

Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*

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Abstract

Corticosteroids, the primary circulating vertebrate stress hormones, are known to potentiate the actions of thyroid hormone in amphibian metamorphosis. Environmental modulation of the production of stress hormones may be one way that tadpoles respond to variation in their larval habitat, and thus control the timing of metamorphosis. Thyroid hormone and corticosteroids act through structurally similar nuclear receptors, and interactions at the transcriptional level could lead to regulation of common pathways controlling metamorphosis. To better understand the roles of corticosteroids in amphibian metamorphosis we analyzed the developmental and hormone-dependent expression of glucocorticoid receptor (GR) mRNA in the brain (diencephalon), intestine and tail of *Xenopus laevis* tadpoles. We compared the expression patterns of GR with expression of thyroid hormone receptor beta (TR β). In an effort to determine the relationship between nuclear hormone receptor expression and levels of ligand, we also analyzed changes in whole-body content of 3,5,3'-triiodothyronine (T₃), thyroxine, and corticosterone (CORT). GR transcripts of 8, 4 and 2 kb were detected in all tadpole tissues, but only the 4 and 2 kb transcripts could be detected in embryos. The level of GR mRNA was low during premetamorphosis in the

brain but increased significantly during prometamorphosis, remained at a constant level throughout metamorphosis, and increased to its highest level in the juvenile frog. GR mRNA level in the intestine remained relatively constant, but increased in the tail throughout metamorphosis, reaching a maximum at metamorphic climax. The level of GR mRNA was increased by treatment with CORT in the intestine but not in the brain or tail. TR β mRNA level increased in the brain, intestine and tail during metamorphosis and was induced by treatment with T₃. Analysis of possible crossregulatory relationships between GRs and TRs showed that GR mRNA was upregulated by exogenous T₃ (50 nM) in the tail but downregulated in the brain of premetamorphic tadpoles. Exogenous CORT (100 nM) upregulated TR β mRNA in the intestine. Our findings provide evidence for tissue-specific positive, negative and crossregulation of nuclear hormone receptors during metamorphosis of *X. laevis*. The synergy of CORT with T₃ on tadpole tail resorption may depend on the accelerated accumulation of GR transcripts in this tissue during metamorphosis, which may be driven by rising plasma thyroid hormone titers.

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Introduction

The primary morphogen controlling amphibian metamorphosis is thyroid hormone, which regulates gene transcription by binding to proteins that are members of the nuclear receptor superfamily (Shi 2000). Two genes designated alpha and beta code for thyroid hormone receptors (TRs) in vertebrates (Forrest 1994, Mangelsdorf *et al.* 1995). In *Xenopus laevis*, TR α mRNA increases shortly after hatching and remains relatively constant throughout the larval period and metamorphosis (Yaoita & Brown 1990). By contrast, TR β expression is low during premetamorphosis

before the acceleration of thyroid activity, but then increases dramatically throughout prometamorphosis, reaching a maximum at metamorphic climax (Yaoita & Brown 1990). The expression of TR β in *X. laevis* is induced by thyroid hormone (i.e. it is autoinduced; Tata 1994) and the *X. laevis* TR β gene contains at least one functional thyroid hormone response element in the proximal promoter region (reviewed by Shi 2000).

Corticosteroids produced by the interrenal glands (amphibian homologue of the mammalian adrenal cortex) are implicated in the positive control of metamorphosis (Kikuyama *et al.* 1993, Hayes 1997). Corticosteroids, like

thyroid hormone, regulate gene transcription by binding to nuclear receptors. Plasma corticosteroid concentrations rise markedly during metamorphic climax in several anuran species (Jaffe 1981, Krug *et al.* 1983, Jolivet-Jaudet & Leloup-Hatey 1984, Kikuyama *et al.* 1986) and in the urodele *Ambystoma tigrinum* (Carr & Norris 1988). This rise in plasma corticosteroids is largely synchronous with the rise in thyroid hormone production (Kikuyama *et al.* 1993, Denver 1998). Several investigators have shown that corticosteroids can synergize with thyroid hormone to accelerate tadpole metamorphosis (see Kikuyama *et al.* 1993, Hayes 1997) and at least two mechanisms have been proposed for this effect. For example, treatment with corticosteroids increases nuclear 3,5,3'-triiodothyronine (T_3) binding capacity in tailfin of two anuran species (Niki *et al.* 1981, Suzuki & Kikuyama 1983b). Another mode of action may be through the regulation of tissue deiodinases, where corticosteroids can enhance conversion of thyroxine (T_4) to T_3 , the more biologically active hormone, and decrease the degradation of T_3 (Galton 1990).

There is biochemical evidence for the presence of corticosteroid receptors in tadpole tissues (reviewed by Kikuyama *et al.* 1993) but there are currently no data on the expression or hormonal regulation of corticosteroid receptor genes during metamorphosis. Gao *et al.* (1994) isolated a cDNA for the *X. laevis* glucocorticoid receptor (GR) and showed, using Northern blot analysis, that the gene is first expressed during late embryogenesis; however, they did not analyze GR expression during metamorphosis. Csikos *et al.* (1995) isolated partial cDNA clones for a putative *X. laevis* mineralocorticoid receptor (MR). They detected a low level of MR mRNA in whole tadpoles at Nieuwkoop and Faber (NF) stage 60 (Nieuwkoop & Faber 1956) by an RNase protection assay, but were unable to detect MR transcripts by Northern blotting (we are also unable to detect MR mRNA in *X. laevis* tadpoles by Northern blotting, R J Denver, unpublished observations).

In the present study we analyzed the developmental and hormone-dependent expression of mRNA for the *X. laevis* GR in three tissues (brain, intestine and tail), and compared this with expression patterns of $TR\beta$. The level of GR mRNA in tadpole brain (the region of the diencephalon) was low during premetamorphosis, rose during prometamorphosis and then again in the juvenile frog. GR gene expression was strongly increased in tail throughout prometamorphosis and metamorphic climax. We found that positive or negative regulation by the respective ligand, or crossregulation of nuclear hormone receptor gene expression was tissue-specific. While $TR\beta$ was upregulated by its ligand in all three tissues examined, corticosterone (CORT) increased GR mRNA only in the intestine. Crossregulation studies showed that GR mRNA is negatively (brain) or positively (tail) regulated by exogenous T_3 , and $TR\beta$ is upregulated by CORT in tadpole intestine. Our results also show that tadpoles

concentrate T_3 from the environment, resulting in tissue concentrations of the hormone that are 4–6 times the environmental concentration. We discuss the implications of these findings for doses of T_3 commonly used to induce *X. laevis* metamorphosis.

Materials and Methods

Animals and hormone treatments

We obtained adult *X. laevis* from Xenopus I (Dexter, MI, USA) and spawned them in the laboratory by injecting into the dorsal lymph sac 1 μ g gonadotropin-releasing hormone agonist ([des-Gly¹⁰,D-His(Bzl)⁶]-LH-RH ethylamide; Sigma Chemical Co., St Louis, MO, USA). We reared tadpoles at 21–23 °C on a 12 h light:12 h darkness photoperiod in charcoal-purified, pH-adjusted water and fed them a suspension of pulverized rabbit chow. Hormones were added directly to the aquarium water. T_3 was dissolved in a small volume of 0.01 M NaOH and diluted 100-fold in 0.6% saline. Hormone stocks were then diluted ~300 000-fold in the aquarium water to give final concentrations of 5 or 50 nM. CORT was dissolved in 100% ethanol and this stock was then diluted in the aquarium water to give a final concentration of 100 or 500 nM (the final concentration of ethanol was 0.0001%).

Tadpoles were staged based on the system of Nieuwkoop & Faber (1956). Tadpoles were killed in 0.1% benzocaine before tissue collection. Animal rearing and dissection procedures were done in accordance with the rules and regulations of the University Committee on the Use and Care of Animals at the University of Michigan.

Hormone extraction and RIA

Tadpoles were snap frozen and stored at –80 °C before hormone extraction. Thyroid hormones were extracted from whole tadpoles following methods described by Denver (1993, 1998a). CORT was extracted from whole tadpoles following the method of Hayes & Wu (1995) with modifications described by Denver (1998a). Briefly, tadpoles were homogenized in 3–4 volumes of methanol containing 1 mM propylthiouracil for thyroid hormone extraction. For CORT extraction, tadpoles were homogenized in 3–4 volumes of ethyl acetate. For estimation of recoveries, 1000 c.p.m. [¹²⁵I] T_3 or 3000 c.p.m. [³H]CORT were added to each extract. Recoveries ranged from 35 to 60% for [¹²⁵I] T_3 and 30 to 45% for [³H]CORT. The validation of these methods for the extraction and recovery of hormones from tadpole tissues is described by Denver (1998a).

The hormones T_3 , T_4 and CORT were measured by RIA in extracts of whole *X. laevis* tadpoles. The T_3 and T_4 RIAs were as described (Mackenzie *et al.* 1978, Denver & Licht 1988, Denver 1998a) and the RIA for CORT was

described by Licht *et al.* (1983). Primary antisera for T_3 and CORT were purchased from Endocrine Sciences (San Diego, CA, USA). Primary antiserum for T_4 was obtained from Dr Viggo Kruse (Denmark). All samples were analyzed in single assays for each hormone.

RNA extraction and Northern blot analysis

Total RNA was extracted from pooled tadpole tissues (eight to ten brains (diencephalon only), five or six intestines or one to three tails) using Trizol reagent (Invitrogen). For Northern blot analysis total RNA (10 μ g) was separated by electrophoresis in a 1% formaldehyde-agarose gel, hydrolyzed in 0.05 M NaOH, 0.01 M NaCl, and transferred to nylon membrane using $20 \times$ SSC as the transfer buffer. Northern blots were stained with methylene blue to verify the integrity of the RNA and to assess RNA loading. Blots were prehybridized in Hybrisol I (Chemicon, Temecula, CA, USA) for 2–4 h and hybridized for 16 h at 42 °C with 32 P-labeled probes prepared from cDNAs provided by Yun-Bo Shi (*X. laevis*; National Institutes of Health, USA) TR β and ribosomal protein L8; rpL8) and Olivier Destree (*X. laevis* GR; Utrecht, The Netherlands). cDNAs were labeled with [32 P]dCTP by random priming (Roche). Blots were washed with $2 \times$ SSC, 0.5% SDS at room temperature for 10 min, then $0.25 \times$ SSC, 0.1% SDS at 65 °C for 1 h before exposure to X-ray film for 1–14 days. Blots were stripped by boiling in $1 \times$ TE (10 mM Tris, 1mM EDTA;pH 8.0), 1% SDS for 5 min and hybridized with a probe for the rpL8 gene (Shi & Liang 1994) to control for RNA loading.

Data analysis

Tadpole wet weight (body weight; BW) was measured using a digital balance (accurate to 0.01 g). Whole-body hormone contents are expressed as ng hormone/g BW. Previous studies showed that correction of hormone content using either DNA content or wet BW produced identical results (Denver 1993). Following autoradiography, Northern blot data were analyzed using a flatbed scanner and band densities were determined using Scion Image software (version 4.05; Scion Corporation Frederick, MD, USA). Each mRNA data point shown on the graphs represents three or four determinations in which tissues were isolated from tadpoles from different spawns, RNA isolated, and Northern blots prepared. After subtracting background on each Northern blot the densitometric values for GR or TR β were normalized to the rpL8 band. Northern blot data were \log_{10} -transformed to achieve homogeneity of variance before analysis by one-way ANOVA. Expression data are presented in the graphs as the mean percentage of maximum mRNA level (for developmental expression analyses) or the mean percentage of zero time (for the hormone treatment time course analyses). Hormone data were \log_{10} -transformed and

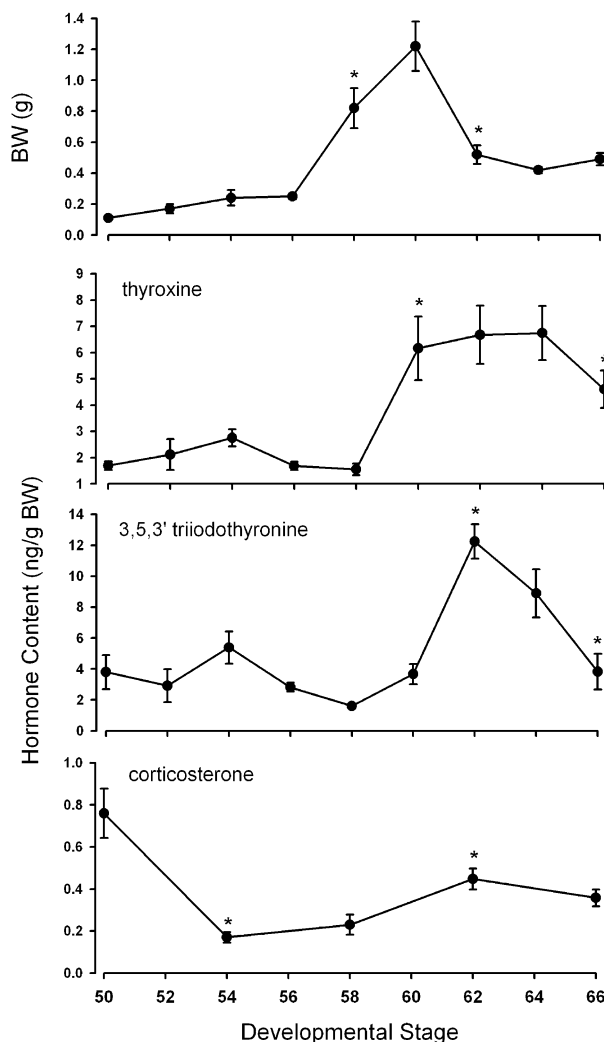


Figure 1 Developmental changes in BW, whole-body T_3 , T_4 and CORT content in *X. laevis* during metamorphosis. Thyroid hormone contents were determined by RIA following organic extraction as described in Materials and Methods. Data in the CORT Figure are modified from Glennemeier & Denver (2002). Each point represents the mean \pm S.E.M. ($n=6-8$ /stage). The asterisks designate significant differences from the preceding developmental stage ($P < 0.05$; Scheffe's test).

analyzed by one-way ANOVA followed by a post-hoc test (Scheffe's multiple contrast test; $P < 0.05$).

Results

Developmental profiles of whole-body T_3 , T_4 and CORT

We measured whole-body content of T_3 and T_4 in *X. laevis* tadpoles from premetamorphosis through to the end of metamorphosis (Fig. 1). This analysis showed the expected activation of the thyroid system characteristic of

anuran metamorphosis. Whole-body T_3 and T_4 content remained low and relatively constant during premetamorphosis and early prometamorphosis. Whole-body T_4 showed a strong increase (4-fold) from NF stage 58 to stage 60 and remained elevated throughout metamorphosis ($P < 0.001$; ANOVA). Mean whole-body T_4 content declined after metamorphic climax, and although the mean was approximately twice that of premetamorphic values, this difference was not statistically significant. Peak whole-body T_4 content (~ 6.5 ng/g BW) equals roughly 7 nM, which compares with a peak plasma concentration of ~ 10 nM as reported by Leloup & Buscaglia (1977) for *X. laevis*. For comparison, peak whole-body T_4 content was 7 and 10 nM in larvae of *Bufo japonicus* (Niinuma *et al.* 1991) and *B. marinus* (Weber *et al.* 1994) respectively.

By contrast, the increase in whole-body T_3 content (3.3-fold) lagged behind changes in T_4 ; whole-body T_3 did not show a statistically significant elevation until stage 62. Whole-body T_3 content remained elevated at stage 64 and declined after metamorphosis to levels seen in pre- and prometamorphic tadpoles ($P < 0.001$; ANOVA). Peak whole-body T_3 content (~ 12 ng/g BW) equals roughly 18 nM, which compares with a peak (NF stage 62) plasma T_3 concentration of 8 nM as reported by Leloup & Buscaglia (1977) for *X. laevis*. For comparison, peak whole-body T_3 content was 6 and 8 nM in larvae of *B. japonicus* (Niinuma *et al.* 1991) and *B. marinus* (Weber *et al.* 1994) respectively.

Data for developmental changes in whole-body CORT content are derived from Glennemeier & Denver (2002). By contrast to whole-body thyroid hormone content, whole-body CORT content was highest during premetamorphosis, declined significantly at the onset of prometamorphosis, but then rose slightly during metamorphic climax (stage 62; $P < 0.0001$; ANOVA).

Developmental expression of TR β and GR mRNAs in brain, intestine and tail during metamorphosis

We compared the developmental expression of TR β and GR mRNAs in tadpole brain, intestine and tail by Northern blotting (Figs 2 and 3). TR β mRNA levels were low and unchanging during premetamorphosis and early prometamorphosis but increased significantly in all tissues during late prometamorphosis ($P < 0.001$; ANOVA). The earliest elevation in brain TR β mRNA level was detected at stage 58. By contrast, TR β mRNA levels increased later in the intestine and tail (stage 60). Maximal expression occurred in the brain at stage 60, in the intestine at stage 62 and in the tail at stage 64. The TR β mRNA levels in the brain of the juvenile frog remained at a late prometamorphic level (compared with stage 58) but returned to premetamorphic levels in the intestine.

Three GR transcripts of 8, 5 and 3 kb were detected by Northern blot in the brain, intestine and tail (blot shown in Fig. 3 for brain only). The 8 kb transcript is not present in

embryos and is expressed only after hatching where it becomes the predominant transcript (data not shown for embryonic expression; see also Gao *et al.* 1994). Similarly, we found that several *X. laevis* cell lines (XTC-2, XL-58, A6, XL-177) expressed only the two smaller GR transcripts (data not shown; see also Spindler & Verrey (1999) for A6 cells).

Expression of GR mRNA in brain showed significant changes during metamorphosis, approximately doubling between premetamorphic and early prometamorphic stages (compare stages 49 and 52; $P < 0.0001$; ANOVA). Brain GR mRNA was then expressed at a constant level until after metamorphosis when it increased approximately 40% above levels seen at metamorphic climax. In the intestine, GR mRNA was expressed at a constant level during metamorphosis. There was a trend towards an increase ($\sim 20\%$) in the mean GR mRNA level at stage 60, which corresponds to the stage when TR β mRNA is strongly upregulated and the intestine begins to remodel (reviewed by Shi 2000). The level of GR mRNA in the juvenile intestine then declined approximately 20% relative to metamorphic levels. The strongest developmental regulation of GR mRNA was seen in the tail where levels increased throughout prometamorphosis and peaked at metamorphic climax (~ 3 -fold above early prometamorphic levels; $P < 0.05$) when the tail is actively resorbing.

Effects of exogenous T_3 on nuclear hormone receptor mRNA levels

Addition of T_3 (50 nM) to the rearing water caused statistically significant increases in TR β mRNA levels in all tissues ($P < 0.0001$ for all; ANOVA; Fig. 4). The kinetics of upregulation was similar among the different tissues; statistically significant increases in TR β transcripts were detected at 16 h in all tissues. It is likely that the upregulation occurred earlier than 16 h, since we and others have detected TR β upregulation by 8–12 h (L P Krain & R J Denver, unpublished observations; Yacota & Brown 1990, Kanamori & Brown 1992, Eliceiri & Brown 1994, Furlow & Brown 1999).

While the kinetics of TR β upregulation was similar in the three tissues analyzed, the dose sensitivity differed. Data for the 50 nM dose of T_3 are shown in Fig. 4. Treatment with 5 nM T_3 produced a similar increase in TR β mRNA in brain and intestine, but did not influence TR β gene expression in the tail (data not shown). Others have documented the relative insensitivity of gene expression in the tadpole tail to exogenous T_3 , which is the last tissue to transform (reviewed by Shi 2000).

By contrast to TR β , T_3 treatment downregulated GR mRNA in the brain ($P < 0.001$; ANOVA; Fig. 5). A significant reduction in brain GR mRNA was detected by 24 h after exposure to T_3 (both 5 and 50 nM T_3 – only the 50 nM dose is shown in Fig. 5). The effect of exogenous

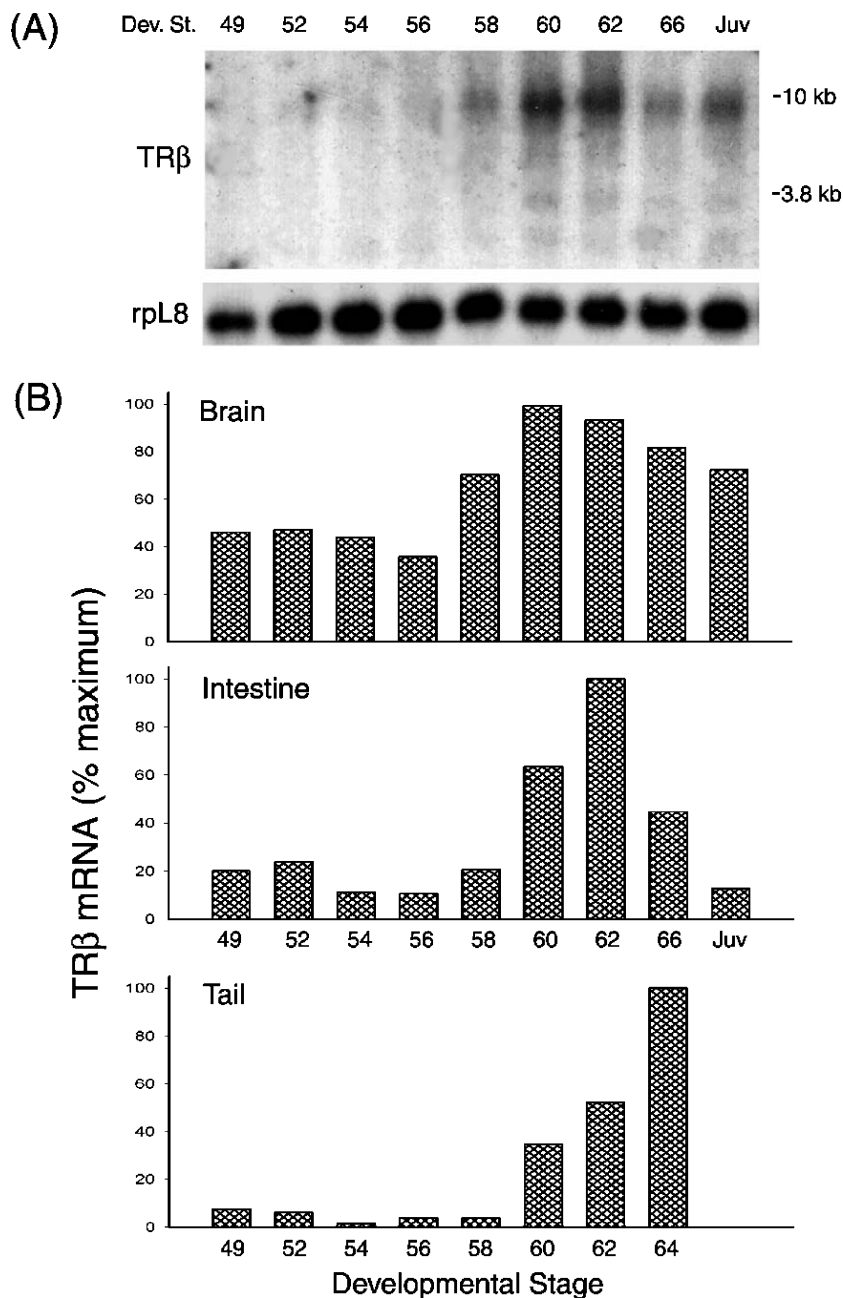


Figure 2 Developmental expression of TRβ mRNA in brain, intestine and tail during metamorphosis. TRβ expression was analyzed by Northern blotting as described in Materials and Methods. A representative Northern blot in brain is shown at the top (A). The graphs (B) show the quantitation of mRNA levels based on densitometric analysis of scanned autoradiograms. TRβ mRNA was normalized to the rpl8 band. Data are expressed as percentage of maximum. Each bar represents the mean of three independent experiments.

T₃ (5 or 50 nM) on GR mRNA levels in the intestine was biphasic, with a transient upregulation followed by a downregulation. The increases in intestinal GR mRNA levels (8–24 h) were not statistically significant but the

decreases at 48 and 72 h were ($P < 0.05$). In the tail, 50 nM T₃ upregulated GR mRNA, with a maximum level of expression at 72 h (~3-fold above the 0 time value; $P < 0.01$). Note that this magnitude of change in

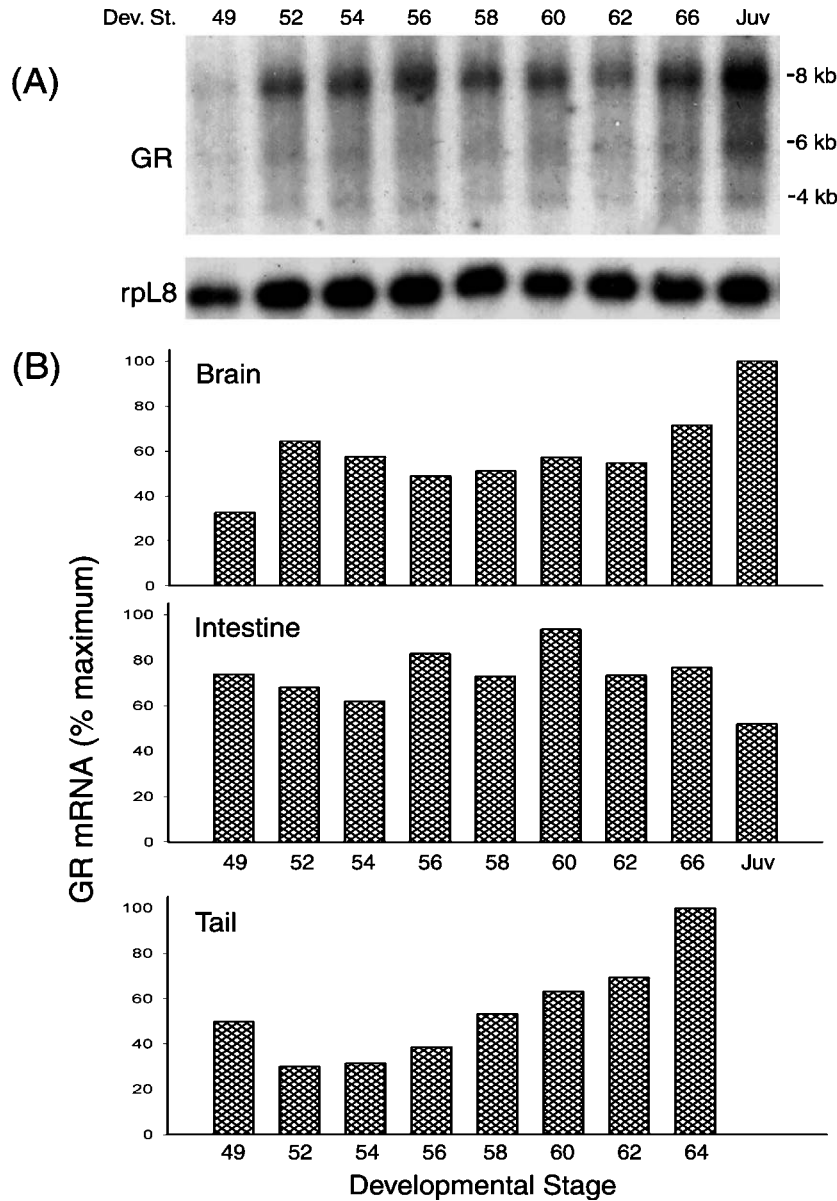


Figure 3 Developmental expression of GR mRNA in brain, intestine and tail during metamorphosis. GR expression was analyzed by Northern blotting as described in Materials and Methods. A representative Northern blot in brain is shown at the top (A). The graphs (B) show the quantitation of mRNA levels based on densitometric analysis of scanned autoradiograms. GR mRNA was normalized to the rpl8 band. Data are expressed as percentage of maximum. Each bar represents the mean of three independent experiments.

gene expression is comparable with that seen in the tail during spontaneous metamorphosis (Fig. 3).

Changes in whole-body T₃ content following exposure to exogenous T₃

It is generally assumed that the tissue content/plasma concentration of thyroid hormones achieved by addition

of hormone to the tadpole-rearing water equals the nominal concentration in the aquarium water i.e. tadpoles equilibrate with their environment. The choice of a 5 nM dose of T₃ in many studies in *X. laevis* is based upon this assumption (Shi & Brown 1993), and the report by Leloup & Buscaglia (1977) that peak plasma T₃ concentration during metamorphosis in this species reaches ~8 nM. To test this assumption we analyzed whole-body T₃ content

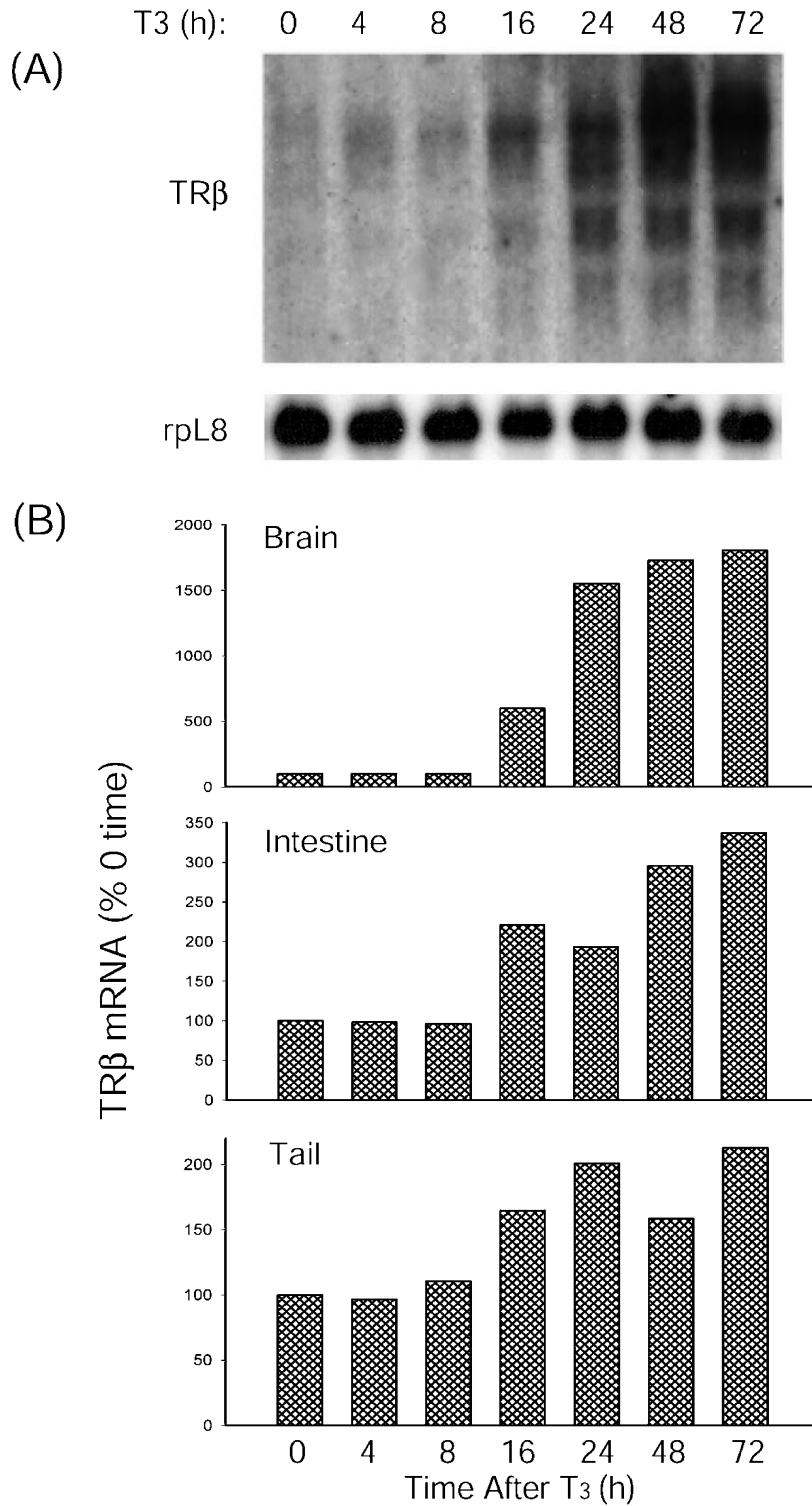


Figure 4 Effects of *in vivo* T₃ treatment on TR β mRNA in brain, tail and intestine. Tadpoles were exposed to T₃ (50 nM in the aquarium water) for various times before harvest for tissue isolation, RNA extraction and Northern blot analysis. Water was changed and the T₃ was replenished every 24 h. A representative Northern blot from brain is shown at the top (A). The graphs (B) show the quantitation of mRNA levels based on densitometric analysis of scanned autoradiograms. TR β mRNA was normalized to the rpL8 band. Data are expressed as percentage of the zero time value. Each bar represents the mean of four independent experiments.

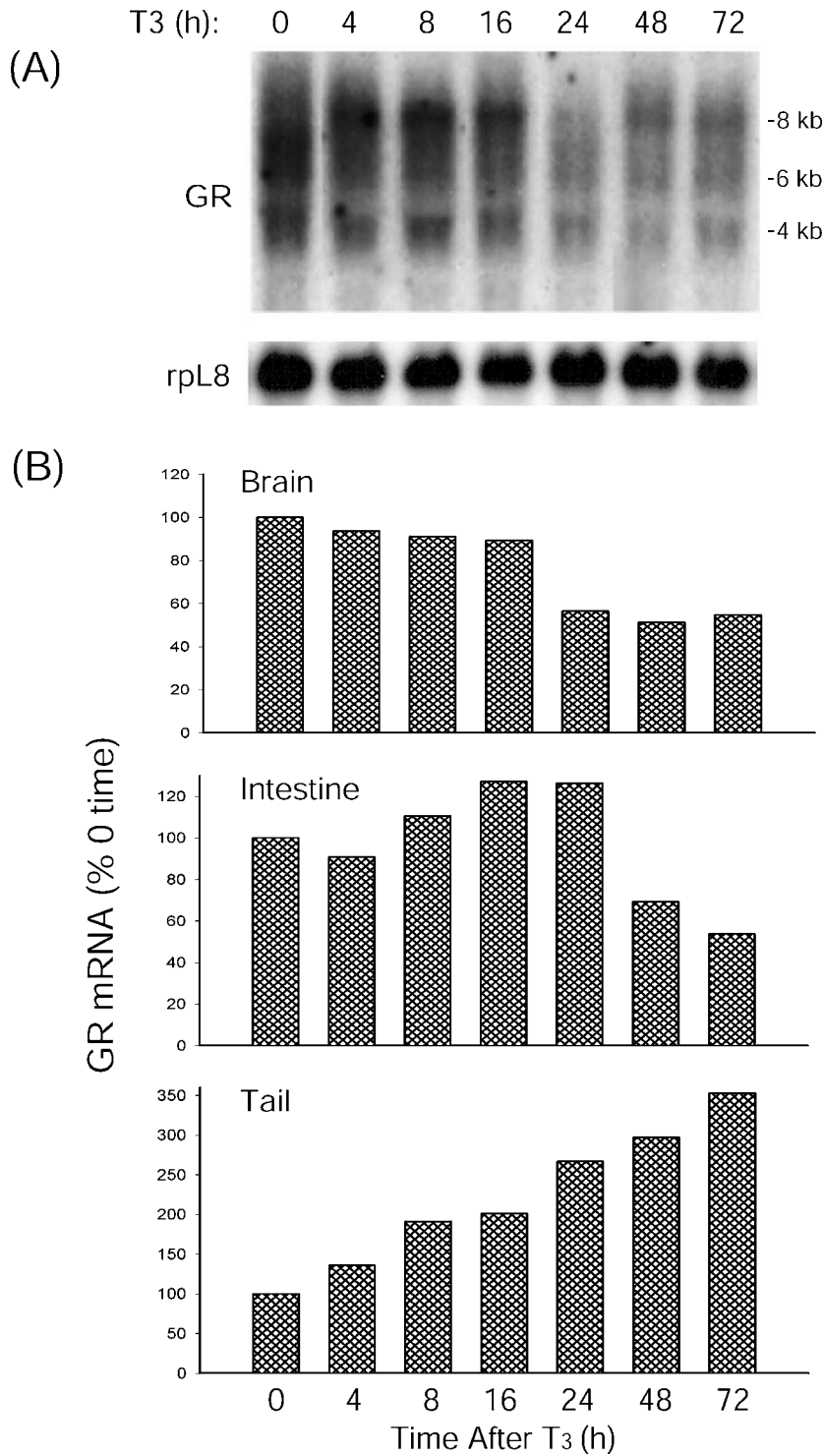


Figure 5 Effects of *in vivo* T₃ treatment on GR mRNA in brain, tail and intestine. Tadpoles were exposed to T₃ (50 nM in the aquarium water) for various times before harvest for tissue isolation, RNA extraction and Northern blot analysis. Water was changed and the T₃ was replenished every 24 h. A representative Northern blot from brain is shown at the top (A). The graphs (B) show the quantitation of mRNA levels based on densitometric analysis of scanned autoradiograms. GR mRNA was normalized to the rpL8 band. Data are expressed as percentage of the zero time value. Each bar represents the mean of four independent experiments.

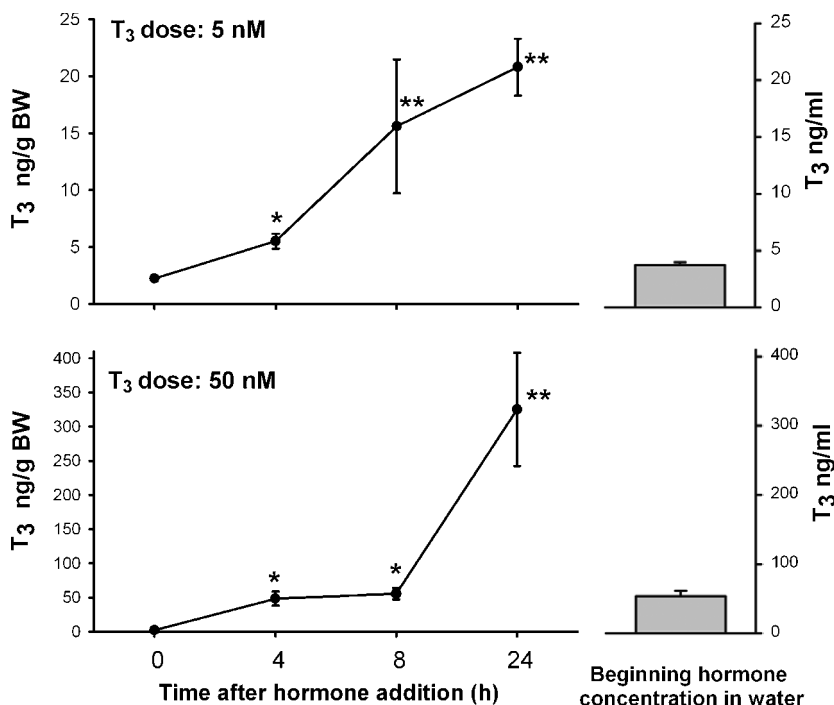


Figure 6 Time-dependent changes in whole-body T_3 content following exposure of premetamorphic tadpoles to two doses of T_3 in the aquarium water. T_3 was added to the aquarium water at time=0 and tadpoles were collected at various times and analyzed for whole-body T_3 content by RIA following organic extraction as described in Materials and Methods. Each point represents the mean ($n=8$ /time point) and vertical bars the S.E.M. *Significantly different from zero time ($P<0.001$); **significantly different from zero time and from the ambient T_3 concentration ($P<0.0001$).

in NF stage 52 tadpoles reared in aquarium water with two different doses of T_3 for various times. The nominal hormone concentrations in the aquarium water achieved were ~ 6 nM (for the intended 5 nM dose) and ~ 70 nM (for the intended 50 nM dose; Fig. 6). Our analysis showed that tadpole tissue hormone content reached environmental concentrations within 4 h of immersion for both doses (Fig. 6; compare line graphs with bars which show the concentration of T_3 in the water.) However, the T_3 tissue content continued to increase and reached a level that was 4–6 times greater than the beginning environmental concentration. The final whole-body T_3 concentration achieved was ~ 24 nM for the low dose and ~ 480 nM for the high dose. Thus, treatment of tadpoles with T_3 by addition to the aquarium water results in pharmacological levels of the hormone in tadpole tissues.

Effects of exogenous CORT on nuclear hormone receptor mRNA levels

In this experiment CORT was added to the rearing water to a final concentration of 100 nM and tadpoles were killed at different times thereafter up to 72 h. The water was not changed during the experiment (i.e. the CORT

was not replenished). No effect of CORT treatment on nuclear receptor mRNA was observed in the brain or tail (data not shown). In the intestine, CORT treatment resulted in a rapid, but transient upregulation of GR and TR β mRNAs (Fig. 7).

Changes in whole-body CORT content following exposure to exogenous CORT

Analysis of whole-body CORT content following addition of CORT to a final concentration of 100 nM (nominal concentration achieved ~ 125 nM) or 500 nM (nominal concentration achieved ~ 550 nM) to the aquarium water showed that while tissue CORT content increased rapidly, tissue content never reached the environmental concentration (Fig. 8). Furthermore, whole-body CORT content declined at 24 and 48 h following addition to the aquarium water. For the 100 nM dose, whole-body CORT content was significantly elevated at 2 h after addition of hormone to the aquarium water ($P<0.001$; ANOVA), continued to rise up to 8 h, but then declined at 24 and 48 h (but remained elevated above the 0 time value). For the 500 nM dose, tissue CORT content was significantly elevated at 2 h after

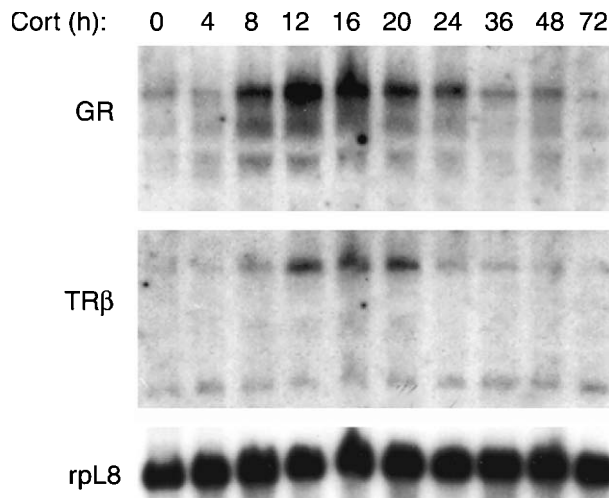


Figure 7 Effects of *in vivo* CORT treatment on GR and TR β mRNA levels in premetamorphic tadpole intestine. CORT was added to the aquarium water to a final concentration of 100 nM at time zero and tadpoles harvested at various times for tissue isolation, RNA extraction and Northern blot analysis. TR β and GR mRNAs were analyzed by Northern blotting as described in Materials and Methods.

addition of hormone to the aquarium water ($P < 0.0001$), but then gradually declined to 48 h (but remained elevated above the 0 time value).

Discussion

Corticosteroids have long been implicated in the positive control of amphibian metamorphosis, but little is known about their mechanism of action. This is the first study to analyze the developmental expression and hormonal regulation of GR mRNA, and to compare GR with TR β gene expression in *X. laevis* during metamorphosis. Our results show that the expression of GR mRNA increases in the brain and tail during metamorphosis. The strong upregulation of GR in the tail leading up to metamorphic climax may provide the molecular basis for corticosteroid enhancement of T₃-dependent tail regression (reviewed by Kikuyama *et al.* 1993). This accumulation of GR transcripts in tail may be mediated by rising plasma thyroid hormone concentrations, since we also found that exogenous T₃ increased GR mRNA in this tissue. Our data support the findings of others that show that there are tissue-specific differences in the timing of the onset of TR β expression and its peak, which may underlie the asynchronous tissue morphogenesis that occurs during spontaneous metamorphosis. Our studies also highlight important methodological issues relating to the administration of hormones in the aquarium water, and assump-

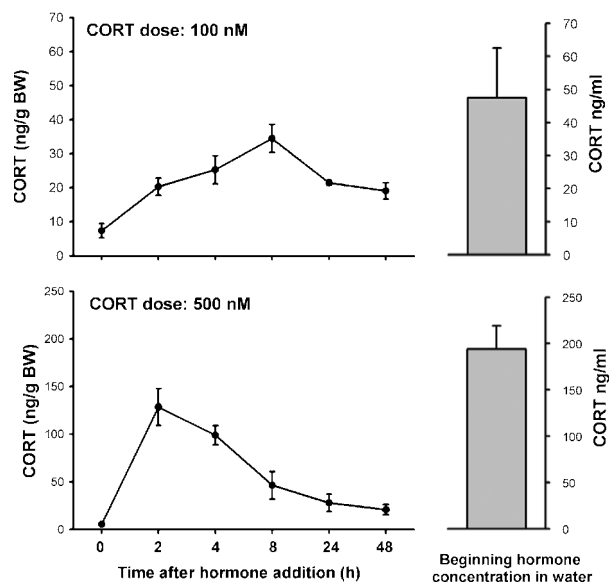


Figure 8 Changes in whole-body CORT content following exposure of premetamorphic tadpoles to two different doses of CORT in the aquarium water. The CORT was added to the aquarium water at time=0 and tadpoles were collected at various times and analyzed for whole-body CORT content by RIA following organic extraction as described in Materials and Methods. Each point represents the mean ($n=8$ /time point) and vertical bars the S.E.M.

tions about tissue hormone content achieved by such administrations.

Developmental patterns of hormone production and nuclear receptor expression

Thyroid hormones and TR β Our analyses of whole-body T₃ and T₄ showed that both hormones exhibited the expected increases during prometamorphosis and metamorphic climax in *X. laevis* as has been described for whole-body thyroid hormone content in other species (Niinuma *et al.* 1991, Weber *et al.* 1994, Denver 1998a) and for plasma thyroid hormone concentrations in *X. laevis* (Leloup & Buscaglia 1977, Tata *et al.* 1993) and other anurans (Regard *et al.* 1978, Mondou & Kaltenbach 1979, Weil 1986). We found that peak tissue hormone contents in *X. laevis* were comparable with those reported for other anurans (Niinuma *et al.* 1991, Weber *et al.* 1994, Denver 1998a). The whole-body T₃/T₄ ratio was highest at stage 62, which is consistent with the upregulation of deiodinase type II (D2) at this stage of development in tadpoles (shown for D2 activity and mRNA in various tissues of tadpoles of *Rana catesbeiana* (Becker *et al.* 1997), also D2 mRNA in *X. laevis* pituitary (R G Manzon & R J Denver, unpublished observations)).

It is worth noting that Leloup & Buscaglia (1977) did not report a significant rise in plasma T₄ or T₃ until NF

stages 59–60. Similarly, Tata (1993) was also unable to detect an increase in plasma T_3 until these late prometamorphic stages. Findings in *X. laevis* are also consistent with measures of plasma T_4 and T_3 in the bullfrog, *R. catesbeiana*, where significant increases in plasma hormone concentration were not observed until late prometamorphosis/early climax (Regard *et al.* 1978). Thus, the published plasma T_3 and T_4 concentrations in tadpoles during metamorphosis are consistent with our measures of whole-body hormone content, which showed significant increases only during late prometamorphosis. However, failure to detect significant elevations in thyroid hormones in tadpole plasma or tissues at earlier developmental stages likely reflects limitations in the sensitivity of the methods used. This conclusion is supported by findings that treatment with goitrogens can block metamorphosis when administered during premetamorphosis or early prometamorphosis (e.g. see Denver *et al.* 1997).

It is now well established that $TR\beta$ mRNA increases during tadpole metamorphosis (Yaoita & Brown 1990, reviewed by Shi 2000). Many of the earlier studies relied on measures of gene expression in whole tadpoles, or gross regions such as the head or 'middle'. In the current study we systematically compared the developmental and hormone-regulated expression of $TR\beta$ mRNA in brain, intestine and tail. We found that the developmental expression of $TR\beta$ mRNA roughly paralleled changes in whole-body thyroid hormone content. However, the timing of the onset of $TR\beta$ expression, and the stage of maximum expression, differed among the tissues analyzed. $TR\beta$ mRNA was highest for each tissue at the time of its major metamorphic change (Shi 2000). Wong & Shi (1995) reported nearly identical patterns of $TR\beta$ gene expression in the intestine and tail as we do. Others have reported highest $TR\beta$ mRNA (and protein) expression at stage 62 in *X. laevis* tadpole 'head' (Kawahara *et al.* 1991, Eliceiri & Brown 1994).

The expression of $TR\beta$ during metamorphosis depends on thyroid hormone (reviewed by Shi 2000). In all *X. laevis* tadpole tissues studied to date $TR\beta$ mRNA is upregulated by its ligand (i.e. it is autoinduced; see Fig. 4; Shi 2000). Sensitivity to the hormone differs among tissues, with the tail being the least sensitive. The difference in the timing of the onset and maximal $TR\beta$ mRNA levels among tissues likely reflects these differences in sensitivity to hormonal stimulation. In the brain, $TR\beta$ mRNA increased slightly before the rise in whole-body T_4 (stage 58 vs 60) and four developmental stages before the rise in whole-body T_3 (at stage 62). As for the inhibitory effects of goitrogens on premetamorphic and early prometamorphic tadpoles mentioned above, the earlier expression of $TR\beta$ (before increases in plasma or tissue thyroid hormone can be detected) further supports the conclusion that the sensitivity of the methods used to assay for thyroid hormones is limiting. Morphological and gene expression data support the view that the brain is

more sensitive to T_3 than most other tadpole tissues (not including the hind limb, which may show the earliest responses to T_3 ; see Kawahara *et al.* 1991, Denver 1998b). $TR\beta$ mRNA levels in the intestine and tail increased at the time when T_4 content was first elevated (stage 60) and continued to increase through metamorphic climax. Maximal $TR\beta$ mRNA level in the intestine (stage 62) was coincident with the peak in whole-body T_3 content, while maximal $TR\beta$ mRNA level in the tail (stage 64) occurred when T_3 was beginning to decline.

CORT and GR In *X. laevis*, CORT was found to be the major corticosteroid in plasma, with lesser amounts of aldosterone (Jolivet-Jaudet & Leloup-Hatey 1984, Kikuyama *et al.* 1993). As we (Glennemeier & Denver 2002) and Kloas *et al.* (1997) reported previously, whole-body CORT is highest during premetamorphosis and drops dramatically at the onset of prometamorphosis. Unlike Kloas and colleagues, we observed a small but significant rise in whole-body CORT at metamorphic climax (stage 62). This rise in whole-body CORT at metamorphic climax is consistent with increased plasma CORT concentrations at this stage as reported by Leloup-Hatey and colleagues for *X. laevis* (Jolivet-Jaudet & Leloup-Hatey 1984, Leloup-Hatey *et al.* 1988) and in other amphibian species (Kikuyama *et al.* 1993). Leloup-Hatey *et al.* (1988) also analyzed changes in CORT content in individual tissues (front and rear legs, tail, intestine, skin and liver) and found increased CORT content in some but not all tissues as metamorphosis proceeded.

Unlike $TR\beta$ mRNA, which is ubiquitously upregulated in tadpole tissues during metamorphosis, the developmental expression of GR mRNA differed among tissues. We found that tail GR mRNA increased throughout prometamorphosis and reached a maximum at metamorphic climax (stage 64). Leloup-Hatey *et al.* (1988) reported a large increase in CORT content in the *X. laevis* tail at the end of metamorphic climax (stage 64), which would be consistent with an increased number of CORT-binding sites (reflected in increased GR mRNA) in the tail at this developmental stage. High-affinity corticosteroid-binding sites have been demonstrated in cytosolic fractions of liver, intestine and tailfin of tadpoles of *X. laevis* (reviewed by Kikuyama *et al.* 1993).

By contrast to the ubiquitous upregulation of $TR\beta$ by its ligand in *X. laevis* tissues, the regulation of GR expression by CORT appears to be tissue-specific. Both up- and downregulation of corticosteroid receptor gene expression by corticosteroids have been reported in mammalian cells (Schmidt & Meyer 1994). In the tadpole intestine we found that GR mRNA was upregulated by CORT, but was unaffected in the brain or tail. Whether this regulation in the intestine has physiological significance is uncertain, since the intestinal GR mRNA level did not change significantly during spontaneous

metamorphosis. The level of brain GR mRNA was low during premetamorphosis but increased significantly during prometamorphosis and remained at a constant level throughout metamorphosis. This increase in brain GR mRNA in early prometamorphosis is correlated with a dramatic drop in whole-body CORT content at this time. Although we did not find that CORT altered GR mRNA in the brain in the current study, other findings of ours suggest that brain GR mRNA may be negatively regulated by corticosteroids (L P Krain & R J Denver, unpublished observations). Negative regulation of GR (and MR) by corticosteroids has been reported in the *X. laevis* kidney cell line A6 (Hagley & Watlington 1996, Spindler & Verrey 1999). Thus, if brain GR is negatively regulated by CORT then the increase in brain GR mRNA at the onset of prometamorphosis could depend on the decline in corticosteroid biosynthesis.

Crossregulation of nuclear hormone receptors

T₃ regulation of GR There are numerous examples of crossregulation of nuclear hormone receptor expression in animals (see Tata 1994). For example, in *X. laevis* liver cells estrogen receptor expression is regulated by thyroid hormone and glucocorticoids (Ulisse & Tata 1994). In the current study we found that T₃ upregulated GR expression in the tail, and this regulation may be consistent with a physiological regulatory relationship, given the developmental pattern of thyroid hormone production and GR mRNA in the tail. Exogenous and endogenous corticosteroids are known to enhance thyroid hormone-induced and spontaneous metamorphosis (Kikuyama *et al.* 1993, Hayes 1997). The increase in tail GR mRNA may be driven by increasing plasma titers of T₃, resulting in increased tissue sensitivity to corticosteroids at metamorphic climax.

By contrast, we found that T₃ downregulated GR expression in the brain. Presently, we do not know if this negative regulation of GR by T₃ in the brain is physiologically relevant, since we did not observe a decline in brain GR mRNA when tissue thyroid hormone content was highest.

CORT regulation of TR β Positive regulation of TR expression by GR is implied by the finding that corticosteroids enhance nuclear T₃ binding capacity in tadpole tail (Niki *et al.* 1981, Suzuki & Kikuyama 1983a). This effect of corticosteroids has been proposed as one mechanism whereby corticosteroids synergize with thyroid hormone to promote morphogenesis (Kikuyama *et al.* 1993). We found that CORT treatment upregulated TR β expression in the intestine but not in the brain or tail of premetamorphic tadpoles. We should note, however, that in tail explant cultures we found that CORT could induce a small but significant increase in TR β mRNA

(E D Hoopfer and R J Denver, unpublished observations). Thus, it is possible that CORT can upregulate TR β expression in the tail *in vivo* as it did in the intestine, but our limit of detection was exceeded in the current study.

Methodological issues

Our findings have highlighted several methodological issues that should be considered when conducting hormone treatments via addition to aquarium water. Animals concentrate T₃ from the environment and the tissue concentration achieved is several-fold greater than would be predicted based on a simple equilibration with the environment (e.g. see Shi & Brown 1993). These results highlight the importance of accounting for the endogenous hormone level achieved when evaluating the physiological significance of gene expression that is induced or repressed by exogenous hormone. Our data support the prediction that to achieve a tissue concentration of T₃ within the physiological range in *X. laevis* one must work with concentrations in the aquarium water between 0.1 and 2 nM.

A second finding is that the increase in tissue content of CORT by tadpole tissues following a single addition of hormone to the aquarium water is transient and decays relatively rapidly. We hypothesize that this phenomenon may account for the transient upregulation of GR and TR β mRNAs in the intestine (Fig. 7). Thus, to maintain constant tissue hormone concentrations one may need to frequently replenish the hormone in the tank (static renewal every 8–10 h) or use a flow-through system.

Conclusions

Corticosteroids are the primary circulating stress hormones in vertebrates, and their production is increased following exposure to environmental stress. These stress hormones are known to synergize with thyroid hormone to promote metamorphosis, and links between the thyroid and stress axes are present at multiple levels (e.g. pituitary regulation, deiodinase expression, etc.; see Denver *et al.* 2002). Thus, a means for modulating the timing of metamorphosis may be to control the production and actions of corticosteroids, which may alter the sensitivity of tadpole tissues to the actions of T₃.

The present study focused on tissue hormone content and nuclear hormone receptor gene expression during spontaneous metamorphosis (i.e. in the absence of stress), but whole-body CORT content (and thyroid hormone content) can increase dramatically in tadpoles in response to environmental stress (Glennemeier & Denver 2002). Such stress-induced alterations in CORT could affect GR or TR β expression, and thus the actions of CORT and T₃ on target tissues during metamorphosis.

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