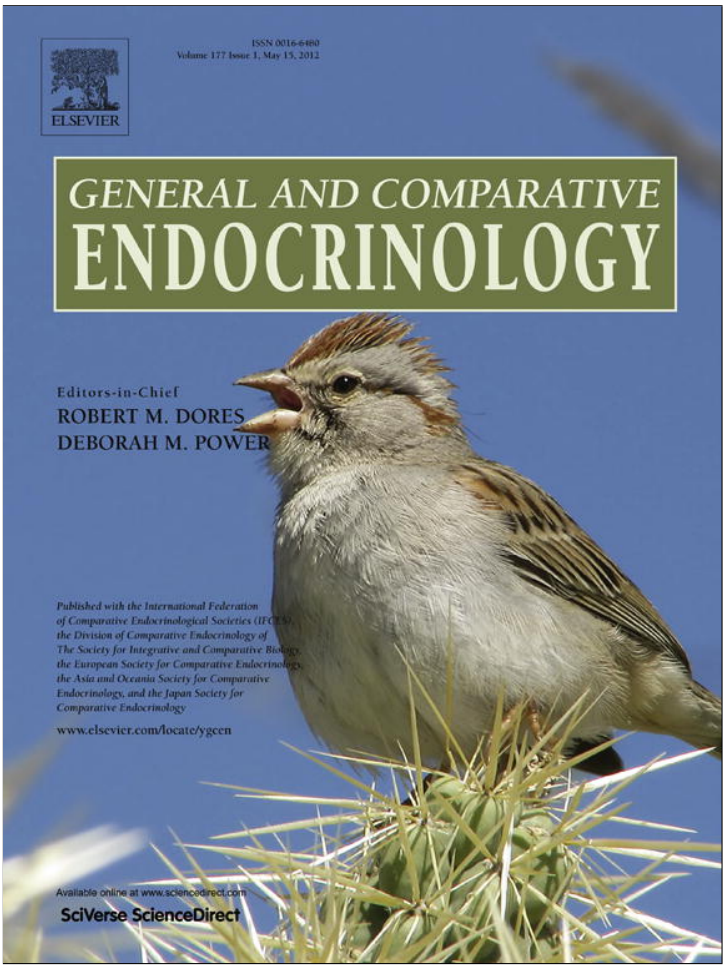

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Developmental expression of sex steroid- and thyroid hormone-related genes and their regulation by triiodothyronine in the gonad-mesonephros of a Neotropical frog, *Physalaemus pustulosus*

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ABSTRACT

Gonadal differentiation in frogs is affected by sex steroids and thyroid hormones (THs); however, the genes controlling differentiation and the molecular effects of THs in the gonad are not clear and have only been investigated in a few anuran species. In this study, we established developmental profiles and TH regulation of sex steroid- and TH-related genes in the gonad-mesonephros complex (GMC) of the túngara frog (*Physalaemus pustulosus*), and compared the results to our previous research in another tropical frog, *Silurana tropicalis*. The developmental profiles allowed us to identify three genes as markers of ovarian development. During metamorphosis, aromatase (*cyp19*), estrogen receptor α, and steroid 5α-reductase 1 (*srd5alpha1*) were higher in the GMC of putative and morphological females. Acute exposure to triiodothyronine (T3) decreased GMC expression of *srd5alpha1* and *cyp19*, while increasing TH-related genes in premetamorphic tadpoles. The regulation of sex steroid-related genes differed significantly from our previous study in *S. tropicalis*. *P. pustulosus* and *S. tropicalis* share ecological, developmental, and reproductive characteristics; however, they are not closely related. These results along with our previous research in the tadpole brain support the hypothesis that evolutionary convergence is not important in understanding differences in the effects of TH on sex steroid-related genes in frogs. Finally, we propose that T3 induces male gonadal development but this can be achieved through different mechanisms depending on the species.

1. Introduction

In amphibians, gonadal (sex) differentiation is influenced by endogenous sex steroids (estrogens and androgens). Both exposure to sex steroids [5,29] and inhibition of sex steroids synthesis [10,43] affects gonadal development and sex ratios in frogs. Despite these observations, the molecular mechanisms that control differentiation of the gonad are not completely understood. The enzyme aromatase (cyp19), responsible for the conversion of testosterone and androstenedione into estradiol and estrone respectively, seems to be a key regulator of ovarian development in some frogs (e.g., *Silurana tropicalis*: [10]; *Rana rugosa*: [37]; *Xenopus laevis*: [50]), whereas there is no consensus about the genes regulating testicular development. We previously used *S. tropicalis* to investigate the role of *cyp19* in gonad development because it has many advantages as a laboratory organism; however, one important limitation of that study was our inability to determine the sex of tadpoles by visual inspection because the gonads are small and translucent, even at metamorphic climax [10,14] (but see [44] for a recent method of genotyping *S. tropicalis*). This limitation reduces the extent of analysis and conclusions about the mechanisms of gonadal differentiation. In contrast, in the Neotropical frog, *Physalaemus* (=*Engystomops*) *pustulosus* (we follow the taxonomic recommendation of [21] and retain use of *Physalaemus*), the morphology of the gonad is visible at metamorphic climax (Gosner stage 42) [12]; therefore, *P. pustulosus* may be an amenable anuran species to examine gene expression in the developing gonad.

In addition to sex steroids, thyroid hormones (THs) are thought to be involved in testicular development and function in some

Abbreviations: 5α-DHT, 5α-dihydrotestosterone; ar, androgen receptor; *cyp19*, Aromatase; dio2, dio3, deiodinases type 2 and 3; dmrt-1, doublesex and mab-3 related transcription factor 1; eralpha, erbeta, estrogen receptor α and β; GMC, gonad-mesonephros complex; G, Gosner stage; rpl8, ribosomal protein L8; THs, thyroid hormones; tralpha, trbeta, thyroid hormone receptor α and β; T3, triiodothyronine; srd5alpha1 and srd5alpha2, steroid 5α-reductase type 1 and 2.

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frogs, fishes, and mammals [2,22,27,39,52]; nevertheless, the action of TH in the gonad have only been investigated in mammals (reviewed in [8,52]). To our knowledge, the only study of TH effects on the developing frog gonad is from our laboratory. In our previous study, we found that TH-related genes (TH-receptors and deiodinases) are expressed in the gonad-mesonephros complex (GMC) during metamorphosis in *S. tropicalis* [14]. Exposure of premetamorphic tadpoles to triiodothyronine (T3; the bioactive form of THs) increased mRNA levels of androgen- related genes, steroid 5α-reductases (*srd5alpha1* and *srd5alpha2*), that code for the enzymes involved in the synthesis of 5α-dihydrotestosterone (5α-DHT), while decreasing expression of one of the estrogen receptors (*erbeta*). These results are in agreement with the masculinising effect of THs in frogs [22,27]. *P. pustulosus* belongs to the Leiuperidae family [24] and is not closely related to *S. tropicalis* (Pipidae family) [20]; *Silurana* is more basal than *Physalaemus* and the two have not shared a common ancestor for approximately 200 million years [47]. Since both species have similar developmental and reproductive characteristics, however, we previously hypothesised that they might exhibit similar T3 regulation of sex steroid-related genes in the brain compared to the North American frog, *Rana pipiens* (Ranidae family), to which *Physalaemus* is substantially more closely related to than it is to *Silurana* [12]. The regulation of genes by T3 in the tadpole brain was more similar between the more closely related species (*P. pustulosus* and *R. pipiens*) than between the ecologically more similar species (*P. pustulosus* and *S. tropicalis*) [12]. Thus common ancestry rather than evolutionary convergence appears to be more important in understanding variation in T3 regulation of sex steroid-related genes among these three taxa. Based on our previous work in the brain, in this study we hypothesised that the effects of T3 on sex steroid-related genes in the GMC differs between *P. pustulosus* and *S. tropicalis*.

In order to identify genes associated with gonadal development in *P. pustulosus*, we first established profiles of genes related to THs (receptors and deiodinases), sex steroids (receptors and synthesis enzymes), and sex differentiation in the GMC during tadpole development. In the second part, we assessed the regulation of these genes by T3 in *P. pustulosus* to study the effects of THs on gonad differentiation and directly compared the results to our previous research in *S. tropicalis*.

2. Materials and methods

2.1. Animals and tissue collection for developmental profiles

Fertilised eggs of *P. pustulosus* were obtained from three pairs of frogs from a colony at the University of Texas at Austin [48] and reared as described in [12]. Staging followed the Gosner (G) developmental table [23]. Gonad-mesonephros complex with surrounding muscle tissue (GMC) were dissected from tadpoles at stages G30 (premetamorphosis; foot paddle stages), G36 (prometamorphosis; hind limb development), and G42 (metamorphic climax; forelimb emergence). GMC samples were preserved in RNAlater as described by the manufacturer (Ambion, Austin, TX, USA) and analysed individually for all stages of development ($n = 6–10$). Animal care and treatment protocols were in accordance with the guidelines of the Institutional Animal Care and Use Committee, University of Texas at Austin (IACUC protocol No. 08101701).

2.2. Cloning dmrt-1

A species-specific cDNA sequence for doublesex and mab-3 related transcription factor 1 (*dmrt-1*) was cloned from *P. pustulosus* GMC cDNA using nested PCR. Mammalian and non-mammalian nucleic acid sequences for *dmrt-1* were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and aligned using ClustalW (EMBL-EBI; http://www.ebi.ac.uk/clustalw/). Nested primers were designed against conserved regions of the *dmrt-1* sequence using Primer 3 (http://frodo.wi.mit.edu/primer3/). Primer sequences were for the first set: forward: 5′-ttctgtatgtggmgggaht-3′ and reverse 5′- artgcatyctgtactgkgagc-3′ (amplicon size: 595 bp) and for the second set: forward 5′- gcarcargcycaagaaga-3′ and reverse: 5′- tttcatctgccaytgrbttc-3′ (amplicon size: 451 bp). PCR amplification was performed using the Mastercycler® gradient Thermal Cycler (Eppendorf, Westbury, NY, 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, Burlington, ON, Canada), and 1 μL of template (cDNA for first round of PCR or PCR product for second round of 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 at 54 °C 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. Amplification products were ligated directly into the 2.1 TOPO® vector (TOPO TA cloning kit, Invitrogen), transformed into *Escherichia coli* (One Shot TOP 10 chemically competent cells, Invitrogen), and sequenced as described in [12]. The partial sequence obtained was deposited on GenBank (accession No. HQ846631) and had 70% nucleotide identity to *S. tropicalis dmrt-1* (accession No. CX931409) and 84% to *Bufo marinus dmrt-1* (accession No. FJ697175).

2.3. Triiodothyronine exposure

Premetamorphic tadpoles (G 32–34) were exposed to three nominal concentrations of T3 (0.5, 5, 50 nM; Sigma, Oakville, ON, Canada) or a dimethyl sulfoxide (DMSO; 0.005% final concentration in the tanks; Sigma) solvent control for 48 h, as previously described in [12]. Chemical additions were not renewed during the exposure period. The density in all the tanks was 1 tadpole/L. Treatment with T3 had no effect on mortality and 100% survivorship was observed in all treatment groups. At the end of the exposure, tadpoles were anesthetised by immersion in clove oil and euthanised by decapitation. GMC were dissected, preserved in RNAlater (Ambion) and pooled (two GMC per pool; $n = 8$ pools) before RNA isolation.

2.4. RNA isolation and cDNA synthesis

Samples were homogenised with an MM301 Mixer Mill (Retsch, Newton, PA, USA) set to 20 Hz for 4 min. Total RNA for the developmental profile and T3 exposure samples was obtained from brain using the RNeasy Micro Kit (including the DNase treatment set) as described by the manufacturer (Qiagen, Mississauga, ON, Canada). Concentrations of isolated RNA (in RNase free water) were determined using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.) and stored at −80 °C. 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 the samples, we modified the reverse transcriptase reaction to be carried out at 42 °C for 90 min (compared to the manufacturer's standard protocol of 50 min) in order to increase the cDNA yield. The cDNA products were diluted 40 or 80-fold prior to PCR amplification.

2.5. Real-time RT-PCR

Simplex real-time RT-PCR assays described in detail in [12] were used to measure mRNA levels of estrogen receptors α and β

(*eralpha*, *erbeta*), *cyp19*, androgen receptor (*ar*), TH-receptors α and β (*tralpha*, *trbeta*), deiodinases type 2 and 3 (*dio2*, *dio3*), *srd5alpha1*, *srd5alpha2*, and the reference gene ribosomal protein L8 (*rpl8*) in the GMC during development and after T3 exposure. In addition, primers for *dmrt-1* (forward: 5′ gctgctctcaggctacaagtg-3′; reverse: 5′-ccacgacaaagtcagaagtgc-3′; amplicon size: 132 bp) were designed using Primer 3 and optimised following the same protocol as in [12]. For *dmrt-1*, 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), 260 nM of each primer set (Invitrogen), 0.25× SYBR Green I Dye (Molecular Probes, Eugene, OR, USA), and 5 μL of diluted cDNA template. The thermocycle program for *dmrt-1* included an enzyme activation step at 95 °C (15 min) and 45 cycles of 95 °C (15 s), 58 °C (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.

For all of the assays, samples were run in duplicate using the Mx3005P real-time PCR System (Stratagene, La Jolla, CA, USA) along with negative template controls where RNase-free water was added to the reaction instead of the template and negative reverse transcriptase controls where RNase-free water was added to the cDNA synthesis reaction instead of the enzyme. Relative mRNA levels of target genes within each sample were obtained using the relative standard curve method. The standard curves were generated using equal parts of cDNA of G42 male and female samples (for the developmental profiles), and using equal parts of cDNA from each treatment including control (for the T3 exposure). Reaction efficiencies were 90–110% with an $R^2 \geqslant 0.990$. Expression of the reference gene *rpl8* decreased significantly during development (∼1.7-fold) and after T3 exposure (∼1.5-fold; data not shown). In our previous studies in *S. tropicalis*, we were not able to find suitable reference genes to normalise data from developmental profiles or T3 treatments (e.g., [11,13]). In those cases, we normalised the gene expression data to RNA content [30], which consists of dividing the gene expression data obtained by real-time RT-PCR by the amount of RNA used in the cDNA synthesis reaction (i.e., 1 μg). For the developmental profiles, data are presented as fold change relative to stage G30 and for the T3 exposure data are presented relative to the control group.

2.6. Statistical analyses

All statistical analyses were carried out using S-Plus (Insightful Corporation, Seattle, WA, USA) with significance set at $p < 0.05$. For all the analyses, data were first tested for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene's test). When these assumptions were not met, data were transformed as required (i.e., Log_{10}, square root). At stages G30 and 36, the gonads are very small; therefore, a morphological sex could not be unequivocally assigned by visual inspection at these early stages. In order to examine differences between sexes early in development, we predicted the sex of the tadpoles using a statistical method. A cluster analysis (using k-means) of *cyp19* was used to divide the data into two groups and assign one of the two sexes (putative male or female) to each tadpole at each stage of development. We chose *cyp19* mRNA because in birds, newts, and several species of fish, levels are sexually dimorphic in the developing gonad and it has been proposed as a molecular marker of ovarian development [3,19,25,32,35]. Using these putative (G30 and 36) and morphological (G42) sexes, differences in the expression of all the genes in the GMC with regards to sex and stage of development were examined using two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc multiple comparisons test (when warranted). Using the putative and morphological sexes, a discriminant analysis was performed to investigate which transcripts or combination of transcripts in the GMC could predict sexes in *P. pustulosus* tadpoles, as has been reported for European sea bass [4].

For the T3 exposure, statistical differences between treatment and control groups were examined using one-way ANOVA followed by Bonferroni's pair-wise comparison between treatment and control. If the ANOVA assumptions (i.e., normality and homogeneity of variance) could not be achieved even after data transformation, data were tested non-parametrically using Kruskal–Wallis' test.

3. Results

3.1. Developmental profiles and sex differences in the GMC

Developmental profiles of sex steroid- and TH-related genes were established in the GMC during tadpole development by sampling at three different stages: G30 (premetamorphosis; foot paddle stage), G36 (prometamorphosis; hind limb development), and G42 (metamorphic climax; forelimb emergence) (Figs. 1–3). The main morphological characteristics and changes over development are shown in Fig. 1.

We assigned sex to stage G42 tadpoles visually prior to dissection of the GMC for gene expression. In the GMC, *cyp19* mRNA levels were clearly sexually dimorphic (250-fold greater in G42 females compared to males; $p < 0.001$) and the cluster analysis confirmed that the morphological sexes belonged to two clearly differentiated groups (Fig. 1A). Therefore, at G42 sexes could be easily determined by *cyp19* mRNA levels. We then decided to assign putative sexes to the tadpoles at G30 and 36 using the results of the cluster analysis of *cyp19* mRNA. Assigned and morphological sexes were used to test for sex differences in all of the target genes. In the case of *eralpha*, sex differences were detected at all three stages of development (Fig. 1B), with higher mRNA levels in females compared to males (G30: 1.7-fold; G36: 2.8-fold; G42: 1.8-fold; $p < 0.05$; Fig. 1B). For *srd5alpha1*, significant differences were detected between the sexes at stage G36 and 42, with higher levels in females relative to males (1.4-fold for both stages; ANOVA; $p < 0.05$; Fig. 2A). Gene expression in the GMC for the other targets was the same in females and males, both putative and morphological (Figs. 1–3).

With the exception of *srd5alpha2*, all genes were detected in the GMC at each of the three stages of development. Levels of *srd5alpha2* were very low (real-time RT-PCR threshold cycle, $C_t > 37$) and could not be reliably measured. For *cyp19*, each sex showed different trajectories: female *cyp19* mRNA increased 14-fold during the developmental period examined, while in males, it decreased by 4-fold (Fig. 1A). In contrast, *eralpha* (1.7- and 6.0-fold; Fig. 1B), *dmrt-1* (3.5- and 7.0-fold; Fig. 2C), and *trbeta* (2.0- and 30-fold; Fig. 3B) mRNA levels increased with each developmental stage. In the case of *erbeta* (Fig. 1C), *ar* (Fig. 2B) and *dio3* (Fig. 3D), mRNA levels remained relatively constant between stages G30 and 36 and only increased significantly at G42. Levels of *erbeta* increased 270-fold, *ar* 4.0-fold, and *dio3* 50-fold at stage G42 relative to G30. The profile of *dio2* was unique among these genes, as mRNA levels decreased from stage G30 to 36 (2.0-fold) and then increased from G36 to 42 (7.5-fold; Fig. 3C). Only *srd5alpha1* (Fig. 2A), and *tralpha* (Fig. 3A) showed no appreciable change during development at the stages analysed. From these results, the genes that showed the maximum expression changes during development were *erbeta* followed by *dio3* and *trbeta*.

Using the putative and morphological sexes, we used a discriminant analysis to determine which genes, or which combination of genes, could be used as sex markers in *P. pustulosus* tadpoles

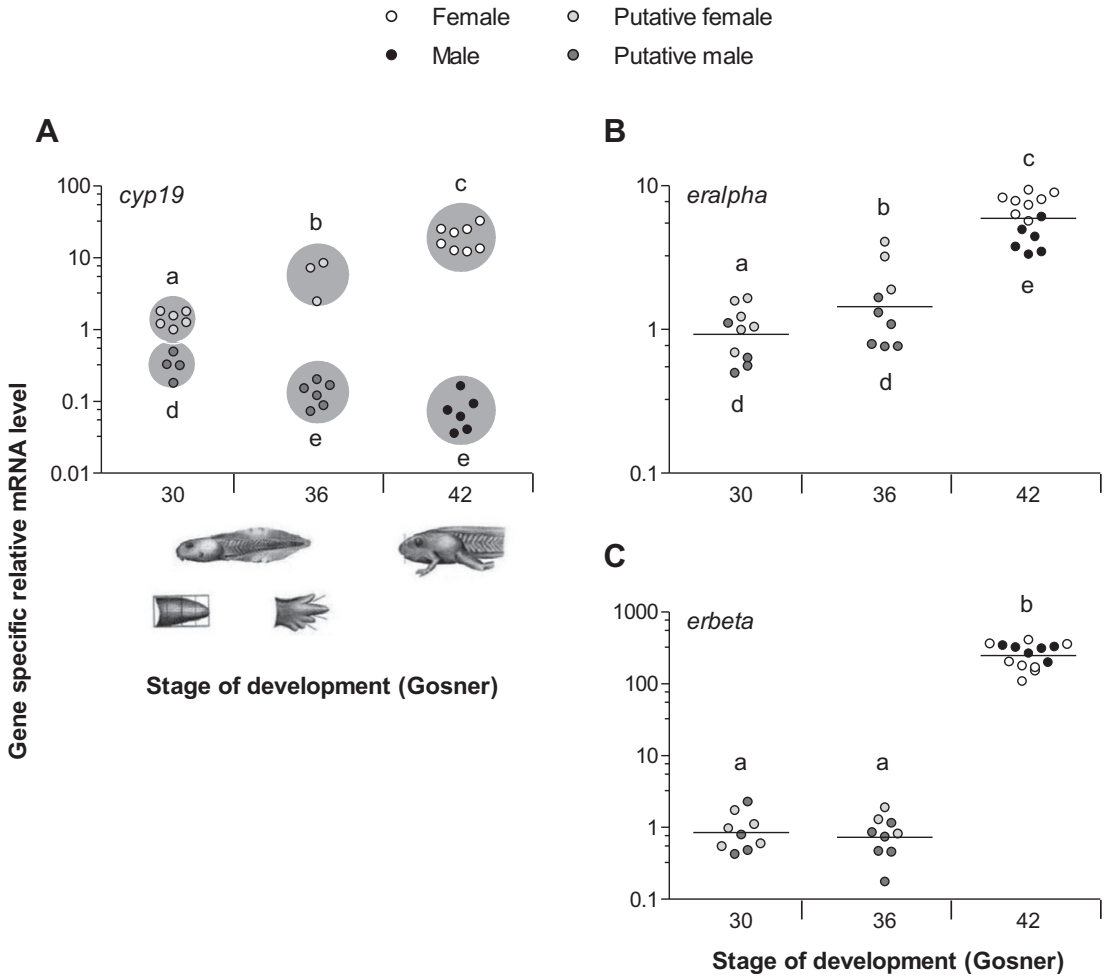

Fig. 1. Developmental profiles of estrogen-related genes in the gonad-mesonephros complex (GMC) during *Physalaemus pustulosus* metamorphosis. Transcript levels of *cyp19* (A), *eralpha* (B), and *erbeta* (C) were measured from stage G30 until 42. Levels of mRNA are expressed relative to stage G30 and are normalised to RNA content. Individual GMC samples are presented along with the grand mean (B,C). Grey circles (A) denote the two groups found with the cluster analysis of *cyp19* (each circle is centered on the mean of each group). Different letters indicate statistically significant differences between developmental stages and between sexes (two-way ANOVA followed by Bonferroni's post hoc multiple comparisons test; $n = 6–10$; $p < 0.05$). In the case of *erbeta*, no differences were detected between sexes at any stage of development, therefore letters indicate differences between stages alone. Main morphological characteristics are shown for each stage of development [23]. Note that the logarithmic scales of the y-axis vary between genes.

(Table 1). Of the nine transcripts (not including *cyp19*), only *eralpha* and *srd5alpha1*, significantly predicted sex, each one alone and in combination with each other (Table 1). None of the TH-related genes predicted sex better than expected by chance (i.e., 50% sexing accuracy; $p > 0.4$; data not shown). Expression of *eralpha* correctly predicted sexes in 60% of cases, while *srd5alpha1* predicted 75% of cases for both sexes. In no case was a combination of genes more effective than the individual genes alone at predicting sexes. When taking stage of development into account, sexing accuracy using *eralpha* improved from 60% to 87% for both sexes, while accuracy using *srd5alpha1* remained relatively similar (81% compared to 75% for both sexes).

3.2. Effects of T3 exposure in the GMC

In order to study TH- regulation of transcripts in the GMC, we exposed premetamorphic tadpoles (G 32–34) to T3 for 48 h. The exposure affected the expression of two sex steroid-related transcripts (Fig. 4), *erbeta* and *srd5alpha1* (*erbeta*: $F_{3,28} = 18.59$; $p < 0.001$; *srd5alpha1*: $F_{3,28} = 4.12$; $p = 0.015$) and the four TH-related genes measured (Fig. 5; *tralpha*: $F_{3,28} = 20.31$; $p < 0.001$; *trbeta*: $F_{3,28} = 294.96$; $p < 0.001$; *dio2*: $F_{3,28} = 40.07$; $p < 0.001$; and *dio3*: Kruskal–Wallis $\chi^2 = 28.10$; $p < 0.001$). Levels of *erbeta* significantly increased at 5 and 50 nM T3 relative to control (2.8- and 6.5-fold respectively; post hoc test $p < 0.01$; Fig. 4C), while *srd5alpha1* mRNA significantly decreased at 50 nM T3 (1.3-fold; post hoc test $p < 0.05$; Fig. 4D). Exposure to 5 and 50 nM T3 resulted in significant increases in *tralpha* (1.7- and 1.9-fold; post hoc test $p < 0.001$; Fig. 5A) and *dio2* (2.3- and 4.0-fold; post hoc test $p < 0.001$; Fig. 5C). T3 exposure resulted in concentration-dependent increases in *trbeta* (post hoc test $p < 0.001$; Fig. 5B) and *dio3* (post hoc test $p < 0.01$; Fig. 5D). Levels of *cyp19* mRNA were highly variable within treatments but were not statistically affected by exposure to T3 ($F_{3,28} = 1.93$; $p = 0.15$; Fig. 4A). No changes were detected in *eralpha* ($F_{3,28} = 1.75$; $p = 0.19$; Fig. 4B), *ar* ($F_{3,28} = 0.78$; $p = 0.51$; Fig 4E), and *dmrt-1* ($F_{3,28} = 0.12$; $p = 0.94$; Fig. 4F) mRNA levels after exposure to T3.

4. Discussion

4.1. Developmental profiles and identification of sex markers in the GMC

Sex steroids have important effects on gonadal development in anurans; however, the sex differentiation genes and the molecular mechanisms involved are not completely understood and have

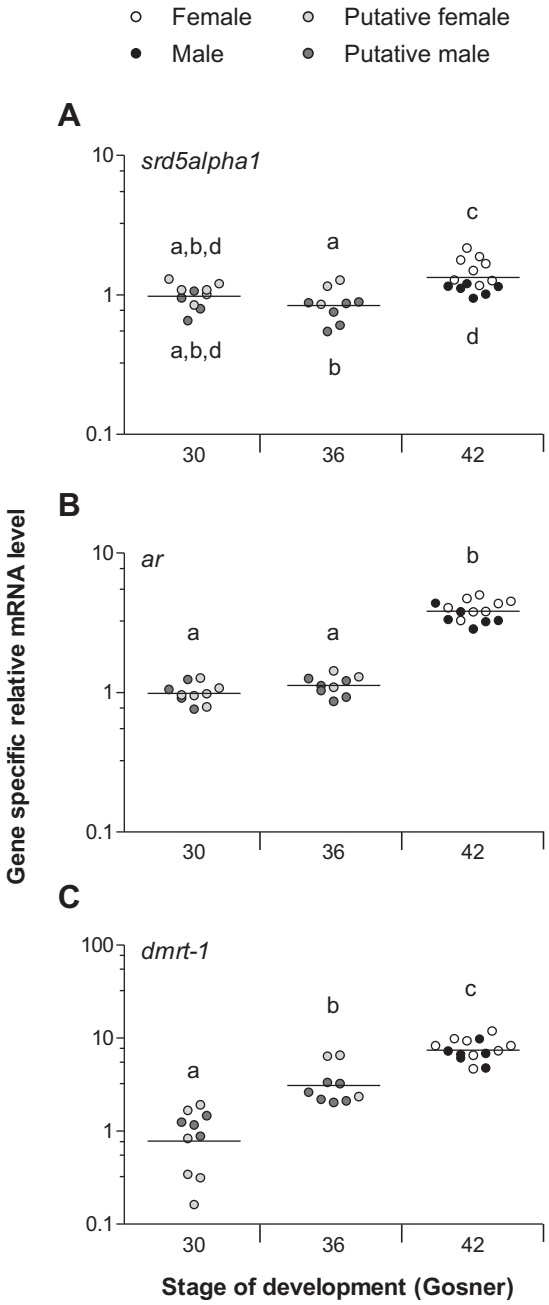

Fig. 2. Developmental profiles of sex differentiation- and androgen-related genes in the gonad-mesonephros complex (GMC) during *Physalaemus pustulosus* metamorphosis. Transcript levels of *srd5alpha1* (A), *ar* (B), and *dmrt-1* (C) were measured from stage G30 until 42. Levels of mRNA are expressed relative to stage G30 and are normalised to RNA content. Individual GMC sample are presented along with the grand mean. Different letters indicate statistically significant differences between developmental stages and between sexes (two-way ANOVA followed by Bonferroni's post hoc multiple comparisons test; $n = 6–10$; $p < 0.05$). In the case of *ar* and *dmrt-1*, no differences were detected between sexes at any stage of development, therefore letters indicate differences between stages alone. Note that the logarithmic scales of the y-axis vary between genes.

only been studied in a few frog species. In this study, genes related to sex steroids and sex differentiation were measured in the GMC during tadpole development in *P. pustulosus*. At G42, the gonads were clearly visually differentiated into either male or female; therefore, gonadal differentiation in *P. pustulosus* takes place during tadpole development. This is also the case of some frog species from the Pipidae and Ranidae families, for which histological analyses demonstrate that gonadal differentiation is complete at metamorphic climax, and in many cases differentiation takes place during premetamorphosis between stage G26 and 36 (*S. tropicalis*: [16]; *X. laevis*: [33]; *Rana temporaria*: [41]; *Rana pipiens*: [46]). In contrast, in other species such as *Bufo boreas* (Bufonidae family) and *Pyxicephalus adpsersus* (Ranidae family), the gonads are not completely differentiated until after metamorphosis (reviewed in [28]).

Three transcripts (i.e., *cyp19*, *eralpha*, and *srd5alpha1*) in the GMC were identified as potential molecular markers of ovarian development and of the sex of tadpoles. At G42, *cyp19* mRNA was significantly higher in females than in males (~250-fold difference). The cluster analysis demonstrated that *cyp19* levels in the GMC were also sexually dimorphic as early as G30, a time when the gonads are not yet visible. In *S. tropicalis*, *cyp19* levels in the GMC showed significant variation between individuals [14]; however, morphological confirmation of the sexes was not available for comparison. This significant sexual dimorphic expression pattern has been observed in other frogs; in *Rana rugosa* [37], *Rana sylvatica* (Navarro-Martin et al., unpublished results), and *X. laevis* [50], *cyp19* mRNA levels are significantly higher in females compared to males during tadpole development. In addition, in *R. rugosa*, cyp19 activity has been confirmed to be higher in undifferentiated gonads of females compared to males [31]. An important difference between the frogs studied to date is the magnitude of the difference in *cyp19* levels between the sexes. At metamorphic climax, sex differences in *cyp19* are in the order of 10-fold in *X. laevis* [50], 100-fold in *R. sylvatica* (Navarro-Martin et al., unpublished results), and 250-fold in *P. pustulosus* (present study). In sexually mature *S. tropicalis* adults, sex differences in *cyp19* are in the 50-fold range [14]. The physiological significance of these differences remains to be fully elucidated. Nonetheless, these studies strongly suggest that levels of *cyp19* mRNA can be used as reliable indicator of the sex of a tadpole in different frog species from different families. This is not surprising since high levels of gonadal *cyp19* seem to be required for ovarian differentiation across vertebrates, including in fish [3,25], newts [35], turtles [45], and birds [32].

In vertebrates (e.g., fish, birds and mammals) and invertebrates (e.g., flies and nematodes), homologous genes to the anuran *dmrt-1* are involved in testicular differentiation [34]. Interestingly, in the GMC, mRNA levels of *dmrt-1* were similar in males and females in *P. pustulosus*. In addition, during anuran gonadal development, *dmrt-1* mRNA is not sexually dimorphic in species from other families, *R. rugosa* [38], *Bufo marinus* [1], and *S. tropicalis* [14], suggesting that *dmrt-1* might not be involved in testicular differentiation in anurans. In *S. tropicalis* adults, however, *dmrt-1* mRNA is higher in testes than in ovaries, suggesting that dmrt-1 may still play a role in testicular function [14].

Levels of *eralpha* were between 1.7- and 2.8-fold higher in females than in males (putative and morphological). Higher levels of *cyp19* in females could result in higher estrogen levels which in turn could increase *eralpha* transcription and estrogenic effects relative to males. Indeed, in other frog species, *eralpha* is autoinduced by estrogens (*X. laevis*: [5,49]; *R. pipiens*: [15], and this regulation is mediated by the presence of an estrogen responsive element (ERE) in the promoter region of the *eralpha* gene [36]. Therefore, we suggest that in the GMC *eralpha* mRNA may be under the influence of estrogen levels and thus related to *cyp19* levels.

In anurans, 5α-DHT induces male gonadal differentiation (e.g., *X. laevis*: [5]; *Rana clamitans*: [7]), but surprisingly, mRNA levels of *srd5alpha1*, one of the enzymes responsible of 5α-DHT synthesis, were significantly higher in females than in males at stages G36 and 42. Sex differences in *srd5alpha1* mRNA were also observed in adult gonads of *S. tropicalis*, expression being 5-fold higher in ovaries than in testes [14]. There were, however, no significant sex differences in *srd5alpha1* during development in *R. rugosa* and *X. laevis* [37,50]. In premetamorphic *R. rugosa* tadpoles, *srd5al-*

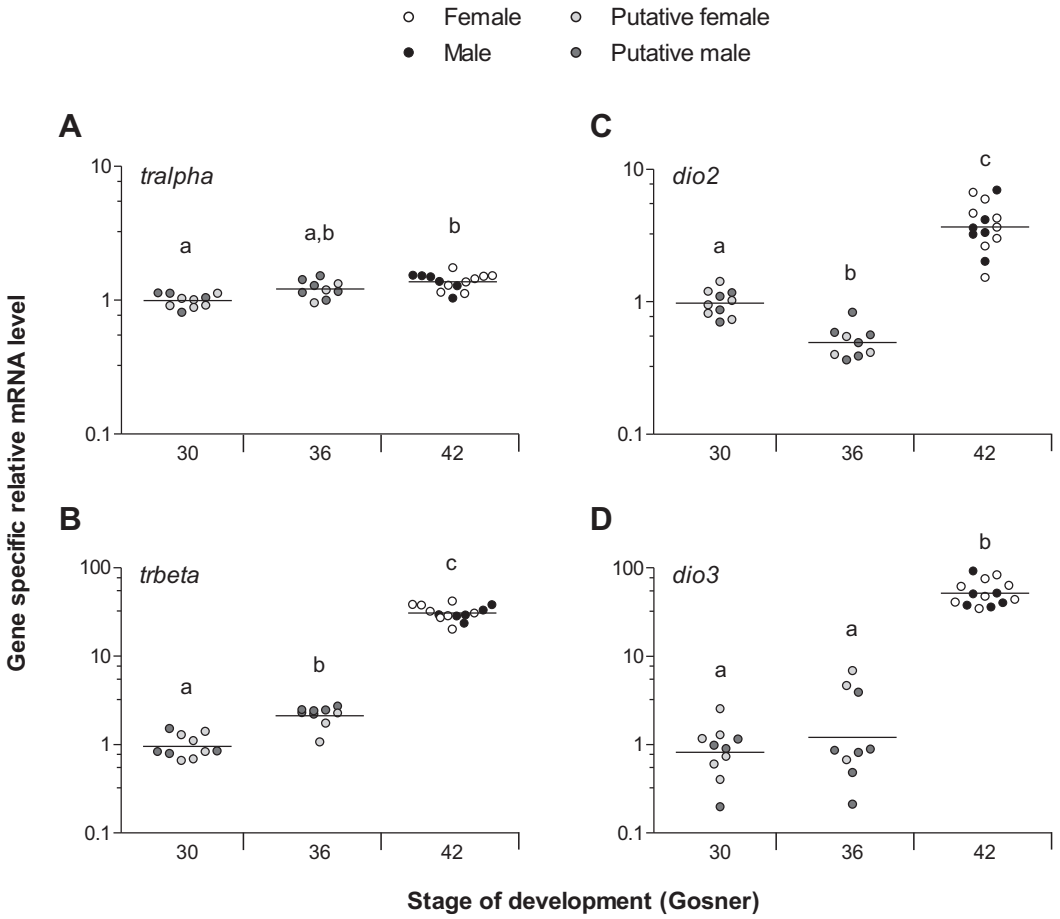

Fig. 3. Developmental profiles of thyroid hormone-related genes in the gonad-mesonephros complex (GMC) during *Physalaemus pustulosus* metamorphosis. Transcript levels of *tralpha* (A), *trbeta* (B), *dio2* (C), and *dio3* (D) were measured from stage G30 until 42. Levels of mRNA are expressed relative to stage G30 and are normalised to RNA content. Individual GMC samples are presented along with the grand mean. Different letters indicate statistically significant differences between developmental stages. In the case of TH-related genes, no differences were detected between sexes at any stage of development (two-way ANOVA followed by Bonferroni's post hoc multiple comparisons test; $n = 6–10$; $p < 0.05$). Note that the logarithmic scales of the y-axis vary between genes.

Table 1
Results of the discriminant analysis to determine sexing accuracy using stage of development, individual and/or combined gonad-mesonephros transcript levels in *Physalaemus pustulosus*. Sexes were assigned using a cluster analysis of *cyp19* levels and by visual inspection at G42.

Variable(s) in the model	Wilks' λ	*F* statistic	*p*-value	Sexing Accuracy	
				Females	Males
Stage	0.94979	0.793	0.462	82% (14/17)	37.5% (6/16)
eralpha	0.8424	5.799	0.022	58.8% (10/17)	62.5% (10/16)
erbeta	0.97757	0.688	0.413	50% (8/16)	62.5% (10/16)
srd5alpha1	0.63933	17.488	<0.001	82% (14/17)	68.8% (11/16)
dmrt-1	0.9884	0.364	0.551	58.8% (10/17)	50% (8/16)
ar	0.96988	0.963	0.334	47% (8/17)	62.5% (10/16)
eralpha + stage	0.3859	15.384	<0.001	94% (16/17)	81.2% (13/16)
srd5alpha1 + stage	0.51481	9.1104	<0.001	76.5% (13/17)	87.5% (14/16)
eralpha + *srd5alpha1*	0.63713	8.543	0.0012	82% (14/17)	68.8% (11/16)
eralpha + *srd5alpha1* + stage	0.3180	15.011	<0.001	88.2% (15/17)	81.2% (13/16)

pha1 expression in the GMC is ~2-fold higher in females compared to males, although this was not statistically significant according to the authors [37]. These results are similar to the data for *P. pustulosus* where we documented a statistically significant 1.4-fold difference. Another function of the srd5alpha enzymes is the conversion of progesterone into 5α-reduced metabolites such as allopregnanolone, a neurosteroid also produced in peripheral organs such as the gonads and adrenal gland in rats [9]. In mammals, allopregnanolone is widely studied for its effects on brain function, such as the effects on mood and depression [51], and neurogenesis [6]. Interestingly, in rats, plasma levels of allopregnanolone are higher in females compared to males [9]. Measuring steroid hormone production in the tadpole gonad will be required to determine the function of higher *srd5alpha1* expression in female gonads with respect to males.

In *P. pustulosus*, expression of *srd5alpha2* was very low and it was not possible to measure it in the GMC. Similarly, in the brain, *srd5alpha2* mRNA was not detected during metamorphosis [12].

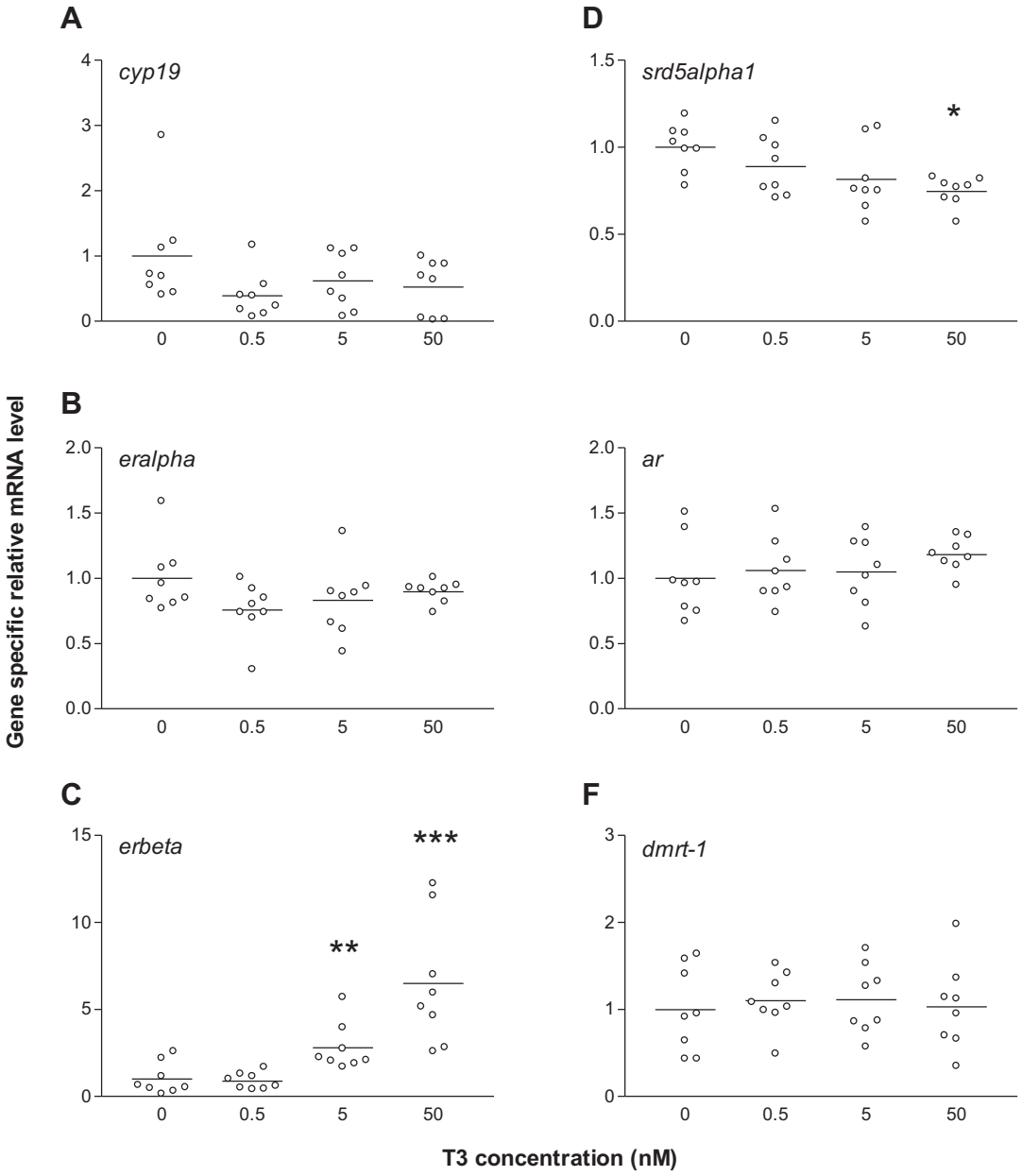

Fig. 4. Effects of T3 exposure on the expression of sex differentiation- and sex steroid-related genes in the gonad-mesonephros complex (GMC) of *Physalaemus pustulosus*. Premetamorphic tadpoles (G 32–34) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h. Effects of T3 on *cyp19* (A), *eralpha* (B), *erbeta* (C), *srd5alpha1* (D), *ar* (E), and *dmrt-1* (F) are presented. Data are presented as fold changes relative to control and are normalised to RNA content. Sample points are presented along with the mean. Asterisks represent significant differences in mRNA levels from the control group (one-way ANOVA followed by Bonferroni's pair-wise comparison between treatment and control; two GMC per pool; $n = 8$ pools; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Note that the scales of the y-axis vary between genes.

These results are in marked contrast to previous studies in *X. laevis* [50] and *S. tropicalis* [14], the only studies that have measured *srd5alpha2* in the GMC and brain in amphibians. This suggests that *srd5alpha2* expression and regulation in *P. pustulosus* is different to what has been observed in the two species of the Pipidae family, and further work is needed to elucidate both the conserved and potentially divergent roles of srd5alpha enzymes in anurans.

4.2. Effects of T3 on transcript levels in the GMC

The second question we addressed in this study was whether T3 could regulate sex steroid-, sex differentiation-, and TH-related genes in the GMC in *P. pustulosus*. During development, *cyp19* seems to be a key regulator of gonadal differentiation in vertebrates [10,17,18,26,43]. In *P. pustulosus*, exposure to T3 did not statistically affect *cyp19* mRNA in the GMC, even though *cyp19* levels in the T3 treatment groups were on average lower with respect to control (between 1.6- and 2.6-fold; Table 2). In order to directly compare results with *S. tropicalis*, we followed the same protocol as in [14]; premetamorphic tadpoles (between G32 and 34) were exposed to T3 and GMC samples were pooled for gene expression analysis. However, according to the developmental profile, *cyp19* mRNA is already sexually dimorphic by G30 in *P. pustulosus*. The high variation in *cyp19* expression within T3 treatments could be due to the effect of pooling GMC samples and may indicate sex differences in response to T3. In goldfish gonads, T3 decreases the expression of gonadal *cyp19* but the effects and mechanisms vary depending on the sex of the fish [40]. Future research should

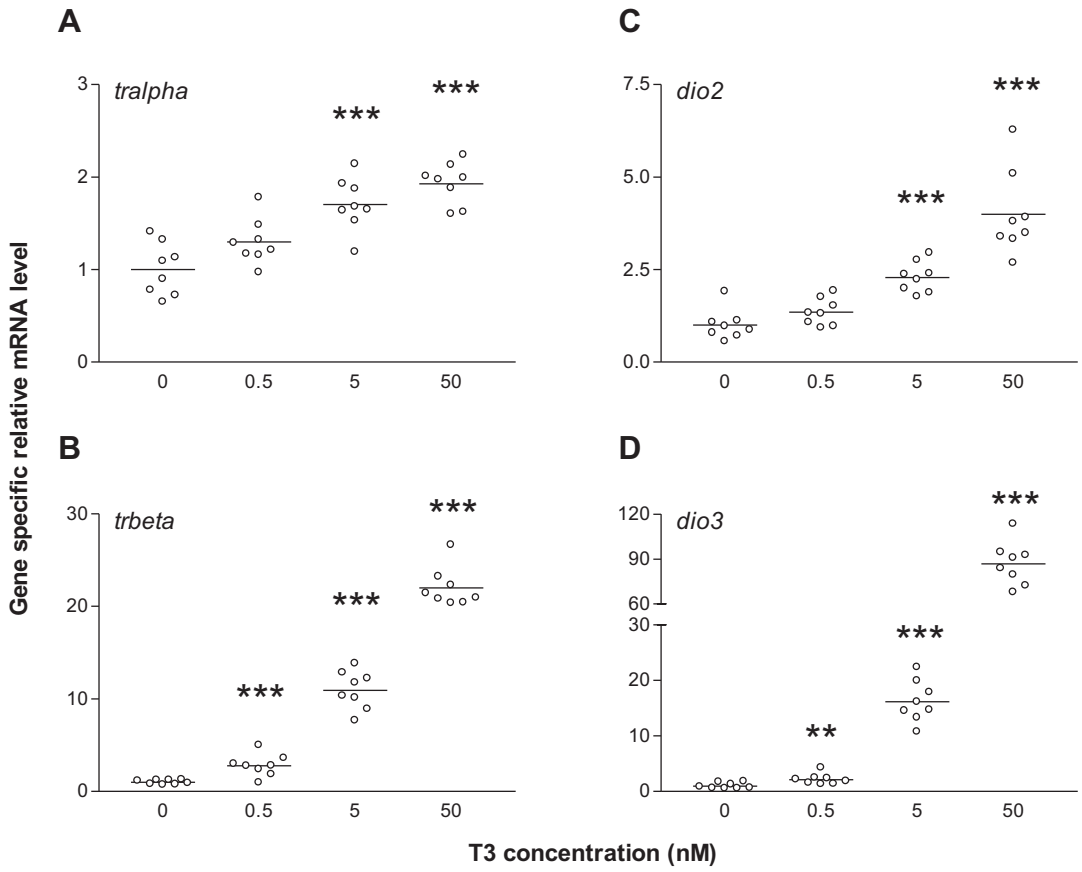

Fig. 5. Effects of T3 exposure on the expression of thyroid hormone-related genes in the gonad-mesonephros complex (GMC) of *Physalaemus pustulosus*. Premetamorphic tadpoles (G 32–34) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h. Effects of T3 on *tralpha* (A), *trbeta* (B), *dio2* (C), and *dio3* (D) are presented. Data are presented as fold changes relative to control and are normalised to RNA content. Sample points are presented along with the mean. Asterisks represent significant differences in mRNA levels from the control group (one-way ANOVA followed by Bonferroni's pair-wise comparison between treatment and control; two GMC per pool; $n = 8$ pools; $^{**}p < 0.01$; $^{***}p < 0.001$). Note that the scales of the y-axis vary between genes.

Table 2
Gene expression changes in the gonad-mesonephros complex after T3 exposure in *Silurana tropicalis* and *Physalaemus pustulosus*. Premetamorphic tadpoles were exposed to T3 (0.5, 5, 50 nM) for 48 h. For each target gene, the average fold change with respect to control (0 nM T3) and normalised to RNA content is presented along with an arrow indicating the direction of change. Asterisks (*) denote statistically significant differences from control (one-way ANOVA; $p < 0.05$). Dashes indicate no differences, dl = detected at very low levels.

Target	*S. tropicalis*[a]			*P. pustulosus*[b]		
	0.5	5.0	50	0.5	5.0	50
eralpha	–	–	–	–	–	–
erbeta	1.3↓	1.7↓*	2.1↓*	–	2.8↑*	6.5↑*
cyp19	–	–	–	2.6↓	1.6↓	1.9↓
dmrt-1	–	–	–	–	–	–
srd5alpha1	1.6↑*	1.9↑*	1.9↑*	–	1.2↓	1.3↓*
srd5alpha2	1.8↑*	3.6↑*	3.9↑*	dl	dl	dl
ar	1.5↑*	1.7↑*	1.9↑*	–	–	–
tralpha	1.8↑	1.6↑*	1.5↑	1.3↑	1.7↑*	2.0↑
trbeta	6.2↑*	6.5↑*	6.8↑*	2.8↑*	11↑*	22↑*
dio2	2.4↑*	6.3↑*	8.7↑*	1.4↑	2.3↑*	4.0↑*
dio3	1.6↑	5.4↑*	15↑*	2.0↑*	16↑*	87↑*

[a] Results from [14].
[b] Results from the present study.

take into account sex differences when studying T3 actions on the developing gonads of *P. pustulosus*.

Exposure to 50 nM T3 decreased *srd5alpha1* mRNA levels. This is exactly opposite to the observed changes in *S. tropicalis*, where higher *srd5alpha1* and *srd5alpha2* mRNA levels were observed after T3 [14]. The developmental profile of *srd5alpha1* in *P. pustulosus* suggests that in addition to synthesising androgens (i.e., converting testosterone into 5α-DHT), the enzyme may be involved in other reduction reactions, such as those involving progesterone. Females at stages G36 and 42 had a higher *srd5alpha1* expression relative to males. The difference in GMC *srd5alpha1* expression between male and females (1.4-fold) was very similar to the decrease in *srd5alpha1* mRNA in the GMC of premetamorphic tadpoles after T3 exposure (1.3-fold), suggesting that the GMC after T3 treatment resembles more a male than a female GMC. These results in combination with a low *cyp19* in certain tadpoles could indicate that T3 may also promote some aspects of masculinisation in *P. pustulosus*.

In the case of TH-related genes, all transcripts were expressed in the GMC during development and were positively regulated by T3. These results are similar to the effects of T3 in the brain of *P. pustulosus* [12] and to the developmental profiles and T3 regulation of TH-related genes in *S. tropicalis* GMC [14]. No differences between sexes were observed in the expression of TH-related genes either at metamorphic climax or in the putative sexes during development. Therefore, we suggest that the TH-related genes measured in this study are not direct regulators of gonadal differentiation in anurans. However, the results indicate that the GMC is a target of THs and that some of the effects of T3 in the gonad could be exerted via TH receptors (tr).

As we hypothesised, we found that the effects of T3 on sex steroid-related transcripts in the GMC differ between *P. pustulosus* and *S. tropicalis*. Although the effects of T3 in the GMC have only being investigated in these two species, the overall effect of T3 ap-

pears to be similar to the brain, for which we previously compared results for three anuran species [12]. In the anuran brain, we proposed that by regulating the expression of androgen or estrogen synthesis enzymes, T3 can increase the ratio of androgens to estrogens. The results of the current study indicate that a similar effect might also be present in the tadpole GMC. In *S. tropicalis*, T3 exposure increases GMC expression of androgen-related genes (*srd5alpha1*, *srd5alpha2*, and *ar*; Table 2), which may be part of a pathway to induce testicular development. In contrast, in *P. pustulosus*, T3 reduced mRNA levels of *srd5alpha1* and in some tadpoles, also reduced *cyp19* (Table 2). In *P. pustulosus*, decreases in the GMC expression of *cyp19* and *srd5alpha1* after T3 treatment may indicate altered or reduced ovarian development. Therefore, it seems that there are at least two possible and distinct mechanisms by which T3 may promote testicular development in tadpoles. Future studies should test this hypothesis and investigate whether the effect of T3 on sex steroid-related gene expression is direct or indirect in the GMC (e.g., performing shorter exposures to T3 and analysing the promoter of the genes affected by T3 exposure).

In conclusion, expression of *cyp19* can be used as indicator of ovarian development and the sex of developing *P. pustulosus* tadpoles. These results provide supporting evidence that levels of estrogen are important regulators of gonadal differentiation in frogs [5,10,29,42,43,53]. On the other hand, the target genes associated with testicular development in vertebrates (i.e., *srd5alpha* and *dmrt-1*) did not seem useful as molecular markers of male development in *P. pustulosus*. The magnitude of *cyp19* sexual dimorphism suggests that *P. pustulosus* is a good Neotropical candidate species to study the process of gonadal differentiation. Along with the brain study [12], these results provide important basic information for future studies on sexual development and metamorphosis. Finally, the two species comparison suggests that T3 may contribute to male gonadal development in anurans via two different mechanisms. Further research on additional frog species representative of the various anuran families and life-history strategies, and the physiological consequences of the regulation of GMC gene expression will help clarify the role of THs in anuran gonadal development.

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