

Regulation of pituitary thyrotropin gene expression during *Xenopus* metamorphosis: negative feedback is functional throughout metamorphosis

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Abstract

Several hypotheses have been proposed to explain the increase and sustained expression of pituitary thyrotropin (TSH) in the presence of elevated plasma thyroid hormone (TH) concentrations at metamorphic climax in amphibians. It has been proposed that the negative feedback of TH on TSH is inoperative until metamorphic climax, and that it is established at this time by the upregulation of pituitary deiodinase type II (DII); DII converts thyroxine (T_4) to 3,5,3'-triiodothyronine (T_3). However, earlier investigators, using indirect measures of TSH, reported that TH negative feedback on TSH was functional in premetamorphic tadpoles. In an effort to understand pituitary TSH regulation during amphibian metamorphosis, we analyzed multiple pituitary genes known or hypothesized to be involved in TSH regulation in tadpoles of *Xenopus laevis*. Tadpole pituitary explant cultures were used to examine direct negative feedback on TSH mRNA expression. Negative feedback is operative

in the early prometamorphic tadpole pituitary and both T_3 and T_4 can downregulate TSH mRNA expression throughout metamorphosis. The expression of both DII and TH receptor βA mRNAs increased during development and peaked at climax; however, these increases coincided with similar increases in deiodinase type III, which inactivates TH. Moreover, corticotropin-releasing factor (CRF) receptors, CRF binding protein and thyrotropin-releasing hormone receptor type 2 mRNA expression also peaked at climax. Our data suggest that the regulation of TSH is more complex than the timing of DII expression, and likely involves a balance between stimulation of TSH synthesis and secretion by neuropeptides (e.g. CRF) of hypothalamic or pituitary origin, increased pituitary sensitivity to neuropeptides through upregulation of their receptors, and intrapituitary TH levels.

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Introduction

Thyroid hormone (TH) is known to be the primary hormonal stimulus for amphibian metamorphosis (see Kikuyama *et al.* 1993, Shi 2000). However, the neuroendocrine mechanisms responsible for the obligatory rise in TH production during metamorphosis are still poorly understood. It is well established that the hypothalamus and pituitary gland regulate tadpole thyroid activity, but the precise molecular controls of the hypothalamic–pituitary–thyroid axis remain to be elucidated. Earlier studies showed that hypophysectomy prior to late prometamorphosis resulted in metamorphic stasis (Dodd & Dodd 1976) and abolished ^{131}I uptake by the thyroid gland. These effects could be reversed by treatment with tadpole pituitary extract or mammalian thyrotropin (TSH; Dodd & Dodd 1976). Furthermore, passive immunization of *Rana catesbeiana* tadpoles with an antiserum to bovine TSH prevented spontaneous metamorphosis (Eddy &

Lipner 1976). A biologically active TSH was purified from bullfrog pituitaries by MacKenzie and Licht (1978) but, to date, no amphibian TSH has been characterized biochemically. However, cDNAs for TSH β and the α -glycoprotein subunit (α -GSU) have been isolated from *Xenopus laevis* (Buckbinder & Brown 1993) and *R. catesbeiana* (Okada *et al.* 2000). In both *X. laevis* and *R. catesbeiana*, pituitary TSH mRNA expression increases during prometamorphosis in parallel with increasing plasma TH concentrations, which supports earlier findings of an increase in pituitary TSH bioactivity during metamorphosis (Dodd & Dodd 1976, Kikuyama *et al.* 1993).

Negative feedback by TH on pituitary TSH biosynthesis and secretion is a universal phenomenon among vertebrates (Licht & Denver 1990, Cohen *et al.* 2000). Direct negative feedback by TH on pituitary TSH secretion was demonstrated using *in vitro* bioassays in adults of two frog species (*R. pipiens*: S Pavgi, personal communication; *R. ridibunda*: Jacobs & Kuhn 1992) but similar studies have

not been conducted in tadpoles. If negative feedback is operative in tadpoles it is puzzling that they are able to mount a sustained rise in TSH production in the face of greatly elevated circulating TH concentrations (Leloup & Buscaglia 1977, White & Nicoll 1981). Either negative feedback is not functional during pre- and prometamorphosis, or there is a strong hypothalamic drive for TSH production that overcomes the negative feedback exerted by the elevated plasma TH concentrations. Etkin and colleagues (Etkin *et al.* 1965, Etkin 1966, 1968) hypothesized that TH exerts a maturational effect on the hypothalamus, median eminence and pituitary gland, and this form of positive feedback is responsible for the sustained rise in plasma TH concentrations that drive metamorphosis. Etkin (1968) also proposed that negative feedback on pituitary TSH does not develop until metamorphic climax. More recently, Huang and colleagues (2001) proposed that negative feedback on pituitary TSH in *X. laevis* develops only at metamorphic climax, coincident with the onset of deiodinase type II (DII) expression. However, they did not directly test whether feedback was in fact absent before climax, and a considerable body of data suggests that negative feedback on pituitary TSH is functional in the premetamorphic tadpole. Kaye (1961) provided evidence that negative feedback can operate from a very early stage of development; i.e. tadpoles of *R. pipiens* treated with TH at Taylor Kollros stage III exhibited depressed thyroidal ^{131}I uptake. Others showed that treatment of premetamorphic tadpoles with goitrogens induced enlargement of the thyroid and degranulation of pituitary thyrotropes (Goos *et al.* 1968a,b, see also Dodd & Dodd 1976). Conversely, replacement with thyroxine (T_4) reversed the effects of goitrogen treatment on the thyrotropes, suggesting that negative feedback on TSH was operative in the premetamorphic tadpole (Goos *et al.* 1968b, Kurabuchi *et al.* 1992). Buckbinder and Brown (1993) reported an elevation in TSH β mRNA in the pituitary of a premetamorphic tadpole treated with the goitrogen, methimazole.

In the present study we used pituitary explant cultures derived from *X. laevis* tadpoles at different developmental stages to determine whether and when TH is capable of exerting negative feedback on TSH gene expression. Our data show that TH can act directly on the tadpole pituitary from early prometamorphosis through metamorphic climax to downregulate TSH gene expression; i.e. negative feedback does not abruptly form at metamorphic climax as suggested by Huang and colleagues (2001). Our expression analyses of pituitary genes that are known or suspected to be involved in TSH regulation support Etkin's maturational hypothesis, whereby the hypothalamic-pituitary unit develops in response to rising plasma TH titers. Moreover, our data suggest that the pituitary gland expresses the genes necessary for autostimulation of TSH synthesis and secretion (i.e. corticotropin-releasing factor (CRF) and thyrotropin-releasing hormone (TRH)) perhaps via an autocrine or paracrine mechanism.

Materials and Methods

Animal husbandry

X. laevis tadpoles were obtained by in-house breeding or were purchased from Xenopus I (Dexter, MI, USA). Tadpoles were raised in dechlorinated tap water (20–22 °C) under 12 h light:12 h darkness conditions and were fed Frog Brittle (Nasco, Fort Atkinson, WI, USA) which was available *ad libitum*. Developmental stages were assigned according to the method of Nieuwkoop and Faber (1956) (NF stage). All procedures involving animals were conducted in accordance with the guidelines of the University Committee on the Care and Use of Animals of the University of Michigan.

Pituitary explant culture

Pituitary explant methods were based on those described by Denver (1988) with modifications. *X. laevis* tadpoles were reared in aquarium water containing oxytetracycline (10 mg/l) for 16–20 h before being killed. For tissue harvest, tadpoles were anesthetized by immersion in 0.01% benzocaine (Sigma, St. Louis, MO, USA), pituitaries were removed and placed two per well in 24-well tissue culture dishes containing 1 ml ice cold amphibian strength Dulbecco's modified Eagle's medium (DMEM; diluted to 0.66 mammalian strength to account for the lower osmotic pressure of amphibian body fluids). The DMEM contained 10 mM HEPES, 3 mM NaHCO_3 , 0.1% bovine serum albumin, 0.01% bacitracin, 0.005% Tween 80, and antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA). Each tissue culture well contained two pituitaries to ensure sufficient tissue for analysis.

Tissue culture was conducted with shaking, in a dark, humidified chamber under an atmosphere of 95% O_2 and 5% CO_2 . Tissues were cultured for 20 h prior to the initiation of hormone treatments to allow for stabilization of TSH synthesis and secretion (see Denver 1988). Following this 20 h pre-incubation period, 250 μl DMEM containing an appropriate concentration of TH was added to the wells. Control groups received 250 μl DMEM without TH. Pituitaries were incubated for an additional 24 h before harvest and RNA extraction. Dose sensitivity of TH negative feedback on TSH gene expression was tested at three different developmental stages: early prometamorphosis (NF stage 56), mid-prometamorphosis (NF stage 59) and metamorphic climax (NF stage 62). Pituitaries were cultured with different doses (final concentration of 0, 1, 10, or 100 nM) of 3,5,3'-triiodothyronine (T_3) (Sigma) or thyroxine (T_4) (Sigma) in separate experiments. Each hormone treatment at each of the developmental stages was carried out in triplicate. At the termination of each explant experiment pituitaries were immediately extracted for RNA and stored at -80°C for analysis by Northern blotting.

RNA extraction and analysis

Tissues were collected into 800 µl Trizol reagent (Invitrogen). Glycogen (20 µg; Invitrogen) was added and RNA was extracted according to the manufacturer's protocol. The RNAs were stored in RNase-free water at -80 °C prior to analysis by either Northern blotting or reverse-transcription polymerase chain reaction (RT-PCR).

Northern blotting

Northern blots were prepared following methods described previously (Denver *et al.* 1997). All probes were labeled with [³²P]dCTP by random priming (Amersham Biosciences, Piscataway, NJ, USA). Blots were initially probed with a *X. laevis* TSHβ cDNA (Buckbinder & Brown 1993) then stripped and reprobed with a *X. laevis* α-GSU cDNA (Buckbinder & Brown 1993), followed by the ribosomal protein L8 cDNA (rpL8; Shi & Liang 1994). Analysis of rpL8 allowed for normalization for RNA loading as it is constitutively expressed throughout metamorphosis and its expression is not altered by TH treatments (Shi & Liang 1994). Blots were hybridized using ULTRAhyb (Ambion, Austin, TX, USA) and washed following the manufacturer's protocol. Blots were then exposed to BioMax X-ray film (Eastman Kodak, Rochester, NY, USA) for 16–48 h prior to densitometric analysis using Scion Image (see below). Individual replicate RNA samples from each treatment were analyzed on a single gel and each treatment was replicated three times (i.e. three separate gels were analyzed).

Semi-quantitative RT-PCR analysis of pituitary gene expression during metamorphosis

Semi-quantitative RT-PCR was employed to compare the expression of 13 different genes in tadpole pituitaries throughout metamorphosis (see Table 1 and Figs 4–7). Total RNA was extracted from a pool of 5 pituitaries from each NF developmental stage: 52, 54, 57, 59, 62, and 64. RNA extractions and RT reactions for each of the six developmental stages studied were conducted simultaneously and the experiment was replicated three times. The RT reaction was conducted on the entire RNA sample and included a 30-min DNase digestion step adapted from Huang *et al.* (1996). Briefly, 1.5 U RNase-free DNase I (Roche, Indianapolis, IN, USA), 20 U RNase inhibitor (Roche) and 4 µl first-strand buffer (Invitrogen; 250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM MgCl₂) were added to each RNA sample to a final volume of 15 µl. The digestion proceeded for 30 min at 37 °C and samples were then heat-inactivated at 75 °C for 5 min. The RT reaction was conducted using SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) according to the manufacturer's protocol

using 250 ng of random hexamer primers (Invitrogen) and a 10-min denaturation step at 70 °C. Each RT reaction was diluted with 180 µl nuclease-free water for a final concentration of 0.025 pituitary equivalents of reverse transcribed RNA (cDNA) per microliter. PCR for several genes conducted on 10 independent minus RT reactions failed to amplify any gene products, thus confirming that there was no genomic DNA contamination in our RT reactions.

PCR amplification and developmental gene expression analysis

PCR was conducted using HotStar Taq DNA polymerase following the manufacturer's protocol (Qiagen, Valencia, CA, USA). Deoxyoligonucleotide primer sequences used for each of the 13 genes analyzed are listed in Table 1. Reaction volumes were 25 or 50 µl, and contained 0.05–0.15 pituitary equivalents of reverse transcribed pituitary RNA. Subsequent to an initial 15-min denaturation and activation step at 95 °C, PCR cycling conditions were as follows: 94 °C for 45 s; 52–63 °C for 45 s; 72 °C for 60 s. Reactions were cycled 26–42 times depending on the target gene, followed by extension at 72 °C for 10 min (Table 1). The number of cycles used for each gene was empirically determined using a linear amplification range beginning at 21 cycles and increasing to 45 cycles in 3 cycle increments. The midpoint of the linear part of the amplification curve was chosen as the optimal number of cycles for each gene (see Table 1). Individual PCR reactions were conducted for each of the 13 genes examined. For each gene, RT-PCR analysis of the six developmental stages was conducted simultaneously and analyzed on a single gel. Three separate RNA pools were analyzed from each developmental stage. PCR products were electrophoresed in a 1.5% agarose gel and the bands stained with ethidium bromide. Stained bands were captured digitally using a closed-circuit television (CCT) camera and densitometric analyses of these data were conducted using Scion Image (see below).

Data and statistical analyses

Autoradiographic films were digitized using a flatbed scanner. Images of ethidium bromide-stained gels were captured using a CCT digital camera. Densitometric analysis was conducted using Scion Image software (v. 3.0 Scion Corporation, Frederick, MD, USA) following adjustment for differences in background. Band densities were expressed as the optical density (OD) × mm².

Statistical analyses were conducted using the SAS computer program (v. 8.0, SAS Institute Inc., Cary, NC, USA). All statistical analyses on densitometric data were conducted on the raw, untransformed data. Pituitary explant culture data were analyzed for statistically significant differences in TSHβ and α-GSU expression using a blocks design ANOVA, with each experimental replicate

Table 1 Primer sequences and polymerase chain reaction (PCR) conditions employed to amplify 13 different genes from *X. laevis* pituitary RNA using semiquantitative reverse transcription PCR

Gene	Primer (sense above, antisense below)	PCR product (bp)	Annealing temperature (°C)	No. of cycles	GenBank accession number
rpL8	CACAGAAAGGGTGCTGCTAAG CAGGATGGGTTTGTCAATACG	477	63	34	U00920
α -GSU	GGTTCAGGGATGCCAGAAATGTA GAGGGAAGGACAGCCAAGAAACA	326	63	26	L07619
TSH β	GCCAGGCTGTCTCAGTCTGTTTTTC GCCGAGATATTGAGGCTCGAATGGTTT	356	63	26	L07618
TR β A	GGCAACAGACTTGGTTTTGG AAGTCCACTTTCCACCCTC	275	57	36	M35359
DII	AGCCATAAATAGCCTGCCCTGTG GGAACCTTAACCTTTGGGACTGC	278	57	34	AF354707
DIII	TACATCGTCTGGAGGGCAAAG TTATTCTCGCAATGACATCTGAACC	490	61.1	35	L28111
TRH	TTCTCTGGTGGTTGTGCTTCT ATCTCCTCTGGAATCGTTTACC	248	57.6	35	X64056
TRH-R1	TTCTTTCTGTCCCGTCTTAC GTGTATTCTCAGACATTGGGTTTAT	481	52.5	41	AF305620
TRH-R2	TGCCATTGTGCTCATATTACAGA TCCAGCGGTACCAAGACCATT	407	57	39	AF305621
CRF	TCTCCTGCCTGCTCTGTCCAA CTTGCCATTTCTAAGACTTCACGG	321	61.1	35	S50096
CRF ₁	GATCTAAATAGCAGGATGCTGTTGGC GCCGTTACAGGTGACATTCTGTAC	288	62.6	35	Y14036
CRF ₂	GTACCTCCCACATCCCACAGCTTAC GACGCCAGGTTCCATTCTCAAAGC	308	62.6	37	Y14037
CRF-BP	TGACTCCTGCTTCCAGACCT TGACCTTGTAATGCTCCCCAC	452	61	39	U41858

rpL8, ribosomal protein L8; α -GSU, α glycoprotein subunit; TSH β , thyrotropin β subunit; TR β A, thyroid hormone receptor β A; DII, deiodinase type II; DIII, deiodinase type III; TRH, thyrotropin-releasing hormone; TRH-R1, TRH receptor type 1; TRH-R2, TRH receptor type 2; CRF, corticotropin-releasing factor; CRF₁, CRF receptor type 1; CRF₂, CRF receptor type 2; CRF-BP, CRF-binding protein.

representing a single block. Data were analyzed following removal of variation due to RNA (and subsequent cDNA) loading using the level of rpL8. Following acceptance of the entire ANOVA model as statistically significant, the data were tested for any statistically significant stage-by-treatment effects. Stage-by-treatment effects were not observed, thus the data for all three developmental stages were pooled and the ANOVA model was used to test for differences between experimental groups following the removal of the variation due to developmental stage. This step in the ANOVA model facilitated a threefold increase in sample size, thus increasing the statistical power of the model. The Dunn-Šidák multiple comparison method (a modification of the sequential Bonferroni test) was used to test for pair-wise differences between experimental groups (Ury 1976, Sokal & Rohlf 1981). Means were accepted as statistically significant if $P < 0.05$. The mean α -GSU and TSH β gene expression data from the 3 replicates of each experimental group and stage are presented graphically as a percentage of the expression in the control group

following normalization for differences in rpL8 gene expression for both dose-response experiments.

A blocks design ANOVA, with each experimental replicate representing a single block, was used to test for statistically significant differences in pituitary gene expression during metamorphosis. All statistical analyses were conducted on raw untransformed, densitometric data. This model was used to identify statistically significant differences between metamorphic stages for 12 different genes following correction for differences in starting total RNA as determined by rpL8 expression. The Dunn-Šidák multiple comparison test was used to test for pair-wise differences between developmental stages (Ury 1976, Sokal & Rohlf 1981). Differences between means were accepted as statistically significant if $P < 0.05$. The means of three experimental replicates for each gene studied are presented graphically as a percentage of the developmental stage with the highest expression level, following normalization for differences in rpL8.

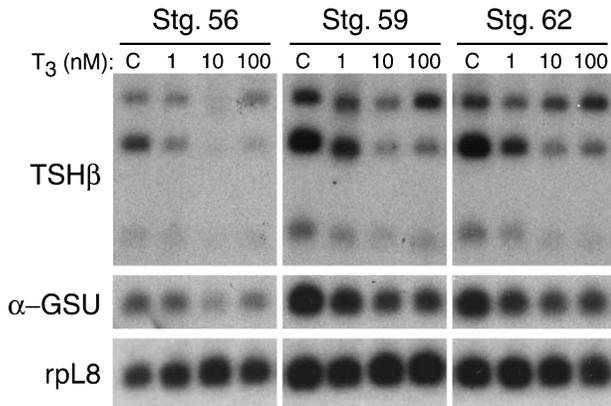


Figure 1 A representative Northern blot of thyrotropin β (TSH), α -glycoprotein subunit (α -GSU) and ribosomal protein L8 (rpl8) mRNA expression by cultured *X. laevis* tadpole pituitaries at three different Nieuwkoop and Faber (1956) stages (Stg.). Following a 20-h preincubation period to stabilize TSH gene expression and secretion, pituitaries were incubated in the presence of 0, 1, 10 or 100 nM triiodothyronine (T_3) for 24 h before harvest.

Results

Pituitary explant experiments

In vitro dose–response experiments were conducted to determine whether negative feedback on pituitary TSH gene expression is operative throughout metamorphosis, and whether the dose sensitivity to T_3 or T_4 changes with development. Overall, both T_3 and T_4 lowered TSH expression by pituitaries cultured *in vitro*, independent of developmental stage. Negative feedback inhibition on TSH β and α -GSU expression by TH was observed at all stages tested (i.e. NF stages 56, 59, 62; Figs 1 – 3). No significant stage-by-treatment effect was observed for either T_3 (TSH β : $P=0.6278$, $F=0.78$, degrees of freedom (df)=8; α -GSU: $P=0.0958$, $F=1.98$, df=8) or T_4 (TSH β : $P=0.2326$, $F=1.43$, df=8; α -GSU: $P=0.7134$, $F=0.67$, df=8); that is, the sensitivity of pituitaries from stage 56, 59 and 62 tadpoles to TH did not differ significantly between stages. Despite the lack of a statistically significant stage-by-treatment effect, pituitaries from early prometamorphic tadpoles (i.e. stage 56) tended to be more sensitive to both T_4 and T_3 than the other stages tested, with respect to the downregulation of TSH β expression. Following exposure to 1 nM T_3 , TSH β mRNA levels in stage 56 pituitaries were 32% of control levels in comparison with 66 and 83% of control levels for stages 59 and 62 respectively (Figs 1 and 2). A similar trend, although also not significant, suggesting that stage 56 pituitaries were most sensitive to TH was observed following treatment with 10 and 100 nM T_3 as well as 1 and 10 nM T_4 (Fig. 2). For reference, peak whole body and serum T_3 and T_4 concentrations in *X. laevis* tadpoles range from 8 to 12 nM and from 7 to 10 nM respectively (Leloup & Buscaglia 1977, Krain & Denver 2004). Thus, TH

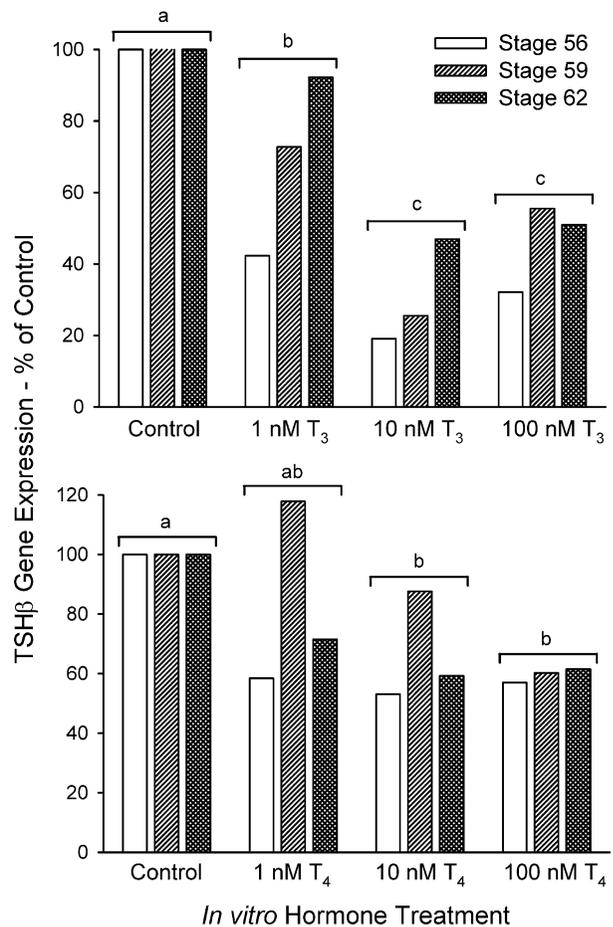


Figure 2 Thyrotropin β (TSH β) gene expression by cultured pituitaries derived from *X. laevis* tadpoles at three different Nieuwkoop and Faber (1956) stages. Pituitaries were preincubated for 20 h to allow for stabilization of TSH gene expression and secretion, and then incubated in the presence or absence of triiodothyronine (T_3) or thyroxine (T_4) for an additional 24 h before harvest. TSH β mRNA levels were normalized to the housekeeping gene ribosomal protein L8 (rpl8). Values are expressed as a percentage of control levels and are the mean of 3 replicates. Analysis of variance indicated significant differences between experimental groups (T_3 : $P=0.0010$, $F=7.12$, df=4; T_4 : $P=0.0011$, $F=6.29$, df=4; corrected for differences in stage) and developmental stages (T_3 : $P=0.0052$, $F=6.93$, df=2; T_4 : $P=0.0015$, $F=8.40$, df=2; corrected for differences in experimental group); however, no stage-by-treatment interaction effect was observed (T_3 : $P=0.6278$, $F=0.78$, df=8; T_4 : $P=0.2326$, $F=1.43$, df=8). Pair-wise differences (Dunn-Šidák multiple comparison test) were accepted as statistically significant if $P<0.05$. Experimental groups labeled with different letters indicate statistically significant differences. All statistical analyses on densitometric data were conducted on the raw untransformed data following removal of variation due to RNA (and subsequent cDNA) loading using the level of rpl8.

concentrations within the normal physiological range for *X. laevis* tadpoles suppress pituitary TSH expression. The negative effects of TH on α -GSU expression were similar

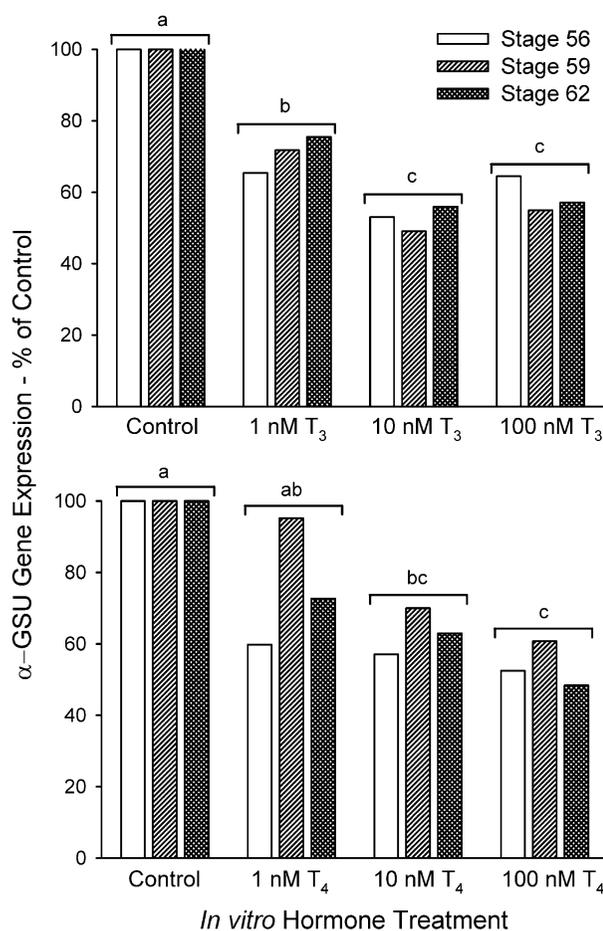


Figure 3 Alpha glycoprotein subunit (α -GSU) gene expression by cultured pituitaries derived from *X. laevis* tadpoles at three different Nieuwkoop and Faber (1956) stages. Pituitaries were preincubated for 20 h to allow for stabilization of α -GSU gene expression and secretion, and then incubated in the presence or absence of triiodothyronine (T_3) or thyroxine (T_4) for an additional 24 h before harvest. α -GSU mRNA levels were normalized to the housekeeping gene ribosomal protein L8 (rpL8). Values are expressed as a percentage of control levels and are the mean of 3 replicates. Analysis of variance indicated significant differences between experimental groups (T_3 : $P < 0.0001$, $F = 17.36$, $df = 4$; T_4 : $P < 0.0001$, $F = 12.77$, $df = 4$; corrected for differences in stage) and developmental stages (T_3 : $P = 0.0012$, $F = 9.16$, $df = 2$; T_4 : $P = 0.0002$, $F = 12.18$, $df = 2$; corrected for differences in experimental group); however, no stage-by-treatment interaction effect was observed (T_3 : $P = 0.0958$, $F = 1.98$, $df = 8$; T_4 : $P = 0.7134$, $F = 0.67$, $df = 8$). Pair-wise differences (Dunn-Šidák multiple comparison test) were accepted as statistically significant if $P < 0.05$. Experimental groups labeled with different letters indicate statistically significant differences. All statistical analyses on densitometric data were conducted on the raw untransformed data following removal of variation due to RNA (and subsequent cDNA) loading using the level of rpL8.

for all three developmental stages examined with no evidence for differences in sensitivity among stages (Fig. 3).

The absence of any significant stage-by-treatment interaction in the ANOVA model permitted the pooling of all stages examined to test for statistically significant differences between experimental groups (i.e. thyroid hormone treatment dose). Both T_4 and T_3 significantly reduced TSH β (T_4 : $P = 0.0011$, $F = 6.29$, $df = 4$; T_3 : $P = 0.0010$, $F = 7.12$, $df = 4$) and α -GSU (T_4 : $P < 0.0001$, $F = 12.77$, $df = 4$; T_3 : $P < 0.0001$, $F = 17.36$, $df = 4$) expression levels (Figs 2 and 3); however, pituitaries were approximately 10 times more sensitive to T_3 than T_4 . Consistent with data from rodent TSH-secreting tumor cells (Shupnik *et al.* 1985), TSH β was downregulated to a greater extent than α -GSU. The lowest TH treatment dose tested was 1 nM; this dose of T_3 significantly downregulated both TSH β and α -GSU expression. However, a treatment dose of 10 nM T_4 was required to reduce either TSH β or α -GSU expression levels below control values. Consistent with pituitaries being more responsive to T_3 than T_4 treatments was the observation that the decline in TSH expression levels tended to be greater following treatment with T_3 than with T_4 . The lowest TSH β expression levels were observed following treatment with 10 nM T_3 (24% of control values). In contrast, the T_4 concentration that produced the greatest reduction in TSH β expression levels was 100 nM, where expression levels were 59% of control values (Fig. 2). A similar trend was observed for α -GSU expression with 10 nM T_3 being as effective at lowering expression levels as 100 nM T_4 (Fig. 3). Maximal decreases in TSH β and α -GSU expression levels were as follows: 10 nM T_3 reduced TSH β expression to 24% of control levels, whereas α -GSU expression was reduced to only 52% of control levels following the same treatment.

Developmental expression of pituitary genes

Thyrotropin, thyroid hormone receptor βA and TH deiodinases
We observed a strong and steady increase (fivefold) in TSH β and α -GSU expression levels from stage 52 to peak values at stage 59 after which expression levels declined slightly (TSH β : $P = 0.0017$, $F = 11.52$, $df = 5$; α -GSU: $P = 0.0015$, $F = 11.99$, $df = 5$; Fig. 4). Pair-wise multiple comparison tests detected significant increases in TSH β and α -GSU expression levels between stages 52 and 54, as well as 54 and 59. However, the decline in TSH β and α -GSU expression following the peak values at stage 59 was not statistically significant (Fig. 4).

Thyroid hormone receptor βA (TR βA) expression levels were at their lowest during premetamorphosis (stages 52 and 54), increased throughout prometamorphosis (stages 57 and 59), peaked at metamorphic climax (stage 62) and remained elevated at stage 64 (Fig. 5). Overall, the observed changes in TR βA expression levels during metamorphosis were statistically significant ($P = 0.0357$, $F = 4.58$, $df = 5$); however, multiple comparison tests did not identify any significant pair-wise differences ($P > 0.05$) between specific developmental stages (Fig. 5).

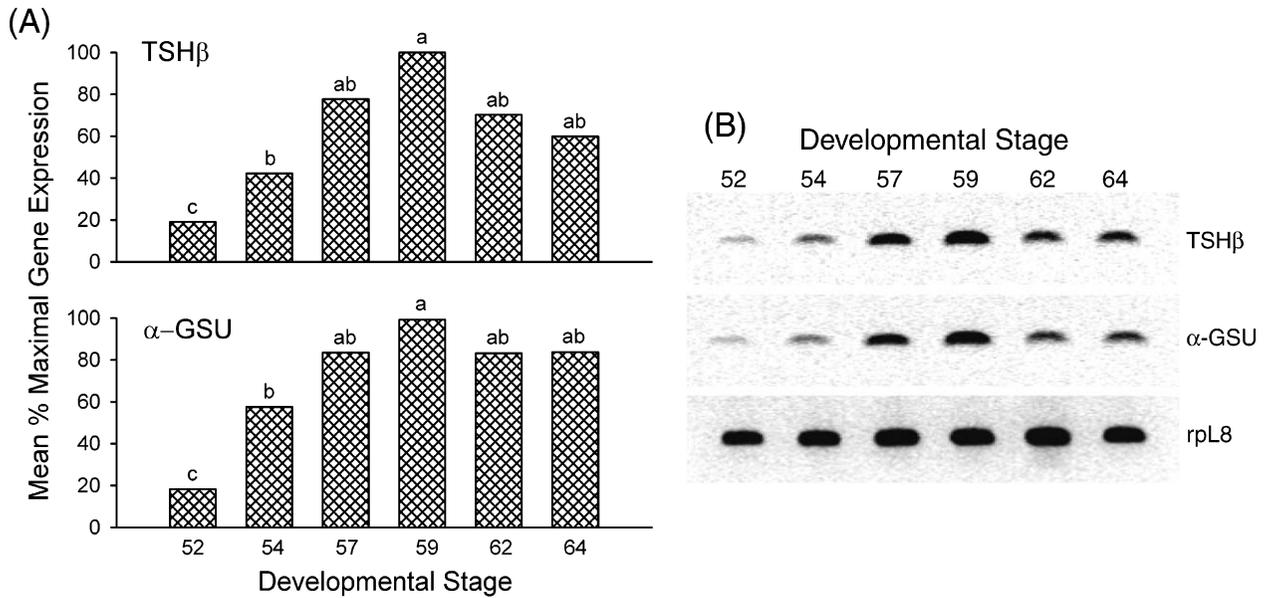


Figure 4 Pituitary thyrotropin β (TSH β) and α -glycoprotein subunit (α -GSU) mRNA expression during metamorphosis of *X. laevis*. RNAs were analyzed by semi-quantitative RT-PCR as described in Materials and Methods. (A) Normalized densitometric values for TSH β and α -GSU mRNAs. Values represent the mean percent maximal gene expression relative to the developmental stage with the highest expression for 3 replicates (5 pituitaries per replicate). Data were normalized for variation in RNA (and subsequently cDNA) loading using ribosomal protein L8 (rpL8) expression. Significant differences between stages were observed for both TSH β ($P=0.0017$, $F=11.52$, $df=5$; ANOVA) and α -GSU ($P=0.0015$, $F=11.99$, $df=5$; ANOVA). Pair-wise differences (Dunn-Šidák multiple comparison test) were accepted as statistically significant if $P<0.05$. Experimental groups labeled with different letters indicate statistically significant differences. All statistical analyses on densitometric data were conducted on the raw untransformed data following removal of variation due to RNA (and subsequent cDNA) loading using the level of rpL8. (B) Representative ethidium bromide-stained gels (negative image) following RT-PCR are shown.

The developmental expression patterns of the iodothyronine deiodinases type II and III (DII and DIII respectively) followed a trend similar to that observed for TSH and TRH β expression. Expression levels of the two deiodinases increased in parallel throughout metamorphosis and remaining elevated at the end of metamorphosis (Fig. 5). In the case of DIII gene expression, the observed differences were significant ($P=0.0004$, $F=15.1$, $df=5$), but in the case of DII they were marginally nonsignificant ($P=0.0581$, $F=3.28$, $df=5$). See Fig. 5 for pair wise differences in DIII gene expression between developmental stages.

Neuropeptides, neuropeptide receptors and CRF binding protein
Both CRF and TRH genes were expressed in the tadpole pituitary throughout metamorphosis, but significant differences between stages were not detected (CRF: $P=0.0966$, $F=2.65$, $df=5$; TRH: $P=0.1149$, $F=2.45$, $df=5$; Figs 6 and 7 respectively). Despite the lack of statistical significance, TRH expression in the pituitary appeared to show a biphasic pattern. TRH expression increased from stage 52 to 57, decreased briefly at stage 59, only to increase slightly at stage 62 and finally decrease again at stage 64 (Fig. 7).

Pituitary expression of the CRF binding protein (CRF-BP) tended to increase throughout prometamorphosis, peak at climax and decline at the end of metamorphosis; however, these differences were not statistically significant ($P=0.2148$, $F=1.77$, $df=5$; Fig. 6). Pituitary expression of CRF receptor types 1 and 2 (CRF $_1$ and CRF $_2$ respectively) increased steadily from stage 52 to stage 59 and remained elevated through stage 64 (Fig. 6). Although the Dunn-Šidák multiple comparison test did not identify any pair-wise differences, the ANOVA model indicated that the CRF $_2$ mRNA expression levels varied significantly ($P=0.0312$, $F=4.15$, $df=5$) with metamorphic stage. It is also noteworthy that the differences in CRF $_1$ mRNA expression levels between stages were marginally nonsignificant ($P=0.0571$, $F=3.31$, $df=5$).

In contrast to the parallel increase in expression of CRF $_1$ and CRF $_2$ throughout metamorphosis, TRH receptors 1 and 2 (TRH-R1 and TRH-R2) exhibited divergent patterns of expression in tadpole pituitaries. TRH-R1 mRNA tended to decrease towards the end of metamorphosis (stages 62 and 64), although this was not statistically significant ($P=0.8288$; $F=0.41$, $df=5$). On the other hand, expression levels of TRH-R2 were relatively low during stages 52 and 54, increased threefold by stage

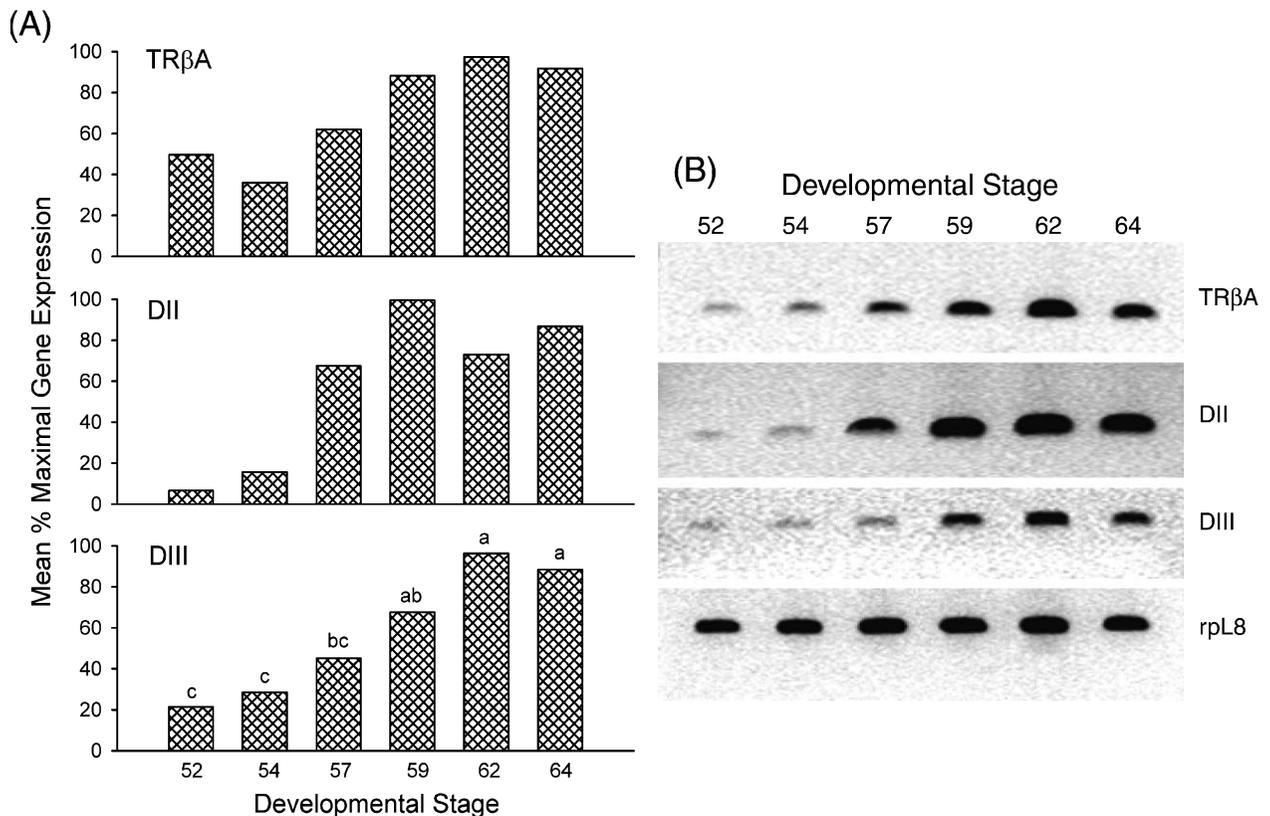


Figure 5 Pituitary thyroid hormone receptor β A (TR β A), deiodinase type II (DII) and deiodinase type III (DIII) mRNA expression during metamorphosis of *X. laevis*. RNAs were analyzed by semi-quantitative RT-PCR as described in Materials and Methods. (A) Normalized densitometric values for TR β A, DII and DIII. Values represent the mean percent maximal gene expression relative to the developmental stage with the highest expression for 3 replicates (5 pituitaries per replicate). Data were normalized for variation in RNA (and subsequently cDNA) loading using ribosomal protein L8 (rpL8) expression. Significant differences between stages were observed for TR β A ($P=0.0357$, $F=4.58$, $df=5$; ANOVA), and DIII ($P=0.0004$, $F=15.10$, $df=5$; ANOVA). Differences in DII expression were marginally nonsignificant ($P=0.0581$, $F=3.28$, $df=5$; ANOVA). Pair-wise differences (Dunn-Sidak multiple comparison test) were accepted as statistically significant if $P<0.05$. Experimental groups labeled with different letters indicate statistically significant differences. The absence of letters for TR β A and DII indicates that no significant pair-wise differences were detected. All statistical analyses on densitometric data were conducted on the raw untransformed data following removal of variation due to RNA (and subsequent cDNA) loading using the level of rpL8. (B) Representative ethidium bromide-stained gels (negative image) following RT-PCR are shown.

57 and remained elevated through stage 64 ($P=0.0031$, $F=18.49$, $df=5$). Pair-wise multiple comparison tests indicated that the increase in TRH-R2 expression between stages 54 and 57 was statistically significant (Fig. 7).

Discussion

We used pituitary explant cultures to test whether T_4 or T_3 can act directly on the *X. laevis* tadpole pituitary gland to suppress TSH gene expression, and to assess whether this negative feedback changes during metamorphosis. Our results clearly show that treatment with physiological concentrations (Leloup & Buscaglia 1977, Krain & Denver 2004) of either T_4 or T_3 can act directly on pituitaries from stage 56, 59 and 62 *X. laevis* tadpoles to suppress TSH

mRNA expression; hence, negative feedback at the level of the pituitary is active from early prometamorphosis (Figs 2 and 3). We observed no significant differences between stages with respect to the effects of TH treatment on TSH mRNA levels, although pituitaries derived from stage 56 tadpoles tended to exhibit the greatest negative feedback response (Figs 2 and 3). This is the first study to clearly demonstrate that TH can act directly on the tadpole pituitary throughout prometamorphosis and metamorphic climax to negatively regulate TSH. Indirect evidence supporting the presence of negative feedback early in tadpole development dates back to the early 1960s. Studies conducted on both *R. pipiens* (Kaye 1961) and *X. laevis* (Dodd & Dodd 1976) showed that treatment with TH suppressed thyroidal activity as measured by a decrease in iodide uptake by the thyroid gland. Similarly, treatment of premetamorphic tadpoles with goitrogens resulted in

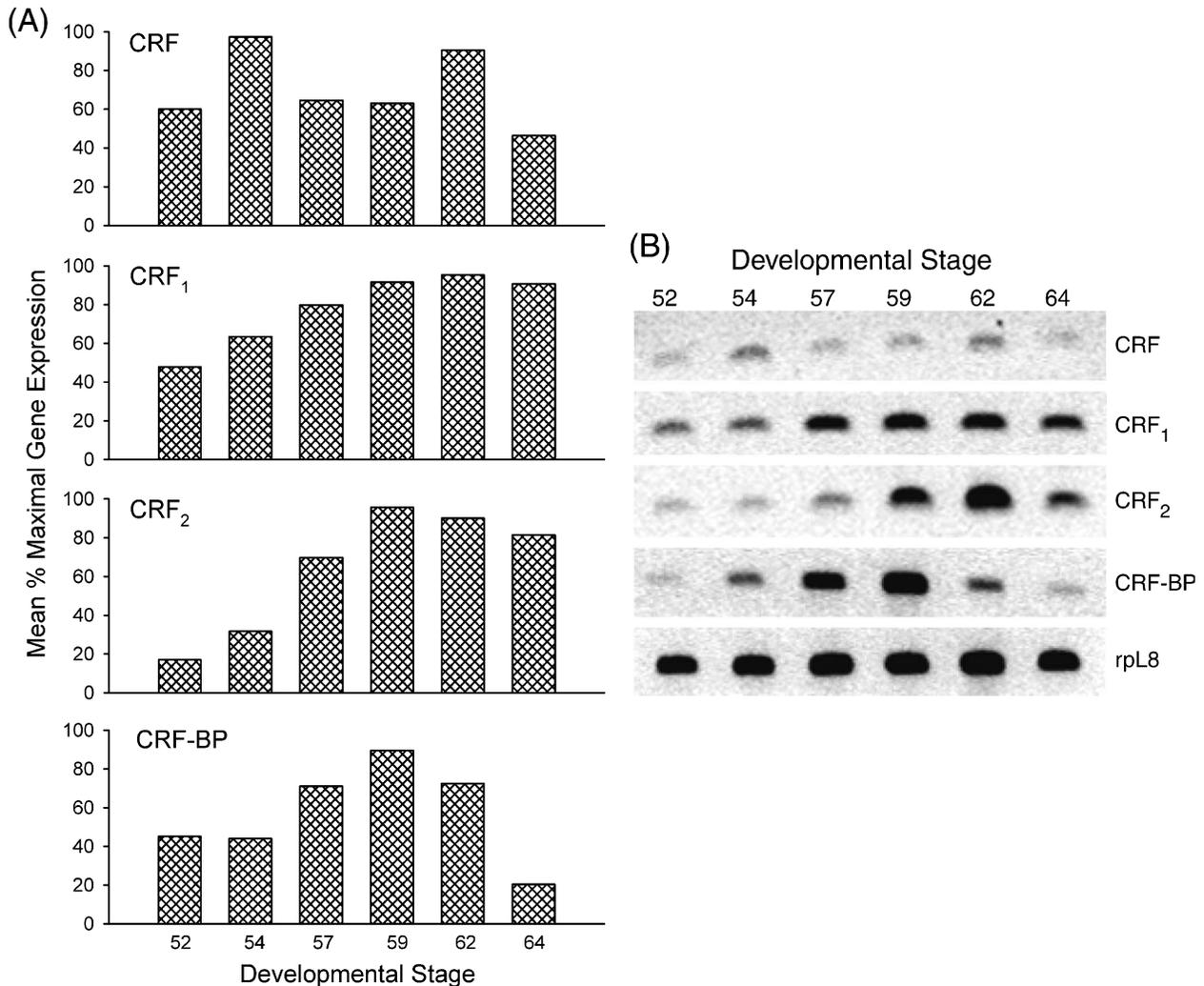


Figure 6 Pituitary corticotropin releasing factor (CRF), CRF receptor type 1 (CRF₁), CRF receptor type 2 (CRF₂) and CRF binding protein (CRF-BP) mRNA expression during metamorphosis of *X. laevis*. RNAs were analyzed by semi-quantitative RT-PCR as described in Materials and Methods. (A) Normalized densitometric values for CRF, CRF₁, CRF₂ and CRF-BP. Values represent the mean percent maximal gene expression relative to the developmental stage with the highest expression for 3 replicates (5 pituitaries per replicate). Data were normalized for variation in RNA (and subsequently cDNA) loading using ribosomal protein L8 (rpl8) expression. Significant differences between stages were observed for CRF₂ ($P=0.0312$, $F=4.15$, $df=5$; ANOVA), but differences were marginally nonsignificant for CRF₁ ($P=0.0571$, $F=3.31$, $df=5$; ANOVA). However, statistically significant differences were not observed for either CRF ($P=0.0966$, $F=2.65$, $df=5$; ANOVA) or CRF-BP ($P=0.2148$, $F=1.77$, $df=5$; ANOVA). Statistically significant ($P<0.05$), pair-wise differences (Dunn-Sidak multiple comparison test) were not detected. All statistical analyses on densitometric data were conducted on the raw untransformed data following removal of variation due to RNA (and subsequent cDNA) loading using the level of rpl8. (B) Representative ethidium bromide-stained gels (negative image) following RT-PCR are shown.

hypertrophy of the thyroid gland (Hanaoka 1967, Dodd & Dodd 1976, Goos 1978). More recently, it was shown that treatment of early prometamorphic *X. laevis* tadpoles with the goitrogen, methimazole, elevated pituitary TSH mRNA levels (Buckbinder & Brown 1993, Huang *et al.* 2001). Taken together, the accumulated data lead to the rejection of the hypothesis that negative feedback is turned on specifically at metamorphic climax (see Huang *et al.* 2001).

Huang and colleagues (2001) recently addressed the issue of thyroidal regulation of the pituitary gland during amphibian metamorphosis and the establishment of negative feedback. In their paper, they claim that TH negative feedback on the pituitary of *X. laevis* is established at stage 62 (metamorphic climax) and that this feedback is causally related to the upregulation of DII. They based their conclusions on several lines of evidence: the demonstrated roles of DII and DIII in the differential timing of cell and

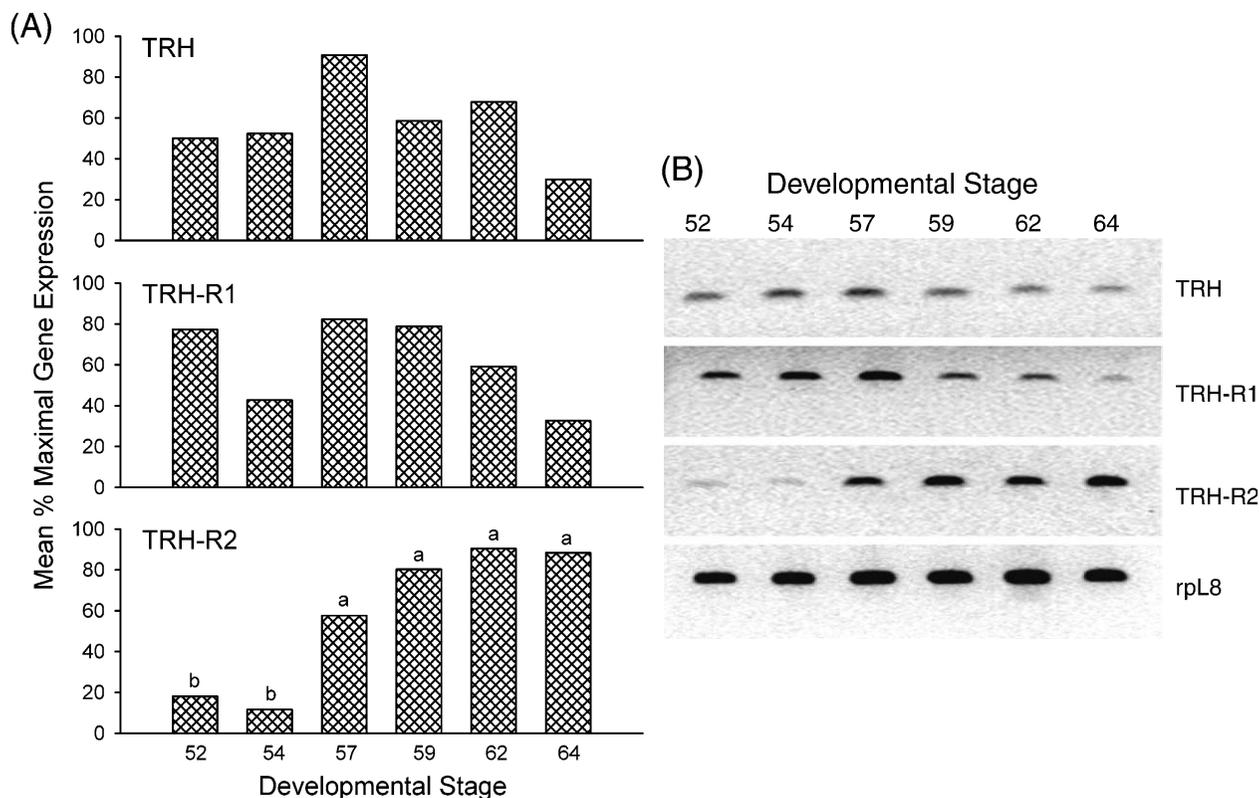


Figure 7 Pituitary thyrotropin releasing hormone (TRH), TRH receptor type 1 (TRH-R1), and TRH receptor type 2 (TRH-R2) mRNA expression during metamorphosis of *X. laevis*. RNAs were analyzed by semi-quantitative RT-PCR as described in Materials and Methods. (A) Normalized densitometric values for TRH, TRH-R1 and TRH-R2. Values represent the mean percent maximal gene expression relative to the developmental stage with the highest expression for 3 replicates (5 pituitaries per replicate). Data were normalized for variation in RNA (and subsequently cDNA) loading using ribosomal protein L8 (rpl8) expression. Significant differences between stages observed for TRH-R2 ($P=0.0031$, $F=18.49$, $df=5$; ANOVA). However, statistically significant differences were not observed for either TRH ($P=0.1149$, $F=2.45$, $df=5$; ANOVA) or TRH-R1 ($P=0.8288$, $F=0.41$, $df=5$; ANOVA). Pair-wise differences (Dunn-Šidák multiple comparison test) were accepted as statistically significant if $P<0.05$. Experimental groups labeled with different letters indicate statistically significant differences. All statistical analyses on densitometric data were conducted on the raw untransformed data following removal of variation due to RNA (and subsequent cDNA) loading using the level of rpl8. (B) Representative ethidium bromide-stained gels (negative image) following RT-PCR are shown.

tissue morphogenesis (Becker *et al.* 1997, Kawahara *et al.* 1999, Huang *et al.* 2001); the presence of DII mRNA at stage 62 of development but not at stage 56 (Huang *et al.* 2001); the observation that TSH mRNA expression levels increase during metamorphosis, peak at climax and then decline rapidly (Buckbinder & Brown 1993); and the claim that T_4 does not downregulate TSH β expression (Huang *et al.* 2001). However, as discussed earlier, our study along with several earlier studies clearly show that negative feedback is active early in prometamorphosis. Moreover, we showed that DII mRNA is present in pituitaries from early prometamorphic *X. laevis*. Thus, our data suggest that a mechanism other than the absence of DII expression in pre- and prometamorphic tadpole pituitaries is responsible for the sustained rise in TSH expression during prometamorphosis and metamorphic climax.

If the hypothesis that the upregulation of DII at metamorphic climax is responsible for the establishment of negative feedback were correct, one would predict that the late prometamorphic and climax stages (i.e. stage 59 and 62 respectively) would be most sensitive to TH negative feedback, particularly for T_4 . We observed the opposite trend in our pituitary explant culture studies, with stage 56 pituitaries being more sensitive to T_3 and, perhaps more importantly, to T_4 than either stage 59 or 62 pituitaries (Figs 2 and 3). The downregulation of TSH expression by T_4 suggests that DII is either active in the pituitary throughout prometamorphosis, or the conversion of T_4 to T_3 via DII is not required for negative feedback. Our pituitary developmental gene expression analysis shows that DII mRNA is present at stage 52, increases steadily to peak levels at stage 59 and remains elevated through stage 64 (Fig. 5). At first glance it would appear

that our data differ from those reported by Huang and colleagues (2001), i.e. that DII is only expressed at metamorphic climax. However, they examined DII expression only at stages 56 and 62 (and sample sizes were not reported), and they used the less sensitive method of Northern blotting. Our data indicate that, like several other genes in the pituitary, the expression of both DII and DIII increase in parallel with TH levels and TSH expression throughout metamorphosis (Fig. 5). Deiodinases likely play an important role in the regulation of intrapituitary TH concentrations, with DII and DIII working in concert to ensure appropriate TH levels. However, our data do not support the hypothesis that either gene plays a dominant role in sustaining the rise in thyroidal activity during metamorphosis, or that the expression of DII 'switches on' negative feedback at metamorphic climax.

To further address whether negative feedback is somehow activated at climax, we reevaluated the expression of pituitary TSH during *X. laevis* metamorphosis. In contrast to the data reported by Buckbinder and Brown (1993), we did not observe a sharp decline in TSH expression at metamorphic climax (Fig. 4). These data further argue against an abrupt onset of negative feedback at metamorphic climax. Okada and colleagues (2000) also failed to detect a sharp decline in TSH expression immediately after metamorphic climax in *R. catesbeiana*. In their study, a decline in TSH expression was not observed until Taylor-Kollros stage XXIV, which is equivalent to NF stage 65 in *X. laevis* development, and is the final stage of metamorphosis. Consistent with our finding that TSH expression does not decline until after stage 64, we also found that whole body T_4 content does not decline until metamorphosis is completed at stage 66 (Krain & Denver 2004).

Taken together, the findings of our study and those of others show that negative feedback is active throughout metamorphosis and there is no abrupt change in either the negative feedback response or deiodinase mRNA expression in the pituitary gland at metamorphic climax. If the lack of TH negative feedback on pituitary TSH is not responsible for facilitating the sustained rise in thyroidal activity during prometamorphosis then what mechanisms might underlie the development of the high level of thyroidal activity necessary for the completion of metamorphosis? One mechanism, first proposed by Etkin and colleagues (Etkin *et al.* 1965, Etkin 1966) involves the maturation of hypothalamic neurosecretory nuclei and the median eminence, where neurohormones are delivered to the pituitary portal circulation. This hypothesis is supported by both morphological and gene expression data (Etkin *et al.* 1965, Etkin 1966, Denver 1996). It is also possible that the sensitivity of the pituitary gland to neurohormonal stimulation develops during prometamorphosis in parallel with the development of the hypothalamus and median eminence. Such maturation of the

neuroendocrine system would result in the increased drive for TSH production.

It is well established that an intact hypothalamus and pituitary are required for metamorphosis. Metamorphic climax can be prevented by ablation of the hypothalamic primordia, ecotopic transplantation of the pituitary gland or disruption of the hypothalamic-pituitary portal system by physical barriers (reviewed by Denver 1996). In mammals and other higher vertebrates, TRH is the primary regulator of TSH synthesis and secretion, and thus of the thyroid axis. In amphibians, TRH does not regulate thyroid secretion in larvae, but it can stimulate TSH secretion in adults (see Denver 1996, Okada *et al.* 2004). There is now considerable evidence that shows that CRF functions as a larval amphibian TSH releasing factor (reviewed by Denver *et al.* 2002). Okada and colleagues (2004) recently demonstrated, using an homologous TSH radioimmunoassay, that TRH has no effect, but CRF is a potent secretagogue for TSH on pituitaries derived from bullfrog tadpoles. Furthermore, CRF-like peptides can modulate amphibian metamorphosis through the stimulation of both the thyroid and interrenal axes (reviewed by Denver *et al.* 2002). Our studies show that the expression of the mRNAs for the CRF receptors (CRF₁ and CRF₂) increased in the tadpole pituitary throughout prometamorphosis, with a peak at stage 59, and continued expression at this peak level through stage 64 (Fig. 6). This finding suggests that the sensitivity of the tadpole pituitary to CRF increases during metamorphosis. Recently, De Groef and colleagues (2003) showed that CRF₂ expression is localized to the thyrotropes in the chick pituitary, while CRF₁ is expressed only in corticotropes. The secretion of TSH by the chick pituitary gland is stimulated by CRF, and De Groef and colleagues (2003) provided pharmacological evidence that this stimulation is mediated by CRF₂. We hypothesize that a similar expression pattern of CRF receptors exists in the tadpole pituitary, although we have not yet tested this.

For TRH, while the levels of TRH-R1 mRNA were relatively constant, we observed a strong increase in pituitary TRH-R2 mRNA during prometamorphosis. Three putative TRH receptors have been cloned in *X. laevis* (Bidaud *et al.* 2002). Recently, Lu and colleagues (2003) found that the TRH-R1 exhibits high affinity for TRH and TRH analogs similar to the mouse TRH-R1. By contrast, the frog TRH-R2 exhibited lower affinity for TRH analogs, but the hierarchy of binding was similar to the mammalian TRH receptors (Lu *et al.* 2003). Interestingly, the receptor designated TRH-R3 exhibited very low affinities for all TRH analogs tested, which led the authors to conclude that the putative *X. laevis* TRH-R3 is a receptor for another peptide ligand (Lu *et al.* 2003). Galas and colleagues (2003) found, using *in situ* hybridization histochemistry, that while TRH-R1 and TRH-R2 mRNAs are expressed in the *X. laevis* pars distalis, the 'TRH-R3' mRNA is only expressed in the pars

intermedia. We did not analyze the expression of this 'TRH-R3' gene in our study. The increased expression of TRH-R2 in the tadpole pituitary gland during metamorphosis suggests the possibility that the gland increases its sensitivity to TRH at this time. While TRH does not affect tadpole TSH secretion, it has been hypothesized that TRH could contribute to the rise in pituitary prolactin production that occurs during late prometamorphosis (White & Nicoll 1981, Norris 1989, Buckbinder & Brown 1993).

Interestingly, we discovered that mRNAs for both CRF and TRH were expressed in the tadpole pituitary gland throughout metamorphosis (Figs 6 and 7), suggesting a possible autocrine or paracrine role for these neuropeptides. Paracrine and autocrine regulation of pituitary TSH and corticotropin (ACTH) by the intrapituitary expression of neuropeptides has been reported in humans and rats (Pagesy *et al.* 1992, Bruhn *et al.* 1998, Giraldi & Cavagnini 1998). TRH mRNA was localized to rat pituitary somatotropes (Bruhn 1994a,b,c) and TRH of pituitary origin can modulate both the secretion of TSH and the biosynthesis of pituitary TRH respectively (Bruhn *et al.* 1998). Similarly, CRF mRNA was localized to the corticotropes in the rat pituitary (Thompson 1987, Giraldi & Cavagnini 1998) and there is evidence for the regulation of ACTH secretion by CRF of pituitary origin (Giraldi & Cavagnini 1998).

In summary, our data clearly show that negative feedback at the level of the pituitary is active from early prometamorphosis in *X. laevis*, and that both T₄ and T₃ can suppress pituitary TSH mRNA expression throughout metamorphosis. We found that the components necessary for the pituitary gland to respond to neurohormones are expressed at the highest levels during the peak phase of thyroidal activity. Taken together with the observation that the hypothalamic neurosecretory neurons and the median eminence, the structure necessary for the delivery of neurohormone to the pituitary, develop during prometamorphosis under the influence of TH, our data support the hypothalamic drive hypothesis for the sustained production of TSH during amphibian metamorphosis.

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