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 (TRβ). 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. TRβ 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 TRβ 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.


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, TRα mRNA increases shortly after hatching and remains relatively constant throughout the larval period and metamorphosis (Yaoita & Brown 1990). By contrast, TRβ expression is low during premetamorphosis before the acceleration of thyroid activity, but then increases dramatically throughout metamorphosis, reaching a maximum at metamorphic climax (Yaoita & Brown 1990). The expression of TRβ in X. laevis is induced by thyroid hormone (i.e. it is autoinduced; Tata 1994) and the X. laevis TRβ 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 (T3) 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 (T4) to T3, the more biologically active hormone, and decrease the degradation of T3 (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, where corticosteroids can enhance conversion of thyroxine (T4) to T3, the more biologically active hormone, and decrease the degradation of T3 (Galton 1990).

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 TRβ. The level of GR mRNA in tadpole brain (the region of the diencephalon) was low during premetamorphosis, rose during metamorphosis and then again in the juvenile frog. GR gene expression was strongly increased in tail throughout metamorphosis 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 TRβ 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 T3, and TRβ is upregulated by CORT in tadpole intestine. Our results also show that tadpoles concentrate T3 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 T3 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 µg gonadotropin-releasing hormone agonist ([des-Gly10,pro-Has(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. T3 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 0·001%).

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. [125]IT3 or 3000 c.p.m. [3H]CORT were added to each extract. Recoveries ranged from 35 to 60% for [125]IT3 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 T3, T4 and CORT were measured by RIA in extracts of whole X. laevis tadpoles. The T3 and T4 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 T₃ and CORT were purchased from Endocrine Sciences (San Diego, CA, USA). Primary antiserum for T₄ 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 µg) 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 × 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 32P-labeled probes prepared from cDNAs provided by Yun-Bo Shi (X. laevis; National Institutes of Health, USA) TRβ and ribosomal protein L8; rpL8) and Olivier Destree (X. laevis GR; Utrecht, The Netherlands). cDNAs were labeled with [32P]dCTP by random priming (Roche). Blots were washed with 2 × SSC, 0.5% SDS at room temperature for 10 min, then 0.25 × 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 × 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 TRβ were normalized to the rpL8 band. Northern blot data were log₁₀-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 log₁₀-transformed and 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 T₃, T₄ and CORT

We measured whole-body content of T₃ and T₄ 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 T₃ and T₄ content remained low and relatively constant during premetamorphosis and early premetamorphosis. Whole-body T₄ 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 T₃ 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 T₃ 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 T₃ content was 7 and 10 nM in larvae of Bufo japonicus (Ninuma et al. 1991) and B. marinus (Weber et al. 1994) respectively.

By contrast, the increase in whole-body T₃ content (3.3-fold) lagged behind changes in T₄; whole-body T₃ did not show a statistically significant elevation until stage 62. Whole-body T₃ 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 T₃ content (~12 ng/g BW) equals roughly 18 nM, which compares with a peak (NF stage 62) plasma T₃ concentration of 8 nM as reported by Leloup & Buscaglia (1977) for X. laevis. For comparison, peak whole-body T₃ content was 6 and 8 nM in larvae of B. japonicus (Ninuma 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 metamorphosis, declined significantly at the onset of prometamorphosis, and declined after metamorphosis to levels seen in premetamorphic tadpoles (P<0.0001; ANOVA). The strongest developmental regulation of GR mRNA was seen in the tail where levels increased throughout metamorphosis and peaked at metamorphic climax (~3-fold above early prometamorphic levels; P<0.005) when the tail is actively resorbing.

**Developmental expression of TRβ and GR mRNAs: in brain, intestine and tail during metamorphosis**

We compared the developmental expression of TRβ and GR mRNAs in tadpole brain, intestine and tail by Northern blotting (Figs 2 and 3). TRβ mRNA levels were low and unchanged during premetamorphosis and early prometamorphosis but increased significantly in all tissues during late prometamorphosis (P<0.0001; ANOVA). The earliest elevation in brain TRβ mRNA level was detected at stage 58. By contrast, TRβ 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 TRβ 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 (~20%) in the mean GR mRNA level at stage 60, which corresponds to the stage when TRβ 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 metamorphosis and peaked at metamorphic climax (~3-fold above early prometamorphic levels; P<0.05) when the tail is actively resorbing.

**Effects of exogenous T₃ on nuclear hormone receptor mRNA levels**

Addition of T₃ (50 nM) to the rearing water caused statistically significant increases in TRβ 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 TRβ 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 TRβ upregulation by 8–12 h (L P Krain & R J Denver, unpublished observations; Yaoita & Brown 1990, Kanamori & Brown 1992, Eliceri & Brown 1994, Furlow & Brown 1999).

While the kinetics of TRβ upregulation was similar in the three tissues analyzed, the dose sensitivity differed. Data for the 50 nM dose of T₃ are shown in Fig. 4. Treatment with 5 nM T₃ produced a similar increase in TRβ mRNA in brain and intestine, but did not influence TRβ gene expression in the tail (data not shown). Others have documented the relative insensitivity of gene expression in the tadpole tail to exogenous T₃, which is the last tissue to transform (reviewed by Shi 2000).

By contrast to TRβ, T₃ 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 T₃ (both 5 and 50 nM T₃ — only the 50 nM dose is shown in Fig. 5). The effect of exogenous
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 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.
gene expression is comparable with that seen in the tail during spontaneous metamorphosis (Fig. 3).

Changes in whole-body $T_3$ content following exposure to exogenous $T_3$

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 $T_3$ in many studies in X. laevis is based upon this assumption (Shi & Brown 1993), and the report by Leloup & Buscaglia (1977) that peak plasma $T_3$ concentration during metamorphosis in this species reaches $\sim 8$ nM. To test this assumption we analyzed whole-body $T_3$ content

![Figure 3](image_url)

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.
Figure 4 Effects of in vivo T3 treatment on TRβ 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. TRβ 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.
in NF stage 52 tadpoles reared in aquarium water with two different doses of T₃ 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 T₃ in the water.) However, the T₃ tissue content continued to increase and reached a level that was 4–6 times greater than the beginning environmental concentration. The final whole-body T₃ concentration achieved was ~24 nM for the low dose and ~480 nM for the high dose. Thus, treatment of tadpoles with T₃ 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 TRβ 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 addition to the aquarium water (P<0.0001; ANOVA) and remained elevated up to 48 h.
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\(\beta\) 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\(\beta\) 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 assumptions about tissue hormone content achieved by such administrations.

Developmental patterns of hormone production and nuclear receptor expression

Thyroid hormones and TR\(\beta\) 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 T3 until these late prometamorphic stages. Findings in X. laevis are also consistent with measures of plasma T4 and T3 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 T3 and T4 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 Denyer et al. 1997).

It is now well established that TRβ mRNA increases during tadpole metamorphosis (Yaota & 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 TRβ mRNA in brain, intestine and tail. We found that the developmental expression of TRβ mRNA roughly paralleled changes in whole-body thyroid hormone content. However, the timing of the onset of TRβ expression, and the stage of maximum expression, differed among the tissues analyzed. TRβ mRNA was highest for each tissue at the time of its major metamorphic change (Shi 2000). Wong & Shi (1995) reported nearly identical patterns of TRβ gene expression in the intestine and tail as we do. Others have reported highest TRβ mRNA (and protein) expression at stage 62 in X. laevis tadpole ‘head’ (Kawahara et al. 1991, Eliceiri & Brown 1994).

The expression of TRβ during metamorphosis depends on thyroid hormone (reviewed by Shi 2000). In all X. laevis tadpole tissues studied to date TRβ 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 TRβ mRNA levels among tissues likely reflects these differences in sensitivity to hormonal stimulation. In the brain, TRβ mRNA increased slightly before the rise in whole-body T4 (stage 58 vs 60) and four developmental stages before the rise in whole-body T3 (at stage 62). As for the inhibitory effects of goitrogens on premetamorphic and early prometamorphic tadpoles mentioned above, the earlier expression of TRβ (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 T3 than most other tadpole tissues (not including the hind limb, which may show the earliest responses to T3; see Kawahara et al. 1991, Denyer 1998b). TRβ mRNA levels in the intestine and tail increased at the time when T4 content was first elevated (stage 60) and continued to increase through metamorphic climax. Maximal TRβ mRNA level in the intestine (stage 62) was coincident with the peak in whole-body T3 content, while maximal TRβ mRNA level in the tail (stage 64) occurred when T3 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 & Denyer 2002) and Kloas et al. (1997) reported previously, whole-body CORT is highest during premetamorphosis and drops dramatically at the onset of metamorphosis. 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 TRβ 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 metamorphosis 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 TRβ 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 metamorphosis and remained at a constant level throughout metamorphosis. This increase in brain GR mRNA in early metamorphosis 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 TRβ 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 TRβ 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 TRβ mRNA (E D Hoopfer and R J Denver, unpublished observations). Thus, it is possible that CORT can upregulate TRβ 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 TRβ 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 TRβ expression, and thus the actions of CORT and T3 on target tissues during metamorphosis.
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References

Becker KB, Stephens KC, Davey JC, Schneider MJ & Galton VA 1997 The type 2 and type 3 iodothyronine deiodinases play important roles in coordinating development in Rana catesbeiana tadpoles. *Endocrinology* **138** 2989–2997.


Forrest D 1994 The erbA thyroid hormone receptor genes in development of the central nervous system. *Seminar in Cancer Biology* **5** 167–176.


Kanamori A & Brown DD 1992 The regulation of thyroid hormone receptor beta genes by thyroid hormone in *Xenopus laevis*. *Journal of Biological Chemistry* **267** 739–745.


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Ulisse S & Tata JR 1994 Thyroid hormone and glucocorticoid independently regulate the expression of estrogen receptor in male Xenopus liver cells. Molecular and Cellular Endocrinology 105 45–53.


Wong J & Shi YB 1995 Coordinated regulation of and transcriptional activation by Xenopus thyroid hormone and retinoid X receptors. Journal of Biological Chemistry 270 18479–18483.