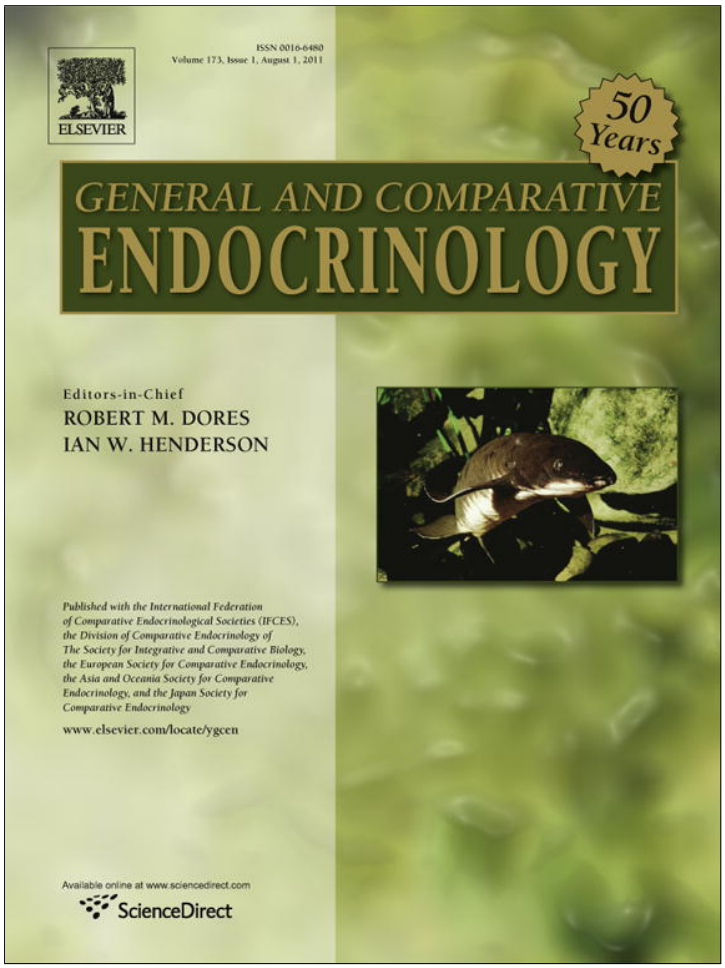

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Higher thyroid hormone receptor expression correlates with short larval periods in spadefoot toads and increases metamorphic rate

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ABSTRACT

Spadefoot toad species display extreme variation in larval period duration, due in part to evolution of thyroid hormone (TH) physiology. Specifically, desert species with short larval periods have higher tail tissue content of TH and exhibit increased responsiveness to TH. To address the molecular basis of larval period differences, we examined TH receptor (TR) expression across species. Based on the dual function model for the role of TR in development, we hypothesized that desert spadefoot species with short larval periods would have (1) late onset of TR expression prior to the production of endogenous TH and (2) higher TR levels when endogenous TH becomes available. To test these hypotheses, we cloned fragments of TRα and TRβ genes from the desert spadefoot toads *Scaphiopus couchii* and *Spea multiplicata* and their non-desert relative *Pelobates cultripes* and measured their mRNA levels in tails using quantitative PCR in the absence (premetamorphosis) or presence (natural metamorphosis) of TH. All species express TRα and TRβ from the earliest stages measured (from just after hatching), but *S. couchii*, which has the shortest larval period, had more TRα throughout development compared to *P. cultripes*, which has the longest larval period. TRβ mRNA levels were similar across species. Exogenous T3 treatment induced faster TH-response gene expression kinetics in *S. couchii* compared to the other species, consistent with its higher TRα mRNA expression and indicative of a functional consequence of more TRα activity at the molecular level. To directly test whether higher TRα expression may contribute to shorter larval periods, we overexpressed TRα via plasmid injection into tail muscle cells of the model frog *Xenopus laevis* and found an increased rate of muscle cell death in response to TH. These results suggest that increased TRα expression evolved in *S. couchii* and contribute to its higher metamorphic rates.

1. Introduction

Spadefoot toads are a closely related group of anurans that display extreme variation in larval period duration [4,17]. The group exhibits the shortest to some of the longest known larval periods among amphibians [14,27]. New World spadefoot toad genera (*Scaphiopus* and *Spea*) live mostly in xeric environments while Old World spadefoot toads (*Pelobates*) live in mediterranean climates or temperate forests [25,43]. Even though interspecies variation in larval period can be partially attributed to the effects of the dramatically different environments [28], New World taxa exhibit intrinsically shorter larval periods compared to Old World taxa when reared under identical laboratory conditions [4]. Specifically, *Scaphiopus* had the shortest larval period of all three genera, *Spea* is intermediate, and *Pelobates* has the longest (16 vs. 24 vs. 35 days, respectively).

Because thyroid hormone (TH) controls metamorphosis in amphibians [12,38], TH physiology is likely central to the explanation for how the extremely short larval periods evolved in New World spadefoot toads [7]. The simplest explanation for accelerated metamorphosis in desert species would be increased levels of circulating TH or earlier production of TH via changes in the hypothalamic-pituitary-thyroid (HPT) axis. Indeed, it has been shown in spadefoot toads that desert species with shorter larval periods have higher TH-tissue content in the tail during metamorphosis compared to non-desert species [5]. However, because of cellular factors influencing TH signaling (see below), these results may not reflect plasma levels and thus cannot distinguish between central and peripheral control as the site of evolutionary change underlying larval period differences. Stronger evidence, from *in vitro* tail tip assays, exists for altered peripheral control as a mechanism for achieving shorter larval periods [5]. Tail tips from three spadefoot species shrank at rates correlating with their varied larval period durations when exposed to the same concentrations of TH, indicating species differences in TH responsivity. These *in vitro* results indicate that evolution of peripheral control mechanisms contributed to increased rates of metamorphic change.

Peripheral control of metamorphic tissue transformation is achieved through the combinatorial effects of cellular factors influencing TH signaling [40]. For tissues to respond to the TH

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signal, TH (typically the inactive version T4) must first enter the cell via TH-transporters [22,46]. Once inside the cell, deiodinase enzymes can either activate T4 to make the active form T3 or inactivate T4 and T3 [1,18]. T4 and T3 bind cytoplasmic TH-binding proteins, which may reduce the levels of free TH within the cell [39]. Finally, T3 must bind to TRs in the nucleus to exert its effects [41]. TRs function as transcription factors by binding to TH-response elements (TREs) of DNA sequences as heterodimers with retinoid X receptors (RXRs) [47]. This heterodimer complex alters gene expression through the recruitment of cofactors, initiating the gene cascade events that lead to the morphological changes associated with metamorphosis [7]. Evolutionary changes in the expression levels of any of these TH-signaling proteins could potentially alter the amount of TH signaling, thereby giving rise to altered rates of tissue transformation and larval period duration [2].

As TRs are central to TH signaling and metamorphosis [9], altered TR expression among species was a prime candidate for an evolutionary change that could explain the faster tail shrinkage and by extension the shorter larval period duration in *Scaphiopus couchii*. Frogs, like all vertebrates, have two TR genes, TRα and TRβ [49]. These TR genes have very different temporal expression profiles, where TRα expression begins shortly after hatching and increases in tissues as they remodel. TRβ expression is low throughout premetamorphosis and has some embryonic effects [20] but becomes strongly upregulated during tissue transformation in response to the presence of TH [48]. The difference in TH-inducibility between TRα and TRβ is due to the presence of a TRE in the TRβ promoter [34]. Both TRs are believed to have overlapping molecular roles in transcription, though some differences have been detected [11].

The dual function model for the role of TR in development provides a framework for understanding how evolutionary changes in the expression of TR may influence the timing and rate of metamorphosis [7]. In the absence of TH, TRs function to repress expression of TH-response genes, while in the presence of TH they activate transcription of these same genes. This dual function is based on the interaction of TRs with different types of cofactors. Before endogenous TH levels rise, unliganded TRs are bound to corepressors, such as N-CoR (nuclear receptor corepressors) and SMRT (silencing mediator for retinoid and thyroid hormone receptors), and this complex inhibits the transcription of TH-response genes [35,45]. Once endogenous TH levels begin to increase, the binding of T3 leads to a conformational change in TR, resulting in an affinity for the binding of coactivators such as SRC (steroid receptor coactivator) 1, 2, and 3, and inhibiting the binding of corepressors. Coactivators then acetylate and methylate histones, which promotes transcription of TH-response genes [21,26,30–32].

To examine the potential role of TRs in the evolution of accelerated metamorphosis, we cloned partial gene sequences of TRα and TRβ from *S. couchii*, *Spea multiplicata*, and *Pelobates cultripes* and compared their mRNA expression in whole bodies during early development and in tails during metamorphic development in each species. Because endogenous TH is undetectable during premetamorphic development [5], TRs are expected to function as repressors of metamorphic genes. If a species had a delay in detectable TRs during this time, they would potentially have less repression of metamorphic genes, which could lead to a faster onset of metamorphosis, as seen in experiments in *Xenopus* [37]. Thus, we hypothesized that TRs will first become detectable at later developmental stages in species with shorter larval periods. Because TH-response gene expression levels depends on TR expression levels and the induced rate of metamorphosis depends on exogenous hormone concentration [8,38], more TRs during metamorphosis would enable a faster or higher change in expression of metamorphic genes, and ultimately lead to a shorter larval period. Thus, we also hypothesized that species with shorter larval periods will have greater TR expression levels during natural metamorphosis when TH is present.

2. Materials and methods

2.1. Animal care and treatment

Adult *S. couchii* and *S. multiplicata* were collected from southeastern Arizona and southwestern New Mexico during the summer of 2007. Spawn (a mixture of six clutches) and adult *P. cultripes* were collected from Doñana National Park, Spain in November 2007 and shipped to the University of Cincinnati. Adult spadefoot toads were maintained in approximately 15–20 cm soil in large plastic tanks with screened lids, fed *ad libidum* crickets dusted with vitamins (Nekton-Rep-Color) and sprinkled with water as needed. Induction of breeding to obtain tadpoles was described previously [4]. Tadpoles were reared in aerated 400 liter stock tanks at 28 °C with a 12L/12D cycle, and were fed *ad libidum* ground rabbit chow twice daily. Water was changed as needed. Tadpoles of *Xenopus laevis* were bred from laboratory stock and reared to NF stage 56 [29]. The care and treatment of the animals used in this study were in accordance with the protocols approved by the University of Cincinnati Institutional Animal Care and Use Committee.

2.2. Tissue collection

Tadpoles were removed from stock tanks at predetermined developmental stages and anesthetized in benzocaine prior to tissue collection. For early stages (Gosner 23–28) [19], whole tadpoles were snap frozen on dry ice, and samples were pooled to ensure sufficient material for RNA isolation. For later stages (Gosner 32, 35, 38, 42, 44), tadpoles were first anesthetized in benzocaine, then tails were excised and snap frozen on dry ice. Some *S. couchii* and *S. multiplicata* tail samples were pooled due to their small size (no *P. cultripes* tails were pooled).

Two T3 doses were used with Gosner 31 tadpoles: 8 nM (a minimum dose to induce a response across spadefoot toad genera in a tail tip assay [5] and 50 nM (a potentially saturating dose based on studies done in *Xenopus*), as well as untreated tadpoles. T3 was dissolved in 0.5 N NaOH as a 1 mM stock with a final concentration of 2.5×10^{-5}–4×10^{-6} N NaOH in the rearing water depending on T3 dose. Because tadpoles vary greatly in size, we adjusted the water volume for the T3 treatments based on the average tadpole body mass of the species at stage 31 [4] to ensure that the same amount of TH per gram body mass was available across species. Thus, *S. couchii* tadpoles were kept at a density of five tadpoles per tank in 1 L of water, *S. multiplicata* tadpoles were kept at a density of one tadpole per tank in 1 L of water, and *P. cultripes* tadpoles were kept at a density of one tadpole per tank in 4 L of water. Water and hormone were changed daily. Tadpoles were not fed during T3 treatment. Tails were harvested as described above at 0, 12, 24, 48, 72, 120, and 168 h post-T3 induction. Control tadpoles were harvested at 0, 72, and 120 h, and no significant differences were found among time points in the control animals for each species (data not shown). Species varied in their ability to survive exposure to T3 levels, and within a given treatment and species all individuals died within 24 h of each other. *S. couchii* tadpoles died between 48 and 72 h of 50 nM T3 exposure and between 72 and 96 h of 8 nM T3 exposure. *S. multiplicata* tadpoles died between 48 and 72 h of 50 nM T3 exposure and between 120 and 144 h of 8 nM T3 exposure. *P. cultripes* tadpoles died between 72 and 96 h of 50 nM T3 exposure and between 168 and 192 h of 8 nM T3 exposure. Due to their small size, five *S. couchii* tails were pooled to

make one sample. No *S. multiplicata* or *P. cultripes* samples were pooled. All samples were stored at −80 °C.

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from frozen samples using Trizol reagent following the manufacturer's protocol (Invitrogen). Sample homogenization was done using a Fisher Scientific Power Gen 125 for up to 1 min per sample. RNA was resuspended using RNase-free water and stored at −80 °C. RNA concentrations were measured using a Nanodrop ND-1000 Spectrophotometer. cDNA was synthesized using 2 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit following the manufacturer's protocol (Applied Biosystems). Two microliters of neat or 10-fold diluted cDNA were used in quantitative PCR for TRs and rpL8, respectively.

2.4. Cloning spadefoot TRα, TRβ, and rpL8

Three micrograms total RNA from *S. multiplicata* Gosner stage 44 tail, *S. couchii* Gosner stage 42 tail and whole body, and *P. cultripes* Gosner stage 42 head isolated using TriZol (Invitrogen) was used to make cDNA as above. One microliter of cDNA per 20 μL reaction or 2 μL per 50 μL reaction was used to PCR amplify fragments of TRα, TRβ, and rpL8 using high fidelity Taq polymerase (PrimeSTAR HS DNA polymerase, Takara) with the listed primers and PCR conditions (Tables 1A and 1B). Final primer concentration was 0.5 μM and there were 35 cycles for each reaction. DRB20b/21b were previously designed for *Xenopus* rpL8 [6]. DRB182 and DRB185 were designed to amplify TRs from *Lepidobatrachus* (C. Infante, unpublished). DRB203, 206, 207, 208 are degenerate primers designed by hand using alignments of TRα, TRβ, or rpL8 sequences from *X. laevis, Spea hammondii,* and sequenced human, mouse, chick, and zebrafish genomes. PCR products were gel purified (QiaQuick Gel Extraction Kit, Qiagen) and sequenced in both directions with the same primers used for PCR amplification. PCR products from three to four individuals per gene per species were sequenced.

2.5. Quantitative PCR

Quantitative PCR (qPCR) with Taqman FAM-labeled probes was carried out to quantitate expression levels of TRα, TRβ, and the housekeeping gene rpL8 in single-plex reactions using an Applied Biosystems 7300 Real Time PCR System, Applied Biosystems Universal PCR Master Mix, RNase-free water, and primer-probe sets from Applied Biosystems. Primer-probe sets were used for each gene that can bind identical sequences of DNA for all three species (Table 2). The final primer/probe concentrations were 0.9 and 0.25 μM, respectively and determined by Applied Biosystems optimized for use with their Custom TaqMan Gene Expression Assay Service and Universal PCR Master Mix. The amplified region of each Taqman primer/probe set crossed an intron/exon boundary (based on the *X. tropicalis* genome sequence) to mitigate amplification of contaminating genomic DNA in the qPCR reactions. Serial dilutions of *S. couchii* stage 42 (climax of metamorphosis) whole body cDNA were used as standards (*P. cultripes* stage 42 whole body cDNA standards gave parallel results), where slopes ranged from −3.42 to −3.87 (a slope of −3.54 reflects 100% amplification efficiency) and R^2 values averaged 0.998 (range (0.996–0.999). In every reaction, no template controls were used and failed to detect any reaction product contamination. All samples were run in duplicate. qPCR conditions were as follows: 50 °C 2 min, 95 °C 10 min, then 40 cycles of 95 °C 10 s, 60 °C 1 min.

Table 1B
Primer sequences.

Gene and direction	Primer name and sequence
TRα forward	DRB182 5′ CCAGACAGCGAGACCCTAAC
TRα reverse	DRB206 5′ GGRCAYTCNACYTTCATRTG[a]
TRβ forward	DRB203 5′ TGYAARTAYGARGGNAARTGYGT
TRβ reverse	DRB185 5′ TAGGAGCCTGCCCAATATCTTC
rpL8 forward	DRB20b 5′ CGTGGTGCTCCTCTTGCCAAG
rpL8 reverse	DRB21b 5′ GACGACCAGTACGACGAGCAG
rpL8 forward	DRB207 5′ ATGGCTACATCAAGGGNATTGTGAAAG
rpL8 reverse	DRB208 5′ TTGCGACCAGCTGGRGCATC

[a] Also binds TRβ.

2.6. Tail muscle injections

The control expression plasmid for tail injection pDPHGHG-HS4 was based on vectors previously published Ref. [33] and engineered using gene synthesis (DNA20.com) and standard cloning methods. To make the TRα expression plasmid pDPHGHTRa-HS4, *X. laevis* TRα was cloned into the AgeI/EcoRI sites of pDPHGHG-HS4 replacing one of the GFPs. Before injection, NF stage 55–57 tadpoles were given a 5 min. heat shock at 33 °C. The next day, tadpoles were anesthetized in benzocaine and injected into the tail muscle with 0.5 μL of 2 μg/μL plasmid DNA, control plasmid on the left side of the tadpole and TRα plasmid on the right, based on previous methods [10,36]. In brief, needles were loaded with DNA in water containing 0.05% w/v fast green dye (Sigma) to visualize site of injection (the dye dissipated within hours) and held in place with a Drummond micromanipulator in the forth and sixth myomeres from the hind limb insertion point to a depth of 300–500 μm. Injection was carried out with a Picospritzer III (Parker Instruments) under 40 lbs of pressure with a 200–400 ms pulse. Beginning the same day as injection (Day 0), expression of the exogenous genes was induced by daily 1 h heat shocks at 33–34 °C [15]. On Day 3, daily treatment of 0 or 2 nM T3 was begun. On Days 3–17, tails were imaged under a fluorescence dissecting microscope, and the number of GFP-positive cells was counted. Within each T3 and plasmid injection treatment, 3–5 tadpoles were injected for a total of 20–50 GFP-positive cells.

Table 1A
Primers, PCR settings, and product sizes for amplifying TRα, TRβ, and rpL8 from *S. couchii*, *S. multiplicata*, *P. cultripes*, and GenBank accession numbers.

Gene	Species	Primers	PCR settings	Product size	Accession numbers
TRα	*S. couchii*	DRB182/206	94C 30s-52C 30s-68C 40s	374 bp	HQ682068
TRα	*S. multiplicata*	DRB182/206	94C 30s-45C 30s-65C 40s	374 bp	HQ682069
TRα	*P. cultripes*	DRB182/206	94C 30s-50C 30s-68C 40s	374 bp	HQ682070
TRβ	*S. couchii*	DRB203/185	94C 30s-45C 30s-65C 40s	305 bp	HQ682073
TRβ	*S. multiplicata*	DRB203/185	94C 30s-45C 30s-65C 40s	305 bp	HQ682072
TRβ	*P. cultripes*	DRB203/185	94C 30s-50C 30s-68C 40s	305 bp	HQ682071
rpL8	*S. couchii*	DRB20b/21b	94C 30s-52C 30s-68C 40s	576 bp	HQ682076
rpL8	*S. multiplicata*	DRB207/208	94C 30s-45C 30s-65C 40s	589 bp	HQ682074
rpL8	*P. cultripes*	DRB20b/21b	94C 30s-52C 30s-68C 40s	576 bp	HQ682075

Table 2
Primer and probe sequences used for single-plex quantitative PCR with expected product size in base pairs (bp).

Gene	Forward primer	Reverse primer	FAM-labeled probe	Product
TRα	TCCTCAGACCGTACGGGTTT	CAGGCCCCAATCATGCG	TCGCAAACACAACATTC	165 bp
TRβ	GGAACCAGTGCCAAGAATG	TCATCCAAGACCAAGTCTGTTG	CGCTTCAAAAAGTG	123 bp
rpL8	CACAATCCTGAAACCAAGAAAACCA	CCACACCACGGACACGT	AAGGCCAAGAGAAACT	193 bp

2.7. Statistical analyses

Statistical analyses were done using JMP 7.0 statistical software. Normality of all data was tested using a Fit Distribution and Fitted Normal test. Data that were not normal were log-transformed and then re-tested. Homogeneity of variances of all data was tested using an UnEqual Variances *F* test. In the presence of significant heterogeneity, values were log-transformed and re-tested. Within each stage or time point, the averaged technical duplicates for each gene were analyzed by ANOVA followed by a Tukey–Kramer post hoc test for significant differences among species at $\alpha = 0.05$. In addition, differences within species across stages or time points were similarly analyzed. Data that were not normal or had significant heterogeneity of variances that persisted after log transformation were analyzed pairwise using the nonparametric Wilcoxon tests.

3. Results

To compare TR expression across spadefoot species, portions of TRα, TRβ, and rpL8 from three to four individuals per gene per species were PCR amplified and sequenced (Supplementary Fig. 1). We sequenced 316–334 bases (depending on species) of the TRα ligand binding domain, 286 bases of the TRβ DNA binding domain, and 505–542 bases of the housekeeping gene rpL8. The nucleotide and protein identities were ⩾85% for TRα, ⩾ 90% for TRβ, and ⩾85% for rpL8 among spadefoot species. Taqman primer/probe sets for quantitative PCR were designed to bind regions of nucleotide sequence identity for each gene across all three spadefoot species (Supplementary Fig. 1). Very few sequence polymorphisms were identified (Supplementary Fig. 1), and none were found in the primer/probe regions. In addition, the amplified region of each Taqman primer/probe set crossed an intron/exon boundary (based on the *X. tropicalis* genome sequence) to mitigate amplification of contaminating genomic DNA in the qPCR reactions.

To compare TR across species during early development, we measured whole-body TRα and TRβ mRNA expression during premetamorphic development (Gosner stages 23–28). Because rpL8 values were significantly decreased at Gosner stage 28 for *P. cultripes* and *S. multiplicata*, we compared species from Gosner stages 23–27. All species had detectable TRα and TRβ starting at the earliest stage, Gosner 23 (Fig. 1). Also, all species significantly increased TRα levels to a similar degree (1.7- to 2.2-fold change from stage 23–27), though *S. multiplicata* was the only species to significantly increase its TRβ expression (2-fold change) from stage 23–27. *S. multiplicata* and *S. couchii* had significantly more TRα mRNA expression (3- to 4-fold more) than *P. cultripes* at each stage (Fig. 1A). For TRβ, *P. cultripes* had 50% more expression than the other species, though the overall levels of TRβ at this stage were very low (Fig. 1B).

To compare TR expression across species during natural metamorphosis, we measured TRα and TRβ mRNA expression from tails at Gosner stages 32, 35, 38, 42, and 44. The rpL8 values were significantly higher within *S. couchii* at stage 32 compared to the other stages and significantly lower at stage 44 for *P. cultripes* and *S. multiplicata*. Thus, we compared species at stages 35 (beginning of metamorphosis, TH levels begin to rise), 38 (TH levels are at their midpoint), and 42 (climax of metamorphosis and TH levels). We used tail tissue instead of whole body samples because previous work demonstrating variance in responsivity between the three species was done using tail tissue [5]. The <2-fold changes in TRα levels from stage 35–42 were not significant in any of the three species, but *S. couchii* had significantly more TRα compared to *P. cultripes* across these stages (Fig. 2A). In contrast, all species significantly increased TRβ expression and had comparable TRβ levels (Fig. 2B).

To compare TH signaling through the TRs at the molecular level across species, we administered exogenous T3 to premetamorphic tadpoles of all three species beginning at Gosner stage 31, well before endogenous TH levels become detectable in the body. We used two T3 doses, 8 nM T3 was the minimum to induce a response in tail tissue across spadefoot toad genera [5] and 50 nM T3 was used as a potentially saturating dose. For all species, TRα and TRβ expression levels significantly changed in response to both doses of T3 (Figs. 3 and 4). As during natural metamorphosis, there were no significant differences in TH-induced TRα or TRβ expression between *S. multiplicata* and *P. cultripes*, regardless of T3 dose or receptor isoform. Similarly, all three species had similar fold changes in TRα across time points (2- to 3-fold change), but *S. couchii* had a greater level TRα mRNA expression (3- to 4-fold more) compared to the other species (Figs. 3A and 4A). Unlike in natural metamorphosis, TRβ levels were greater in *S. couchii* (up to 2- to 3-fold more) compared to *S. multiplicata* and *P. cultripes* by 12 h for both T3 treatments (Figs. 3A and 4A). Significant differences were maintained throughout the treatments for TRα. In addition, the typical decline in TRβ expression occurred earlier and more dramatically in *S. couchii* compared to the other species at the 8 nM dose (Fig. 4B). The rpL8 values did not significantly change in *P. cultripes* or *S. multiplicata* but significantly and gradually decreased in *S. couchii* across the time points (Figs. 3C and 4C).

To directly evaluate the possible effect of increased TR expression on the rate of metamorphic change, we overexpressed TRα in tail muscle cells in *X. laevis* and compared their rate of disappearance relative to control muscle cells. Digital images of tails injected with control and TRα plasmids (Fig. 5A) and treated with or without 2 nM T3 were taken daily for 2 weeks so that the disappearance of individual cells could be recorded (Fig. 5B). In the absence of T3, cells did not disappear after use of either the control or TRα expression plasmids. However, in the presence of 2 nM T3, cells with TRα overexpression disappeared about twice as quickly compared to controls (Fig. 5C).

4. Discussion

To examine the role of TR underlying the evolution of accelerated metamorphosis in desert spadefoot toads, we compared the levels of TRα and TRβ mRNA expression during early premetamorphic development in whole bodies. According to the dual function model [7], the role of TR in premetamorphosis prior to circulating TH is to repress genes important for metamorphosis [37]. Therefore, we hypothesized that early TR expression to achieve a shorter larval period should be delayed in *S. couchii*, which has the fastest metamorphosis, because metamorphic genes would have higher levels of expression in the absence of TR repression. However,

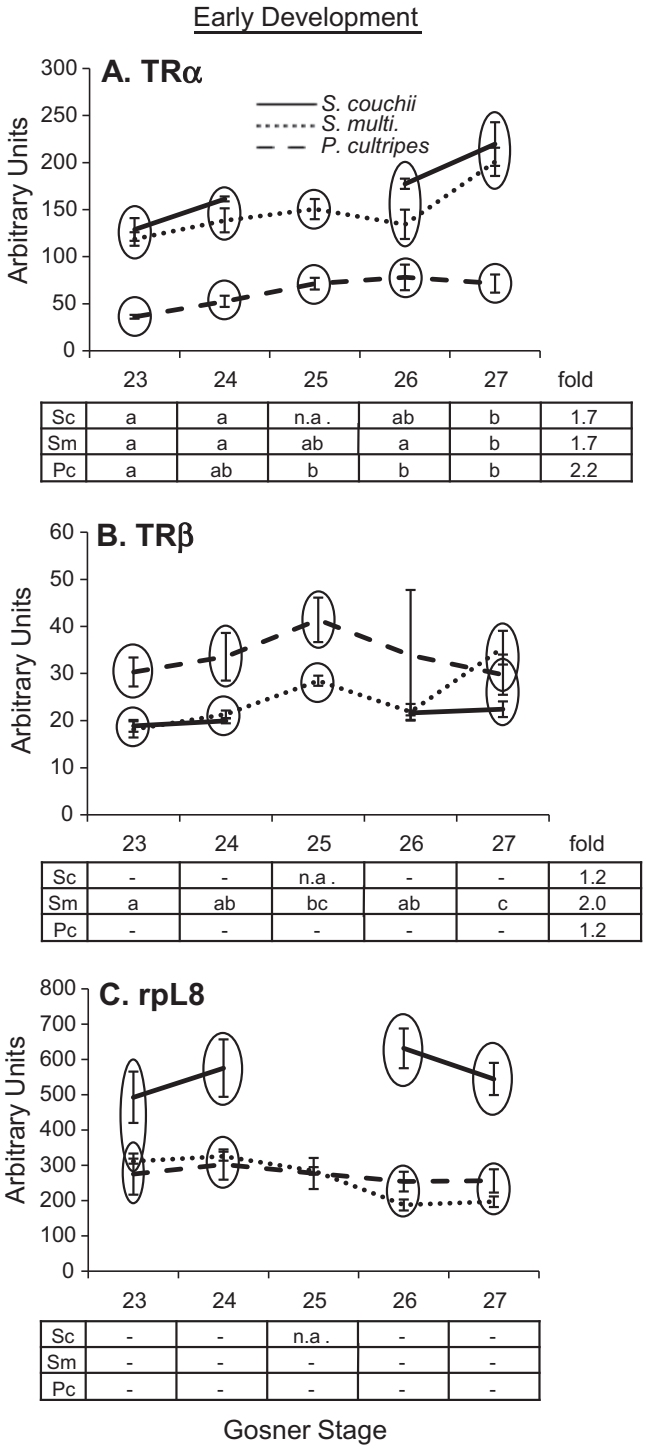


Fig. 1. Quantitation of TRα and TRβ mRNA expression during early development in spadefoot toads. cDNA was prepared from RNA isolated from whole bodies of *P. cultripes, S. multiplicata* and *S. couchii* tadpoles at stages 23–27 and then analyzed by qPCR using (A) TRα, (B) TRβ, and (C) rpL8 primer/probe sets. Data points indicate means and SE, $n = 4–5$. Circles around data points indicate significance groups determined by Tukey–Kramer post hoc tests across species within a stage. Lack of circles indicates lack of significant differences. Letters in the tables represent significance groups across stages within a species. Numbers in the tables indicate the greatest fold changes in gene expression within a species relative to stage 23 for each gene. n.a. = not applicable, because *S. couchii* lacks stage 25 [3].

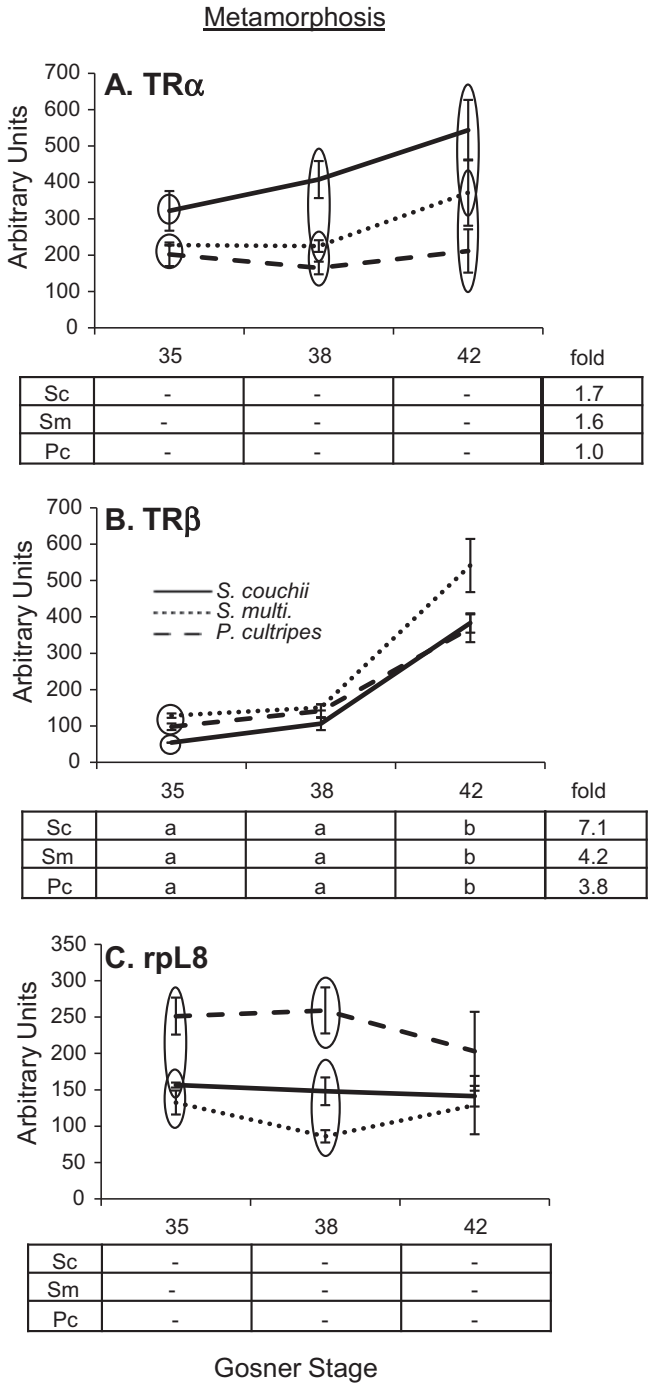
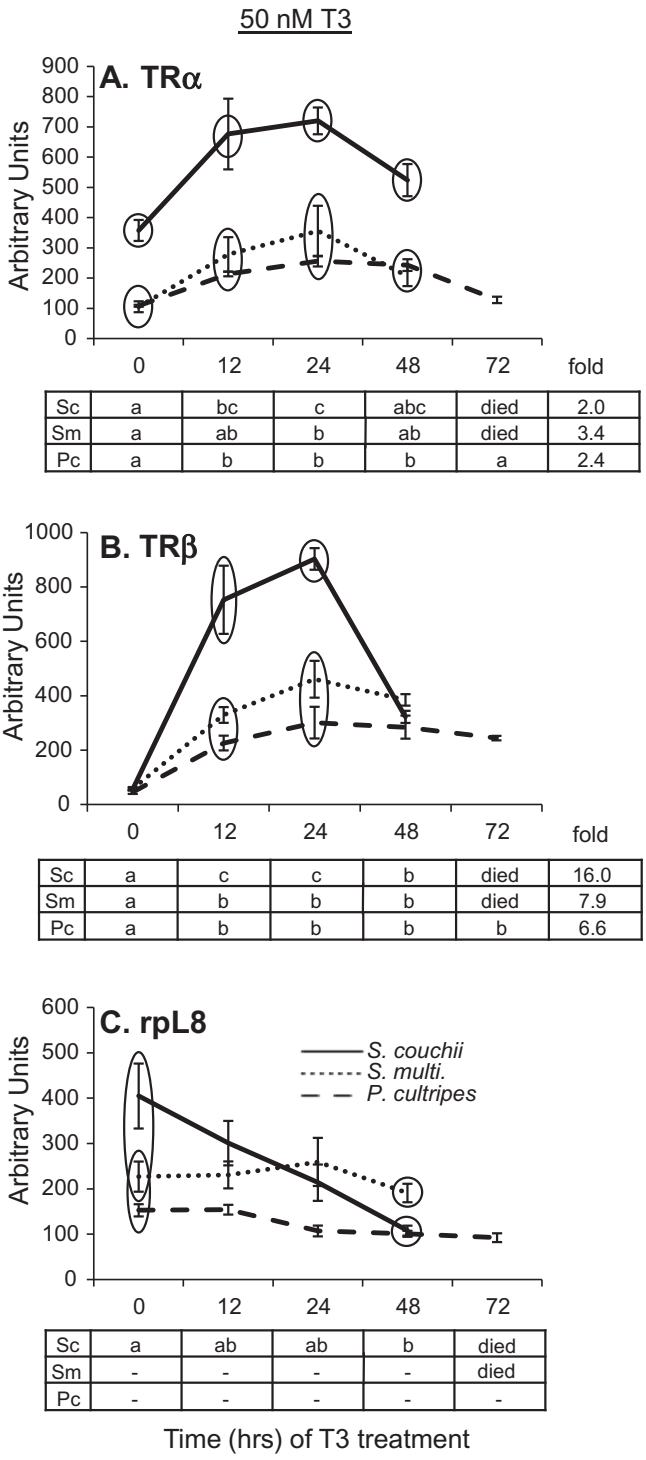


Fig. 2. Quantitation of TRα and TRβ expression during natural metamorphosis in spadefoot toads. cDNA was prepared from RNA isolated from tails of *P. cultripes, S. multiplicata*, and *S. couchii* tadpoles at stages 35, 38, and 42 and analyzed by qPCR using (A) TRα, (B) TRβ, and (C) rpL8 primer/probe sets. Data points indicate means and SE, $n = 4–7$. Circles around data points indicate significance groups determined by Tukey–Kramer post hoc tests across species within a stage. Lack of circles indicates lack of significant differences. Letters in the tables represent significance groups across stages within a species. Numbers in the tables indicate the greatest fold changes in gene expression within a species relative to stage 35 for each gene.

mRNA for both TR isoforms was detectable in each species at all of the early stages that we sampled (Gosner 23–28), failing to support our hypothesis. Furthermore, the sum of TR expression levels are likely higher during premetamorphosis in *S. couchii* because TRα mRNA levels were about 3-fold higher and TRβ mRNA levels were only 30% lower compared to *P. cultripes* (Fig. 1A and B).

During metamorphosis in the presence of TH, the dual function model says the role of TR is to induce genes important to initiate metamorphosis. Also, because the rate of metamorphosis is

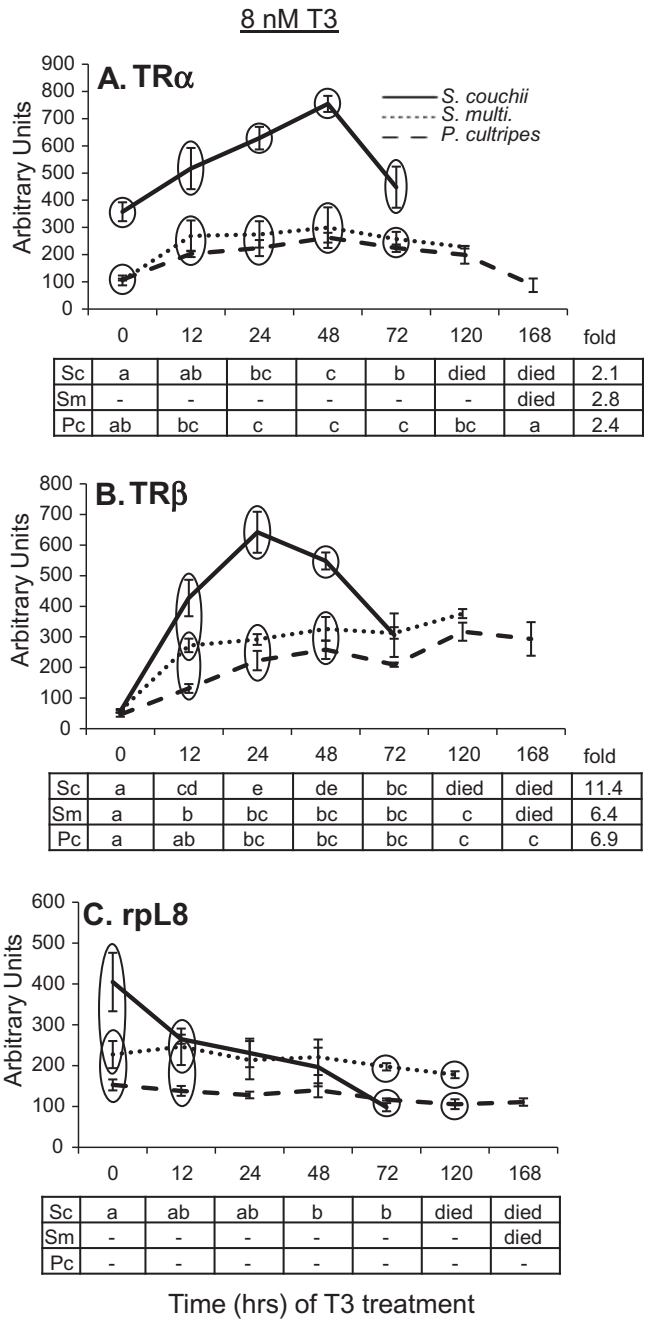


Sc	a	bc	c	abc	died	2.0
Sm	a	ab	b	ab	died	3.4
Pc	a	b	b	b	a	2.4

Sc	a	c	c	b	died	16.0
Sm	a	b	b	b	died	7.9
Pc	a	b	b	b	b	6.6

Sc	a	ab	ab	b	died	
Sm	-	-	-	-	died	
Pc	-	-	-	-	-	

Fig. 3. Quantitation of TRα and TRβ expression during 50 nM T3-induced metamorphosis in spadefoot toads. Gosner stage 31 *P. cultripes, S. multiplicata*, and *S. couchii* tadpoles were reared in water with 50 nM T3 for 0–72 h. cDNA was prepared from RNA isolated from tails harvested at the indicated time points and analyzed by qPCR using (A) TRα, (B) TRβ, and (C) rpL8 primer/probe sets. Data points indicate means and SE, $n = 3–6$. Circles around data points indicate significance groups determined by Tukey–Kramer post hoc tests across species within a time point. Lack of circles indicates lack of significant differences. Letters in the tables represent significance groups across time points within a species. Numbers in the tables indicate the greatest fold changes in gene expression within a species relative to time 0 for each gene.



Sc	a	ab	bc	c	b	died	died	2.1
Sm	-	-	-	-	-	-	died	2.8
Pc	ab	bc	c	c	c	bc	a	2.4

Sc	a	cd	e	de	bc	died	died	11.4
Sm	a	b	bc	bc	bc	c	died	6.4
Pc	a	ab	bc	bc	bc	c	c	6.9

Sc	a	ab	ab	b	b	died	died
Sm	-	-	-	-	-	-	died
Pc	-	-	-	-	-	-	-

Fig. 4. Quantification of TRα and TRβ expression during 8 nM T3-induced metamorphosis in spadefoot toads. Gosner stage 31 *P. cultripes, S. multiplicata*, and *S. couchii* tadpoles were reared in water with 8 nM T3 for 0–168 h. cDNA was prepared from RNA isolated from tails harvested at the indicated time points and analyzed by qPCR using (A) TRα, (B) TRβ, and (C) rpL8 primer/probe sets. Data points indicate means and SE, $n = 3–6$. Circles around data points indicate significance groups determined by Tukey–Kramer post hoc tests across species within a time point. Lack of circles indicates lack of significant differences. Letters in the tables represent significance groups across time points within a species. Numbers in the tables indicate the greatest fold changes in gene expression within a species relative to time 0 for each gene.

positively correlated with TH concentration added exogenously [12], more signaling through TR likely increases the metamorphic rate. Thus, we measured TRα and TRβ mRNA expression in tails to test the hypothesis that during natural metamorphosis, *S. couchii* has more TRα and/or TRβ enabling greater TH/TR signaling leading to its faster rate of metamorphosis. *S. couchii* had significantly higher TRα expression levels compared to the other species, consistent with our hypothesis. Surprisingly, TRβ expression profiles were similar during metamorphosis across species. The higher

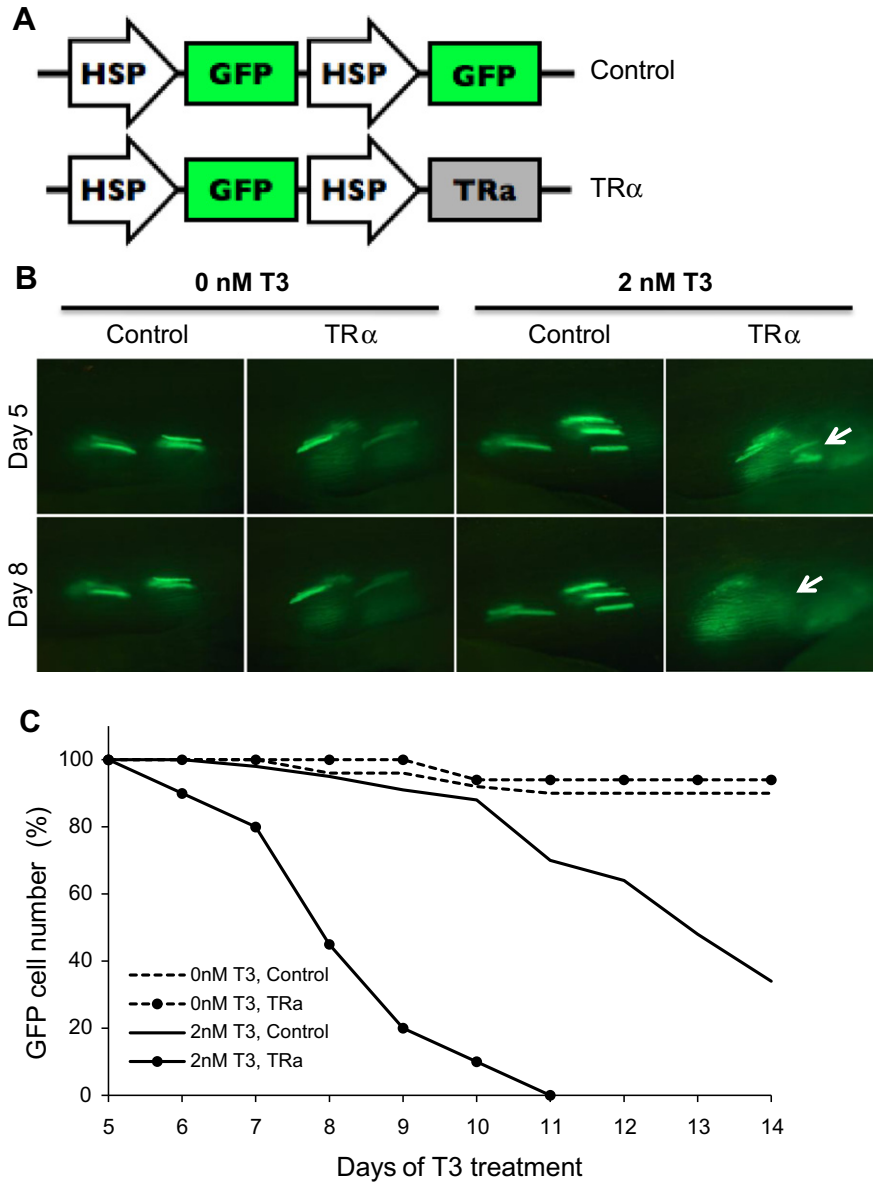


Fig. 5. Overexpression of TRα increases the rate of T3-induced metamorphic change. (A) Diagram of control and TRα expression constructs. Heat shock promoters (HSPs) regulate expression of two GFP (green fluorescent protein) genes in the control expression plasmid, and HSPs regulate expression of one GFP and one TRα in the TRα expression plasmid. (B) Exemplar images of GFP expressing muscle cells. Tadpoles were injected with control and TRα expression plasmids in the left and right side of the tail muscle respectively and heat shocked, T3-treated, and photographed daily. The number of visible GFP expressing cells due to heat shock increased until Day 5. On Day 8, all cells remained visible, except in T3-treated tadpoles injected with the TRα expression plasmid. White arrows indicate examples of muscle cell disappearance between Day 5 and 8. (C) Quantification of GFP expressing muscle cell disappearance. GFP expressing cells were monitored daily for two weeks, and the number of visible cells stopped increasing on Day 5 for all treatments and thus cell disappearance was quantified relative the peak number on Day 5, $n = 20–50$. In the absence of T3, GFP expressing cells remained visible for 2 weeks. In the presence of 2 nM T3, GFP expressing cells disappeared nearly twice as fast in muscle cells overexpressing TRα compared to controls.

levels of TRα may enable the TRβ levels in *S. couchii* to be achieved in a fraction of the time compared to the other species. Autoinduction of TR seems to be a critical component of metamorphosis [44], as the level of TR is not sufficient to bind thyroid hormone response elements in all TH-response genes at pre-induction TR levels [8]. Thus, a species that has a head start, i.e., higher premetamorphic TR levels, may require less time to achieve sufficient levels of TR required for the full TH gene regulation cascade.

We measured TR mRNA as a proxy for comparing the more biologically relevant protein activity levels across species. However, appropriate comparison of mRNA levels across species requires careful consideration. As in other studies [13,42], we found that rpL8 values were not constant across stages or treatments. Thus, we did not normalize TR to the housekeeping gene rpL8 and restricted our analyses to where rpL8 values were not different among stages for the three species (Figs. 1 and 2), though this was not possible for species comparisons across T3 treatments (Figs. 3 and 4). Furthermore, rpL8 levels varied significantly across species, precluding its use in normalization even during stages when its levels did not vary within species. Our results are therefore reported as TRα mRNA levels relative to total RNA, because cDNA from all three species was prepared using 2 μg of RNA and the same volume of cDNA was used in each qPCR reaction.

For various reasons, this metric (TR mRNA per unit total RNA) used in isolation does not guarantee a comparable level of TR protein activity per cell. Potential differential processing of mRNA into protein, post-translational modifications, and/or protein turn over across stages or treatments within a species or across species,

reduces the reliability of mRNA as a proxy for protein activity comparisons across species. Even if we compared protein levels across species, genome size differences may negatively influence the appropriateness of such comparisons. The genome size differs by three fold among species (*P. cultripes* has four picograms DNA per haploid genome, and *S. multiplicata* and *S. couchii* have 1.3 ("Animal Genome Size Database" http://www.genomesize.com/)). Genomes that are larger in size are also likely to have more non-coding DNA, and the amount of TRE binding sites in these different genomes is unknown. Because TRs bind to TREs to induce gene expression, divergent amounts of TREs in non-coding regions of genomes could alter effective levels of TRs, such that bigger genomes may require more TR per cell to achieve the same level of gene expression compared to cells with fewer non-productive TR binding sites. Thus, it is possible that neither mRNA nor protein expression level comparisons across species would guarantee relevant differences in TR activity across species.

Because of these difficulties, we used a second approach to deduce whether TR protein activity may differ among species, namely a TH-response gene induction assay where different TR protein activity levels may underlie altered TH-response gene expression kinetics. In *Xenopus* upon administering T3, TRβ expression is induced, reaches a peak, and then declines [38]. We hypothesized that the expression profile of TRβ in *S. couchii* would be compressed in time, consistent with its higher TRα mRNA expression levels compared to the other spadefoot species, i.e., TRβ expression would go up and back down faster. At the higher 50 nM T3 dose, we found that the expression profiles for TRβ were similar across species in that TRβ expression was significantly up regulated by 12 h, peaked and 24 h, and began to decline by 48 h. On the other hand, the lower dose of 8 nM T3-induced an earlier significant increase in TRβ in *S. couchii* and *S. multiplicata* compared to *P. cultripes*, 12 h vs. 24 h. Also, the peak TRβ value was achieved by 24 h in *S. couchii* and 120 h in the other species, and TRβ levels started to decrease only in *S. couchii* over the T3 treatment period. In addition, *S. multiplicata* and *P. cultripes* significantly differ in larval period duration but do not have significant differences in TR expression or TRβ induction characteristics. However, though not significant, the TR values for *S. multiplicata* were generally intermediate between *S. couchii* and *P. cultripes*, corresponding to larval period differences [4].

The species differences we observed in gene induction after TH exposure are consistent with species differences in TR protein activity, but other factors may also be involved. For the induction assay, we used T3, rather than T4, ruling out any contributions to TH/TR signaling from deiodinase type 2 (converts T4 to T3). However, species differences in expression of cytoplasmic TH-binding proteins, deiodinase type 3 (degrades T3), TH-transporters, and TR-associated transcription factors, as well as TRs, may contribute to species differences in TH signaling/gene induction activity [2]. Although our study cannot discriminate among the potential contributions of these factors, these data indicate that one or more of these factors have been altered in *S. couchii* to explain the increase in TH-response gene expression. In combination with TRα mRNA differences, the TH-response gene induction comparisons point to a contributing role for TRα in larval period differences among species.

When comparing results from natural and induced metamorphosis, the TRβ expression profiles were similar across species during natural metamorphosis, whereas after T3 induction the TRβ profile of *S. couchii* achieved higher levels compared to the other species. In addition, the fold change in TRβ expression levels was higher after T3 induction than during natural metamorphosis for all three species. In contrast, the differences in TRα profiles among species were maintained in natural and induced metamorphoses, i.e., TRα levels in *S. couchii* were higher at all time points compared to the other species. However, as with TRβ, the fold change was greater after T3 treatment compared to natural metamorphosis for all three species. The higher fold changes in induced metamorphosis may reflect supraphysiologic amounts of T3, which may induce to higher levels TRα (a TH indirect response gene) and TRβ (a TH direct response gene). The greater TRβ induction in induced metamorphosis only in *S. couchii* may be due to different premetamorphic levels of corticosterone among species. Corticosterone peaks at metamorphosis and synergizes with TH to increase the expression levels of many TH-response genes [16,23,24]. *S. couchii* may have more corticosterone to synergize with exogenous T3 at stage 31 when the T3 treatments were carried out compared to the other species (Kulkarni and Buchholz, unpublished).

Our TR expression and T3-induction results indicated a correlation between higher TRα expression and shorter larval periods among spadefoot toads. To directly test the effects of TR expression levels on rates of metamorphic change, we measured the rate of T3-inudced disappearance of tail muscle cells with and without overexpressed TRα in *X. laevis*. In the absence of T3, muscle cells did not disappear, even with TRα overexpression. However, muscle cells died after T3 treatment as during natural metamorphosis, but the rate of disappearance was greater in muscle cells overexpressing TRα. Thus, species differences in TRα expression levels may contribute different rates of metamorphosis among species.

5. Conclusions

We show that TRα, and not TRβ, mRNA expression levels are higher in the desert spadefoot species *S. couchii* compared to its relatives that have longer larval periods. *S. couchii* also has faster TH-response gene induction kinetics that, in combination with its higher TRα mRNA levels, suggests this species has greater TR protein activity during metamorphosis. The effect of increased TR activity on metamorphic rate was examined in the model frog *X. laevis*, where we showed that the rate of T3-induced metamorphic change was higher when TRα was overexpressed in tail muscle cells. The higher expression of TRα in the absence of TH in *S. couchii* is expected to increase tissue sensitivity and responsivity to TH once it is released in circulation enabling faster gene induction kinetics underlying the more rapid rate of tissue transformation during metamorphosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2011.05.013.

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