissue resorption. Genes up-regulated by CORT (**Figure 2**, CORT-only genes) are associated with energy production in mitochondria and metabolic processes. Again, it makes sense that an increase in GCs increases the expression of genes involved in gluconeogenesis to regulate energy requirements for altered metabolism during stress and provide sufficient energy for the acceleration of metamorphosis. Genes synergistically up-regulated when CORT and T3 were present together (**Figure 2**, synergistic induced genes) include GO terms associated with intracellular protein transport, vesicle-mediated transport, protein localization, and cellular localization. Finally, genes that are down-regulated by T3 and CORT co-treatment (**Figure 2**, synergistic repressed genes) are linked with negative regulation of development and cell differentiation. Globally, these results are consistent with the action of the two hormones to promote tail resorption. It is important to note, however, that the proportion of genes that emerge from GO analysis is small relative to the number of differentially expressed genes. Thus, the ontology analysis results do not reflect all the functions represented by the differentially expressed genes. There remains, therefore, an important part of the data for which we are not yet in a position to define the biological function.

TH and GC Crosstalk: Mechanism of Gene Regulation

The number of patterns of TH- and GC-response gene regulation (**Figure 2**) suggests that multiple molecular mechanisms likely exist to provide this gene regulation diversity. Research into these mechanisms of interest benefits from knowledge that T3 and GCs act directly through nuclear receptors that initiate gene regulation cascades of induced transcription factors (20, 121). Identifying direct response genes for each hormone is a key element of on-going research. TH and GCs regulate gene expression via hormone response elements (HRE) that interact with the promoter of hormone direct target genes (23, 122). The identification of such HREs is difficult because of the complexity of these elements (123). First, HRE sequences may be partially degenerate, engendering numerous false positives identified by sequence analysis algorithms. Second, the presence of an HRE sequence does not guarantee the binding of the receptor, presumably because the chromatin organization around an HRE can dictate the accessibility of the HRE to receptors. Finally, the HRE position relative to the promoter of the target gene can be near or far upstream or downstream of the gene and also within the gene (124). Despite advances in knowledge and computer algorithms, currently only experimentation can allow the identification of HREs (123).

To date, only 12 direct T3-response genes have an identified HRE in *Xenopus tropicalis* (125), including *thrβ*, *klf9*, *thibz* (a transcription factor), the metalloproteinases *mmp11* and *mmp13*, and *dio3*. Such information is still not available for GC-response genes, except for *klf9*. Note that all known HREs are positive, resulting in up-regulation of the gene in response to the hormone. HREs can be either positive or negative, but the existence of negative HREs remains to be established. Likewise, the existence of an "antagonism module" which would harbor HREs for each hormone but with opposite effect on transcription to explain antagonistic gene regulation interactions between TH and GCs, is not known. However, antagonistic interactions may be indirect due to induced transcription factors or chromatin modifiers affecting hormone response gene expression. Indeed, *sox3*, *dot1L*, and *de novo DNA methyltransferase 3* are direct T3 response genes that themselves affect chromatin structure and gene expression (126–128).

CONCLUSIONS

Numerous explorations into the hormonal control of frog metamorphosis have revealed the powerful effects of TH on nearly every tissue in the tadpole body. These studies have also identified limitations in our ability to replicate these developmental events using exogenous TH. These limitations may be artifacts of the experimental hormone treatments, or TH may indeed be insufficient to accomplish all of the developmental changes of metamorphosis. The relative ease of eliminating TH to study its role in development is contrasted with the difficulty of selectively removing other hormones, as yet unachieved for GCs, aldosterone, prolactin, that may also play a role in natural development. The advent of gene disruption technologies to produce loss of function mutations in pituitary hormones, steroid synthesizing enzymes, and hormone receptors opens the door for continued advances to understand the roles of other hormones besides TH involved in the complex endocrine mechanisms that control post-embryonic development in amphibians.

AUTHOR CONTRIBUTIONS

LS and DB have conceived the presented idea and contributed to the writing of the paper.

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REVIEW
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Contaminant and Environmental Influences on Thyroid Hormone Action in Amphibian Metamorphosis

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Aquatic and terrestrial environments are increasingly contaminated by anthropogenic sources that include pharmaceuticals, personal care products, and industrial and agricultural chemicals (i. e., pesticides). Many of these substances have the potential to disrupt endocrine function, yet their effect on thyroid hormone (TH) action has garnered relatively little attention. Anuran postembryonic metamorphosis is strictly dependent on TH and perturbation of this process can serve as a sensitive barometer for the detection and mechanistic elucidation of TH disrupting activities of chemical contaminants and their complex mixtures. The ecological threats posed by these contaminants are further exacerbated by changing environmental conditions such as temperature, photoperiod, pond drying, food restriction, and ultraviolet radiation. We review the current knowledge of several chemical and environmental factors that disrupt TH-dependent metamorphosis in amphibian tadpoles as assessed by morphological, thyroid histology, behavioral, and molecular endpoints. Although the molecular mechanisms for TH disruption have yet to be determined for many chemical and environmental factors, several affect TH synthesis, transport or metabolism with subsequent downstream effects. As molecular dysfunction typically precedes phenotypic or histological pathologies, sensitive assays that detect changes in transcript, protein, or metabolite abundance are indispensable for the timely detection of TH disruption. The emergence and application of 'omics techniques—genomics, transcriptomics, proteomics, metabolomics, and epigenomics—on metamorphosing tadpoles are powerful emerging assets for the rapid, proxy assessment of toxicant or environmental damage for all vertebrates including humans. Moreover, these highly informative 'omics techniques will complement morphological, behavioral, and histological assessments, thereby providing a comprehensive understanding of how TH-dependent signal disruption is propagated by environmental contaminants and factors.

Keywords: thyroid hormone, environmental contaminant, endocrine disruptor, frog tadpole, metamorphosis, environmental factors, transcriptomics, genomics

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INTRODUCTION

Thyroid hormone (TH) signaling is a cornerstone of molecular events that mediate the profound morphological changes characteristic of early vertebrate development (1). The obligate requirement for TH is perhaps best exemplified by metamorphosing anuran amphibians for which the essential stimulation by TH initiates transitions from larval to juvenile stages under conducive

environmental conditions (2). Amphibians undergo complex and comprehensive morphological changes as functionally athyroid premetamorphic tadpoles progress through prometamorphosis (with concurrent, increasing endogenous TH levels) and into juvenile frogs after metamorphic climax (**Figure 1**) (4). These changes encompass the coordinated maturation and remodeling of organs, *de novo* generation of limbs, regression of the tail, and the consequent alteration in behavior, diet, and niche as most aquatic tadpoles develop into more terrestrial-dwelling frogs (**Figure 1**) (5).

TH production is controlled by the hypothalamic-pituitary-thyroid (HPT) axis (**Figure 2**). The hypothalamus stimulates the pituitary with corticotropin releasing factor (CRF) to release thyroid stimulating hormone (TSH). TSH promotes the synthesis of TH in the follicular cells of the thyroid gland (2). The central dogma of TH signaling is that the newly synthesized prohormone thyroxine (T_4) is transported from the thyroid gland by transporter proteins (e.g., transthyretin). Once at the destination peripheral tissue, T_4 is converted into its more active form, 3,3′,5-triodothyronine (T_3), by the enzymatic activity of deiodinases (**Figure 2**). Additionally, the bioactivity of T_4, without conversion, has recently been demonstrated (6–9). TH binds its TH receptors (TRs), TRα, and TRβ, which are constitutively bound to cognate receptor elements that regulate genes sensitive to TH. Metamorphosis is initiated in anurans upon TH production, which stimulates gene expression cascades and subsequent proteomic and metabolomic alterations (**Figure 2**) (10, 11). TH metabolism is regulated through various enzymatic activities (glucuronidation, sulfation, and deiodination), which can target the hormone for degradation and thereby modulate TH activation of gene expression (**Figure 2**). For more detailed descriptions of thyroid hormone production, activity, and metabolism, the reader is encouraged to consult the following publications and the references therein (2, 12–15).

The spatiotemporal control of TH-dependent molecular and physiological activities during metamorphosis is particularly sensitive to abiotic and xenobiotic perturbations. Although the mechanism of molecular interference is not known for most adverse exposures, disruption can potentially target any aspect of TH synthesis, activity, and metabolism (**Figure 2**). Such disruptions include the exposure of premetamorphic tadpoles to exogenous TH, which results in a precocious induction of metamorphosis that can be exploited to experimentally assess toxicant perturbations during this developmental period (2).

In the present review, we discuss the effects of chemical and environmental disruptors of metamorphic TH signaling on anuran amphibians. Anurans are particularly tractable for the study of TH disruption due to the absolute necessity for TH to initiate metamorphosis, and consequently, the well-demarcated developmental transitions in amphibians (11). Chemical disruption of anuran metamorphosis almost exclusively originates from anthropogenic sources: industry, agriculture,

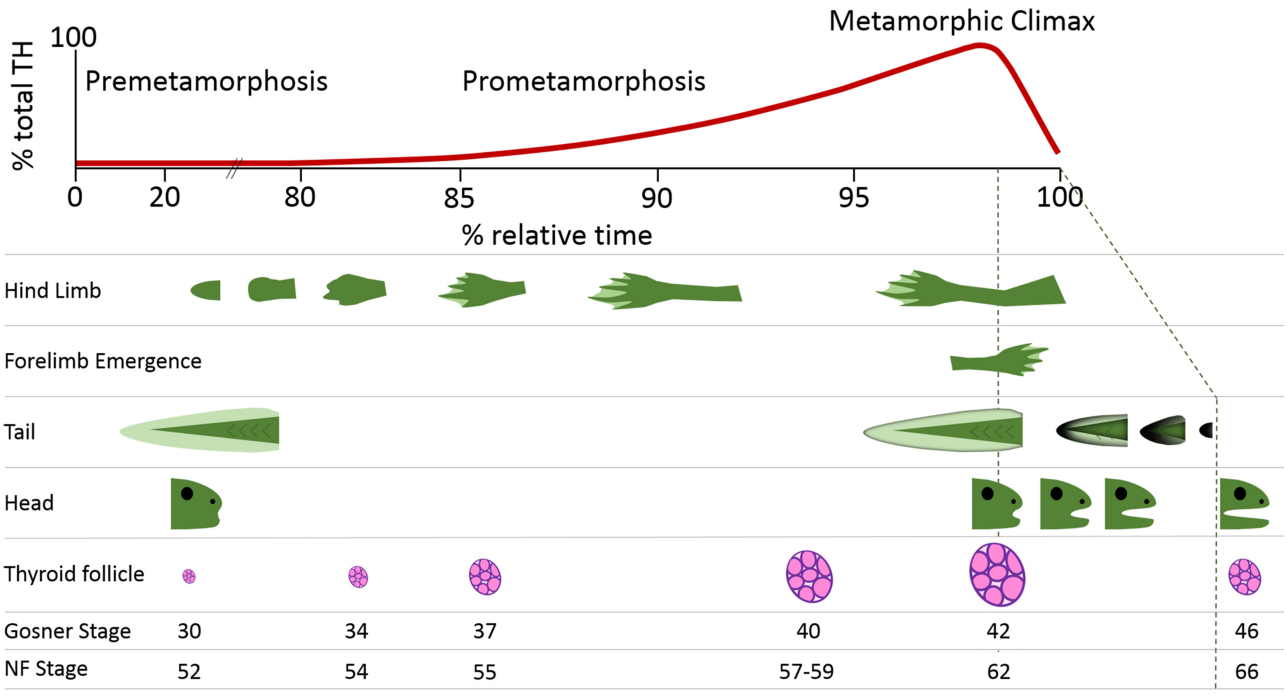

FIGURE 1 | Thyroid hormone (TH) levels and key morphological hallmarks during frog postembryonic development. Amphibian metamorphosis is a postembryonic process driven by TH signaling. The free-swimming tadpole (0% relative time) has virtually undetectable levels of TH. The morphological changes that occur in the development of a tadpole to a juvenile frog (100% relative time) are inextricably aligned to internal rises in TH levels. These rising TH levels lead to progression through the stages of development, which can be seen through morphometric measurements including hindlimb development, forelimb emergence, tail regression, head shape changes, and thyroid follicle production. The Gosner and Nieuwkoop and Faber (NF) staging system comparisons are from Just (3).

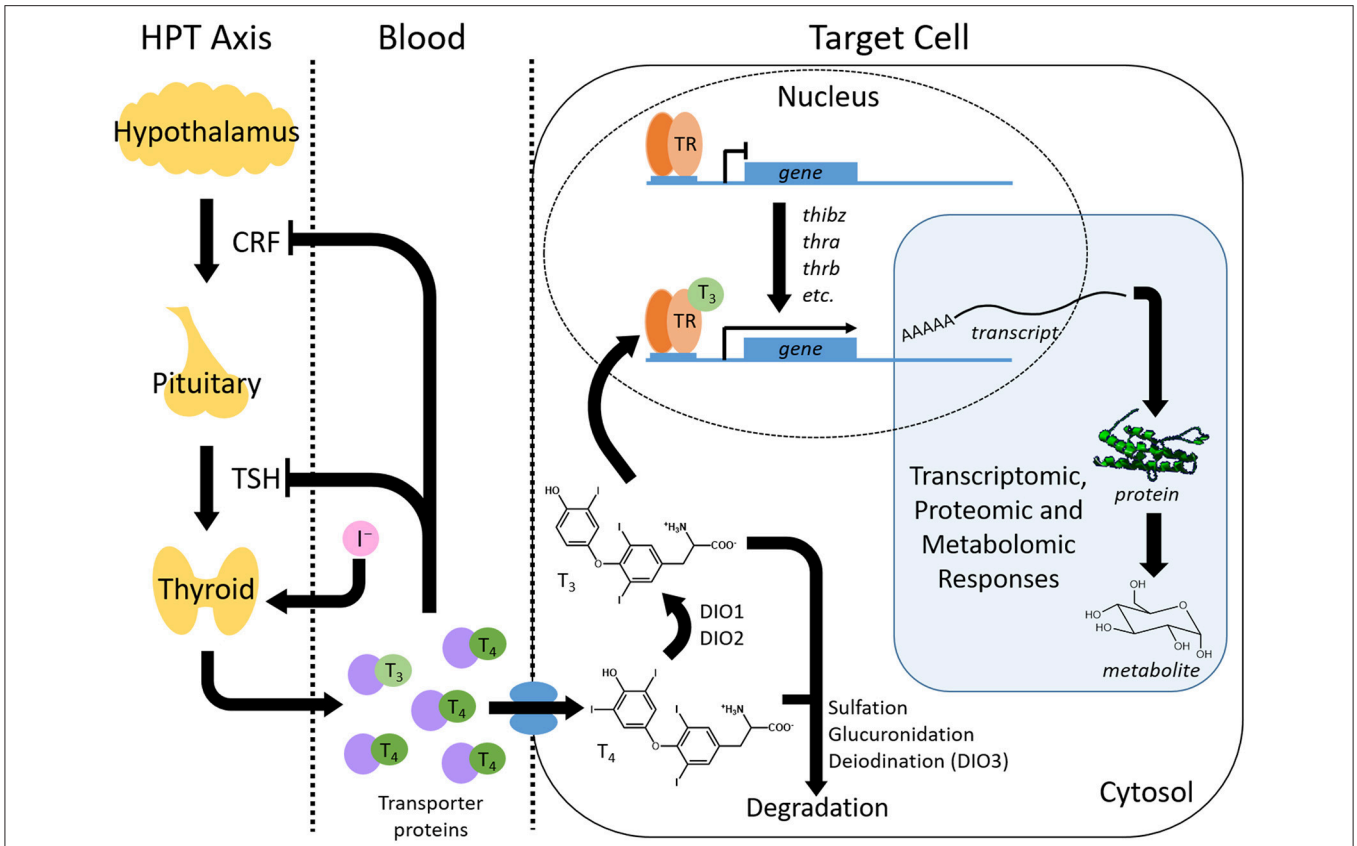

FIGURE 2 | Overview of thyroid hormone (TH) production, transport, activity and regulation. The thyroid hormone signaling pathway involves a complex interplay between TH synthesis, transport, signal transduction, and catabolism. TH is synthesized within the hypothalamus-pituitary-thyroid (HPT) axis where the pituitary is stimulated to release thyroid stimulating hormone (TSH) by corticotropin releasing factor (CRF) from the hypothalamus. TSH induces the production of thyroxine (T_4) and, in lesser amounts, triiodothyronine (T_3) from the thyroid gland. The production of TH self-regulates through a negative feedback loop that inhibits further CRF and TSH production. TH travels through the blood via transporter proteins to peripheral tissues where it is imported into target cells. Here, T_4 is converted to T_3 through deiodinases (DIO), although T_4 can bind to receptors as well. Binding of THs to TH nuclear receptors (TR) leads to the activation of TH response genes. This change in transcript abundance results in downstream proteomic and metabolomic responses that produce the phenotypic changes resulting from the TH signal. The TH signal is also regulated within the cell by catabolism that includes processes such as sulfation, glucuronidation and deiodination.

pharmaceuticals, and personal care products (PPCPs; **Figure 3**). Additionally, environmental factors, including temperature variations and ultraviolet radiation, have demonstrated effects on metamorphosis (**Figure 3**). Numerous studies have examined the effects of single chemical, complex chemical mixtures, or environmental exposures on amphibian morphology during metamorphosis and we focus our discussion on those that have additionally demonstrated a TH-dependence of these effects. Adverse toxicant and environmental exposures can compromise other endocrine and molecular signaling pathways beyond TH, with sub-lethal physiological consequences for reproductive success, behavior, and broader dysfunction (16–19). We have restricted our discussion to select representatives from each of the major classes listed above and regret being unable to undertake an exhaustive review of all the excellent work done on TH disruptors.

The adoption of molecular biology techniques to assess the perturbation of TH-dependent metamorphosis has complemented conventional morphological characterizations and provided further insight into the sensitive responses of TH-induced gene expression (**Figure 1**) (20, 21). We discuss how the application of quantitative polymerase chain reaction (qPCR), DNA microarrays, next generation sequencing and other 'omics techniques can ascertain TH disruption through the timely detection of biomarkers prior to the manifestation of morphological phenotypes (11, 22, 23). A list of TH-responsive gene transcripts mentioned in the current review is presented in **Table 1**.

PHARMACEUTICALS AND PERSONAL CARE PRODUCTS

Pharmaceutical and personal care products (PPCPs) are an abundant source of diverse anthropogenic contaminants in global aquatic and terrestrial environments (24, 25). Increasing evidence links TH disruption in frogs with a variety of PPCPs, some of which are highlighted below and summarized in **Table 2**.

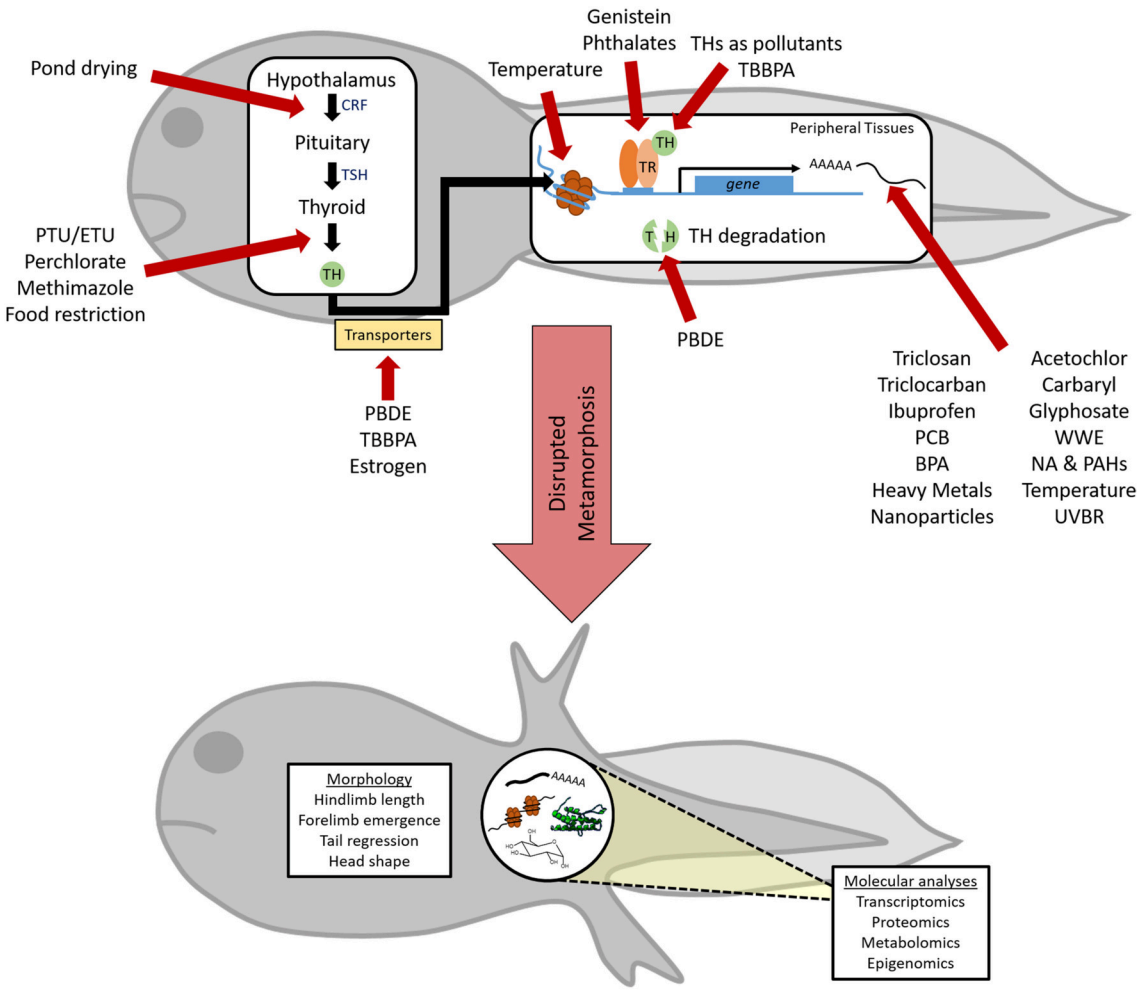

FIGURE 3 | Perturbation of thyroid hormone (TH)-dependent amphibian metamorphosis by xenobiotic and abiotic exposures. Chemical and/or environmental factors can disrupt TH action at multiple points along this pathway (red arrows), although little is known about the specific mechanism of action for many factors. Due to its absolute reliance on proper TH signaling, metamorphic endpoints can be used to reveal the TH-disrupting capabilities of these factors. However, a more complete understanding of endocrine disruption and insight into modes of action can be achieved through the use of advanced techniques to assess alterations in the transcriptome, proteome, metabolome, and epigenome within metamorphosing tadpoles. CRF, corticotropin releasing factor; TSH, thyroid stimulating hormone; TR, TH receptor; PTU, propylthiouracil; ETU, Ethylenethiourea; TBBPA, Tetrabromobisphenol A; PBDE, polybrominated diphenyl ethers; PCB, polychlorinated bisphenols; BPA, bisphenol A; NA & PAHs, napthenic acid and polycyclic aromatic hydrocarbons; WWE, wastewater effluent; UVBR, ultraviolet B radiation.

THs as Pollutants (T_3/T_4)

THs can be found as pollutants in environmental water systems. As thyroid medication is the third-most prescribed drug in Canada for women aged 25–66, TH can be found in municipal wastewater (37). Brown and Wong measured the concentrations of T_4 at a wastewater treatment plant in Winnipeg, Canada and found a range from 60 to 79 ng/L (~0.1 nM) with T_4 persisting through the treatment phases (38). The majority of recent studies examining precocious metamorphosis induced by THs have used physiological levels (e.g., 10–50 nM). More recently, however, studies have shown that premetamorphic tadpoles are competent to respond to lower, more environmentally-relevant levels of T_3 and T_4 found in wastewater (6, 7). Maher et al. found that in *Rana* [*Lithobates*] (*R.*) *catesbeiana dio2* and *cebp1* are responsive to as little as 0.05 nM T_4 in the brain and back skin, respectively (7). Slightly higher concentrations of 0.1 nM T_3 and 0.5 nM T_4 led to an increased number of TH-responsive transcripts such as *thrb, thibz, klf9,* and *rlk1* in the back skin, brain, intestine, liver, and tail fin (**Table 2**). In the same species, Jackman et al. found that olfactory epithelium exposed to 0.5 nM T_4 also exhibited a significant increase in *thrb, thra, and thibz* (7). The responsiveness of TH-linked transcripts to environmentally-relevant levels of THs indicates that these low concentrations may be enough to affect metamorphosis. An early study demonstrating TH-induced metamorphosis found that premature induction resulted in mortality when TH amounts were greater than environmental levels (7).

TABLE 1 | List of gene names utilized and their abbreviations.

Abbreviation	Gene name
ahrl	Aryl hydrocarbon receptor-like
app	Amyloid β precursor protein
asl	Argininosuccinate lyase
cebp1	CCAAT enhancer binding protein 1
cebp2	CCAAT enhancer binding protein 2
cebpd	CCAAT enhancer binding protein Δ
cps1	Carbamoyl phosphate synthetase 1
crhbp	Corticotropin-releasing factor-binding protein
dio1	Deiodinase 1
dio2	Deiodinase 2
dio3	Deiodinase 3
fap	Fibroblast activation protein α
heket	Heket
hsp30	Heat shock protein 30
ipo	Importin
klf9	Krüppel-like factor 9 formerly referred to as *bteb*
krt1	Cytokeratin type 1
mbp	Myelin basic protien
mct8	Monocarboxylate transporter
mmp2	Matrix metalloproteinase 9, formerly known as *gelatinase A*
mmp9	Matrix metalloproteinase 9, formerly known as *gelatinase B*
nfic	Nuclear factor I/C
oatp1c1	Solute carrier organic anion transporter family member 1c1
otc	Ornithine transcarbamylase
pcna	Proliferating cell nuclear antigen
pparg	Peroxisome proliferator activated receptor γ
prlr	Prolactin receptor
rlk1	Rana larval keratin I
rxrg	Retinoid X receptor γ
ssh	Sonic hedgehog
st3	Stromelysin 3 also known as matrix metalloprotein 11 (*mmp11*)
thibz	TH induced bZip protein formerly referred to as *TH/bZip*, *b/Zip*, *gene 8*, or *gene 9*
thra	TRα
thrb	TRβ
timp2	Tissue inhibitor of metalloproteinases 2
trip4	TR interactor 4
tsha	Thyroid stimulating hormone α
tshb	Thyroid stimulating hormone β
ttr	Transthyretin

Exposure to T_3 is also associated with behavioral changes in which tadpoles lose the ability to detect a predator cue (36). Surprisingly, comparable T_4 exposures had no effect on this behavioral endpoint (36). Molecular analyses of the olfactory epithelium using qPCR and RNA-seq methods revealed that this tissue was extraordinarily sensitive to both hormones and, while many gene responses were shared between the two hormones, a substantial number were unique to each hormone with T_3 significantly affecting a ¼ more contigs than T_4 (6, 7). Notable differences in sensory perception, potassium ion transport, DNA repair, mitochondrial energetics and transcription/RNA processing gene ontologies provide some insight into the different effects of these hormones (36). These studies accentuate that the two TH contaminants should be treated separately when looking at responses to environmentally-relevant levels of THs.

Propylthiouracil and Ethylenethiourea

6-Propylthiouracil (PTU) is a TH synthesis antagonist that is clinically used to treat hyperthyroidism. Ethylenethiourea (ETU) is also an anti-thyroidal compound that, similar to PTU, inhibits thyroid peroxidase, the enzyme that synthesizes TH (39). *Xenopus* (*X.*) *laevis* tadpoles independently exposed to PTU and ETU had inhibited metamorphic progression (30, 40). *X. laevis* tadpoles exposed to ETU at stage 51 exhibited delays and arrest of natural metamorphosis, as measured by forelimb emergence (21). Histological aberrations in thyroid gland formation were evident with increased glandular size and follicle size and partial colloid depletion following exposures to ETU and PTU (21, 30). Elevated abundance of *tsha* and *tshb* transcripts were measured by qPCR in the pituitary tissue of tadpoles exposed to ETU (21). Similar metamorphic delays and aberrant thyroid gland histology were also observed in *X.* (*Silurana*) *tropicalis* and *R. rugosa* tadpoles following PTU exposures (41, 42).

Early prometamorphic *X. laevis* tadpoles (Niewkoop and Faber [NF] stage 54) exposed to 20 mg/L PTU did not have significantly altered *thra*, *thrb,* or *klf9* transcript abundance in the brain, hindlimb or tail (31, 43). MAGEX cDNA array analysis of naturally metamorphosing *X. laevis* tadpoles at NF stage 54 exposed to PTU recorded a greater number of transcripts with decreased abundance than increased abundance in the brain at 24, 48, and 96 h post-treatment (**Table 2**) (32). Differential transcription was ontologically associated with transcriptional regulation at 24 h and at 96 h, transcription, hormonal regulation and structural proteins (32). Correspondence analysis was used to identify possible metamorphic biomarker candidates and qPCR analyses confirmed the increased expression of myelin basic protein (*mbp*) and myelin proteolipid protein (*plp*) in the brain upon PTU exposure (**Table 2**) (32). Using similar experimental conditions, the PTU-dependent effects were further examined in the *X. laevis* hindlimb and tail (34). Seven transcripts were identified by cDNA arrays to have differential abundance in the hindlimb at 24 and 96 h post-exposure and were associated with hormonal regulation and structural proteins at 24 h and protein processing, transcription, and transport and binding at 96 h (**Table 2**) (34). Using cDNA arrays, 4 transcripts were detected to have differential levels in the tail at 48 h and were linked to transcription, cell growth control, and transport and binding ontologies (**Table 2**) (34). Potential biomarkers were screened using qPCR and cytokeratin type I (*krt1*) transcripts were elevated significantly in both the hindlimb and tail (**Table 2**) (34).

Naturally metamorphosing *X. laevis* tadpoles exposed to ETU exhibited developmental arrest and aberrant thyroid histology: goiter formation, colloid depletion and follicular cell hypertrophy and hyperplasia (35). Treatment with this goitrogen induced significant decreases in *thrb*, *klf9*, *pcna*, *mcm2*, *kif2C,* and increased *dapl1* transcript abundance in the brain as measured by qPCR (35). ETU treatment also resulted in increased *tshb*

TABLE 2 | Summary of PPCP effects on morphological and molecular endpoints for amphibians undergoing both natural and TH-induced metamorphosis.

			Metamorphosis		Morphological/ Behavioral	Molecular			
Category	**Chemical**	**Species**	**Natural**	**Induced**	**Results**	**Tissue**	**Technique**	**Result**	**References**
Antimicrobial	Methyl Triclosan	*R. catesbeiana*	Y			C-fin[d]	qPCR	↑*rlk1, thrb*	(26)
	Triclocarban	*R. catesbeiana*	Y			C-fin	qPCR	↓*rlk1*	(26)
	Triclosan	*Pseudacris regilla*		T_4	↑ metamorphic rate	Brain	qPCR	↑*pcna, thra, thrb*	(27)
				T_4		Tail	qPCR	↑*thra;* ↓*thrb*	(27)
		R. catesbeiana		T_3	↑ metamorphic rate	Brain	qPCR	↑*pcna, thrb*	(28)
				T_3		C-fin	qPCR	no effect on *hsp30, rlk1, thrb*	(28)
				T_3		Tail	qPCR	↓*thrb*	(28)
		X. laevis		T_3		XTC-2 cell line	qPCR	↑*klf9, thra, thrb*	(28)
Estrogen	E_2[a]	*R. catesbeiana*	Y			Olfactory epithelium	qPCR	↑*st3, thibz*	(6)
	E_2[b]	*R. catesbeiana*	Y			Olfactory epithelium	RNA-seq (52,699 contigs)	Δ 267: 112 overlap with TH	(6)
Pharmaceutical	Ibuprofen	*R. catesbeiana*	Y	T_3		C-fin	qNPA[c],qPCR	↑*ahrl, cebp2, dio3;* ↓*prlr*	(29)
				T_3		Liver	qNPA,qPCR	↑*asl, thra, thrb*	(29)
				T_3		Liver	cDNA Array (MAGEX; 434 genes)	Δ 27: transcription, calcium transport, proteolysis, cell cycle, protein phosphorylation	(29)
			Y			Liver	cDNA Array (MAGEX; 434 genes)	Δ 26: oxygen transport, arginine metabolism, urea production	(29)
	Methimazole	*X. laevis*	Y		↓ metamorphic rate	Brain	qPCR	↓*app, thra*	(30–33)
			Y			Brain	cDNA Array (MAGEX; 434 genes)	[24 h] ↑4↓35: chromatin structure, signal transduction, transcription; [48 h] ↑12↓7: cell growth control, chromatin structure, structural, signal transduction, transcription; [96 h] ↑4↓30: apoptosis/protein processing, cell growth control, chromatin structure, hormonal regulation, metabolism, signal transduction, structural, transcription, translation, transport/binding	(32)
			Y			Hindlimb	qPCR	↑*ipo, krt1;* ↓*thra*	(34)
			Y			Hindlimb	cDNA Array (MAGEX; 434 genes)	↑11: cell growth control, hormonal regulation, protein processing, signal transduction, structural, transcription, transport/binding	(34)
			Y			Tail	qPCR	↓*ipo*	(34)
			Y			Tail	cDNA Array (MAGEX; 434 genes)	↑4↓1: hormonal regulation, structural, protein processing, signal transduction	(34)

(Continued)

TABLE 2 | Continued

			Metamorphosis		Morphological/ Behavioral	Molecular			
Category	**Chemical**	**Species**	**Natural**	**Induced**	**Results**	**Tissue**	**Technique**	**Result**	**References**
	Propylthiouracil	*X. laevis*	Y		↓ metamorphic rate	Brain	qPCR	↑*mbp*, ↑*plp*	(31, 32)
			Y			Brain	cDNA Array (MAGEX; 434 genes)	[24 h] ↑3↓36: transcription; [48 h] ↑8↓11; [96 h] ↑9↓29: transcription, hormonal regulation, structural	(31, 32)
			Y			Tail	qPCR	↑*krt1*	(31, 34)
			Y			Tail	cDNA Array (MAGEX; 434 genes)	[48 h] Δ 4: transcription, cell growth control, transport/binding	(31, 34)
			Y			Hindlimb	qPCR	↑*krt1*	(31, 34)
			Y			Hindlimb	cDNA Array (MAGEX; 434 genes)	[24 h] Δ 7: hormonal regulation, structural; [96 h] Δ 7: protein processing, transcription, transport/binding, signal transduction	(31, 34)
	Ethylenethiourea	*X. laevis*	Y		↓ metamorphic rate	Brain	qPCR	↑*dapl1;* ↓*thrb, pcna, mcm2, kif2C*	(35)
			Y			Pituitary	qPCR	↑*tshb*	(35)
			Y			Thyroid tissue	qPCR (60 gene screen)	↑43↓6: TH synthesis, secretion, metabolism; protein synthesis and transport, growth arrest, apoptosis, cellular stress responses	(35)
Thyroid hormone	T_3[b]	*R. catesbeiana*		T_3		Olfactory epithelium	RNA-seq (52,699 contigs)	Δ 38,830: sensory perception, DNA repair, mitochondrial energetics, transcription and RNA processing, endoplasmic reticulum	(6)
	T_3[a]	*R. catesbeiana*		T_3		Back skin	qPCR	↑*cebp1, klf9, thibz;* ↓*rlk1*	(7)
				T_3		Brain	qPCR	↑*dio2, klf9;* ↓*rlk1*	(7)
				T_3		Intestine	qPCR	↓*klf9, rlk1, thrb*	(7)
				T_3		Liver	qPCR	↑*cebp1*	(7)
				T_3	↓ predator cue avoidance	Olfactory epithelium	qPCR	↑*dio2, heket, st3, thibz, thra, thrb*	(6, 36)
				T_3		Tail fin	qPCR	↑*thibz*	(7)
	T_4[b]	*R. catesbeiana*		T_4		Olfactory epithelium	RNA-seq (52,699 contigs)	Δ 31,439: sensory perception, potassium ion transport, DNA repair, mitochondrial energetics, transcription and RNA processing	(6)
	T_4[a]	*R. catesbeiana*		T_4		Back skin	qPCR	↑*cebp1, klf9, thibz*	(7)
				T_4		Brain	qPCR	↑*dio2*	(7)
				T_4		Intestine	qPCR	↑*thra*	(7)
				T_4		Liver	qPCR	↑*thrb*	(7)
				T_4		Olfactory epithelium	qPCR	↑*dio2, heket, st3, thibz, thra, thrb, trpv1*	(6)

[a]*Contaminant at environmental levels where physiological level data was collected.*
[b]*Contaminant at physiological levels.*
[c]*Quantitative nuclease protection assay.*
[d]*Cultured tail fin assay.*

transcripts in the pituitaries. A qPCR candidate biomarker screening was performed on thyroid tissue and 49 of 60 genes had significantly differential abundance following ETU exposure compared to the controls (35). Of these, 43 genes had increased transcript abundance, while six were decreased. These ETU-induced differential transcripts were ontologically associated with the synthesis, secretion, and metabolism of THs, protein synthesis and transport, growth arrest, apoptosis, and cellular stress responses (35).

Methimazole

Methimazole is an established disruptor of amphibian HPT axis function and has been frequently used as a metamorphosis inhibitor (30). Similar to PTU and ETU, methimazole is a goitrogen and anti-thyroid drug that affects TH signaling by inhibiting thyroid peroxidase (44). Exposure to methimazole for 14 days during metamorphosis resulted in a significantly decreased metamorphic rate in pre- and prometamorphic *X. laevis* tadpoles and thyroid gland hypertrophy and follicular cell hyperplasia (**Table 2**) (30, 33). The molecular effects of up to 72 h of methimazole exposure on early prometamorphic *X. laevis* tadpoles were queried by qPCR analysis of known TH-regulated genes. Zhang et al. found a significant decrease in *thra* and *app* gene expression in the brain; a decrease in *thra* and increase in *ipo* and *krt1* mRNAs in the hindlimb; and a decrease in *ipo* transcripts in the tail (**Table 2**) (36, 38, 39). Helbing et al. used cDNA arrays to further evaluate the pathways affected by methimazole in *X. laevis* tadpoles (**Table 2**) (32, 34). In the brain, an increase of 20 and decrease of 76 gene transcripts related to transcription, hormonal regulation, and structural pathways was observed (32). In the hindlimb, the 11 increased transcripts were related to cell growth control, hormonal regulation, protein processing, signal transduction, structural, transcription, and transport/binding pathways. The tail had four increased and one decreased transcript that were related to hormonal regulation, structural, protein processing and signal transduction pathways (34). Ontological analyses of differentially affected brain transcripts were associated with apoptosis/protein processing, cell growth control, chromatin structure, hormonal regulation, metabolism, signal transduction, structural, transcription, translation, and transport/binding pathways with qPCR analysis revealing an increase in *ipo* and *krt1* and a decrease in *thra* mRNA levels (**Table 2**) (34).

Estrogen

The steroid hormone and TH axes are closely related. As the synthesis of both endocrine hormones is controlled through hypothalamic-pituitary axes and both bind nuclear receptors that stimulate gene expression cascades, it is unsurprising that there is some cross-talk between these two pathways. The majority of studies that have looked at the effects of 17β-estradiol (E_2) or the synthetic estrogen 17α-ethinylestradiol (EE_2) on metamorphosis have found a decreased metamorphic rate (**Supplementary Table 1**) (32–35, 44) [reviewed by Hayes (45)]. However, Frieden & Naile found accelerated tail reduction in *Bufo* (*B.*) *bufo* upon exposure to estrone (E_1) (46). How estrogens influence TH signaling is not completely understood. In adult *R. ridibunda*, E_2 decreases plasma T_3 and T_4 (47), although this may not occur during metamorphosis. Brande-Lavridsen et al. found that during metamorphosis in *R. temporaria*, there was no significant difference in total or free T_3 upon exposure (48). However, Yamauchi et al. found that both E_2 and the synthetic estrogen diethylstilbestrol could competitively bind with recombinant *X. laevis* and *R. catesbeiana* transthyretins; TH transporter proteins (49) (**Supplementary Table 2**). The thyroid itself was found to show no change in number of follicles or overall thyroid volume, although there was a decreased follicular cell height upon exposure to EE_2.

To determine the response of the gene program, Jackman et al. investigated the transcriptomic effects of E_2 in the olfactory epithelium of *R. catesbeiana* and found none of the classic TH-response genes, such as *thra, thrb, thibz,* or *dio2* changed upon an acute exposure to either environmentally-relevant or higher levels of E_2 (6). This is corroborated by Bulaeva et al. who exposed *R. sylvatica* to much higher levels of E_2 and still saw no significant response of *thrb* (50). With more in-depth RNA-seq analysis, Jackman et al. found 112 significantly changing contigs that also responded to exposure to T_3 and/or T_4 (6). However, compared to almost 45,000 contigs that respond to exposure to TH, this cross-talk signaling is quite minimal. As estrogens are found throughout our wastewater systems (51), it is imperative to determine the mechanism by which estrogens are affecting with TH signaling and proper development.

Triclosan and Triclocarban

Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol; TCS] is a bactericidal and antifungal agent that is ubiquitously incorporated into thousands of industrial and consumer products including clothing, toys, cleaning supplies, personal care products (i.e., soap, shampoo, toothpaste, etc.), and surgical soaps and sutures (52, 53) with 10.5 million pounds produced globally in 2015 (53). Triclosan and triclocarban (TCC), another widely used antibacterial in PPCPs, are the most common, broad-spectrum antimicrobial agents used in household items and PPCPs (54). While sewage treatment removes most triclosan, it still contaminates sewage effluent and, consequently, aquatic environments (24). The U.S. Food and Drug Administration banned the use of TCS, TCC, and 17 other antimicrobials in personal wash products in 2016 to minimize the exacerbation of bacterial resistance and health risks, including endocrine disruption (54, 55). TCS has structural similarity to TH and disruption of TH action in frogs provided some of the earliest evidence of this endocrine disruption.

Low and environmentally-relevant amounts of TCS can affect different aspects of TH signaling in amphibians (30, 41, 51–57). Exposure of premetamorphic *R. catesbeiana* tadpoles to environmentally-relevant amounts of triclosan can induce altered growth and transcript responses that are exacerbated upon T_3-induced metamorphosis (28). The combinatorial effects of TCS and T_3 on tadpoles resulted in greater body mass reductions and precocious metamorphosis. These phenotypic changes were accompanied and preceded by changes to TH-responsive gene expression (28). Expression of *thrb* was transiently decreased in the tadpole tail at 48 h, while the

brain had increased expression of *thrb* and proliferating cell nuclear antigen transcripts (*PCNA*). Under comparable TCS ± T_3 treatments, cultured *X. laevis* XTC-2 cells had increased expression of *thra, thrb, and klf9* after exposure to both chemicals, supporting the developmentally-sensitive TCS effects in different anuran species (28). Recent work demonstrated that *X. tropicalis* exposed to TCS levels considered safe in drinking water developed metabolic pathologies resembling prediabetes and produced progeny exhibiting delayed metamorphosis and diminished reproductive success (58).

Adaptation of the Amphibian Metamorphosis Assay for the Pacific tree frog, *Pseudacris* (*P.*) *regilla*, (TREEMA) revealed comparable morphological and molecular disruption by TCS when administered in conjunction with T_4 (27). By the second day of exposure, TCS enhanced the T_4-stimulated increases in *thra, thrb, and pcna* in the tadpole brain and disrupted expression of TH-responsive genes in the tail (**Table 2**) (27). The earliest morphological effects of TCS and T_4 exposures were evident at day 4 with increased foot paddle formation and later impairments in developmental stage progression. Tadpoles exposed to both TCS and T_4 also had accelerated development and increased hindlimb length/snout-vent length ratio (27). Like other anurans, the perturbed metamorphic profile in *P. regilla* is indicative of disrupted developmental coordination (27). Exposure of *X. laevis* tadpoles to TCS resulted in increased *thrb* mRNA in the tail fin after 21 days followed by thyroid gland hypertrophy at 32 days (**Table 2**) (56, 57, 59, 60).

Methyl triclosan (mTCS) is a bacterial metabolite of TCS and is more persistent in the environment than TCS, which is readily degraded by photolysis (61). This metabolite, along with TCS and TCC, were tested using premetamorphic *R. catesbeiana* cultured tail fin (C-fin) assays. TCS did not affect TH-responsive *rlk1* or *thrb* transcript abundance, but did increase *hsp30* levels (**Table 2**) (26). mTCS exposure increased both *rlk1* and *thrb* transcripts in the absence of T_3 (26), suggesting that some, but not all, of the TCS activity observed in intact animals may be due to the conversion to mTCS. TCC exposure caused a reduction in *rlk1* transcripts and an increase in *hsp30* mRNA (**Table 2**) (26), indicating a TH-like activity of this antimicrobial agent.

Ibuprofen

Ibuprofen is a commonly used non-steroidal anti-inflammatory analgesic that is now a prevalent component of complex municipal wastewater effluents that permeate aquatic environments (62, 63). Ibuprofen is primarily considered to act through prostaglandin synthesis inhibition, however, it can also interfere with multiple regulatory pathways (29, 64). Little is known about the effects ibuprofen can have on aquatic organisms during sensitive developmental periods, which is concerning given the multiplicity of molecular pathways ibuprofen targets and its abundance in global freshwater environments.

Exposure of *R. catesbeiana* tadpoles to environmentally-relevant concentrations of ibuprofen disrupted TH-stimulated metamorphic reprogramming of the liver transcriptome and in C-fin assays (**Table 2**) (29). MAGEX cDNA microarray analyses of tadpole livers exposed to ibuprofen and T_3 detailed molecular pathways affected by these combined exposures: transcription, calcium transport, proteolysis, cell cycle, and protein phosphorylation. Additionally, ibuprofen treatment affected pathways related to oxygen transport, arginine metabolism and urea production (29). Ibuprofen exposure of T_3-stimulated tadpoles enhanced the upregulated expression of *thra* and *thrb*. Quantitative nuclease protection assay analysis of C-fin cultures showed that ibuprofen exposure alone could increase expression of *dio3*, while both ibuprofen and T_3 treatment resulted in an increase in *hsp30* transcripts, indicating potential tissue-specific responses (29). Ibuprofen can also affect transcriptional programs in the tail fin and back skin of *R. catesbeiana* under temperature-dependent, T_3-stimulated conditions and this is further discussed below (65).

INDUSTRIAL AND AGRICULTURAL CHEMICALS

Polychlorinated Bisphenols (PCBs)

Polychlorinated bisphenols (PCBs) are ubiquitous environmental contaminants that were widely used in capacitors and transformers between 1929 and 1979 (66). Concern about the endocrine disrupting potential of PCBs resulted in their import and use being banned in North America by 1979. However, the extreme environmental persistence and bioaccumulation of PCBs continue to plague us (66). With the effects of PCBs on TH homeostasis well-characterized (67), there was a clear need to investigate the effect of these compounds on amphibian metamorphosis.

As the toxicity of PCBs is typically due to bioaccumulation over time, Gutleb et al. examined the effects of ingested PCBs in *R. temporaria* and *X. laevis* after an exposure of either 10 days or several weeks (68). They found that dietary exposure to a technical mixture of PCBs, clophen A50, decreased metamorphic rate in both species after 10 days. Furthermore, exposures to PCB 126 decreased the rate of metamorphosis after several weeks (**Supplementary Table 1**). In a later study, Gutleb et al. showed that immersion in PCB 77 and apolar sediment extracted from PCB-contaminated ponds significantly reduced the rate of metamorphosis in *X. laevis* (**Supplementary Table 1**) (69). Gutleb et al. confirmed these effects using a *X. laevis* thiourea-synchronized metamorphosis assay and a 60 day dietary exposure. In this study, they found that clophen A50 and an apolar sediment extract from polluted ponds decreased the rate of metamorphosis (**Supplementary Table 1**) (70).

To assess the effects of PCB exposure on TH-mediated gene expression, Lehigh et al. examined the toxicity of another technical mixture of PCBs, A1254 (71). qPCR analysis of pooled mRNA from *X. laevis* tadpoles showed that A1254 exposures decreased *dio2* and *dio3* expression and increased *ttr* expression (**Table 3**). These results, in combination with the previous studies performed by Gutleb et al., show that mixtures of PCBs exhibit significant effects on TH-driven amphibian metamorphosis (**Table 3**).

TABLE 3 | Summary of industrial and agricultural chemical effects on morphological and molecular endpoints for amphibians undergoing both natural and TH-induced metamorphosis.

			Metamorphosis		Morphological	Molecular			References
Category	**Chemical**	**Species**	**Natural**	**Induced**	**Result**	**Tissue**	**Technique**	**Result**	
Flame retardants	A1254 (technical PCB mixture)	*X. laevis*	Y		↓ metamorphic rate	Whole tadpole	qPCR	↑*ttr*; ↓*dio2, dio3*	(71)
	BDE-47	*X. laevis*	Y		↓ metamorphic rate	Brain	qPCR	↓*dio2, klf9, mct8, oatp1c1, thra, thrb, tshb*	(72, 73)
	TBBPA	*Pelophylax nigromaculata*	Y			Intestine	qPCR	↑*mmp2, thibz*	(74)
				T_3		Intestine	qPCR	↓*klf9, mmp2, ssh, thibz, thrb*	(74)
		Pseudacris regilla		T_3	↑ metamorphic rate	Brain	qPCR	↑*thra*	(75)
				T_3		Tail	qPCR	↑*mmp9*; ↓*pcna*	(75)
		X. laevis		T_3	↓ metamorphic rate	Head	qPCR	↓*thibz, thrb*	(76–80)
			Y			HeLa cells	GAL4-luciferase reporter assay with *Xenopus* TRα ligand binding domain	↓activation	(77)
				T_3		Hindlimb	qPCR	↓*dio2, st3, thrb*	(80)
				T_3		Intestine	qPCR	↓*dio2, klf9, mmp2, thrb*	(80)
				T_3		Tail	qPCR	↓*cebpd, dio3, klf9, st3, thrb*	(79)
				T_3		Transgenic tadpoles	*thibz*-luciferase reporter	↓activation	(78)
				T_3		Transgenic tadpoles	*thibz*-GFP reporter	↓activation	(77)
Isoflavonoid	Genistein	*R. catesbeiana*	Y			C-fin	qPCR	↓*thrb*	(81)
				T_3		C-fin	qPCR	↓*thrb*	(81)
				T_3	↓ tail regression	Tail tip culture	qPCR	↓*thrb*	(82)
				T_3		Tail tip culture	Western blot	↓tyrosine phosphorylation	(82)
				T_3		Tail tip culture	Western blot	↓activity of protein kinase C	(82)
				T_3		Tail tip culture	Western blot	↓TRα phosphorylation levels	(82)
				T_3		Tail tip culture	Western blot	↑TRα	(82)
Metals	Cd	*B. gargarizans*	Y		↓ metamorphic rate	Whole tadpole	qPCR	[5 μg/L] ↑*thra*; [50 μg/L] ↓*thra*; [100 μg/L] ↓*dio2 thrb*	(83)
	Cd/Te quantum dots	*R. catesbeiana*		T_3		C-fin	qPCR	↓*rlk1, thrb*	(84)

(Continued)

TABLE 3 | Continued

			Metamorphosis		Morphological	Molecular			References
Category	**Chemical**	**Species**	**Natural**	**Induced**	**Result**	**Tissue**	**Technique**	**Result**	
	Cu	*B. gargarizans*	Y		↓ metamorphic rate	Whole tadpole	qPCR	↑*dio3;* ↓*dio2, thra, thrb*	(85)
	Hg	*B. gargarizans*	Y		↓ metamorphic rate	Liver	qPCR	↓*dio2, thra, thrb*	(86)
	Nanosilver	*R. catesbeiana*		T_3		C-fin	qPCR	↓*rlk1, thrb*	(84)
	Nanosilver	*X. laevis*	Y			Liver	cDNA Array (MAGEX, 497 genes)	[Premet[a]] ↑3↓4: Myotube cell development, Protein binding, Proteolysis, Oxidative stress, ATP biosynthesis, Transcriptional regulation, Cell cycle arrest; [Promet[b]] ↑12↓4: Regulation of transcription, Nervous system development	(87)
Pesticide	Acetochlor	*R. catesbeiana*	Y			Brain	qPCR	↑*thra, thrb*	(88)
				T_3		C-fin	qPCR	↑*thrb*	(89)
				T_3		Tail	qPCR	↑*thra, thrb*	(88)
		X. laevis		T_3	↑ metamorphic rate	Tail	qPCR	↑*thra, thrb*	(90)
				T_3		Tail	cDNA Array (MAGEX, 420 genes)	Altered pathways: transcription factors, apoptotic proteins, signaling molecules, enhanced expression of T_3-responsive genes	(90)
	Carbaryl	*R. clamitans*	Y			Brain	qPCR	↑*thra, thrb*	(91)
			Y			Brain	cDNA Array (MAGEX, 420 genes)	Altered pathways: transcription, cell growth control, signal transduction	(91)
			Y			Tail	qPCR	↑*thra*	(91)
	Roundup Original and Transorb	*R. pipiens*	Y		↑ metamorphic rate	Tail	qPCR	↑*thrb* (premets, not metamorphic climax)	(92)
Plastics Additive	BPA	*X. laevis*	Y	T_3	↓ tail regression	Cultured tail	PCR	↓*thrb*	(93)
				T_3	↓ metamorphic rate	Intestine	qPCR	↓*mmp2, st3, thibz, timp2*	(94)
				T_3		Intestine	DNA microarray (Affymetrix)	↓T_3 signaling pathways	(94)
	Dicyclohexyl phthalate	*X. laevis*		T_3		XL58 cell line	*thibz*-luciferase reporter; qPCR	↓activation; ↓*thrb*	(95)
	Di-n-butyl phthalate	*X. laevis*		T_3	↓ metamorphic rate	Head		↑*tshα*, tshβ; ↓*thrb*, RXRγ	(96)
				T_3		XL58 cell line	*thibz*-luciferase reporter; qPCR	↓activation; ↓*thrb*	(95)
	Mono-n-butyl phthalate	*X. laevis*		T_3	↓ metamorphic rate	Head		↑*tsha, tshb*; ↓*rxrg*, *thrb*	(96)
						Head	Bisulfite sequencing	↓methylation in *thrb* promoter	(96)
	N-butylbenzyl phthalate	*X. laevis*		T_3		Whole tadpole		↓*thrb*	(95)
				T_3		XL58 cell line	*thibz*-luciferase reporter; qPCR	↓activation; ↓*thrb*	(95)

[a] *Premetamorphic tadpoles.*
[b] *Prometamorphic tadpoles.*

Perchlorate

Perchlorates, such as ammonium perchlorate, potassium perchlorate, and sodium perchlorate, are well-known as powerful oxidizing agents, which has led to their widespread usage in explosives such as rocket propellants, fireworks, and signal flares (97). They are also used to treat TH diseases (98) as perchlorates competitively inhibit the uptake of iodine by the sodium-iodide symporter, leading to lack of iodine for the production of THs (99). Unfortunately, due to its widespread industrial use, perchlorate is a persistent pollutant. As amphibians have an almost identical TH system to humans, it is unsurprising that perchlorates also affect their TH-regulated processes [reviewed by Carr and Theodrakis (100)], leading to a decreased metamorphic rate (31, 35, 50, 101–103). Chronic exposures to environmental levels of perchlorate decrease T_4 in *X. laevis*, both *in vivo* (104) and *in vitro* (105). This indirectly results in the enlargement of the thyroid glands as well as hyperplasia and hypertrophy of thyroid follicles due to the lack of negative regulation of TSH (20, 35, 104, 106, 107). Predictably, the decrease in T_4 levels also leads to decreased metamorphic rates (35, 101, 102, 106, 107).

The involvement of the TH-induced gene expression program in this metamorphic delay seems to be organ-dependent. Using cDNA array analyses of acute exposures of sodium perchlorate in *X. laevis,* Helbing et al. found that the brain was the most responsive with a maximum of 39 responsive genes involved mostly in transcription, transport/binding, apoptosis/protein processing, and structure (**Table 3**) (32). *Tshb* mRNA significantly increased after 48 h, suggesting an acute exposure already leads to dysregulation of the negative feedback loop. The cDNA array only indicated 8 and 4 responsive genes in the tail and hindlimb, respectively (34), indicating that these tissues may be less responsive to acute exposures of perchlorate. However, in chronic exposures of environmentally-relevant levels of perchlorate, there is a more consistent response. Flood & Langlois (108) observed decreased TH-responsive genes, *thra*, and *thrb*, in the liver of *X. tropicalis* chronically exposed to potassium perchlorate. A similar result was seen in the brain of *X. laevis* chronically exposed to sodium perchlorate (**Table 3**) (35). Bulaeva et al. (50) found that *R. sylvatica* had decreased *thrb* transcript levels in the tail and liver, which could be continually observed even 40 days after a 2 week exposure to sodium perchlorate, indicating that the effects from perchlorate may be persistent and possibly irreversible.

Brominated Flame Retardants (BFRs)

Brominated flame retardants (BFRs) have been and continue to be ubiquitously incorporated into a variety of items to confer fire resistance (109). These materials include textiles, plastics, electronic circuitry, wood, paper, dust, and inadvertently in the 1970's, livestock feed (109–111). Roughly 5,000,000 metric tons of bromine are produced worldwide annually, with demand increasing each successive year (111). BFRs include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD). Depending upon the mechanism by which BFRs are integrated within materials, BFRs can be classified as brominated monomers, reactive (i.e., TBBPA) or additive (i.e., PBDE, HBCD). BFRs can readily leach from materials if they are not strongly chemically bound to the composite polymer, thereby contaminating the environmental biota, leading to mortality, compromised development and other toxicity-dependent pathologies among animal populations. A growing concern is that increasing amounts of BFRs have been found in the environment throughout different trophic levels, including humans, underscoring the need to better understand the biological implications of BFRs (111). Many BFRs are lipophilic and this facilitates their persistent bioaccumulation in the biota of both aquatic and terrestrial environments (112). Due to the deleterious effects of penta- and octa-BDE BFRs and PBBs, they have since been banned, which has spurred the development of novel BFRs (111). However, the environmental effects of these novel BFRs, which are not limited to TBBPA derivatives, are under increasing scrutiny (110, 113). Herein, we review BFRs that have a demonstrated effect on amphibian metamorphosis (**Table 3**).

Polybrominated Diphenyl Ethers (PBDE)

PBDEs are widely disseminated throughout invertebrates, vertebrates, sediments, and diverse environments, including Arctic marine biota (72). PBDEs can readily accumulate and magnify within trophic levels (114). Mammalian biotransformation of PBDEs to hydroxylated metabolites by cytochrome P450 enzymes result in products that are more toxic than the parent congeners. As previously reviewed, these metabolites can disrupt thyroid homeostasis via several mechanisms including: decreased free and total TH through the competitive binding of thyroid transport proteins and perturbed TH metabolism through glucuronidation, sulfation, and deiodination (72). Notably, there are strong structural similarities between THs and PBDEs.

X. laevis tadpoles (NF stage 50) fed 1,000 or 5,000 μg/g of a commercial mix of PBDE congeners, DE-71, exhibited significant inhibition of metamorphosis as displayed by delayed limb development and tail resorption, lack of pigmentation and head shape changes (72). No major cellular or morphological differences of the thyroid gland were observed following histological analyses. Intraperitoneal injections of DE-71 and BDE-47, but not BDE-99, resulted in delayed metamorphosis through significant reductions in tail resorption (72). Both BDE-47 and BDE-99 are major congeners of DE-71. Although the morphological results of this study implied the disruption of TH activity, such involvement could not be conclusively ascertained.

R. pipiens tadpoles fed lower, environmentally relevant amounts of DE-71 at Gosner stage 25 to stage 42 had delayed metamorphic climax by 22–36 days (3, 115). The elimination of PDBEs following depuration was studied in *R. pipiens* tadpoles that had consumed environmentally-relevant concentrations of DE-71 for 50 days at Gosner stage 25. Following 28 days of depuration, tadpoles had removed more than 94% of PBDE congeners from their bodies (114). The ability to eliminate PBDEs from tissues can vary according to life stage. Metamorphosing frogs (Gosner stage 42–46) were unable to eliminate PBDEs following depuration, however, juvenile frogs

eliminated 89.7% of PBDEs over a 70 day depuration (114). Wild *R. limnocharis* adult frogs found proximal to contaminated e-waste recycling sites similarly showed reduced PBDE levels following 54 days of depuration (116).

A link between PDBE-altered amphibian metamorphic morphology and disrupted TH metabolism was demonstrated by the treatment of *X. laevis* tadpoles with increasing concentrations of BDE-47 (73). After a 21 day BDE-47 dietary exposure, tadpoles exhibited reduced developmental stage progression and decreased hindlimb length. Histological analysis of the thyroid gland showed decreased follicular epithelial cell height and a smaller thyroid lobe area in tadpoles exposed to BDE-47 (73). Corresponding reductions in hindlimb length were observed in *X. tropicalis* tadpoles following BDE-47 exposure (117). BDE-99 exposure in *X. tropicalis* similarly resulted in slower developmental stage progression and reduced hindlimb length (117). qPCR analyses in *X. laevis* to assess transcriptomic changes in the tails and livers of stage-matched tadpoles between NF stage 52 to 56 found tissue-specific TH-dependent regulation (73). No significant differences were observed in tail *thra*, *thrb*, *dio1*, or *dio2* transcripts. However, the brain was sensitive to BDE-47 treatment and significant reductions were observed in *thra*, *thrb*, *klf9*, *tshb*, *dio2*, *mct8,* and *oatp1c1* mRNA (73). The diversity of affected transcripts underscores the broad extent to which thyroid metabolism is adversely affected by BDE-47.

Tetrabromobisphenol A (TBBPA)

Tetrabromobisphenol A (2,2′,6,6′-tetrabromo-4,4′-isopropylidenediphenol; TBBPA) is one of the most abundantly used BFRs, with 150,000 metric tons produced each year. Although the majority of TBBPA is covalently bound within polymer materials, ∼10–20% can leach into the proximal environment (118, 119). As such, TBBPA is found dispersed within environments around the world and in the tissues of affected organisms (112, 119). TBBPA was introduced as a replacement for PBDEs, in part due to their comparatively short half-life in mammals (120). However, TBBPA has been detected in environmental samples and humans, including breast milk (121). TBBPA bears structural similarity to T_4 and binds to human transthyretin more strongly than T_4 (122), but is weak competitor to T_3 for binding TRα in rat (123). TBBPA is also reported to disrupt T_3 binding to TRs in rat (123).

TBBPA antagonizes tail resorption during TH-mediated metamorphosis in the wrinkled frog, *R. rugosa*, and the T_3-associated gene expression of *thrb* and *thibz* in *X. laevis* (76, 123). TBBPA can also act as a TH agonist during metamorphosis in *P. regilla* (75). These contradictory findings may reflect unique endocrine sensitivities due to differential anuran metamorphic trajectories (124). *P. regilla* tadpoles (Gosner stages 30–31) exposed to 10 nM TBBPA had increased tail regression and *mmp9* expression following T_3-induced metamorphosis. MMP9 is a metalloproteinase involved in the deconstruction of the extracellular matrix and is required for tail resorption (125). Following 100 nM TBBPA exposure in the context of T_3-stimulated metamorphosis, *thra* mRNAs were significantly increased in the brain relative to TBBPA exposure alone while the abundance of *pcna* transcripts was decreased in the tail (75).

Fini et al. demonstrated that *X. laevis* tadpoles (NF stage 45) can rapidly take up radiolabeled TBBPA and retain the parent TBBPA and its biotransformed metabolites (TBBPA-glucuronide, TBBPA-sulfate, TBBA-glucuronide-sulfate), while only gradually releasing them (77). TBBPA was shown to be the principal agent of antithyroidal activity, not its biotransformed congeners. TBBPA, but not its metabolites, impaired T_3-induced regression in tadpole head size and gills (77). Moreover, by using transgenic tadpoles containing a *thibz* promoter-green fluorescent protein (*thibz*-GFP) TH-response reporter construct, 10 μM TBBPA, not its sulfate conjugates, inhibited T_3-induced GFP expression (77). TH metabolizing enzymes, deiodinases, UDP-glucuronyl transferases and sulfotransferases were not affected by TBBPA with or without T_3 induction. A GAL4-luciferase reporter assay using the *Xenopus* TRα ligand binding domain transiently transfected into HeLa cells demonstrated that TBBPA can effectively compete with T_3 binding. However, sulfated TBBPA conjugates lack this T_3 displacement capacity (77). Independent studies in *X. laevis* recapitulated similar finding of TBBPA inhibition of T_3-mediated reductions in head area, hindlimb length and decreased apoptosis and epithelial folds within intestines (78–80). Additionally, various groups described the restricted activation of a *thibz* response element-luciferase reporter assay and the reduction of tissue-specific gene expression of *thrb*, *st3*, *klf9*, *cebpd, mmp2, dio2,* and *dio3* transcripts upon TBBPA inhibition of T_3-induced metamorphosis (**Table 3**) (78–80).

TBBPA is proposed to have developmental stage-specific effects on *X. laevis* metamorphosis, potentially related to endogenous levels of TH. During pre- and prometamorphosis, endogenous levels of TH are low and TBBPA exposure was associated with increased hindlimb length and the promotion of development. However, during metamorphic climax when TH amounts are maximal, developmental stage transitions were delayed (80). An additional potential confounder may be the amount of TBBPA that metamorphic anurans are exposed to (74). Molecular analysis of *Pelophylax (P.) nigromaculatus* intestines showed that tadpoles exposed to low concentrations of TBBPA (1 nM) had agonistic effects on T_3-induced expression of TH-response genes (**Table 3**). In contrast, higher TBBPA concentrations (100–1,000 nM) had antagonistic effects in the same experimental paradigm (74). The molecular mechanisms by which TBBPA may act as both an agonist and antagonist of tissue-specific development while endogenous TH levels vary need to be ascertained.

Bisphenol A (BPA)

Bisphenol A (4,4′ isopropylidenediphenol; BPA) is a widely used monomer in the manufacture of polycarbonate plastics, epoxy resins and food containers. More than 2.2 million metric tons of BPA were globally produced in 2009. Since the 1930's, BPA was known to be xenoestrogenic and growing concerns about the exposure of humans to BPA culminated in the US Food and Drug Administration banning BPA from baby bottles in 2012 (126). Despite debates between food and drug administrations and researchers about the endocrine disrupting effects of BPA, this monomer has been implicated in a plethora of etiologies

including diabetes, obesity, and hypothalamic neuroendocrine dysfunction. Early developmental periods are also ostensibly sensitive to the effects of BPA (127–129).

BPA is found ubiquitously throughout the environment, soils, surface waters, sewage, and more. Detoxification of BPA within organisms occurs through glucuronidation and the biotransformed oxidative metabolites that result can have greater endocrine disrupting effects than the parent BPA or analog (130). While the effects of BPA on estrogenic dysregulation are well-studied, BPA can also affect signaling pathways of THs, androgens, and glucocorticoids (130). BPA exposure inhibits amphibian metamorphosis by targeting TH signaling and is extensively reviewed in Heimeier and Shi (131).

X. laevis embryos exposed to BPA displayed delayed metamorphosis by 2–4 stages at NF stages 52–54 (**Table 3**) (93). Tadpoles exposed to BPA had similarly delayed natural and T_4-induced metamorphosis. Cultured tadpole tails treated with BPA had repressed T_3-induced tail shortening and had BPA-inhibited *thrb* expression in the presence and absence of T_4 stimulation (93).

Twenty-one day exposure of *X. laevis* tadpoles to BPA concentrations that were equivalent to human infant exposures also protracted T_3-induced metamorphosis by 8 stages and stalled intestinal development (**Table 3**) (94). By 4 days, however, maladaptive molecular effects were observed in the reduced expression of early T_3-responsive genes, *st3* and *thibz*, and the late responders, *mmp2* and *timp2*, in the intestine following combined BPA and T_3 exposures. An oligo DNA microarray analysis of the intestinal transcriptome confirmed that BPA antagonizes the expression of genes involved in T_3 signaling pathways (**Table 3**) (94).

Genistein

Genistein is a plant-synthesized isoflavinoid found in high amounts in soy products (132). As a phytoestrogen, the endocrine disrupting capabilities of this compound have been well-studied for estrogen signaling [reviewed by Henley and Korach (133)]. However, its effects on TH signaling have been far less studied. Ji et al. acutely exposed premetamorphic *R. catesbeiana* tadpoles to T_3 and then cultured the tail tips in the presence or absence of genistein to determine the effects of this contaminant on TH-induced metamorphic changes (82). Exposure to genistein led to the ablation of tail tip regression seen upon exposure to only T_3. This morphological response is correlated with a decreased abundance of the *thrb* transcript (**Table 3**). In support of this finding, Hinther et al. also found decreased *thrb* upon exposure of cultured tail fin of *R. catesbeiana* to genistein, both induced and not induced by T_3 (**Table 3**) (81). A possible mechanism by which TH signaling is being disturbed is through modulation of phosphorylation pathways. Genistein is a tyrosine protein kinase inhibitor (134), which is demonstrated in this amphibian model by leading to reduced overall tyrosine phosphorylation in T_3-exposed *R. catesbeiana* tail tips cultured with genistein (82). As tyrosine phosphorylation of protein kinase C (PKC) is known to increase the activity of this kinase (135), the decreased tyrosine phosphorylation induced by genistein is correlated with negative PKC activity. It is postulated that this phosphorylation pathway impacts TH signaling through PKC serine phosphorylation of TRα. Upon acute exposure to T_3, there is a significant increase in serine phosphorylation in *R. catesbeiana* tail tips, which can be reversed with PKC inhibitors (82). This response is attenuated by exposure to genistein, which likely leads to the observed decrease in the TH response gene *thrb*. Genistein can also affect thyroid peroxidase function in mammalian systems [reviewed by Doerge and Sheehan (136)]; however, whether this affects TH signaling in amphibians has yet to be determined. Further studies are needed to determine the role of phosphorylation pathways in cellular level TH signaling and whether other areas of the greater TH signaling pathway are affected by this contaminant.

Phthalates

Phthalates are plasticizers added to increase the flexibility of plastics. These contaminants can be found in the air, soil, freshwater, and saltwater (137–139). The ubiquity of phthalates in the environment is concerning as they have shown to have TH disrupting effects [reviewed by Mathieu-Denoncourt et al. (140)]. Using a T_3-activated *X. laevis* reporter cell system (**Table 3**), Sugiyama looked at the effects of five different phthalates on T_3 signaling within the constructed cells (**Table 3**) and found di-n-butyl phthalate, n-butylbenzyl phthalate and dicyclohexyl phthalate caused a decrease in activity (95). These TH-disrupted responses were all associated with a decrease in endogenous *thrb* mRNAs in the reporter cells. N-butylbenzyl phthalate also led to decreased *thrb* with a T_3-induced whole tadpole exposure. In line with these findings, Shen et al. found that chronic exposure of *X. laevis* tadpoles to di-n-butyl phthalate and its metabolite mono-n-butyl phthalate resulted in decreased *thrb* (96).

The mechanism by which phthalates disrupt TH signaling within the cell likely involves the regulation of TRs. Using a TR-mediated reporter gene assay, Shen et al. found that dibutyl phthalate, mono-n-butyl phthalate, and di-2-ethylhexyl phthalate demonstrated TRβ agonist activity (141). As TRs have various methods by which they can be regulated, Shen et al. queried the involvement of the TR corepressor silencing mediator for retinoid or TH receptors (SMRT) in the phthalate-dependent TR regulation and found that both di-n-butyl phthalate and mono-n-butyl phthalate increased the interaction between SMRT and TR in a mammalian two-hybrid assay (**Table 3**) (96). Furthermore, in the amphibian system, decreased methylation of the promoter region of *thrb* was found upon exposure to mono-n-butyl phthalate, which could be involved in TR-mediated regulation of the *thrb* gene. However, the same result was not seen with di-n-butyl phthalate, indicating potential differences in phthalate response (96). The involvement of other epigenetic mechanisms, such as histone post-translational modification, has yet to be elucidated. In contrast to the aforementioned studies, Mathieu-Denoncourt found that chronic exposure to monomethyl phthalate, a dimethyl phthalate metabolite, led to an increased metamorphic rate in *X. tropicalis* that associated with no TH response gene expression changes (**Supplementary Table 1**) (142). This suggests that various phthalates may have different mechanisms of disruption and/or

the timing of TH response gene effects have differing response kinetics that were not captured in the study. Further work on these substances on a broader range of amphibian species is warranted.

Metals

Metals acting as environmental contaminants stem from a variety of natural and anthropogenic sources (143). Heavy metals are notable environmental endocrine disrupting chemicals (EDCs) and can dysregulate TH-driven amphibian metamorphosis upon exposure.

Cadmium (Cd) exposure has been shown to significantly decrease metamorphosis in *B. americana* (144), as well as completely block completion of metamorphosis in other amphibians like *Pleurodeles waltl* (145). There is a significant correlation between Cd concentration and decreasing rates of metamorphosis in *X. laevis* (146). Furthermore, the effects of Cd exposure are exacerbated in male *X. laevis* tadpoles when the environmental pollutant estradiol-17β (E_2) is present (147).

Sun et al. observed significant decreases in *dio2*, *thra*, and *thrb* transcripts following Cd exposures in *B. gargarizans* at concentrations an order of magnitude lower than previously reported to decrease metamorphic rate (83). At the lowest Cd concentration, an increase in *thra* expression was observed, but this may be due to using *actb* as a single normalizer, which can be TH-responsive (87). Thyroid histology revealed significant follicular cell hyperplasia in the cadmium-exposed animals.

Copper is naturally ubiquitous in the environment and influxes of anthropogenic copper occur due to soil disturbances or agricultural runoff (148). In several Ranidae species and *B. gargarizans*, chronic exposure to copper can significantly delay the rate of metamorphosis (**Table 3**) (85, 148, 149). Wang et al. showed that copper exposure in *B. gargarizans* significantly increased *dio3* expression and significantly decreased *dio2*, *thra*, and *thrb* expression at copper concentrations greater than what caused metamorphic delay (85). Although a transcriptional response is expected at lower concentrations, it is possible that measurements were done too late to observe significant changes in TH-related transcription as tadpole exposures commenced at Gosner stage 26 and transcript quantification did not occur until stages 42 and 46. Copper exposure also induced follicular cell hyperplasia in the thyroid gland.

Chronic mercury exposure exhibited a similar phenomenon in *B. gargarizans* as did copper; metamorphosis was delayed at lower concentrations than what caused significant decreases in *dio2*, *thra*, and *thrb* expression and induced follicular cell deformation in the thyroid gland (**Table 3**) (86). Again, transcript measurements were performed much later than the initial exposure such that lower concentration transcript effects may have been missed.

Other metals that resulted in a delay in metamorphosis include lead (Pb) in *R. pipiens* (**Table 3**), iron (Fe; ionized or ore particulates) or manganese (Mn) in *R. catesbeiana*, and depleted uranium (U) in *X. laevis* tadpoles (**Table 3** and **Supplementary Table 1**) and further research on their effects on TH signaling is needed (150–152).

Nanoparticles

Several metals have been manufactured as constituents of nanoparticles. Nanoparticles are any particles that have at least one dimension <100 nm (153). These nanoparticles possess unique properties compared to their ionic counterparts that make them highly desirable for wide use in industrial and medical applications. However, this has led to significant environmental contamination by nanoparticles and the endocrine disrupting potential of nanoparticles has been well-documented (153). As nanoparticles have unique aggregation and surface charge distributions, their exposure often results in different endocrine disrupting effects compared to their corresponding metal ions (154). It is important to study the endocrine disrupting potential of metal ions and nanoparticles separately as the effects of one are not necessarily predictive of the other. Nevertheless, few studies directly compare the effects of nanoparticle and metal ion exposures in the same study. Further complications in comparing the effects of nanoparticle and constituent ion exposures arise from differences in experimental conditions and species studied.

Chronic exposure to zinc, copper, and titanium oxide nanoparticles can delay metamorphosis in *X. laevis* tadpoles (155–158). However, titanium oxide-based nanoparticles or their ionic counterparts had no effect on TH signaling in the *R. catesbeiana* C-fin assay (159). Nanoparticle interference significantly decreased the rate of metamorphosis in *R. sylvatica* tadpoles chronically exposed to nanogold (**Supplementary Table 1**) (160).

Specific gene targets of nanoparticle endocrine disruption were investigated by Hinther et al. using a *R. catesbeiana* C-fin assay and 48 h exposures (84). They found that exposure to silver nanoparticles or Cd telluride quantum dots in combination with T_3 significantly decreased the expression of the TH-responsive genes: *rlk1* and *thrb* (**Table 3**). The extent of TH-mediated gene disruption arising from 28 day nanosilver exposures was further evaluated by Carew et al. in pre- and prometamorphic *X. laevis* tadpoles (87). They found that, while exposure did not alter the overall rate of metamorphosis, there were transient perturbations of leg length and snout/vent length that were pre- or prometamorph-specific. Using a MAGEX cDNA array and qPCR performed on liver tissue extracted from these tadpoles, they identified 3 induced and 4 repressed transcripts in premetamorphs and 12 induced and 4 repressed transcripts in prometamorphs exposed to nanosilver (**Table 3**) (87). Of these, *mmp9*, *pparg*, and *trip4* have linkages to TH signaling pathways.

Pesticides

Acetochlor

Acetochlor [2-chloro-*N*-(ethoxy-methyl)-*N*-(2-ethyl-6-methylphenyl) acetamide] is a widely used preemergent herbicide and persistent organic pollutant that contaminates groundwater (161). More than 10 million kg of acetochlor are used per year in the United States, with surface water concentrations ranging from median levels of 2.7 nM (730 ng/L) to as high as 10 nM (2.7 μg/L) within the 80th percentile of measurements sampled in the Midwestern United States (162, 163). Acetochlor can induce TH-dependent dysfunction and other pathologies in a variety of aquatic species (164–167).

In combination with other pesticides, acetochlor may contribute to altered comorbid fungal infections in amphibians (168).

Concurrent treatment of premetamorphic *R. pipiens* tadpoles with acetochlor and T_3 resulted in the acceleration of metamorphosis as evidenced by precocious forelimb emergence (169). As priming tadpoles with T_3 prior to acetochlor treatment did not cause accelerated metamorphosis, it was concluded that acetochlor was interacting with T_3 in a TR-independent manner to elicit precocious development (169).

R. catesbeiana tadpoles exposed to environmentally relevant concentrations of acetochlor (10 nM) did not affect *thrb* expression in tail fin biopsies (89). However, the combined treatment of acetochlor with T_3 caused a synergistic increase in *thrb*, which concurred with earlier morphological findings of accelerated metamorphosis (89). Acetochlor induced the upregulation of *thra* and *thrb* in the brains of athyroid premetamorphic *R. catesbeiana* tadpoles and these increases were amplified upon exogenous T_3 treatment (88). These results suggest a tissue-specific sensitivity to acetochlor. The *thra*/*thrb* transcript ratios were also altered and these transcript changes were not associated with any effects on escape behavior following acetochlor treatment (88).

Understanding of the TH-dependent molecular mechanisms disrupted by acetochlor was refined by cDNA microarray studies in *X. laevis*. Crump et al. demonstrated that changes in gene expression precede the morphological changes of T_3-induced accelerated metamorphosis ensuing from acute and environmentally-relevant acetochlor exposures (90). After 48 h, acetochlor exposure caused a T_3-mediated increase in *thra* and *thrb* and the overall magnification of genes otherwise upregulated by T_3 (90). Of interest is that genes normally downregulated by T_3 showed an attenuated response in the presence of acetochlor, suggesting that acetochlor perturbs mechanisms of transcriptional regulation (**Table 3**). Such impairment of transcription implies that acetochlor may disrupt epigenetic modes of regulation (90).

During prometamorphosis, endogenous levels of TH naturally increase and acetochlor exposure caused an accumulation of *thra* and *thrb* transcripts in tail fin biopsies from *R. catesbeiana* tadpoles. The brains of these acetochlor-treated prometamorphic tadpoles were assessed after a 59 day depuration period and no significant differences were observed in *thra* and *thrb* transcripts, although the ratios between them were altered at higher acetochlor concentrations (88). No major developmental changes were observed either in forelimb emergence, tail regression or mouth development (88).

Carbaryl

Carbaryl belongs to the carbamate class of insecticides and is commonly used in agricultural and home garden applications to control insect populations (170). Though presumed to have low toxicity, carbamates have structural similarities to organophosphate insecticides and can modify acetylcholinesterases, which has important implications for neurotransmission (171, 172). Carbaryl exposure can limit the resistance of amphibians to parasitic infection and its toxicity is exacerbated by previous Ranavirus infection of *R. sylvatica* (173, 174). Of outstanding interest are the implications for metamorphosing organisms in carbaryl-treated areas.

R. clamitans tadpoles exposed to environmentally relevant carbaryl concentrations did not have altered metamorphosis according to morphological metrics: tadpole development and time to metamorphosis (91, 175). However, both short- and long-term alterations in gene expression were observed in brain and tail tissues of tadpoles acutely exposed to carbaryl at 8 and 16 weeks post-hatching (**Table 3**) (91). Gosner stage 25 tadpoles exposed to carbaryl for 3 days at 16 weeks post-hatching had higher *thra* and *thrb* expression in the brain at Gosner stage 46. Greater *thrb* expression was also observed in tadpoles exposed at 8 weeks post-hatching (91). DNA microarray analysis highlighted the persistent transcript effects of carbaryl on altered brain pathways that included transcription, signal transduction and cell growth control. Immediately following carbaryl exposure, *thra* is increased in the tadpole tail (91). Pesticide exposures during such sensitive early developmental periods have potential consequences for fitness and health of the organism during its lifespan.

Glyphosate and Surfactants

Glyphosate is a commonly used herbicide for both domestic and agriculture applications around the world. Many commercially available formulations, such as Roundup®, contain glyphosate, which is rendered more toxic due to the inclusion of surfactants, whose toxicity can be influenced by pH, temperature, and species and developmental stage of exposed organisms (176, 177).

Several North American amphibians (*R. clamitans*, *R. pipiens*, *R. sylvatica,* and *B. americana*) exposed to glyphosate, different commercial herbicides and the surfactant polyethoxylated tallowamine (POEA) exhibited varying sensitivities depending on developmental stage and species (92). Glyphosate alone did not elicit deleterious effects, but in combination with POEA in Roundup Original® and Roundup Transorb®, metamorphic defects were observed, particularly in *R. pipiens*, which was sensitive to these exposures (**Table 3**). Consequent to exposures at Gosner stage 25, tadpoles exhibited increased time to metamorphosis. Gonadal abnormalities were also observed as was tail damage that included necrosis, blistering, and abnormal growth (92). As observed with other disruptions to TH signaling, molecular aberrations were observed prior to phenotypic changes. At stage 25, but not 42, increases in *thrb* expression resulted from exposure to Roundup Original® and Roundup Transorb® (92). However, newer glyphosate herbicide formulations that do not include POEA are less toxic, making them more promising potential alternatives for agricultural and domestic use.

COMPLEX MIXTURES

Although there is considerable focus on the effects of individual toxicants on TH activity, such chemicals do not persist alone in the environment. Mixture effects arising from the combination of different toxicants can result in TH-dependent disruptions not predicted by the individual chemical constituents (178).

Metal Mixtures

Heavy metals exhibit increased toxicity as a consequence of mixture effects (179). Dorchin and Shanas examined the endocrine disrupting potential of a mixture of metals (Cu, Pb, and Ni) in concentrations comparable to that of runoff from busy highways (180). Exposure to this metal mixture significantly decreased the metamorphic rate of *Bufo* (*B.*) *viridis* tadpoles (**Table 4**) (180). A similar effect of metal mixtures was observed in *Limnodynastes peronei*, which exhibited a decreased rate in metamorphosis after being exposed to coal-mine wastewater containing low metal amounts (**Table 4**) (185).

Wastewater Effluents

Wastewater effluents (WWE) are complex mixtures that can contain contaminants from agricultural, industrial, and domestic sources and hence, can disrupt TH function. A primary source of contamination comes from PPCPs in human waste. Although wastewater goes through extensive filtration prior to dispersal, TH disruption still ensues from effluent exposures (36). The TH disruption potential of WWE was first examined in 2009 when Sowers et al. found that a 50% dilution of municipal WWE significantly decreased the rate of *R. pipiens* metamorphosis (**Table 4**) (186). A delay in metamorphosis was also observed in *R. catesbeiana* after exposure to pond water that had been a receptacle for municipal WWE (**Table 4**) (187).

Searcy et al. examined the effects on TH-mediated metamorphic gene expression within *X. laevis* tadpole *ex vivo* tail tip cultures exposed to WWE (184). Using oligo microarray and qPCR analyses, they found that WWE and T_3 exposures significantly increased the expression of TH-sensitive genes: *thrb*, *dio2*, *crhbp,* and *fap* (**Table 4**). The *in vivo* effects of WWE on TH-linked gene expression was also demonstrated by Castillo et al. in a transgenic *X. laevis* harboring a *thibz*-GFP reporter construct that was activated by WWE exposure (**Table 4**) (188).

As wastewater treatments do not completely eliminate EDCs, Wojnarowicz et al. assessed the removal of EDCs by three methods of wastewater filtration using the C-fin assay (182). Despite clearing conventional contaminants, all three treatments produced WWEs that increased TH-sensitive gene expression (*thibz*, *thra*, *thrb*) upon exposure (**Table 4**). The treatment types also had conflicting results in their ability to clear TH signaling effects depending upon the season in which the WWEs were collected (**Table 4**) (182). In a later study, Wojnarowicz et al. demonstrated the inefficiency of municipal wastewater treatment plants by showing that there is little difference in the endocrine-disrupting potential of WWE to that of the original influent using TH-mediated molecular endpoints and C-fin assays (**Table 4**) (183).

The considerable compositional variation within WWEs poses a challenge when assessing their endocrine disrupting potential. Heerema et al. generated a wastewater standard composed of common PPCPs to evaluate the exposure effects of the simulated WWE and test the efficiency of wastewater treatment systems (36). After filtration using an anaerobic membrane bioreactor (AnMBR), the standard WWE induced a significant upregulation of TH-sensitive *thibz* in the olfactory epithelium of *R. catesbeiana*

TABLE 4 | Summary of contaminant mixture effects on morphological and molecular endpoints for amphibians undergoing both natural and TH-induced metamorphosis.

		Metamorphosis		Morphological/Behavioral		Molecular			
Chemical Mixture	**Species**	**Natural**	**Induced**	**Results**	**Tissue**	**Technique**	**Result**	**References**
Bunker crude oil	*X. laevis*	Y			Whole tadpoles	qPCR	[0.25 g/L oil WAF] ↓*ppar*g, *thrb*; [25 g/L oil WAF] ↑*pparg, dio2*	(181)
Refinery oil	*X. laevis*	Y			Whole tadpoles	qPCR	↑*ppar*g; ↓*thrb*	(181)
Wastewater	*R. catesbeiana*		T_3		C-fin[a]	qPCR	↑*thibz, thra, thrb*	(182, 183)
		Y		↓ predator cue avoidance	Olfactory epithelium	qPCR	↑↓*thibz*[b]; ↑*heket*; ↓*dio2*	(6, 36)
	X. laevis		T_3	↑ metamorphic rate	Cultured tail	qPCR	↑*crhbp, dio2, fap, thrb*	(184)

[a]*Cultured tail fin assay.*
[b]*Varied response due to complex mixtures and treatment techniques.*

tadpoles. This suggests that the effluent was influencing TH-dependent pathways (**Table 4**). This study also assessed the behavioral effects, particularly predator cue avoidance, associated with WWE exposure. Once a tadpole is exposed to T_3, it will stop responding to a simulated predator cue (36). WWE exposure mimicked the effects of T_3 signaling in the olfactory epithelium and decreased predator cue avoidance (36). As a follow-up to this work, Jackman et al. showed that membrane enhanced biological phosphorous removal (MEBPR) performed better at removing EDCs from WWE than AnMBR (6). However, both effluent types resulted in the perturbation of TH-responsive gene transcript levels in the olfactory epithelium. TH agonist activity was observed in the AnMBR WWE and antagonist activity from MEBPR WWE, likely reflective of the influent source material (**Table 4**).

Petroleum Oil Products

Oil spills from a variety of sources can contaminate freshwater systems, thereby affecting the local biota (189). Major toxic components of oils, such as napthenic acid (NA) and polycyclic aromatic hydrocarbons (PAHs), are dispersed within water-soluble fractions after a spill (190, 191). NA and PAHs act as EDCs in amphibians. NA can directly reduce the rate of metamorphosis in *X. tropicalis* and *R. pipiens* and PAHs from tar-based pavement sealers can significantly reduce the rate of metamorphosis in *X. laevis* (**Supplementary Table 1**) (192, 193). The endocrine disrupting potential of these compounds seems to be quite persistent in the environment as *R. sylvatica* tadpoles exposed to pond water from wetlands proximal to reclaimed oil sands had significantly altered T_3/T_4 ratios and had accelerated or delayed rates of metamorphosis depending on the age of the reclaimed wetland (**Supplementary Table 1**) (194).

The effects of NA and PAHs on TH-sensitive gene expression was evaluated by exposing *X. laevis* tadpoles to simulated oil spill conditions using water accommodated fractions (WAF). WAFs were prepared using bunker crude oil or refinery oil (181). Bunker crude WAF exposures resulted in a significant decrease in *dio2* and *thrb* and differential expression of *pparg* at various WAF concentrations (**Table 4**). Refinery WAF exposures resulted in a significant decrease in *thrb* expression and a significant increase in *pparg* expression (**Table 4**). Therefore, water soluble components of oil spills can adversely affect TH-sensitive gene expression critical for amphibian metamorphosis.

EFFECTS OF ENVIRONMENTAL FACTORS

Temperature

Temperature serves as an important environmental cue for seasonality changes. As such, poikilothermic anurans have evolved to allow this environmental factor to serve as a critical cue in their developmental program. The role of temperature in modulating developmental timing is clearly demonstrated during natural metamorphosis when warmer temperatures lead to increased endogenous T_3 and thereby a faster metamorphic rate (**Supplementary Table 2**) (195–198). Along with the increase in TH levels, there is an upregulation of TH-regulated transcripts (including *thra*, *thrb*, *thibz*, *dio2*, and *dio3*) that initiate the metamorphic program (**Table 5**) (195, 202).

Conversely, metamorphic rate slows as temperatures decrease and can be halted altogether at 4–5°C (**Supplementary Table 1**) (203, 207, 208). Although premetamorphic tadpoles will not undergo precocious TH-induced metamorphosis at cold temperatures, a TH-induced memory is established whereby the metamorphic program resumes when permissive temperatures are attained, even when no TH signal remains (207). This cold temperature arrest is observed at the transcriptomic level (**Table 5**) (65, 203, 204, 209). Hammond et al. induced metamorphosis at 5°C in premetamorphic *R. catesbeiana* tadpoles through T_3 exposures and assessed transcript responses in the brain, liver, back skin, tail fin, and lung (65). Across all tissues, *thrb* did not show the rapid induction observed at permissive temperatures. Transcripts encoding the transcription factor *thibz*, however, were upregulated in response to T_3 in all tissues, although this was not found in the liver by Suzuki et al. (**Table 5**) (203). Other TH response genes, including *dio2*, *dio3, cebp1*, *klf9*, and transcripts encoding urea cycle and energy metabolism enzymes showed varied responses across tissues, indicating there may be a tissue-specific response to T_3 in cold temperatures (65, 202–204). Cold temperatures also inhibited the T_3-induction of plasma glucose and decreased lipid polyunsaturation consistent with an effect on energy metabolism in tadpoles (**Table 5**) (203).

Regulation of chromatin structure is postulated to be a mechanism by which this differential gene expression occurs at permissive and non-permissive temperatures [reviewed by Hammond et al. (210)]. Using chromatin immunoprecipitation, Mochizuki et al. found that upon T_3 exposure of *R. catesbeiana* at 4°C compared to 28°C, there was decreased association of positive transcription histone H3K36 marks within two known temperature responding genes in the liver: *thrb* and *cebp1* (**Table 5**) (204). How histone post-translational modifications may differentially regulate genes with upregulated transcription in cold temperatures and simultaneously establish a molecular memory of the TH signal to be activated under permissive conditions is unknown. As changing climate becomes an increasing threat to declining frog populations, it is critical to understand the effect that temperature has on the proper regulation of TH signaling during development.

Ultraviolet B Radiation

Ultraviolet B radiation (UVBR) is becoming a growing concern as stratospheric ozone levels deplete (211). Paired with an increasing penetration of UVBR into the water column (212), embryo and tadpole stages, which reside in aquatic environments, are at a greater risk. Metamorphic or developmental consequences of UVBR exposure on these more sensitive stages varies depending on species and life stage exposures [reviewed in Croteau (205)]. Blocked or delayed postembryonic development is the most commonly seen defect upon UVBR exposure (**Supplementary Table 1**) (213, 214). Croteau et al. examined the relationship between UVBR-induced developmental delays in *R. pipiens* and whether it may be related to the disruption of TH signaling (206). Exposure

TABLE 5 | Effects of environmental factors on morphological and molecular endpoints for amphibians undergoing both natural and TH-induced metamorphosis.

			Metamorphosis		Morphological	Molecular			
Category	**Treatment**	**Species**	**Natural**	**Induced**	**Results**	**Tissue**	**Technique**	**Result**	**References**
Food restriction	Low food	*R. sylvatica*	Y		↓ metamorphic rate	Tail	qPCR	↓*thrb*	(80)
Pond drying	Decreasing water	*Pelobates cultripes*	Y		↑ metamorphic rate	Body without tail	qPCR	↑*thrb*	(199)
		R. temporaria	Y		↑ metamorphic rate	Liver	cDNA array (MAGEX 497 genes)	↑37↓1: cell cycle, transcription, integral to membrane	(200, 201)
			Y			Liver	qPCR	↑*cps1, thrb*	(200)
Temperature	High temperature	*R. catesbeiana*	Y			Liver	qPCR	↑*dio2, dio3, thibz, thrb*	(195)
				T_3		Liver	qPCR	↑*thra, thrb*	(202)
	Low temperature	*R. catesbeiana*		T_3	↓ tail regression	Back skin	qPCR	↑*klf9, thibz, thra;* ↓*dio2*	(65)
				T_3		Brain	qPCR	↓*dio3, thra, thrb*	(65)
				T_3		C-fin[a]	qPCR	↓*thra, thrb*	(65)
				T_3		C-skin[b]	qPCR	↓*thra, thrb*	(65)
				T_3		Liver	qPCR	↓*cps1, dio2, dio3, mmp11, nfic, otc, several energy metabolism transcripts, thibz, thra, thrb*	(65, 202–204)
				T_3		Liver	ChIP	↓H3K36me3 in *thrb* gene	(204)
				T_3		Liver	Thin layer chromatography	↓lipid polyunsaturation	(203)
				T_3		Lung	qPCR	↑*klf9, thibz*	(65)
				T_3		Plasma	Mutarotase-glucose oxidase method	Inhibit T_3-induction of glucose	(203)
				T_3		Tail fin	qPCR	↑*thibz*	(65)
UVBR	UVBR	*R. pipiens*	Y			Tail	qPCR	↓*dio2;* ↑*dio3*	(205, 206)

[a]*Cultured tail fin assay.*
[b]*Cultured back skin assay.*

to UVBR showed no effect on total body T_3 or brain CRF levels, indicating that synthesis of TH through the HPT axis is unlikely affected, although total T_4 was not measured (206). Rather, UVBR effects may act locally on peripheral tissues. Increased *dio3* found in the tail during stages preceding the observed morphological delay may cause decreased local TH through enhanced turnover (**Table 5**). There is also decreased expression of *dio2*, which may act to further regulate the activity of THs through decreased conversion of T_4 to T_3 (2). This local response highlights the need to look at various tissues as their response to UVBR may differ leading to uncoordinated metamorphosis. As levels of UVBR are expected to rise, it is imperative to determine its mechanism of action in TH disruption, especially for sensitive life stages like postembryonic development.

Photocycle: Light-Dark Cycle Implications

Endocrine systems are entrained to the circadian clock. The TH axis is no exception, with THs following a rhythmic 24 h cycle (215). As photoperiod, along with temperature, is an environmental cue for seasonality changes, it is not surprising that many studies have found that the light:dark (L:D) cycle has an impact on metamorphic rate (**Supplementary Table 1**) (216–218). In the majority of studies, increasing photophase (light phase) of a 24 h L:D cycle or decreasing cycle length increases metamorphic response to TH stimulation (218). However, when the 24 h cycle is not maintained, the L:D ratio is no longer indicative of metamorphic rate. In a case where Wright et al. found decreased tail reduction with an increased photophase, there was 18L:12D, which surpasses the standard 24 h cycle (219).

The mechanism by which the L:D cycle alters TH-induced metamorphosis is poorly understood. It has been determined that altering the L:D cycle leads to differences in the fluctuating rhythm of T_4 [reviewed by Wright (215)] (**Supplementary Table 2**). However, under any L:D cycle, there is an inextricable rise in T4 as development progresses until metamorphic climax (220). This indicates that the alteration in metamorphic rate is not due to a disruption in TH concentration. It is more likely that a disruption in the circadian rhythms of THs may lead to different interactions with agonists and antagonists (220, 221). It remains to be determined how this variation in cycling affects TH responses at the transcriptomic level, which may provide a better mechanistic understanding of how the L:D cycle impacts metamorphic timing.

Pond Drying

Many tadpole species reside in ephemeral bodies of water. Loss of these temporary habitats is fatal to water-dwelling tadpoles; therefore, it is unsurprising that across species, there is a positive correlation between pond desiccation rate and speed of development into terrestrial frogs (**Supplementary Table 1**) (198, 222, 223). The ability to translate this environmental cue to a phenotypic response is proposed to occur through the HPT axis. CRF levels increase in response to habitat desiccation, preceding the morphological observation of hastened metamorphic rate (224). This stress-induced increase in CRF leads to augmented secretion of THs (**Supplementary Table 2**) (199, 224, 225), which can be reversed through exposure to CRF antagonists (225). This increase in activity in the HPT axis leads to accelerated metamorphosis through the downstream regulation of TH response genes. Johansson et al. used cDNA microarray and qPCR analyses to determine the hepatic transcriptomic response to simulated pond drying in *R. temporaria* (**Table 5**) (200). This study found that classic TH response genes, such as *thra* and *thrb*, increase along with decreasing water levels, which corroborates previous findings that decreased water levels lead to increased TH levels and higher expression of *thrb* in the blood (199). More liver-specific TH response transcripts also demonstrated significant changes, including urea cycle enzyme *cps1* (200). The ability of tadpoles to respond to decreasing water levels demonstrates the plasticity of TH-mediated metamorphosis that allows tadpoles to adapt to changing environments.

Food Restriction

Similar to pond drying, availability of other resources, such as food, plays an important role in developmental timing. As metamorphosis often entails a niche transition from aquatic to terrestrial environments, it stands to reason that when resources available in the aquatic habitat are no longer sufficient, it may prompt a transition to a new environment where resources may be greater or competition lower. The impact of food restriction on metamorphosis has varied results (**Supplementary Table 1**) (226–231). Complete starvation and consistently low or actively decreasing food levels leads to increased metamorphic rate in *Scaphiopus* (*S.*) *hammondii* (226), *Phrynobatrachus guineensis* (227), *and R. temporaria* (228). In contrast, consistently low or actively decreasing food sources reduces metamorphic rate in *Hyla cineria* and *Hyla gratiosa* (229, 230). The varied response may be due to the different life histories of the different species. Another contributing factor is the developmental timing of food restriction. D'Angelo et al. determined that there is a critical developmental point, around limb bud formation, before which metamorphosis will be stalled but after which, metamorphosis will be accelerated (232). Bulaeva et al. restricted food for *R. sylvatica* prior to this critical time point and found that the decrease in metamorphic rate coincided with a decrease in *thrb* transcript levels, indicating disrupted TH signaling (**Table 5**) (50). In contrast, histological analysis of the thyroid gland of the tadpoles starved past this critical period give evidence to a short burst of increased secretory activity (232). Evidence of this burst of thyroid activity was corroborated *in vitro* by Wright et al. who found a brief increase in secretion of T_4 in cultured thyroids excised from *R. catesbeiana* tadpoles that were starved for 1 week compared to those that were fed consistently (**Supplementary Table 2**) (233). Boorse and Denver also found increased levels of T_3 and T_4 *in vivo* after food restriction in *S. hammondii* (234). Future studies have yet to determine how this burst of T_4 affects the TH-induced transcriptomic program leading to increased metamorphic rate.

Combined Chemical and Environmental Effects

It is well-established that individual environmental factors play an important role in the proper regulation of TH signaling during metamorphosis. In natural systems, however, temperature, photoperiod, UVBR intensity, and resource restriction (pond-drying and food restriction) effects are inextricably linked. Not only are they influenced by each other, but frogs are simultaneously exposed to all anthropogenic chemicals that enter their habitat. The combinatorial effect of environmental and chemical stressors can be exponentially more detrimental as they may work synergistically to increase toxicity or reduce an organism's capacity to respond to other stressors.

The additive toxicity of UVBR and various chemical contaminants has been well-documented [reviewed by Blaustein et al. (235) and Croteau et al. (205)]; however, the sublethal effects on development have not been as well-studied. Crump et al. found that environmentally-relevant levels of the estrogenic compound, octylphenol, had a combined effect with UVBR that increased metamorphic rate, unlike exposure to either factor individually (236). The TH-based mechanism by which this combined effect occurs was further studied by Croteau et al. who observed that at earlier developmental stages, there is a significant increase in *thrb* upon exposure to UVBR combined with octylphenol compared to either factor alone (**Table 6**) (206). This indicated that TH-signaling during metamorphosis is being differentially affected by the combination of chemical and environmental factors.

Increases in metamorphic rate induced by warmer temperatures are compounded by concomitant contaminant exposures that also induce metamorphosis. Freitas et al. exposed *R. catesbeiana* tadpoles to the pesticide diuron and its metabolite, 3,4-dichloroaniline, at 28 and 34°C and observed an increased developmental response to both chemicals at the higher temperature compared to either exposure at the lower temperature or the temperature-matched control (**Table 6**) (195). This combination of exposures increased the expression of *dio2* and the warm temperature plus diuron exposure increased *klf9*, which likely explains the observed change in metamorphic rate.

Contaminants can also have an impact on the ability of environmental cues to regulate developmental timing. For species that overwinter as tadpoles, transitioning while the temperatures are still too low could be fatal. Hammond et al. investigated the impact of two known TH EDCs discussed above, ibuprofen, and TCS, on the temperature-controlled TH response in premetamorphic *R. catesbeiana* tadpoles (**Table 6**) (28, 29, 65). After a 48 h exposure to each contaminant at 5°C, both produced a significant increase in *klf9* in a cultured *R. catesbeiana* back skin biopsy assay (C-skin). In contrast, when tail fin biopsies from the same tadpoles were cultured in a C-fin assay, exposed to the chemicals at 5°C and then shifted into clean media at more permissive temperatures, there was a significant decrease in *thrb* after TCS exposure (**Table 6**). This indicates that both EDCs have the potential to disrupt proper TH signaling during the cold-induced establishment of TH molecular memory. As well, TCS exposure at cold temperatures may be remembered when warmer temperatures occur, potentially leading to detrimental effects throughout metamorphosis.

Natural systems contain combinations of environmental factors and chemical contaminants. It is therefore important to conduct studies with multiple stressors to provide more meaningful information. A changing climate and intensifying UVBR combined with increased anthropogenic contamination are escalating the need to elucidate how these factors influence critical biological systems such as TH signaling in metamorphosis, both independently and in combination with each other.

CONCLUDING CONSIDERATIONS

As our understanding of the disruption of TH-dependent metamorphosis by environmental and chemical perturbations improves, it is apparent that there are several pressing challenges that must be addressed. Anurans are keystone sentinel species that can portend the deleterious and combinatorial effects of contaminants and changing climate effects on all trophic levels within different environmental niches. While it is important to understand the mechanisms affected by a single contaminant, the environmental context in which the exposure occurs must be considered. Within an affected environment, disruption of TH-dependent metamorphosis is rarely, if ever, derived from an isolated contaminant. To this end, the interplay between environmental conditions and complex mixtures should be assessed in tandem to ascertain the cumulative effects on TH-dependent metamorphosis.

Amphibian screening assays have been developed that address the need for the timely detection of contaminants that affect

TABLE 6 | Combined effects of chemical contaminants and environmental factors on morphological and molecular endpoints for amphibians undergoing both natural and TH-induced metamorphosis.

	Treatment			Metamorphosis		Morphological	Molecular			
Category	**Environmental**	**Chemical**	**Species**	**Natural**	**Induced**	**Results**	**Tissue**	**Technique**	**Result**	**References**
Temperature	28°C	Diuron	*R. catesbeiana*	Y			Liver	qPCR	↑*thibz*	(195)
	34°C		*R. catesbeiana*	Y			Liver	qPCR	↑*dio2, klf9*	(195)
	5°C	Ibuprofen	*R. catesbeiana*		T_3		C-skin	qPCR	↑*klf9*	(65)
	5°C	Triclosan	*R. catesbeiana*		T_3		C-skin	qPCR	↑*klf9*	(65)
	5°C to 24°C		*R. catesbeiana*		T_3		C-fin	qPCR	↑*thrb*	(65)
UVBR	UVBR	4-Tert-octylphenol	*R. pipiens*	Y		↓ metamorphic rate	Tail	qPCR	↑*thrb*	(236)

TH-dependent metamorphosis and that accurately reflect *in vivo* changes (237). The *Xenopus* metamorphosis assay (XEMA) was initially conceived to assay the TH-disrupting capacity of compounds and derivatives of XEMA have since been developed (40, 78, 238). Similarly, cell lines and serum-free organ culture techniques, including tail fin (C-fin) and back skin (C-skin), have utility in ascertaining TH disrupting effects (81, 209, 239, 240). Organ culture techniques are particularly useful since they retain the three-dimensional structure of tissues while facilitating a repeated-measures analysis, including the rapid assessment of TH-dependent molecular changes in gene expression. As such, organ cultures provide an informative and complementary counterpart to conventional morphological and histological assessments (89). As changes in gene expression typically precede morphological variations during metamorphosis, transcriptomic assessments provide a more timely and sensitive assessment of altered metamorphic trajectories that may not be readily observed as a pathological phenotype. Non-lethal molecular assessments of tail fin biopsies are additionally well-suited to long-term studies involving repeated measures (84).

Careful consideration should be paid to the selection of amphibian species used to assess the ramifications of environmental contaminants on metamorphic dysfunction. Although *Xenopus* species are widely accepted animal models in research laboratories, their natural habitats, life cycles and physiologies are quite distinct from other anurans, such as Ranids, Hylids, and Bufonids (241). Consequently, physiological responses to environmental or chemical perturbations can differ widely between anurans. Therefore, the adoption of amphibian models that closely resemble native species in affected areas would provide the most meaningful assessments of TH-dependent metamorphic disruptions (242).

The use of qPCR, DNA microarrays, and RNA-seq in conjunction with morphological characterizations have demonstrated the sensitive and differential tissue-specific gene expression arising from environmental or toxicant exposures during TH-dependent metamorphosis (23, 32, 34, 209, 243). As cutting-edge 'omics techniques—transcriptomics, genomics, epigenomics, proteomics, and metabolomics—are increasingly utilized with bioinformatics in ecotoxicology studies, it will be possible to elucidate the mechanisms affected by TH-disrupted metamorphosis in a comprehensive manner (210, 244–247). Considerable progress has been made in recent years with the sequencing of the *X. laevis*, *X. tropicalis, Nanorana parkeri,* and *R. catesbeiana* genomes and increasing numbers of transcriptomes; all of which are invaluable resources (247–251). Thoughtful consideration of the species, tissue-specificity, and developmental stage observed; the timing and duration of exposure and study conditions will be indispensable in establishing large-scale studies for meaningful meta-analyses.

Additional attention should be paid to the examination of metabolized derivatives that may be more potent than the parent congener. Biotransformed derivatives can be generated through metabolic activities within the affected organism or by physical transformation in the environment (for instance, weathering or photo-oxidation) (252–254). Compared to the parent congener, activated derivatives may consequently be more stable, better able to mimic or target different aspects of TH regulation (i.e., TH receptors, metabolizing enzymes, etc.) or be rendered more lipophilic, which would facilitate their uptake or excretion. The mechanisms underlying increased derivative toxicity, whether they are independent of or compromise TH regulation, is an important area of study.

With more than 70% of ∼7,000 extant amphibian species threatened and declining around the world, there is an urgent need to address how anthropogenically-derived environmental disruptions are affecting vulnerable species (255). Humans are not impervious to the deleterious changes affecting wildlife; metabolomic studies demonstrated that metabolites altered during anuran metamorphosis are also associated with human disease outcomes (245). Moreover, a recent study demonstrated that TH-related gene expression and early brain development were altered in *X. laevis* following exposure to concentrations of chemicals (including TCS, phthalates, pesticides, and others) detected in human amniotic fluid (256). Given the developmental parallels between TH-dependent amphibian metamorphosis and mammalian postembryonic development, it is apparent that exposures negatively affecting amphibians will also impair human health (257). As sobering as these ramifications are, such deleterious outcomes are not necessarily irrevocable if timely remediation actions are taken. The genomic plasticity afforded by epigenetic alterations, while able to endure maladaptive stresses, similarly has the posited capacity to adapt to remediation. Such potentially ameliorative effects merit further investigation that would be best addressed using the genomics-based approaches discussed in conjunction with morphological analyses. Remediation efforts will require understanding the complexity of the ecological stresses and the interplay between complex toxicant mixtures and changing environmental conditions. The unique sensitivity of anurans to TH ideally positions them as indicators for not only metamorphic and developmental effects, but also for the fitness and reproductive success of all vertebrates that depend upon TH function.

AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualization, literature research and interpretation, writing, and editing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00276/full#supplementary-material

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Corrigendum: Contaminant and Environmental Influences on Thyroid Hormone Action in Amphibian Metamorphosis

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A Corrigendum on

Contaminant and Environmental Influences on Thyroid Hormone Action in Amphibian Metamorphosis

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In the original article, there was a mistake in **Figure 1** and the corresponding figure legend as published. The progression of tail regression and mouth sculpting relative to metamorphic timing was incorrectly depicted and there was an error in the reference in the figure legend. The corrected **Figure 1** appears below.

The corrected **Figure 1** legend is "Thyroid hormone (TH) levels and key morphological hallmarks during frog postembryonic development. Amphibian metamorphosis is a postembryonic process driven by TH signaling. The free-swimming tadpole (0% relative time) has virtually undetectable levels of TH. The morphological changes that occur in the development of a tadpole to a juvenile frog (100% relative time) are inextricably aligned to internal rises in TH levels. These rising TH levels lead to progression through the stages of development, which can be seen through morphometric measurements including hindlimb development, forelimb emergence, tail regression, head shape changes, and thyroid follicle production. The Gosner and Nieuwkoop and Faber (NF) staging system comparisons are from Just (3)."

In the original article, reference 3 was incorrectly written as Gosner KL. A Simplified Table for staging anuran embryos and larvae with notes on identification. *Herpetologica*. (1960) 16:183–90. It should be Just, JJ, Kraus-Just J, Check DA. Survey of chordate metamorphosis. In: Gilbert LI, Frieden E, editors. *Metamorphosis*. Boston, MA: Springer (1981). p. 265–326.

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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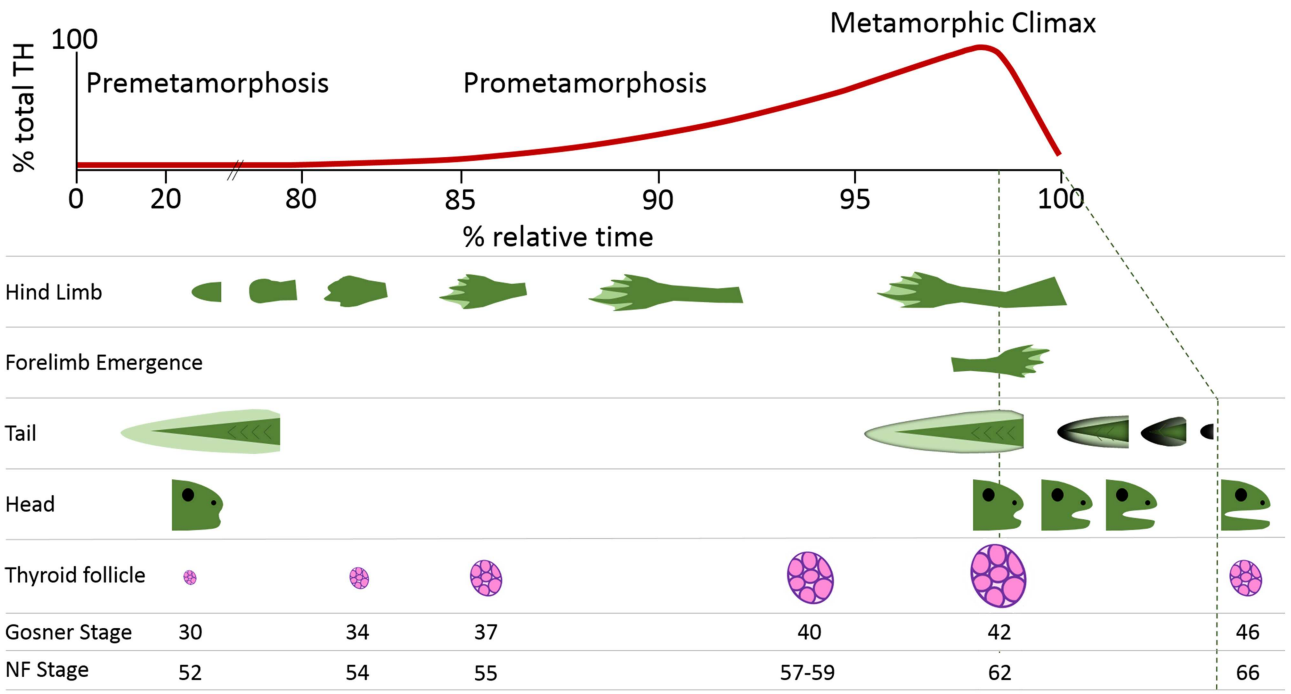

FIGURE 1 | Thyroid hormone (TH) levels and key morphological hallmarks during frog postembryonic development. Amphibian metamorphosis is a postembryonic process driven by TH signaling. The free-swimming tadpole (0% relative time) has virtually undetectable levels of TH. The morphological changes that occur in the development of a tadpole to a juvenile frog (100% relative time) are inextricably aligned to internal rises in TH levels. These rising TH levels lead to progression through the stages of development, which can be seen through morphometric measurements including hindlimb development, forelimb emergence, tail regression, head shape changes, and thyroid follicle production. The Gosner and Nieuwkoop and Faber (NF) staging system comparisons are from Just (3).

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ORIGINAL RESEARCH
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Evolutionary Conservation of Thyroid Hormone Receptor and Deiodinase Expression Dynamics *in ovo* in a Direct-Developing Frog, *Eleutherodactylus coqui*

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Direct development is a reproductive mode in amphibians that has evolved independently from the ancestral biphasic life history in at least a dozen anuran lineages. Most direct-developing frogs, including the Puerto Rican coquí, *Eleutherodactylus coqui*, lack a free-living aquatic larva and instead hatch from terrestrial eggs as miniature adults. Their embryonic development includes the transient formation of many larval-specific features and the formation of adult-specific features that typically form postembryonically—during metamorphosis—in indirect-developing frogs. We found that pre-hatching developmental patterns of thyroid hormone receptors alpha (*thra*) and beta (*thrb*) and deiodinases type II (*dio2*) and type III (*dio3*) mRNAs in *E. coqui* limb and tail are conserved relative to those seen during metamorphosis in indirect-developing frogs. Additionally, *thra, thrb,* and *dio2* mRNAs are expressed in the limb before formation of the embryonic thyroid gland. Liquid-chromatography mass-spectrometry revealed that maternally derived thyroid hormone is present throughout early embryogenesis, including stages of digit formation that occur prior to the increase in embryonically produced thyroid hormone. *Eleutherodactylus coqui* embryos take up much less 3,5,3′-triiodothyronine (T_3) from the environment compared with *X. tropicalis* tadpoles. However, *E. coqui* tissue explants mount robust and direct gene expression responses to exogenous T_3 similar to those seen in metamorphosing species. The presence of key components of the thyroid axis in the limb and the ability of limb tissue to respond to T_3 suggest that thyroid hormone-mediated limb development may begin prior to thyroid gland formation. Thyroid hormone-dependent limb development and tail resorption characteristic of metamorphosis in indirect-developing anurans are evolutionarily conserved, but they occur instead *in ovo* in *E. coqui*.

Keywords: embryo, direct development, thyroid hormone, amphibians, evolution, metamorphosis, maternal effects, life history

INTRODUCTION

Direct development, a distinctive life-history mode in amphibians and other animals, has evolved in anurans multiple times from the ancestral biphasic life history; it characterizes many hundreds of living species (1). Even though direct-developing frogs typically lack both a free-living aquatic larval stage and a discrete, post-hatching metamorphosis, many species display a cryptic metamorphosis before hatching: adult-specific features, such as limbs, form precociously in the egg, and numerous tadpole-specific features are present initially but then are lost [**Figure 1**; (2, 3)]. Because such changes in frogs with indirect development are mediated by thyroid hormone (TH), the primary regulator of metamorphosis (4), evolutionary change in thyroid axis function and timing may underlie the numerous heterochronies observed between direct-developing and indirect-developing species (5–9). Yet, there have been few attempts to precisely delineate the role of this or other pertinent physiological mechanisms.

Embryonic development of direct-developing frogs, as seen in the Puerto Rican coquí, *Eleutherodactylus coqui*, appears to comprise a mosaic of TH-independent and TH-dependent features. We use the term "embryonic" to describe all *in ovo* development in *E. coqui*, although this period encompasses both the initial formation of major organ systems as well as the patterning, morphogenesis and growth that follows. Many of the latter events correspond to metamorphic changes in biphasic anurans. It was once thought that embryonic development in direct-developing species was primarily TH-independent (5). However, subsequent studies with exogenous T_3 and with TH-synthesis inhibitors suggested at least a partial role for TH in terminal stages of limb development as well as tail resorption (6, 10). In *E. coqui*, for example, treatment with exogenous T_3 causes precocious tail resorption but has little to no effect on limb elongation (11). Similarly, treatment with methimazole, a TH-synthesis inhibitor, inhibits only tail resorption and late stages of limb elongation but does not affect early limb differentiation or digit formation (8). The apparent TH-independence of early stages of limb development is correlated with the fact that limb bud, paddle and digit formation occur prior to formation of the embryonic thyroid gland [**Figure 1**; (12, 13)]. Thus, limb development in *E. coqui* comprises two periods: limb bud differentiation and paddle and digit morphogenesis, which precede formation of the thyroid gland and may be TH independent; and limb growth and elongation, which follow thyroid gland formation and are TH dependent. Experiments with TH-synthesis inhibitors, however, can only address the role of TH in the second period. The presumed TH independence of the first period remains to be verified experimentally.

All organs in the body are exposed to roughly the same concentration of circulating TH, primarily in the form of thyroxine (T_4) and lower concentrations of 3,5,3′-triiodothyronine [T_3; (14, 15)]. Hereafter, we use the term TH to refer to both T_4 and T_3. However, tissue-specific differences in uptake, metabolism, and action provide for diverse effects of TH in different tissues. Thus, tissue-specific changes in TH metabolism and action likely contribute to the heterochrony of developmental events observed in direct-developing anurans relative to biphasic species. Alternatively, the principal locus of change in hormonal control may involve a shift in the source of THs and when they are present in the embryo. Maternally derived TH is present at early developmental stages of all vertebrates examined so far. In most vertebrates, maternal TH is in the yolk; in most mammals, maternal TH can pass from mother to fetus via the placenta or milk. Yet, the role of maternally derived TH in amphibian embryos is poorly understood (16–18). If maternally derived THs are present in early embryos of *E. coqui*, they could influence limb development prior to formation of the embryonic thyroid gland. Finally, three different deiodinase enzymes control cellular metabolism of T_4 in target tissues. In amphibians, two types of deiodinases play major roles during development. Deiodinase enzyme type II (Dio2) converts T_4 into T_3, which has at least 10 times greater affinity for TH receptors (TRs) than T_4. Deiodinase type III (dio3) converts T_4 to both T_2 and reverse triiodothyronine (rT_3), which are unable to bind TRs in most species. Thyroid hormones act by binding to two TR subtypes, designated alpha (α) and beta (β), to activate or repress transcription of TH target genes. Contrasting expression patterns of TRs and deiodinases may in part underlie the diverse, tissue-specific effects of TH in *Xenopus* species (19–26), and it is likely that changes in the temporal or spatial expression of deiodinases or TRs influence TH competence and action in target tissues in *E. coqui*.

Here we tested the hypothesis that developmental changes in TR and deiodinase mRNAs in developing *E. coqui* limb and tail, and in whole body TH content are conserved relative to those seen during metamorphosis in indirect-developing frogs. We also investigated whether *E. coqui* tissues are capable of responding directly to T_3 action by mounting gene regulation responses similar to those seen in metamorphosing species. Taken together, our data support the hypothesis that limb development and tail resorption in *E. coqui* (8, 12) are mediated by conserved components of TH signaling. Additionally, our results suggest that maternal TH could facilitate limb development prior to formation of the embryonic thyroid gland.

MATERIALS AND METHODS

Animal Care

Live adult *Eleutherodactylus coqui* were field-collected from introduced populations in Hilo, Hawaii, with the permission of the U.S. Fish and Wildlife Service (permits EX-14-06, EX-16-07, and EX-17-11). They were brought to Harvard University and maintained as a breeding colony in the Hanken laboratory (IACUC protocol #99-09-03); embryos were obtained following spontaneous matings. Following removal of the overlying chorion with watchmaker forceps in 2% cysteine (pH 8.5) in 10% Holtfreter solution, embryos were reared in 10% Holtfreter solution in Petri dishes at 22.5°C. Embryos were staged according to the normal table of Townsend & Stewart (TS; 1985), which defines 15 stages from fertilization (1) to hatching (15). Following internal fertilization, the adult female deposits embryos at TS stage 1.

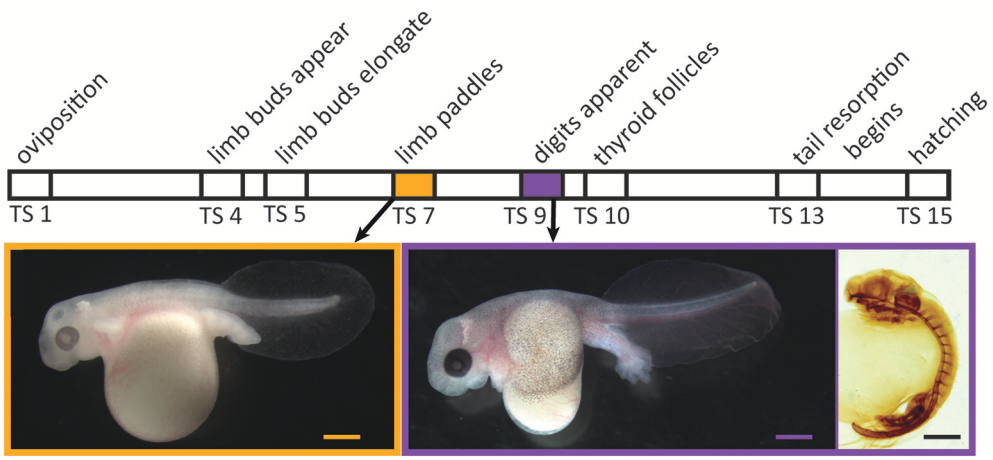

FIGURE 1 | Relative timing of several developmental events during embryogenesis in *Eleutherodactylus coqui*. Images depict live TS stage 7 **(Left)** and stage 9 **(Middle)** embryos removed from overlying egg membranes, and a TS stage 9 embryo **(Right)** stained for type II collagen, which reveals the cartilaginous skeleton and notochord. Scale bars, 1 mm.

Molecular Cloning and Sequence Validation

Partial cDNAs for *dio2*, *dio3*, *thra*, *thrb*, *ribosomal protein L8* (*rpL8*), *thyroid hormone induced bZip protein* (*thibz*), and *alpha-actinin 4* (*actn4*) (Genbank accession numbers MK784754, MK784753, MK784748, MK784749, MK784751, MK784750, MK784755) were isolated by PCR with exact primers (**Table 1**) using cDNA generated from RNA isolated from whole TS stage 13 embryos, and the resultant DNA fragments were subcloned into the pCR II plasmid. Exact primers for *dio2*, *dio3*, *thra*, *thrb*, *rpL8*, and *thibz* were designed from predicted full-length cDNA sequences provided by L. Sachs, N. Buisine, and G. Kerdivel (personal communication), while *actn4* primers were designed from genomic sequences provided by A. Mudd, R. Harland, and D. Roksahr (personal communication). We also subcloned a partial cDNA for *krüppel-like factor 9* (*klf9*) by degenerate PCR (oligonucleotide primers designed using CODEhop) using the same cDNA described above (Genbank accession number MK784752). The sequences of the subcloned partial cDNA fragments were confirmed by direct DNA sequencing and by comparing them against the full-length cDNAs provided by the investigators listed above.

Prior to the full-length predicted cDNA sequences becoming available, oligonucleotide primers for SYBR-based reverse transcriptase quantitative PCR (RTqPCR) were designed based on the available mRNA sequences on Genbank for *thra* and *thrb,* and the previously cloned *rpL8* [Genbank accession numbers AF201957.1 and AF201958.1; (8), **Table 1**]. For probe-based quantitative PCR (qPCR), primers and probes for *actn4* were designed from the partial cloned cDNA sequence while *dio2*, *dio3*, *thra*, *thrb*, *rpL8*, *thibz*, and *klf9* were designed based on the full-length sequences from other investigators listed above (Genbank accession numbers MK784763, MK784762, MK784757, MK784756, MK784760, MK784758, MK784759, MK784761).

Whole Body Extraction and Quantification of Iodothyronines Using LC-MS/MS

The iodothyronines T_3, rT_3, T_4, and T_2 were quantified from whole *E. coqui* embryos throughout development. Because embryos were not dissected from the yolk, all measurements include embryo and yolk TH content. Animals at different stages were anesthetized and snap frozen until extraction and LC-MS/MS analysis. Unfertilized oocytes were dissected from the ovaries of a newly sacrificed female and snap frozen. Between 15 and 20 embryos (∼ 600 mg) were pooled to make one biological replicate. Three or four biological replicates were used for each developmental stage. Tissues were extracted for thyroid hormone analysis as described by Denver (27, 28) with the following modifications: stable isotope-labeled T_3 and T_4 ($^{13}C_6$ T_3 and T_4, Sigma) were used as an internal standard to correct for differences in extraction efficiency, and solid phase extraction with a Supel-Select SCX cartridge (60 mg 3 mL, Sigma) was used to further purify the extracted tissue. After conditioning the cartridge with 3 mL methanol (HPLC Grade, Sigma) and equilibrating it with 5 mL of 2% formic acid in water (HPLC Grade, Sigma), the sample was loaded, rinsed first with 3 mL 2% formic acid in water and then with 3 mL methanol, and finally eluted with 2 mL of freshly prepared 5% ammonium hydroxide in methanol. It was then evaporated to dryness under nitrogen flow and resuspended in 100 µl of 0.1% formic acid in methanol. Samples were measured at the Harvard Small Molecule Mass Spectrometry facility by using gradient liquid-chromatography mass-spectrometry (LC-MS/MS). Ten microliters of samples were injected on a C18 column (Kinetex 2.6 µm, 100 Å pore size, 150 × 2.1 mm, Phenomenex) in an Agilent 1290 HPLC coupled with an Agilent 6460 Triple Quad Mass Spectrometer. See **Supplementary Information** for the LC and MS parameters (**Supplementary Tables 1, 2**). Calibration curves were made in 0.1% formic acid in methanol with pure standards and the same amount of internal standard as the samples. Quantification

TABLE 1 | Degenerate PCR, exact PCR, and qPCR primers and probes for *Eleutherodactylus coqui* and *Xenopus tropicalis*.

Gene	Type	Species		Sequence	Probe sequence	Amplicon size (bp)
thibz	qPCR	*E. coqui*	F	GAGGGTCAAACGCCAGTATT	TGAAGGGTGCTATAAAGTAGCTGAT	72
			R	GTCCGGGTCTGTGTAATGTC		
klf9	qPCR	*E. coqui*	F	CAAGTCCTCCCACCTCAAAG	CCCACTACAGAGTGCATACAGGTGA	65
			R	CATGTGCATGGAAATGGACG		
rpL8	qPCR	*E. coqui*	F	CTGGAGGTGGACGTATTGAC	ACCCATTCTGAAGGCAGGTCGT	68
			R	TCTTGGCCTTGTACTTGTGG		
dio2	qPCR	*E. coqui*	F	ACACAGTTACCTCAACAGGG	TGCAATCTGATCTCCCAGGAGCA	87
			R	AACAGTGTGGAACATGCAGA		
dio3	qPCR	*E. coqui*	F	GCAGCCCAGCAGTATTATCA	CGTGGAGGACATGCGTTTAACCC	95
			R	CACATGGGTGGTCTCGTTTA		
thra	qPCR	*E. coqui*	F	ACTACATCAACCACCGCAAA	CCCACTTCTGGCCTAAGCTCCT	81
			R	CAATCATGCGCAAGTCAGTC		
thrb	qPCR	*E. coqui*	F	GCAGCCCAGCAGTATTATCA	TCAAATGTTGTGCCTGCGGCT	95
			R	GTGATCACCATGGGAGATGG		
actn4	qPCR	*E. coqui*	F	AAGCCATCTCTGAAGTCCTC	AGTGCCAGCCTTCCTCAGGTG	80
			R	TTTCACGGCTTGGTGTAACT		
rpL8	qPCR	*E. coqui*	F	GACCAGAGTAAAGCTGCCTTCT	SYBR	95
			R	TTGTCAATACGTCCACCTCCAG		
thra	qPCR	*E. coqui*	F	CGACAAAATCACCCGAAATCAGT	SYBR	78
			R	GACAAGGTCCATTGCCATGC		
thrb	qPCR	*E. coqui*	F	CTTGCGCCTCTTTTCTCTGTTT	SYBR	76
			R	CAGATCTGGTTTTGGATGACAGC		
klf9	Degenerate	*E. coqui*	F	GGSTGTGGCAAAGTYTAYGGSAA		215
			R	TTGGTYAARTGRTCRCTCCTCAT		
rpL8	Exact	*E. coqui*	F	GACATTATCCATGATCCAGGCCG		616
			R	CAGTCTTTGTACCGCGCAGACG		
dio2	Exact	*E. coqui*	F	GAGTGTGGACCTGTTGATCACT		745
			R	TTTCTGTTCCATCCACTGTCGT		
dio3	Exact	*E. coqui*	F	TGCAAACTTCTCAAACAGGTGG		716
			R	TTCCTCAGTTCAGCGATCTTGT		
thra	Exact	*E. coqui*	F	AGAGCCAGATGAAAAGAGGTGG		801
			R	CTGTCAGGATCGTAACGCACA		
thrb	Exact	*E. coqui*	F	CTAGCAGCATGTCAGGGTACAT		779
			R	TACCACCCCTAGTCCTCCATTT		
actn4	Exact	*E. coqui*	F	GAAACAGCAGCGGAAGACTTTC		619
			R	CTTCTTATCAGGACGAGCGGTG		
thibz	Exact	*E. coqui*	F	CTCCATGATTCAACTCCACCCA		961
			R	CGTAGTGAGGGTGAGACAACAA		
thibz	qPCR	*X. tropicalis*	F	AAGAGACGCAAGAACAACGA	AGAAGCGCCGGGCGGGGGA	111
			R	GAGTCGGGCATTCTCTTCAA		
klf9	qPCR	*X. tropicalis*	F	AGTCTTCCCACCTTAAAGCC	ACGCCCTTTTCCGTGTACGTGGCCT	106
			R	GTCAACTCATCGGAACGAGA		
eef1a1	qPCR	*X. tropicalis*	F	CTTGACTGCATTTTGCCACC	AGCCTCTGCGTCTGCCTCTGCAGG	112
			R	GTCTCCACACGACCAACTG		
dio3	qPCR	*X. tropicalis*	F	CGGTGCCTACTTTGAGAGAC	TACCAGGGAGGGCGGGGGCC	94
			R	CCGAGATCTTGTAGCCTTCC		
thrb	qPCR	*X. tropicalis*	F	TTGATGATACCGAAGTCGCC	TCGCCCTGGCCTCACTAGTGTGGAGA	102
			R	AACCTTCCTGGCACTTTTCT		
actn1	qPCR	*X. tropicalis*	F	CAAAGTGCTGGCTGTCAATC	AGCTGGCCAGTGATCTGCTGGAGTGG	105
			R	TCTAACCAAGGGATTGTGCG		

results with a signal-to-noise (S/N) ratio >10 were used for the statistical analysis. Results with a ratio between 3 and 10 (purple type; **Supplementary Table 3**) were included in the graph (**Figure 2**) but not used in the statistical analysis; those with a ratio below 3 were not used (red type; **Supplementary Table 3**). We normalized iodothyronine content to the weight of the tissue extracted.

Quantitative PCR

Dechorionated embryos were anesthetized by immersion in 10% Holtfreter solution with drops of 2% neutral-buffered MS-222 added until the embryos no longer responded to toe pinches (between 30 and 60 s). Limbs and tails were dissected and homogenized in TriZol reagent (Invitrogen) and kept at −20°C until RNA isolation. Total RNA was isolated following the manufacturer's protocol within 3 weeks of homogenization. Because qPCR primers did not span exon-exon boundaries, genomic DNA was removed with an Ambion DNA-*free* kit (cat. #AM1906). Controls with no reverse-transcriptase verified that removal of genomic DNA was complete. Total RNA was quantified with a Qubit Fluorometer 3.0 and checked for purity on a Nanodrop spectrophotometer. For SYBR Green RTqPCR assays, 200 ng of total RNA was used for input for each reaction. For probe-based qPCR, 660 ng of total RNA for each sample was synthesized into cDNA with iScript Reverse Transcriptase Supermix for RT-qPCR (BioRad). Complementary DNA was kept at −20°C until the qPCR assay was performed. mRNA levels were analyzed with either Ssoadvanced Universal Probes Supermix (BioRad) or an iTaq Universal SYBR Green One-Step kit (BioRad) on a CFX384 machine. See **Supplementary Data** for qPCR cycling conditions. Optimal qPCR conditions were determined with temperature gradient and cDNA dilutions for dynamic range of input. Standard curves showed high efficiency of reaction (90–105%), and R^2 was equal to or >0.98 for all primer sets. No template controls showed no amplification. All oligonucleotides are listed in **Table 1**. All SYBR and probe-based qPCR experiments were done in simplex. The relative mRNA levels were determined as described by Schmittgen and Livak (29). For the developmental expression studies, target-gene expression was normalized to the reference gene *rpL8*, which did not show significant variation across development [*rpL8* mRNA values are given in **Supplementary Table 5**; see also (8)]. In the *in vivo* and the tissue explant T_3 response experiments, *E. coqui* target gene mRNA levels were normalized to the reference genes *rpL8* and *actn4*, which was unaffected by T_3 treatment. Small, statistically insignificant changes in reference gene mRNAs could have led to a small underestimation of the effect of T_3 in these experiments.

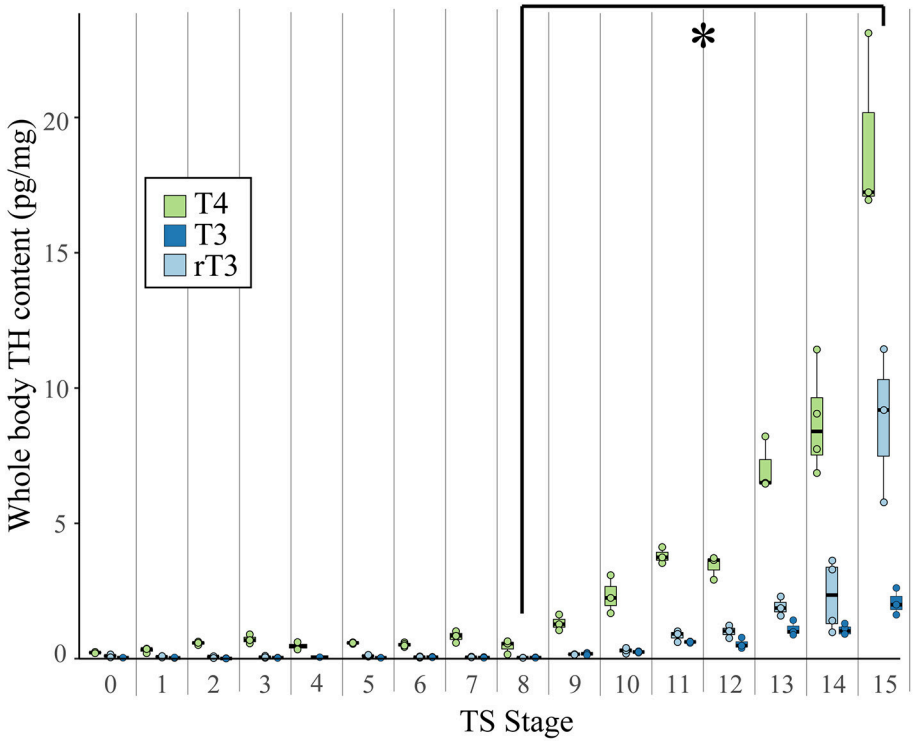

FIGURE 2 | Whole body content of T_4, T_3, and rT_3 in pooled *E. coqui* embryos at TS stages 1–15 and in unfertilized oocytes (TS 0) as quantified by LC-MS/MS. Whole body content of iodothyronines was normalized to sample weight; between 15 and 20 embryos were pooled to generate one biological replicate. Each value depicted in the graph is based on two-to-four replicates. Values based on fewer than three replicates are not included in the statistical analysis. All three iodothyronines increased significantly between TS stages 8 and 15 (*post-hoc* Dunn's test; $p < 0.05$), indicated by the asterisk (*). See **Supplementary Data** for a complete list of significant pairwise differences. Each boxplot represents median and range of the data.

For *Xenopus tropicalis*, qPCR primers and probes for *thrb*, *klf9*, *thibz*, *dio3*, *elongation factor 1 alpha* (*eef1a1*) and *alpha-actinin 1* (*actn1*) were designed from publicly available sequences (Genbank accession numbers XM_012964865.2, NM_001113674.1, XM_018092557.1, NM_001113667.2, NM_001016692.2, and NM_001079198.1). For tissue explant experiments, *X. tropicalis* target gene expression was normalized to *eef1a1* and *actn1*.

Treatment of *E. coqui in vivo*

Eleutherodactylus coqui embryos were dechorionated into 10% Holtfreter solution at least 24 h prior to immersion in T_3. One mM stock T_3 in DMSO or 0.01 N NaOH was diluted to make 50 nM T_3 in 10% Holtfreter solution. We chose 50 nM T_3 because it has been shown to induce tail resorption in *E. coqui* (8), and a 46-h timepoint to allow enough time for induction of T_3 response genes. We chose TS stage 9 embryos because the last third of limb development is TH-dependent (8), but TS stage 9 is still prior to thyroid gland activation. T_3 treatment solutions were refreshed every 8–12 h. After 46 h (n = 12–14 TS-9 embryos), dechorionated embryos were anesthetized as described above and limbs and tails were dissected, from which total RNA was extracted using TriZol reagent.

Measurement of Environmental T_3 Uptake in *X. tropicalis* and *E. coqui*

To determine if *E. coqui* embryos are capable of taking up TH from their surrounding environment, we immersed dechorionated TS stage 9 *E. coqui* embryos or NF 51–55 *X. tropicalis* tadpoles in 30 mL (*E. coqui*) or at least 500 mL (*X. tropicalis*) 10% Holtfreter solution with either 1 nM (n = 4–6 biological replicates/treatment) or 50 nM (n = 3–4 biological replicates/treatment) stable isotope-labeled T_3. We chose TS stage 9 *E. coqui* embryos to match the *in vivo* T_3 treatment experiments and selected *X. tropicalis* tadpoles with developing limbs with similar morphology to *E. coqui* TS stage 9. Approximately twenty *E. coqui* individuals (600 mg tissue) or two tadpoles were pooled were pooled to make one biological replicate. Tadpoles were either ordered from Xenopus1 (Ann Arbor, Michigan, U.S.A.) or derived from the Hanken lab colony. Stock 100 μg/mL stable isotope-labeled T_3 was diluted to either 1 or 50 nM T_3. After either 8 or 24 h in 1 nM labeled T_3 solution or 46 h in 50 nM T_3 solution, *X. tropicalis* tadpoles and *E. coqui* embryos (with yolk removed) were anesthetized with neutral-buffered 2% MS-222, rinsed three times in PBS and snap frozen until extraction. On average, *E. coqui* embryos were more densely packed in T_3 solution (5.9 mg tissue per mL media) than *X. tropicalis* tadpoles (2.0 mg tissue per mL media); however, *E. coqui* embryos are routinely cultured in these conditions with no ill effects. Tissue was extracted as described above. Because we measured whole body content of stable isotope-labeled T_3 as a proxy for T_3 uptake, we used 25 ng of stable isotope-labeled rT_3 as an internal standard to correct for extraction efficiency.

Tissue Explant Culture and T_3 Treatments

To further investigate if thyroid axis components in the *E. coqui* limb and tail are functional, we cultured *E. coqui* and *X. tropicalis* limb and tail explants (30, 31), treated them with T_3, and assayed gene expression. We treated NF stage 52–54 (32) *X. tropicalis* tadpoles and TS stage 9 *E. coqui* embryos with 50 U/mL of penicillin-streptomycin added to aquarium or Petri dish solution for 24 h prior to dissection. Tadpoles and embryos were terminally anesthetized and dipped into 70% ethanol to sterilize the epidermis before dissection. Four *X. tropicalis* and two *E. coqui* individuals were pooled to make a single biological replicate of each species. Tissues were dissected into ice-cold 1:1.5-diluted Leibowitz-15 media (Gibco) containing 50 U/mL penicillin-streptomycin, 50 mg/mL gentamicin and 10 mM HEPES. Prior to T_3 treatment tissues were cultured overnight in media supplemented with insulin (500 ug/mL) on a laboratory bench at room temperature (21°C) with gentle shaking (50 rpm). The next morning, stock T_3 was diluted in 0.01 N NaOH and added to the media to a final concentration of 50 nM. Media and T_3 were changed every 8–12 h. After treatment for 8 or 46 h, limb and tail explants were rinsed three times in phosphate-buffered saline (PBS) and homogenized in TriZol. RNA was isolated according to the manufacturer's protocol.

Statistical Analysis

Statistical analyses of qPCR data were done with RStudio version 1.0.136 and visualized with ggplot2 (https://ggplot2.tidyverse.org/). Developmental timeline qPCR and iodothyronine content data followed a non-normal distribution as determined by Q-Q plots and the Shapiro-Wilk test; Levene's test determined that TH content data additionally had unequal variance. Log_{10}-transformed data were not normally distributed. Therefore, a Kruskal-Wallis test was used to determine if there were significant differences among groups, and a *post-hoc* Dunn's test with the Benjamini and Hochberg (BH) correction was used to identify stages that differ from each other while adjusting for multiple comparisons. We performed a least squares regression on T_4, T_3, and rT_3 data sets to investigate possible differences in iodothyronines kinetics during development. For the developmental timeline qPCR data, statistical tests were performed on data pooled from two independent experiments (see **Supplementary Data** for data from each experiment). For *in vivo* and *in vitro* T_3 treatment experiments, Student's *t*-test was used to identify significant differences between T_3-treated groups and controls.

RESULTS

Predicted Proteins of Isolated *E. coqui* cDNAs Contain Conserved Domains

Most isolated cDNAs contained functional domains of orthologous proteins. The predicted *E. coqui* TRα and TRβ sequences cover amino acids 11–281 (65%), and amino acids 9–273 (69%) of the orthologous *X. tropicalis* proteins, respectively. Both predicted TR protein sequences contain the DNA-binding domain and most of the ligand-binding domain. Alignments show that the predicted protein sequence of the *E. coqui* TRα DNA-binding domain has 97% identity to the *X. tropicalis* DNA-binding domain, while the TRα ligand-binding domain shared between the predicted *E. coqui* and *X. tropicalis* sequences

are 98% identical. The DNA-binding domain of the predicted *E. coqui* TRβ sequence is 100% identical to the DNA-binding domain in *X. tropicalis* TRβ, and the ligand-binding domain is 95% identical. The predicted partial *E. coqui* Dio2 sequence covers amino acids 2–254 (98%) of *X. tropicalis* Dio2 and the partial *E. coqui* Dio3 sequence covers amino acids 7–252 (90%) of *X. tropicalis* Dio3. Additionally, the predicted protein sequence of both *dio2* and *dio3* isolated cDNAs contain the selenocysteine site and the thioredoxin domain. Both thioredoxin domains share 86% identity with the orthologous *X. tropicalis* thioredoxin domain. The partial predicted amino acid sequence of *E. coqui Klf9* covers amino acids 194–264 (25%) of *X. tropicalis Klf9* and contains the three characteristic zinc-finger domains (100% identity) in the C-terminus of *X. tropicalis Klf9*. The isolated *E. coqui thibz* sequence covers amino acids 159–335 (53%) of *X. tropicalis* NFIL3-like (synonym for *thbzip*) and lacks the highly conserved basic leucine zipper domain. Even without the highly conserved basic leucine zipper domain, the predicted *E. coqui* protein sequence still clusters with other orthologous NFIL3-like proteins, rather than with other proteins with the basic leucine zipper domain (NFIL3 and CREB1) in maximum likelihood trees of these three orthologous vertebrate proteins (data not shown). Similarly, the other partial predicted *E. coqui* sequences cluster with other orthologous genes rather than with other closely related proteins containing similar domains (data not shown). We confirmed all isolated *E. coqui* cDNAs against the full-length transcript provided by investigators listed in the methods. Finally, we also performed BLASTx and BLASTn searches with the isolated *E. coqui* cDNA sequences. All cloned sequences have high similarity to predicted orthologous genes in frog species and other vertebrates (**Supplementary Table 4**).

Changes in Whole Body Iodothyronine Content During Embryonic *E. coqui* Development

Using LC-MS/MS, we detected the iodothyronines T_4, T_3, and rT_3 in unfertilized oocytes and at every stage of development (**Figure 2**). Thyroxine content (pg/mg body weight) was highest, followed by rT_3 and then T_3. We detected T_2 only at TS stages 14 and 15, when hatching occurs, and at this point, T_2 content was less than all other iodothyronine content and ranged between 0.04 and 0.78 pg/mg body weight (**Supplementary Table 3**). The three quantifiable iodothyronines were low and relatively constant up to TS stage 8, after which stage they showed statistically significant increases [T_3: Kruskal-Wallis rank sum test, $X^2 = 43.2$ (df = 15), $p < 0.001$; T_4: Kruskal-Wallis rank sum test, $X^2 = 43.7$ (df = 15), $p < 0.001$; rT_3: Kruskal-Wallis rank sum test, $X^2 = 39.7$ (df = 14), $p < 0.001$]. Whole body content of all three iodothyronines showed statistically significant increases between stages 8 and 13 (*post-hoc* Dunn's test; $p = 0.048$, 0.033, 0.035 for T_3, T_4, and rT_3, respectively). The velocity of change was slower for rT_3 and T_3 compared with T_4. Stage was a significant predictor for all three iodothyronines [T_3: $F = 70.8$ (df = 45), $p < 0.001$; T_4: $F = 54.2$ (df = 46) $p < 0.001$; rT_3: $F = 23.7$ (df = 43), $p < 0.001$]. Although all iodothyronines are positively correlated with stage, the velocity of change was slower for rT_3 and T_3 (slope of least squares regression (LSR) line, b = 0.2964 and 0.1009, respectively) compared to T_4 (LSR, b = 0.7984). Tissue content of all three iodothyronines was highest at TS 15. Note also that oocytes and early embryos (TS 0–5) of *E. coqui* have large yolk deposits, which may increase the S/N ratio and cause an underestimation of iodothyronine content in the embryo and yolk at these stages.

Changes in Thyroid Hormone Receptor and Deiodinase mRNA Levels in the Embryonic Tail

Both *thra* and *thrb* mRNAs in the *E. coqui* tail showed statistically significant changes during development [**Figure 3A**; *thra*: Kruskal-Wallis rank sum test, $X^2 = 20.18$ (df = 4), $p < 0.001$; *thrb*: Kruskal-Wallis rank sum test, $X^2 = 26.78$ (df = 4), $p < 0.001$]. *Thyroid hormone receptor α* and *thrb* mRNA in the tail bud are approximately equal at TS stage 5 (**Figure 3A**). *Thyroid hormone receptor α* mRNA in the tail at hatching is between 2.1- and 4-fold higher than the early tail (TS stages 5 and 7, *post-hoc* Dunn's test, $p = 0.002$ and 0.03, respectively). *Thyroid hormone receptor β* mRNA follows a similar pattern—it increased 4-fold between the onset of tail resorption (TS 13) and hatching (TS 15)—although *thra* increased only 1.8-fold over the same interval (**Figure 3A**). *Thyroid hormone receptor β* mRNA at hatching (TS 15) is between 18- and 47-fold higher than in the early tail (TS stage 5 and TS stage 7, *post-hoc* Dunn's test, $p < 0.001$ and $p = 0.002$, respectively).

Deiodinase type II and *dio3* mRNAs significantly changed during tail development (**Figure 3B**; *dio2*: Kruskal-Wallis rank sum test, $X^2 = 17.37$ (df = 4), $p = 0.002$; *dio3*: Kruskal-Wallis rank sum test, $X^2 = 26.11$ (df = 4), $p < 0.001$). Patterns of deiodinase mRNA in the developing tail were essentially the opposite of those seen in the limb. *Deiodinase type II* mRNA was low throughout tail development and resorption but rose almost 10-fold as hatching neared (TS 15; **Figure 3B**). At hatching (TS 15), *dio2* mRNA was higher than at TS 5, 7 and 13 (*post-hoc* Dunn's test, $p = 0.001$, 0.029, and 0.031, respectively). *Deiodinase type III* mRNA increased 27-fold between TS 5 and 13 (*post-hoc* Dunn's test, $p < 0.001$) and then decreased steeply (11-fold) between the onset of tail resorption and hatching (*post-hoc* Dunn's test, $p = 0.007$). Repeated experiments demonstrate the similar patterns of *thra*, *thrb*, *dio2*, and *dio3* expression (**Supplementary Figure 1**).

Changes in Thyroid Hormone Receptor and Deiodinase mRNA Levels in the Embryonic Hind Limb

Both *thra* and *thrb* mRNAs in the *E. coqui* hind limb showed statistically significant changes during development (**Figure 4A**; *thra*: Kruskal-Wallis rank sum test, $X^2 = 20.66$ (df = 4), $p < 0.001$; *thrb*: Kruskal-Wallis rank sum test, $X^2 = 25.36$ (df = 4), $p < 0.001$). The level of *thra* mRNA was greater than *thrb* mRNA in the limb bud until TS 10, when the *thra* mRNA level began to decrease and continued to decline through hatching (**Figure 4A**). The peak *thra* mRNA level at TS 10 coincides with the appearance of thyroid follicles (13); *thra* mRNA in the hind

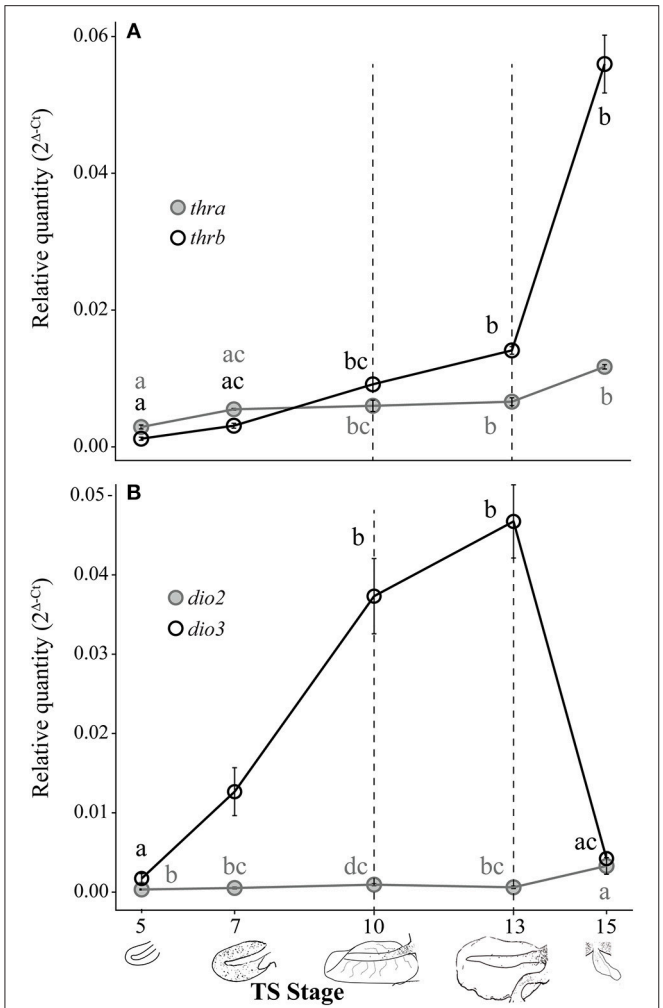

FIGURE 3 | Relative *thra* and *thrb* mRNA levels **(A)** and *dio2* and *dio3* mRNA levels **(B)** in the pre-hatching tail of *E. coqui*. Dashed vertical line at TS stage 10 marks when thyroid follicles are first visible in histological sections; the line at TS 13 indicates the onset of tail resorption. Drawings on the x-axis depict tail growth and resorption before hatching. Each expression value is represented as a circle centered on the mean of 3–7 individuals ± SE. Lower-case letters in gray (*thra* and *dio2*) and black (*thrb* and *dio3*) indicate significant pairwise differences between groups (*post-hoc* Dunn's test, $p < 0.05$). See **Supplementary Data** for a complete list of pairwise comparisons.

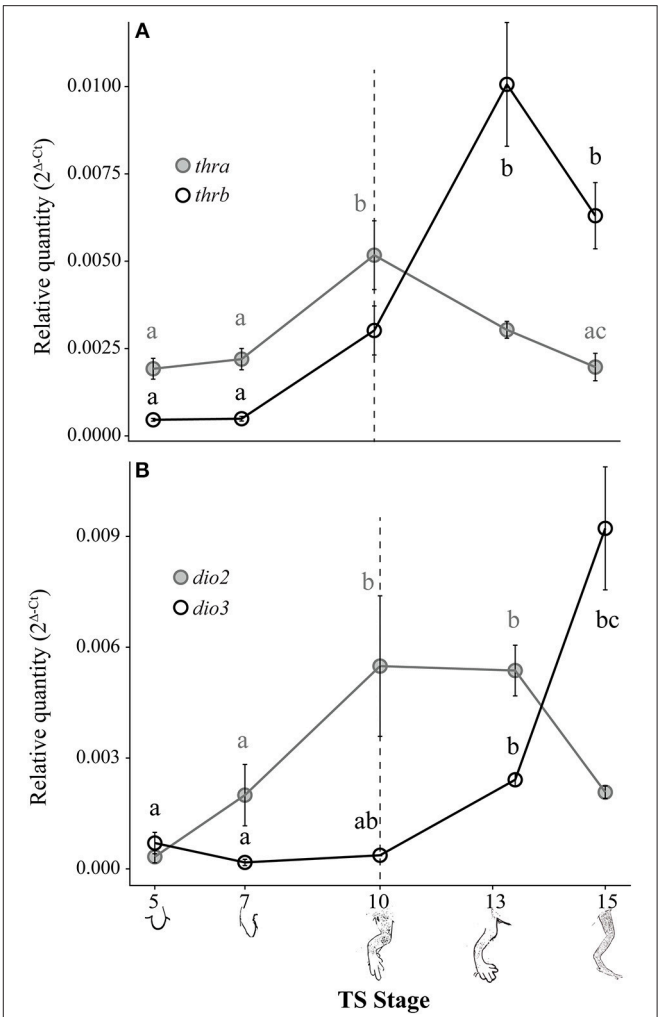

FIGURE 4 | Relative *thra* and *thrb* mRNA levels **(A)** and *dio2* and *dio3* mRNA levels **(B)** in the pre-hatching hind limb of *E. coqui*. Dashed vertical line at TS stage 10 marks when thyroid follicles are first visible in histological sections. Drawings on the x-axis depict sequential formation of the limb. Each expression value is represented as a circle centered on the mean of 5–7 hind limb pairs ± SE. Lower-case letters in gray (*thra* and *dio2*) and black (*thrb* and *dio3*) indicate significant pairwise differences between groups (*post-hoc* Dunn's test, $p < 0.05$). See **Supplementary Data** for a complete list of pairwise comparisons.

limb at this stage was significantly higher than in the limb bud at TS 5 (*post-hoc* Dunn's test, $p = 0.001$), in the limb paddle at TS stage 7 ($p = 0.009$) and in the fully formed froglet limb at TS 15 (*post-hoc* Dunn's test, $p = 0.002$). At hatching, *thra* mRNA level was lower than *thrb* mRNA levels. Between paddle (TS 7) and toepad formation (TS 13), *thrb* mRNA rose ∼21-fold to a peak at TS 13. At TS 13, *thrb* expression was significantly higher than in the limb bud and paddle (**Figure 4A**; TS 5 and 7; *post-hoc* Dunn's test, $p < 0.001$ and p = 0.001, respectively). *Thyroid hormone receptor β* mRNA drops almost 1.5-fold between TS 13 and hatching.

Deiodinase type II and *dio3* mRNAs both showed statistically significant but contrasting patterns throughout limb development [**Figure 4B**; *dio2*: Kruskal-Wallis rank sum test, $X^2 = 18.65$ (df = 4), $p < 0.001$; *dio3*: Kruskal-Wallis rank sum test, $X^2 = 25.76$ (df = 4), $p < 0.001$]. *Deiodinase type II* mRNA increased 16-fold between limb bud (TS 5) and digit formation (TS 10) and remained at this level through subsequent limb growth (TS 13; *post-hoc* Dunn's test, $p = 0.007$ and $p < 0.001$, respectively). *Deiodinase type II* mRNA decreased 2.6-fold between TS 13 and hatching to the level originally present in the newly formed limb bud (e.g., TS 5). *Deiodinase type III* mRNA remained low throughout most of limb development, but it increased 25-fold between the initial formation of thyroid follicles (TS 10) and hatching (TS 15; *post-hoc* Dunn's test, $p = 0.001$). Repeated experiments show the same general

contrasting mRNA expression patterns for *dio2*, *dio3*, *thra*, and *thrb* (**Supplementary Figure 2**).

Exogenous T_3 Induced Gene Expression Responses in the TS 9 *E. coqui* Tail, but Not the Limb

To determine if *E. coqui* tissues are capable of mounting a gene regulation response to exogenous T_3, we performed *in vivo* T_3 treatments (**Figure 5A**). Immersion of TS 9 *E. coqui* embryos in 50 nM T_3 for 8 h caused a significant induction of *klf9* (Student's *t*-test, $t = 5.61$ (df = 21.74), $p < 0.001$) and *thibz* (Student's *t*-test, $t = 6.20$ (df = 12.42), $p < 0.001$) in the tail. Immersion in 50 nM T_3 for 46 h additionally significantly induced *thrb* mRNA (**Supplementary Figure 4**). In contrast, the identical treatment significantly increased only *thibz* expression (**Figure 5B**; Student's *t*-test, $t = 3.11$ (df = 18.92), $p = 0.006$) in the limb.

E. coqui Embryos Took up Significantly Less T_3 From the Environment Than Did *X. tropicalis* Tadpoles

Because previous studies suggested that *E. coqui* limbs are insensitive to TH, and because we observed a weak TH response in our *in vivo* experiments, we wanted to confirm that immersion in T_3 increased tissue content of T_3. We quantified stable isotope-labeled T_3 tissue content after immersing *X. tropicalis* tadpoles or *E. coqui* embryos in stable isotope-labeled T_3 solution under three conditions. We chose 50 nM T_3 and 46 h treatment to match the *E. coqui in vivo* T_3 response experiments. We also chose two conditions that represent relevant time points from previous studies of larval *Xenopus* species: (1) treatment with 1 nM T_3 for 8 h is sufficient for *X. tropicalis*' whole body T_3 content to surpass the T_3 concentration in the surrounding media (33), and (2) treatment with 1 nM T_3 for 24 h is sufficient to induce gene expression responses in *X. tropicalis* (31, 34). After immersing *E. coqui* in 1 nM labeled T_3 for 8 and 24 h, we detected endogenous T_3 but not labeled T_3. However, we detected labeled T_3 in *X. tropicalis* tissue at both 8 and 24 h (**Table 2**). We detected stable isotope-labeled T_3 in both *E. coqui* and *X. tropicalis* tissue following 46-h treatment with 50 nM T_3. Total content of labeled T_3 in *X. tropicalis* tissue was ∼63 times that found in *E. coqui* tissues [**Table 2**, Student's *t*-test, $t = -3.20$ (df = 2.00), $p = 0.085$]. Additionally, *X. tropicalis* has ∼ 875 times more stable isotope-labeled T_3 than endogenous T_3 content. In contrast, stable isotope-labeled T_3 in *E. coqui* is approximately equal to endogenous T_3 content.

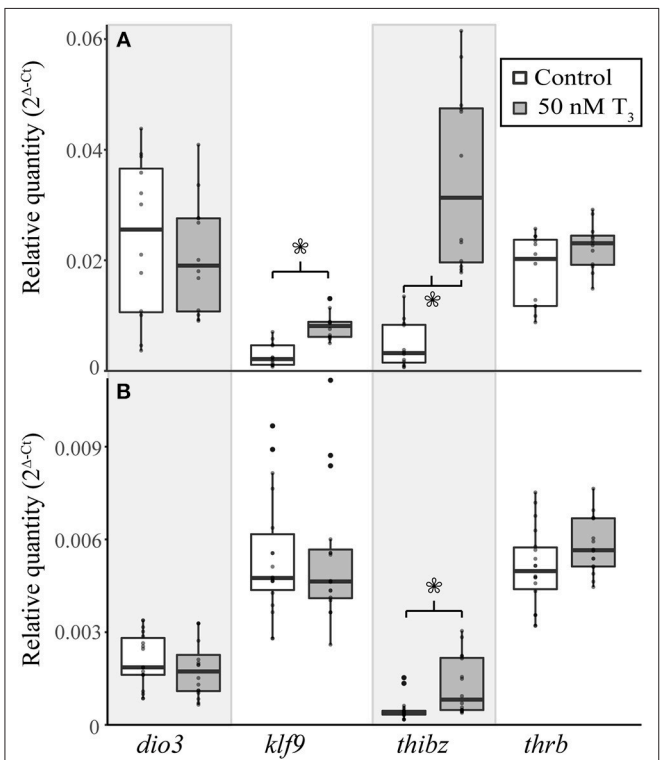

FIGURE 5 | Exogenous treatment with 50 nM T_3 for 8 h induces gene expression in the tail of *E. coqui* embryos at TS stage 9 **(A)**, but not in limbs at the same stage **(B)**. Boxes and whiskers depict the median and range of 12–16 individuals from two independent experiments. Asterisks indicate a significant change in expression (Student's *t*-test, $p < 0.05$).

Exogenous T_3 Strongly Induced T_3 Response Genes in TS Stage 9 *E. coqui* Limb Explants

Treatment with 50 nM T_3 for 8 h significantly increased *dio3* [Student's *t*-test, $t = 8.40$ (df = 4.00), $p = 0.001$), *klf9* (Student's *t*-test, $t = 14.41$ (df = 4.18), $p < 0.001$], *thibz* [Student's *t*-test, $t = 9.64$ (df = 4.01), $p < 0.001$], and *thrb* [Student's *t*-test, $t = 8.26$ (df = 4.39), $p < 0.001$] mRNAs in explants of *X. tropicalis* tail (**Figure 6A**). The same treatment caused a significant increase in *dio3* [Student's *t*-test, $t = 3.49$ (df = 3.00), $p = 0.040$], *klf9* [Student's *t*-test, $t = 13.66$ (df = 3.08), $p < 0.001$], and *thibz* [Student's *t*-test, $t = 21.50$ (df = 2.07), $p = 0.002$] mRNAs in *X. tropicalis* limb explants (**Figure 6B**). Similarly, exogenous T_3 increased *dio3* [Student's *t*-test, $t = 2.56$

TABLE 2 | Nieuwkoop and Faber stage 51–55 *Xenopus tropicalis* tadpoles have more labeled T_3 tissue content than do TS stage 9 *E. coqui* embryos after immersion in labeled T_3 for 8, 24, or 46 h.

Species	Labeled T_3 concentration (nM)	Timepoint (h)	Labeled T_3 pg/mg	T_3 pg/mg
X. tropicalis	1	8	1.079 ± 0.19	0.095 ± 0.02
E. coqui	1	8	0.000 ± 0.00	0.236 ± 0.01
X. tropicalis	1	24	1.371 ± 0.08	0.018 ± 0.01
E. coqui	1	24	0.000 ± 0.00	0.245 ± 0.04
X. tropicalis	50	46	30.436 ± 9.37	0.035 ± 0.01
E. coqui	50	46	0.483 ± 0.27	0.447 ± 0.20

Each value represents the mean of 3–6 individuals ± standard error.

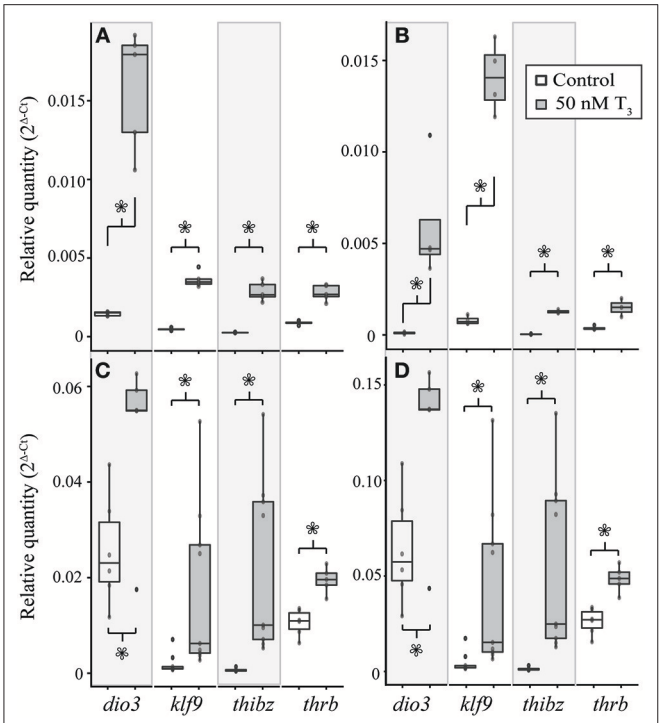

FIGURE 6 | Treatment with 50 nM exogenous T_3 for 8 h induces *deiodinase type III* (*dio3*), *krüppel-like factor 9* (*klf9*), *thyroid hormone induced bZip protein* (*thibz*), and *thrb* expression in NF stage 52–54 *X. tropicalis* tail **(A)** and limb **(B)** explants and in TS stage 9 *E. coqui* tail **(C)** and limb explants **(D)**. Asterisks indicate a significant increase in mRNA levels (Student's *t*-test, $p < 0.05$).

(df = 6.47), $p = 0.040$], *klf9* [Student's *t*-test, $t = 2.67$ (df = 8.22), $p = 0.028$], *thibz* [Student's *t*-test, $t = 3.54$ (df = 8.00), $p = 0.008$], and *thrb* [Student's *t*-test, $t = 5.38$ (df = 8.62), $p < 0.001$] mRNAs in explants of *E. coqui* tail (**Figure 6C**). *Deiodinase type III* [Student's *t*-test, $t = 2.61$ (df = 9.80), $p = 0.027$], *klf9* [Student's *t*-test, $t = 6.11$ (df = 8.05), $p < 0.001$], *thibz* [Student's *t*-test, $t = 6.49$ (df = 8.00), $p < 0.001$], and *thrb* [Student's *t*-test, $t = 7.70$ (df = 8.80), $p < 0.001$] increased after the same treatment in *E. coqui* limb explants (**Figure 6D**).

In both species, the magnitude of increase for all genes was greater in the limb than in the tail (**Table 3**). The same trends were observed after treating tissue explants with 50 nM T_3 for 46 h (**Supplementary Figure 5**). In tail explants, T_3 induced fold changes of a similar order of magnitude for *thibz* (between 42- and 44-fold) and *thrb* (between 1.8- and 3.8-fold), but not for *dio3* and *klf9*; in *E. coqui*, *dio3*, and *klf9* mRNAs increased 2- and 9.9-fold, respectively, while *dio3* and *klf9* mRNAs increased 11.9- and 12.5-fold in *X. tropicalis*. In limb explants, *dio3* and *thibz* mRNA differed by an order of magnitude between species. *Deiodinase type III* mRNA increased 58.8-fold in *X. tropicalis* limb explants, while *dio3* mRNA increased 3.7-fold in *E. coqui* limb tissue. *Thyroid hormone induced bZip protein* mRNA increased only 37-fold in *X. tropicalis* limb explants, while *dio3* mRNA increased 180-fold in *E. coqui* limb explants.

TABLE 3 | Induction of *deiodinase type III* (*dio3*), *krüppel-like factor 9* (*klf9*), *thyroid hormone induced bZip protein* (*thibz*), and *thyroid hormone receptor β* (*thrb*) in tail and limb explants of NF stages 52–54 *Xenopus tropicalis* and TS stage 9 *Eleutherodactylus coqui* after treatment with 50 nM T_3 for 8 h.

		Average fold increase	
Species	**Gene**	**Tail**	**Limb**
X. tropicalis	*dio3*	11.9	58.8
	klf9	12.5	17.8
	thibz	43.8	37.1
	thrb	3.8	4.0
E. coqui	*dio3*	2.0	3.7
	klf9	9.9	21.3
	thibz	42.0	180.0
	thrb	1.8	3.0

Values represent the average fold increase above control (vehicle-treated) levels.

DISCUSSION

In this study we show that the core TH signaling components are evolutionarily conserved in *Eleutherodactylus coqui* limb and tail tissue. We also show that developmental patterns of *thra*, *thrb*, *dio2,* and *dio3* mRNAs, and whole-body TH content in *E. coqui* closely match those reported during metamorphosis of *Xenopus* species. We also find maternal T_4, T_3, and rT_3 in unfertilized eggs and early embryos of *E. coqui*, which may mediate TR signaling prior to embryonic thyroid gland formation. This is the first published report of TH metabolites and maternally derived TH in a direct-developing frog. Additionally, we demonstrate that *E. coqui* tissues show robust gene expression responses to exogenous T_3 similar to those seen in metamorphosing species. *Eleutherodactylus coqui* embryos take up much less T_3 from the environment compared with *X. tropicalis*. This difference likely explains the relatively weak and variable gene expression responses seen *in vivo* in *E. coqui*, and was likely a significant confounding factor for previously published results.

Developmental Profiles of Whole Body Iodothyronine Content

Temporal dynamics of whole-body iodothyronine content in direct-developing *E. coqui* mirror those described for indirect-developing frogs, which retain the ancestral biphasic life history: *Scaphiopus hammondii* (28), *Rana catesbeiana* (35), *Bufo marinus* (36), *Bufo japonicus* (37), and *Xenopus laevis* (33). Anuran metamorphosis comprises three successive stages: premetamorphosis, when little to no TH is present; prometamorphosis, when TH concentrations slowly rise; and a rapid metamorphic climax characterized by a peak in TH concentrations. The temporal profile of TH content in embryonic *E. coqui* similarly defines three successive periods: (1) Low TH content characterizes the first half of development, prior to thyroid follicle formation (TS 1–8). (2) After thyroid follicles appear, TH content gradually rises until tail resorption began (TS 9–12). (3) TH content dramatically increases, with a peak in TH at or just prior to hatching (TS 13–15). In addition to amphibians,

many other vertebrates experience peak concentrations of TH at life history transitions—at hatching in precocial birds (38), at the larval-to-juvenile transition in several fish species (39–41), at ∼14 days post-partum in rats and mice (42, 43), and at birth in humans (44).

Thyroid hormones are present throughout early embryogenesis and the subsequent period of pre-hatching development in *E. coqui* (TS 1–9), beginning up to eight days before thyroid follicles can be detected histologically (13). These hormones are almost certainly maternal in origin. Similarly, T_4 and T_3 have been detected in yolk and gastrulating embryos of four other anuran species—*Bufo marinus* (36), *Rana catesbeiana* (35), *Bombina orientalis* (45), and *Xenopus laevis* (16). Early *Xenopus tropicalis* embryos express key TH signaling components (46). Indeed, TH signaling is also functional in the *Xenopus* tadpole central nervous system (CNS) before thyroid gland formation (16, 18). Maternally derived TH has a conserved role in vertebrate CNS development (47) and embryogenesis (17, 48, 49). Therefore, it seems likely that direct-developing frogs require maternal TH for normal neural development, as do most vertebrate species, although we do not evaluate that hypothesis here.

Maternal TH may regulate limb development occurring before the differentiation of the embryonic thyroid gland in direct-developing frogs. In metamorphosing anurans, TH signaling is required for terminal limb differentiation (22), but the initial stages of limb development are TH-independent. For example, tadpoles immersed in methimazole, a TH-synthesis inhibitor, develop a long limb-bud-like structure (24), and thyroidectomized tadpoles develop calcification centers in the hind limb (50, 51). In *E. coqui*, the limb bud proliferates and digits develop prior to the appearance of embryonic thyroid follicles (TS stages 9–10) [**Figure 3**; (8, 13)]. Two hypotheses could account for this observation: (1) *E. coqui* relies on maternal TH, rather than embryonically produced TH, to regulate early stages of digit patterning and growth (TS 6–9); or (2) paddle and digit formation in *E. coqui* proceed independently of TH. Our data show that requisite components of TH signaling are present at this time. Future investigation should evaluate the functional role of TH during this critical developmental period. A switch from embryonic to maternally synthesized TH for the regulation of early limb development, if it occurred, could explain the heterochronic shift in limb development and would represent an evolutionary novelty in direct-developing species.

Thyroid Hormone Receptor α, thrb, Dio2, and Dio3 mRNA Expression Patterns During Development and T3 Response in the Embryonic Tail

Tail resorption in *Xenopus tropicalis* occurs late in metamorphosis and is mediated by TRβ (52). Because tail resorption in *E. coqui* occurs late in embryogenesis and requires T_3 (8), we expected that *thra, thrb, dio2,* and *dio3* mRNA dynamics in the *E. coqui* tail would mirror those described in *Xenopus*. Our results support this hypothesis: in the *E. coqui* tail, a rise in *thrb* expression coincides with the rise in embryonic TH content, consistent with a role for *thrb* in mediating tail resorption.

Deiodinase type II and *dio3* mRNA expression patterns in the developing *E. coqui* tail are also similar to those described in indirect-developing species in which these deiodinase enzymes are critical for coordinating metamorphosis (20). Elevated *dio3* expression protects the tail from an early apoptotic response to T_3 until metamorphic climax in *Xenopus* (26); *E. coqui* tail resorption also begins at TS 13, when *dio3* expression significantly decreases. Although they serve different functions, the tail serves a critical role in both species: the larval *Xenopus* tail is a critical locomotor organ, whereas the embryonic *E. coqui* tail functions in respiration. In both species, maintenance of the tail is accomplished in part by *dio3* inactivation of T_4 and T_3.

Given the conservation of mRNA dynamics in the *E. coqui* tail, we wanted to determine whether the tissue could respond to exogenous T_3. In *Xenopus* species, treatment with exogenous T_3 induces transcription of direct T_3 response genes *dio3*, *klf9*, *thibz*, and *thrb* (19, 53–56). Exogenous T_3 induces significant increases in the mRNA of three of these T_3 response genes, *klf9*, *thibz*, and *thrb*, supporting the hypothesis that TH signaling components are conserved and mediate tail resorption in *E. coqui*.

Thyroid Hormone Receptor α, thrb, Dio2, and Dio3 mRNA Expression Patterns During Development and T_3 Response in the Embryonic Hind Limb

Thyroid hormone receptor α, *thrb, dio2*, and *dio3* mRNA expression patterns parallel those described in *Xenopus* species in the period leading up to and during metamorphosis (33, 57). In indirect-developing frogs, TRα has a critical role in controlling post-embryonic developmental timing (58–60) and in promoting proliferation in the hind limb during metamorphosis (61–63). Constitutive *thra* expression supports a proliferative and competence-establishing role for TRα in *E. coqui*. In the *E. coqui* limb, a rise in *thrb* expression coincides with the rise in embryonic TH content, consistent with TRβ autoinduction and tissue sensitization to TH described in *Xenopus* (64).

The tissue-specific patterns of *dio2* and *dio3* underlie the differential sensitivity of limb and tail tissue in metamorphosing frogs. *Deiodinase type II* expression is constitutive in the developing limb of *Xenopus laevis*, causing the limb to be sensitive to small amounts of T_3 produced during premetamorphosis (23). Similarly, elevated *dio2* expression in *E. coqui* limbs throughout most of limb development, including several days prior to formation of the embryonic thyroid gland, supports a role for TH-mediated limb development and growth.

In indirect-developing species, including *Xenopus* and spadefoot toads (*Scaphiopus*), concentrations between 1 and 10 nM T_3 are sufficient to promote precocious metamorphosis, tail resorption, and gene expression responses in limbs and tail (31, 65, 66). However, previous studies report that the

E. coqui limb has no morphological response to high doses of exogenous T_3 (11). Our study is the first to characterize mRNA expression changes in a direct-developing frog species in response to exogenous T_3. Treatment of *E. coqui* embryos with exogenous T_3 prior to formation of the thyroid follicles increases expression of four direct T_3 response genes in the tail, consistent with studies in *Xenopus* species (19, 53–55). However, limbs of the same embryos do not respond to T_3, despite the high dose administered (50 nM T_3). The lack of response previously observed in direct-developing species may be confounded by an inability of T_3 to reach the limb tissue. We observe a weak induction of T_3 response genes in TS stage 7 limbs, a full two days before *E. coqui* begins to produce TH (**Supplementary Figure 3**). It is possible that this response occurs because the adult epidermis is not yet fully formed and T_3 is better able to penetrate into the tissue, or because there is less endogenous T_3 present at TS 7 than at TS 9. In either case, the ability to respond to T_3 prior to thyroid gland formation is similar to biphasic species; tadpoles are also TH competent as soon as they hatch. Finally, the similar robust gene regulation response induced in *E. coqui* and *X. tropicalis* limb explants suggests that the limb tissue itself is similarly competent in both species. Overall, these data support the hypothesis that TH plays a role in *E. coqui* limb development and may do so prior to formation of the embryonic thyroid gland.

Here we support previous claims that later stages of limb development in *E. coqui* are TH-dependent but we additionally show that TH-signaling components are present during earlier stages, and that *E. coqui* limb tissue is sensitive to T_3. *Eleutherodactylus coqui* eggs are provisioned with maternally derived TH, which may mediate organogenesis before differentiation and activity of the embryo's own thyroid gland. Altogether, our data suggest that the TH-mediated molecular module active during post-hatching metamorphosis in indirect-developing frogs has been shifted prior to hatching in direct-developing species.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Harvard Faculty of Arts and Sciences Institutional Animal Care and Use Committee. The protocol was approved by the Harvard Faculty of Arts and Sciences Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

ML designed experiments and performed experiments, interpreted data, and wrote the manuscript. RD and JH contributed to experimental design, edited the manuscript, and discussed data interpretation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00307/full#supplementary-material

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REVIEW
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Teleost Metamorphosis: The Role of Thyroid Hormone

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In most teleosts, metamorphosis encompasses a dramatic post-natal developmental process where the free-swimming larvae undergo a series of morphological, cellular and physiological changes that enable the larvae to become a fully formed, albeit sexually immature, juvenile fish. In all teleosts studied to date thyroid hormones (TH) drive metamorphosis, being the necessary and sufficient factors behind this developmental transition. During metamorphosis, negative regulation of thyrotropin by thyroxine (T4) is relaxed allowing higher whole-body levels of T4 that enable specific responses at the tissue/cellular level. Higher local thyroid cellular signaling leads to cell-specific responses that bring about localized developmental events. TH orchestrate in a spatial-temporal manner all local developmental changes so that in the end a fully functional organism arises. In bilateral teleost species, the most evident metamorphic morphological change underlies a transition to a more streamlined body. In the pleuronectiform lineage (flatfishes), these metamorphic morphological changes are more dramatic. The most evident is the migration of one eye to the opposite side of the head and the symmetric pelagic larva development into an asymmetric benthic juvenile. This transition encompasses a dramatic loss of the embryonic derived dorsal-ventral and left-right axis. The embryonic dorsal-ventral axis becomes the left-right axis, whereas the embryonic left-right axis becomes, irrespectively, the dorsal-ventral axis of the juvenile animal. This event is an unparalleled morphological change in vertebrate development and a remarkable display of the capacity of TH-signaling in shaping adaptation and evolution in teleosts. Notwithstanding all this knowledge, there are still fundamental questions in teleost metamorphosis left unanswered: how the central regulation of metamorphosis is achieved and the neuroendocrine network involved is unclear; the detailed cellular and molecular events that give rise to the developmental processes occurring during teleost metamorphosis are still mostly unknown. Also in flatfish, comparatively little is still known about the developmental processes behind asymmetric development. This review summarizes the current knowledge on teleost metamorphosis and explores the gaps that still need to be challenged.

Keywords: thyroid hormones, metamorphosis, teleost, morphogenesis, asymmetry

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INTRODUCTION

Teleosts (ray-finned fish) constitute the most diversified vertebrate group (1). They comprise more than 23,000 species and occupy a wide range of aquatic habitats, morphologies, behavior, and physiology. Most teleosts develop indirectly, i.e., between the end of embryonic development and sexually immature juvenile stages, they assume a transitional larval form where rudiments of all organs are already present, although not mature. Also, teleost larvae can present different ecologies and physiologies from their adult form.

It is only at the transition from larvae to juvenile that the metamorphosis occurs, and where the larva develops into a fully formed fish identical to the adult form but still sexually immature. In most teleosts, with both symmetric and asymmetric morphologies, this developmental transition is orchestrated by thyroid hormones (TH) that are the sufficient factors necessary for larvae to undergo metamorphosis. Production of TH occurs in the thyroid gland and consists of the prohormone thyroxine (T4) and the active hormone triiodothyronine (T3). Conversion, transport, and binding of T3 to its cognate receptors are tightly regulated at the cellular level since TH needs to be in a strict physiological range (2).

The involvement and dependence of TH for teleost metamorphosis were initially found in flatfish (3–15). Later it was also found that in most symmetric teleost species, metamorphosis occurs and parallels the developmental landmarks seen in flatfishes. The pre-metamorphic stage is characterized by lower whole-body levels of T4 and T3 and lower expression of *thyrotropin* (*tshb*), *thyroglobulin* (*tg*), *deiodinase 2* (*dio2*), and *thyroid hormone receptor beta* (*thrb*) and higher expression of *deiodinase 3* (*dio3*) (**Figure 1**). As soon as metamorphosis started, T4 and T3 increase together with increased expression of *thsb*, *tg*, *thrb,* and *dio2* and decreased *dio3* expression (**Figure 1**). The levels of T4 and T3 and expression of *tshb*, *tg*, *thrb*, *dio2* peak at the climax of metamorphosis, whereas *dio3* expression attains its lowest expression levels (**Figure 1**). As metamorphosis terminates the levels of T4 and T3 and markers of gene expression return to pre-metamorphic levels (15–24) (**Figure 1**). So far the observed markers and stages of metamorphosis are conserved between teleosts and anurans, clearly showing that this is a homologous developmental process regulated by TH (**Figure 1**).

The evidence today points to TH regulation of most organ maturation and developmental processes that occur during teleost metamorphosis. These changes enable not only a more efficient locomotion and digestion but also physiological and metabolic adaptations that allow the juvenile fish to adapt to their new habitat and lifestyle.

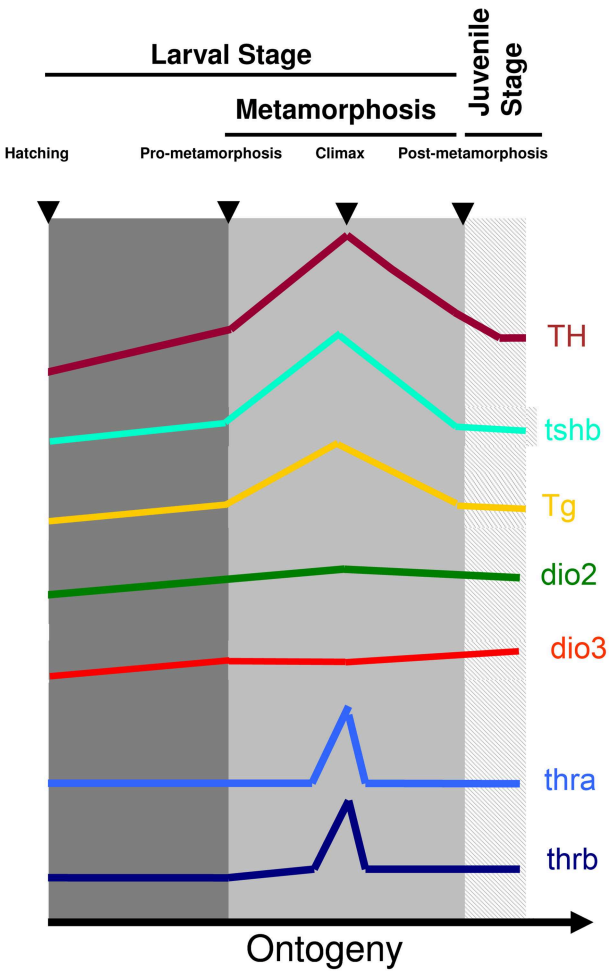

FIGURE 1 | Archetypal profile of T4 and T3 and expression of *tshb*, *tg*, *dio2*, *dio3*, *thra,* and *thrb* genes during teleost metamorphosis. The general observation in teleost species so far indicates that a surge of TH is accompanied by a rise in *tshb* and *tg* expression and the increased expression of TH signaling genes *dio2* and *thyroid hormone receptors* together with a decrease of *dio3*. As soon as metamorphosis terminates TH levels decrease concomitantly with decreased levels of expression of *tshb* and *tg* and *dio2* and *thyroid hormone receptors*, whereas *dio3* levels increase to pre-metamorphic levels. Figure adapted from (15) with permission from Elsevier.

CENTRAL REGULATION OF METAMORPHOSIS

One of the outstanding characteristics of anuran and teleost metamorphosis, in comparison to other developmental events, is the existence of a central regulation at the organismal level together with organ/tissue/cell-specific regulation of TH signaling. This regulation enables metamorphosis to occur when appropriate environmental conditions are achieved. A better example remains unknown where the factor that regulates each developmental event is also regulated at the central organismal level so that increased serum concentration can drive specific cellular developmental events.

Given the importance of TH in the regulation of a wide range of molecular pathways, their production by the thyroid gland is tightly controlled by the hypothalamic-pituitary-thyroid (HPT) axis, which ensures homeostasis of TH serum levels. This serum TH homeostasis is achieved in different ways in vertebrates

so far studied. In adult mammals, hypothalamic thyrotropin-releasing hormone (TRH) is released into the hypophyseal portal system and regulates the production of thyrotropin (TSHb) by the pituitary gland, that in turn regulates TH production by the thyroid gland (25). In adult reptiles and birds, the hypothalamic factor, corticotropin-releasing hormone (CRH), has a more prominent role in regulating thyrotropin (TSHb) secretion and T4 serum levels than TRH (26, 27). However, in teleosts, the current knowledge suggests species-specific regulation of the HPT axis (28–34). The observation that in adult cyprinids, Leptin, Galanin, β-endorphin, and neuropeptide Y (NPY) can regulate *in vitro* pituitary *tshb* expression (**Figure 2**), suggests that in teleosts pituitary-thyroid regulation may occur by hypothalamic factor/s other than TRH or CRH or by other, non-hypothalamic, endocrine factors (34) (**Figure 2**). Some studies in teleosts suggest that hypothalamic inhibition rather than stimulation by an unidentified factor might constitute the primary mechanism for HPT-axis regulation (35–37). In flatfish, even considering the known involvement of TH in metamorphosis, the knowledge on the regulation of the HPT-axis and the underlying neuroendocrine regulation remains very scarce.

During post-embryonic development, TH are the necessary and essential factors regulating metamorphosis. TH exerts their effect through a whole-body and tissue/cell-specific manner, both in anurans and flatfishes (3–5, 27, 28, 38, 39). In metamorphosing anurans, the central regulation of metamorphosis involves both the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamus-pituitary-adrenal gland axis (HPA), more commonly associated with stress (**Figure 2**). Environmental cues, such as decreased water levels, stimulate the HPA axis, and the release of CRH, which acts via its receptor, CRHr2, in pituitary thyrotrophs to enhance TSHb secretion, TH production and triggering metamorphosis [revised in (27); **Figure 2**]. A common feature of anurans and flatfishes is the simultaneous increase in *tshb* expression and TH serum levels during metamorphosis. In anuran metamorphosis, the set-point of the HPT axis is modulated by the action of CRH on pituitary thyrotrophs, so that high levels of serum TH do not repress *tshb* expression [revised in (27)]. In adult teleosts and during metamorphosis, TH are the main regulators of pituitary *tshb* expression, pointing to a central negative feedback mechanism at the level of the pituitary and the thyroid gland (28, 31, 32, 34, 40, 41) (**Figure 2**). During flatfish metamorphosis, hypothalamic inhibition is relieved, and the negative feedback on pituitary *tshb* by plasma TH adjusts to a higher set-point (28, 30, 41). Goitrogens block sole (*Solea senegalensis*) metamorphosis, indicating that the negative feedback loop between the thyroid and the pituitary gland is functional in larvae well before metamorphosis (28, 41, 42). Evidence on how the flatfish hypothalamus regulates the HPT-axis remains elusive. Recent work on flatfish metamorphosis, suggests that hypothalamic thyrotroph regulation may not exist at all during sole metamorphosis (41) (**Figure 2**). In sole larvae, blocking of metamorphosis after methimazole treatment (that blocks iodination of Tg and T4 production) did not change the temporal and spatial expression of *trh* and *crh,* suggesting that these neuroendocrine factors are not involved in sole metamorphosis. As a whole, the evidence raises fundamental questions about hypothalamic regulation of TH during metamorphic development in teleosts. The way *tshb* expression is regulated to induce metamorphosis of symmetric teleosts is even less well-known, and a great gap of knowledge exists on how the onset of metamorphosis occurs in these teleosts. It is still one of the great open questions in teleost metamorphosis.

Collectively, these evidences highlight a conserved mode of action in teleosts and an integrated response of the larval organism to the signal of TH. First, *tshb* level increases whole organism TH content that in turn gives rise to local tissue/cell responses. These are mediated locally by *dio2* and *dio3* ratios (discussed below) and increased *thrb* expression that allows the initiation of the metamorphic program and the morphogenetic changes in sensitive tissues. As in anurans, in teleost, metamorphosis *thrb* is considered to be the major *TH receptor* mediating metamorphic cell responses. However, there is little to none functional evidence in teleosts to support this view. This consideration derives from evidence in anurans [revised in (27)] and the existence, by whole-body analysis, of a peak in expression of *thrb* at the climax of metamorphosis in several teleosts (13–16, 43, 44). Since in most teleost species there are at least three different *TH receptor* genes (*thraa*, *thrab,* and *thrb*), it is likely that different *TH receptors* are involved in specific metamorphic events. The most likely scenario is that in some cells/tissues, metamorphic morphogenetic events are regulated by *thraa*, *thrab,* or *thrb* or even a combination of all or some *TH receptors* in a given cell/tissue. Nonetheless, in sole metamorphosis, the asymmetric development of the pseudomesial bone (discussed below) is correlated only with the asymmetric expression of *thrb*, strongly suggesting that, at least for this metamorphic event, *thrb* is the main effector of TH function (45). The actual scenario on the TH receptors mediating each metamorphic event is not clear and more work is necessary to elucidate this question in teleost metamorphosis.

MORPHOLOGICAL, MORPHOGENETIC, AND PHYSIOLOGICAL CHANGES DURING TELEOST METAMORPHOSIS

The word metamorphosis derives from the ancient Greek, where it means a change in form. In teleosts, just as in anurans, the larvae undergo such morphological changes that the overall shape of the animal gives rise to the juvenile form. In flatfish, this developmental event results in a dramatic morphological change. The symmetric flatfish larva develops into an asymmetric juvenile that is characterized by the migration of one eye to the opposite side of the head and the tilting of the body axis toward the migrating eye side. In the end, the primary body axis changes with regards to the original embryonic established dorsal-ventral and left-right axis (LRA). The lateral ocular side becomes dorsal, the blind lateral side becomes ventral, and the dorsal and ventral sides become left or right. The metamorphosed flatfish juvenile becomes a benthic animal in contrast to the pelagic larvae from which it arises. Its form adapts perfectly to life in the bottom of

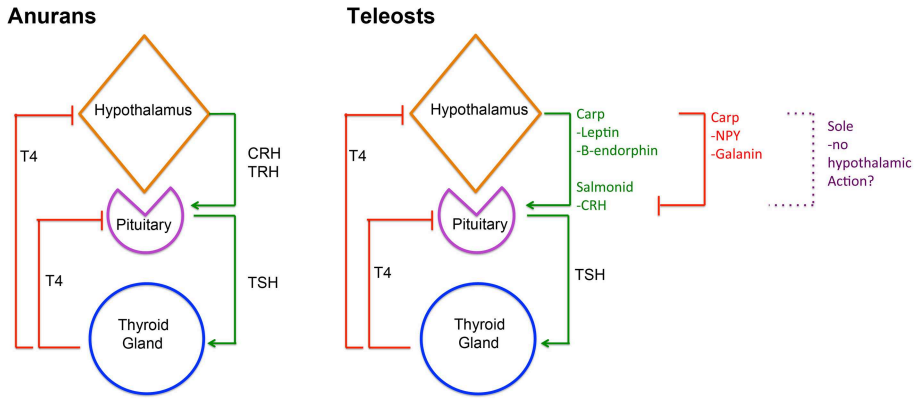

FIGURE 2 | Diagram depicting HPT-axis regulation in anurans and teleosts. In anurans, hypothalamic derived CRH and/or TRH are involved in regulating pituitary TSHb expression and secretion that in turn regulates T4 production in the thyroid gland and consequently serum levels. Inversely, serum T4 then negatively regulates CRH/TRH and TSHb expression and secretion. In teleosts, HPT-axis regulation is more diverse and likely reflects species specificity. In salmonids, CRH seems to regulate pituitary TSHb expression and secretion, whereas in carp leptin and β-endorphin seem to assume that role. Notably, in carp, Galanin, and NPY seem to repress TSHb expression and secretion. Remarkably, in sole metamorphosis, neither TRH nor CRH seems to be involved in regulating pituitary TSHb raising questions about the role of the hypothalamus in HPT-axis regulation in these teleosts. Despite this, in all teleosts studied so far, the negative feedback loop between thyroid gland T4 and pituitary TSHb is present and conserved.

aquatic environments where it uses stealth to prey and escape predators. The morphogenetic changes in symmetric teleosts are more subtle and do not change the embryonic derived body axis of the animal.

Nonetheless, in both teleost groups, they render the post-metamorphic juvenile a more hydrodynamic efficient form (46–50), reducing drag and enabling faster and less energy consumption during locomotion. These hydrodynamic changes are accompanied by a change in swimming mode with a passage from a larval C-shape movement to a more S-shape movement (48, 50). As the larvae undergo metamorphosis, these changes preconize an adaptation of form to fit a new function/lifestyle. To enable these changes, all the major organ systems in the larvae develop in response to higher metamorphic TH-levels.

AXIAL MUSCLE

Simultaneously with the change in locomotion, TH during metamorphosis reshapes the constitution of muscle fibers and the myotome. In most teleosts studied so far, a generalized increase in muscle hyperplasia occurs in the myotomes at the start of metamorphosis, that afterwards, gives way to increased muscular hypertrophy. This shift in muscle growth is more evident in the most epa- and hypaxial regions of the myotomes of metamorphic larvae, where there is a steep increase in the number of new muscle fibers developing (51–55, 55–66). During seabream (*Sparus aurata*) metamorphosis, several sarcomeric genes undergo isoform switching that are likely involved in modulation of locomotion (55, 55–57, 63, 64, 67). The increase in thyroid hormones during metamorphosis correlates with mRNA splicing events of *troponin T* (*tnnt*) genes in *S. aurata*, *Paralichthys olivaceus* (summer flounder), *Solea solea*, *Scophthalmus maximus* (turbot), and *Hyppoglossus hyppoglossus* (halibut) (59, 60, 68–70). Similar responses to T3 of other sarcomeric genes and proteins, like *myosin heavy chain* (71) and *Ca-ATPase* (72), are found in mammals. Some teleost species, during metamorphosis, develop a teleost-specific axial muscle denominated pink muscle that presents biochemical and functional characteristics intermediate of white and red muscle (54, 56, 73–76).

Notably, studies on halibut metamorphosis show that *dio2* and *3* play a critical role in these muscular developmental changes. The enzymes, *dio2* and *3*, are expressed in the same hyperplasic cells in the most epaxial and hypaxial regions of the myotome of early metamorphosing larvae, indicating that a tight regulation of cellular TH is essential for this cell proliferation. From the climax of metamorphosis onwards, the epa- and hypaxial cells co-expressing *dio2* and *dio3* become scattered throughout the myotome and resemble muscle pioneer cells (39). This evidence shows that TH is involved in axial muscle development during and after teleost metamorphosis. Nonetheless, more detailed studies are still needed to directly demonstrate the exact role of TH in teleost skeletal muscle development. Notably, the TH-dependent changes in skeletal muscle development in teleosts mirror some of the effects of TH in mammalian skeletal muscle development, where Dio2 and Dio3 activity is pivotal in the regulation of TH-action [revised in (77)].

BLOOD

Together with locomotion changes and the joint development of axial muscle during metamorphosis, there are also changes in gas-exchange. In most teleosts, blood develops fairly soon during embryonic development (78, 79). However, embryonic/larval erythrocytes are not fully mature. Most of the gas-exchanges in larvae until metamorphosis occur by simple diffusion through the skin (80). It is only at metamorphosis that erythrocytes can efficiently retrieve CO_2 from cells and deliver oxygen to

the tissues. TH stimulates and promotes the switching in the expression of *alpha-globin* genes from larval to adult isoforms (5, 81–84). This switching enables a more efficient gas exchange in juvenile rainbow trout in comparison to larval animals, with greater Bohr effect in adult haemoglobins (82). During amphibian metamorphosis, there is a similar regulation of haemoglobins, and the evidence seems to suggest that this event occur so that the metamorphosed animal can adapt to the new post-metamorphic environment (85, 86).

Taken together, gas-exchange at the gill level becomes more prominent as metamorphosis progresses. This transition in gas-exchange is especially important since the skin becomes more complex, stratified and impermeable to gases (80, 87, 88) as a direct consequence of TH-driven skin development during metamorphosis.

SKIN

The skin of teleost larvae is composed only of an epidermal layer and has little or no stratification. As the larvae undergo metamorphosis, the skin develops into a stratified epithelium with a multi-layered epidermis and dermis, which is formed by an acellular layer of collagen that is invaded by fibroblasts (87–92). At the same time, the adult pigmentation pattern starts to develop (93–97). Interestingly, in flatfish pigmentation becomes asymmetric when eye migration terminates. However, melanocyte precursors cells are found symmetrically distributed in the animal, but only the ones on the ocular side differentiate into melanocytes (95, 96). It is still unclear how these cells respond asymmetrically to TH. In zebrafish, as metamorphosis comes to an end, scales start to develop from specialized fibroblasts that have invaded the collagen lamella (98).

In teleost skin, keratinocytes are direct targets of TH. Before metamorphosis, halibut larval keratinocytes only express *dio3* but as soon as the larvae enter the climax of metamorphosis, *dio2* becomes highly expressed (39). Together with this increase of *dio2* expression, keratinocytes start to lose their larval morphology, larval keratin isoforms are repressed and apparently, no epidermal keratin is expressed in adult body skin keratinocytes (87, 99, 100). Notably, as keratinocytes differentiate toward an adult phenotype, they lose a distinct basal keratin bundle that is present only in larval cells (87).

During the much better studied amphibian metamorphosis, it is known that both cellular and molecular changes in the skin are dependent on TH and gave rise to fully stratified skin after metamorphosis (101–103), thus paralleling the observed events in teleost skin during metamorphosis. The most striking difference between teleost and anurans during skin metamorphic development is the apparent lack of expression of post-metamorphic keratin isoforms in teleost skin [88, 103, 104].

Taken together, TH during metamorphosis gives rise to an orchestrated series of developmental events in the skin of teleosts that make this organ develop from a simple to a complex structure that is a more impermeable and selective barrier. Importantly, these changes give rise to a dramatic shift in the physiology of the larvae since gas exchanges switch from the skin to the gills (80).

SKELETAL DEVELOPMENT

In mammals, TH is an important factor in bone development and homeostasis [revised in (104)]. So far, this aspect of metamorphosis has not received much attention in teleosts. Teleost skeleton develops significantly during metamorphosis. During the larval stage, most cartilaginous structures are already present, but most of the dermal derived bones are still absent. Most dermal derived bones develop as metamorphosis starts, and most of the cartilaginous derived bone starts to transit from their cartilaginous scaffold into an ossified definitive structure during metamorphosis (11, 45, 105–118).

Especially notable during metamorphosis is the development of the dermal derived structures of the axial skeleton, namely the vertebral bodies. These dermal derived structures start to ossify as soon as metamorphosis begins. Simultaneously, the neurocranium starts its ossification at this stage from the mesenchyme tissue surrounding the brain. Together with the resorption of the larvae fin-fold, the fin rays begin to develop and ossify, enabling the full development of the fins after metamorphosis (11, 105–107, 110–118).

One interesting aspect of how skeletal development during metamorphosis can be adjusted to allow better adaptations to the habitat conditions of juveniles, comes from *Oreochromis mossambicus* (*Mozambique tilapia*). In this species rearing of metamorphosing larvae at higher temperatures promotes carnivore-oriented skull development, whereas lower temperatures reared larvae developed omnivore oriented structures (116). Although the exact role of TH signaling is unknown, these observations highlight that during tilapia metamorphosis, skeletal development is adjusted to match the specific environmental needs posed to the larvae.

However, most of the studies on the role of TH on teleost skeletal development are descriptive. Some reports show that changes in TH signaling might correlate with abnormal skeletal development in *S. senegalensis* (113). Also, TH signaling is essential for asymmetric development of the flatfish specific pseudomesial bone (45). Despite these advances, still much remains to be found about the exact role and the genetics of TH signaling regulating bone development during teleost metamorphosis.

GASTROINTESTINAL-TRACT

One of the key features of anuran metamorphosis is the development of the gastrointestinal (GI) tract. During metamorphosis, the tadpole gut shortens and modifies to allow a shift from herbivorous to carnivorous feeding [revised in (119)]. In mammals, TH is essential for the development of the gut, where *Thra* plays a crucial role in GI cell differentiation (120–122). During teleost metamorphosis, the larvae GI tract is also home to a series of significant morphological, molecular and functional changes (9, 123–131). In Atlantic halibut, Japanese

flounder and summer flounder metamorphosis leads to higher compartmentalization of the GI tract. In these flatfish, the development of the stomach and an increased length, folding, and regionalization of the intestine is observed. Accompanying these morphological events is the beginning of the expression of *pepsinogen* (126, 129–131). Similar development of the GI tract is observed in the symmetrical teleost *Dicentrarchus labrax* (sea bass) (124, 128). One key feature of teleost intestine development at metamorphosis is the increase in microvilli area, globulet and secretory cells (124, 126, 128, 131). In halibut, one of the key features of metamorphic stomach development is the synchronous establishment of molecular and cellular components in such a way that stomach function starts at the climax of metamorphosis (126).

Together with the digestive tract changes, other organs involved in digestion respond to increased TH-levels during teleost metamorphosis. In the zebrafish pancreas at metamorphosis, β-cell differentiation and *insulin* expression increase and respond positively to exogenous TH treatment, whereas the opposite is observed for *glucagon* expression (132). In Senegalese sole, early in metamorphosis, the intestine and liver start to express *apolipoprotein A-I* that is involved in cholesterol metabolism (133). This evidence suggests that during metamorphosis, TH regulates many of the developmental events in most GI-tract organs to allow a novel metabolic and homeostatic program to start and take full advantage of the novel food sources in the post-metamorphic habitat.

FLATFISH METAMORPHOSIS ASYMMETRIC HEAD DEVELOPMENT

Of all teleosts, flatfishes display the most dramatic morphological manifestation of metamorphosis. It was considered, since its discovery, that these teleosts become asymmetric at metamorphosis (134). The fossil record shows that eye migration is one of the early events taking place during flatfish metamorphic evolution (135, 136). Nonetheless, evidence shows that, besides skin pigmentation, asymmetric metamorphic development observed at the body level is located in the most anterior third of the head that encompasses the eye field (11, 39, 45, 59, 87, 115, 117, 118, 137, 138).

Evidence suggested that sub-dermal cell proliferation was the factor responsible for driving asymmetric eye migration during flatfish metamorphosis (139). However, in these experimental conditions of sub-ocular colchicine-inhibited cell proliferation, the eye was still able to migrate (>50%), albeit not as much as in normal developing larvae (139). This evidence suggested that, although cell proliferation is a factor involved in flatfish metamorphic eye migration, it is not the major mechanism that drives it. Later evidence in Senegalensis sole shows that asymmetric sub-ocular development and ossification of the pseudomesial bone (that develops only during flatfish metamorphosis and ventral to the migrating eye) is the major driving force of eye migration during metamorphosis (45). In methimazole treated sole larvae eye, migration was impaired, clearly showing that TH is the sufficient and necessary factor behind eye migration. Also, in these larvae, neither pseudomesial ossification nor sub-dermal cell proliferation was observed. This evidence shows that sub-ocular asymmetric ossification and sub-dermal proliferation are both TH-driven events (45). Additional evidence using sub-ocular injection of apyrase [dermal bone differentiation inhibitor (140)] in pre-metamorphic sole larvae prevented pseudomesial ossification and most of the eye migration (only 5–15% migration observed). These pieces of evidence show that pseudomesial ossification is the major force driving eye migration and asymmetric head development in sole metamorphosis (45). Given that pseudomesial bone asymmetric development at metamorphosis is a common event in all flatfish (11, 115, 117, 137, 138), the findings in sole (45) argue that this is the mechanism by which asymmetric TH-driven head development occurs in all flatfish. Together, pseudomesial asymmetric ossification and cell proliferation are essential for proper eye migration in flatfish (45, 139). In this scenario, asymmetric flatfish eye migration itself is a passive event where the eye, the eye socket, ocular muscles, and the ocular nerve are all pushed to the opposite side of the head by asymmetric ossification of the pseudomesial bone together with sub-ocular cell proliferation (45, 139). Moreover, this event compresses the mesenchyme tissue in-between the eyes that also differentiates into bone, giving rise to the paramesial bone (11, 39, 45, 59, 87, 115, 117, 118, 137, 138). The tissue mechanics underlying this process are still unknown.

The pseudomesial bone arises from an asymmetric TH-responsive center, characterized by the co-expression of *dio2* and *thrb,* that increase in expression as metamorphosis progresses and terminate as soon as metamorphosis is completed (45). In the TH-responsive center, TH regulates *dio2* in a non-canonical fashion. Rather than increased concentrations of TH leading to decreased *dio2* expression, as is observed in symmetric developing structures in the sole head; in the asymmetric TH-responsive center, *dio2* expression is dependent and positively regulated by TH (45). Nonetheless, it is still not known which are the genetic factors behind this asymmetric sub-ocular expression pattern of *dio2* during metamorphosis.

GENETIC REGULATION OF FLATFISH METAMORPHIC EYE MIGRATION

The genetic regulation of asymmetric head development in flatfish remains an elusive matter. It was suggested that flatfish metamorphic asymmetry is related to embryonic left-right (LR) asymmetric development by the *Nodal-Lefty-Pitx2* pathway (141). In both *P. olivaceus* and *Verasper variegatus*, asymmetric habenular expression of *pitx2* was found during metamorphosis of these flatfish (141). Also, it has been argued that asymmetric *pitx2* habelunar expression during metamorphosis led to the decreased size of the blind side habenula lobe that in turn generated a torsion force that would lead to both optic nerve and eye migration (141). However, no asymmetric nerve endings were shown to emerge after this asymmetric event. These observations raise questions about how *pitx2* asymmetric habenular expression can give rise to asymmetric pseudomesial

ossification and cell proliferation that are the driving forces of flatfish eye migration (45, 139). Tentative efforts were also carried out to understand if blocking of *pitx2* signaling, via ouabain exposure during embryogenesis, could lead to random migration of the eye. The reversal of normal eye migration occurred in a small proportion of individuals (∼6%), but the gross majority of these larvae metamorphosed normally (141).

Given that flatfish metamorphic eye migration is exclusively dependent on TH, it is still not known if *pitx2* expression in *P. olivaceus* and *V. variegatus* is dependent on TH. Moreover, *Nodal-Lefty-Pitx2* embryonic signaling was independent of putative *pitx2* asymmetric metamorphic development (141), further raising concerns about how this embryonic mechanism could lead to asymmetric metamorphic development. Notably, habenular asymmetry is not exclusive of flatfish and is also found in other bilateral teleosts (142), suggesting that the asymmetry in the habenula of *P. olivaceus* and *V. variegatus* might not be related to metamorphosis. Moreover, a detailed mapping of the nervous system during sole metamorphosis only found asymmetric differences in the olfactory epithelia, olfactory bulb and in the most anterior diencephalon that is encompassed by the eye field (45). Remarkably it was found that the olfactory epithelia and bulb only became asymmetric if there was eye migration but not in its absence. These pieces of evidence strongly suggest that the asymmetric metamorphic development of these brain structures was due to the force constraints brought about by pseudomesial asymmetric ossification. Consequently, eye migration is not directly caused by TH-action on these structures. Curiously, in zebrafish embryogenesis, it was found that maternal TH are involved in establishing *pitx2* expression in the developing pituitary but how or if this has any consequence in metamorphic *pitx2* expression in zebrafish or any other teleost species remains unknown (143).

Up until now, the most detailed data available on asymmetric flatfish head metamorphic development only sheds light on how TH regulates asymmetric pseudomesial ossification that leads to eye migration (45). Several efforts have been made using next-generation transcriptomic approaches to solve this question (144–146). However, no definitive answer was obtained even using these high-throughput strategies. Alves et al. (147) sequenced the head transcriptome of metamorphosing halibut and found that the most significant differentially expressed transcripts (DET) were related to TH production. The whole-body transcriptome of metamorphosing Japanese flounder reports that the top DET were for genes related to bone development (145). Transcriptomics confirm that increased TH production is a crucial event in teleost metamorphosis and that bone development is a central aspect of teleost metamorphosis as already shown by several studies (11, 45, 105–118). While this manuscript was in revision, a new study analyzing the transcriptome of dissected heads of metamorphosing sole larvae was published. In this study, it was also not possible to identify a single gene responsible for asymmetric head development during flatfish metamorphosis (148). Instead, it was reported that in the head of sole larvae undergoing metamorphosis, several different genetic cascades function in a time and stage-specific manner. The earliest changes are related to hormonal production, followed by protein and mRNA processing, cell cycle regulation, nuclear organization, and finally, DNA replication. Collectively, this evidence argues that metamorphosis in sole head occurs in a stepwise manner with earlier genetic cascades promoting the next stage of developmental changes. Also, this study shows that earlier occurring metamorphic events are at the organismal level while later events are at the cellular level, suggesting that metamorphosis progresses from changes in the organism to specific cellular responses (148). Nonetheless, neither study was able to identify a single gene or genetic mechanism behind asymmetric eye migration in flatfishes. These findings argue that asymmetric head development can be due to a combination of different flatfish specific genetic pathways that together give rise to asymmetric head development.

TH AND SALMONID SMOLTIFICATION

In most teleosts, a single surge in TH is found and related to the larval to juvenile transition, known as metamorphosis (discussed above). However, in salmonids, a second peak of T4 also occurs just before spontaneous smoltification (24, 149). TH treatment increases survival of salmonids after SW transfer (150), further arguing that TH are involved in salmonid smoltification. The evidence seemed to suggest that T4 levels are directly related to the adaptability of salmon to seawater (SW) and parr fish that are not able to adapt to SW present hypothyroidism (150). Also, TH are involved in the acquisition of the specific morphological changes that come with smolting like silvering and behavior responses like downstream migration (151).

Little is known about the tissue-specific developmental events driven by TH during the parr to smolt transition in salmonids. An early study reported that brain and hepatic T4 levels increase before plasma levels, whereas muscle T4 content decreased as soon as plasma T4 levels increased (152). Nonetheless, the biological consequences of different organ T4 content and timing are still unknown. A more recent study provided evidence showing THs are involved in both the light response and gill seawater adaptation observed in salmons undergoing smoltification (153). In this study, Lorgen et al. (153) show that different *dio2* paralogs are involved in different tissue responsivity to TH, but the exact consequences of this differential TH cell signaling remain elusive.

Nonetheless, this evidence suggests that TH might have a role in integrating environmental cues that allow for the specific adaptions needed for seawater transition and that these changes are brought about by complex peripheral regulation of TH metabolism. Notably, T4 levels increase in wild smolts, whereas in hatchery smolts T4 levels remain stable (154), suggesting that the role of TH in salmonid smoltification is complex and likely linked to environmental cues. Albeit this evidence, and in contrast to metamorphosis that is considered a larval to juvenile transition, TH are not the necessary and sufficient factors regulating smolting. In both anuran and teleost metamorphosis, T4 and T3 increase to give rise to this developmental transition. However, instead in salmon smoltification, T4 but not T3 levels increase (24, 149, 152,

154). Taken together the evidence seems to suggest that TH participate, together with other endocrine factors [revised in (155)], in the transition from parr to smolt but they are not the essential factor triggering smoltification. As a whole, salmonid smoltification is likely to be a developmental transition specific of this class of teleost that is mediated by several endocrine factors including TH.

FUTURE PERSPECTIVES

The evidence so far obtained on teleost metamorphosis highlight the role of TH as an integrative factor able to give rise to a series of synchronized developmental changes across the entire organism. These prepare the organism for the physiological and ecological challenges of juvenile animals. Despite recent advances, teleost metamorphosis is still an understudied developmental event. Important questions at the core of this developmental process are still not answered. These include: (1) how central regulation of the onset of metamorphosis is achieved and; in the case of flatfish metamorphosis, (2) which are the TH-dependent genetic and cellular mechanisms behind asymmetric head development. Other aspects like the role of TH metabolites other than T4 and T3 during metamorphosis are mostly unstudied.

Also, a recent study implicated genetic divergence in *tshb* paralogs locus in mediating and modulating TH signaling and physiological adaptation in three spine-sticklebacks marine and freshwater ecotypes (156). Although no exact molecular mechanism or developmental/metamorphose-related implications of divergent TH signaling were found between the two ecotypes, these evidences also highlight the potential capacity of TH for promoting speciation in teleosts (156).

The advent and fast pace of development of new technologies will allow for better discrimination of the genetic events during teleost metamorphosis and TH signaling in general. Also, more powerful imaging technologies for large specimens open the door for discoveries and better insight into morphological development observed during teleost metamorphosis.

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The author confirms being the sole contributor of this work and has approved it for publication.

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Thyroid Hormone Receptor Alpha Is Required for Thyroid Hormone-Dependent Neural Cell Proliferation During Tadpole Metamorphosis

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Thyroid hormone (T_3) plays several key roles in development of the nervous system in vertebrates, controlling diverse processes such as neurogenesis, cell migration, apoptosis, differentiation, and maturation. In anuran amphibians, the hormone exerts its actions on the tadpole brain during metamorphosis, a developmental period dependent on T_3. Thyroid hormone regulates gene transcription by binding to two nuclear receptors, TRα and TRβ. Our previous findings using pharmacological and other approaches supported that TRα plays a pivotal role in mediating T_3 actions on neural cell proliferation in *Xenopus* tadpole brain. Here we used *Xenopus tropicalis (X. tropicalis)* tadpoles with an inactivating mutation in the gene that encodes TRα to investigate roles for TRα in mitosis and gene regulation in tadpole brain. Gross morphological analysis showed that mutant tadpoles had proportionally smaller brains, corrected for body size, compared with wildtype, both during prometamorphosis and at the completion of metamorphosis. This was reflected in a large reduction in phosphorylated histone 3 (pH3; a mitosis marker) immunoreactive (ir) nuclei in prometamorphic tadpole brain, when T_3-dependent cell proliferation is maximal. Treatment of wild type premetamorphic tadpoles with T_3 for 48 h induced gross morphological changes in the brain, and strongly increased pH3-ir, but had no effect in mutant tadpoles. Thyroid hormone induction of the direct TR target genes *thrb*, *klf9*, and *thibz* was dysregulated in mutant tadpoles. Analysis of gene expression by RNA sequencing in the brain of premetamorphic tadpoles treated with or without T_3 for 16 h showed that the TRα accounts for 95% of the gene regulation responses to T_3.

Keywords: thyroid hormone (T3), metamorphosis, *Xenopus*, neurogenesis, development, knockout (KO)

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INTRODUCTION

Thyroid hormone (T_3) plays several key roles in neurological development of vertebrates, influencing dendrite and axon development, synaptogenesis, myelination, cell migration, proliferation, and differentiation (1–3). Thyroid hormone deficiency during fetal and early postnatal life leads to severe, irreversible neurodevelopmental defects in mammals, a condition in humans known as cretinism (1, 4).

The hormone is also essential for development in non-mammalian vertebrates, which is perhaps best exemplified by the dependence of amphibian tadpole metamorphosis on T_3 (5). Tadpoles of *Xenopus* species are important model organisms for investigating the molecular and cellular mechanisms of T_3 action during vertebrate development (6).

Thyroid hormone regulates gene transcription by binding to ligand-activated transcription factors (T_3 receptors; TRs) (7, 8). All jawed vertebrates that have been studied have two TR genes, designated alpha and beta. In mammals, multiple TR isoforms originate from the two genes, some that bind T_3 and are functional, others that do not bind T_3. They are produced by differential promoter usage or mRNA processing (3); similar TR protein diversity has not been described in amphibians. Mammalian and amphibian TR genes exhibit cell type and developmental stage-specific expression patterns, which supports that the subtypes/isoforms serve different functions in development and physiology (7, 9, 10). The mRNAs for the *Thra* gene, which codes for the functional isoform TRα1, is widely distributed in rodent brain from early development through adulthood, and most studies support that this protein is the major TR subtype involved with brain development (7). By contrast, mRNAs for *Thrb* (coding for TRβ1 and TRβ2, among several other isoforms) are expressed in the brain during postnatal life, and have a more limited distribution in the brain, being restricted to the retina, cochlea and hypothalamus, and also the pituitary gland (11–13).

The expression patterns of TR genes in *Xenopus* species have broad similarities to mammals. For example, *thra* mRNA is detected in the tadpole shortly after hatching and continues to be produced at a high, mostly constant level through metamorphosis in all tissues that have been studied (5). By contrast, *thrb* mRNA is not detected until the beginning of metamorphosis, at which time it is autoinduced by the rising plasma T_3 titer. In tadpole brain, *thra* mRNA shows wide distribution; it is detected in virtually all cells in tadpole brain, and exhibits particularly strong signal in neurogenic zones (10, 14). The *thrb* mRNA was detected by *in situ* hybridization histochemistry (ISHH), and the protein by immunohistochemistry (IHC) only after treatment with T_3, and analysis of TRβ in tadpole brain showed that the protein is found in specific brain nuclei throughout the brain, predominantly outside of neurogenic zones lining the ventricles (10).

Several lines of evidence in mammals support that TRα is critical for mediating T_3 action on neurogenesis (15), oligodendrocyte differentiation (16), and astrocyte maturation (17). Previously, we showed that the TRα selective agonist CO23 induced brain cell proliferation, while the TRβ-selective agonists GC1 and GC24, used at concentrations that preferentially activate TRβ, had no effect (10). In the current study we used gene knockout technology to investigate a role for TRα in mediating T_3 action on cell proliferation and gene regulation in tadpole brain. Mitosis is low in the premetamorphic tadpole brain, then it increases strongly and peaks during prometamorphosis, and this depends on endogenous T_3 (10). We compared the volume and gross morphology of the tadpole brain, and we analyzed mitosis by IHC for phosphorylated histone 3 (pH3) which is a marker for M phase of the cell cycle. We conducted RNA-sequencing (RNA-seq) on a micro-dissected region of the tadpole brain that contains the preoptic area/thalamus/hypothalamus, and which houses neurosecretory neurons that project axons to the median eminence to control pituitary hormone secretion; this tadpole brain region is highly dependent on T_3 for its development (10, 18, 19). We also conducted time course analyses of gene regulation responses to T_3 in wild type and mutant tadpole brain.

MATERIALS AND METHODS

Animal Care and Treatment

We obtained wildtype (WT) *Xenopus tropicalis* frogs from NASCO and reared them in dechlorinated tap water at 25C under a 13L:11D photoperiod, and fed them frog brittle (NASCO, Fort Atkinson, WI) or Sera Micron plankton food (tadpoles only). We obtained the heterozygous *thra* mutant frog line (TRαM5) from Dr. Yun-Bo Shi. This line was created using TALENS targeting exon 4 (the third coding exon) of the *X. tropicalis thra* gene, leading to the generation of a mutation that results in a predicted non-sense protein after amino acid 54, which is just before the first zinc finger of the DNA binding domain that begins at amino acid 60 (20). Here we designate the homozygous mutant frog line $thra^{mt-exon4}$ to indicate the presence of an inactivating mutation in exon 4, leading to a functional knockout.

We generated homozygous $thra^{mt-exon4}$ tadpoles by crossing heterozygous mutant frogs. Tadpoles were staged using the developmental staging system of Nieuwkoop and Faber (21) (NF). We used PCR-based genotyping to identify homozygous $thra^{mt-exon4}$ tadpoles as previously described (22). We tail clipped tadpoles at NF stage 48, and lysed the tissues in 30 μL Quick^{TM}Extraction buffer at 65°C for 15 min, followed by heating to 94°C for 5 min. To analyze the wild type *thra* allele we used forward primer F_{wt} 5′- AGCTATCTG GACAAAGACGAGCCG-3′, and for the mutant allele we used forward primer F_{m5} 5′-ACATCCCCAGCTATCCCCA GCTATG-3′. The common reverse primer was 5′-GCAA ACTTTTTGGCTCAGAGGCCAC-3′. We used the same PCR program for both primer sets: 94°C 30 s, 68°C 60 s for 35 cycles.

For treatment of tadpoles with 3,5,3′-triiodothyronine (T_3; sodium salt, Sigma-Aldrich, St. Louis, MO), we dissolved the hormone in 0.01N NaOH to make a 500 μM stock solution, then we added the stock to the aquarium water to a final concentration of 5 nM T_3 for all experiments; controls received an equivalent amount of 0.01N NaOH. For the RNA-seq experiment, we treated NF stage 54 tadpoles with vehicle or T_3 added to the aquarium water for 16 h before harvesting tissues for RNA isolation. For time course analyses using RTqPCR we treated NF stage 54 tadpoles with T_3 for 0, 8, 16, or 42 h before harvesting tissues for analysis. For morphometric analyses and immunohistochemistry for

cell proliferation we treated tadpoles with T_3 or vehicle for 42 h before tissue harvest. All procedures involving animals were conducted under an approved animal use protocol (PRO00006809) in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Michigan.

For morphometric analyses we euthanized tadpoles or newly metamorphosed frogs by immersion in 0.1% benzocaine, then recorded body weight and body length (using calipers; snout-tail tip for tadpoles, snout-vent for frogs) before dissecting the head and fixing it in 4% paraformaldehyde dissolved in MOPS buffer (0.1 M MOPS, 10 mM EGTA, 1 mM $MgSO_4$, pH 7.4; Sigma-Aldrich) at 4°C overnight. We then dissected the fixed tadpole brain and captured images using a Leica stereoscope. All images were captured at the same magnification (10 X) and illumination settings. We used ImageJ software to quantify brain length, width and height for untreated NF stage 56 and 66 animals ($n = 6$ or 8/genotype, NF 56 or 66, respectively). We calculated brain volume by multiplying brain length, width and height, and normalized each animal's brain volume to its body length. We also analyzed brain morphology of NF stage 54 tadpoles after treatment with 5 nM T_3 for 48 h; here we measured brain length and width ($n = 5$/treatment and genotype).

Total RNA Extraction, RNA Sequencing, and Reverse Transcriptase Quantitative PCR (RTqPCR)

We micro-dissected the region of the tadpole brain containing the preoptic area/thalamus/hypothalamus for isolation of RNA as previously described (23). For each biological replicate we pooled tissue from 5 tadpoles. We isolated total RNA from tadpole brain using Tri Reagent (Sigma-Aldrich, St. Louis, MO) and the Direct-zol RNA kit (Zymogen) following the manufacturers' instructions. The quality of the RNA was analyzed using a Bioanalyzer, and libraries were prepared by the University of Michigan DNA Sequencing Core. We used 3 biological replicates for each genotype and hormone treatment, for a total of 12 samples sequenced on two lanes using the Illumina Hiseq-4000 platform with 50 nt read length single-end sequencing. We conducted quality processing on raw reads using FastQC (v0.11.7), and we mapped aligned reads to the *X. tropicalis* genome (v4.1) using Bowtie. We conducted gene-level read counts using RSEM to count the number of reads overlapping each of 22,820 custom-annotated *X. tropicalis* genes. This custom annotation (the MNHN gene model) was built by Nicolas Buisine and Laurent Sachs based on their unpublished high-throughput RNA paired-end tag sequencing to identify the 5′ and 3′ ends of transcripts that were expressed in different *X. tropicalis* tissues, including the brain. We then conducted differential expression analysis using limma.

We used RTqPCR to validate gene expression changes identified by RNA-seq, and also for analysis of the kinetics of gene transcription responses to exogenous T_3 in the two genotypes. We synthesized cDNA from 1 μg total RNA using the High Capacity cDNA Synthesis Kit (Applied Biosystems Inc., Foster City, CA). We conducted real-time qPCR using ABsolute Blue qPCR SYBR Low ROX Mix (ABgene Thermo Scientific, Surrey, UK) and Fast 7500 Real-Time PCR System (ABI) or StepOne Real Time PCR Systems (Life Technologies). We designed oligonucleotide primers to span exon-exon boundaries using the program Prime Blast (NCBI) (**Table 1**). For most genes we used a relative quantification method (24, 25) to compare mRNA levels by generating standard curves for each gene using a pool of cDNA. To compare levels of *thra* and *thrb* mRNAs in brains of tadpoles of the two genotypes we used an absolute quantification method. We constructed standard curves using the plasmids pCR-TOPO-xtTRα and T7TS-xtTRβ that contain full-length cDNAs for *thra* and *thrb*, respectively.

TABLE 1 | Oligonucleotide primer sequences used for reverse transcriptase quantitative polymerase chain reaction.

Gene name	DNA sequence*
cga F	GCATGTGCTCCATTCCTTTCC
cga R	GGCCTTTCCGAGCCTTTTTG
cbx7 F	TGCCATTGGAGAGCAAGTGT
cbx7 R	TGCTGTATTTGGGAGGCCAG
crebrf F	TGGGCTTTGAGATGCCTCAG
crebrf R	TTGCTAGGAGGTCTGTGCTC
dio3 F	CGCGGCTTGGATGTCATTGC
dio3 R	GCTCCAGTGACACGCACCTT
ef1a1 F	CGGAACTACCCTGCTGGAAG
ef1a1 R	GGCAAAGGTAACCACCATGC
egln3 F	GACCGACTGGTGATGCTCTG
egln3 R	CGTTGGATTGTCCACATGGC
igf2 F	GTTTGTGGAGACAGGGGCTT
igf2 R	CAGCTCCGGAAGCAACATTC
klf9 F	GGCACAGGTGTCCTTATGCT
klf9 R	AAGGGCGTTCACCTGTATGG
nr3c2 F	GTCCACTATTTCGAGCCCAG
nr3c2 R	GGCGTTACTCCAGGAAGGAAT
pim3 F	CGGTGTACACGGATTTTGACG
pim3 R	ACGGTTGCTGATCTTCCATGA
st3 F	GGTTATGTGTGGCGCCTTCG
st3 R	AATGGGAAAGGGCCCAGAGG
thibz F	CCAAGGGAAACGGGTGGCTT
thibz R	GTGCCACCTCTGCGGAAAGT
thra F	AAATGCATTGCCGTTGGCAT
thra R	GCCGCTCTCGATTCTCTTCA
thrb F	TAGTTAATGCGCCTGAGGGTG
thrb R	TGCTCGGAGGGACATGATCT
traf3 F	GTTCGACTAGCCCACAATGC
traf3 R	TCCCTCCTGCAGCAGTTATC
tshb F	TGTGCTTACTGCCTTGCCAT
tshb R	CAGCCAGGAATGGTCACTGT
znf395 F	CATGTACAAGTGCCTGTGGC
znf395 R	CTCTCTTGCGCTGGTCAGAA

*F, forward; R, reverse. Sequences given are 5′ to 3′.

The two cDNA inserts were of similar size (*thra*-1936 nt; *thrb*-2223 nt). We normalized mRNA levels to the reference gene *ef1α*, which did not change after treatment with T_3 (data not shown).

Immunohistochemistry (IHC)

We conducted IHC on tadpole brain to detect mitotic cells using a rabbit antiserum raised against human pH3 (phosphorylated histone 3, Millipore), which marks cells in M phase of the cell cycle (10). We analyzed untreated tadpole brains from the two genotypes (n = 4–7/genotype) at NF stage 56 when cell proliferation is highest during metamorphosis (10). We also treated NF stage 54 tadpoles of the two genotypes with T_3 for 42 h (n = 4–5/genotype and treatment), which induces a robust cell proliferation response in WT tadpole brain (10). We euthanized animals by immersion in 0.1% benzocaine, then dissected the head and fixed it in 4% paraformaldehyde in MOPS buffer at 4°C overnight. After fixation we dissected the brain from the skull, saturated the tissue in 30% sucrose overnight at 4°C, embedded it in OCT compound (Fisher Scientific), snap-froze the sample, and stored at −80°C until processing. We produced transverse, 14 μm cryosections, dried them for 2 h at 42°C, and then stored the slides at −80°C until analysis. For IHC, we first washed the cryo-sections in Tris-buffered saline plus 0.1% tween-20 (TBST) three times, then blocked with 10% normal goat serum diluted in TBST for 1 h at room temperature. We incubated the sections with primary antibody (1:500 dilution) overnight at 4°C, and then washed with TBST three times. We then detected primary immune complexes by incubating with a secondary antibody conjugated with Cy3 (1:1,000 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at room temperature for 2 h, washed three times in TBST, and mounted the sections using a mounting medium that contained DAPI for counterstaining (Vector Laboratories).

We captured digital micrographic images at 100X using an Olympus IX81 inverted microscope (Olympus, Tokyo, Japan) and a Retiga 1300R Fast digital video camera (QI Imaging, Tuscon, AZ). We carefully matched sections for anatomical level following the *Xenopus* brain atlas developed by Tuinhof et al. (26), with modifications by Yao et al. (27). Images were analyzed in a blinded manner. We randomly selected 5 sections per brain within the region bound by the anterior preoptic area and hypothalamus/thalamus using a random number generator, and we manually counted cells immunoreactive for pH3 (pH3-ir) (10).

Statistics and Data Analysis

We analyzed data using the computer program SYSTAT (v. 13; Systat Software, San Jose, CA). Differences between treatments were analyzed by one-way ANOVA followed by Fisher's least significant difference (Fisher's LSD) *post hoc*-test, by two-way ANOVA, or by Student's independent sample t-test ($\alpha = 0.05$). Derived values were Log_{10}-transformed before statistical analysis if the variances were found to be heterogeneous. We used linear regression analysis to analyze time course gene expression data.

RESULTS

Brain Size and Cell Proliferation Are Reduced in *thra$^{mt-exon4}$* Animals

We compared brain size in WT and *thra$^{mt-exon4}$* tadpoles at NF stage 56, the developmental stage when T_3-dependent neural cell proliferation is maximal (10), and in newly metamorphosed frogs (NF stage 66). The NF stage 56 *thra$^{mt-exon4}$* tadpoles were 25% smaller (body length from snout to tip of tail) than WT animals, but their brain volume was 46% smaller (**Figure 1A**). Brain volume, corrected for body length was 28% smaller in *thra$^{mt-exon4}$* tadpoles compared with WT. The NF stage 66 (newly metamorphosed frogs) *thra$^{mt-exon4}$* animals were 8% smaller (snout to vent length) than WT animals, but their brain volume was 28% smaller (**Figure 1B**). Brain volume corrected for body length was 22% smaller in newly metamorphosed *thra$^{mt-exon4}$* frogs compared with WT (**Figure 1B**).

Our earlier findings showed that cell proliferation in *Xenopus* tadpole brain peaks at NF stage 56 (10). To investigate if the smaller brain size in NF stage 56 *thra$^{mt-exon4}$* tadpoles was correlated with reduced cell proliferation, we conducted immunohistochemistry for pH3, a marker for cells in M phase of the cell cycle. This analysis showed a significant (50.5%) reduction in pH3-immunoreactive cell nuclei in *thra$^{mt-exon4}$* tadpole brains compared with WT (**Figure 1C**).

TRα Is Required for T_3-Dependent Morphological Changes, and Induction of Cell Proliferation in Tadpole Brain

Treatment of NF stage 54 (early prometamorphic) WT tadpoles with T_3 (5 nM for 42 h) caused significant changes in tadpole brain morphology, decreasing brain length by 28%, and increasing brain width by 24% (**Figures 2A,B**). By contrast, exogenous T_3 had no significant effect on brain morphology in *thra$^{mt-exon4}$* tadpoles. Our previous work showed that exogenous T_3 can induce cell proliferation in NF stage 54 *Xenopus* tadpole brain, measured by BrdU incorporation or pH3-ir. We saw the expected increase in pH3-ir after T_3 treatment in WT tadpoles (∼2.7 fold increase), but in *thra$^{mt-exon4}$* tadpoles T_3 treatment reduced pH3-ir by ∼30% compared with vehicle-treated animals (**Figure 2C**).

Thyroid Hormone Receptor α Is the Major TR Expressed in Tadpole Brain

Using RTqPCR with absolute quantification, we found that baseline *thra* mRNA was ∼12.5 times higher than baseline *thrb* mRNA, confirming that TRα is the major TR subtype in tadpole brain (**Figure 3A**). The baseline *thra* mRNA was significantly lower in *thra$^{mt-exon4}$* tadpole brain compared with WT (21% lower), while the baseline *thrb* mRNA was significantly higher in *thra$^{mt-exon4}$* (∼2 fold; **Figure 3A**). Treatment with T_3 for 42 h had no significant effect on *thra* mRNA level in either genotype; whereas, T_3 treatment caused a large induction of *thrb* mRNA in the brains of WT (7.9 fold increase) tadpoles, and a significant but lower induction in *thra$^{mt-exon4}$* animals (3.2 fold increase).

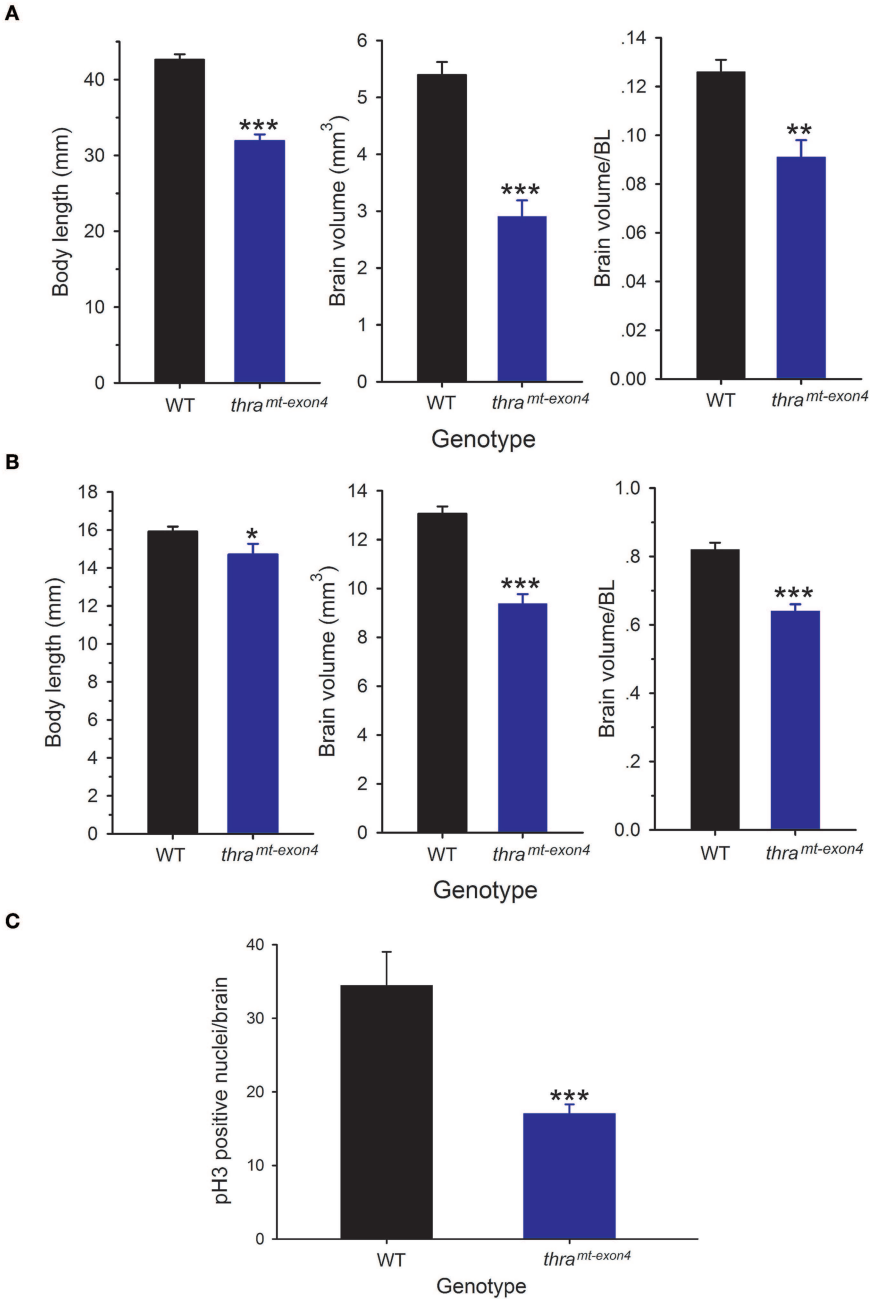

FIGURE 1 | Brain size and cell proliferation is reduced in *thra*$^{mt-exon4}$ animals. We measured body length and brain volume in wild type (WT) and *thra*$^{mt-exon4}$ animals at two stages of development. Brain volume was measured on fixed brains as described in the Materials and Methods. Bars are the mean ± SEM. **(A)** Comparison of body length and brain volume of WT and *thra*$^{mt-exon4}$ prometamorphic tadpoles (NF stage 56; $n = 6$/genotype). **(B)** Comparison of body length and brain volume of WT and *thra*$^{mt-exon4}$ newly metamorphosed frogs (NF stage 66; $n = 8$/genotype). **(C)** Comparison of phosphorylated histone 3 (pH3) immunoreactive nuclei in the brain of WT and *thra*$^{mt-exon4}$ NF stage 56 tadpoles ($n = 4$–6/genotype). *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$, unpaired Student's *t*-test.

The Vast Majority of Gene Regulation Responses to Exogenous T_3 Are Lost in *thra*$^{mt-exon4}$ Tadpole Brain

We used RNA sequencing to analyze T_3-dependent changes in gene transcription in tadpole brain after exposure to exogenous T_3 for 16 h, comparing WT and *thra*$^{mt-exon4}$ (NF stage 54). We chose this time point for analysis because it should capture most or all direct T_3-dependent gene regulation responses, including the delayed immediate early genes (23, 28, 29). We identified 3,481 unique mRNAs that were induced or repressed in WT vs. 419 in *thra*$^{mt-exon4}$ (*thra*$^{mt-exon4}$ only 12% of WT), and 178 genes that overlapped between the

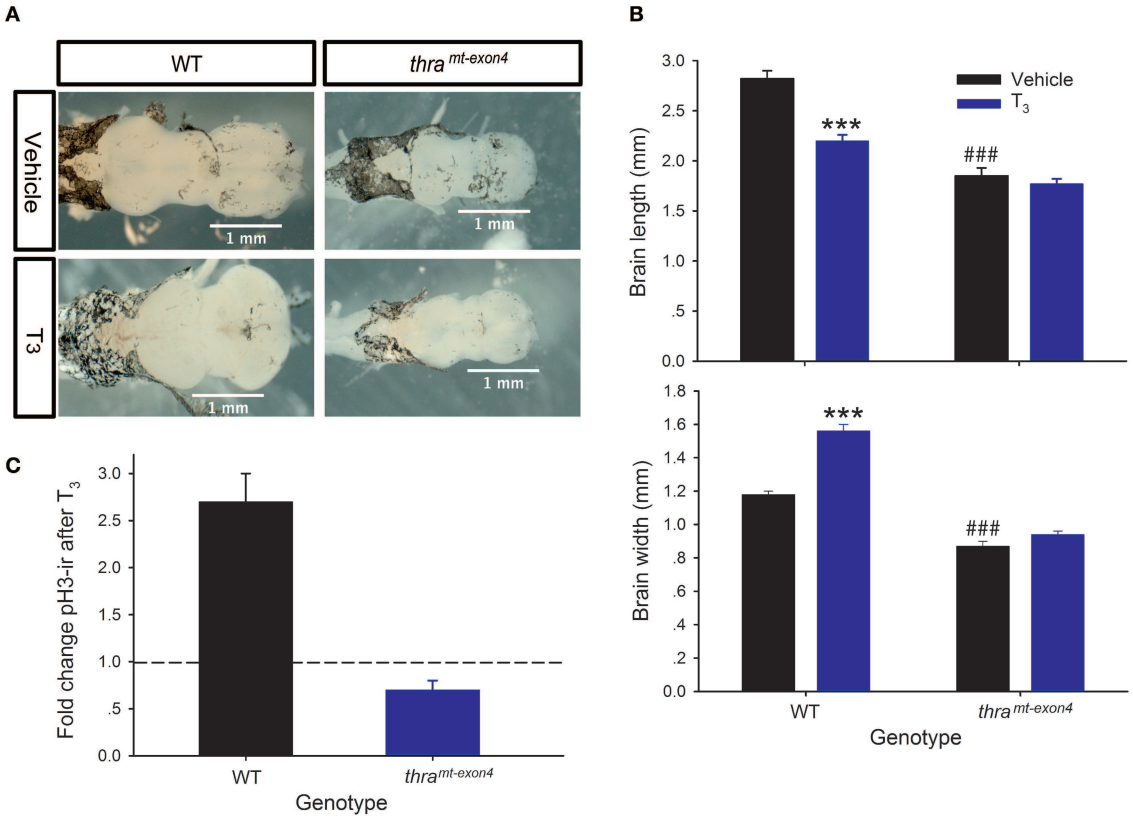

FIGURE 2 | Thyroid hormone receptor α is required for T_3-dependent morphological changes, and induction of cell proliferation in tadpole brain. We treated wild type (WT) and *thra*$^{mt-exon4}$ premetamorphic (NF stage 54) tadpoles with T_3 (5 nM) or vehicle added to the aquarium water for 42 h, then measured brain size and phosphorylated histone 3 immunoreactivity (pH3-ir) in the brain as described in the Materials and Methods. Bars are the mean ± SEM. **(A)** Exogenous T_3 caused dramatic morphological changes in the brains of WT, but not *thra*$^{mt-exon4}$ tadpoles. Shown are dorsal images of fixed tadpole brains. **(B)** Exogenous T_3 decreased length, and increased width of WT tadpole brain, but had no effect in *thra*$^{mt-exon4}$ animals (***$p < 0.0001$, unpaired Student's *t*-test). In vehicle-treated tadpoles, the baseline brain length and width were both smaller in *thra*$^{mt-exon4}$ compared with WT (###$p < 0.0001$, unpaired Student's *t*-test). **(C)** Treatment with T_3 strongly increased pH3-ir in WT ($p < 0.0001$, unpaired Student's *t*-test), but decreased it in *thra*$^{mt-exon4}$ tadpole brain ($p < 0.0001$). Shown is the fold change in total brain pH3-ir nuclei in each genotype. The dashed line indicates fold change of 1.0 = no change.

two genotypes (**Figure 3B**). That is, only 5.1% (178/3481) of genes regulated by T_3 in WT were regulated in the brain of *thra*$^{mt-exon4}$ animals. Interestingly, 241 genes (57.5% of all T_3 regulated genes in *thra*$^{mt-exon4}$) were regulated by T_3 only in *thra*$^{mt-exon4}$ animals. The RNA-seq dataset has been deposited in the Gene Expression Omnibus archive at the National Center for Biotechnology Information (GEO accession #GSE130816).

We investigated the kinetics of transcriptional activation by T_3 of three well-known direct T_3 response genes, *thrb*, *klf9*, and *thibz*. These genes are included in the 178 genes that overlap between WT and *thra*$^{mt-exon4}$. All three genes were strongly induced at the 8 h time point in WT tadpole brain, and their mRNAs remained elevated (i.e., they reached a maximum by 8 h: *thrb* and *klf9*) or continued to increase (*thibz*) through 42 h (**Figure 3C**). By contrast, although all three genes were induced by T_3 in the brains of *thra*$^{mt-exon4}$ tadpoles, the level of induction at 8 h was significantly lower than in WT for all three genes. The kinetics of gene induction, measured as the normalized mRNA level at four time points, and analyzed by linear regression, showed significantly slower kinetics for all three genes in *thra*$^{mt-exon4}$ animals (slopes of curves 0 vs. 8 h; *thrb*: WT = 0.95, *thra*$^{mt-exon4}$ = 0.37; *klf9*: WT = 0.94, *thra*$^{mt-exon4}$ = 0.34; *thibz*: WT = 0.56, *thra*$^{mt-exon4}$ = 0.09).

We also compared the induced and repressed gene lists between wild type and *thra*$^{mt-exon4}$ tadpoles. This showed that only 5.9% (109 of 1,855) of the genes induced by T_3 in WT were regulated after loss of *thra* (**Figure 4A**). Many of the well-known T_3-induced genes are in this gene list (**Supplemental Table 1**), suggesting that TRβ is sufficient to mediate T_3 regulation of a core set of TH response genes. However, in comparing the level of gene induction by T_3 of these 109 genes ($\log_2$ fold change >0.5), we found that the majority of these genes exhibited impaired responses to the hormone (**Supplemental Table 1**); four examples that we validated by RTqPCR are shown in **Figure 4B**. While exogenous T_3 caused significant increases in mRNA levels in both genotypes, the

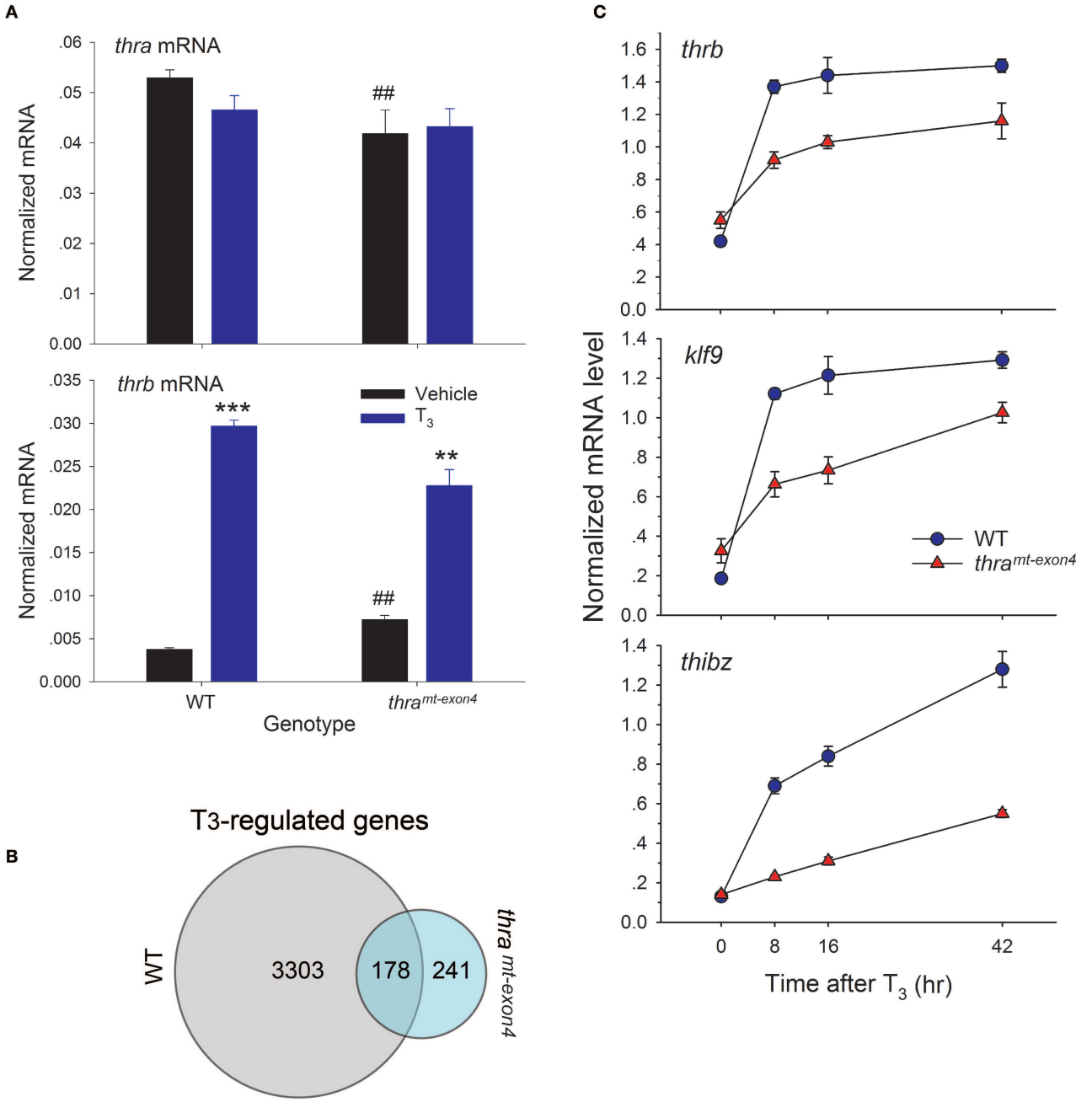

FIGURE 3 | Gene transcription responses to exogenous T_3 are impaired in *thra*$^{mt-exon4}$ tadpole brain. We treated wild type (WT) and *thra*$^{mt-exon4}$ premetamorphic (NF stage 54) tadpoles with T_3 (5 nM) or vehicle added to the aquarium water for different times, then measured target gene mRNA levels in brain (region of the preoptic area/thalamus/hypothalamus) by RTqPCR using absolute quantification (for *thra* and *thrb*, panel A) or relative quantification (genes in panel B). **(A)** Thyroid hormone receptor α is the major TR subtype expressed in tadpole brain. Comparison of baseline mRNA levels, and effects of T_3 treatment for 42 h on *thra* and *thrb* mRNA levels in WT and *thra*$^{mt-exon4}$ tadpole brain. Bars represent the mean ± SEM (n = 4/genotype/treatment). Note that the baseline (vehicle-treated) *thra* mRNA level is 14 times greater than *thrb* mRNA in WT tadpole brain. The baseline *thra* mRNA was significantly lower, while the *thrb* mRNA was significantly higher in *thra*$^{mt-exon4}$ compared with WT tadpole brain (##$p < 0.001$, Student's unpaired t-test). Treatment with T_3 had no effect on *thra* mRNA, but strongly induced *thrb* mRNA in both genotypes (**$p < 0.001$, ***$p < 0.0001$, Student's unpaired t-test). **(B)** Venn diagram representing the numbers of T_3-regulated genes determined by RNA-seq conducted on RNA isolated from brains of wild type and *thra*$^{mt-exon4}$ NF stage 54 tadpoles treated with or without T_3 for 16 h. **(C)** Time course of induction by T_3 in tadpole brain of mRNAs for three direct T_3 response genes, *thrb*, *klf9*, and *thibz* (note that these three genes are included in the 178 genes that overlap between WT and *thra*$^{mt-exon4}$). Points represent the mean ± SEM (n = 4/genotype/time point). The mRNA level for each of the genes was elevated at 8 h in both genotypes ($p < 0.001$, ANOVA), and the slope of each curve was significantly different between WT and *thra*$^{mt-exon4}$ ($p < 0.0001$, linear regression analysis).

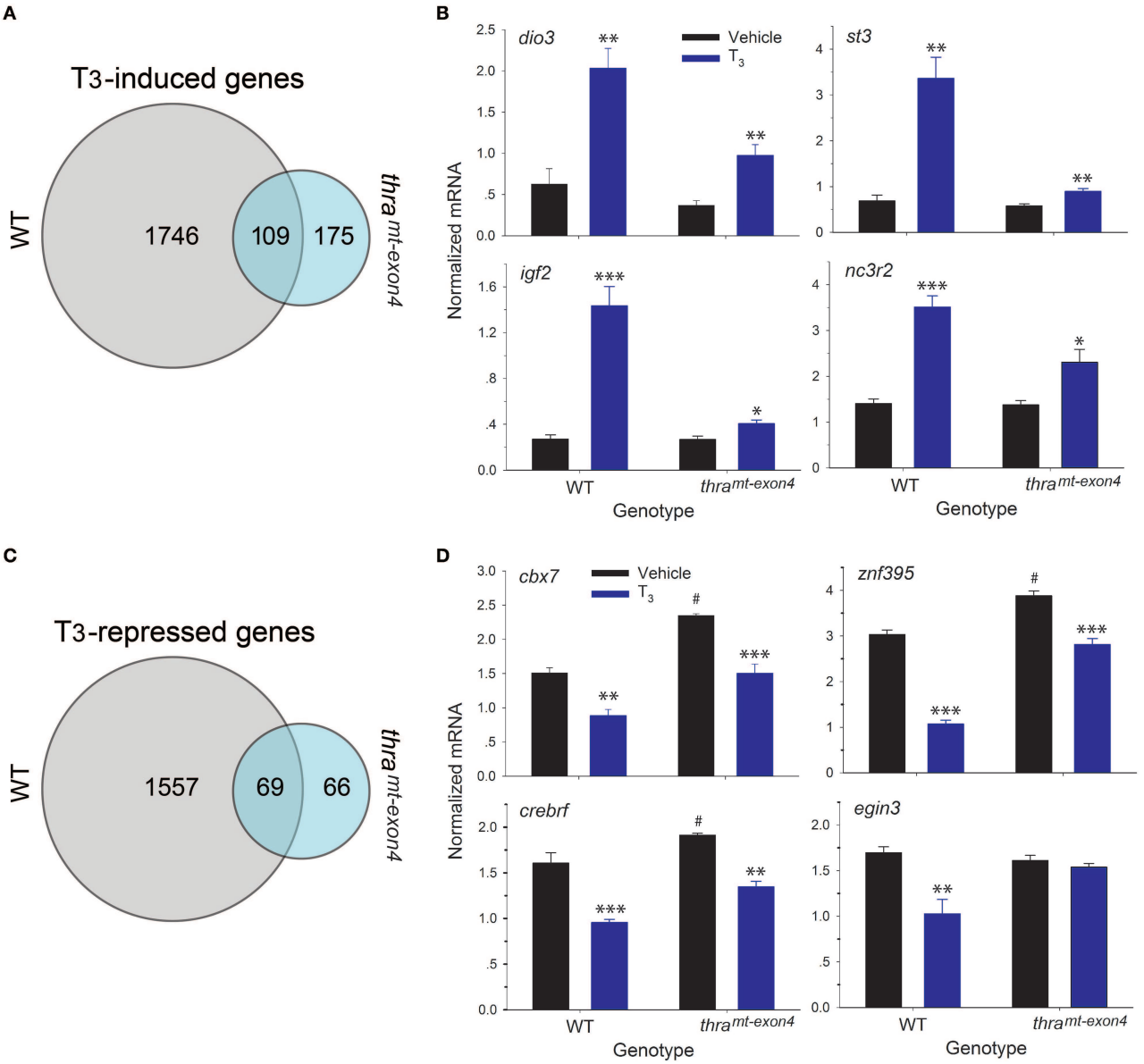

FIGURE 4 | RNA sequencing shows that the majority of gene regulation responses to exogenous T_3 are lost in $thra^{mt-exon4}$ tadpole brain. We treated wild type (WT) and $thra^{mt-exon4}$ premetamorphic (NF stage 54) tadpoles with T_3 (5 nM) added to the aquarium water for 16 h, then analyzed mRNA levels in brain (region of the preoptic area/thalamus/hypothalamus) by RNA-sequencing or RTqPCR using relative quantification. **(A)** Venn diagram representing the numbers of T_3-induced genes determined by RNA-sequencing conducted on RNA isolated from tadpole brains treated with or without T_3. **(B)** Validation of gene regulation for four T_3-induced genes found within the overlap between the WT and $thra^{mt-exon4}$ gene sets (109 genes). **(C)** Venn diagram representing the numbers of T_3-repressed genes determined by RNA-sequencing conducted on RNA isolated from tadpole brains treated with or without T_3. **(D)** Validation of gene regulation for four T_3-repressed genes found within the overlap between the WT and $thra^{mt-exon4}$ gene sets (69 genes). Bars represent the mean ± SEM (n = 4/genotype/treatment). For comparisons of vehicle with T_3 treated: $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$, Student's unpaired t-test. For comparisons of baseline mRNA levels (vehicle treated) among the two genotypes: $^{\#}p < 0.01$, Student's unpaired t-test.

level of gene induction was reduced in $thra^{mt-exon4}$ compared with WT (fold change; *dio3*: WT = 3.2, $thra^{mt-exon4}$ = 2.6; *st3*: WT = 4.9, $thra^{mt-exon4}$ = 1.5; *igf2*: WT = 5.3, $thra^{mt-exon4}$ = 1.5; *nc3r2*: WT = 2.5, $thra^{mt-exon4}$ = 1.7). There were 169 genes that were induced by T_3 in $thra^{mt-exon4}$ but not in WT.

For T_3-repressed genes, only 4.2% (69 of 1,626) of the genes repressed by T_3 in WT were regulated after loss of *thra* (**Figure 4C**). For genes common to WT and $thra^{mt-exon4}$, the majority had elevated baseline mRNA levels (fold change; *dio3*: WT = 3.2, $thra^{mt-exon4}$ = 2.6; *st3*: WT = 4.9, $thra^{mt-exon4}$ = 1.5; *igf2*: WT = 5.3, $thra^{mt-exon4}$ = 1.5; *nc3r2*: WT = 2.5,

$thra^{mt-exon4} = 1.7$). In comparing the level of gene repression by T_3 of these 70 genes (log_2 fold change > -0.5), we found that many of these genes exhibited reduced responses to the hormone (**Supplemental Table 1**); three examples that we validated by RTqPCR are shown in **Figure 4D**. While exogenous T_3 caused significant decreases in mRNA levels in both genotypes, the level of gene repression was slightly reduced in $thra^{mt-exon4}$ compared with WT (% change relative to baseline; *cbx7*: WT $= -70.2\%$, $thra^{mt-exon4} = -56\%$; *znf395*: WT $= -184\%$, $thra^{mt-exon4} = -38\%$; *crebrf*: WT $= -68\%$, $thra^{mt-exon4} = -42\%$). Also shown in **Figure 4D** is one example of a gene (*egin3*) that was repressed by T_3 in WT (% change relative to baseline: -65%), but was not regulated in $thra^{mt-exon4}$; also, this gene's baseline mRNA level was not different among the two genotypes. There were 50 genes that were repressed by T_3 in $thra^{mt-exon4}$ but not in WT.

Lastly, we identified 10 core cell cycle control genes in the list of genes regulated by T_3 in WT, and then looked for these genes in the T_3 regulated list from $thra^{mt-exon4}$ tadpoles. This showed several cyclins and cyclin-dependent kinases and an E2F transcription factor were regulated by T_3 in WT brain (**Table 2**). However, none of these genes were changed by T_3 in $thra^{mt-exon4}$ tadpole brain. We will provide a full gene ontology and pathway analysis of T_3-regulated genes in wild type tadpole brain in a future publication.

Thyroid hormone receptors, in the absence of ligand, are resident in chromatin bound to DNA, where they generate a closed chromatin state and repress gene transcription by recruiting corepressor complexes (8). Unliganded TRs function to repress gene transcription in premetamorphic tadpoles prior to endogenous TH synthesis, and this action is hypothesized to be important for maintaining the tadpole stage, a life cycle stage important for growth and dispersal (i.e., the dual-function model) (30). We hypothesized that loss of TRα would lead to de-repression of genes that are typically induced by T_3, which would be reflected in increased baseline mRNA levels for these genes in $thra^{mt-exon4}$ tadpoles. Our RNA-seq analysis found 212 genes whose baseline mRNA levels were increased in $thra^{mt-exon4}$ tadpoles compared to WT; however, only 74 of these genes (35%) were regulated by T_3 in WT (**Figure 5A**). Two examples of these genes analyzed by RTqPCR are shown in **Figure 5B** (fold change in baseline in $thra^{mt-exon4}$, *pim3*: 1.6; *traf3*: 1.5; see also *thrb* in **Figure 3A**). Of the 212 genes, 138 exhibited increased baseline mRNA levels $thra^{mt-exon4}$, but these genes were not regulated by T_3 in WT.

We also found 325 genes whose baseline mRNA level was reduced in $thra^{mt-exon4}$ tadpoles compared with WT (**Figure 5C**). Of the 325 genes, 78 (25%) corresponded to genes that were repressed by T_3 in WT. The remaining 247 genes with elevated baseline mRNA levels in $thra^{mt-exon4}$ were not regulated by T_3 in WT. Two examples of these genes analyzed by RTqPCR are shown in **Figure 5D**, one that was repressed by T_3 in WT (*tshb*: change in baseline in $thra^{mt-exon4} = -66\%$) and one that was not significantly affected by T_3 in WT (*cga*: change in baseline in $thra^{mt-exon4} = -51\%$). These two genes code for subunits of the glycoprotein hormone thyroid stimulating hormone (TSH; *tshb*—TSHβ, *cga*—common glycoprotein subunit alpha); note that the region of the tadpole brain that we dissected for RNA-seq analysis contained the pituitary gland.

TABLE 2 | Core cell cycle control genes regulated by T_3 in wild type by not in $thra^{mt-exon4}$ premetamorphic tadpole brain*.

Gene name	Gene symbol	Induced (I) or repressed (R)
Cyclin J	ccnj	I
Cyclin D2	ccnd2	I
Cyclin-dependent kinase 2	cdk2	I
Cyclin-dependent kinase 8	cdk8	I
Cyclin-dependent kinase 11b	cdk11b	I
Cyclin-dependent kinase 13	cdk13	I
E2F transcription factor 6	e2f6	I
Cyclin-dependent kinase 19	cdk19	R
Cyclin I	ccni	R
Cyclin G2	ccng2	R

*Data from RNA sequencing conducted on wild type and $thra^{mt-exon4}$ NF stage 54 tadpole brain (diencephalon) treated with or without T_3 (5 nM) for 16 h.

DISCUSSION

Here we show that TRα is required for T_3-dependent cell expansion in *Xenopus* tadpole brain during metamorphosis, which supports previous findings that used non-genetic approaches (10). The *thra* mRNA is 135 times more abundant than *thrb* mRNA, supporting that this is the major TR subtype in premetamorphic tadpole brain (10, 31). Furthermore, we found that TRα is required for 95% of the gene regulation responses to T_3 in premetamorphic tadpole brain. Genes that were regulated by T_3 in both genotypes exhibited impaired gene regulation responses in TRa knockout tadpole brain, both in their kinetics and magnitude of induction.

In the unliganded state, TRs are resident in chromatin where they recruit co-repressors to generate a compact chromatin state, and repress gene transcription. Hormone binding to TRs results in the exchange of co-repressors for co-activators, which generates an open chromatin environment promoting gene transcription (8). Current evidence supports that TRs have two general roles in tadpoles related to their repressor and activator functions, which is referred to as the dual function model (30, 32–34). During premetamorphosis, before the thyroid gland is developed and producing T_3, when existing TRs are in the unliganded form, TRs (predominantly TRα) repress gene transcription required for transformation of the tadpole into the juvenile adult, thereby maintaining the tadpole stage. When T_3 production rises during prometamorphosis, the aporeceptor is converted to a transcriptional activator, recruiting histone modifying enzymes that generate an open chromatin structure required for active transcription. The TRα is hypothesized to be necessary to establish competence of cells to respond to T_3 (30).

Earlier findings from our laboratory showed that mitosis in neurogenic zones of the tadpole brain increased dramatically at the beginning of metamorphosis, and reached a peak at NF stage 56, which correlated with rising plasma T_3 concentration (10). Neural cell proliferation can be induced

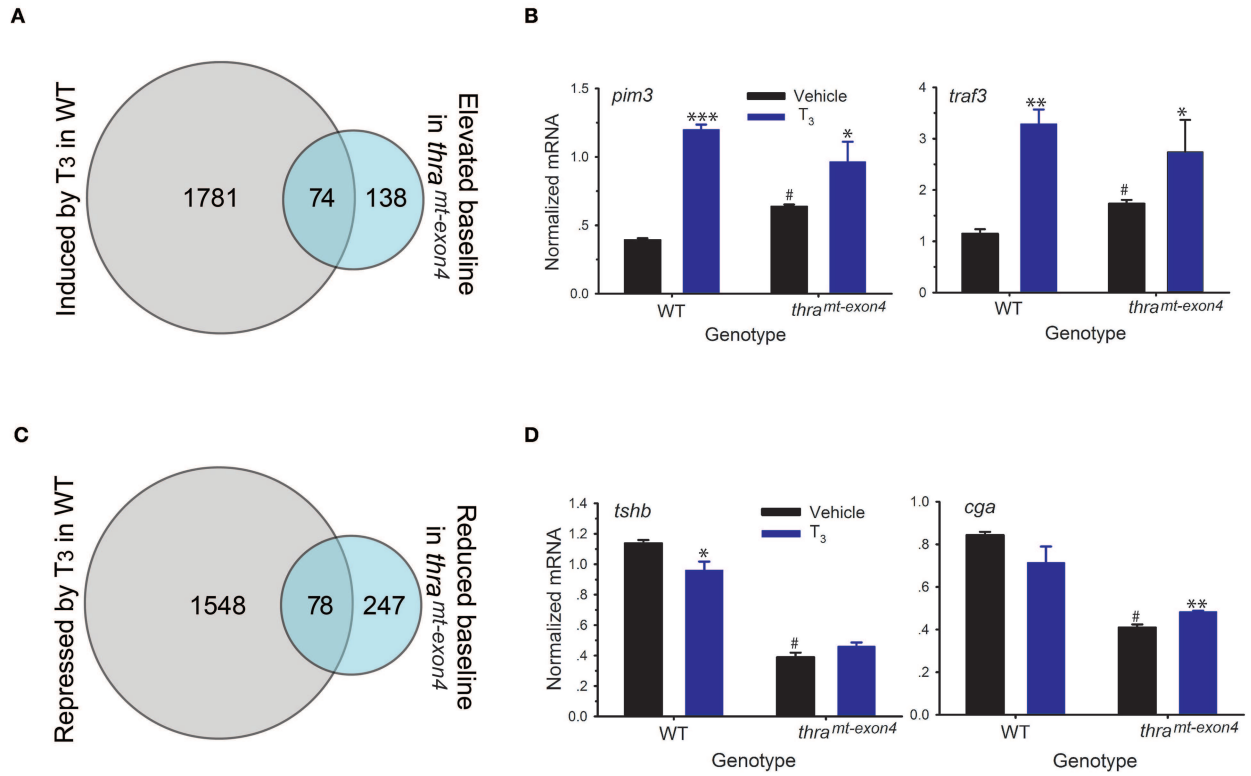

FIGURE 5 | Effects of loss of TRα on baseline gene transcription in premetamorphic tadpole brain. We treated wild type (WT) and $thra^{mt-exon4}$ premetamorphic (NF stage 54) tadpoles with T_3 (5 nM) added to the aquarium water for 16 h, then analyzed mRNA levels in brain (region of the preoptic area/thalamus/hypothalamus; including the pituitary) by RNA-sequencing or RTqPCR using relative quantification. **(A)** Venn diagram representing the number of T_3-induced genes in WT and the number of genes whose baseline mRNA level was elevated in $thra^{mt-exon4}$ tadpoles, determined by RNA-sequencing conducted on RNA isolated from tadpole brains treated with or without T_3. **(B)** Validation of gene regulation for two T_3-induced genes found within the overlap between the T_3 induced WT and elevated baseline in $thra^{mt-exon4}$ gene sets (74 genes). **(C)** Venn diagram representing the number of T_3-repressed genes in WT and the number of genes whose baseline mRNA level was reduced in $thra^{mt-exon4}$ tadpoles, determined by RNA-sequencing conducted on RNA isolated from tadpole brains treated with or without T_3. **(D)** Validation of gene regulation for two T_3-repressed genes found within the overlap between the T_3 repressed WT and reduced baseline in $thra^{mt-exon4}$ gene sets (78 genes). Bars represent the mean ± SEM (n = 4/genotype/treatment). For comparisons of vehicle with T_3 treated: $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$, Student's unpaired t-test. For comparisons of baseline mRNA levels (vehicle treated) among the two genotypes: $^{\#}p < 0.01$, Student's unpaired t-test.

precociously by the addition of T_3 to the aquarium water of premetamorphic tadpoles (NF stage 50–52); whereas, we found that cell proliferation in prometamorphic tadpole brain was reduced to premetamorphic levels by treatment with the goitrogen methimazole, which blocks the endogenous rise in plasma T_3. We also provided strong evidence that the action of T_3 on neural cell proliferation is mediated by TRα. For example, using ISHH and IHC, we found that TRα is highly expressed in neurogenic zones of tadpole brain, with strongest expression in proliferating cells. Furthermore, treatment with the TRα selective agonist CO23 induced mitosis. By contrast, we found that TRβ was expressed outside of neurogenic zones where neural cells undergo migration and differentiation, and treatment of tadpoles with two TRβ selective ligands, GC1 and GC24, at concentrations that preferentially activate TRβ, failed to induce cell proliferation (10, 35). These findings, using pharmacological agents combined with histochemistry, support the view that the TRα mediates hormone action on mitosis in tadpole brain.

Our current findings using mutant tadpoles deficient in TRα provide additional support for this model. We found that the $thra^{mt-exon4}$ tadpoles had proportionally smaller brains corrected for body size, both during prometamorphosis when cell proliferation is maximal, and in the newly metamorphosed juvenile frog. This may be explained by our finding that early prometamorphic $thra^{mt-exon4}$ tadpoles had fewer pH3-positive cell nuclei in their brains compared with WT. These differences between the two genotypes is likely due to complete impairment of T_3 dependent cell proliferation, since we found that premetamorphic $thra^{mt-exon4}$, unlike WT tadpoles, were totally resistant to exogenous T_3 effects on gross morphological changes, and pH3-ir in the brain. Taken together with our previous work (10), these new findings provide strong support for an essential role for TRα in T_3-dependent neurogenesis in *Xenopus* tadpole brain.

We saw a small (∼30%), but statistically significant decrease in pH3-ir after T_3 treatment in $thra^{mt-exon4}$ tadpole brain. This may be due to the upregulation of *thrb* in the absence of

the proliferative actions of liganded TRα, since several lines of evidence support that TRβ functions to limit cell proliferation, and induce cell differentiation. The level of *thrb* mRNA in tadpole brain is low or non-detectable during premetamorphosis, then rises during prometamorphosis in response to increasing plasma T_3 titer, and peaks at metamorphic climax when most cells in the brain have exited the cell cycle, migrated, and are in the process of differentiating and maturing (10, 23). Experiments using the TRβ specific T_3 analog GC1 support the view that TRβ functions in cell differentiation and apoptosis (10, 36). Thyroid hormone receptor β is expressed predominantly outside of neurogenic zones in tadpole brain (10), consistent with a role in cell migration and differentiation. We hypothesize that the reduction in pH3-ir that we observed in $thra^{mt-exon4}$ tadpole brain after treatment with T_3 is due to the actions of TRβ, which is autoinduced in $thra^{mt-exon4}$ tadpole brain as it is in WT (see **Figure 3A**). With the loss of TRα, the ability of T_3 to induce cell proliferation is lost; whereas, induction of TRβ may promote neural progenitors to exit the cell cycle and to differentiate.

Our results support that TRα is the major TR subtype expressed in tadpole brain, and its loss leads to profound deficits in gene regulation responses to T_3. Until now, analysis of gene regulation in *thra* mutant tadpoles was limited to a handful of known T_3 regulated genes (20, 22, 37, 38). Here we used RNA-seq after treatment with T_3 to provide an unbiased, transcriptomic analysis of the effects of loss of TRα on gene regulation in tadpole brain. This showed that only 5.1% of the genes regulated by T_3 in WT tadpole brain were similarly regulated in $thra^{mt-exon4}$ animals. Furthermore, genes that were regulated by T_3 in both genotypes exhibited impaired responses to the hormone in $thra^{mt-exon4}$ tadpole brain. For three well-known direct T_3 response genes (*thrb*, *klf9*, *thibz*), we saw impaired kinetics in response to the hormone, and also a reduction in the maximal level of gene induction. This suggests that, while this subset of genes can be regulated by TRβ, and indeed, their delayed response likely reflects the time required to autoinduce *thrb* and produce functional TRβ protein, TRα is required for the initial and maximal response to the hormone. Taken together, the data support that TRα is required for cells to become competent to respond to the hormone, and to maintain a sustained response (30).

Interestingly, we found that 57.5% of the genes regulated by T_3 in $thra^{mt-exon4}$ were only regulated in the mutant genotype and not in WT. This may be explained by TRα having a normal function of counteracting T_3 actions on gene transcription mediated by TRβ, which are unmasked when TRα is lost. This is supported by findings in the mammalian pituitary thyrotrophic cell line Tα1T, where TRα is recruited to the *Tshb* gene promoter only after knockdown of TRβ (39).

The loss of TRα also affected the baseline mRNA level of some genes (212 increased, 325 decreased in $thra^{mt-exon4}$). However, only 35% of the genes whose baseline mRNA levels were increased after loss of TRα were induced by T_3 in WT. The increase in baseline mRNA levels of T_3 regulated genes after loss of TRα is likely due to loss of the repressor actions of unliganded TRα, as has been shown previously (20, 22, 37, 38). Interestingly, baseline transcription (vehicle treated) of a subset of genes (325) was reduced in the absence of TRα, supporting that this unliganded nuclear receptor is necessary to support gene transcription in the absence of hormone. Of these genes, 24% were also repressed by T_3 in WT. Taken together, our findings support that TRα is not only required for gene regulation responses to T_3 in tadpole brain, but also plays an important role in maintaining normal baseline gene transcription. This supports studies conducted on other tadpole tissues that showed dysregulation of baseline gene transcription in TRα knockout tadpoles (20, 22, 37, 38).

Lastly, our RNA-seq analysis provides a molecular basis for the loss of T_3-dependent cell proliferation that we saw in $thra^{mt-exon4}$ tadpole brain. We found 10 core cell cycle control genes that were induced by T_3 in WT, but not in $thra^{mt-exon4}$ tadpole brain. These included several cyclins, cyclin-dependent kinases and an E2F transcription factor. Taken together, our findings support the view that the TRα is the major TR subtype expressed in tadpole brain, and it is required for T_3 action on cell proliferation. The vast majority of genes regulated by T_3 in tadpole brain, both induced and repressed, depend on TRα.

DATA AVAILABILITY

The datasets generated for this study can be found in GEO, GSE130816.

ETHICS STATEMENT

All procedures involving animals were conducted under an approved animal use protocol (PRO00006809) in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Michigan.

AUTHOR CONTRIBUTIONS

LW designed and conducted experiments, analyzed data, and helped to write the manuscript. CH conducted experiments, analyzed data, and helped to write the manuscript. CS analyzed data and helped to write the manuscript. RD designed experiments, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00396/full#supplementary-material

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REVIEW
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Thyroid Hormone Distributor Proteins During Development in Vertebrates

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Thyroid hormones (THs) are ancient hormones that not only influence the growth, development and metabolism of vertebrates but also affect the metabolism of (at least some) bacteria. Synthesized in the thyroid gland (or follicular cells in fish not having a discrete thyroid gland), THs can act on target cells by genomic or non-genomic mechanisms. Either way, THs need to get from their site of synthesis to their target cells throughout the body. Despite being amphipathic in structure, THs are lipophilic and hence do not freely diffuse in the aqueous environments of blood or cerebrospinal fluid (in contrast to hydrophilic hormones). TH Distributor Proteins (THDPs) have evolved to enable the efficient distribution of THs in the blood and cerebrospinal fluid. In humans, the THDPs are albumin, transthyretin (TTR), and thyroxine-binding globulin (TBG). These three proteins have distinct patterns of regulation in both ontogeny and phylogeny. During development, an additional THDP with higher affinity than those in the adult, is present during the stage of peak TH concentrations in blood. Although TTR is the only THDP synthesized in the central nervous system (CNS), all THDPs from blood are present in the CSF (for each species). However, the ratio of albumin to TTR differs in the CSF compared to the blood. Humans lacking albumin or TBG have been reported and can be asymptomatic, however a human lacking TTR has not been documented. Conversely, there are many diseases either caused by TTR or that have altered levels of TTR in the blood or CSF associated with them. The first world-wide RNAi therapy has just been approved for TTR amyloidosis.

Keywords: albumin, development, evolution, phylogeny, thyroid hormones, thyroxine-binding globulin, transthyretin, vertebrates

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THYROID HORMONES

Thyroid hormones (THs) are fundamentally involved in the regulation of growth, development, and overall metabolism, particularly of the CNS. Despite being amphipathic in structure, THs are lipophilic compounds and readily partition between the lipid phase and the aqueous phase with a ratio of about 20,000:1 (1). Therefore, THs are not freely diffusible in the aqueous environments of the blood and cerebrospinal fluid (CSF).

THs are considered evolutionarily "old" hormones, as THs and their derivatives impact the metabolism of not only vertebrates but also bacteria (2), ascidians, tunicates, and other invertebrate species [for review see Holzer et al. (3)]. Furthermore, the endostyle of tunicates can incorporate iodine into tyrosine residues which are then incorporated into proteins, rendering the endostyle the functional precursor (from a TH perspective) to the thyroid gland (4). In amphibians, reptiles, birds, and mammals, THs are synthesized in the thyroid gland, which is a discrete gland located at

the base of the neck. In fish, however, the shape and location of the thyroid gland varies considerably between species e.g. diffuse follicles around the ventral aorta (cyclostomes), or near the branchial arteries of the gills (some teleosts) or a compact gland near the branchial arch (elasmobranchs) (5).

Following synthesis in the thyroid gland (or follicles, as for fish), the THs are secreted via TH transmembrane transporters into the blood. In vertebrate species studied to date, most TH secreted by the thyroid is in the form of 3,3′,5,5′-tetraiodo-L-thyronine (thyroxine; T4) and less is in the form of 3,3′5-triiodo-L-thyronine (T3) (6) (see **Figure 1**). Due to the lipophilicity of THs (as mentioned above), they preferentially partition into the lipid environment of membranes (11). However, this can be counteracted with the presence of plasma proteins that bind THs and enable distribution of the THs from their site of synthesis to their target cells throughout the body. Thus, these proteins are termed "TH Distributor Proteins" (THDPs) (12). In humans, the THDPs are albumin, transthyretin (TTR), and thyroxine-binding globulin (TBG) (see **Figure 1**). Greater than 99.7% of THs in the blood of mammals is bound to THDPs, rendering a very small fraction in the free form. Because only the free (non-protein bound) THs can enter cells, it is very important that the THDPs regulate the amount of free THs in the blood (and CSF).

THYROID HORMONE DISTRIBUTOR PROTEINS (THDPs)

In humans, the main THDPs are albumin, TTR, and TBG. Albumin is a single polypeptide chain protein with a molecular mass of about 67 kDa that is rich in alpha helical structure (see **Figure 1**). At physiological pH, albumin has a heart shape globular structure but under acidic pH adopts an elongated "cigar conformation" (these conformational changes are reversible). Albumin comprises about half the total protein in blood (∼40 g/l) and can bind many compounds weakly: THs, fatty acid, drugs etc. (13). TTR is a 55 kDa homotetrameric protein rich in beta sheet structure and can be considered a dimer of dimers which come together with a central channel that has two TH binding sites [Blake et al. (14); see **Figure 1**], although under physiological conditions only one site is filled due to negative co-operativity (9). TTR is present in healthy adult blood at about 0.25 mg/l and can also bind up to two molecules of retinol-binding protein, which in turn bind retinol (15). Thus, TTR distributes two ligands for nuclear hormone receptors. TBG is a 54 kDa monomeric protein that has only a single site for TH binding and is highly glycosylated (see **Figure 1**). Whilst due to its structure TBG can be considered a serpin (serine protease inhibitor), it does not actually function as a serpin. Albumin, TTR, and TBG each have differing affinities and on/off rates for T4 and for T3 [for greater details and discussion, see (16)]. In general, albumin binds quite weakly, TTR has intermediate affinity and TBG has the highest affinity for THs. In mammals, each of these three THDPs binds T4 with higher affinity than T3. This provides a buffering like system, for maintaining the free level of THs [see (12)]. The range is from the concentration of free T4 in blood up to the maximum solubility of T4 at pH 7.4. Some text books claim that the reason for hydrophobic signal molecules being bound to protein in the blood is due to poor solubility of the hydrophobic signal compound in the blood, but this is not true. The maximum solubility of T4 at pH 7.4 is 2.3 μM (17) i.e., 100,000 times the concentration of free T4 in human blood (24 pM). The function of THDPs in enabling TH distribution was shown by a set of elegant experiments by Mendel et al. (11): rat livers perfused with T4 in the absence of THDPs resulted in T4 partitioning into the first cells they came in contact with; whereas when livers were perfused with T4 together with THDPs, this resulted in a uniform distribution of T4 throughout the liver and T4 also in the perfusate.

Albumin and TBG have higher affinity for T4 than for T3 in all species studied. However, this is not the case for TTR: in all studied species of birds, reptiles, amphibians, and fish [with the notable exception of sea bream, where TTR had similar affinity for T3 and T4 (18)],TTR has higher affinity for T3 than for T4 (19–24). Only in mammals, does TTR have higher affinity for T4 than for T3 [for a detailed discussion on how and why this may have occurred during evolution, see (25)].

DISTRIBUTION OF THDPs IN ADULT VERTEBRATES

Not all vertebrates have all three THDPs in their blood. There are clear patterns based on various groups of vertebrates, during both ontogeny and phylogeny. The traditional way of identifying THDPs in blood of various species was analyzing serum or plasma directly for radioactively-labeled T3 or T4 binding to proteins. The discussion in this paragraph relates to data collected in that way. Of the ∼150 species of adult vertebrates studied, all had albumin as a THDP in their blood (26–30). For some groups of animals, albumin was the only THDP. In general: fish, amphibians, and reptiles and monotremes (echidna and platypus) and some polyprotodont marsupials. For another set of animals, both albumin and TTR were present in blood. In general: birds, diprotodont marsupials, and some eutherians ("placental mammals"). The final set had all three THDPs. This group only comprised some eutherian mammals but we could not discern a clear pattern within eutherians for presence/absence of TBG [previous studies had suggested TBG was present in "larger mammals" but this no longer holds true e.g., (28)]. In general, there has been an increase in TH distribution capacity during vertebrate evolution, both in the number of THDPs and in light of each "new" THDP having higher affinity for THs than the previous i.e., albumin (original THDP with weak affinity for THs) then TTR (second THDP appearing during evolution, with higher affinity for THs than albumin) then TBG [third THDP appearing, with higher affinity for THs than TTR; see (31)].

More recently, with the ever-expanding number of genomes and transcriptomes that are accessible via publicly available databases, it has been possible to identify genes and mRNA or expressed sequence tags (ESTs) corresponding to proteins of interest. This approach can be valuable for identification of low

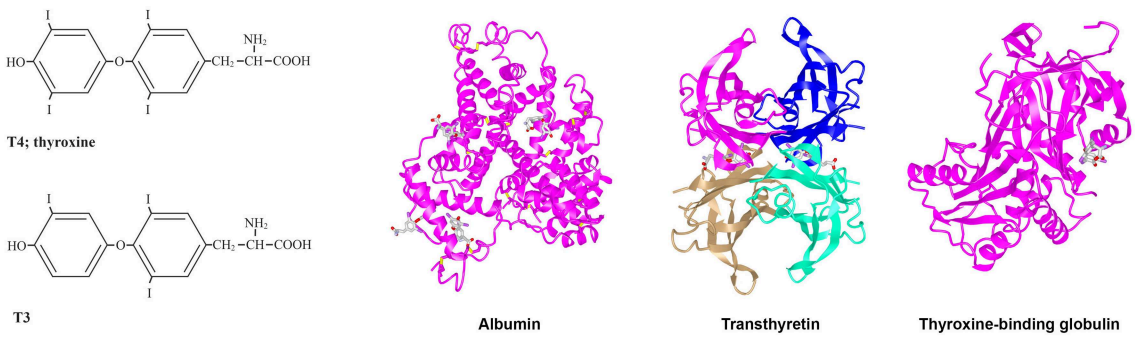

FIGURE 1 | Structures of T4, T3, albumin, TTR, and TBG. **(Left)** Structures of T3 and T4. **(Right)** 3D protein structures and T4 binding sites of the three THDPs in humans: albumin (7), transthyretin (8), and thyroxine-binding globulin (7–10).

abundance proteins. Whilst this can be an alternative approach to identification of proteins being synthesized in a given tissue of a particular species at a defined stage of life, this can also generate many "false positives" if only considered superficially. It is very time-consuming to check each transcript for complete integrity (full length, lack of internal stop codons etc.). Never the less, this approach has been used successfully to identify TTR in many species of fish (32). Given the huge diversity of fish (>25,000 species), the data presented in **Table 1** that were collected the "traditional" way, are probably overly simplistic and not truly representative of the actual situation in nature. "Omics" approaches are required to further investigate the distribution of TTR synthesis in each group of fish and the corresponding ontogenic analyses. Furthermore, the variation in piscine TTRs to date has already revealed that some fish TTRs bind T3 with higher affinity than T4 whereas others bind both ligands with similar affinity (see above).

For those animals with three THDPs, TTR is responsible for most of the delivery (bioavailability) of T4 (40). This is in contrast to the previously held belief that because TBG binds about 75% of T4 in blood (albumin binds about 10% and TTR binds about 15%), that TBG is responsible for the delivery of T4. We think of the situation as analogous to Goldilocks and the Three Bears (41): albumin binds so weakly that it is not very efficient in distributing T4; TBG binds so tightly that it is not efficient in releasing T4 (more like a storage reservoir in the blood); but due to the combination of off rates and capillary transit times, TTR is responsible for most of the delivery of T4 to tissues (42).

TABLE 1 | Vertebrate species with additional THDP with higher affinity for TH in blood during development.

	Development	Adult	Reference (for each species)
Fish: *Sparus aurata*	TTR	Albumin	(22, 33)
Oncorhynchus massou	Albumin		(34)
Salmo salar			(35)
Oncorhynchus tshawytscha			(35)
Amphibians: *Xenopus laevis*	TTR Albumin	Albumin	(36)
Rana catesbeiana			(20)
Reptile: *Crocodylus porosus*	TTR Albumin	Albumin	(35)
Mammals			
Marsupials—Polyprotodonta: *Sminthopsis crassicaudata*	TTR Albumin	Albumin	(35)
Diprotodonta: *Macropus eugenii*	TBG TTR Albumin	TTR Albumin	(35) (37)
Eutherians—Rodentia: *Rattus norvegicus*	TBG TTR	TTR Albumin	(38)
Mus musculus	Albumin		(39)

Adapted from Richardson et al. (35).

THDPs IN BLOOD DURING VERTEBRATE DEVELOPMENT

The pattern of THDPs in blood during development differs from that in adults (see below). In general, it was revealed that there was an additional THDP present during specific stages of development, compared to adulthood. For example, in two species of salmon, where albumin is the only THDP in adults, TTR was also present at smoulting (35) and in juvenile fish (22, 33, 34). Whereas, adult amphibian had only albumin as a THDP in blood, around the time of metamorphosis TTR was also present (20, 23, 36). Juvenile saltwater crocodiles were found to have TTR in addition to albumin, and the polyprotodont marsupial (fat-tailed dunnart) had TTR in addition to albumin during development (35). The diprotodont marsupial tammar wallaby had a TBG-like protein during development in addition to the albumin and TTR present in adults (37). All vertebrates have a transient surge in TH levels at a specific stage in

development (6). The additional THDPs appear to coincide with the elevated TH levels in blood (35). This provides an augmented TH distribution capacity at the time when TH levels in blood are elevated during development (see **Table 1**). In this table, we consider only plasma proteins. However, whilst plasma proteins are responsible for the majority of TH distribution in homeotherms, some fish are known to have the bulk of their THs distributed in the blood by lipoproteins (43) and a small proportion of THs in human blood (44). Oviparous animals (including some fish, amphibians, reptiles, birds, monotremes, and some invertebrates) also synthesize another lipoprotein: vitellogenin, an egg yolk precursor protein synthesized in the liver. Vitellogenin is a TH binding protein. In females, vitellogenin levels cycle according to estrogen levels. However, TH can regulate the levels of vitellogenin by inducing estrogen receptor alpha (45).

THDPs IN THE CSF

THs must cross the blood-brain barrier or the blood-CSF barrier in order to enter the CNS. These two barriers have been studied most extensively in mammals but some information is known about other vertebrates also. However, to the best of our knowledge, the quantitative contribution of each pathway (crossing the blood-brain barrier or crossing the blood-CSF barrier, relative to the other) for TH entering the brain is not yet known.

The blood-CSF barrier is formed by the tight junctions between the epithelial cells of the choroid plexus. The choroid plexus is a villous structure located in the lateral, third and fourth ventricles of the brain and is responsible for secreting about 70% of the CSF (46). In adults, the major protein synthesized and secreted by the choroid plexus from studied species of mammals (eutherians, marsupials, and monotremes), birds, and reptiles is TTR (24, 29, 47–50). This TTR is secreted toward the CSF and not into the blood (51) and has been implicated in moving T4 (but not T3) from the blood across the choroid plexus into the CSF (1, 52, 53). The major protein synthesized and secreted by the choroid plexus of amphibians is a lipocalin, specifically: prostaglandin D synthetase (54, 55) also known as Cpl1 (55) and β-trace (56). This protein has a calyx structure which could be used for binding small hydrophobic molecules and thus could have been a functional precursor to TTR, although possibly not transporting THs.

TTR GENE EXPRESSION IN THE CHOROID PLEXUS DURING DEVELOPMENT

In those species studied, the choroid plexus has the highest concentration of TTR mRNA per tissue weight compared to other tissues in the body e.g., 11- to 22-fold higher than in the liver [see (57)]. However, the timing for the maximal TTR mRNA levels in the choroid plexus differs between animals. Animals who are fairly independent soon after birth/hatching (e.g., chickens and sheep) are described as precocial and their brains are further developed at birth compared to altricial animals, whose brains are less developed at birth and are dependent on their mothers (e.g., rats, mice, marsupials). Precocial animals were found to have the peak of TTR mRNA in their choroid plexus before birth, whereas altricial animals had the peak of TTR mRNA in the choroid plexus after birth (58). For both groups of animals, the peak in TTR mRNA is just prior to the maximal growth rate in the brain. Given that the blood-brain barrier starts to develop when the first blood vessel grows into the brain (59) and that the choroid plexus develops faster than other parts of the brain, producing most of the CSF (and thus regulating its composition), and the peak of TTR mRNA just prior to the maximal growth rate of the brain, it follows that the choroid plexus-derived TTR could have a significant role in moving T4 from the blood into the CSF (57).

Whilst TTR is the only THDP known to be synthesized in the CNS (to the best of our knowledge, albumin and TBG are exclusively synthesized in the liver), this does not mean that albumin and TBG are absent from the CSF. In adult mammals, the protein concentration of the CSF is about 0.43 g/l compared to that in blood of about 70 g/l (60). Plasma proteins are present in the CSF at a concentration inversely proportional to their Stokes radius (61). Thus, whilst albumin is not synthesized in the CSF, it is present in the CSF; similarly for TBG in species where TBG is synthesized by the liver. It follows that, for example, although the choroid plexus of fish and amphibians do not synthesize TTR, their CSF would contain some albumin. Similarly, the CSF in reptiles and birds contains albumin and TTR; and the CSF of humans contains albumin, TTR, and TBG. However, the ratio of TTR to albumin in the CSF is very different to that in the blood: whereas in the blood albumin comprises ∼50% total protein and TTR comprises ∼0.4% total protein, in the CSF albumin comprises ∼40% total protein and TTR comprises about 4% total protein (45). Thus, the TTR to albumin ratios and TH distribution kinetics would differ significantly. TTR is the main carrier of TH in the CSF (62, 63). Presumably, during the peak in TH concentration in blood during development, when the liver is synthesizing an "additional" THDP, some of that protein will enter the CSF. To date, it is unknown if TH levels in the CSF peak when (or soon after) the TH levels in the blood peak. Indeed, it might not be a reasonable question to consider, as the concentration of proteins and other molecules is not consistent throughout the CSF (46). In contrast to the blood, which mixes within minutes and is fairly homogeneous, the CSF flows in a directional "pipeline-like" manner and measurements of concentrations of its components differ depending on the sampling site [see (12)].

BIOAVAILABILITY OF THs TO TARGET TISSUES

THs can exert both genomic and non-genomic actions. Regardless of which type of action a given TH will have, it is still required to get from its site of synthesis to its target tissue/cell and this is mediated via the THDPs: TTR, albumin, and TBG (depending on the species and stage of development).

Once THs have arrived at their target cell and have dissociated from the THDP, they are able to enter cells via TH

transmembrane transporter proteins. These TH transmembrane transporters belong to the family of solute carriers and those known to move THs into and out of cells are the monocarboxylate transporters MCT8 and MCT10; L-amino acid transporters LAT1 and (depending on the species) LAT2; and organic anion transporter peptide OATP1C1 [for review see (64)]. Of these, only MCT8 and MCT10 are exclusive for the transmembrane transport of THs.

As mentioned above, the majority of TH secreted from the thyroid gland is in the form of T4 and around 80% of T3 is generated by local deiodination in target cells e.g., various regions of the brain produce differing proportions of T3 via local deiodination (65). Deiodination is carried out by a family of deiodinase enzymes, each of which can remove a specific iodine atom from a TH. Deiodinases can be classified by their broad reactions as either Outer Ring Deiodinases or Inner Ring Deiodinases, according to the position of the iodine atom being removed. Deiodinases can also be classified via their structures (amino acid sequences), locations and substrate preferences: Dio1, Dio2, and Dio3 [for review see (66)]. Deiodinases can either activate or inactivate THs within a cell. Whereas, genomic pathways are regulated mainly by T3, non-genomic pathways may be regulated by a greater number of TH derivatives (67).

IMPACT OF THDPs IN HUMAN DISEASE

Albumin, TTR, and TBG are negative acute phase plasma proteins (68) i.e., following stress, illness, surgery, or injury, their rates of synthesis in the liver decrease. This is thought to result in a transient increase in free TH in blood, which can then enter cells and direct anabolic reactions to restore health and homeostasis. Humans lacking either albumin (www.albumin.org) or TBG [see (69)] have been reported and were essentially without overt symptoms. Until now, no human lacking TTR has been reported. Could lack of TTR be incompatible with human life? Could this be due to TTR being the main protein synthesized and secreted by the choroid plexus or due to TTR being the main source of delivery of THs to tissues?

On the other hand, albumin and TBG have very few diseases associated with them: analbuminaemia [(70), www.albumin.org] and a variant of TBG in Australian Aborigines which has low affinity for THs (71), whereas TTR has major diseases associated with it: the various forms of TTR amyloidosis. Familial Amyloidotic Polyneuropathy (FAP) is a late onset autosomal dominant form of amyloidosis. More than 100 point mutations have been associated with causing TTR FAP (72). This is a significant number, as the polypeptide chain has only 127 amino acids in total (TTR is a homo-tetramer). In addition, wild type TTR can also form amyloid: Senile Systemic Amyloidosis. This occurs most frequently in the hearts of elderly men (73). The cause for wild type TTR to spontaneously form amyloid in the heart is currently unknown.

The first approved anti-TTR amyloid drug to come onto the market was Tafamidis (trade name Vyndaqel), a compound designed by Jeff Kelly and colleagues (74). Very recently, Patisiran (trade name ONPATTRO) has been approved by the European Commission and the United States Food and Drug Administration for the treatment of TTR amyloidosis. This is the first world-wide approved RNAi therapeutic! Apparently, patients receiving this RNAi therapy did not show deficiencies in thyroid or vitamin A metabolism. This could be due to only a small proportion of TTR circulating in blood having a TH or RBP-retinol bound.

An increasing body of knowledge is building around associations of altered TTR concentrations in the blood and/or CSF and a variety of diseases such as Alzheimer's Disease (75–77), rheumatoid arthritis (78, 79), schizophrenia (80, 81), preeclampsia (82–84), Guillain-Barre syndrome (85–87), and depression (88), and references in Alshehri et al. (41).

CONCLUSION

Whether TH actions are via genomic or non-genomic pathways, THs need to get from their site of synthesis to their sites of action via THDPs in the blood and CSF. The network of THDPs is augmented during the developmental surge in THs in blood, providing increased distribution capacity. The THDPs have distinct profiles during both ontogeny and phylogeny, such that very fine regulation of free TH available to enter cells is highly controlled. In adult humans, lack of albumin or TBG are tolerated, yet a single point mutation in TTR can lead to disease. Humans lacking TTR have not yet been identified. The implication is that lack of TTR in humans is incompatible with life. This could be due to TTR being responsible for most delivery of TH to tissues or due to its role in moving TH from the blood into the CSF via the choroid plexus. Abnormal levels of TTR in humans are increasingly being associated with a variety of diseases. It is unknown if the altered TTR levels are a cause or a consequence of these diseases.

AUTHOR CONTRIBUTIONS

Overall structure was suggested by SJR. SAR and IG wrote most drafts with guidance from MP and NO. SJR edited the final version.

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REVIEW
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Pesticides With Potential Thyroid Hormone-Disrupting Effects: A Review of Recent Data

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Plant Protection Products, more commonly referred to as pesticides and biocides, are used to control a wide range of yield-reducing pests including insects, fungi, nematodes, and weeds. Concern has been raised that some pesticides may act as endocrine disrupting chemicals (EDCs) with the potential to interfere with the hormone systems of non-target invertebrates and vertebrates, including humans. EDCs act at low doses and particularly vulnerable periods of exposure include pre- and perinatal development. Of critical concern is the number of pesticides with the potential to interfere with the developing nervous system and brain, notably with thyroid hormone signaling. Across vertebrates, thyroid hormone orchestrates metamorphosis, brain development, and metabolism. Pesticide action on thyroid homeostasis can involve interference with TH production and its control, displacement from distributor proteins and liver metabolism. Here we focused on thyroid endpoints for each of the different classes of pesticides reviewing epidemiological and experimental studies carried out both in *in vivo* and *in vitro*. We conclude first, that many pesticides were placed on the market with insufficient testing, other than acute or chronic toxicity, and second, that thyroid-specific endpoints for neurodevelopmental effects and mixture assessment are largely absent from regulatory directives.

Keywords: thyroid hormones, pesticides, endocrine disruptors, organochlorine, organophosphates, pyrethroids, neonicotinoids, neurodevelopment

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BACKGROUND

Plant Protection Products (PPPs) (herein referred to as pesticides) are used to control noxious pests and disease causing organisms including insects, fungi and unwanted plants (1) (EU Commission[1]). A major shift from inorganic pesticides, such as lead arsenate, to synthetic organic chemicals occurred in the late 1940s. These approaches revolutionized pest control efficiency to such a degree that synthetic pesticides were rapidly integrated into the ongoing industrialization of agriculture, public health programs and use by individuals. The revolution in industrial farming increased crop production and quality, with ensuing global production of synthetic pesticides escalating at a yearly rate of 10% from the 1950s, reaching upwards of 3 million tons by the turn of the century and 4 million tons in 2016 (2–4). Along with increasing production volumes, numerous synthetic pesticides appeared on the market, often due to development of acquired resistance by target species and/or regulatory restrictions brought by health or environmental concerns. The first synthetic pesticides introduced in the 1940s were the **organochlorine pesticides (OCP)**, followed by **organophosphates (OP)** in the 1960s and **carbamates** in the 1970s. **Pyrethroid** production

[1]https://ec.europa.eu/food/plant/pesticides_en

began in the 1980s, with the more recent compounds such as **neonicotinoids** and **phenylpyrazoles** being added in the 1990s (5).

Despite the early warnings of Rachel Carson in 1962, the intensity of pesticide use placed an increasing burden on the environment with limited consideration for the full extent of consequences. Acute or chronic exposure to a variety of pesticides occurring through cutaneous, respiratory or dietary routes are supported by current human biomonitoring efforts as well as studies on wild-life (6, 7). Simultaneously, a substantial body of evidence confirming the adverse health effects and ecological impact of PPP (including biocides) has been accumulating since the 1960s when pesticides, once heralded as a "magic bullet," made usage more controversial.

Carson's premonition of the impact of the organochlorine insecticide DDT[2] on eggshell thinning in birds was later shown to be caused by endocrine-mediated effects, specifically, the estrogenic action of DDT's main metabolite p,p′-DDE[3] (8, 9). The term "endocrine disruptor" gained traction in the early 90s when used to describe the interference of several man-made chemicals, including certain pesticides, on estrogen, androgen, thyroid and steroid pathways (10, 11). While the acute toxic effects of pesticides on target, non-target species or occupational workers are quite well-documented (12–14), the effects of low doses relevant to concentrations assessed in foodstuffs and the environment are relatively neglected. Traditionally, regulatory systems rely on dose-response models and determine a threshold for safe levels of exposure, but potentially overlook the presence of non-monotonic dose-response curves below the toxicological no-observed-adverse-effect level (NOAEL) (15). This issue is particularly important given that many pesticides act as endocrine disrupting chemicals (EDCs), capable of interfering with natural hormones even at low doses and hence affect the normal development and function of multiple organs (16–19). Additionally, EDCs may exert specific effects during sensitive time-windows of development with adverse health outcomes occurring later in life—bringing the matter of timing of exposure during vulnerable periods, such as prenatal or postnatal life, increasingly into focus in both regulatory and fundamental research (20, 21).

OVERVIEW OF THE HYPOTHALAMUS-PITUITARY-THYROID AXIS

During the last 30 years insights into the role of thyroid hormones (TH) at different levels of biological organization have contributed to a better physiological understanding of their function. In humans, TH is essential for the development of the brain, inner ear, eye, heart, kidneys, bone and skeletal muscle, amongst other tissues (22), but also for fine regulation of energy metabolism (23). The essential role of TH during vertebrate development has been amply reviewed (24–26), however neurodevelopment warrants special attention. Strikingly, THs are crucial for normal brain development which is dramatically illustrated by cretinism, a syndrome induced by a severe lack of TH or iodine during embryo-fetal and post-natal development (27). In addition, disruption at any of the multiple levels along the hypothalamus-pituitary-thyroid axis (HPT-axis) axis during this vulnerable period of development, particularly early pregnancy (28, 29) can lead to deleterious effects on offspring IQ. These effects can be exerted through modifications of TH levels in the blood stream and within specific tissues, with subsequent modulation of TH-dependent actions in the nervous system (transcription, proliferation, neurogenesis, gliogenesis, migration), resulting potentially in altered brain structure and behavior (19, 28)[4].

To summarize TH production and physiology very succinctly, TH synthesis in the thyroid colloid requires iodine, which circulates as iodide ion. In the thyroid gland, iodide is combined with the amino acid tyrosine to produce thyroxine (T4) or triiodothyronine (T3). Synthesis of THs (T4 and T3) is tightly controlled by the HPT-axis. The hypothalamus produces thyrotropin-releasing hormone (TRH), which triggers production of thyroid stimulating hormone (TSH) by the anterior pituitary. TSH is released into the bloodstream and binds to receptors on thyroid follicular cells of the thyroid gland. TSH stimulates iodide uptake mediated by the sodium/iodide symporter (NIS). After oxidation of iodide by thyroperoxidase (TPO), organification of iodide which consists of incorporation of iodide into thyroglobulin (Tg) is required to produce precursors of T3 and T4 (30). THs exert negative feedback on their upstream regulators thereby controlling hormone levels. Increases in T3 or T4 inhibit production of TSH and TRH by the pituitary and hypothalamus, respectively. Inversely, as T3 and T4 decrease, TSH and TRH genes are activated (31). In the bloodstream, THs are almost entirely bound to serum distributing proteins, such as transthyretin (TTR), albumin or thyroxin-binding globulin (TBG). Less than 0.001% of total T4 and T3 are available as free T4 or T3 (FT4 or FT3) and can enter cells through trans-membrane-transporters. Several transporters carry THs, including monocarboxylate transporters (MCTs), several members of the organic anion-transporting polypeptide (OATP) family (32, 33) and the heterodimeric L-type amino acid transporters (LATs). Intracellular TH availability is regulated in a dynamically and tightly coordinated manner by specific deiodination processes. Deiodinases type 1and 2 (D1 and D2) activate THs whereas D3 carries out inactivation of T4 and T3 (34). Lastly, THs can either positively or negatively regulate gene transcription via the thyroid hormone receptor (THR) by binding to thyroid hormone response elements (TREs) located on the promotors of target genes or even via direct modulation of gene expression (35). THRs are encoded by the thyroid hormone receptor α (THRA) and thyroid hormone receptor β (THRB) genes each with specific transcriptional responses (36, 37).

Clinical assessment of thyroid function requires measurement of TSH. Elevated levels of TSH (and most often) simultaneously low TH indicate hypothyroidism, whereas suppressed TSH and

[2]dichlorodiphenyl trichloroethane (DDT).

[3]p,p'-dichlorodiphenyl dichloroethylene (p,p'-DDE).

[4]World Health Organization.

high TH suggest a hyperthyroid condition. Subclinical hypo- and hyperthyroidism are characterized by TH values in the reference range and, respectively, high or low TSH (38). TSH is currently considered to be the most sensitive indicator of thyroid status, although assays and interpretations of thyroid function tests are not always straightforward, especially in the case of discordant results, notably with T4 or FT4 (39, 40).

Epidemiological studies report associations between TSH and/or TH as a function of exposure to numerous persistent as well as non-persistent pesticides. Similarly, experimental evidence suggests that pesticides may act as thyroid disruptors, affecting the HPT axis at several levels: central regulation, iodine uptake, production and distribution of THs, or binding of TH to membrane transporters or receptors (41–43). As a consequence, pesticide disruption of the HPT axis is increasingly scrutinized in Europe and in the United-States. In 2013, the European Food Safety Agency (EFSA) reported that 101 of the 287 pesticides they examined had the potential to interfere with thyroid function (44). As to the socio-economic costs of EDCs, in 2015 the neurobehavioral deficits (principally IQ loss) and neurodevelopmental diseases including attention deficit/hypoeractivity disorder (ADHD), induced by three substances with endocrine disrupting properties and acting principally on thyroid signaling, was estimated at 157 billion euros in the European Union (EU) (29). Of this 157 billion, 120 billion were attributed to organophosphate pesticides, such as chlorpyrifos.

Several reviews have shown that many xenobiotics from different chemical classes, including PPPs, are potential TH axis disrupting compounds acting at different levels of the HPT-axis (45, 46) but none has recently addressed different pesticide classes. Current knowledge regarding the full impact of pesticides on human thyroid function is still limited (47–49). To fill this gap, we reviewed the latest research on thyroid-related endpoints associated with old and newly formulated pesticides. The strategy is shown in **Figure 1** and is detailed in the Methods section as well as in **Supplementary Material**.

METHODS

A literature search of scientific literature was carried out using PubMed (on the 21st of March 2019) for references on pesticides and thyroid endpoints. Studies that contained both a pesticide term and a TH outcome in the title and/or abstract were included. A total of 232 articles were retrieved.

Search terms combined an extensive list of thyroid endpoints and pesticide-related terms varying from broad (e.g., insecticides) to more specific (e.g., permethrin) terms. Chemical groups of pesticides were based on those widely used and we added certain substances from the EU's Pesticides Database[5] Yale MeSh Analyser[6] was used to add specific Mesh terms possibly omitted during preliminary searches. Searches compiled two parenthetical terms (TH outcome/pesticides) with an AND operator. These parenthetical terms contained sub-terms linked to each other via OR operators. The final search query is consultable in **Table S1**.

As the major aim of this review was to provide recent research conducted on pesticide exposure with thyroid-related adverse outcomes, the search in PubMed was limited to the last 5 years and involved two screening stages. A first selection was conducted on the title and abstract, and the second screening was based on full-text analysis. When uncertainty arose regarding the eligibility of a publication from its abstract, the full-text version of the article was retrieved to ensure that there was no inappropriate selection, e.g., "organochlorine" includes a diverse family of chemicals, some of which are not used as pesticides. The study selection was restricted to English-language articles. After analysis of title and abstract, 140 publications were retained for further analysis based on the full article. After excluding research that failed to meet the inclusion norms, a total of 46 publications was selected. The flowchart of the literature selection is presented in **Figure 1**. The references retrieved are organized by eligibility and classes of pesticides in an excel file accessible in **Table S1**.

In order to provide a more exhaustive overview, reference lists of included articles were also screened by title and abstract in order to include additional relevant articles without restrictions on their date of publication.

ORGANOCHLORINE PESTICIDES

OC pesticides (OCPs) are chlorinated hydrocarbon compounds, which were used extensively worldwide from the 1940s to the 1960s. They are considered to be the first generation of synthetic broad-spectrum pesticides, of which the most notable representatives are the insecticide DDT and the fungicide Hexachlorobenzene (HCB). OCPs represent a large class of chemicals which can be divided into dichlorodiphenylethanes (DDT, dicofol, and methoxychlor), hexachlorocyclohexanes (HCHs such as HCB, chlordane, lindane), cyclodienes (aldrin, dieldrin, endrin, endosulfan, heptachlor), and toxaphene, a complex mixture of highly chlorinated bornanes, as well as mirex and its derivative chlordecone (50–52). Their action stems from their capacity to alter ion exchange in nerve axons in the peripheral and central nervous system, resulting in decreased action potentials but the precise mechanisms of action are diverse and remain mostly unknown. Cyclodienes have the added effect of competitively binding to the GABA-A receptor (53).

DDT was widely applied to both US military and civilian populations during the Second World War to combat malaria, typhus and other insect-borne diseases (54). Given its efficiency and low-cost, DDT was excessively applied in the agricultural sector for crop and livestock protection, as well as in homes and gardens (54). In the 1950s, production reached 100,000 tons per year in the US (55) until Carson's Silent Spring (56) brought OCP's toxic effects on non-target wild-life species as well as humans to the fore of public scrutiny. Public concern led to the creation of the US EPA in 1970 and shortly after, the ban of many OCPs for agricultural use. In 2001, the international Stockholm Convention singled out 12 compounds as Persistent Organic Pollutants (POPs), 9 of which were OCPs

[5]http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database
[6]http://mesh.med.yale.edu/

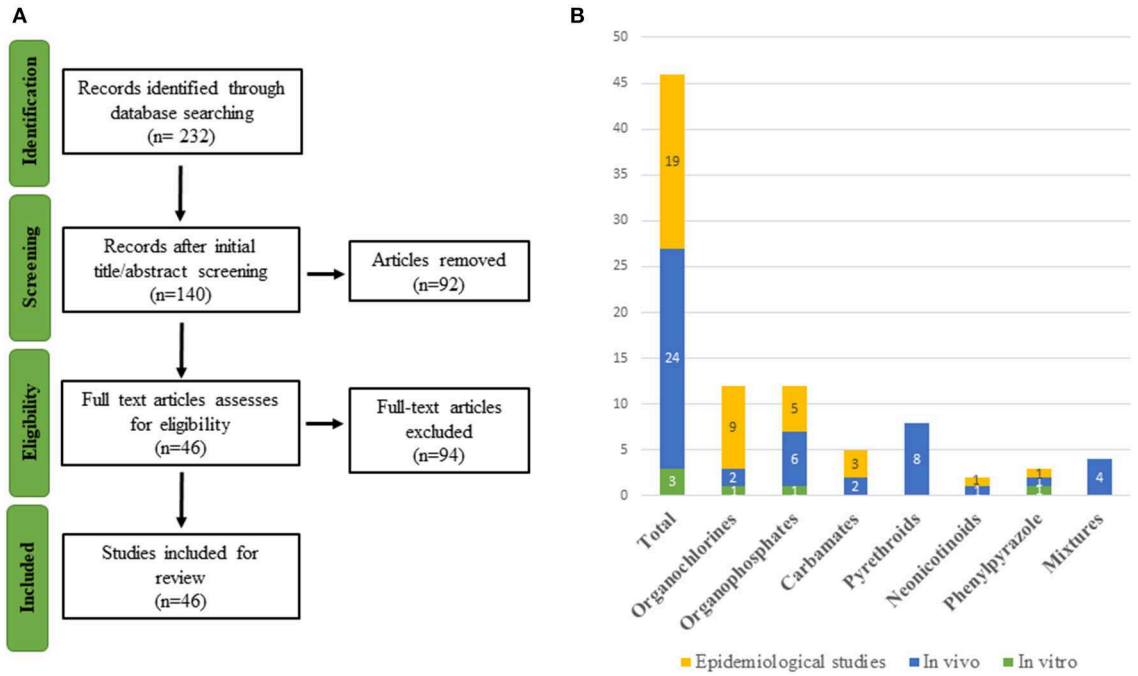


FIGURE 1 | (A) Flowchart of study selection. **(B)** Graphical representation of the 46 included studies organized by compound and type of study.

[UNEP (57), "12 initial POPs"]. Despite the ban, OCs remain a widespread environmental pollutant due to their resistance to environmental degradation, bioaccumulation in lipid-rich tissues, and biomagnification in the food chain (58, 59).

Epidemiological Evidence of Thyroid Disruption

Because DDT, HCB, and other OCPs have been associated with impaired neurodevelopment and neurocognition in infants and children (60–69), numerous epidemiological studies have investigated whether these adverse effects could be mediated, at least in part, by disruption of the HPT axis in either pregnant women or newborns (70–72, 72–81). An overview of the epidemiological organochlorine studies can be found in **Table 1**.

A Canadian birth-cohort study ($n = 101$) studied several OCs in pregnant women and observed that p,p′-DDE, the main metabolite of DDT, HCB and a constituent of chlordane were negatively associated with total T3 (TT3) levels, and β-HCH with FT4 (78). At 12 weeks of pregnancy, higher concentrations of p,p′-DDE in maternal serum ($n = 157$) was associated with lower FT4 levels and higher TSH levels (82). In an exploratory cross-sectional study of 17 OCPs in neonates in China, Luo et al. (77) examined cord plasma concentrations ($n = 115$) of HCHs, p,p′-DDE and methoxychlor, and reported a negative association with FT4 levels. Other OCPs, such as aldrin and dieldrin, sum of DDTs and its metabolites, as well as the sum of OCPs were correlated with increases in TSH levels. In a small study of a farming population in northern Thailand ($n = 39$), cord serum levels of p,p′-DDT and p,p′-DDE were negatively associated with cord serum TT4 (71).

In a study on POPs in Korea ($n = 104$) by Kim et al. (76), β-HCH, chlordanes, DDT, and p,p′-DDE measured in mothers or in cord serum were associated with either decreased TH levels or increased TSH levels. Specifically, maternal p,p′-DDE was associated with decreased FT3, FT4, and TT4 in cord serum and was identified as a predominant determinate of bloodspot TSH with an interquartile range (IQR) increase of p,p′-DDE accounting for a 19% increase of TSH. Additional evidence of thyroid disruption was found in cord serum, with p′p-DDE associated with increased bloodspot TSH and decreased TT3. Maternal β-HCH was associated with decreased FT3 and TT3 in cord blood, while cord β-HCH was associated with increased bloodspot TSH. In cord serum, HCH was negatively associated with TT4. Maternal chlordanes were negatively associated with both cord fT4 and TT4 levels, and chlordanes in cord serum were positively associated with TSH.

A study in Belgium ($n = 198$) reported that, in cord plasma, HCB was associated with decreased FT3 and FT4, and p,p′DDE with decreased FT4, however no significant variations were detected for TSH (79). In a study on infants born in a HCB-polluted area in Spain ($n = 70$), Ribas-Fitó et al. (73) focused on TSH for determination of thyroid status. While no relationship was found for HCB, β-HCH, and p,p′-DDE were associated with higher TSH concentrations in plasma of neonates. Moreover, β-HCH tended to be negatively associated with TT3 ($P < 0.065$) and TT4 ($P < 0.081$) in placentas ($n = 58$) (83) and positively with TSH ($p = 0.09$) in cord serum ($n = 453$) (72).

Freire et al. (75), analyzed placenta samples ($n = 220$) from a male birth cohort in Spain and, out of 17 OCPs assessed, p,p′DDE and HCB in placenta presented a close-to-significant positive ($p = 0.09$) or negative association ($p = 0.09$) with cord blood

TABLE 1 | Parameters of epidemiological data retrieved in the review – Organochlorines.

									Thyroid hormone		Pesticide									
Chemical class	**Study**	**Collection**	**Country**	**City**	**Population**	***N***	**Mean age (years)**	**Male-Female**	**Matrix**	**Time**	**Matrix**	**Time**	**Pesticide name**	**Mean concentration**	**TSH**	**TT3**	**FT3**	**TT4**	**FT4**	**Hypothyroidism**	**Other observations**
EPIDEMIOLOGY—ORGANOCHLORINES																					
Organochlorine	Takser et al. (78)	–	Canada	Southwest Quebec	Pregnant women and newborns	149	Maternal age: 27	0–100%	Serum and cord blood	1st, 2nd trimester, and delivery	Plasma and cord blood	1st, 2nd trimester, and delivery	Oxychlordane	1st trimester: 20 ng/L 2nd Trimester: 30 ng/L Delivery: 30 ng/L Cord blood: 10% detected	ns	ns	–	–	ns	–	TT3 levels during pregnancy were reduced in women exposed to five pollutants (PCB-138, PCB-153, PCB-180, p,p′-DDE, and HCB)
													trans-Nanochlor	1st trimester: 30 ng/L 2nd Trimester: 40 ng/L Delivery: 50 ng/L Cord blood: 14% detected	ns	ns	–	–	ns	–	
													cis-Nanochlor	1st trimester: 0% detected 2nd Trimester: 1% detected Delivery: 20% detected Cord blood: 0% detected	ns	↓ during pregnancy	–	–	ns	–	
													Mirex	1st trimester: 19% detected 2nd Trimester: 15% detected Delivery: 20% detected Cord: blood: 1% detected	ns	ns	–	–	ns	–	
													HCB	1st trimester: 40 ng/L 2nd Trimester: 60ng/L Delivery: 60ng/L Cord blood: 20 ng/L	ns	↓ during pregnancy	–	–	ns	–	
													β-HCH	1st trimester: 30 ng/L 2nd Trimester: 40 ng/L Delivery: 50 ng/L Cord blood: 1% detected	ns	ns	–	–	↓ during pregnancy	–	
													DDT	1st trimester: 10 ng/L 2nd Trimester: 30 ng/L Delivery: 40 ng/L Cord blood: 11% detected	ns	ns	–	–	ns	–	
													p,p′-DDE	1st trimester: 380 ng/L 2nd Trimester: 430 ng/L Delivery: 470 ng/L Cord blood: 160 ng/L	ns	↓ during pregnancy	–	–	ns	–	
Organochlorine	Lopez-Espinosa et al. (82)	2003–2005	Spain	Valencia	Pregnant women	157	Maternal age: 30	0–100%	Serum	12 weeks of pregnancy	Serum	12 weeks of pregnancy	p,p′-DDE	1.3 ng/L	↑	ns	–	–	↓ during pregnancy	–	–
Organochlorine	Luo et al. (77)	November 213–June 2014	China	Hospitals in Henan	Pregnant women	115	Maternal age: 26.62	0–100%	Cord plasma	At birth	Cord plasma	At birth	a-HCH	0.24 ng/mL	ns	–	ns	–	ns	–	–
													β-HCH	0.62 ng/mL	ns	–	ns	–	ns	–	–
													g-HCH	0.31 ng/mL	ns	–	ns	–	ns	–	–
													d-HCH	0.63 ng/mL	ns	–	ns	–	ns	–	–
													Sum HCHs	0.0062 ng/mL	ns	–	ns	–	↓	–	–
													DDE	1.91 ng/mL	ns	–	ns	–	↓	–	–
													DDD	0.09 ng/mL	ns	–	ns	–	ns	–	–

(Continued)

TABLE 1 | Continued

Chemical class	Study	Collection	Country	City	Population	*N*	Mean age (years)	Male-Female	Thyroid hormone		Pesticide										
									Matrix	Time	Matrix	Time	Pesticide name	Mean concentration	TSH	TT3	FT3	TT4	FT4	Hypothyroidism	Other observations
													DDT	4.89 ng/mL	ns	–	ns	–	ns	–	–
													Sum DDTs	0.0201 ng/mL	↑	–	ns	–	ns	–	–
													Aldrin	8.58 ng/mL	↑	–	ns	–	ns	–	–
													Dieldrin	6.23 ng/mL	↑	–	ns	–	ns	–	–
													Endrin	0.49 ng/mL	ns	–	ns	–	ns	–	–
													Sum Drins	0.0412 ng/mL	↑	–	ns	–	ns	–	–
													Endosulfan I	2.62 ng/mL	ns	–	ns	–	ns	–	–
													Endosulfan sulfate	0.26 ng/mL	ns	–	ns	–	ns	–	–
													Sum endosulfan	0.0071 ng/mL	ns	–	ns	–	ns	–	–
													Methoxychlor	0.98 ng/mL	ns	–	ns	–	↓	–	–
													Heptachlor	1.45 ng/mL	ns	–	ns	–	ns	–	–
													Sum OCPs	0.0812 ng/mL	↑	–	ns	–	ns	–	–
Organochlorine	Asawasinsopon et al. (71)	2003–2004	Thailand	Mae Rim District of Chiang Mai Province	Pregnant women and newborns	39	Maternal age: 23.8	46.2–53.8%	Cord serum	At delivery	Cord serum	At delivery	p,p'-DDT p,p'-DDE	77.7 ng/g 742 ng/g	ns ns	– –	– –	↓ ↓	ns ns	– –	–
													p,p'-DDD	89.1 ng/g	ns	–	–	ns	ns	–	
													o,p'-DDE	46.6. ng/g	ns	–	–	↓	ns	–	
													o,p'-DDT	17.1 ng/g	ns	–	–	ns	ns	–	
													Dieldrin	94.9 ng/g	ns	–	–	ns	ns	–	
													Heptachlor	37.1 ng/g	ns	–	–	ns	ns	–	
													Heptachlor epoxide	38.8 ng/g	ns	–	–	ns	ns	–	
POPs	Kim et al. (76)	February and December 2011	Korea	Seoul, Anyang, Ansan, Jeju	Pregnant women and newborns	148	Median maternal age: 33	0–100%	Umbilical cord serum + bloodspot newborn	Delivery and 2 days after birth	Cord blood and maternal serum	Delivery	Sum HCHs	Cord blood: 10.4 ng/g	ns	ns	ns	ns	ns	–	–
													Sum HCHs	Maternal serum: 9.4 ng/g	ns	ns	ns	ns	ns	–	–
													β-HCH	Cord blood: 7.5 ng/g	ns	ns	ns	ns	ns	–	–
													β-HCH	Maternal serum: 7.5 ng/g	ns	↓	↓	ns	ns	–	–
													Sum CHDs	Cord blood: 2.6ng/g	↑	ns	ns	ns	ns	–	–
													Sum CHDs	Maternal serum:3.9 ng/g	ns	ns	ns	ns	↓	–	–
													Sum DDTs	Cord blood: 65.2 ng/g	ns	ns	ns	ns	ns	–	–
													Sum DDTs	Maternal serum: 62.3 ng/g	↑ bloodspot	ns	ns	ns	ns	–	–
													p,p'-DDE	Cord blood: 63 ng/g	↑ bloodspot	ns	ns	ns	ns	–	–
													p,p'-DDE	Maternal serum:55.2 ng/g	↑ bloodspot	ns	ns	ns	ns	–	–
													HCB	Cord blood: 12.7.0 ng/g	ns	ns	ns	↓	ns	–	–
													HCB	Maternal serum: 5.5 ng/g	ns	ns	ns	ns	ns	–	–
Organochlorine	Maervoet et al. (79)	2002–2004	Belgium	Flanders	Pregnant women	198	Maternal age: 29.4	0–100%	Cord blood	Delivery	Cord blood	Delivery	p,p´-DDE	0.37 ng/mL	ns	–	ns	–	↓	–	–
													HCB	0.05 ng/mL	ns	–	↓	–	↓	–	
Organochlorine	Ribas-Fitó et al. (73)	1997–1999	Spain	Flix (catalonia)	Newborns	70	Not reported	Not reported	Plasma	3 days after birth	Cord serum	Delivery	p,p´-DDE	Not reported	↑	–	–	–	–	–	–
													HCB	Not reported	ns	–	–	–	–	–	–
													β-HCH	Not reported	↑	–	–	–	–	–	–

(Continued)

TABLE 1 | Continued

									Thyroid hormone		Pesticide									
Chemical class	**Study**	**Collection**	**Country**	**City**	**Population**	***N***	**Mean age (years)**	**Male-Female**	**Matrix**	**Time**	**Matrix**	**Time**	**Pesticide name**	**Mean concentration**	**TSH**	**TT3**	**FT3**	**TT4**	**FT4**	**Hypothyroidism**	**Other observations**
Organochlorine	Li et al. (83)	1997–2001	Denmark	Copenhagen	Placenta samples	58	Maternal age: 30.4	100% placenta's of boys	Placenta	Delivery	Placenta	Delivery	Sum 25 OCPs β-HCH methoxychlor	77.7 ng/g 9.18 ng/g 0.008 ng/g	– – –	ns ↓ $p < 0.081$ ns	– – –	ns ↓ $p <$ 0.065 ns	– – –	– – –	Additionally measured rT3: methoxychlor were inversely associated with rT3
Organochlorine	Lopez-Espinosa et al. (72)	2004–2006	Spain	Valencia	Pregnant women and newborns	453	30	54.7–45.3%	Dry blood spot	3 days after birth	Cord serum	Delivery	p,p'-DDT p,p'-DDE HCB β-HCH	8.0 ng/g 197 ng/g 75 ng/g 20 ng/g	ns ns ns ↑ $p =$ 0.09	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –
Organochlorine	Freire et al. (75)	2000–2002	Spain	Southern spain	Pregnant women and neonates	220	Maternal age: 31.8	100–0%	Cord blood	Delivery	Placenta	Delivery	o,p'-DDT p,p´-DDT p,p´-DDE o,p'-DDD Sum DDTs Endosulfan-I Endosulfan-II Endosulfan-diol Endosulfan-ether Endosulfan-sulfate Endosulfan-lactone Sum Endosulfans Aldrin Endrin Dieldrin Lindane HCB Methoxychlor Mirex	0.86 ng/g 1.25 ng/g 2.01 ng/g 1.91 ng/g 4.16 ng/g 0.73 ng/g 1.37 ng/g 2.10 ng/g 0.23 ng/g 0.93 ng/g 1.14 ng/g 4.02 ng/g 0.82 ng/g 2.53 ng/g 1.05 ng/g 0.41 ng/g 1.02 ng/g 1.20 ng/g 1.15 ng/g	ns ns ↑ $p =$ 0.09 ns ns ns ns ns ns ↓ ns ns ns ↑ ns ns ↓ $p =$ 0.09 ns ns	– – – – – – – – – – – – – – – – – – –	– – – – – – – – – – – – – – – – – – –	– – – – – – – – – – – – – – – – – – –	– – – – – – – – – – – – – – – – – – –	–
Organochlorine	Dufour et al. (81)	2013–2016	Belgium	Liege	Pregnant women and newborns	221	29.2	52.8–47.2%	Dry blood spot	3 days after birth	Cord serum	Delivery	HCB β-HCH Trans-Nanochlor p,p'-DDE	0.0% detected 0.5% detected 0.0% detected 24.1% detected	– - – Boys: ↓	– – – –	– – – –	– – – –	– – – –	– – – ns	–
Organochlorine	Dallaire et al. (74)	1993–1996	Canada	Nunavik r (Quebec)	Pregnant women and neonates	410	Maternal age: 23	48.1–51.9%	Cord serum	Delivery	Cord plasma	Delivery	HCB	140 ng/L	ns	ns	–	–	↑	–	–
		1993–1997	Canada	Lower North Shore of the St. Lawrence River (Quebec)	Pregnant women and neonates	260	Maternal age: 25	48.5–51.5%	Cord serum	Delivery	Cold plasma	Delivery	HCB	150 ng/L	ns	ns	–	–	↑	–	

(Continued)

TABLE 1 | Continued

									Thyroid hormone		Pesticide										
Chemical class	**Study**	**Collection**	**Country**	**City**	**Population**	***N***	**Mean age (years)**	**Male-Female**	**Matrix**	**Time**	**Matrix**	**Time**	**Pesticide name**	**Mean concentration**	**TSH**	**TT3**	**FT3**	**TT4**	**FT4**	**Hypothyroidism**	**Other observations**
Organochlorine	Cordier et al. (80)	2004–2007	Guadeloupe	University Hospital Pointe-à-Pitre and the General Hospitals of Basse-Terre	Mother-child cohort	111	Maternal age: 30.7	0–100%	Child serum	At 3 months of age	Cord blood and breast milk samples	Cord blood: at delivery—breast milk: 3 months after delivery	Chlordecone	Median—cord blood: 0.14μg/L	Boys: ↑	–	ns	–	Boys: ns	–	–
															ns	–	ns	–	Girls: ns	–	–
														Breast milk	ns	–	Boys: ↓	–	Boys: ns	–	–
															ns	–	Girls: ↓	–	Girls: ↓	–	–
Organochlorine	Alvarez-Pedrerol et al. (70)	1997–1999	Spain	Island of Menorca	Children	259	Maternal age: 33	47.9–52.1%	Serum	At 4 years of age	Serum	At 4 years of age	p,p'-DDT	0.06 ng/mL	ns	↓	–	–	ns	–	–
													p,p'-DDE	0.88 ng/mL	ns	ns	–	–	ns	–	
													HCB	0.32 ng/mL	ns	ns	–	–	ns	–	
													β-HCH	0.22 ng/mL	ns	↓	–	–	ns	–	
Organochlorine	Meeker et al. (84)	January 2000 and May 2003	North-America	Boston	Men	341	36	100–0%	Serum	Cross-sectional	Serum	Cross-sectional	p,p'-DDE	236 ng/g	↓	↑	–	–	↑	–	–
													HCB	15.6 ng/g	ns	↓	–	–	ns	–	–
Organochlorine	Bloom et al. (85)	2000–2002	North-America	Upper Hudson river communities	Women	48	63.2	0–100%	Serum	Cross-sectional	Serum	Cross-sectional	Sum DDT	3.59 μg/L	ns	↑	–	↑	ns	–	–
Organochlorine	Blanco-Munoz et al. (86)	July-October 2004 and December 2004–May 2005	Mexico	States of Mexico and Morelos	Floriculture workers (men)	136	32.7	100–0%	Serum	Longitudinal	DDE an DDT in serum and DAP metabolites in urine	Longitudinal study	DDE	6.14 and 4.71 ng/ml in rainy and dry seasons	ns	↑	–	↑	–	–	–
Organochlorine	Rathore et al. (87)	1997–1998	India	Jaipur	Women visiting the Thyroid Clinic	123	37	0–100%	Serum	Cross-sectional	Serum	Cross-sectional	Sum OC	18.83 ppm depleted T4 vs. 14.68 normal T4	ns	ns	–	ns	–	ns	–
													Total DDT (pp'DDE+pp'DDT+pp'DDD)	8.43 ppm depleted T3 vs. 6.91 normal T4	ns	ns	–	ns	–	ns	
													Total HCH (α,β,?)	3.82 ppm depleted T4 vs. 3.86 normal t4	ns	ns	–	ns	–	ns	
													Dieldrin	5.38 ppm in depleted T4 group vs. 2.5 normal T4	ns	ns	–	↓	–	↑	
													Hepatchlor	1.18 ppm in depleted T4 vs. 1.41 normal T4	ns	ns	–	ns	–	ns	
Organochlorine	Rylander et al. (88)	Not clearly indicated	Sweden	Swedish east coast, off the Baltic Sea	Fishermen	196	59	100–0%	Serum	Cross-sectional	Serum	Cross-sectional	p'p'-DDE	580 ng/g lipid	↑	–	–	–	ns	–	Also measured FSH, LH, estradiol, and testosterone Negative association: p,p'-DDE and estradiol level.

(Continued)

TABLE 1 | Continued

									Thyroid hormone		Pesticide									
Chemical class	**Study**	**Collection**	**Country**	**City**	**Population**	***N***	**Mean age (years)**	**Male-Female**	**Matrix**	**Time**	**Matrix**	**Time**	**Pesticide name**	**Mean concentration**	**TSH**	**TT3**	**FT3**	**TT4**	**FT4**	**Hypothyroidism**	**Other observations**
Organochlorine	Schell et al. (89)	1995–2000	North America	St. Lawrence River with territory in New York States, in Ontario and Quebec Canada	Mother–youth pairs	232	Youth: 17.6	–	Serum	Cross-sectional	Serum	Cross-sectional	HCB	Non-breast fed: 0.03 ppb breast-fed: 0.04	ns	ns	–	–	↓	–	Breast-fed adolescents had higher levels of p,p'-DDE
													p-p'-DDE	Non-breast fed: 0.31 ppb breast-fed: 0.41	ns	ns	–	–	ns	–	
Organochlorine	Goldner et al. (48)	1993–1997 (Phase 1), 1999–2003 (Phase 2)	North America	Iowa, North Carolina	Female spouses of workers involved in Agricultural Health Study	16,529	47.2+HH109:L118	0–100%	Self-reported thyroid disease	Self-reported thyroid disease	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Aldrin	–	–	–	–	–	–	↑	–
													Chlordane	–	–	–	–	–	–	↑	–
													DDT	–	–	–	–	–	–	↑	–
													Heptachlor	–	–	–	–	–	–	↑	–
													Lindane	–	–	–	–	–	–	↑	–
Organochlorine	Lerro et al. (92)	June 2010–September 2013	North-America	Iowa or North Carolina	Pesticide applicators	679	Not indicated	100–0%	Serum	Cross-sectional	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Aldrin	–	↑	ns	–	↓	–	↑	–
													Chlordane	–	ns	ns	–	ns	–	ns	–
													DDT	–	ns	ns	–	ns	–	ns	–
													Heptachlor	–	ns	ns	–	ns	–	ns	–
Organochlorine	Piccoli et al. (90)	2012–2013	Brazil	Farroupilha, Serra gaucha, South Brazil	Agricultural workers	275	42	56.4–43.6%	Serum	Cross-sectional	Serum	Cross-sectional	HCH, HCB, heptachlor epoxide A, heptachlor epoxide B, heptachlor, transnonachlor, DDT, DDE, DDD, p,p'-DDD, endosulfan I, endosulfan II, aldrin, endrin, dieldrin, methoxychlor, mirex, pentachloroanisole	Many subject were below limit of detection, therefore no mean	Sum: ↓	↑	–	–	↑	–	–
Organochlorine	Shrestha et al. (91)	1991–1997	North-America	North Caroline and Iowa	Pesticide applicators	35,150	Median age 62	97.9–2.1%	Self-reported thyroid disease	Self-reported thyroid disease	Detailed self-reported us of pesticides	Detailed self-reported us of pesticides	Aldrin	Aldrin	nm	nm	nm	nm	nm	↑(attained age)	nm
													Heptachlor	Heptachlor	nm	nm	nm	nm	nm	↑(attained age)	nm
													Lindane	Lindane	nm	nm	nm	nm	nm	↑(attained age)	nm
													Chlordane	Chlordane	nm	nm	nm	nm	nm	↑ (all participants)	nm
Organochlorine	Goldner et al. (49)	1993–1997 (Phase 1), 1999–2003 (Phase 2)	North America	Iowa, North Carolina	Male private applicators (mainly farmers) in AHS	22,246	45.6	100–0%	Self-reported thyroid disease	Self-reported thyroid disease	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Chlordane	–	–	–	–	–	–	↑	–
													DDT	–	–	–	–	–	–	↑	–
													Heptachlor	–	–	–	–	–	–	↑	–
													Lindane	–	–	–	–	–	–	↑	–
													Toxaphene	–	–	–	–	–	–	↑	–

TSH levels, respectively, and the cyclodiene endrin was associated with higher odds of increased TSH. In addition, the metabolite endosulfan-sulfate was related to lower TSH levels.

In contrast to previous studies, cord blood p′p-DDE levels ($n = 221$) were negatively associated with TSH levels when only male newborns were considered (81). Dallaire et al. (74) reported that prenatal exposure to HCB was positively associated with FT4 levels in newborns from two fish-eating populations in Quebec ($n = 260$ and $n = 410$). The authors considered, however, that nutrient confounders including iodine and selenium, may have mitigated the antagonistic effect of OCPs on TH. In 4 year old children ($n = 259$) which were followed in a Spanish birth cohort for examination of TH function, p,p′-DDT and β-HCH concentrations in serum were associated with decreased TT3 (70).

Chlordecone was banned in the USA in 1976 but was widely used until the 1993 in the French Caribbean (Martinique and Guadeloupe). In 2015, a longitudinal birth-cohort ($n = 111$) study in Guadeloupe investigated the effects of prenatal and postnatal exposure during breastfeeding. The authors reported that prenatal exposure was associated with increased levels of TSH in boys, while postnatal exposure determined at 3 months was associated with decreased FT3 levels in both genders, and FT4 levels in girls (80).

Taken together, the majority of mother/child cohort results show that OCPs may exert significant TH inhibitory effects. While several studies in new-borns and children are often considered unsatisfactory due to the lack of significant and concordant effects in both TH and TSH levels, given the high variability of each parameter, effects on either one can be considered to indicate a hypothyroid-like effect of many organochlorines.

When considering adults, results are more contradictory with some studies reporting effects more in line with hyperthyroidism in men (84), aging women (85) and occupational workers (86), while others found that OCPs were related to clinical or subclinical hypothyroidism in women (87), elderly men (88), adolescents (89) and pesticide applicators (48, 49, 90–92). Moreover, an analysis of data on several organochlorines acquired from the United-States cross-national survey NHANES[7] did not find an effect of p,p′-DDE on TT4 in adults due to inconsistent results in both sampling cycles (1999–2000 and 2000–2001), and associations with TSH were not significant (93).

In vivo Evidence of Thyroid Disruption From Animal Studies

In vivo studies have proposed several potential mechanisms underlying thyroid disruption by OCPs, majoritarily for DDT (63). Liu et al. (94) exposed male rats for 10 days to p,p′-DDE and reported a reduction in serum TT4 and FT4 along with decreased levels of transthyretin proteins responsible for T4 transport, upregulation of hepatic enzymes involved in T4 clearance as well as increased hippocampal thyroid hormone receptor mRNA. The effects observed encapsulate previously suggested modes of action of OCs. Many OCs may act by mimicking TH, binding to TRs along the HPT-axis, decreasing bioactivity of TH via increased clearance, and/or reducing binding to transport proteins due to their structural similarities to TH (95). Moreover, long-term exposure to low doses of DDT resulted in altered physiology and cytophysiological changes in the follicular epithelium of the thyroid gland to compensate for reduced secretion in thyrocytes (96, 97). Likewise, HCB disruption of the HPT-axis is characterized by decreased T4 in male rats, increases in hepatic T4-UDPGT activity along with increased T4 conversion to T3 in the thyroid and liver (98) and competitive inhibition of T4 binding to transporters by its major metabolite pentachlorophenol (PCP) (99, 100).

In vitro Evidence of Thyroid Disruption

In vitro, a first line of evidence was the antagonistic action of DDT in a TSH-induced cAMP production assay (101), the principal second messenger required for thyroid gland activation. Rossi et al. (102, 103) investigated this mechanism of action and suggested that DDT could modify the lipid organization of the cell membrane and induce production of extracellular vesicles containing membrane bound TSH receptors (TSHR), thereby inducing failure of the TSH receptor to internalize and prolong TSHR-cAMP signaling. In addition, HCB was shown to reduce viability and inhibit cell cycle progression of FRTL-5 rat thyroid cells along with increased mRNA levels of transforming growth factor-beta (TGF-β1) known to inhibit cell growth in thyroid epithelial cells (104). In turn, modulation of TGF-β1 expression can have repercussions on thyroid function via regulation of thyroid specific genes such as those encoding NIS, thyroglobulin, thyroperoxidase, and the TSH receptor (105).

To conclude on OCPs, multiple mechanisms have been suggested to explain the reduction in circulating THs and/or the increase in TSH. These include displacement from distributor proteins, increased hepatic metabolism, and indirect effects on thyroid function.

ORGANOPHOSPHATE PESTICIDES

Organophosphates (OPs) pesticides (OPPs) are one of the two main classes of acetylcholinesterase inhibitors, the other being carbamates. During World War II, OPs were used for warfare as a human nerve gas agent, inducing seizures and, at high doses, respiratory arrest (106). Ultimately, OPs were adapted as insecticides because they act on insects via the same mechanism at relatively low doses. The adverse effects of OPs have been widely studied since the 1970s. Remarkably, even low-level prenatal exposure to various OPs, notably chlorpyrifos, can impede normal fetal brain development (107, 108). Despite the ban on in-house use in industrialized countries, chlorpyrifos, malathion, and diazinon continue to be extensively used for crop protection (109). After ingestion, most OP pesticides are metabolized, producing different dialkyl phosphate (DAP) metabolites which can be eliminated via urine within 24 h. As DAP metabolites can originate from different OPs, urinary DAP levels give an overview of general OP exposure rather than being a specific biomarker of a certain OP (110–112).

[7]National Health and Nutrition Examination Survey.

Epidemiological Evidence of Thyroid Disruption

An epidemiological study conducted in California observed that prenatal, but not postnatal, levels of urinary DAP metabolites ($n = 329$) were associated with weaker intellectual development in 7-year-old children, highlighting the vulnerability of the developing fetus (113). In 2012, prenatal low-dose chlorpyrifos (CPF) exposure was associated with differences in brain morphology (107) but no TH related endpoints were measured. Authors of a small cross sectional study ($n = 66$) conducted on children in Indonesia, retrieved questionnaires, analyzed urine samples and measured classical thyroid related endpoints. Six different DAPs, originating from degradation of potentially 28 OPs, were measured in morning spot urine samples. The mean TSH level in children who tested DAP positive was significantly higher than those children with undetectable levels of DAP (114).

In adults, as for OCPs, less data is available. In an extensive prospective health study ($n = 30{,}003$), agricultural spouses were monitored and followed. The authors found that exposure to organophosphate insecticides was a risk factor for developing several hormonally-related cancers, including breast, thyroid, ovary and lymphoma (115). Further, a study conducted in China ($n = 325$) demonstrated that DAP levels in urine of pregnant women were positively associated with FT4 concentration and negatively with TSH levels, warranting further investigation on the consequences of OP exposure on thyroid function during pregnancy (116). An overview of TH-related epidemiological organophosphate studies can be found in **Table 2**.

In vivo Evidence of Thyroid Disruption From Animal Studies

In vivo, a mouse study reported that a 3-day administration of CPF at doses under the concentration required for inhibition of brain acetylcholesterinase, could induce histological and histo-morphometrical thyroid effects, resulting in hypothyroidism in dams. In the F1, exposed during pre- (3 days) and post-natal (additional 3 days) periods, long-term reductions in serum T4 levels were measured in males 150 days after birth (117). Despite the concentration levels of CPF in this study being much higher than the levels estimated in children (118), these results may still be of significance due to potential additive effects that are likely to occur from continuous exposure to a variety of organophosphorus compounds (see section Mixtures). In addition, Mie et al. (119) reported that the manufacturers' report that was submitted for authorization of chlorpyrifos was misleading as significant effects of exposure on brain development were omitted from conclusions. Specifically, cerebellum height and brain weight were reduced at intermediate doses. As TH is essential for Purkinje cell differentiation in the cerebellum, a thyroid disrupting effect could be hypothesized.

CPF interferes with maturation of surgeonfish (*Acanthurus triostegus*) through a thyroid hormone-dependent process. By inhibiting TH levels, chlorpyrifos disrupted metamorphosis and reduced the ability of juveniles to graze algae, an important factor in coral reef maintenance (120). Another fish study conducted a 21-day exposure of OP monocrotophos (MCP) to goldfish (*Carassisu auratus).* Exposure concentrations were 0.01, 0.10, and 1.00 mg/L. Expression profiles of the HPT axis-responsive genes were altered in the liver, brain, and kidneys, and plasma levels of T3 were decreased (121). This study was repeated in 2018 (122) using lower concentrations of MCP (0, 4, 40, and 400 μg/L) and a different exposure protocol (2-, 4-, 8-, and 12-days of exposure) and confirmed the TH disrupting properties of MCP. Also, an economically important teleost species, the Caspian roach (*Rutilus rutilus)* appears to be vulnerable to organophosphate exposure. Research has shown that when young Caspian roach were exposed to environmentally relevant concentrations of diazinon for 9 h, levels of TSH, T4 and T3, were significantly reduced. Furthermore, cortisol and glucose levels were significantly increased. These alterations of physiology might impact survival rates leading to restocking failures through dwindling numbers of juveniles released into river estuaries (123). Ortiz-Delgado et al. (124) investigated malathion exposure effects in the Senegalese sole, *Solea senegalensis,* a flatfish whose morphology, obtained gradually through metamorphosis, is TH-dependent. By exposing fish to malathion (another OPP), the authors demonstrated the sensitivity of this process to this pesticide that is still widely used in developing countries.

In vitro Evidence of Thyroid Disruption

Qiu et al. (125) used FRTL-5 cells to investigate effects of malathion on TH biosynthesis and showed decreased TSH receptor expression. Toxicogenomics from *in vitro* experiments aim to identify molecular patterns able to predict *in vivo* adverse outcomes. This strategy responds to the urgent need for a rapid mechanism-based strategy in risk assessment. In line with this concept, transcriptome analysis was conducted on immortalized rat thyrocytes that were exposed to either CPF or ethylenethiourea (ETU) in order to define *in vivo* gene signatures and mechanisms of toxicity. They showed non-monotonic dose response curves for both compounds along with common and distinct effects on thyroid toxicity, including altered growth of thyrocytes after chemical exposure to either ETU or CPF. Gene expression based on *in vivo* experiments fell short of fully recapitulating *in vitro* predictions because of compensatory and feedback loop mechanisms that are active *in vivo*. Despite this limitation, *in vitro* toxicogenomics managed to predict modes of action with longer exposure times. Notably, interference with thyrotrope growth was the main mechanism identified (126).

To conclude on the diverse mechanisms underlying actions of different OPPs on thyroid equilibrium. Both malathion and CPF could affect thyrotrope production, leading to TH lowering effects. However for CPF, even though the brain effects. On the other hand, for chlorpyrifos, even though the brain effects have been clearly demonstrated in rats and the TH lowering effects are well-identified in fish, the actual mode of action remains to be clarified *in vivo*.

Glyphosate

Glyphosate is an OP compound used worldwide as a broad-spectrum herbicide. It binds and competitively inhibits the activity of enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involved in the shikimic acid pathway (127) only found in plants and micro-organisms (128). Glyphosate usage has

TABLE 2 | Parameters of epidemiological data retrieved in the review—organophosphates.

									Thyroid hormone		Pesticide									
Chemical class	**References**	**Collection**	**Country**	**City**	**Population**	***N***	**Mean age (years)**	**Male-Female**	**Matrix**	**Time**	**Matrix**	**Time**	**Pesticide name**	**Mean concentration**	**TSH**	**TT3**	**FT3**	**TT4**	**FT4**	**Hypothyroidism**	**Other observations**
Epidemiology—Organophosphates																					
Organophosphate	Suhartono et al. (114)	March–May 2015 and August–October 2015	Indonesia	Agricultural area, brebes destrict	Children from elementary school	66	9.2	52%-48%	Serum	Cross-sectional	Morning spot urine	Cross-sectional	6 DAP metabolites	Not indicated	↑	-	-	-	ns	↑ with positive urinary ogranophosphate pesticide metabolites	-
Organophosphate	Lerro et al. (115)	1993–1997	North-America	Iowa and North Carolina	Spouses of pesticide applicators	30,003	-	0%-100%	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	-	-	-	-	-	-	↑ risk with OP use for serval hormonally-related cancers including thyroid cancer
Organophosphate	Wang et al. (116)	April 2011-December 2013	China	Southern coastal area of Laizhou Wan (Bay) of Bohai Sea in Shandong Province	Pregnant women	325	28.4	0%-100%	Serum	cross-sectional	Urinary	cross-sectional	6 DAP metabolits: Sum DAP	-	↓	ns	ns	ns	↑	-	-
													DMP	9.81 μg/L	ns	ns	ns	ns	ns	-	-
													DMTP	0.79 μg/L	ns	ns	ns	ns	ns	-	-
													DEP	5.00 μg/L	ns	ns	ns	ns	ns	-	-
													DETP	0.78 μg/L	ns	ns	ns	ns	ns	-	-
50 different pesticides	Shrestha et al. (91)	1991–1997	North-America	North Caroline and Iowa	Pesticide applicators	35,150	Median age 62	97.9%-2.1%	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Diazinon	-	-	-	-	-	-	↑(attained age)	-
													Dichlorvos	-	-	-	-	-	-	↑(attained age)	-
													Malathion	-	-	-	-	-	-	↑(attained age)	-
													Glyphosate	-	-	-	-	-	-	↑(attained age)	-
50 different pesticides	Goldner et al. (91)	1993–1997	North-America	North Caroline and Iowa	Pesticide applicators	22,246	45.6	100%-0%	Self-reported thyroid disease	Self-reported thyroid disease	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Diazinon	-	-	-	-	-	-	↑	-
													Malathion	-	-	-	-	-	-	↑	-
													Glyphosate	-	-	-	-	-	-	ns	-
Total of 33 pesticides (16 herbicides, 13 insecticides, two fungicides, two fumigants)	Lerro et al. (92)	June 2010-September 2013	North-America	Iowa or North Carolina	Pesticide applicators	679	Not indicated	100%-0%	Serum	Cross-sectional	Detailed self-reported use of pesticides.	Detailed self-reported use of pesticides.	Chlorpyrifos	-	ns	ns	-	ns	-	ns	-
													Diazinon	-	ns	ns	-	ns	-	ns	-
													Fonofos	-	ns	ns	-	ns	-	ns	-
													Malathion	-	ns	ns	-	ns	-	ns	-
													glyphosate	-	ns	ns	-	ns	-	ns	-

increased tremendously since Monsanto introduced glyphosate-tolerant crop varieties in 1996 (namely, sugar beet, canola, cotton, maize, alfalfa) to be used in conjunction with Roundup, the glyphosate-based formulation (129). The median half-life of glyphosate in the field is reportedly 47 days and the primary breakdown products are aminomethylphosphonic acid (AMPA) and glyoxylate (130).

Epidemiological Evidence of Thyroid Disruption (Three Studies)

A prospective cohort study of licensed pesticide applicators in North Carolina and Iowa with 35,150 male and female participants demonstrated that self-reported use of glyphosate was associated with increased risk of hypothyroidism (91). However, these findings are not consistent with results obtained from the same cohort study conducted by Goldner et al. (49) and Lerro et al. (92).

In vivo Evidence of Thyroid Disruption

Surprisingly, few studies retrieved were found to be relevant to glyphosate exposure and thyroid endpoints. In a first i*n vivo* study, female pregnant Wistar rats were exposed to either 5 or 50 mg/kg/day Roundup®Transorb (Monsanto) from gestation day 18 to post-natal day (PND) 5. Blood and tissues samples from heart, liver, pituitary and hypothalamus were collected for hormonal, metabolomics or gene expression analysis at PND 90. In every tissue collected, TH-related genes were observed to be differentially expressed in exposure groups compared to the control group. Levels of TSH were decreased in exposure groups, however levels of both T3 and T4 were unaffected. This curious lack of TH hormone effects, despite TSH and target gene changes, may reflect changes in TSH set-point resulting from differential gene programming in rats during the fetal period (131).

Due to the removal of patent protection for glyphosate in 2000, many new glyphosate-based herbicides arrived on the pesticide market. Each of these formulations have a slightly altered surfactant mixture and chemistry, making testing procedures even more complicated. The second *in vivo* study, exposed four different North American amphibian species *(Rana clamitans, Rana pipiens, R. sylvatica*, and *Bufo americanus)* to glyphosate and the surfactant polyethoxylated tallowamine (POEA), and six different glyphosate-based formulations. Disruption of the HPT-axis was investigated by measuring time to metamorphosis and expression of *thrb*. Glyphosate alone and formulations lacking POEA were the least toxic, however *R. pipiens* tadpoles showed delayed metamorphosis and decreased snout-vent length at the peak of metamorphosis after exposure to either POEA or glyphosate formulations containing POEA. These effects may be linked to the increased *thrb* mRNA levels observed in the same exposure conditions. This study underlines the need for surfactant composition to be taken into consideration in the evaluation of risk assessment of glyphosate-based herbicides (132). In another frog study (*Lithobates sylvaticus*), glyphosate-formulated exposure alters brain gene expression for *thrb* and *dio3* enzyme, with different alterations depending on the stage (133).

Clearly, how and at which levels glyphosate has the potential to interfere with TH equilibrium in different species has not yet been fully examined. However, one area that remains to be investigated is microbiome metabolism. As human, vertebrate and invertebrate microbiomes express the EPSPS enzyme, it is plausible that microbiome status is modified by short or long-term glyphosate exposure.

CARBAMATES

Carbamates are widely used in agriculture, principally as insecticides, but also as herbicides and fungicides. Although they differ chemically from organophosphates, they act similarly by inhibiting the acetylcholineresterase enzyme (AChE) at the level of neuronal synapses. AChE is responsible for the rapid hydrolytic degradation of the neurotransmitter ACh into inactive products at neuromuscular junctions. In general, the AChE inhibition by carbamates is reversible in contrast with OPs (134). We focused here on the subgroup of dithiocarbamates in which, both oxygen atoms are replaced by sulfur. Dithiocarbamates can be sub-divided into two major groups: ethylenebisdithiocarbamates (EBDC) which includes maneb, zineb, and mancozeb, and dimethyldithiocarbamates (DMDC) consisting of ferbam, ziram and thiram. ETU is one of the major metabolites of EBDCs in mammals (135) whereas carbon disulfide is a metabolite found after *in vivo* DMDC treatment (136). As the thioureas can inhibit thyroid peroxidase, a thyroid gland enzyme essential for TH production, pesticides that generate ETU metabolites are of particular concern (55).

Epidemiological Evidence of Thyroid Disruption

Chronic exposure to EBDCs mainly concerns agricultural and industrial workers but also the general population which may be continuously exposed to residues present in food (137). An overview of the epidemiological carbamate studies can be found in **Table 3**. In Costa Rica, mancozeb is applied weekly on banana plantations by light aircraft. Urinary ETU concentrations of pregnant women living in the vicinity of plantations (n = 451) were more than five times higher than concentrations reported in general populations (138). In 2017, a large epidemiological study was conducted in Taiwan to investigate the association between hypothyroidism and anticholinesterase pesticide poisoning (organophosphate and carbamate). A total of 10,372 subjects poisoned by anticholinesterase pesticides were compared to 31,116 reference subjects between 2003 and 2012. Analysis demonstrated that exposed subjects had significantly increased risk for hypothyroidism (139). A smaller study was conducted on 177 occupationally exposed male workers in Italy and confirmed the thyroid disrupting effect of mancozeb (140).

In vivo Evidence of Thyroid Disruption From Animal Studies

A rat study conducted in 1985 demonstrated that both EBDCs maneb and zineb could affect endogenous TRH at the pituitary or hypothalamic level and therefore inhibit TSH secretion (141).

TABLE 3 | Parameters of epidemiological data retrieved in the review—carbamates.

									Thyroid hormone		Pesticide											
Chemical class	**References**	**Collection**	**Country**	**City**	**Population**	***N***	**Mean age (years)**	**Male-Female**	**Matrix**	**Time**	**Matrix**	**Time**	**Pesticide name**	**Mean concentration**	**TSH**	**TT3**	**FT3**	**TT4**	**FT4**	**Hypothyroidism**	**Other observations**
Epidemiology—Carbamates																					
Carbamates	Van Wendel de Joode et al. (138)	March 2010 and June 2011	Costa rica	Matina County, Limon	Pregnant women	415	24	0–100%	nm	nm	Urine	ETU	Main metabolite of mancozeb: ETU	4.2 μg/L	nm	nm	nm	nm	nm	nm	ETU concentration > than 5 times ↑ than those reporterd for other general populations
Carbamates and organophosphate	Huang et al. (139)	2003–2012	Taiwan	Nationwide	ACPP subjects and non-ACPP* population-based	ACPP = 10,372 non-ACPP = 31,116	54.27	72.13–27.87%	nm	nm	Comparison anitocholestinerase pesticide poisoning with non-pesticide poisoning subjects		Anticholinesterase pesticide poisoning		nm					↑ risk	nm
Carbamates	Medda et al. (140)	July–August	Italy	Chianti area and Bolzano province	occupationally exposed grapevine workers (male)	177	44.6	100–0%	Serum, urinary	Cross-sectional	Plasma	Cross-sectional	ETU	12.2 μg/L	ns	nm	↑	↓	↑	nm	Additionally urinary iodine and thyroglobulin was measured in serum but no significant results were obtained

At the level of the thyroid gland, Mancozeb was shown to reduce TPO activity, consequently altering both weight and histopathology of the thyroid gland, with acute high dose exposure resulting in a hypothyroid status in rats (142, 143).

A zebrafish study exposed embryos to 0.001–10 μM thiram, at various developmental stages for a short duration of time (1 h). Thiram exposure increased *dio3* mRNA expression a 12 hpf and decreased *tpo* mRNA expression at 48 hpf. In addition, delayed hatching, increased mortality, and skeletal defects were observed. The extent to which the disruption of the HPT axis contributed to these specific adverse outcomes needs further investigation (144).

In vitro Evidence of Thyroid Disruption

The effects of ziram, thiram, zineb, and ETU have been investigated *in vitro*, on Chinese hamster ovary cells transfected with the human *TPO* gene. Zineb (50 μM) inhibited the iodinating activity of TPO at a 10-fold higher concentration than its metabolite, ETU, indicating that ETU is the ultimate toxicant *in vivo*. No effect was observed with DMDCs, thiram and ziram (145).

To conclude on carbamates, a major concern is the effects of many metabolites on TPO activity. A major gap to be filled is that there is little epidemiological data available on exposure levels in the environment and general population.

PYRETHROIDS

Pyrethrins are found in Chrysanthemum flowers and serve as a natural system of protection against insects (146). One major limitation of organic pyrethrins is their fast photo-degradation leading to limited usage in agriculture. Hence, researchers established a more stable synthetic compound, labeled pyrethroids (PYRs), based on the chemistry of natural pyrethrins. The chemical structures of PYRs are comparable across the group and retain the essential acid/alcohol configuration of pyrethrins. Both groups block normal nerve impulses by preventing closure of voltage-gated sodium channels in axonal membranes, thereby paralyzing and eventually killing the organism (147).

Currently, PYRs represent a major class of insecticides worldwide, with more than 14% of the total pesticide market in 2005 (148). The most common PYRs include allethrin, bifenthrin, cyfluthrin, λ cyhalothrin, cypermethrin, deltamethrin, permethrin, d-phenothrin, fenvalerate, resmethrin, and tetramethrin (149). Two frequent metabolites, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropne carboxylic acid (DCCA) and 3-phenoxybenzoic acid (3-PBA), are non-specific metabolites of several different PYR insecticides (150).

The major route of exposure for humans is through ingestion of food items treated with PYRs (151). Even though PYRs have relative short half-lives ranging from 3- to 96-days in soil (152), they are found in environmental samples (153, 154), human samples (155) and food (156), probably as a result of excessive and repeated use. Given PYRs structural resemblance to T3 and T4, PYRs insecticides are suspected to act as TH disruptors (150).

An overview of the epidemiological PYR studies can be found in **Table 4**.

Epidemiological Evidence of Thyroid Disruption

As determined by urinary levels of metabolites to estimate internal exposure, human exposure to PYRs is widespread (155). Pregnant women are of particular concern given the vulnerable window of prenatal exposure to pesticides that could lead to impairment of normal development of offspring. The most commonly detected PYR metabolite is 3-PBA, a breakdown product of several PYR, that has been repeatedly measured in urine of pregnant women. However, no association was found between maternal TH serum levels and PYR exposure (157). Concealed behind this outcome remain important facts that should be taken into consideration: (i) both PYRs and their metabolites have the capacity to induce antagonistic effects on TRs (ii) they are able to transfer to the placenta (150, 158, 159). It might be prudent to carry out more regular measurements of TH levels in women exposed to PYRs and eventually examine offspring for TH related effects.

In vivo Evidence of Thyroid Disruption From Animal Studies

In adult rats, two separate *in vivo* studies observed alterations of TH levels in serum using different PYRs and distinct exposure protocols (160, 161), suggesting that PYRs are potential thyroid disruptors. In another rodent study, pregnant mice were orally administered a PYR (fenvalerate) throughout pregnancy on a daily basis. Quantitative analysis of mRNA in the placenta of exposed mice showed a reduction of TRα1 and TRβ1 transcripts. Additionally, fetal intrauterine growth retardation (IUGR) was observed in offspring that were exposed during embryonic development. Following the observation of altered maternal THs together with IUGR, one may hypothesize that disruption of placental TR mRNA could explain poor fetal intrauterine growth (162). Furthermore, a study using lizards (*Eremias argus)* as a model organism showed that a 21-day exposure to λ -cyhalothrin alters the expression of TH-related genes in the liver (163). A zebrafish study demonstrated that permethrin, one of the most frequent pyrethroids, significantly increased expression of major thyroid signaling genes (thyroid hormone receptors, deiodinases, thyroid-stimulating hormone) as well as transthyretin (TTR) protein in zebrafish larvae, after a single 3-day embryonic exposure. Additionally, the same research group showed that permethrin has the potential to alter TTR activity by docking to TTR's active pocket (164). This research group also identified bifenthrin and λ-cyhalothrin as disruptors of the HPT axis in zebrafish embryos. The majority of the genes examined related to the HPT axis (*tpo, dio1, dio2, thra, thrb, ttr)* were found to be upregulated (165). Another zebrafish study tested two PYRs (permethrin and β-cypermethrin) and three metabolites: 3-phenoxybenzoic alcohol (PBCOH), 3-phenoxybenzaldehyde (PBCHO), and 3-phenoxybenzoic acid (PBCOOH). The study demonstrated that both PYRs and their metabolites exert effects on TH signaling, locomotor behavior and development of

TABLE 4 | Parameters of epidemiological data retrieved in the review—pyrethroids.

									Thyroid hormone			Pesticide								
Chemical class	**Reference**	**Collection**	**Country**	**City**	**Population**	***N***	**Mean age (years)**	**Male-Female**	**Matrix**	**Time**	**Matrix**	**Time**	**Pesticide name**	**Mean concentration**	**TSH**	**TT3**	**FT3**	**TT4**	**FT4**	**Hypothyroidism**	**Other observations**
Epidemiology—Pyrethroids																					
Pyrethroids	Zhang et al. (157)	2009–2011	Japan	University Hospital Tokyo	Pregnant women	231	34.1	0–100%	Serum	10-12th week of pregnancy	Urine	10-12th week of pregnancy	3-PBA	0.363μg/g	ns	-	-	-	ns	-	Also measured thyroid binding globuline but no significant results were obtained

embryonic zebrafish. The observed thyroid disruption may play a role in the aberrant larval development (166).

An additional factor that should be taken into consideration is that fish are vulnerable to changes in temperature attributable to global climate change and that the increase in temperature might exacerbate the effect of chemical exposure. One study conducted by Giroux et al. (167) investigated both the effects of higher temperature and pyrethroid (bifenthrin) exposure on TH signaling. To better understand the possible interaction between pesticide exposure and temperature on salmon development, researchers reared the fish at different temperature (11, 16.4, and 19°C) and exposed them at different concentrations (0, 0.15, and 1.5 μg/L) of bifenthrin for 96 h. Final results revealed decreased survival with increasing temperatures. Following pesticide exposure, TH levels were either significantly increased in the case of juvenile exposure in warm water and tended to decrease during fry stages at low temperatures. These adverse sub-lethal effects could have long-term consequences for populations, such as those featured in this study in California which are affected by both a seasonal rainstorm runoff containing pyrethroid pesticides and increasingly warmer waters.

In vitro Evidence of Thyroid Disruption

An *in vitro* research reported that an array of PYR insecticides (cycloprothrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, etofenprox, fenvalerate, permethrin, and tetramethrin) and their prevalent metabolite, 3-PBA, have the potential to disrupt TH signaling. By making use of a receptor-mediated luciferase reporter gene assay, Diu et al. (150) demonstrated that the aforementioned PYRs, of similar chemical structure, exert antagonistic action on the TH receptor and therefore impede the TH axis. The potential for PYRs and their metabolite to interact with androgen or estrogen receptor was also investigated. Interestingly, results suggested that, in the case of estrogen signaling, the metabolites rather than their parent compound should be given greater concern as they were up to 1,000-times more potent in their interaction with the receptor (150).

To conclude on pyrethrins, their structural resemblance to THs, with the fact that *in vitro* and *in vivo* animal studies demonstrate clear interference with TH homeostasis and action argues for more caution in their use and more intense scrutiny of their long-term effects.

PHENYLPYRAZOLE

Fipronil, the most representative synthetic pesticide of the phenylpyrazole family, is a broad-spectrum insecticide and acaricide. This "second generation" pesticide is widely used in crop protection in urban areas for insect control and in veterinary practice for its efficiency against domestic animal ectoparasites (168). As fipronil usage has increased rapidly since its introduction in 1993—accounting for approximately 10% of the global pesticide market (169, 170), it has also become a widespread environmental contaminant detected in both soil and water (171, 172), indoor and outdoor dust (173) as well as in various food matrices as highlighted by the recent egg contamination scandal [(174, 175)[8]] Fipronil was partially restricted in 2013 and prohibited for outdoor use in most EU nations during flowering periods after its suspected involvement in the historical mass mortalities of honey bees (176). However, this decision was recently overturned by the European court due to insufficient impact assessment (177). Fipronil is still used as a biocide for ants and cockroaches as pests in the EU, but there has not been an application for its re-registration for use as a systemic pesticide in agriculture (178). Initially introduced after increasing resistance to organophosphate, pyrethroid and carbamate pesticides (170), its neurotoxicity arises from non-competitive blocking of gamma-aminobutyric acid (GABA)-gated chloride channels resulting in excessive neuronal stimulation and death. Its specificity for target organisms derives from its higher affinity for invertebrate GABA-A and GABA-C receptors than those of mammals (169, 179, 180).

Adverse effects were found in numerous non-target organisms including beneficial insects such as bees and termites, and vertebrates including fish, reptiles, birds, and mammals (168). In addition to hepatotoxic, nephrotoxic, anti-reproductive, and cytotoxic effects (180). Alarmingly, the primary metabolite fipronil-sulfone and the photodegradate fipronil-desulfinyl are both biologically active and are considerably more toxic, persistent, bioaccumulative, and less selective than the parent compound (181–187). Since fipronil is rapidly metabolized into the sulfone metabolite which has a longer half-life (208 h instead of 8.5 h in rats) and a six-fold greater binding capacity to vertebrate GABA receptors than fipronil (181), fipronil-sulfone has been suggested as the main mediator for fipronil-induced toxicity (180, 187, 188).

Epidemiological Evidence of Thyroid Disruption

In human studies, serum fipronil-sulfone, the main metabolite of fipronil, was inversely correlated with serum TSH levels in factory workers manufacturing veterinary drugs containing fipronil, suggesting a central inhibitory effect on TSH secretion (189). While exposure of the general population is lower than occupational exposure, fipronil exposure can nonetheless affect sensitive populations such as newborn infants. In a pregnancy-birth cohort study, maternal fipronil-sulfone was reported to be placentally transferred to newborn infants along with exposure levels inversely correlated with FT3 levels in both cord blood and newborns (169). Furthermore, fipronil-sulfone concentrations were negatively associated with 5-min Apgar scores (a method used for assessing new born status immediately after birth and a biomarker of developmental vulnerability) (190). An overview of the epidemiological fipronil studies can be found in **Table 5**.

In vivo Evidence of Thyroid Disruption From Animal Studies

Exposure to fipronil caused tumors in rats via hypertrophy of thyroid follicles (191) and altered the integrity of follicular cells, thyroid tissue and even the chemical composition of the colloid in mice (186, 192). Moreover, fipronil exposure decreased

[8]https://www.bbc.com/news/world-europe-40878381

TABLE 5 | Parameters of epidemiological data retrieved in the review—phenylpyrazole.

Chemical class	References	Collection	Country	City	Population	*N*	Mean age (years)	Male-Female	Thyroid hormone		Pesticide		Mean concentration	TSH	TT3	FT3	TT4	FT4	Hypothyroidism	Other observations
									Matrix	Time	Matrix	Time	Pesticide name							
Epidemiology—Phenylpyrazole																				
Phenylpyrazole	Herin et al. (189)	2008	France	factory manufacturing fipronil-containing veterinary drugs	Workers of factory	159	34.1	51% - 49%	Serum	Cross-sectional	Serum	Cross-sectional	Fipronil	Mean concentration	ns	-	-	ns	ns	-	-
													Fipronil sulfone	7.79 mg/L	↓	-	-	ns	ns	-	-
Phenylpyrazole	Kim et al. (169)	March 2013–July 2015	South-Korea	Inje University Ilsan Paik Hospital	Pregnant women and matching biological fathers	169 participants, 59 mother-neonate pairs and 51 matching biological	32.08 age at delivery	100%-100%	Cord blood	At birth	Serum mother—serum father—cord blood	Delivery	Fipronil	Below levels of detection	ns	ns	ns	ns	ns	-	-
													Fipronil sulfone	Geometric mean fipronil sulfone—maternal serum: 0.744 ng/mL—paternal serum: 1.163 ng/mL—cord blood serum: 0.525 ng/mL	ns	Infantile fipronil sulfone levels were inversely associated with cord blood T3	Infantilens fipronil sulfone levels were inversely associated with cord blood FT3	ns	ns	-	-

circulating TH levels in rats, at least partially mediated by increased clearance following elevated activity and expression of phase II hepatic enzymes (186, 187, 193, 194).

In vitro Evidence of Thyroid Disruption

Using *in vitro* reporter gene assays, fipronil-sulfone showed antagonistic activity via TRβ in a dose-dependent manner, suggestive of another potential mechanism of action for the thyroid disrupting effect of fipronil (195).

NEONICOTINOIDS

Neonicotinoids are a relatively new class of broad-spectrum insecticides discovered in the 1980s and widely used today in agriculture for their systemic properties (170), but also in commercial, residential and veterinary settings (196). The global insecticide market, previously dominated by carbamates, organophosphates and pyrethroids was reshaped in 2008 when neonicotinoids represented over a quarter of the market. In 2008, the neonicotinoid imidacloprid (IMI) was the world's largest selling insecticide, second only to the herbicide glyphosate (170, 197). Currently, the main neonicotinoids on the market are imidacloprid, thiamethoxam, thiacloprid, clothianidin, acetamiprid, nitenpyram, and dinotefuran, however the first three were recently banned for outdoor use in Europe during flowering, following demonstrated risks to bees and other pollinators. Structurally similar to the natural insecticide nicotine—historically used for centuries as an early insecticide, neonicotinoids have enhanced selectivity and potency for the insect nicotinic acetylcholine receptor (nAChR), compared to vertebrate nAChR subtypes, and relatively poor penetration of the mammalian blood–brain barrier, translating into apparent reduced health risks for mammals, birds and fish (196, 198). The insecticidal activity of neonicotinoids originates from binding to nAchRs, triggering a large influx of cations in the postsynaptic membrane of nerve cells in the central nervous system, causing excessive excitatory neurotransmission which results in paralysis and death (198).

Epidemiological Evidence of Thyroid Disruption

Although there is no epidemiology of TH levels in humans exposed to neonicotinoids, there is one report of reduced IQ as a function of prenatal exposure to neonicotinoids and pyrethyroids (199).

In vivo Evidence of Thyroid Disruption

Pandey and Mohanty (142) examined potential thyroid disrupting effects in wild male finches exposed to IMI under laboratory conditions. They reported altered thyroid histopathology including increases in thyroid weight and volume, hypertrophy and hyperplasia of epithelial and stromal cells, decreases in plasma T4, T3 and TSH levels. It should be noted that a commercial formulation of IMI was utilized which contains multiple potentially bioactive ingredients (200).

In vitro Evidence of Thyroid Disruption

A TH-dependent proliferation assay (T-screen) with GH3 cells was employed to investigate the thyroid disrupting potential of common neonicotinoids, but none of the 7 neonicotinoids tested had any effect on TH-dependent proliferation (200) in the absence of T3. However, Xiang et al. (201) showed that IMI exerted agonist effects in a GH3 luciferase reporter gene assay mediated via TR which was further characterized using human TRβ in a Surface Plasmon Resonance biosensor technique to measure binding kinetics. Moreover, *in silico* molecular docking (MD) analysis predicted that IMI may compete with T3 for binding with TR by forming hydrogen bonds with the imidazole group (202).

Overall, despite their widespread use, relatively few studies have investigated the potential thyroid disrupting effects of neonicotinoids, but many have examined the effects of neonicotinoids in mixtures containing various classes of pesticides, which is more in line with the reality of environmental exposure.

MIXTURES

Humans are simultaneously exposed to myriads of chemicals that may act in concert to induce mixture effects. Currently, risk assessment is focused on examining chemicals individually, without taking into account potential toxic effects of low-dose combinations of EDCs. Frequently, in agricultural practice, multiple pesticides are preferentially used to reduce resistance to an individual pesticide and enhance efficacy (203). In the case of combined exposure with one or more families of different pesticides, several modes-of-action could be involved. Furthermore, each chemical may interfere with another, resulting in complex dose-response interactions (204). It is important to mention that in most cases EDC responses, as well as hormones, do not show the typical monotonic dose responses classically used in toxicology studies but rather follow non-monotonic dose responses (NMDR) (16). A first and widely used model in toxicology is the dose addition model (DA). DA assumes that toxicity can be expected if the sum of the concentration of each compound of the mixture is high enough to surpass the threshold of toxicity of the mixture, regardless of the fact that the concentration of each compound is below its own effect threshold (205). A conventional term for threshold is the No Observed Adverse Effect Levels (NOAELs). NOAELs are used to deduce regulatory threshold values applying an uncertainty factor, typically of 100 resulting from a factor of 10 to take into account interspecies extrapolations and another factor of 10 considering intra-species variability (206). Another concept is the principle of Response Addition (RA) used to assess the toxicity of a mixture containing compounds with different modes of action (207). Consequently, models integrating the concepts of DA and RA are needed to investigate complex mixtures of chemicals with both similar and dissimilar modes of action, a so-called "integrated-addition model" (208). Several studies have undertaken a "whole mixture approach." In this approach a combination of chemicals is considered as a single compound even if specific effects of each component are not investigated (209, 210). While studying complex mixtures is necessary, extrapolating from one mixture to another is difficult knowing that small changes in composition can possibly lead to differences in outcomes (211). An example of application of whole mixture approach is that the *in vivo* identified animal model dose-response relationship of the mixture can determine a reference dose (RfD) associated with an adverse health outcome *in vivo.* A statistical measure of "sufficient similarity" of the RfD can be used to compare the RfD with EDC exposure levels assessed in the human population to generate a "similar mixture risk indicator" in order to identify people at risk. The systematic integration of experimental animal studies and epidemiological studies may improve the scientific understanding of implicated health effects and improve risk assessment of EDCs (212).

In vivo Evidence of Thyroid Disruption

A study exposed rats for 4-days to different TH synthesis inhibitors (pesticides: thiram, pronamide, and mancozeb) and stimulators of T4 liver clearance [polyhalogenated aromatic hydrocarbons (PHAHs): 2 dioxins, 4 dibenzofurans; and 12 PCBs including dioxin-like and non-dioxin like polychlorinated biphenyls (PCBs)], and compared the decrease in T4 serum levels with the three aforementioned models' predictions (response addition, dose addition and integrated addition as explained above). Rats exposed to highest dose of the mixture demonstrated a 45% reduction of serum T4. Results from this study support both the dose- and integrated-addition model as they provided a better prediction than the response-addition model (213). Another study conducted by Wu et al. (214) observed synergistic effects between the organophospate triazophos (TRI) and the neonicotinoid, imidacloprid, when investigating combined toxicities in zebrafish. mRNA levels of *dio1, dio2,* and *tsh* were more affected in joint exposure compared to their individual pesticide exposure, underlining the importance to incorporate joint toxicity studies. Due to specific action of each compound, a mixture of the insecticide IMI in co-exposure with the fungicide mancozeb (MCZ) is frequently used in the field. Therefore, another mixture study used this combination to mimic real-life exposure for both wild animals and human beings. One focus of this study was the assessment of differences in body weight of mice exposed via lactation to low doses of the two pesticides. First, *in silico* molecular docking (MD) was used to predict the probable binding modes of pesticides with THRs (alpha and beta). MD showed both compounds could compete with active TH for TR binding. No effect on body weight was seen with individual exposure whereas low dose exposure to a combination of both pesticides induced significant gain in relative body weight. This increase in weight might be a consequence of TH imbalance as, additionally, significant decreases in THs (both T3 and T4) and increases of TSH in blood plasma were observed in mice exposed to the mixture (202). Another study exposed rats to the four most widely used OP insecticides (dichlorvos, dimethoate, acephate, and phorate) in agriculture in China and identified that these pesticides elicit a joint toxic response. Rats were given the mixture via drinking water for a period of 24 weeks and plasma was

analyzed by metabolomics. Levels of iodotyrosine, an early precursor to T4, were decreased, suggesting that exposure to an OP mixture can alter thyroid gland function (215). As there is increasing emphasis on the use of blood-based biomarkers of adverse outcomes in safety assessment, other studies have evaluated targeted metabolite profiles after exposure to different chemical groups (193). Given the "one health" concept, with human health being related to that of biodiversity and the environment, non-target organisms of pesticides are a concern for human well-being. A study on the seasonally breeding finch, *Amandava amandava* demonstrated that when co-exposed to a dithiocarbamate (mancozeb/MCZ) and a neonicotinoid IMI, both thyroid homeostasis and reproductive axis were affected. A 30-day exposure to this mixture via food intake, at concentrations even lower than environmental levels, changed thyroid weight and volume, with decreased T3 and T4 plasma levels (216, 217).

Overall, thyroid-disrupting chemicals are less investigated than estrogen or androgen disrupting compounds, thus very few studies have centered on investigating TH-related endpoints of pesticide mixtures.

DISCUSSION

Evidence of PPP Thyroid Disrupting Effects

A significant increase in many neurodevelopmental and neurodegenerative diseases in both adults and children has drawn attention to the potential role of environmental factors, including pesticides (218). Despite the relatively well-known effects of different classes of pesticides on the HPT axis (44, 219), numerous knowledge gaps and mechanistic insights remain between specific thyroid hormone-related endpoints and adverse neurodevelopmental parameters. In this review, after applying a defined methodology, we focused on 45 recent papers that investigated the association of TH disruption and pesticide exposure. A large number of these pesticides are associated with TH axis disruption either in humans or in experimental models.

Epidemiological Data

In most mother/child cohorts, organochlorine pesticides were associated with either an increase in TSH, a decrease in circulating TH or both (see section Background and **Table 1**). These pesticides represent the first generation and the majority is banned. However, as they are so persistent, they remain in human fluids and the environment as legacy pesticides, adding to the potential effects of newer pesticides. This second generation pesticides reviewed here, includes, the acetylcholine esterase inhibitors, includes organophosphates (including glyphosate and chlorpyrifos) and carbamates (sections Overview Of The Hypothalamus-Pituitary-Thyroid Axis and Methods). Their main mode of action causes the accumulation of acetylcholine at the synaptic region. Their presence in blood or urine during early development has been associated with thyroid dysfunction (114), increase of hypothyroidism (91, 139, 140), brain function impairment (107, 113), and increase in hormone-dependent cancer (115), which can be related to homeostatic imbalance.

Interestingly, for the last generation of pesticides examined, only Fipronil belonging to the phenylpyrazole family was associated with a TSH decrease (189) in adults or a fT3 decrease level in newborns (169). No other associations were identified with pyrethroids or neonicotinoids despite several disruptive effects on the thyroid axis having been reported in *in vivo* animal studies, along with description of plausible mechanisms *in vitro* (see sections Pyrethroids, Phenylpyrazole, Neonicotinoids). However, the lack of epidemiological studies on TH and neonicotinoid exposure needs to be re-emphasized.

To understand the apparent discrepancy between epidemiological and animal models, some parameters need to be taken into account: (i) a major difference between previous and modern pesticide formulations is the shorter half-life and lower bio-accumulative potential of newer compounds. This implies that active compounds are not necessarily present at the time of sampling. (ii) If absent or below the detection limit, a specific biomarker or metabolite needs to be measured. In all cases, specific analytical methods need to be developed and validated. (iii) We are all exposed to a multitude of chemical and pesticide residues which are not necessarily measurable due to their short half-lives (see following section Underestimated Parameters in Epidemiological Studies). An example, although not a pesticide, is tetrabromobisphenol A(TBBPA) (220). Therefore, combined effects may not be apparent in epidemiological data whereas, in a more systematically controlled experiment, significant effects of a single compound or mixture may be revealed. Moreover, as we are exposed to a multiple of chemicals at a certain time point and geographical location, one has to be extremely cautious about associations made with any single chemical and on a given study population. To date, most data sets rely on basic biomarkers such as TH level measurements and too few studies investigate actual mixture effects.

Underestimated Parameters in Epidemiological Studies

(1) Joint exposures

Few epidemiological studies address mixture effects. This is because current risk assessment focuses on single molecules, with interactions between chemicals rarely taken into consideration. Mixture exposure, representing more realistic scenarios, is unduly underestimated.

First, legacy pesticides that have been overwhelmingly studied as single compounds are not considered as parts of mixtures we are currently exposed to. Second, synergy, commonly defined as the effect of multiple compounds working in combination that is greater than the expected additive effect of individual chemicals, is rarely considered (221). Further, EDCs can also exert opposing effects on the same axis and cross-talk between different axes has been well-established (222).

One of the principal aims of epidemiological studies is to clarify complex pictures. Thus, one goal is to determine if an association exists with the lowest occurring parameter. The effects of chemical exposure can be manifold, with pathways induced by banned, persistent or legacy PPP, potentially masking adverse health endpoints through overlapping and, possibly, overlooked mechanisms. Further, absence of association is not necessarily synonymous with chemical inertness.

(2) Long lasting effects and sensitization

Pesticides may not exert an obvious adverse effect at the moment of exposure, but effects may be delayed or one exposure may sensitize the organism to a later occurrence, incurring a fitness cost, which might only be seen when a second stressor appears or later in life.

Different scenarios can be envisaged. The following examples may not refer specifically to pesticides but serve to illustrate the point. First, alterations of the immune system can be implicated. Recent studies revealed modifications in immune responses following exposure to a mixture of molecules used for fracking and found in drinking water. This has been shown in mouse and amphibian models following developmental exposure to a "fracking" mixture. Prior exposure resulted in increased mortality when the animals had to cope with an otherwise benign viral infection (223, 224). These altered immune responses echo epidemiological studies such as in the Faeroe islands reported by Grandjean and Andersen (225). They showed that children exposed to perfluorinated compounds displayed reduced antibody responses to a standard vaccination protocol.

Second, other environmental factors may act as secondary stressors, e.g., another xenobiotic affecting the same or another essential physiological pathway or the same xenobiotic at a different time-point, to which one can add climate change and/or reduced insect populations during bird migration. All of these parameters need to be borne in mind when attempting to understand the more far reaching effects of pesticides on non-target species, including humans.

Evidence for *in vivo* and *in vitro* Mechanisms of Action

One could summarize that organochlorine and organophosphates were designed to stably block enzymes used in the biochemical processes of the pest targeted. Most novel pesticides were designed to address problems of accumulation (most pronounced in compounds classified in the Stockholm convention as persistent organic pollutants (POPs), pesticide-induced pest resistance and high mammalian toxicity at low doses. In theory, these second generation molecules can be used at higher concentrations, as well as being more specific and biodegradable. However, their impact on non-target organisms are often neglected and only toxicity assays were (and still are) required before release on the market. As illustrated by the OP chlorpyrifos, pesticides may have cryptic effects at sub-lethal concentrations (119, 120, 226).

All classes of pesticides have been associated with disruptive effects of the HPT axis at various levels determined by *in vitro* and/or *in vivo* studies (see **Figure 2**). Strikingly, none of these actions are currently included in assays required before a PPP is marketed. One must note that detection of disruption at a given level is insufficient to identify adverse action of all pesticides (even within different pesticide classes). While the abnormality might only be transient, persistence may be due to underlying thyroid dysfunction. More, as shown for OCPs, some chemicals may have opposite effects on circulating TH. Interestingly in Freire et al. (75), an endosulfan metabolite was associated with a decrease in TSH whereas other OCPs were associated with an increase in TSH. While *in vivo* studies suggest a mechanism based on increased clearance by hepatic activation of phase II enzymes, *in vitro* studies suggest direct action on TH synthesis at the thyroid gland level highlighting the complementarity of approaches.

Both the EFSA scientific report (44) on cumulative assessment groups of pesticides and annex A of the guidance document for the identification of EDCs, state that all molecules acting on the liver, increasing metabolism or modifying deiodinase 1 activity with consequent TH levels modifications, can be considered as TH axis-disrupting compounds. This feature represents a large proportion of pesticides and is a significant step toward identifying chemicals affecting the thyroid axis. Hence, pesticides may also exert effects beyond TH levels and the thyroid gland, with displacement from distributor protein and increased liver metabolism (e.g., fipronil-sulfone, see **Figure 2** for a detailed review on level of actions of different pesticides on TH axis).

A Need for Improved Testing Strategy, Risk Assessment, and Updated Legislation

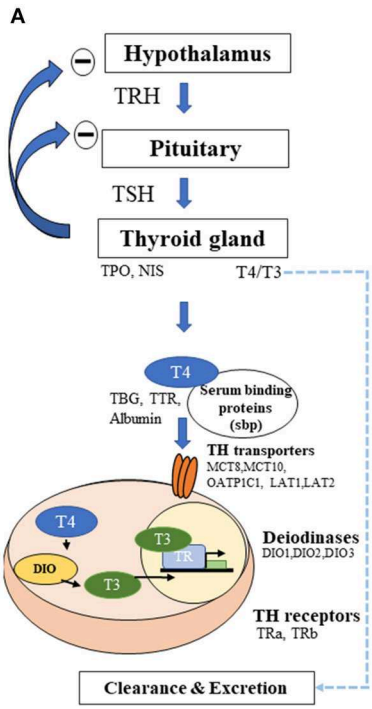
As Milner and Boyd already discussed in 2017, "pesticidovigilance" is something that needs to be developed. One of the possible strategies is to transpose what is currently carried out in pharmacovigilance for drugs, to PPPs or other classes of chemicals. This step would be beneficial for human, animal and environmental health (227).

Improved Testing

The vast majority of the available validated assays by EPA or OECD were developed from former reproductive or toxicological assays enriched with endocrine related endpoints, i.e., TH levels measurements and thyroid gland histology. Therefore, these assays were not specifically designed to detect endocrine sensitive or specific endpoints. The central nervous system is one of the major targets of TH and therefore, TH-axis disruptors. Consequently, we sorely need better brain endpoints for potential effects of pesticide exposure taking into account the multiple levels of potential interference. In the brain, cell proliferation, neural stem cell differentiation, neuronal migration, synaptogenesis or myelination can all be influenced by TH availability. For example, in neonatal rats, prenatal exposure to the anti-thyroid compound PTU at low doses induces failure in neuronal migration—an event more sensitive than circulating TH levels (228). Given this overall lack of sensitivity, the EU commission is currently funding three large-scale projects (ATHENA, ERGO, and SCREENED) to develop innovative and specific endpoints for TH axis disruption, specifically with a focus on brain endpoints.

Risk Assessment

The report by Demeneix and Slama for the EU parliament in early 2019 highlighted the steps necessary for efficient risk management in terms of protecting Health and environment:



	Organochlorines	Organophosphates	Carbamates
		MCP: ↓ *trh* mRNA in hypothalamus (Zhang et al. 2014; goldfish) ↑ *trh* mRNA in brain (Zhang et al. 2018; goldfish)	Zineb: ↓ TRH (Laisi et al. 1985; rat)
	DDT: ↓ TSH-induced cAMP (Santini et al., 2003) ↓ TSHR activation (Rossi et al., 2018)	MCP:↓ TSH, ↑ *tshb* mRNA in brain (Zhang et al. 2014; goldfish) ↓↑ *tshb* mRNA in brain (Zhang et al. 2018; goldfish) Glyphosate: ↓ TSH (Souza et al., 2017; rats) Diazinon: ↓ TSH (Katuli et al., 2014; caspian roach) Malathion: ↓ TSHR mRNA (Qiu et al., 2017)	Maneb: ↓ TSH (Laisi et al. 1985; rat) Zineb: ↓ TSH (Laisi et al. 1985; rat)
	p,p'-DDE: ↓ TT4, FT4 (Liu et al., 2011; rats) DDT: ↑↓ TSH & TH Altered histo/morphology (Yaglova et al., 2014, 2017; rats) HCB: ↓ T4 (Alvarez et al., 2005; rats) ↓ FT4, ↓ TT4, ↑TSH (Van Raaij et al.,1993; rats) ↑ TGF-b1 mRNA (Chiappini et al., 2014) PCP: ↓ FT4, TT4 (Van Raaij et al.,1993; rats)	CPF: ↓ T4 (De Angelis et al., 2009; mice) ↓ T3 (Holzet et al. 2017; *surgeonfish*) MCP: ↓T3 (Zhang et al., 2014; goldfish) ↓T3 (Zhang et al., 2018; goldfish) Diazinon: ↓ T3, ↓ T4 (Katuli et al. 2014; Caspian roach) MCP: ↓ T3, ↓ T4 (Zhang et al., 2014; goldfish)	Mancozeb: ↓ T4, ↓ TPO activity (Kackar et al. 1997) ↓ T4, ↑↓ T3, ↑↓ TSH, Altered thyroid histology (Pandey et al. 2015) Thiram: ↓ *tpo* mRNA (Chernick et al., 2018; zebrafish) Zineb, ETU: ↓ TPO iodinating activity (Marinovich et al., 1997)
	p,p'-DDE: ↓ TTR (Liu et al., 2011; rats) HCB &PCP : ↓ binding of T4 to sbp (Van Raaij et al.,1993)	MCP: ↑ *ttr* mRNA in liver (Zhang et al., 2014; goldfish) ↑ *ttr* mRNA in liver (Zhang et al., 2018; goldfish)	
		Glyphosate: ↓ Mct8, Oatp1c1 mRNA in hypothalamus, pituitary (De Souza et al., 2017; rats)	
	HCB: ↑ DIO1 activitiy in thyroid & total body (Alvarez et al.,2005; rats)	MCP: ↑↓ *dio2,dio3* mRNA in liver (Zhang et al., 2018; goldfish) ↑ *dio1,dio2, dio3* mRNA in liver; ↑ *dio2, dio3 in brain* (Zhang et al., 2014; goldfish) Glyphosate: ↓ Dio2, Dio3 mRNA in hypothalamus (De Souza et al., 2017; rats)	Thiram: ↑ *dio3* mRNA (Chernick et al.2018; Zebrafish)
	p,p'-DDE: ↑ TRa1 & TRb1 mRNA in hippothalamus (Liu et al. 2011; rats)	Glyphosate: ↑ TRa1, TRb1 mRNA in liver, pituitary (De Souza et al., 2017; rats) ↑ *thrb* (Howe et al., 2004; frogs) ↑ *thrb* (Lanctôt et al., 2014; frog)	
	p,p'-DDE: ↑ EROD, ↑ PROD, ↑ UDPGT (Liu et al., 2011; rats) HCB: ↑ T4-UDPGT activity (Alvarez et al., 2005)		

B

	Pyrethroids	Neonicotoids	Phenylpyrazoles
	Permethrin: ↑ *tshb* mRNA (Tu et al., 2016a; zebrafish) ↓ *tshb* mRNA (Xu et al.,2019; zebrafish) Bifenthrin: ↑ *tshb* mRNA (Tu et al., 2016b; zebrafish) Cypermethrin: ↓ *tshb* mRNA (Xu et al.,2019; zebrafish)	Imidacloprid: ↓TSH (Pandey & Mohanty, 2015; finches)	
	Fenvalerate: ↑ T3, ↑ T4 (Kaul et al., 1996; rats) ↑ T3 (Girray et al., 2010; rats) Cyhalothrin: ↑ T3, ↑ T4 (Chang et al., 2017; lizards) Altered histo/morphology (Chang et al., 2017; lizards) Bifenthrin: ↑ T3, ↓ T4 (Tu et al., 2016b; zebrafish) ↑↓ T3, ↑↓ T4 (Giroux et al., 2019; salmons) Cyhalothrin: ↑ T3, ↑ T4 (Tu et al., 2016b; zebrafish) Permethrin: ↑ T3, ↑ T4 (Tu et al., 2016a; zebrafish) ↑ *tpo* mRNA (Tu et al., 2016a; zebrafish)	Imidacloprid: ↑ T4, ↑ T3 Altered histo/morphology (Pandey & Mohanty, 2015; finches)	Fipronil: Altered histology Altered colloid composition (Ferreira et al., 2012; mice) ↓ T3, ↓ T4, ↑ TSH (Leghait et al.,2009; rats) ↓ T3, ↓ T4, (Moser et al., 2015; rats) ↓ T3, ↓ T4 (Roques et al., 2012; mice)
	Permethrin: ↑TTR & *ttr* mRNA (Tu et al., 2016a; zebrafish) Bifenthrin: ↑ *ttr* mRNA (Tu et al.,2016b; zebrafish) Cyhalothrin:↑ *ttr* mRNA (Tu et al.,2016b; zebrafish)		
		Imidacloprid: ↓TR assay ↑ TR binding (Xiang et al., 2017)	Fipronil-sulfone: ↓ TR assay (Lu et al., 2015)
	Permethrin: ↑ *dio1*, ↑ *dio2* mRNA (Tu et al., 2016a; zebrafish) Permethrin, Cypermethrin: ↓ *dio2* mRNA (Xu et al.,2019; zebrafish) Cyhalothrin: ↓ dio1 mRNA (Tu et al., 2016b; zebrafish) ↑ *dio1,dio2* mRNA in liver & brain (Chang et al., 2017; lizards) Cyhalothrin & Bifenthrin: ↑ *dio2* mRNA (Tu et al.,2016b; zebrafish)		
	Fenvalerate: ↓ TRa1, ↓ TRb1 mRNA in placenta (Wang et al., 2017; mice) Cyhalothrin: ↑ *tra*, ↑ *trb* mRNA in liver & brain (Chang et al.2017; lizards) Bifenthrin, Cyhalothrin: ↑ *TRa* mRNA (Tu et al.,2016b; zebrafish) Permethrin: ↑ *TRa*,↑ *TRb* mRNA (Tu et al., 2016a; zebrafish) Permethrin, Cypermethrin: ↑ *TRa, TRb* mRNA (Xu et al.,2019; zebrafish) PYRs: ↓ TR assay (Du et al.; 2010)		Fipronil: ↑ UDPGT activity (Leghait et al., 2009; rats) ↑ *Ugt1a1, Sult1b1* & *Gsta2* mRNA in liver (Roques et al., 2013; mice) ↑ T4 clearance ↑ UDPGT activity, ↑ *Ugt1a1, Sult1b1* mRNA in liver (Roques et al., 2012; mice)
	Bifenthrin: ↑ UGT1ab mRNA (Tu et al.,2016b; zebrafish) Cyhalothrin: ↑ *udp*, ↑ *sult* mRNA in liver (Chang et al.2017; lizards)		

FIGURE 2 | Mode(s) of action of pesticides reported by *in vivo* (in blue) and *in vitro* (in green) articles. **(A)** Mode(s) of action of 1st and 2nd generation of pesticides (organocholorines, organophosphates, and carbamates). **(B)** Mode(s) of action of newer pesticide families (pyrethroids, neonicotinoids, and phenylpyrazoles). TRH, thyrotropin-releasing hormone; TSH, thyroid stimulating hormone; T4, thyroxine; T3, triiodothyronine; TPO, thyroperoxidase; NIS, sodium/iodide symporter; TBG, thyroxin binding globulin; TTR, transthyretin; MCT, monocarboxylate transporters (mct8, mct10); OATP1C1, organic anion-transporting polypeptide 1c1; LAT, large neutral amino acid transporter (lat1 and lat2); DIO, deiodinases (dio1, dio2, dio3); TR, thyroid hormone receptor (thra, thrb); UGT1ab, UDP-glucuronyltransferase 1ab; UDPGT, UDP-glucuronyltransferase; SULT, sulfotransferase; EROD, ethoxy-resorufin-O-deethylase; PROD, pentoxy-resorufin-O-deethylase; GSTA2, glutathione S-transferase alpha 2; cAMP, Cyclic adenosine monophosphate; TGF-b1, transforming growth factor b1; DDT, dichlorodiphenyl trichloroethane; p,p′DDE, p,p′-dichlorodiphenyl dichloroethylene; HCB, hexachlorobenzene; PCP, pentachlorophenol; MCP, monocrotophos; CPF, chlorpyrifos; ETU, ethylenethiourea.

(1) a hazard definition followed by the (2) availability of a guidance document to allow reproducible application of the definition (3) specific validated assays (4) test requirements and (5) integration of the results in a risk management strategy (229). Only steps 1, 2 and 5 are fulfilled for PPPs. Current risk assessment cannot integrate specific endpoints and mixture effects, though innovative approaches have been developed to overcome these limitations for compounds other than pesticides (230).

Legislation

A definition for EDCs and criteria relative to PPP and biocides was adopted in 2018 in the EU. A guidance document on identification of endocrine disruptors for PPP and biocides was approved in June 2018 (231). Nevertheless, major gaps still exist in order to appropriately regulate PPP, essentially attributable to the current lack of specific endpoints. Recognition of the effects of mixtures is making progress as highlighted by EFSA submitting a document for public consultation on the establishment of cumulative assessment groups of pesticides for their effects on thyroid signaling (232).

CONCLUSION

The recent data reviewed here show that in humans, TSH, T3, and T4 measurements are still the most common endpoints evalutated in order to address thyroid disruption and pesticide exposure. Epidemiological data reveal that legacy pesticides (Organochlorine, organophosphate, carbamates) are more often associated with thyroid axis disruption than modern compounds (pyrethroids, neoinicotinoids, and phenylpyrazoles). However, experimental work demonstrates, with both *in vivo* and *in vitro* evidence, that each pesticide class disrupts thyroid homeostasis at different levels. Therefore, one should consider systematizing TH measurements, determining more sensitive brain endpoints for assessing TH disruption and carrying out more detailed longitudinal studies in epidemiology. As the OECD and the EU Commission have underlined, revisiting thyroid axis-disrupting chemicals is a major research priority.

TAKE HOME MESSAGES

- Multiple arguments link environmental causes to the increased incidence of neurodevelopmental disease that many authors have suggested could be related to altered TH signaling.
- Experimental studies show that different classes of pesticides can act at multiple levels on TH signaling.
- Most often, data from epidemiological studies measure levels of TSH and/or TH in blood. These endpoints may not be sensitive enough to detect the small changes that might occur with newer pesticide formulations.
- Hence, there is a need for novel TH and brain specific endpoints that are more sensitive to variations in TH.
- A final point is the need to take the mixture effects (of pesticides and other EDCs) into consideration in risk assessment and regulatory purposes.

AUTHOR CONTRIBUTIONS

ML and J-BF conceptualized and designed the study and conducted pre-screening on abstracts. ML, J-BF, and SC identified the Mesh terms. ML and SC enriched the Mesh Terms and reviewed all the articles and interpreted the data. ML drafted the figure. ML, SC, BD, and J-BF edited the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00743/full#supplementary-material

Table S1 | All articles (223) classified by pesticide class with justification for selection or omission.

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