RESEARCH ARTICLE

Reduced Effects of Thyroid Hormone on Gene Expression and Metamorphosis in a Paedomorphic Plethodontid Salamander

JOURNAL OF EXPERIMENTAL ZOOLOGY PART B MOLECULAR AND DEVELOPMENTAL EVOLUTION

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ABSTRACT

It has been over a century since Gudernatsch (1912, Wilhelm Roux Arch Entwickl Mech Org 35:457–483) demonstrated that mammalian thyroid gland extracts can stimulate tadpole metamorphosis. Despite the tremendous developmental diversity of amphibians, mechanisms of metamorphosis have mostly been studied in a few model systems. This limits our understanding of the processes that influence the evolution of developmental aberrations. Here we isolated thyroid hormone receptors alpha (TRα) and beta (TRβ) from Oklahoma salamanders (*Eurycea tynerensis*), which exhibit permanently aquatic (paedomorphic) or biphasic (metamorphic) developmental modes in different populations. We found that TRα and TRβ were upregulated by thyroid hormone (T_3) in tail tissues of larvae from metamorphic populations, but basal levels of TR expression and T_3 responsiveness were reduced in larvae from paedomorphic populations. Likewise, we found that T_3 treatment resulted in complete loss of larval epibranchials in larvae from metamorphic populations, but little to no epibranchial remodeling occurred in larvae from paedomorphic populations over the same duration. This is the first study to directly demonstrate reduced gene expression and metamorphic responses to T_3 in a paedomorphic plethodontid compared to metamorphic conspecifics, and the first salamander system to show differential expression of thyroid hormone receptors associated with alternative developmental patterns. *J. Exp. Zool. (Mol. Dev. Evol.) 322B:294–303, 2014.*

J. Exp. Zool. (Mol. Dev. Evol.) 322B:294–303, 2014

How to cite this article: Aran RP, Steffen MA, Martin SD, Lopez OI, Bonett RM. 2014. Reduced effects of thyroid hormone on gene expression and metamorphosis in a paedomorphic plethodontid salamander. J. Exp. Zool. (Mol. Dev. Evol.) 322B:294–303.

Evolutionary change in the timing of developmental events (heterochrony) is likely a common mechanism that has produced tremendous morphological diversity across the tree of life (Gould, '77; Alberch et al., '79). Because the endocrine system can coordinate concomitant ontogenetic changes in disparate tissue systems, deviations in endocrine pathways can cause heterochronic shifts simultaneously across the whole organism. A classic example of a major heterochronic shift is the phenomenon of paedomorphosis in salamanders, whereby individuals forego metamorphosis and become reproductively mature in their larval form (Gould, '77; Duellman and Trueb, '86). Most of our knowledge about the genetic and endocrine mechanisms that

Grant sponsor: University of Tulsa; grant sponsor: National Science Foundation; grant number: DEB 1050322.

Conflicts of interest: None.

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Received 3 March 2014; Accepted 8 May 2014

DOI: 10.1002/jez.b.22580

Published online 1 June 2014 in Wiley Online Library (wileyonlinelibrary.com).

underlie paedomorphosis is derived from studies of the laboratory model *Ambystoma* (e.g., axolotls and tiger salamanders; e.g., Galton, '92; Voss and Shaffer, '97; Voss and Smith, 2005; Page et al., 2008, 2009, 2010; Voss et al., 2012). However, there is tremendous developmental diversity in salamanders, including many independent instances of paedomorphosis (Wiens et al., 2005; Bonett et al., 2013, 2014), which could have resulted from a variety of molecular deviations.

Thyroid hormone (TH; 3,5,3-triiodothyronine; T_3) and its less active form thyroxine (3,5,3′,5′-tetraiodothyronine; T_4) ligand to nuclear thyroid hormone receptors (TRs: TRα and TRβ) in target tissues to regulate transcription (Sap et al., '86; Weinberger et al., '86; Shi, '99; Escriva et al., 2000). These hormones are critical for vertebrate development and often show pronounced endogenous increases during metamorphosis (Shi, '99; Laudet, 2011). In metamorphosing amphibians, T_3 and T_4 initiate and regulate morphological transformations such as gill and tailfin (or whole tail) re-absorption, and remodeling of the skeleton (Alberch et al., '85; Hanken and Summers, '88; Shi, '99). There are many possible deviations in the thyroid hormone axis (and/or the interacting stress axis) that could result in the evolution of paedomorphosis (Rosenkilde and Ussing, '96; Laudet, 2011). Two common hypotheses are: (1) lower thyroid activity reduces endogenous thyroid hormone levels (reviewed in Norris and Platt, '73), or (2) target tissues evolve reduced sensitivity to thyroid hormone (Noble, '24; Svob et al., '73; Safi et al., 2006). The latter hypothesis is based on several obligatory paedomorphic lineages that do not show metamorphic changes (to gills or tailfin) in the presence of exogenous T_3 or T_4, yet have functional thyroid glands (Swingle, '22; Noble, '24; Noble and Farris, '29; Svob et al., '73; Safı et al., 2006). At least one obligately paedomorphic species (*Necturus maculosus*) has functional TRs in target tissues (e.g., gills; Safı et al., 2006) that are regulated by TH, but the lack of tissue transformation may be due to a decoupling of TH regulation of downstream target genes that would otherwise result in metamorphosis (Safı et al., 2006; Vlaeminck-Guillem et al., 2006).

The family Plethodontidae is the most species rich and developmentally diverse clade of salamanders (Wake, '66; Chippindale et al., 2004; AmphibiaWeb, 2014). While ancestral plethodontids likely metamorphosed (in their jelly capsule or after a free-living larval stage; Chippindale et al., 2004), there have been multiple independent instances of paedomorphosis in the tribe Spelerpini (Wake, '66; Ryan and Bruce, 2000; Bonett et al., 2014). Several early studies exposed many different lineages of paedomorphic plethodontids to exogenous T_3 or T_4 (Kezer, '52; Dundee, '57, '62; Dundee and Gorbman, '60; Dent and Kirby-Smith, '63). These treatments induced metamorphosis to varying degrees in different species, but these studies were typically based on only few specimens, rarely included untreated controls, and never included comparisons to larvae of closely related metamorphic populations or species (Kezer, '52; Dundee, '57, '62; Dent and Kirby-Smith, '63). Subsequent developmental research on thyroid hormone induced metamorphosis in plethodontids has focused on metamorphic species, but without direct experimental comparisons to related paedomorphs (Alberch et al., '85, '86; Rose, '96; Hickerson et al., 2005). Furthermore, no experiments have examined patterns of transcription during metamorphosis in this family.

The Oklahoma salamander (*Eurycea tynerensis*) is endemic to the Ozark Plateau of eastern North America and has both metamorphic and paedomorphic populations (Bonett and Chippindale, 2004). Paedomorphosis has independently arisen multiple times in *E. tynerensis*, and is associated with streambed substrates that allow access to permanent subterranean water (Bonett and Chippindale, 2006; Emel and Bonett, 2011). Most populations show high genetic isolation from one another, and typically exhibit a single life history mode (paedomorphosis or metamorphosis; Bonett and Chippindale, 2006; Emel and Bonett, 2011).

In this study, we test whether larvae from paedomorphic and metamorphic populations of Oklahoma salamanders exhibit differential sensitivity to thyroid hormone. We examine TH sensitivity at the level of transcription by analyzing expression of thyroid hormone receptor genes alpha (TRα) and beta (TRβ) in tailfin tissue of larval *E. tynerensis*. We focus on these genes because they are regulated by thyroid hormone in other vertebrates, and are necessary for amphibian metamorphosis (Shi, '99; Buchholz et al., 2004, 2006). We tested for prolonged effects of potential TH sensitivity differences by analyzing the timing of metamorphosis of larval to adult epibranchials. Finally, we discuss the implications of variation in thyroid hormone receptor expression and thyroid hormone sensitivity in the development and evolution of larval paedomorphosis in salamanders.

MATERIALS AND METHODS

Animals

We sampled larval Oklahoma salamanders (*E. tynerensis*) from two metamorphic and two paedomorphic populations in the Western Ozarks (Table 1). These four populations were selected to represent extremes in body size and life history within this clade: (1) Meta-Sm, a metamorphic population that metamorphoses at a small body size, (2) Meta-Lg, a metamorphic population that metamorphoses at a large body size, (3) Paedo-Sm, a paedomorphic population that matures at a relatively small body size, and (4) Paedo-Lg, a paedomorphic population that matures at a large body size. Specimens were collected in the field using dip nets in January and February 2012, which is 2–3 months prior to dry summer conditions that may initiate metamorphosis. Specimens were returned to the University of Tulsa and maintained in groups of five or six individuals in 400 mL of filtered tap water in BPA-free containers. Groups were based on population, size, and treatment (see section Experimental design). Specimens were

Table 1. Populations, treatment groups, and body sizes of *E. tynerensis* used in the experiments.

Population: class	Size (g)	Treatment	*n*
Meta-Sm: 1	0.42 ± 0.04	Control	6
Meta-Sm: 2	0.61 ± 0.07	Control	6
Meta-Sm: 1	0.42 ± 0.06	25 nM T3	6
Meta-Sm: 2	0.65 ± 0.11	25 nM T3	6
Meta-Lg: 1	0.39 ± 0.11	Control	6
Meta-Lg: 2	0.57 ± 0.05	Control	5
Meta-Lg: 1	0.35 ± 0.06	25 nM T3	6
Meta-Lg: 2	0.65 ± 0.09	25 nM T3	5
Paedo-Sm: 1	0.33 ± 0.04	Control	6
Paedo-Sm: 1	0.33 ± 0.04	25 nM T3	6
Paedo-Lg: 1	0.39 ± 0.08	Control	6
Paedo-Lg: 2	0.68 ± 0.08	Control	6
Paedo-Lg: 1	0.41 ± 0.04	25 nM T3	6
Paedo-Lg: 2	0.65 ± 0.05	25 nM T3	6

There were two size classes for each treatment group (1 and 2), except for the Paedo-Sm population. However, size classes within treatments were pooled for analyses (see section Results). Body size is in grams (g).
Meta-Sm: Barcelona, Crawford Co., Arkansas.
Meta-Lg: Cookson, Cherokee Co., Oklahoma.
Paedo-Sm: Council Hollow, Ottawa Co., Oklahoma.
Paedo-Lg: Malloy Hollow, Adair Co., Oklahoma.

acclimated to a diurnal incubator on a 10:14 (light:dark) cycle at 18°C for 10 days prior to the start of the experiment and throughout its duration. Salamanders were each fed two to three bloodworms (chironomid larvae) twice a week. Specimens were handled in accordance with institutional IACUC protocols at the University of Tulsa.

Experimental Design

Larvae in the T_3 treatment were exogenously administered 25 nM of 3,3′,5-triiodo-L-thyronine (T_3 Sigma T2752, St. Louis, MO) for 3 weeks. T_3 treated or control water was replaced every 3–4 days throughout the experiment. Forty-eight hours after the initiation of treatment, larvae were anesthetized by immersion in a 0.05% solution of tricane methanosulfate (MS-222). The dorsal portion of the tailfin of each specimen was dissected off with a scalpel and snap frozen on dry ice for gene expression analyses of TRs. Salamanders were awoken with filtered tap water and returned to T_3 treatment (or control) for the duration of the experiment. At 21 days after the start of the experiment, salamanders in some treatment groups showed external signatures of metamorphosis such as resorption of external gills, development of eyelids, and changes in head shape. At this point all specimens were re-anesthetized with MS-222 and preserved in 10% formalin for clearing and staining to determine metamorphic progress.

Thyroid hormone receptors are directly regulated by TH in a diversity of vertebrates including fishes (Liu et al., 2000), amphibians (Kanamori and Brown, '92; Machuca and Tata, '92), and mammals (Suzuki et al., '94). Therefore, when functioning, TR expression should show a relatively early response to thyroid hormone. Our pilot experiments showed that a 48 hr treatment with 25 nM T_3 was effective for upregulating TRs in tailfin tissue of larvae from metamorphic populations of *E. tynerensis* and other metamorphic species of *Eurycea*. Stream adapted salamander larvae, such as *Eurycea*, have relatively small tailfins (Valentine and Dennis, '64; Duellman and Trueb, '86) that begin to resorb early in the process of metamorphosis. Our time frame (48 hr) allowed for comparable tissue harvest among populations, and also the potential for detecting significant upregulation of TRs.

Thyroid Hormone Receptor Isolation and Phylogenetic Analysis

RNA was isolated from tailfin tissues using Trizol Reagent (Invitrogen, Carlsbad, CA), generally following the manufacturer's protocol. RNA concentration was determined using a NanoDrop 8000, and samples were scaled to 100 ng/μL of total RNA. cDNA was synthesized with SuperScript II (Invitrogen) using Oligo DT primers.

Partial transcripts of TRα, TRβ, and a normalizing gene ribosomal protein L8 (rpL8) were amplified via PCR with both general and specific primers. These genes were amplified from the cDNAs of several species of *Eurycea* in addition to our four populations of *E. tynerensis*. PCR products were enzymatically cleaned with EXOSAPIT (USB Corp., Santa Clara, CA). Cycle sequencing reactions were performed with Big Dye v 3.1 (ABI, Applied Biosystems, Inc., Foster City, CA) and sequenced on an ABI 3130xl capillary sequencer at the University of Tulsa. Sequences were deposited on GenBank (TRα: KJ787653–KJ787656; TRβ: KJ787657–KJ787660).

Nucleotide sequences were edited and aligned using Sequencher 4.0 (Gene Codes; Ann Arbor, MI). BLAST (Altschul et al., '90) searches against NCBI database show that these sequences are clearly orthologous to well established thyroid hormone receptor genes (TRα and TRβ) and rpL8 of other vertebrates. The *E. tynerensis* TR nucleotide sequences were translated into amino acid sequences using ExPASy (Artimo et al., 2012), and aligned collectively (TRα and TRβ) using the BLOSSUM protein weight matrix in Clustal W2 (Larkin et al., 2007). The alignment was trimmed to the shortest amino acid sequence collected for *E. tynerensis* for each gene. We also aligned TRα and TRβ sequences from two other salamanders (*Necturus maculosus* and *Ambystoma mexicanum*) and two frogs (*Rana rugosa* and *Xenopus tropicalis*). Salamander TRα and TRβ protein sequences appear to be 408 and 373 amino acids in length, respectively (based on *Necturus* and *Ambystoma*). We isolated minimally 339 amino acids for TRα (83%; *Ambystoma* positions 44–382) and 304 for TRβ (82%; *Ambystoma* positions 16–319) from all four of our populations of *E. tynerensis*. These partial sequences include all of

the TR DNA-binding domain, 11 of the 12 helices of the ligand binding domain, and 20 of the 22 amino acid positions where the TR protein is predicted to contact thyroid hormone (Wagner et al., '95; Weiss and Refetoff, 2000; Fig. S1). Bayesian analysis implemented in MrBayes version 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was used to reconstruct the phylogeny of amphibian TRα and TRβ (together), under the JTT amino acid substitution model (Jones et al., '92). The analysis was run with four chains (three hot and one cold) for 25 million generations, sampling every 1,000 generations. Stationarity was assessed by viewing likelihood values across generations in Tracer version 1.5 (Rambaut and Drummond, 2007). The first five million generations (5,000 trees) were conservatively discarded as burn-in, which was well beyond stationarity. A 50% majority rule consensus was used to summarize the branch lengths and posterior probabilities of nodes support from across the 20,000 post burn-in trees.

Thyroid Hormone Receptor Expression Analysis

Taqman (BHQ1a-6FAM) gene expression assays were developed for TRα, TRβ, and rpL8 (Table 2). Primer and probe binding sites appear to be highly conserved across our four study populations and other divergent species of *Eurycea*. Expression reactions were run using ABI TaqMan Gene Expression Master Mix on an ABI StepOne Plus qPCR machine at the University of Tulsa. Although TRα and TRβ diverged early in vertebrate evolution, the nucleotide sequences of these two genes are alignable and share many conserved regions. To verify the specificity of our thyroid hormone receptor assays we PCR amplified, cloned, and sequenced a large fragment of both TRα and TRβ for *Eurycea* to unequivocally isolate a single transcript of each gene. Our TRα and TRβ gene expression assays only amplify detectable quantities with plasmids that include their respective genes. All experimental samples for a given assay (gene) were run simultaneously on a single qPCR plate with a four point standard curve and negative controls. Expression quantity values (Qtys) were interpolated from CT values (number of cycles) based on the standard curve. The Qtys of both TRα and TRβ were normalized with the Qtys of rpL8, which is ubiquitously expressed and is often used as a normalizing gene for thyroid hormone studies on amphibians (e.g., Mazon and Denver, 2004; Sato et al., 2007). One-way Analyses of Variance (ANOVA) were performed on the log transformed Qty values, and Fisher's Least Square Difference was used to determine significant differences among groups.

Morphological Analysis

Specimens were cleared and stained following Hanken and Wassersug ('81). Briefly, formalin fixed specimens were eviscerated and hydrated in distilled water, and cartilage was stained with alcian blue 8GX (Sigma A3157) for 4 days. Stain was fixed with absolute ethanol, then specimens were gradually rehydrated with distilled water prior to clearing with Trypsin 1:250 (Gibco 27250-018, Grand Island, NY) until specimens were limp. Bones were also stained with alizarin red (VWR VW3611-2) and specimens were ultimately stored in glycerin. During metamorphosis of *Eurycea*, three pairs of cartilaginous larval epibranchials ("gill arches") degenerate and are replaced de novo by a pair of distinct cartilaginous adult epibranchials (Fig. 1). This remodeling process is regulated by thyroid hormone (Alberch et al., '85). Therefore we used degeneration of the cartilaginous larval epibranchials as a morphological marker for thyroid hormone sensitivity and metamorphic timing. We dissected the entire cartilaginous hyobranchial apparatus from each specimen and mounted it on a slide in a solution of glycerin and pectin. Digital images were taken of each slide using Motic Images on a dissecting scope under identical lighting. The area and intensity of staining of the second larval epibranchials were measured using ImageJ (Abramoff et al., 2004), and the measurements of left and right were averaged. To quantify epibranchial remodeling, we determined a larval "epibranchial score" for each specimen by multiplying the average area by average intensity for each individual and normalized it by the specimen's starting body weight. Epibranchial scores ranged from 0 (complete reabsorption of the second larval epibranchial pair) to approximately 150 (second larval epibranchial complete). One-way ANOVAs were used to test for significant variation in epibranchial loss among groups and Fischer's LSD was used to test for significant differences among groups.

Table 2. Primers and probes for quantitative RT-PCR.

Gene	Primers	TaqMan probe
TRα	5′-GGAGGAGATGTTGAAGAGTATGCA-3′	5′-ACCGGCCAGAGCC-3′
	5′-GCTCCCACTCCTCGATGGT-3′	
TRβ	5′-TGTTTTGTGAGCTGCCTTGTG-3′	5′-AGACCAAATCATCCTGC-3′
	5′-TCTCCATGCAGCAGCCTTT-3′	
rpL8	5′-TACGACCACCACCAGCAACAAC-3′	5′-TCGAAGAAGGTGGTGGCATCTGCAA-3′
	5′-GCTCGTGCCTCTGGCAATTATG-3′	

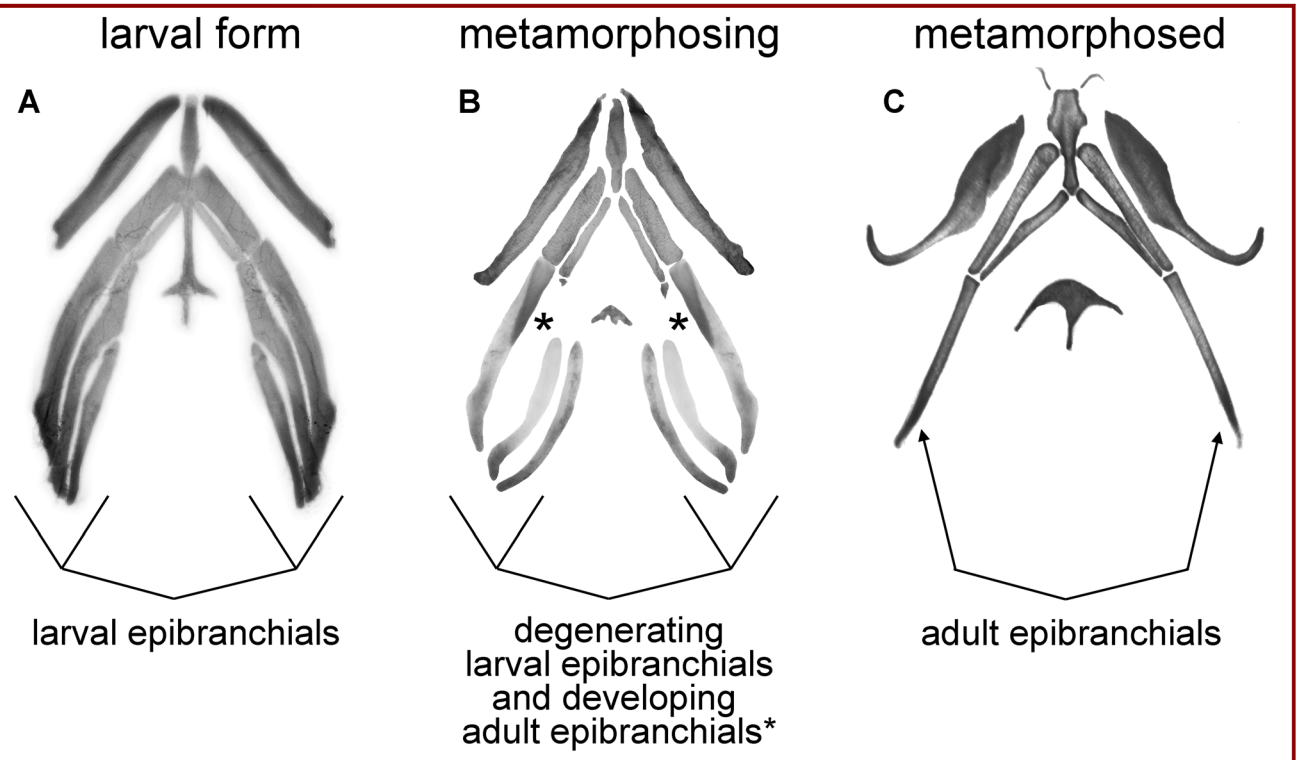

Figure 1. Cartilaginous hyobranchial apparati of larval form, metamorphosing, and metamorphosed *Eurycea tynerensis*. Larvae and paedomorphic adults have three pairs of larval epibranchials (A). During metamorphosis the three pairs of larval epibranchials degenerate and one pair of adult epibranchials forms de novo (B, * indicates adjacent developing adult epibranchials). The middle larval epibranchial (2nd epibranchial) degenerates first and is the structure that we quantified as a morphological marker for metamorphosis. The metamorphosed hyobranchial apparatus (C) shows fully formed adult epibranchials. Apparati were stained with alcian blue and dissected from whole prepped specimens.

RESULTS

Thyroid Hormone Receptor Phylogeny and Variation

Bayesian phylogenetic analysis of amphibian thyroid hormone receptor amino acid sequences shows strong support (posterior probabilities = 1.0) for two clades, which reciprocally define the two well-known paralogs of this gene in vertebrates (TRα and TRβ; Fig. 2). There were nine variable positions among the 1,017 nucleotide sites (339 amino acids) of TRα from the four *E. tynerensis* populations. The nucleotide mutations only resulted in three non-synonymous substitutions in the TRα amino acid sequences of *E. tynerensis* (Fig. S1). These three variable amino acid positions are within the ligand binding domain, but not part of the helices, or at positions where the residues are predicted to directly contact thyroid hormone (Fig. S1). There was only a single variable position in the 912 nucleotides (304 amino acids) of TRβ for *E. tynerensis*, and it was a synonymous substitution (i.e., at the amino acid level this region of TRβ was identical for the four *E. tynerensis* populations).

Thyroid Hormone Receptors Alpha and Beta Expression

We found no significant differences in gene expression between size classes (1 and 2) for any given treatment within populations. We also regressed expression values against body weight and found no relationship within populations or treatment groups. Therefore, we pooled data for all individuals with the same treatment and population (i.e., we combined classes 1 and 2 of the same treatment within populations, Table 1). The following results are based on comparisons of pooled size groups within populations, resulting in a total of eight groups (one control and one T_3 treated group per population). We found significant differences in variation of TRα expression in tailfins among populations and treatments ($F_{(7,70)} = 9.48$, $P < 0.0001$, Fig. 3A). Meta-Sm, Meta-Lg, and Paedo-Lg controls exhibited the same basal levels of expression, but Paedo-Sm was significantly lower than all other controls ($P < 0.01$). Every population showed an average increase in TRα expression with T_3 treatment, but only the metamorphic populations were significantly higher than their respective controls (Meta-Sm $P < 0.002$; Meta-Lg $P < 0.02$). On

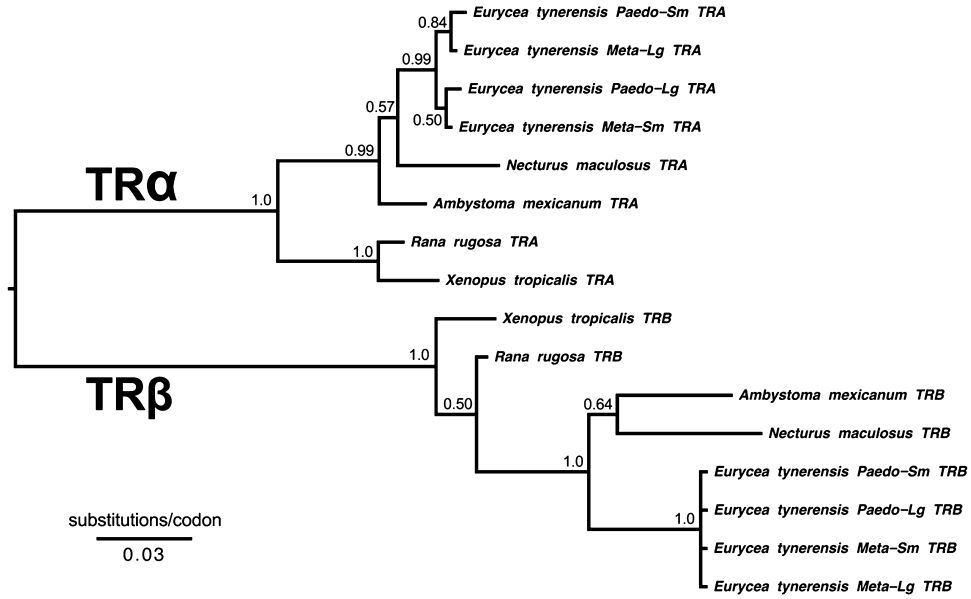

Figure 2. Phylogeny of amphibian thyroid hormone receptors. Fifty percent majority rule phylogram from a Baysian inference analysis of amphibian thyroid hormone receptor amino acid sequences. Numbers subtending the nodes are Bayesian posterior probabilities. The two major clades correspond to thyroid hormone receptors alpha (TRα) and beta (TRβ).

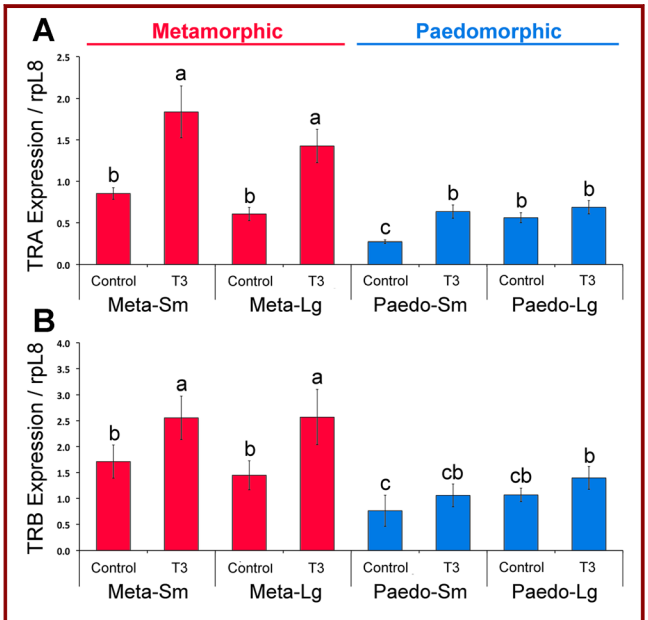

Figure 3. Expression of TRα and TRβ in tailfins of control and T_3 treated *E. tynerensis* larvae from metamorphic and paedomorphic populations. Averages and standard deviations of TRα (A) and TRβ (B) expression quantities (normalized by rpL8) are plotted for each population and treatment group (Control or 25 nM T_3 for 48 hr). Lower case letters indicate significant differences among groups from one-way ANOVA with Fischer's LSD post hoc test ($\alpha < 0.05$).

average, T_3 treated Meta larvae showed two to three times higher TRα expression than treated Paedo larvae, and these comparisons were significant ($P < 0.001$ in each case).

There were also significant differences in TRβ expression in tailfins among populations and treatments ($F_{(7,66)} = 4.74$, $P < 0.0001$, Fig. 3B). TRβ expression patterns were somewhat similar to TRα. Meta-Sm, Meta-Lg, and Paedo-Lg controls exhibited statistically similar basal levels of TRβ expression. Paedo-Sm controls had significantly lower TRβ expression than both Meta controls ($P < 0.001$ in both comparisons), but not lower than Paedo-Lg ($P = 0.110$). All four populations showed increased TRβ expression with T_3 treatment, but only the metamorphic populations were significantly higher than their respective controls (Meta-Sm $P < 0.04$; Meta-Lg $P < 0.01$). On average, T_3 treated Meta larvae showed more than two times higher TRβ expression than Paedo larvae, and these comparisons were significant ($P < 0.01$ in each case).

Epibranchial Remodeling

We found significant differences in epibranchial remodeling among treatments and populations ($F_{(7,69)} = 27.99$, $P < 0.0001$, Fig. 4). T_3 treatment of both metamorphic populations (Meta-Sm and Meta-Lg) resulted in almost complete loss of larval epibranchials ($P < 0.0001$ from all controls and paedomorphic larvae). Paedomorphic populations had varying responses. All Paedo-Sm individuals maintained complete larval epibranchials, and no adult epibranchial development was visible after T_3

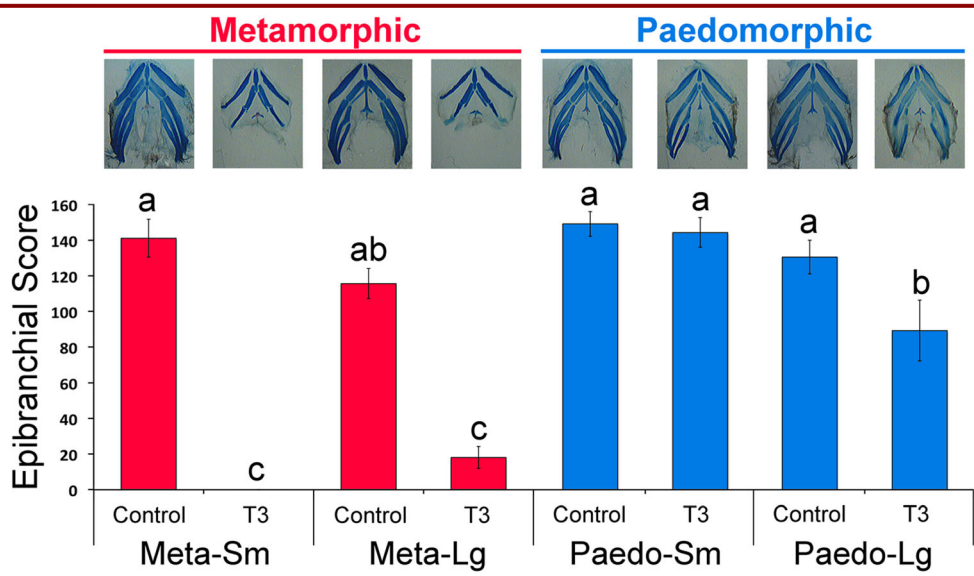

Figure 4. Differential thyroid hormone sensitivity of cartilaginous hyobranchial apparati in larvae from metamorphic and paedomorphic populations of *Eurycea tynerensis*. For each individual, the larval epibranchial score was calculated by the following formula: average area of the second larval epibranchials multiplied by the average intensity of staining, and divided (normalized) by the body weight. Averages and standard deviations of larval epibranchial scores are plotted for each population and treatment group (control or 25 nM T_3 for 21 days). Lower case letters indicate significant differences among groups from one-way ANOVA with Fischer's LSD post hoc test ($\alpha < 0.05$). Images above are representative hyobranchial apparati for each treatment and population. Hyobranchial remodeling during *Eurycea* metamorphosis is shown in Figure 1.

treatment for 21 days. Paedo-Lg larvae showed significant reductions to larval epibranchials compared to their controls, but still considerably less remodeling than T_3 treated larvae from metamorphic populations (Fig. 4). Only 25% of Paedo-Lg larvae were remodeled to a degree comparable to T_3 treated metamorphic larvae. In most Paedo-Lg larvae, juvenile epibranchials were still present and only partially regressed.

DISCUSSION

In this study, we found significant differences in sensitivity to exogenous thyroid hormone among larvae from closely related populations of paedomorphic and metamorphic plethodontid salamanders. We detected lower basal levels of transcription and reduced transcriptional responses to T_3 for both TRα and TRβ in tailfins of larvae from paedomorphic populations compared to those from metamorphic populations. Furthermore, examination of the hyobranchial apparati showed that larvae from paedomorphic populations exhibited reduced or no loss of the 2nd pair of larval epibranchials after continuous T_3 treatment compared to metamorphic populations, which completely lost all three pairs of larval epibranchials. This is the first study to compare thyroid hormone sensitivity and gene expression among closely related salamanders in the developmentally diverse Plethodontidae, and the first salamander system to demonstrate differential TRα and TRβ expression among populations exhibiting alternative life history modes.

Mechanisms of Differential Thyroid Hormone Sensitivity

Previous hypotheses have suggested that larval paedomorphosis is a result of reduced activity of the thyroid gland and consequently lower endogenous thyroid hormone (or thyroxine) levels (reviewed in Norris and Platt, '73). Other hypotheses propose that paedomorphosis may also have arisen from reduced sensitivity of ancestrally metamorphic tissues (e.g., external gills, tailfin, and hyobranchial apparatus) to thyroid hormone or other metamorphic stimulants (Swingle, '22; Noble, '24; Svob et al., '73; Safı et al., 2006). Here we show that applying a potent dose of thyroid hormone (25 nM) to *E. tynerensis* larvae from recently derived paedomorphic populations does not recover metamorphosis at the same rate as larvae from metamorphic populations (Fig. 4). We interpret this result at least in part as differential sensitivity of target tissues to thyroid hormone.

Paedomorphic populations of *E. tynerensis* either showed no significant upregulation of TRα and TRβ or significant responses two to three times lower than TH treated larvae from metamorphic populations. Larvae from metamorphic populations always showed a significant response to TH (Fig. 3). Therefore, reduced thyroid hormone sensitivity of target tissues in paedomorphic

larvae may in part be explained by reduced transcriptional activity of thyroid hormone receptors. Even though paedomorphic *Ambystoma* carrying different QTLs (*Met*$^{1-3}$) show different responses to TH (Voss et al., 2012), there has been no evidence to date that differences in *Ambystoma* life histories are the result of differential expression of TRs. The only direct comparison made to date was between developing brains of metamorphic (*Ambystoma tigrinum*) and paedomorphic (*A. mexicanum*) species using gene expression microarrays (Page et al., 2010). Therefore, our demonstration that TRs in larval tailfin tissue of *E. tynerensis* are differently expressed (and differentially upregulated) among alternative life histories is the first such example in salamanders, but this may not be a universal pattern of urodele paedomorphosis.

We acknowledge that there are many components of the hypothalamic–pituitary–thyroid (HPT), or interacting hypothalamic–pituitary–adrenal (HPA) axis (Denver, 2009), that could be responsible for reduced sensitivity to TH (Rosenkilde and Ussing, '96; Laudet, 2011). Given that TRs are regulated by TH, one of the simplest explanations for differential TR regulation in target tissues is altered endogenous TH levels. Dundee and Gorbman ('60) quantified thyroidal accumulation of radioiodine in paedomorphic *E. tynerensis*, which they interpreted as a "low-level of thyroid hormone synthesis" leading to paedomorphosis. However, this study was based only on adult paedomorphic *E. tynerensis*, and did not include direct comparisons to metamorphic relatives. To date, we have been unable to extract sufficient plasma from larval *E. tynerensis* to conduct T_3 and T_4 assays. Nevertheless, we note that supplying a potent continuous dose of T_3 (25 nM) does not initiate any metamorphic changes in at least one of our paedomorphic populations of *E. tynerensis* (Paedo-Sm; Fig. 4).

Genetic or environmental effects early in development could produce differential TH responses to metamorphic hormones (e.g., TH or CORT; Hu et al., 2008; Voss et al., 2012). The larvae for our experiment developed in the wild, potentially under different environmental conditions (Bonett and Chippindale, 2006; Bonett and Martin, unpublished). Preliminary experiments with *E. tynerensis* larvae completely raised in an identical common garden show increased TR expression in larvae from metamorphic mothers compared to larvae from paedomorphic mothers. Furthermore, preliminary tail culture experiments show that even in isolation from the rest of the endocrine system, TH induced transcription of TRs is reduced in paedomorphic populations compared to metamorphs. Taken together, our preliminary experiments suggest some heritability to patterns of TR expression, but further studies are needed to test potential environmental influences effecting TR expression and metamorphosis in this system.

Divergence from Metamorphosis and Loss of TH Sensitivity

In several obligately paedomorphic families (e.g., Cryptobranchidae, Proteidae, and Sirenidae), thyroid extract or T_3 treatment does not induce metamorphosis (Swingle, '22; Noble, '24; Noble and Farris, '29; Svob et al., '73; Safi et al., 2006), and may only result in partial shedding of skin (e.g., Noble and Farris, '29). In at least *Necturus* (Proteidae) it is clear that TRα and TRβ are functional and active in target tissues, and the lack of transformation of external gills may be due to a decoupling of hormone regulation of downstream target genes that presumably completed metamorphosis in ancestral species (Safi et al., 2006; Vlaeminck-Guillem et al., 2006). Obligately paedomorphic families are highly divergent from extant metamorphic species, and each have exhibited this life history since the Cretaceous (Holman, 2006; Bonett et al., 2013). There have also been multiple independent instances of paedomorphosis in plethodontid salamanders (Wake, '66; Ryan and Bruce, 2000; Bonett et al., 2014), which vary in metamorphic responsiveness from nearly complete metamorphosis (facultative metamorphosis; Kezer, '52) to very limited changes (obligate paedomorphosis; Dundee, '57, '62; this study). How long does it take for a lineage to lose metamorphic sensitivity to thyroid hormone? In our direct comparison of closely related paedomorphic and metamorphic populations of *E. tynerensis*, we find reduced metamorphic sensitivity in paedomorphic larvae compared to metamorphic larvae. Interestingly, we also find differential responses between larvae from two different paedomorphic populations. Based on mitochondrial DNA divergence (Emel and Bonett, 2011), the paedomorphic population that is most closely related to metamorphic populations (Paedo-Lg, Malloy Hollow) was also the most responsive to T_3 with regard to remodeling of larval epibranchials (Fig. 4). Comparatively, the paedomorphic population that is much more divergent from metamorphic relatives (Paedo-Sm, Council Hollow) had lower basal levels of thyroid hormone receptors, and almost no sign of epibranchial remodeling over the same experimental period (Figs. 3 and 4). It is also possible that body size influences TH sensitivity after the evolution of paedomorphosis, even though we did not find differences in TH sensitivity among individuals of different body sizes within populations. A more comprehensive analysis of divergence from metamophosis and body size evolution among the many independently paedomorphic lineages of *E. tynerensis* is needed, which may shed light on the mechanisms of loss of sensitivity to thyroid hormone during the evolution of paedomorphosis in salamanders.

ACKNOWLEDGMENTS

We thank D. Buckley for comments on the manuscript, J. Phillips and A. Trujano for help in the field, and W. Meyers and K. Klein for preliminary work on *Eurycea* thyroid hormone receptors. M. Howery from the Oklahoma Department of Wildlife Conservation and Kelly Irwin from the Arkansas Game and Fish Commission issued permits that made this work possible. Funding was provided by the University of Tulsa and National Science Foundation (DEB 1050322 to R.M.B.).

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