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Developmental Profiles and Thyroid Hormone Regulation of Brain Transcripts in Frogs: A Species Comparison with Emphasis on *Physalaemus pustulosus*

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Key Words

Túngara · Brain · Thyroid hormone · Androgen · Estrogen · Gene expression · Metamorphosis · *Silurana (Xenopus) tropicalis* · *Rana (Lithobates) pipiens* · *Physalaemus* (*Engystomops) pustulosus*

Abstract

In amphibians, thyroid hormones (THs) are considered key regulators of brain remodeling during metamorphosis, while sex steroids (estrogens and androgens) control sexual differentiation and gonadal development. However, these two endocrine axes can interact during tadpole brain development. Previously, we demonstrated that THs affect sex steroid-related gene expression in the developing brain of *Silurana tropicalis* and *Rana pipiens*; however, the gene expression changes differed between species. We chose to study a third anuran species, *Physalaemus pustulosus,* to test new hypotheses about the role of THs in the regulation of brain gene expression. We first established developmental transcript profiles of TH- and sex steroid-related genes in the brain of *P. pustulosus*. Then, following the same protocols as in our previous studies, we investigated triiodothyronine (T3) regulation of brain transcripts in premetamorphic *P. pustulosus* and then compared the results with our previous two studies. In the case of TH-related genes, TH receptor beta *(trbeta)* and deiodinase type 3 *(dio3),* mRNA developmental profiles were similar in the three species and with respect to other species in the published literature. However, the profiles of TH receptor alpha *(tralpha)* and deiodinase type 2 *(dio2)* mRNA revealed differences between anuran species. Among the three anurans we have studied, the direction of the T3 regulation of TH-related genes was overall similar, but the magnitude of gene expression change differed depending on the rate of metamorphosis in a given species. For the sex steroid-related genes, each species exhibited similar developmental profiles but differed in their response to T3. In *P. pustulosus*, T3 reduced the expression of aromatase *(cyp19)* while increasing mRNA levels of androgen and estrogen receptors. These results are similar to previous research in *R. pipiens* but differ from data for *S. tropicalis,* for which we found an increase in androgen synthesis enzymes but no effect on *cyp19*. Together, we propose that T3 has the potential to induce the brain androgen system in anurans. This could be achieved by increasing androgen synthesis enzymes *(S. tropicalis)* or by decreasing estrogen synthesis (due to a decrease in *cyp19* in *P. pustulosus* and *R. pipiens*). In conclusion, we demonstrated that mechanisms of hormone interactions differ between anuran species, but in all cases T3 appears to affect the balance of sex steroids in the brain,

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stimulating the androgen system. We have shown that the regulation of sex steroid-related genes by T3 is more similar among closely related species than species with similar reproductive and developmental characteristics.

Introduction

Research in anuran tadpoles has demonstrated that thyroid hormones (THs) control the remodeling of the developing central nervous system (CNS) during metamorphosis [Denver, 1998; Tata, 2006]. In vertebrates, the CNS is also a target of sex steroids (estrogens and androgens) to control brain sexual development and reproduction [e.g. Pellegrini et al., 2005; Matsuda et al., 2008; Roselli et al., 2009]. During anuran metamorphosis, these two hormone axes interact to regulate gene expression. Previously, we showed that THs (triiodothyronine; T3) affect sex steroid-related transcripts in the brain during tadpole development. In *Rana pipiens,* T3 reduces the expression of aromatase (*cyp19,* the enzyme responsible for the conversion of testosterone into estrogen) mRNA [Hogan et al., 2007]. In contrast, in *Silurana (Xenopus) tropicalis,* T3 does not affect aromatase but positively regulates steroid 5α-reductase type 1 and 2 [*srd5alpha1* and *srd5alpha2*, enzymes responsible for 5α-dihydrotestosterone (5α-DHT) synthesis from testosterone] [Duarte-Guterman and Trudeau, 2010]. These results suggest that the effects of THs on sex steroid-related genes in the brain differ between anuran species. However, there are limitations about the conclusions that can be drawn from a two-species comparison. While they are an important first step, these previous studies do not indicate the range of differences and how these may be distributed phylogenetically in anurans [Garland and Adolph, 1994]. Therefore, in this study we established brain developmental profiles and investigated the effects of T3 on brain gene expression in a third anuran and directly compared this to our previous research.

Studies in frog developmental endocrinology, especially pertaining to metamorphosis and sexual development, have concentrated on using species of the Pipidae (i.e. *S. tropicalis* and *Xenopus laevis*) and Ranidae (e.g. *R. pipiens, Rana catesbeiana,* and *Rana rugosa*) families [Hayes, 1998; Shi, 2000]. Therefore, for this study, the third species we have chosen is *Physalaemus pustulosus;* it belongs to the Leiuperidae family [Grant et al., 2006] and it is evolutionarily separated from the Pipidae *(S. tropicalis)* and Ranidae *(R. pipiens)* families [Ford and Cannatella, 1993]. Although *P. pustulosus* has been extensively used in sexual selection and communication studies [Ryan, 1985, 2010], only very limited information is available regarding tadpole metamorphosis and sexual development.

The current species comparison allowed us to examine whether T3 regulation of brain transcripts is similar among species that share reproductive and developmental characteristics or whether gene regulation is more similar among closely related species. All three species chosen for this project have a tadpole stage but have varying rates of development and reproductive characteristics (table 1). *P. pustulosus* and *S. tropicalis* have similar characteristics and ecological and habitat requirements (table 1 and IUCN [2010]); however, *P. pustulosus* is more closely related to *R. pipiens* than to *S. tropicalis* [Ford and Cannatella, 1993].

Metamorphosis is controlled by THs in all anuran species studied [Shi, 2000], but it has also been suggested that a shorter length of larval development is related to higher sensitivity to THs [Buchholz and Hayes, 2005]. Therefore, we first hypothesized that T3 regulation of brain TH-related genes would be similar (in terms of directions of change) in the three species, but the magnitude of change would vary depending on the rate of metamorphosis. According to the characteristics in table 1, we predicted that *S. tropicalis* and *P. pustulosus* would have a similar regulation of TH-related genes (similar direction and magnitude of change) compared to *R. pipiens* (similar direction but different magnitude of change). Regarding sex steroid-related genes, we hypothesized that T3 regulation would vary depending on the developmental and reproductive characteristics of the tadpoles rather than phylogenetic relationships. We predicted that *S. tropicalis* and *P. pustulosus* would have a similar T3 regulation of sex steroid-related genes (i.e. absence of an effect on *cyp19* but positive regulation of *srd5alpha*) while *R. pipiens* would show a distinct transcript profile (i.e. decrease in *cyp19*). The experimental approach used was the same in all three studies. Studies with *S. tropicalis* are published in Duarte-Guterman and Trudeau [2010] and those with *R. pipiens* are published in Hogan et al. [2007]. Those results are further used herein to compare the mechanisms of crosstalk in the three different species.

We first cloned TH-related genes [TH receptors *(tralpha, trbeta)* and deiodinases *(dio2*, *dio3)*] and sex steroid-related genes [*cyp19*, estrogen receptors (*eralpha, erbeta)*, androgen receptor *(ar)*, and steroid 5α-reductases *(srd5alpha1, sdr5alpha2)*] and established their transcript profiles in the brain during metamorphosis of *P. pustulosus*. Then, premetamorphic tadpoles (Gosner [1960] stage

Table 1. Developmental and reproductive characteristics of *S. tropicalis, P. pustulosus,* and *R. pipiens*

	S. tropicalis	*P. pustulosus*	*R. pipiens*
Anuran family	Pipidae	Leiuperidae	Ranidae
Geographic location	Central Africa	Central and South America	North America
Days to metamorphosis	30–90	20–50	>90
Age at sexual maturity	~16–24 weeks	~8–12 weeks	~2–3 years
Sex-determining mechanism	ZZ/ZW	unknown	XX/XY
Timing of gonad differentiation	Premetamorphosis	Metamorphosis (before stage G42)	Premetamorphosis
Reproduction	Rain season (prolonged breeder)	Rain season (prolonged breeder)	Spring (ephemeral breeder)
Hibernation (overwintering)	No	No	Yes

Information taken from: Amaya et al. [1998], Cannatella [2008], COSEWIC [2009], El Jamil et al. [2008], Force [1933], Humphrey et al. [1950], IUCN [2010], Kochan et al. [2003], Marsh [2001], and personal observations in the Trudeau and Ryan laboratories (days to metamorphosis and *P. pustulosus* timing of gonad differentiation).

32–34; G32–34) were treated with T3 in order to examine the influence of THs on the abundance of specific brain transcripts and compare the results with *S. tropicalis* and *R. pipiens.*

Materials and Methods

Animals

Romero-Carvajal et al. [2009] described in detail the procedures for *P. pustulosus* tadpole and adult care and reproduction in captivity; frogs in the current study were obtained from the same colony at the University of Texas at Austin. Fertilized eggs were obtained from three pairs of frogs, and developmental stages were characterized according to the Gosner developmental table [Gosner, 1960]. Tadpoles were reared in dechlorinated water and fed every 2 days with JurassiDiet pellet food (JurassiPet, Madison, Ga., USA). Room temperature was maintained at 27–28°C and the tank water temperature was 25–26°C. A 12:12 h light:dark cycle was maintained starting at 4:00 a.m. local time. Animal care and treatment protocols were in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC), University of Texas at Austin (IACUC protocol No. 08101701).

Cloning TH- and Sex Steroid-Related Genes

Species-specific cDNA sequences for *tralpha, trbeta, dio2, dio3, eralpha*, *erbeta, cyp19, srd5alpha1,* and *srd5alpha2* were cloned from *P. pustulosus* cDNA or genomic DNA (gDNA). Total RNA was obtained from the head, body, or tail of different individuals at G42 using the RNeasy Micro Kit (Qiagen, Mississauga, Ont., Canada). First strand cDNA synthesis was prepared from 1–2 μg of RNA and 200 ng random hexamer primers using SuperScript II reverse transcriptase as described by the manufacturer (Invitrogen, Burlington, Ont., Canada). gDNA was obtained from a G42 tail following a standard phenol-chloroform-isoamyl extraction protocol. Mammalian and nonmammalian nucleic acid sequences for the target genes were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and aligned using ClustalW (EMBL-EBI; http://www.ebi.ac.uk/clustalw/). Degenerate primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and are presented in online supplementary table 1 (for all online supplementary material, see www.karger.com/doi/10.1159/000331265) PCR amplification was performed using the Mastercycler® gradient Thermal Cycler (Eppendorf, Westbury, N.Y., USA). The PCR mixture (25 μl final volume) contained 1.0× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM forward and reverse primers, 1 U Taq® DNA polymerase (Invitrogen), and 1 μl of template (cDNA, gDNA, or PCR product for nested PCR). The initial denaturation step was performed at 94°C for 3 min to activate the Taq enzyme. This was followed by 40 cycles with a denaturation step at 94°C for 45 s, an annealing step for 30 s, and an extension step at 72°C for 1 min. The final extension step was performed at 72°C for 10 min and samples were immediately cooled at 4°C. The PCR conditions and the template (cDNA or gDNA) used to clone each gene are presented in online supplementary table 1. Amplification products were ligated directly into the 2.1 TOPO® vector (TOPO TA cloning kit; Invitrogen) and transformed into *Escherichia coli* (One Shot TOP 10 chemically competent cells; Invitrogen). Cells were then plated onto LB-agar plates containing standard concentrations of ampicillin and X-Gal and incubated at 37°C for 16 h. Positive colonies were selected and an additional PCR was performed using M13 primers (included in the TOPO TA cloning kit) to ensure that the correct insert size was present in the colonies before sequencing. Colonies were grown overnight at 37°C in LB broth containing ampicillin, and plasmids were purified using the Qiaprep miniprep kit (Qiagen). The purified plasmids (10 μl; at least 3 per gene) were sent to the Ontario Genomics Innovation Centre (Ottawa Health Research Institute, Ottawa, Ont., Canada) for sequencing. We checked the quality of the sequencing using the chromatograms. In the resulting sequences, the number of nucleotide mismatches was minimal (i.e. between 2 and 14 nucleotides per sequence). For each gene, at least three partial sequences were aligned to obtain a consensus sequence which was then deposited in GenBank (accession numbers are presented in online suppl. table 1).

Phylogenetic Analysis

Phylogenetic trees for *cyp19* and *eralpha* were constructed using anuran nucleotide sequences available on GenBank and the

partial sequences we cloned from *P. pustulosus*. Only these two genes were chosen because there are nucleotide sequences published for species of the Ranidae, Pipidae, and Bufonidae families. The other genes were not taken into account for various reasons: (1) published sequences were not available for at least one representative of each family, (2) published sequences were too short for analysis, and/or (3) published sequences did not align with our *P. pustulosus* cloned sequences. Sequence alignment was generated by Muscle [Edgar, 2004] and maximum-likelihood analyses were performed with PhyML (http://www.phylogeny.fr) [Guindon and Gascuel, 2003; Guindon et al., 2005]. Statistical branch support was calculated using 500 bootstraps.

Tissue Collection for Developmental Profiles

Brain samples were taken at different stages of development: G30 (premetamorphosis; foot paddle stages), G36 (prometamorphosis; hind limb development), and G42 (metamorphic climax; forelimb emergence). Tadpoles were anesthetized by immersion in clove oil and euthanized by decapitation. Whole brains were dissected and preserved in RNAlater as described by the manufacturer (Ambion, Austin, Tex., USA). For stages G30 and G36 brains were pooled (2–4 brains per pool, n = 5–8 pools), but for G42 brains were analyzed individually based on morphological sex (n = 8). The sex of tadpoles at G42 was further confirmed by the expression of *cyp19* in the gonad; *cyp19* was 250-fold higher in the gonad-mesonephros complex of morphological females compared to males [Duarte-Guterman et al., unpubl. res.].

T3 Exposure

In order to compare the T3 regulation of TH- and sex steroid-related genes between frog species, we used the same protocol and T3 concentrations described in Duarte-Guterman and Trudeau [2010] for *S. tropicalis* and in Hogan et al. [2007] for *R. pipiens*. In a pilot exposure, we confirmed that the T3 concentrations and length of exposure previously used were also appropriate for *P. pustulosus* (i.e. after 48 h no morphological effect of T3 was observed; after 72 h limb development and resorption of the tail had begun in tadpoles exposed to 5 and 50 nM T3; data not shown). Premetamorphic tadpoles (G32–34 equivalent to NF 53–54) were exposed to three nominal concentrations of 3,3′,5-triiodo-L-thyronine (T3; 0.5, 5, and 50 nM; Sigma, Oakville, Ont., Canada) or a dimethylsulfoxide (DMSO; Sigma) solvent control for 48 h. The final DMSO concentration in the tank was 0.005% in all treatments. The density in all tanks was 1 tadpole/liter of water. Chemical additions were not renewed during the 48-hour period. At the end of the exposure, whole tadpole brains were dissected after the anesthesia. Collected tissues were preserved in RNA-later (Ambion) and pooled (2 brains per pool; n = 8 pools) before RNA isolation.

RNA Isolation and cDNA Synthesis

Total RNA for the developmental profile and T3 exposure samples was obtained from brains using the RNeasy Micro Kit (including the DNase treatment set) as described by the manufacturer (Qiagen). Pooled and individual brain samples were homogenized with an MM301 Mixer Mill (Retsch, Newton, Pa., USA) set to 20 Hz for 3 min. Isolated RNA was resuspended in RNase-free water and concentrations were determined using a NanoDrop spectrophotometer (ND1000; NanoDrop Technologies). Total cDNA was prepared from 1 μg of total RNA and 0.2 μg random hexamer primers using SuperScript II reverse transcriptase (Invitrogen). For all of the samples, the reverse transcriptase reaction was modified to be carried out at 42°C for 90 min (compared to the manufacturer's standard protocol of 50 min) to increase the cDNA yield. The cDNA products were diluted 40- or 80-fold prior to PCR amplification.

Real-Time RT-PCR Assays and Data Analysis

The partial sequences obtained from the cloning and the *ar* sequence published in GenBank (accession No. DQ320626) were used in Primer3 to design gene-specific primer sets for real-time RT-PCR. The specificity of the primer sets was verified by PCR and gel electrophoresis. The appearance of a single product at the expected size and sequencing of these PCR products confirmed that the primers were amplifying the intended product. For all real-time RT-PCR assays, primer concentrations were optimized for a minimum threshold cycle and a maximum change in fluorescence. Primer sequences and optimized conditions are presented in online supplementary table 2. The expression of individual gene targets was analyzed using the Mx3005P real-time PCR system (Stratagene). The simplex reaction consisted of a 25-μl DNA amplification reaction containing 1.0× PCR buffer (Qiagen), 2.5 mM $MgCl_2$ (Qiagen), 200 μM dNTPs (Invitrogen), 100 nM passive reference dye (Stratagene), 1.25 U HotStarTaq (Qiagen), optimized concentrations of each primer set (Invitrogen), 0.25× SYBR Green I Dye (Molecular Probes, Eugene, Oreg., USA), and 5 μl of diluted cDNA template. The thermocycle program included an enzyme activation step at 95°C (15 min) and 45 cycles of 95°C (15 s), 56–60°C (gene specific annealing temperature; 5 s), 72°C (30 s), and 80°C (8 s). After the amplification phase, a denaturation step of 1 min (95°C) was followed by 41 cycles starting at 55°C and increasing 1°C/30 s to generate a dissociation curve to confirm the presence of a single amplicon.

Relative mRNA abundance of target genes was obtained using the standard curve method. The standard curves were generated using a cDNA mix of G42 samples (for the developmental profiles) and using equal parts of cDNA from each treatment including the control (for the T3 exposure). Samples were run in duplicate along with a negative template control (RNase-free water instead of cDNA template) and a negative reverse transcriptase control (cDNA template for which water was added instead of SuperScript II). Reaction efficiencies were 90–110% with an $R^2 \geq 0.990$. As also observed in *S. tropicalis* [Duarte-Guterman and Trudeau, 2010], mRNA levels of the reference gene, ribosomal protein L8 *(rpl8)*, decreased during development in *P. pustulosus* (fig. 2f). Therefore, data for the developmental profiles are presented as fold changes relative to G30 and normalized to the RNA content. This method is one of the strategies to normalize real-time RT-PCR data [Huggett et al., 2005] and consists of dividing the gene expression data obtained by the amount of RNA used in the cDNA synthesis reaction (1 μg; see RNA Isolation and cDNA Synthesis). In previous experiments with *S. tropicalis,* common reference genes, including *rpl8,* used to normalize gene expression data have been affected by T3 [Duarte-Guterman et al., 2010; Duarte-Guterman and Trudeau, 2010, 2011] and, therefore, normalizing the data using reference genes was not possible. In the brain of *P. pustulosus* tadpoles*, rpl8* mRNA was not affected by T3, but in order to be consistent and compare data across studies the data for the T3 exposure is presented relative to the control group and normalized to the RNA content only, and the results for *rpl8* are shown separately in figure 4f.

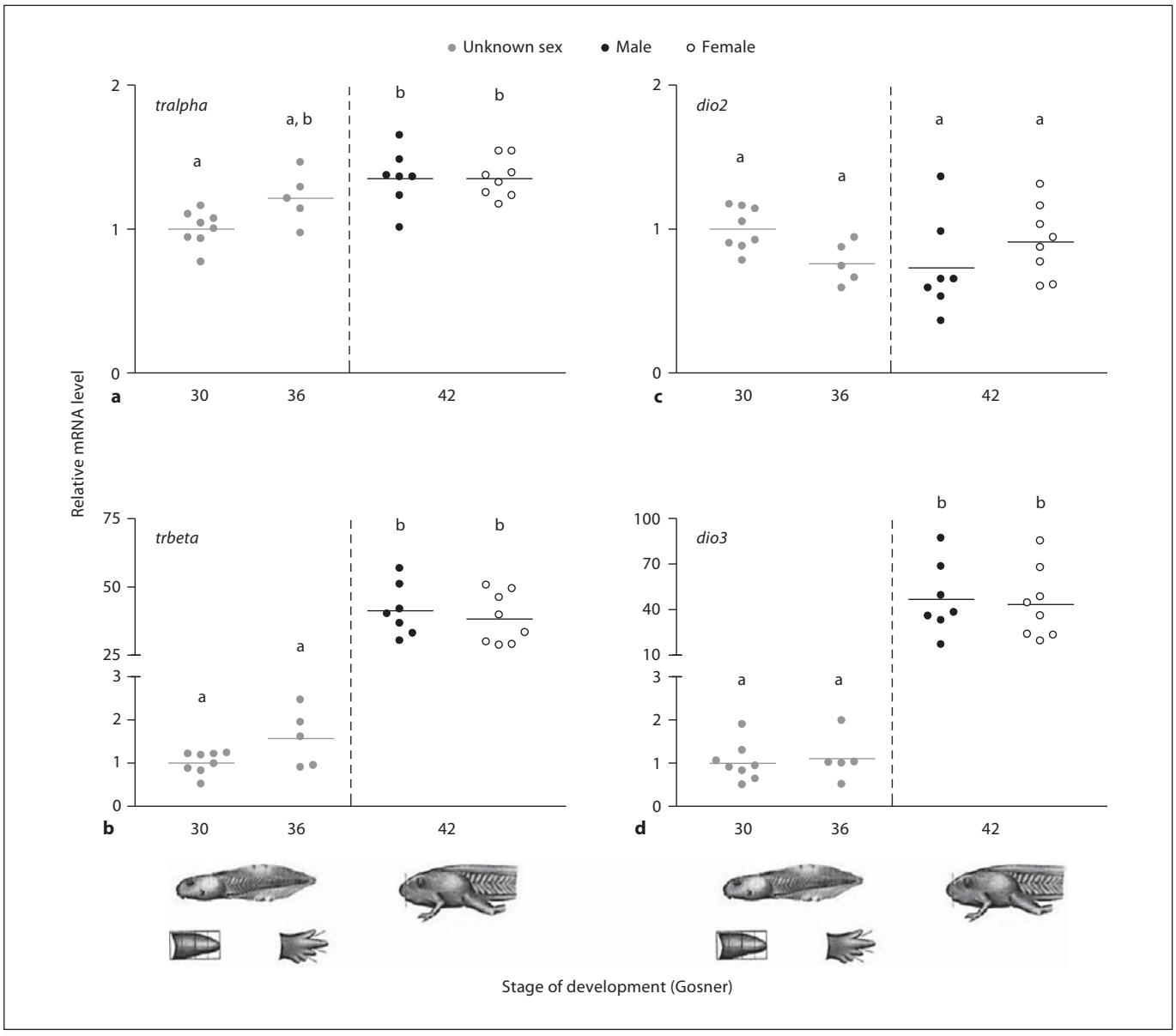

Fig. 1. Brain developmental profiles of TH-related genes during *P. pustulosus* metamorphosis. Transcript levels of *tralpha* (**a**), *trbeta* (**b**), *dio2* (**c**), and *dio3* (**d**) were measured in the brain at G30, G36, and G42. Levels of mRNA are expressed relative to G30 and are normalized to RNA content. Individual sample points are presented along with the mean. Different letters indicate statistically significant differences between stages (one-way ANOVA; n = 5–8; $p < 0.05$). Main morphological characteristics (i.e. whole body and hind limb diagrams [Gosner, 1960]) are included for each stage of development. A vertical dashed line separates the developmental samples from G42 male and female brains. Note that the scale of the y-axes varies between genes.

Statistical Analyses

Statistical analyses were performed using S-Plus 8.0 (Insightful Corporation, Seattle, Wash., USA), and $p < 0.05$ was considered statistically significant. Data for all of the genes were first tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test). When the assumptions were not met, the data were transformed. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc multiple comparisons test between all stages (developmental profiles) or by Bonferroni's pairwise comparison between treatment and control groups (T3 exposure). In the case of the developmental profile of *erbeta,* data were not normally distributed; thus we used the nonparametric Kruskal-Wallis test, and differences between developmental stages were analyzed using individual t tests (for unequal variance) adjusting the p value according to the Bonferroni correction [Field and Miles, 2010].

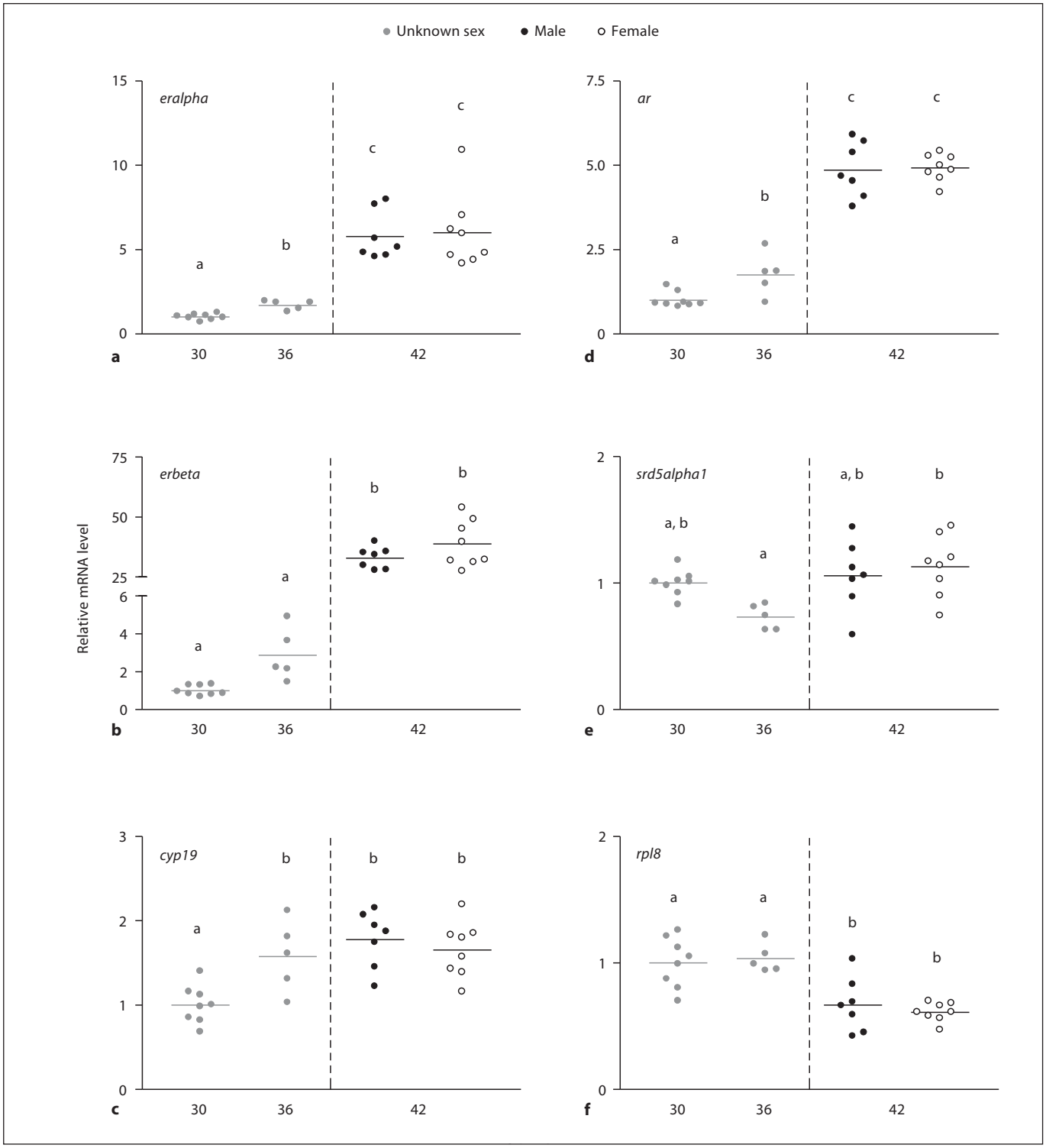

Fig. 2. Brain developmental profiles of sex steroid-related genes during *P. pustulosus* metamorphosis. Transcript levels of *eralpha* (**a**), *erbeta* (**b**), *cyp19* (**c**), *ar* (**d**), and *srd5alpha1* (**e**) were measured in the brain at G30, G36, and G42. Results for the reference gene *rpl8* are also presented (**f**). Levels of mRNA are expressed relative to G30 and are normalized to RNA content. Individual sample points are presented along with the mean. Different letters indicate statistically significant differences between stages (one-way ANOVA; n = 5–8; $p < 0.05$). A vertical dashed line separates the developmental samples from G42 male and female brains. Note that the scale of the y-axes varies between genes.

Table 2. Percent nucleotide identity of *P. pustulosus* target genes analyzed in this study compared to sequences from other species belonging to different anuran families

	Xenopus laevis	*Silurana tropicalis*	*Rana catesbeiana*	*Rana pipiens*	*Rana rugosa*	*Bufo bankorensis*	*Bufo marinus*	*Bufo rangeri*	*Bombina orientalis*	Average
tralpha	88	88	91	–	–	–	–	–	–	89
trbeta	86	86	90	–	89	92	–	–	–	88
dio2	82	82	86	–	–	–	–	–	–	83
dio3	82	82	78	79	–	–	–	–	–	80
eralpha	80	80	–	82	83	–	–	90	83	83
erbeta	79	79	–	–	82	–	–	88	–	82
cyp19	78	78	–	79	80	–	91	–	–	81
ar[a]	81	82	84	84	85	–	–	–	–	83
sdr5alpha1	73	74	–	–	75	–	–	–	–	74
sdr5alpha2	79	85	–	–	–	–	–	–	–	82
Range	73–88	74–88	78–91	79–84	75–89	92	91	88–90	83	

[a] Complete coding sequence (GenBank accession No. DQ320626) [Chakraborty and Burmeister, 2010].

Results

Cloning of Genes in P. pustulosus

Gene fragments for TH- and sex steroid-related genes were cloned from *P. pustulosus* using degenerate primers designed to match motifs of the most conserved regions of these genes. The percent nucleotide identity of the genes obtained in this study in comparison to the homologous genes in other anuran species varies from 73 to 92% and is presented in table 2. TH receptors *(tralpha, trbeta)* are highly conserved (88–89% average identity). The deiodinases *(dio2, dio3),* sex steroid receptors *(eralpha, erbeta, ar), cyp19,* and *srd5alpha2* have about 80–83% identity, while *sdr5alpha1* is the least conserved sequence, with an average identity of 74%. A phylogenetic tree was constructed using the available anuran *cyp19* and *eralpha* mRNA sequences (6–7 sequences per gene) and the homologous mRNA sequence from the salamander *Pleurodeles waltl* as the out-group (online suppl. fig. 1). Based on these two genes, *P. pustulosus* is most closely related to the Bufonidae, and more distantly related to the Pipidae (online suppl. fig. 1 and table 2), which follows the currently accepted relationships between these species [Ford and Cannatella, 1993].

Brain Developmental Profiles during Metamorphosis

Developmental profiles of TH- and sex steroid-related genes were established by sampling at three stages of development (the main morphological characteristics are shown in fig. 1). For all of the genes assessed, with the exception of *srd5alpha2,* transcripts were detected in the brain throughout metamorphosis and no differences in mRNA levels were detected between males and females at metamorphic climax (fig. 1, 2). In the case of *srd5alpha2,* transcripts were not detected in the brain of *P. pustulosus* (i.e. no amplification was detected in the real-time RT-PCR after 45 cycles). For *tr* and *dios* (fig. 1), and *erbeta* and *sdr5alpha1* (fig. 2b, e), no significant differences in brain mRNA levels were observed between G30 and G36 tadpoles. Transcript levels of *eralpha* and *ar* showed similar gradual increases during tadpole development (1.7-fold at G36 and 5.0-fold at G42; fig. 2a, d), while *cyp19* only increased 1.5-fold at G36 relative to G30 and then remained constant between G36 and G42 (fig. 2c). Large increases in mRNA levels at G42 were observed for *trbeta* (40-fold; fig. 1b), *dio3* (44-fold; fig. 1d), and *erbeta* (35-fold; fig. 2b). The genes that remained unchanged throughout metamorphosis were *dio2* (fig. 1c) and *srd5alpha1* (fig. 2e). Expression of the reference gene *rpl8* decreased significantly during development (1.5- to 1.7-fold; fig. 2f). Therefore, the gene expression data was normalized to the RNA content and the results will contribute to further research and validation of *rpl8* as a reference gene in *P. pustulosus.*

Effects of T3 on Brain Gene Expression

Premetamorphic tadpoles (G32–34) were exposed to T3 for 48 h, as previously reported for *S. tropicalis* [Duarte-Guterman and Trudeau, 2010] and *R. pipiens* [Hogan et al., 2007], and transcript levels were measured in the brain. Treatment with T3 had no effect on mortality,

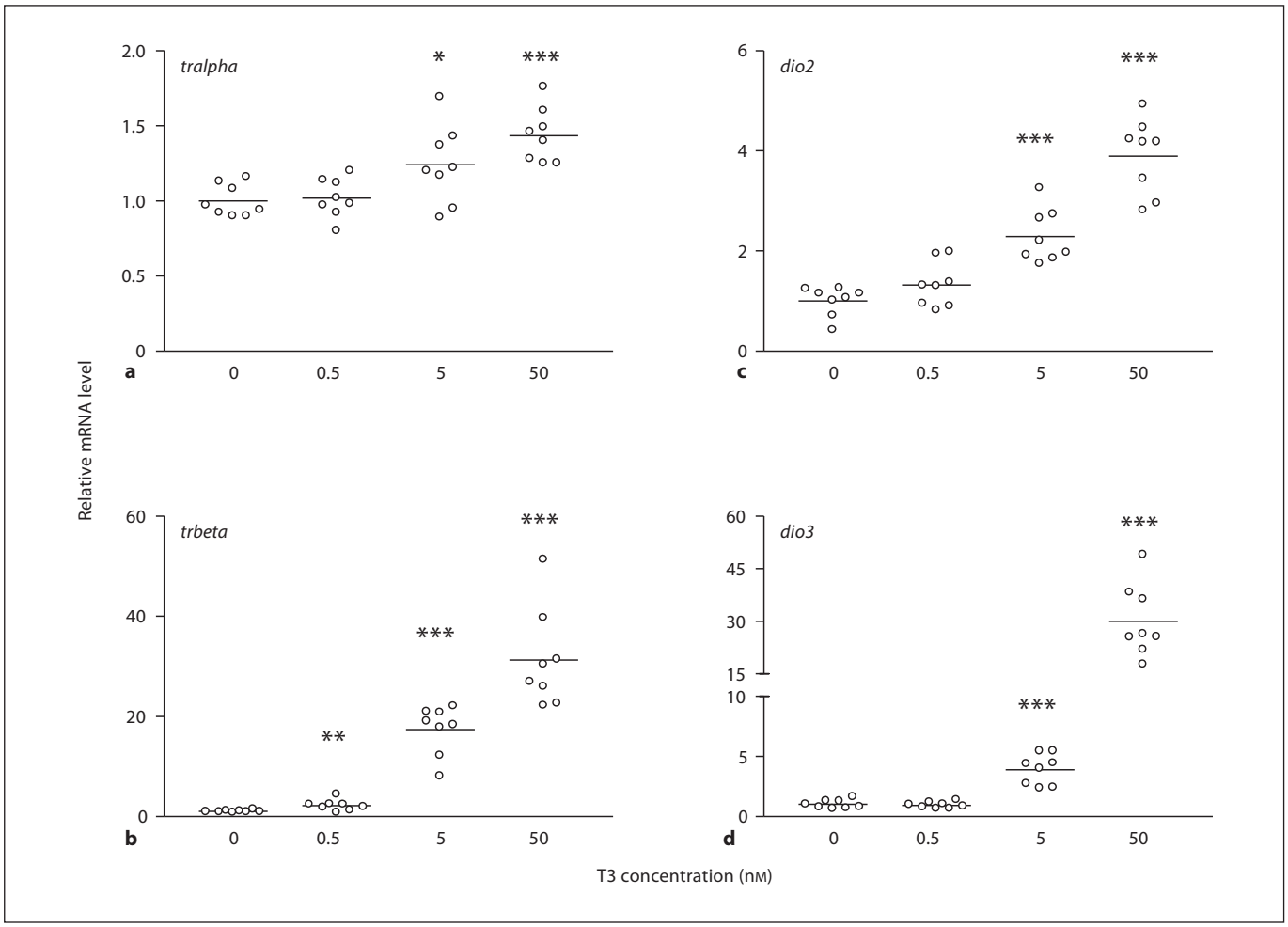

Fig. 3. Effects of T3 exposure on the expression of TH-related genes in the brain of *P. pustulosus*. Premetamorphic tadpoles (G32–34) were exposed to T3 (0, 0.5, 5, and 50 nM) for 48 h and transcript levels of *tralpha* (**a**), *trbeta* (**b**), *dio2* (**c**), and *dio3* (**d**) were measured in the brain. Data are presented as fold changes relative to the control and are normalized to RNA content. Individual sample points are presented along with the mean. Asterisks represent significant differences in mRNA levels from the control group (one-way ANOVA; n = 8; * $p < 0.05$; *** $p < 0.001$). Note that the scale of the y-axes varies between genes.

and 100% survivorship post-exposure was observed in all treatment groups. Real-time RT-PCR analyses revealed that mRNA levels of TH-related genes in the brain were affected by T3 (fig. 3) as follows: *tralpha* ($F_{3, 28} = 10.6$; $p < 0.001$), *trbeta* ($F_{3, 28} = 74.7$; $p < 0.001$), *dio2* ($F_{3, 28} = 35.87$; $p < 0.001$), and *dio3* ($F_{3, 28} = 200.7$; $p < 0.001$). Transcript levels of *tralpha* showed the smallest of changes with increases at 5 nM (1.2-fold; post hoc test $p < 0.05$) and 50 nM of T3 (1.5-fold; post hoc test $p < 0.001$; fig. 3a). Levels of *dio2* and *dio3* were more responsive to exogenous T3. We recorded larger increases at 5 nM (2.0-fold, post hoc test $p < 0.001$, and 4.0 fold, post hoc test $p < 0.001$, respectively) and 50 nM of T3 (4.0-fold, post hoc test $p < 0.001$, and 30-fold, post hoc test $p < 0.001$, respectively; fig. 3c, d). In the case of *trbeta,* T3 induced brain transcript levels in a concentration response manner (2.0- to 30-fold, post hoc test $p < 0.01$; fig. 3b). The expression levels of sex steroid-related genes in the brain were also measured after T3 exposure (fig. 4). T3 exposure affected mRNA levels of *erbeta* ($F_{3, 28} = 35.6$; $p < 0.001$), *cyp19* ($F_{3, 28} = 3.54$; $p = 0.03$), and *ar* ($F_{3, 28} = 3.16$; $p = 0.04$). In the case of *erbeta,* T3 significantly induced transcript levels at 50 nM T3 (3.5-fold, post hoc test $p < 0.001$; fig. 4b), and for *ar* mRNA levels increased at 5 and 50 nM (1.2-fold, post hoc test $p < 0.05$; fig. 4d). Levels of *cyp19* significantly decreased with T3 exposure at 5 and 50 nM (1.4-fold,

Table 3. Gene expression changes in the brain after T3 exposure in three frog species, *S. tropicalis, P. pustulosus,* and *R. pipiens*

Target gene	*S. tropicalis*[a]			*P. pustulosus*[b]			*R. pipiens*[c]		
	0.5	5.0	50	0.5	5.0	50	0.5	5.0	50
eralpha	–	1.7↑*	2.0↑*	–	–	–	–	–	1.5↑*
erbeta	1.5↑	2.4↑*	2.7↑*	–	1.3↑	3.4↑*	na	na	na
cyp19	–	–	–	–	1.4↓*	1.4↓*	–	1.9↓*	1.6↓*
srd5alpha1	1.5↑*	–	–	–	–	–	na	na	na
srd5alpha2	7.5↑*	6.1↑*	5.6↑*	nd	nd	nd	na	na	na
ar	–	–	–	–	1.2↑*	1.2↑*	na	na	na
tralpha	1.4↓	1.7↓	1.8↓*	–	1.2↑*	1.4↑*	–	–	–
trbeta	6.0↑*	10↑*	15↑*	2.0↑*	17↑*	31↑*	–	–	6.1↑*
dio2	2.3↑*	2.7↑*	3.0↑*	1.3↑	2.3↑*	3.9↑*	–	–	2.9↑*
dio3	2.5↑*	22↑*	136↑*	–	3.9↑*	30↑*	–	–	4.0↑*

* Statistically significant differences compared to the control (one-way ANOVA followed by Bonferroni's pairwise comparison; $p < 0.05$). Dashes indicate no differences compared to the control. na = Not measured; nd = not detected.

[a] Results from Duarte-Guterman and Trudeau [2010]. [b] Results from the present study. [c] Results modified from Hogan et al. [2007].

post hoc test $p < 0.05$; fig. 4c). Transcript levels of *eralpha* ($F_{3, 28} = 1.18$; $p = 0.34$) and *srd5alpha1* ($F_{3, 28} = 1.16$; $p = 0.34$) were not affected by the treatments (fig. 4a, e).

Discussion

In this study, we established brain developmental profiles of TH- and sex steroid-related genes and their regulation by T3 in *P. pustulosus.* This allowed us to test hypotheses about the relationship between reproductive and developmental characteristics and gene regulation after T3 exposure because we could compare this new data with two unrelated frog species, *S. tropicalis* and *R. pipiens.* We found that T3 regulates sex steroid-related genes in the brain of *P. pustulosus,* again demonstrating as in previous studies [Hogan et al., 2007; Duarte-Guterman and Trudeau, 2010] that cross-regulation between sex steroid and TH pathways is present in the amphibian brain. As we predicted, T3 regulation of TH-related genes was similar among the three anurans (in terms of direction of change) and the magnitude of change was similar in the species with similar rates of metamorphosis. On the other hand, we found species-specific differences in the effects of exogenous T3 on the expression of sex steroid-related genes in the brain. We rejected our hypothesis that species with similar reproductive and developmental characteristics have similar crosstalk mechanisms and instead found that closely related species have a more similar T3 regulation of sex steroid-related genes.

Developmental Profiles and T3 Regulation of TH-Related Genes

This is the first time that TH- and sex steroid-related transcripts have been measured during development in *P. pustulosus.* The profiles of *tralpha, trbeta,* and *dio3* during metamorphosis are similar to the trends observed in the brain of other anurans, e.g. *X. laevis* [Krain and Denver, 2004], *S. tropicalis* [Wang et al., 2008; Duarte-Guterman and Trudeau, 2010], and *R. pipiens* [Hogan et al., 2007] (fig. 5). Expression of *trbeta* and *dio3* increased during development and also in a concentration-dependent manner after T3 exposure. This suggests that increased expression during development is regulated by rising TH levels. The autoinduction of *trbeta* by T3 in *P. pustulosus* (fig. 3b) has also been observed in the brains of all other premetamorphic tadpoles where this response has been studied. These include *R. catesbeiana* [Helbing et al., 2006], *R. pipiens* [Hogan et al., 2007], *X. laevis* [Krain and Denver, 2004], and *S. tropicalis* [Wang et al., 2008; Duarte-Guterman and Trudeau, 2010] (table 3), indicating a conservation of this mechanism in the Anura. Similarly, *dio3* was also induced by T3 in the *P. pustulosus* tadpole brain (fig. 3d), which suggests the presence of a TH response element in the *dio3* promoter, as has been found in *X. laevis* and *R. catesbeiana* [St Germain et al., 1994; Becker et al., 1995].

In *P. pustulosus, dio2* remained relatively constant during development, which is similar to results in two species of Ranidae [Becker et al., 1997; Hogan et al., 2007] but different from the results of two species of Pipidae

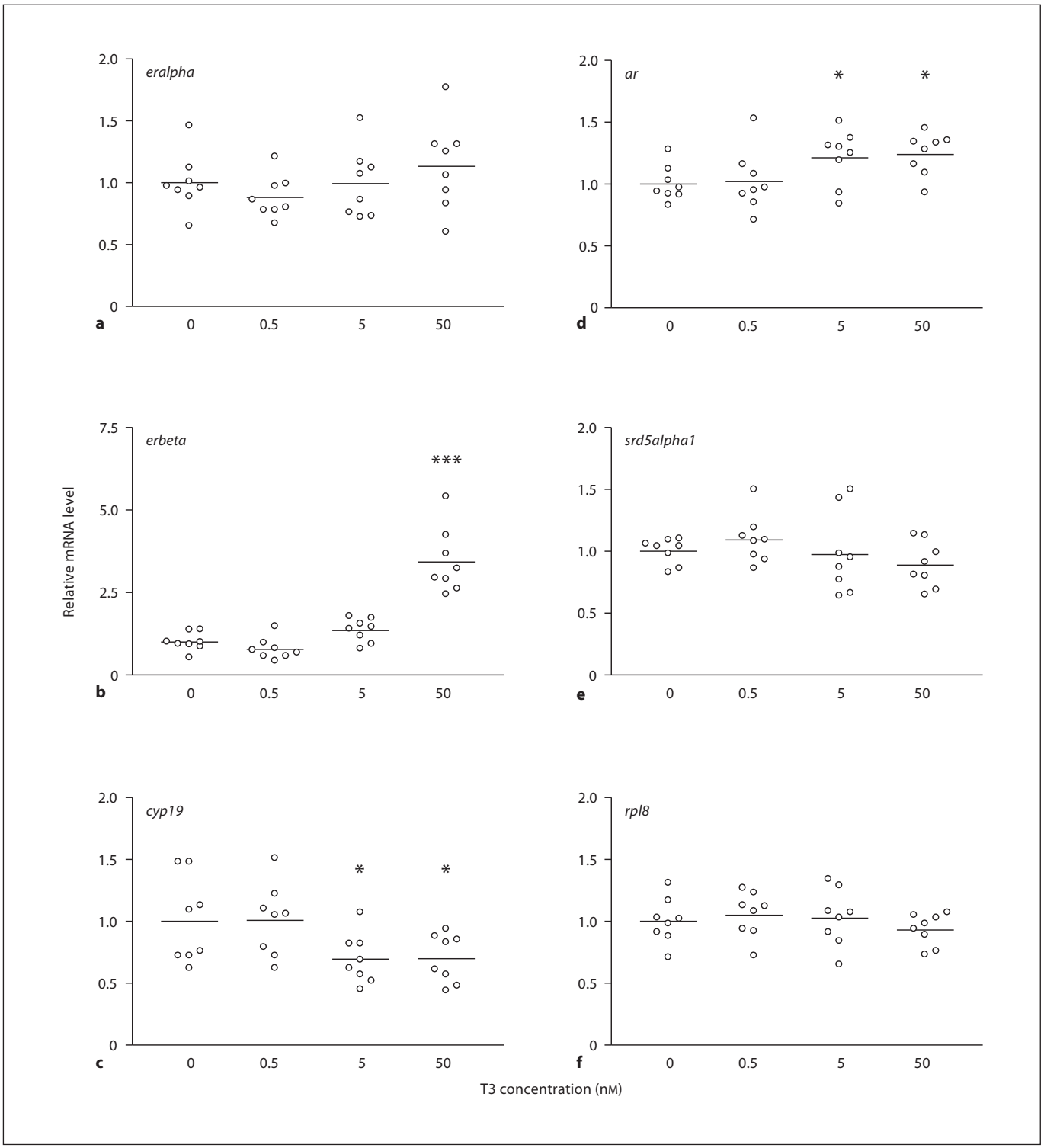

Fig. 4. Effects of T3 exposure on the expression of sex steroid-related genes in the brain of *P. pustulosus*. Premetamorphic tadpoles (G32–34) were exposed to T3 (0, 0.5, 5, and 50 nM) for 48 h and transcript levels of *eralpha* (**a**), *erbeta* (**b**), *cyp19* (**c**), *ar* (**d**), and *srd5alpha1* (**e**) were measured in the brain. Results for the reference gene *rpl8* are also presented (**f**). Individual sample points are presented along with the mean. Data are presented as fold changes relative to the control and are normalized to RNA content. Asterisks represent significant differences in mRNA levels from the control group (one-way ANOVA; n = 8; * $p < 0.05$; *** $p < 0.001$). Note that the scale of the y-axes varies between genes.

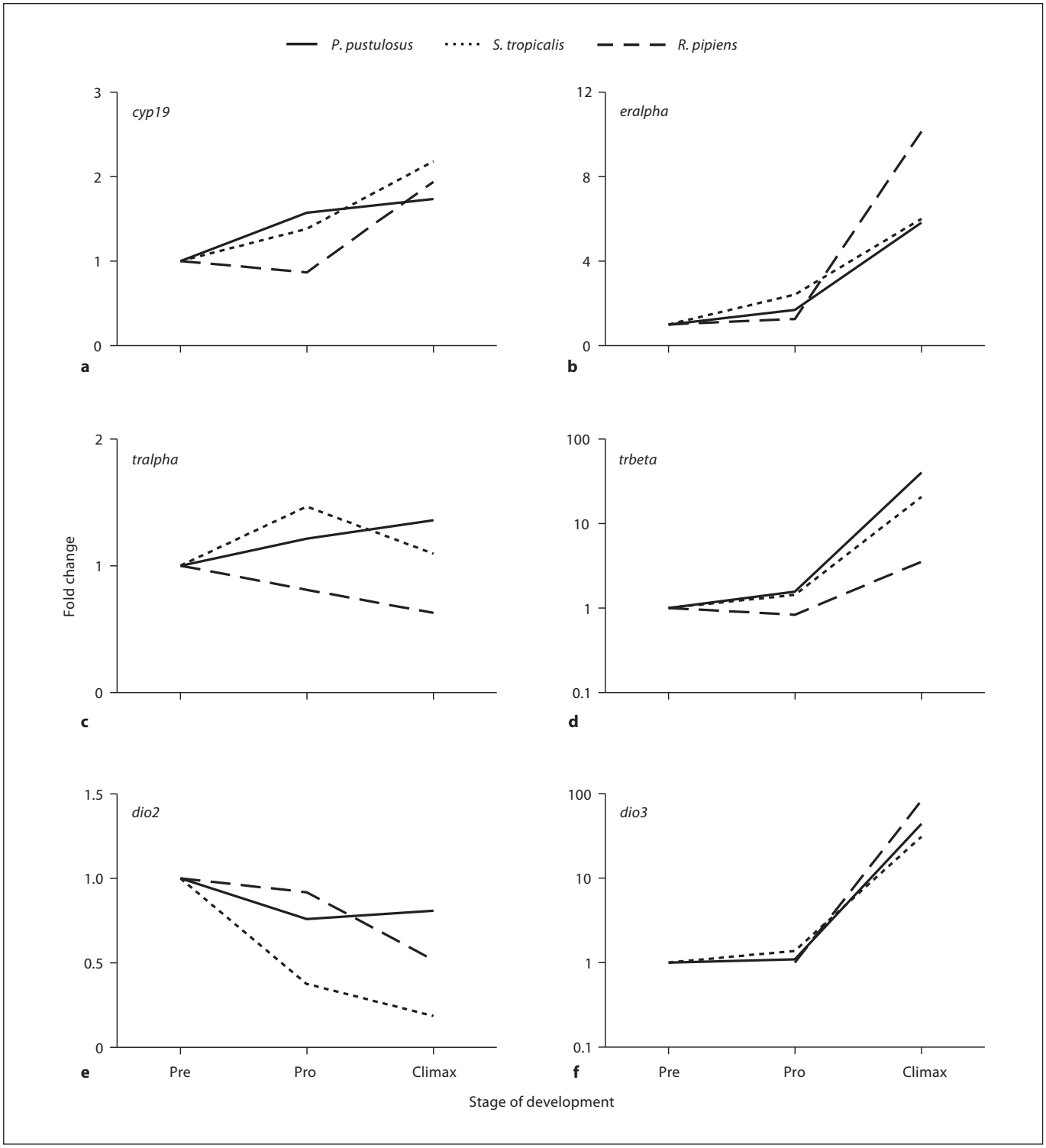

Fig. 5. Sex steroid- and TH-related gene expression profiles in the brain during tadpole development. Three anuran species are presented at three stages of development [premetamorphosis (pre), prometamorphosis (pro), and metamorphic climax]. Data are normalized to RNA content only and presented as fold changes relative to the first stage of development (premetamorphosis), except for *dio3* in *R. pipiens* which was not detected in the brain of premetamorphic tadpoles and is presented relative to prometamorphic levels. Note that the scale of the y-axes varies between genes and is logarithmic in the case of *trbeta* and *dio3*. Data sources: *S. tropicalis* [Duarte-Guterman and Trudeau, 2010], *R. pipiens* [modified from Hogan et al., 2007], and *P. pustulosus* (present study; fig. 1, 2).

[Cai and Brown, 2004; Duarte-Guterman and Trudeau, 2010]. Becker et al. [1997] found that dio2 activity did not change in the brain during development of *R. catesbeiana* tadpoles and *dio2* mRNA did not change at metamorphic climax relative to premetamorphosis in *R. pipiens* [Hogan et al., 2007] (fig. 5e). However, at metamorphic climax, *dio2* decreases in the brain of *X. laevis* [Cai and Brown, 2004] and *S. tropicalis* (~5-fold relative to premetamorphosis; fig. 5e) [Duarte-Guterman and Trudeau, 2010]. On the other hand, the positive regulation of *P. pustulosus dio2* mRNA by T3 is similar to what has been observed in other anurans [Brown, 2005; Hogan et al., 2007; Bonett et al., 2010; Duarte-Guterman and Trudeau, 2010]. Since endogenous THs increase during metamorphosis [Tata, 2006], comparing the developmental profiles and T3 regulation helps us elucidate whether endogenous THs are important regulators of gene expression. In the case of *dio2,* expression increased after T3 treatment but remained constant during metamorphosis. These results suggest that additional factors regulate *dio2* levels in the brain during development. A possible candidate is prolactin, suggested to be an inhibitory factor of metamorphosis by counteracting the effects of THs and by promoting growth [reviewed in Shi, 2000]. In *X. laevis* tadpoles, prolactin regulates the expression of *dio3* in the tail and liver [Shintani et al., 2002] and it blocks the autoinduction of *trbeta* [Baker and Tata, 1992]. Future research should investigate the possibility of prolactin controlling *dio2* expression in the tadpole brain.

In the three anuran species studied, the developmental patterns and T3 regulation were similar for most of the TH-related genes analyzed in the brain. This supports our hypothesis that T3 regulates TH-related gene expression in a similar manner in metamorphosing anurans. Interestingly, the comparative analysis of developmental profiles of TH-related genes in the brain, including data originally reported by Hogan et al. [2007] for *R. pipiens* and by Duarte-Guterman and Trudeau [2010] for *S. tropicalis,* indicates that there are species differences in the magnitude of gene expression change (fig. 5). Developmental expression patterns in *S. tropicalis* and *P. pustulosus* are more similar and sometimes very different from those in *R. pipiens*. For example, in all three species, *trbeta* and *dio3* increase at metamorphic climax, though the fold changes with respect to premetamorphosis vary depending on the species: *trbeta* and *dio3,* respectively, increase ~30-fold and ~35-fold in *S. tropicalis* and *P. pustulosus* or 3.5-fold and ~80-fold in *R. pipiens*. These results do not seem to be in agreement with the phylogenetic relationship between these three species since *P. pustulosus* is more closely related to *R. pipiens* [Ford and Cannatella, 1993] (online suppl. fig.1). Rather, as we hypothesized, these differences may be related to the developmental characteristics of each species (table 1). In spadefoot toads, species with shorter larval periods *(Scaphiopus couchii, Spea multiplicata)* have greater in vitro tissue sensitivity to THs compared to species with slower developmental rates *(Pelobates syriacus)* [Buchholz and Hayes, 2005]. This also appears to be the case here since, compared to *R. pipiens*, both *P. pustulosus* and *S. tropicalis* have shorter larval periods and increased sensitivity to THs as demonstrated by a greater induction in *trbeta* mRNA in the brain at metamorphic climax (relative to premetamorphosis; fig. 5d) and after T3 exposure (table 3). It is thus an interesting hypothesis that interactions between TH and its receptor may affect the evolution of diverse anuran life histories [Buchholz and Hayes, 2005; Buchholz et al., 2006].

Developmental Profiles of Sex Steroid-Related Genes

With the exception of *sdr5alpha2,* all other sex steroid-related transcripts were detected in the brain of *P. pustulosus* tadpoles. In contrast, expression of *sdr5alpha2,* one of the enzymes responsible of 5α-DHT synthesis, has been detected during development in the brain of *S. tropicalis* [Duarte-Guterman and Trudeau, 2010] and *X. laevis* [Urbatzka et al., 2007]. The differences between the species could suggest that *srd5alpha2* expression patterns or biological activities differ between anurans. Previous attempts to clone *srd5alpha2* in *R. pipiens* [Langlois, pers. commun.] and *Rana sylvatica* [Navarro-Martin, pers. commun.] have not succeeded, and to date no other sequences have been published for any other frog species. This could mean that *srd5alpha2* is very lowly expressed in these species and therefore difficult to amplify using degenerate primers. Indeed, in our hands *P. pustulosus srd5alpha2* could only be cloned using gDNA. In contrast, *srd5alpha1* (also involved the synthesis of 5α-DHT) was detected in the tadpole brain during metamorphosis. The regulation of *srd5alpha1* and *srd5alpha2* in the brain also differs between species. In *S. tropicalis,* both *srd5alpha1* and *srd5alpha2* increased with T3, though *srd5alpha2* increased more dramatically [Duarte-Guterman and Trudeau, 2010]. This is different from the case of *P. pustulosus,* where brain *srd5alpha1* was not affected by T3. Future research should attempt to clone the full sequences of the *srd5alpha1* and *srd5alpha2* genes in *P. pustulosus* to better understand species differences in expression and regulation of these enzymes.

In *P. pustulosus* at stage G42, the sexes did not differ in brain transcript levels, similar to previous observations in *S. tropicalis* [Duarte-Guterman and Trudeau, 2010] and *R. pipiens* metamorphs [Duarte-Guterman, unpubl. res.]. However, in adults, brain sex differences in the level of expression of *eralpha, erbeta,* and *ar* have been observed in *P. pustulosus* [Chakraborty and Burmeister, 2010] and *erbeta* and *ar* in *S. tropicalis* [Duarte-Guterman and Trudeau, 2010]. These studies suggest that sexually dimorphic expression of sex steroid receptors only develops after metamorphosis is complete and may contribute to the regulation of sex-specific behaviors [Chakraborty and Burmeister, 2010] or sex differences in steroid feedback to control gonadotropin release.

The developmental profiles and magnitude of changes of *eralpha, erbeta, ar,* and *cyp19* were very similar between *P. pustulosus* and *S. tropicalis* [Duarte-Guterman and Trudeau, 2010] (fig. 5). At metamorphic climax relative to premetamorphosis, *cyp19* increased the least, with only 1.7-fold in *P. pustulosus* and 2-fold in *S. tropicalis*. The most dramatic increase in expression among the sex steroid-related genes was observed with *erbeta*, with 35- and 150-fold at metamorphic climax relative to premetamorphosis in *P. pustulosus* and *S. tropicalis*, respectively. In both species, there are marked differences in the profiles of *eralpha* and *erbeta* suggesting that each gene is differentially regulated during development. Indeed, *eralpha* and *erbeta* respond differently to a range of estrogens in different amphibian species, indicating species-specific differences in ligand sensitivities and the autoregulation of these receptors [Katsu et al., 2010].

In the case of *eralpha*, for which we also have data in *R. pipiens,* the general trend is similar in the three species but, interestingly, the magnitude of change at G42 differs between the species (fig. 5b). At metamorphic climax, *eralpha* increased 5-fold in *P. pustulosus* and 6-fold in *S. tropicalis,* while in *R. pipiens eralpha* increased ~10-fold, relative to premetamorphic levels. Unlike differences in TH-related gene profiles, the physiological causes and consequences of potentially different brain estrogen sensitivities (due to differences in *eralpha* levels) in the brain are unknown. Currently, we do not have a clear understanding of the roles of sex steroids in the larval amphibian brain. Future studies need to establish developmental profiles of the other estrogen receptor, *erbeta,* and androgen-related genes in *R. pipiens* to help elucidate the differences in developmental expression and whether these can be related to the reproductive characteristics of each anuran species.

T3 Regulation of Sex Steroid-Related Genes

The developmental profiles of sex steroid-related genes in the brain were generally similar between anuran species. However, the effects of exogenous T3 on the expression of sex steroid-related genes in the brain differed not only in the magnitude but also in the direction of change. Using the same exposure protocol, we observed that T3 increases brain *eralpha* and *erbeta* in *S. tropicalis* and brain *eralpha* in *R. pipiens* (table 3). Since *eralpha* also increased during metamorphosis, along with endogenous THs, we previously suggested that T3 contributes to this increase in *eralpha* mRNA during development in *S. tropicalis* and *R. pipiens* [Hogan et al., 2007; Duarte-Guterman and Trudeau, 2010]. In *P. pustulosus,* T3 increases *erbeta* but not *eralpha* expression, which suggests that T3 does not have the same regulatory action in this species. These results indicate that although expression patterns of *eralpha* during development are conserved across these anuran species the regulatory controls may differ.

Treatment with T3 decreased *cyp19* mRNA in the brain of *P. pustulosus* with a magnitude similar to that observed in *R. pipiens* (table 3). This negative regulation has been observed in mammals and fish, where T3 suppresses Cyp19 mRNA and activity in gonad (testes and ovaries) in vivo and in vitro cultures: goldfish [Nelson et al., 2010], mice [Cecconi et al., 1999; Catalano et al., 2003], pigs [Gregoraszczuk et al., 1998], and rats [Ulisse et al., 1994; Ando et al., 2001; Hatsuta et al., 2004]. In contrast, in *S. tropicalis,* T3 did not affect brain *cyp19* but instead increased *srd5alpha1* and *srd5alpha2* mRNA (table 3). These results lead us to reject our hypothesis that crosstalk mechanisms are similar among species with similar reproductive and developmental characteristics. Instead, it appears that closely related species, *P. pustulosus* and *R. pipiens*, have a more similar T3 regulation of sex steroid-related genes. We propose that in these three species, T3 exposure results in an increase in the brain androgen-to-estrogen ratio by regulating the expression of androgen or estrogen synthesis enzymes. In *S. tropicalis,* T3 increases the expression of androgen synthesis enzymes *(srd5alpha1* and *srd5alpha2)*, whereas in *R. pipiens* and *P. pustulosus,* T3 reduces the expression of brain *cyp19.* In *P. pustulosus,* the idea of induction of the androgen system is supported by the increase in brain *ar* mRNA after T3 exposure (fig. 4d). It is possible that reduction of *cyp19* results in the accumulation of testosterone, which is known to autoregulate the expression of *ar* in lizards and hamsters [Cardone et al., 1998; Esposito et al., 2002]. Indeed, we have also shown that pharmacological inhibi-

tion of cyp19 results in an increase in whole body *ar* mRNA in *S. tropicalis* larvae [Langlois et al., 2010]. Estrogens and androgens are important regulators of the expression of sex steroid-related genes in anurans and other vertebrates [Balthazart and Ball, 1998; Torres and Ortega, 2003; Diotel et al., 2010; Bagamasbad and Denver, 2011; Hogan et al., unpubl. res.]. However, according to our results, rising levels of T3 may synergize or counteract the effects of sex steroids during brain development in anurans. In future studies, measurements of the protein and/or enzyme activity of sex steroid-related endpoints will be required to fully understand the physiological effects of T3 in the tadpole brain.

In conclusion, the developmental expression and regulation of TH-related genes in *P. pustulosus* are similar to those of other species from divergent anuran families. This gives direct support to the hypothesis that THs similarly control metamorphosis in all frog species. Developmental profiles of sex steroid-related genes in the brain were generally comparable among anurans. Importantly, however, the regulation of sex steroid-related genes by T3 varied considerably between the three species for which there is comparable data. We rejected the hypothesis that the effects of T3 on sex steroid-related transcripts depend on the species' developmental and reproductive characteristics. Future research is required to elucidate the underlying causes of species differences and the physiological consequences of crosstalk between TH and sex steroid systems in the developing anuran brain. This research also represents an important contribution to the establishment of *P. pustulosus,* a phylogenetically divergent neotropical species, as a new laboratory model for studies on metamorphosis, sexual development, and endocrine disruption.

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