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# Corticosteroids disrupt amphibian metamorphosis by complex modes of action including increased prolactin expression

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ABSTRACT

Although thyroid hormones (TH) are the primary morphogens regulating amphibian metamorphosis, other hormones including corticosteroids are known to participate in this regulation. The present study investigated effects of corticosteroids on larval development of the amphibian *Xenopus laevis*. Premetamorphic tadpoles (stage 51) were treated with aldosterone (ALDO; 100 nM), corticosterone (B; 10, 100, 500 nM) and dexamethasone (DEX; 10, 100, 500 nM) for 21 days and organismal responses were assessed by gross morphology determining stage development, whole body length (WBL), and hind limb length (HLL). B and DEX reduced WBL and HLL and caused abnormal development including the lack of fore limb emergence while ALDO treatment showed no significant effect. Gene expression analyses using RT-PCR revealed up-regulation of prolactin (PRL) in brain, but down-regulation of type III deiodinase in tail tissue induced by the glucocorticoids B and DEX. Additionally, stromelysin-3 transcript in tail tissue was decreased by B. ALDO at 100 nM had no effect on mRNA expression, neither in brain nor in tail tissue. These findings indicate that corticosteroids modulate TH-dependent metamorphosis by complex mechanisms that even include indirect effects triggered by increased PRL mRNA expression.



## 1. Introduction

Thyroid hormones (TH) play a critical role for the initiation and regulation of amphibian metamorphosis (reviewed in Brown and Cai, 2007; Furlow and Neff, 2006). The development of the larval tadpole into a juvenile frog includes drastic morphological and physiological changes under the influence of rising TH levels with peak concentrations at metamorphic climax (Krain and Denver, 2004). Synthesis of TH by the thyroid gland is regulated via the hypothalamus–pituitary–thyroid (HPT) axis. Hypothalamic corticotropin-releasing factor (CRF) stimulates synthesis and secretion of thyroid-stimulating hormone (TSH) by the pituitary, which stimulates thyroidal TH synthesis. Circulating TH, in turn, feed negatively back on synthesis and secretion of CRF and TSH (Manzon and Denver, 2004; Denver, 1997). In peripheral tissues, TH action is mediated by TH receptors (TRs), which belong to the superfamily of nuclear hormone receptors (Tata et al., 1993). In the amphibian *Xenopus laevis*, two TR subtypes (TRα, TRβ) have been described. Characterization of their developmental expression profiles revealed that TRα is expressed early during development and shows a rather constitutive expression. In contrast, expression of TRβ is TH-dependent and high TRβ expression is associated with tissue remodelling during later development (Opitz et al., 2006b; Yaoita and Brown, 1990). The very complex metamorphic events are spatiotemporally coordinated and controlled by changes in thyroidal TH synthesis/release, but are also regulated by several mechanisms affecting the local availability of bioactive TH in the transforming tissues. The thyroid gland mainly synthesizes thyroxine (T4), which is converted to the more potent triiodothyronine (T3) via outer ring deiodination (Brown, 2005). Inner ring deiodination of T4 and T3, in turn, results in formation of the inactive iodothyronines reverse T3 and diiodothyronine, respectively. In *X. laevis*, three iodothyronine deiodinases (D1, D2, and D3) have been characterized (Kuiper et al., 2006; Brown, 2005) and tissue-specific expression patterns of D1, D2 and D3 have been related to the coordinated spatiotemporal progression of metamorphosis (Brown, 2005).

Besides TH, other hormonal factors have been found to be involved in the regulation of metamorphosis. A number of studies have provided evidence that corticosteroids can modulate the regulation of metamorphosis by TH (reviewed in Hayes, 1997). In tadpoles, the primary corticosteroids corticosterone (B) and aldosterone (ALDO) are present in considerable amounts already after hatching and characteristic changes of corticosteroid levels have been determined during larval development (Kloas et al., 1997).

Depending on the phase of larval development, treatment with exogenous corticosteroids has been shown to inhibit or accelerate metamorphosis (Hayes, 1995; Galton, 1990; Kikuyama et al., 1983, 1982; Leloup-Hatey et al., 1990; Wright et al., 1994). Inhibitory effects of corticosteroids have been demonstrated when treatment was initiated at premetamorphic stages, when levels of circulating TH are marginal. In

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contrast, treatment of tadpoles at more advanced developmental stages, characterized by a rising TH plasma concentration, resulted in the acceleration of metamorphosis (Hayes, 1997; Hayes et al., 1993). However, different studies of corticosteroid effects on metamorphosis yielded opposite results and mechanisms involved in corticosteroid action are not fully understood. One mechanism by which corticosteroids can modulate TH action in peripheral tissues is via regulation of iodothyronine deiodinase activities. Effects of corticosteroids on the expression and activity of deiodinases have evidenced in various vertebrates (Walpita et al., 2007; Van der Geyten and Darras, 2005; Darras et al., 2002; Galton, 1990). Accordingly, altered ratios of T4 and T3 have been found as consequence of corticosteroid treatment in amphibians (Hayes and Wu, 1995; Leloup-Hatey et al., 1990).

One further possible mode of corticosteroid action is via feedback mechanisms on the expression and secretion of hypothalamic CRF. In larval amphibians, CRF is known to be the primary hypothalamic factor stimulating the thyroid as well as the interrenal axis (Miranda et al., 2000; Denver, 1999; Shi, 1999).

Another important modulator of TH-dependent metamorphosis is the pituitary hormone prolactin (PRL) displaying inhibitory effects on TH-dependent tail resorption and hind limb development (Huang and Brown, 2000; Tata et al., 1991). Results from various studies suggest that effects of PRL are mediated at the level of TR interaction. PRL was shown to inhibit the autoinduction of TRβ mRNA by TH and to affect the expression of other TH-dependent genes (Baker and Tata, 1992; Shintani et al., 2002). During metamorphosis, PRL plasma levels peak at climax stages, that is concurrent with peaking TH plasma levels. Accordingly, one hypothesis regarding the physiological role of PRL during metamorphosis is to contribute to the regulation of TH-induced tissue remodelling by providing an inhibitory factor when TH levels are maximum (Shi, 1999).

A previous study provided first evidence for an involvement of PRL in corticosteroid action (Lorenz et al., 2009). To shed further light on the mechanisms involved in the modulatory effects of corticosteroids on metamorphosis, we examined in the present study organismal responses of *X. laevis* tadpoles treated with the corticosteroids B and ALDO, and the synthetic glucocorticoid dexamethasone (DEX) and performed gene expression analyses in brain and tail tissue.

## 2. Materials and methods

### 2.1. Animals and husbandry

*X. laevis* tadpoles were bred in the animal stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany. Injection of human chorionic gonadotropin (Sigma, Deisenhofen, Germany) into the dorsal lymph sac of adult *X. laevis* was used to induce spawning as described by Kloas et al. (1999). Fertilized eggs as well as hatched larvae were reared in 60-L tanks containing 40 L of reconstituted tap water, which was prepared by dissolving 2.5 g of a commercial marine salt (Tropic marin®; Germany) in 10 L deionized water as described in Opitz et al. (2005). Water temperature was adjusted to 22 ± 1 °C and pH value was 7.0 ± 0.5. Water was permanently aerated by airstones. Tadpoles were fed daily with Sera Micron (Sera, Heinsberg, Germany). On day 7 post fertilization, tadpoles were transferred to 11-L glass tanks containing 10 L of reconstituted tap water with a larval density of 4 individuals/L and raised until stage 51. Developmental stages were determined according to the normal table of Nieuwkoop and Faber (1994).

### 2.2. Experimental design

Tadpoles were treated for a total of 21 days according to the protocol for the conduct of the XEMA (*Xenopus* metamorphosis assay) test (Opitz et al., 2005) with various corticosteroids. In each case, a total of 25 tadpoles (stage 51) were placed in one of two replicate tanks containing ALDO (100 nM; Sigma), B (10, 100, and 500 nM; Sigma), DEX (10, 100, and 500 nM; Sigma) and dimethyl sulfoxide (DMSO; 0,002%; Sigma) as a solvent control (SC). Test solutions were changed out completely three times a week. General test conditions included a 12 h:12 h light:dark cycle, a water temperature of 22 ± 1 °C, pH value of 7.0 ± 0.5 and constant aeration by airstones. Tadpoles were fed daily with increasing amounts of Sera micron ranging from 10 to 20 mg food per animal and day. On experimental days 0, 7, 14, and 21, developmental stage and whole body length (WBL) was determined for all test animals as described by Opitz et al. (2005). In addition, hind limb length (HLL) was measured for all test animals on experimental days 14 and 21. At test termination on experimental day 21, five tadpoles were randomly selected from each replicate tank for sampling of brain tissue (including the pituitary) and tail tissue (a 20 mm piece of the tail tip). Tadpoles were chilled on ice and killed by decapitation. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until further processing.

### 2.3. Semi-quantitative RT-PCR

Total RNA was extracted from brain and tail tissue using Trizol reagent (Invitrogen, Karlsruhe, Germany) as described by Jagnytsch et al. (2006). RNA concentrations were determined by UV-absorbance measurements at 260 and 280 nm using a Spectrafluor Plus microplate reader (Tecan, Crailsheim, Germany). cDNA was reversely transcribed from 1 µg of total RNA using Avian Myeloblastosis Virus reverse transcriptase (AMV-RT; Biometra, Göttingen, Germany) as described by Jagnytsch et al. (2006).

PCR was performed in a 25 µL volume reaction containing 3 µL cDNA (10-fold diluted) as template, 18.7 µL water, 2.5 µL 10× buffer for Taq DNA polymerase (Qbiogene, Germany), 0.2 µL dNTP solution (10 mM of each dNTP, Qbiogene), 2.2 µL of each gene specific primer (50 µM working solution, TIB Molbiol GmbH, Germany) and 0.2 µL Taq DNA polymerase (5 U/µL, Qbiogene). All PCR reactions were carried out in a thermal cycler (Biometra). Thermal cycler protocols included initial denaturation at 94 °C for 4 min followed by cycles of denaturation (94 °C for 40 s), annealing (40 s), and extension (72 °C, 40 s) and a final extension step (72 °C, 10 min). All reactions were optimized for evaluating endpoint amplicon levels within the linear range. Primer sequences, cycle numbers and annealing temperatures used for detection of CRF, TSH (β-subunit), and PRL in brain–pituitary tissue, stromelysin-3 (STR-3) in tail tissue, and elongation factor 1α (EF), TRβ, D1, D2, and D3 in brain and tail tissue are listed in Table 1. Amplified PCR products (5 µL per sample) were

**Table 1**
Gene-specific primer sets and thermal cycler conditions for PCR analysis.

| Target gene | Forward (F) and reverse (R) primer | $T_A$ [°C] | Cycles |
|---|---|---|---|
| EF1α[a] | F: TGC CAT TGT TGA CAT GAT CCC<br>R: TAC TAT TAA ACT CTG ATG GCC | 59 | 17 |
| TRβ[a] | F: GTC GCT TCA AAA AGT GCA TCG<br>R: ACC CTC GGG CGC ATT AAC TAT | 67 | 30 |
| TSHβ[a] | R: AGA GTG CGC TTA CTG CCT TG<br>F: GGT AGG AAA AGA GCG GGT TC | 61 | 25 |
| PRL[b] | F: GTG TGA CTT CCC TAC CAA TAT G<br>R: TAT GGG AAT CCC TGC GAA GGC | 62 | 28 |
| CRF | F: TCT CCT GCC TGC TCT GTC CAA<br>R: CTT GCC ATT TCT AAG ACT TCA CGG | 64 | 27 |
| D1 | F: GAC CCC CAT TCC TCT TCA G<br>R: GCA TTG TTC AGC CGG TGT TCT TA | 58 | 28 |
| D2 | F: GCT CTG GCA AGT GAT GTG<br>R: GTG CTG CCT TTT ATG TTC TT | 61 | 29 |
| D3 | F: GGC CAA GTG GTC ATT CAA GT<br>R: GGA CTG GAC GTG TTG GAA GT | 61 | 29–30 |
| STR-3 | F: CAT TCC GTT ACC CAC TAA GC<br>R: CTG TGA GCC ATA GAA GAA CC | 58 | 30 |

$T_A$, annealing temperature.
[a] Taken from Opitz et al. (2006a).
[b] Taken from Imaoka et al. (2000).

electrophoresed on a 1.5% agarose gel and stained with ethidium bromide (GIBCO/BRL, Eggenstein, Germany). Images of ethidium bromide-stained gels were taken using the GelDoc 2000 system (Bio-Rad, Munich, Germany) and densitometric analyses were performed with Quantity One software (Bio-Rad). Densitometric values for EF were used to normalize values of target transcripts for variations in cDNA loading. For each target transcript, results from duplicate analyses of individual RNA samples were averaged yielding a single value for each RNA sample to be used in statistical analysis. PCR products were extracted from agarose gels using QIAquick kit (Qiagen), and the identity of the PCR products was confirmed by sequence analysis (Sequence Laboratories, Göttingen, Germany).

### 2.4. Statistics

Data obtained from WBL and HLL measurement as well as from densitometric measurements of PCR products were assessed for normality and homogeneity of variances. Data that met these criteria were analyzed by Dunnett's test to compare control data to all other treatment groups. All other data were analyzed using the non-parametric Dunn's multiple comparison test to determine whether significant differences existed between control and the other treatment groups. All statistical analyses were performed using the software Graphpad Prism4 (GraphPad Software, Inc.). Differences were regarded as being significant at $p<0.05$.

## 3. Results

### 3.1. Organismal responses based on gross morphology

Mortality was always below 10%, with the exception of the 500 nM B and 500 nM DEX treatment groups where increased rates of 20% and 16% were observed, respectively (Table 2). Determination of WBL revealed no effect on WBL in the ALDO treatment group, whereas exposure to B and DEX caused a concentration-dependent inhibition of larval growth (Fig. 1). The highest concentration of B significantly reduced WBL after 7, 14 and 21 days ($p<0.001$), respectively. Treatment with 100 nM B reduced WBL after 14 and 21 days ($p<0.001$), whereas 10 nM B did not affect WBL. The two highest concentrations of DEX decreased WBL after 7 ($p<0.05$ for 100 nM; $p<0.001$ for 500 nM), 14 and 21 ($p<0.001$) days of treatment significantly. Treatment with 10 nM DEX had no effect on WBL. Assessment of hind limb growth by measuring HLL revealed no effect of treatment with ALDO at 100 nM. Tadpoles exposed to the two highest concentrations of B showed a concentration-dependent reduction of HLL on experimental day 21 ($p<0.001$). DEX treatment decreased HLL on experimental day 21 in a concentration-dependent manner, with significant effects observed for 100 nM and 500 nM ($p<0.001$) (Fig. 2).

On study day 21, NF stages were determined for all tadpoles. Animals of the SC group developed to NF stages 57–60 (Table 2). Exposure to ALDO did not affect larval development. In contrast, B treatment caused an abnormal development. Regardless of the concentration tested, all tadpoles exposed to B showed a conspicuous narrowing of the head already after 7 days (see Fig. 3A and B). Moreover, within the experimental period of 21 days all tadpoles treated with B failed to show fore limb emergence, which characterizes the transition from developmental stage 57 to stage 58 (Nieuwkoop and Faber, 1994). On the other hand, hind limb development often corresponded to stages >58 (Fig. 3B and D).

**Table 2**
Mortality and stage development of *X. laevis* tadpoles following 21 days of treatment.

| Treatment | Mortality [%] | Stage development [mean ± SD] |
|---|---|---|
| SC | 9 | 57.4 ± 1.1 |
| 100 nM ALDO | 0 | 57.5 ± 1.1 |
| 10 nM B | 0 | a.d. |
| 100 nM B | 5 | a.d. |
| 500 nM B | 20 | a.d. |
| 10 nM DEX | 9 | 57.9 ± 1.1 |
| 100 nM DEX | 5 | a.d. |
| 500 nM DEX | 16 | a.d. |

SC, solvent control; B, corticosterone; ALDO, aldosterone; DEX, dexamethasone; a.d., abnormal development.

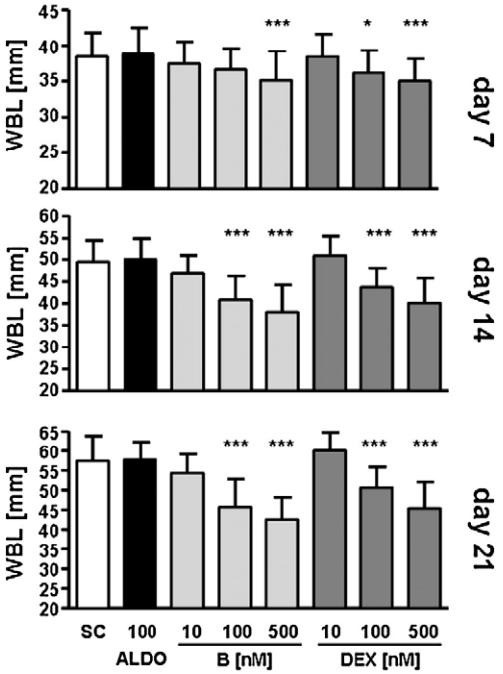


**Fig. 1.** Effects of corticosteroids on larval growth. Whole body length (WBL) was determined for all test animals on experimental days 7, 14 and 21. Tadpoles were treated with aldosterone (ALDO), corticosterone (B), dexamethasone (DEX) and a solvent control (SC). Data are shown as mean values ± standard deviations. Asterisks mark significant differences to the SC (*$p<0.05$, ***$p<0.001$; Dunn's multiple comparison test).

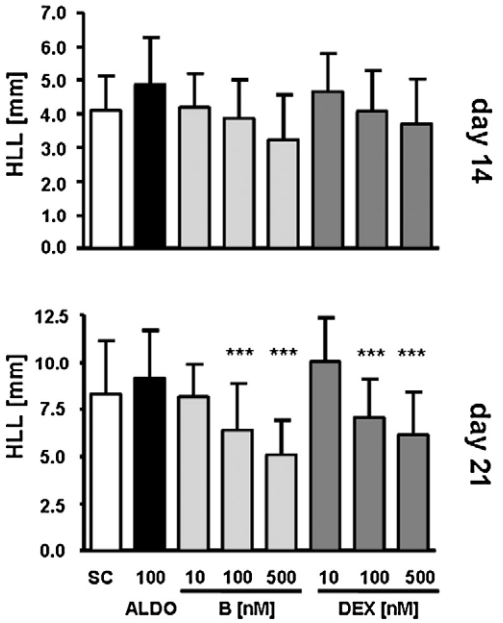


**Fig. 2.** Effects of corticosteroids on hind limb growth. Hind limb length (HLL) was determined for all test animals on experimental days 14 and 21. See Fig. 1 for details on treatment groups. Data are shown as mean values ± standard deviations. Asterisks mark significant differences to the SC (***$p<0.001$; Dunn's multiple comparison test).


**Fig. 3.** Effects of corticosteroids on tadpole gross morphology on experimental day 21. Dorsal and lateral view of a stage 58 tadpole of solvent control group (A and C). Tadpoles treated with corticosterone showed a conspicuous narrowing of the head, lack of fore limb emergence but normal hind limb morphogenesis (B and D).

Despite this effect was not measurable, a more pronounced tail fin was observed. Consequently, the description of developmental stages of B treated tadpoles according to the staging criteria of Nieuwkoop and Faber (1994) was impossible.

Treatment of tadpoles with the synthetic corticosteroid DEX resulted in a similar deviation from normal development (Table 2). However, in contrast to B, treatment with the lowest concentration of DEX (10 nM) did not exhibit such an abnormal development and overall developmental rates in the 10 nM DEX treatment group were not different compared to the SC group.

### 3.2. Gene expression analyses

On study day 21, brain was dissected together with the pituitary and analyzed for treatment-related changes in mRNA expression of several target transcripts using semi-quantitative RT-PCR. Treatment of tadpoles with different corticosteroids had no effect on relative expression of TRβ, TSHβ and CRF compared to SC group (Fig. 4). In addition, no treatment-related changes in mRNA expression were detected for the three deiodinases D1, D2, and D3 (Fig. 5). In contrast, corticosteroid treatments stimulated PRL mRNA expression. Treatment with 100 nM ALDO resulted in a more than 2-fold higher PRL expression but this effect was not statistically significant. Significant up-regulation of PRL mRNA expression was, however, detected for B and DEX at 100 ($p<0.05$) and 500 nM ($p<0.05$ for DEX and $p<0.01$ for B) (Fig. 4).

Furthermore tail tissue sampled on day 21 was analyzed for treatment-related changes in mRNA expression of TRβ, D1, D2, D3 and STR-3. Corticosteroid treatments did not alter relative expression levels of TRβ, D1 and D2, (Fig. 6). However, D3 mRNA expression was decreased in a concentration-dependent manner by B and DEX treatments. These effects were significant for tadpoles exposed to 100

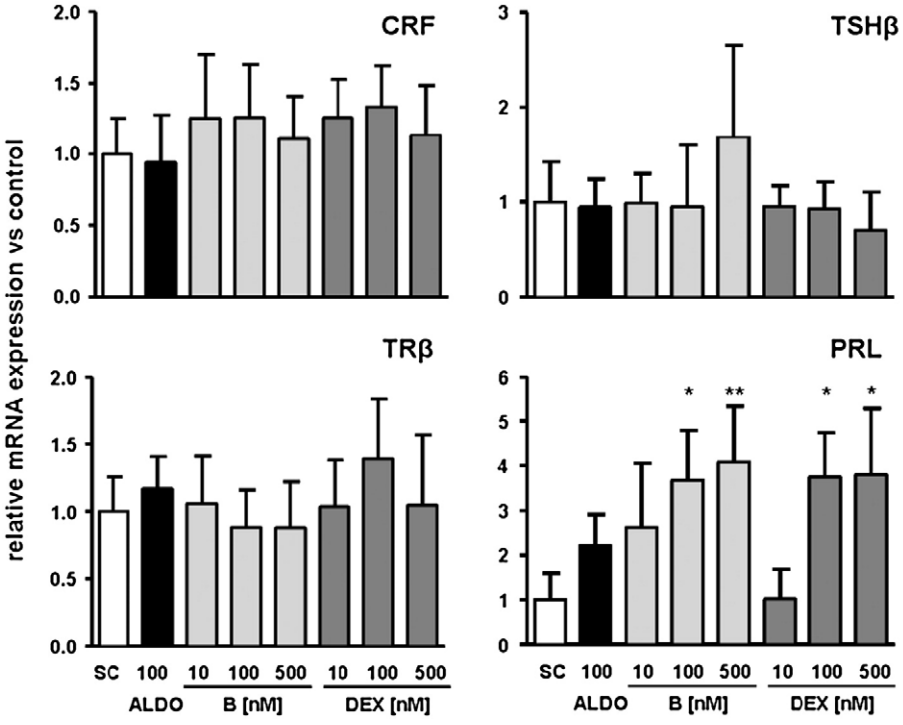


**Fig. 4.** Effects of corticosteroids on mRNA expression of thyroid hormone receptor β (TRβ), corticotropin-releasing factor (CRF), thyroid-stimulating hormone (β-subunit, TSHβ) and prolactin (PRL) in brain of *X. laevis* tadpoles. Tadpoles were treated for 21 days as described in Fig. 1 and brain tissue was sampled on experimental day 21. Treatment-related changes of mRNA expressions were analyzed by semi-quantitative RT-PCR. Values for target genes were normalized to elongation factor 1α values and results are expressed relative to solvent control (SC) group. Data are shown as mean values ± standard deviations ($n=8$). Asterisks mark significant differences to the SC (*$p<0.05$, **$p<0.01$; Dunn's multiple comparison test).

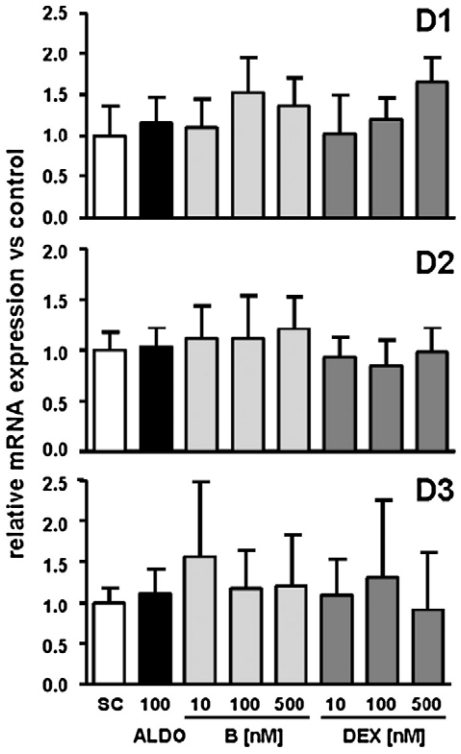


**Fig. 5.** Effects of corticosteroids on mRNA expression of deiodinase type 1, 2 and 3 (D1, D2, and D3) in brain of *X. laevis* tadpoles. Tadpoles were treated for 21 days as described in Fig. 1 and brain tissue was sampled on experimental day 21. Treatment-related changes of mRNA expressions were analyzed by semi-quantitative RT-PCR. Values for target genes were normalized to elongation factor 1α values and results are expressed relative to solvent control (SC) group. Data are shown as mean values ± standard deviations ($n = 8$).

and 500 nM B ($p < 0.001$) as well as for the 500 nM DEX treatment group ($p < 0.05$) (Fig. 6). Moreover, exposure to B resulted in a concentration-dependent reduction of STR-3 mRNA expression, which was significant for the two highest concentrations ($p < 0.01$ for 100 nM and $p < 0.001$ for 500 nM) (Fig. 6).

## 4. Discussion

In anuran amphibians, TH are the primary morphogens regulating metamorphosis (reviewed in Brown and Cai, 2007; Furlow and Neff, 2006). However, numerous studies provided evidence that other hormones including corticosteroids and PRL can modulate TH action during metamorphosis and might have a physiological role in the fine-tuning of TH-dependent tissue remodelling (Shi, 1999). To this end, the role of corticosteroids for the regulation of TH-dependent metamorphosis and the molecular mechanisms involved in corticosteroid action have been discussed controversially. Both amphibian corticosteroids, B and ALDO, play diverse roles for metabolism, osmomineral regulation and metamorphosis (Kloas et al., 1997; Leloup-Hatey et al., 1990). Corticosteroid levels display distinct developmental profiles during metamorphosis with peak levels occurring before and during the phase of metamorphic climax. Dependent on the experimental design and the test organism used, corticosteroids have been shown to exert inhibitory or accelerating effects on metamorphic development in anuran amphibians (reviewed by Denver et al., 2002; Hayes, 1997; Hayes et al., 1993; Galton, 1990; Leloup-Hatey et al., 1990). In previous studies, effects of corticosteroids on the functional activity of the HPT axis as well as on TH action in peripheral target tissues have been demonstrated (Darras et al., 2002; Denver, 1997; Galton, 1990; Hayes and Wu, 1995; Hayes et al., 1993). However, a connection of PRL and corticosteroid action was not discussed so far until a preliminary exposure test with B provided

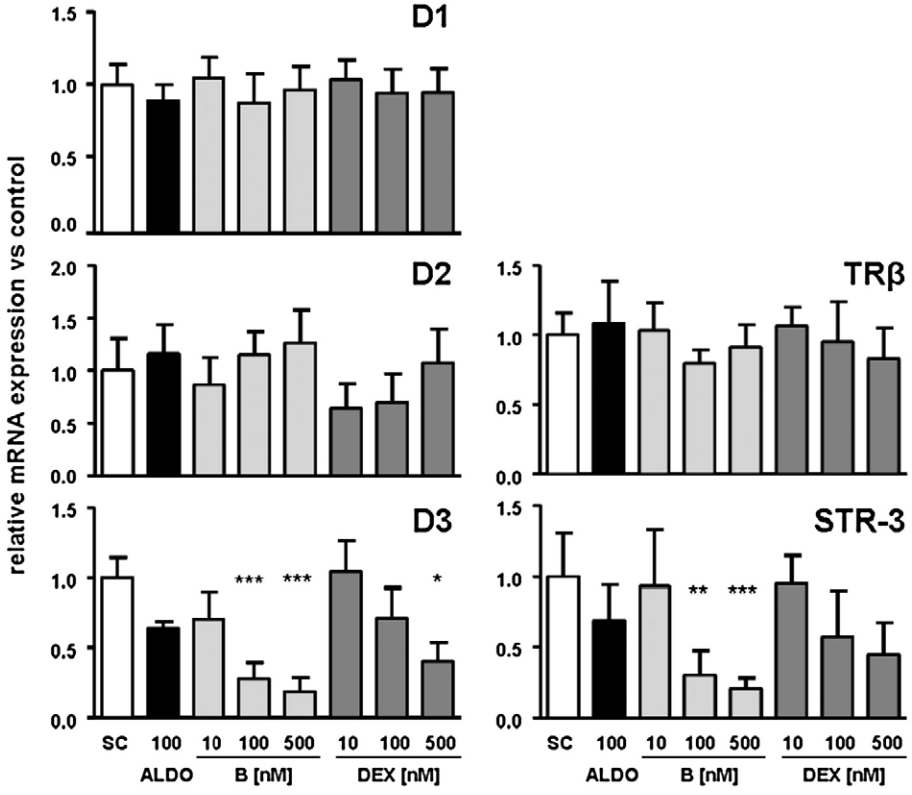


**Fig. 6.** Effects of corticosteroids on mRNA expression of D1, D2, D3, TRβ and stromelysin-3 (STR-3) in tail of *X. laevis* tadpoles. Tadpoles were treated for 21 days as described in Fig. 1 and tail tissue was sampled on experimental day 21. Treatment-related changes of mRNA expressions were analyzed by semi-quantitative RT-PCR. Values for target genes were normalized to elongation factor 1α values and results are expressed relative to solvent control (SC) group. Data are shown as mean values ± standard deviations ($n = 8$). Asterisks mark significant differences to the solvent control (SC) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Dunn's multiple comparison test).

first evidence for a PRL involvement in corticosteroid action (Lorenz et al., 2009).

In the present study, chronic treatment of premetamorphic *X. laevis* tadpoles with the glucocorticoids B and DEX but not with the mineralcorticoid ALDO, which indeed was only tested at 100 nM, caused a marked disruption of the normal progression of metamorphic tissue remodelling. Our morphological observations are in line with findings by Leloup-Hatey et al. (1990). These authors also reported an extensive narrowing of the head within several days after inserting a B implant. Moreover, they described the complete lack of fore limb emergence, which was one of the most striking morphological features characterizing tadpoles treated with B and DEX in the present study. During normal development, perforation of the opercular skin covering the developing fore limbs marks the transition of tadpoles from stage 57 to stage 58 (Nieuwkoop and Faber, 1994). Evaluation of hind limb morphology revealed that hind limb morphogenesis corresponded to stages >58 in most corticosteroid-treated tadpoles. In this regard, it should be noted that the fore limbs showed substantial growth and development but failed to break through the covering skin. Thus, the morphological phenotype resulting from corticosteroid treatment was very different compared to what is observed following treatment of tadpoles with antithyroidal substances causing a delay or the lack of fore limb emergence by arresting metamorphosis (Opitz et al., 2006a). These findings suggest that corticosteroids do not act by simply antagonizing TH function but that the action of corticosteroids is more complex and involves tissue-specific mechanisms of action.

The recent results further confirm previous findings that treatment with B and DEX inhibits tadpole growth (Hayes, 1995). The mechanisms involved in growth retardation caused by B and DEX are unknown. The presence of systemic toxicity at higher concentrations of B and DEX cannot be ruled out given that mortality was increased following treatment with high concentrations of B and DEX.

The effects of B and DEX on hind limb growth were difficult to assess. Absolute hind limb length was significantly reduced in B and DEX treatment groups. Because the observed decrease of HHL corresponded to the reduction of total body length, it might merely reflect the decrease in overall growth rates and might therefore not represent a specific effect on hind limb growth. In fact, assessment of hind limb morphology did not indicate a gross inhibition of hind limb morphogenesis.

To elucidate the mechanisms involved in corticosteroid action on metamorphic development, gene expression analyses of brain and tail tissue were performed. Expression of glucocorticoid receptor mRNA in brain of *X. laevis* tadpoles has been demonstrated by Krain and Denver (2004) indicating that the brain is a target for corticosteroid action in metamorphosing tadpoles. In brain tissue, corticosteroid treatment did not alter expression of several key players involved in TH action including TRβ and the three iodothyronine deiodinases D1, D2, and D3. Regarding TRβ mRNA expression, our results are consistent with results from previous short-term treatment studies reporting no changes in TRβ expression in tadpole brain following 72 h treatment with 100 nM B (Krain and Denver, 2004). To our knowledge, mRNA expression of iodothyronine deiodinases in brain following corticosteroid treatment has not been previously investigated in *X. laevis* tadpoles.

In addition to brain tissue, possible effects of corticosteroids on TH-dependent gene expression were also investigated in tadpole tail, which represents a major TH target at later developmental stages. Again, no treatment-related changes in mRNA expression were detectable concerning TRβ suggesting unchanged levels of bioavailable TH. However, due to rising TH levels during metamorphosis, TRβ mRNA expression becomes a less sensitive marker for changes of TH levels in late prometamorphic stages (Opitz et al., 2006b).

As found in brain tissue, D1 and D2 mRNA of corticosteroid-treated tadpoles did not differ from control levels in tail. However, in contrast to our findings in brain, treatment with B and DEX caused even a concentration-dependent decrease in D3 mRNA expression. The deiodinase encoded by D3 mRNA catalyzes the inner ring deiodination of T4 and T3 to presumably inactive iodothyronines (Brown, 2005). Thus, high expression of functional D3 will protect tissues from TH action. The cytoprotective role of D3 activity in tadpole tail during early stages of metamorphosis has been characterized in several studies (Huang et al., 1999; Wang and Brown, 1993). However, the decreased D3 mRNA expression in tail tissue of tadpoles treated with B and DEX might provide a molecular mechanism to increase the local availability of bioactive T3. As shown in several studies, mRNA of STR-3, a matrix metalloproteinase involved in tail tissue resorption (Berry et al., 1998; Damjanovski et al., 1999; Patterton et al., 1995), represents an early TH-responsive transcript in tadpole tail which strongly increases in late prometamorphic stages (Fu et al., 2006; Wang and Brown, 1993). Interestingly, decreased D3 mRNA levels were accompanied by a likewise reduced STR-3 gene expression in tail of B treated tadpoles. DEX treatment did not significantly decrease STR-3 expression but also tended to result in a decline. Concordantly, B and DEX treated tadpoles revealed no morphological signs indicative of an acceleration of tail resorption as a result of increased TH levels, but were rather characterized by a more pronounced tail fin compared to control animals. Thus, our analysis of a limited set of key players (TRβ, D3, STR-3) involved in TH-dependent tail resorption suggests complex peripheral mechanisms mediating the disruption of tissue remodelling by corticosteroids. The obviously cytoprotective effects in tail of B and DEX treated individuals must be due to other key factors counteracting the dramatic decrease of D3 concomitant with locally high concentrations of bioactive TH. One prime candidate for such a potential counteracting role is PRL.

Additionally, it should also be noted that, based on the impact on D3 and STR-3 mRNA levels and comparing equivalent concentrations, B is the most effective corticosteroid tested, followed by DEX and ALDO.

Beside gene expression in tail as a peripheral TH target tissue, we also determined the expression of several key factors of the HPT axis. In larval amphibians, CRF is the primary regulator of both the thyroid and the interrenal axis (Denver, 1999). Because we reasoned that corticosteroid treatment might affect a negative feedback response at the level of CRF gene expression as we found before (Lorenz et al., 2009), the relative expression of CRF mRNA was analyzed in the different treatment groups. However, in the recent study we could not detect any changes in CRF expression in whole brain homogenates among the different treatments. As shown by Boorse and Denver (2004), CRF mRNA is widely expressed in the amphibian brain. Therefore, the use of whole brain homogenates generally provides a relatively insensitive approach to detect the presumed local changes of CRF mRNA expression in the hypothalamic region.

Previous studies have shown that CRF is a potent stimulator of TSH release in amphibians (Denver, 1993; Okada et al., 2004). In a previous study, we found that B abolished a significant increase of TSH mRNA caused by hypothyroidal treatment (Lorenz et al., 2009). However, the modulatory effect of B treatment on TSHβ mRNA expression was limited to the treatments using hypothyroid tadpoles and treatment with B alone did not result in altered TSHβ mRNA expression. In the present study, we likewise did not detect any changes of TSHβ mRNA levels in the different treatment groups. Further studies are necessary to clarify the mechanisms involved in the modulatory action of B on TSHβ mRNA expression in hypothyroid tadpoles.

Besides corticosteroids, PRL is another important hormone known to modulate TH-dependent amphibian metamorphosis (reviewed in Shi, 1999). Studies using mammalian and chicken pituitary cells demonstrated a stimulatory role of corticosteroids on the transcription of the PRL gene (Fu and Porter, 2004; Camper et al., 1985), and a glucocorticoid response element-like motif was found in the PRL promoter of the rainbow trout (Argenton et al., 1996). These findings

suggest that corticosteroids might also regulate PRL gene expression at the transcriptional level in amphibians. We previously found the first evidence of an involvement of PRL in corticosteroid action on larval development (Lorenz et al., 2009). However, the significant stimulating effect of B was limited to hypothyroid tadpoles, whereas treatment with B alone resulted in a twofold, but not significant increase of PRL mRNA. In the present study, corticosteroid treatments caused a significant up-regulation of PRL mRNA. Whether this increase in PRL mRNA expression also resulted in increased plasma PRL levels is not known, but to our knowledge, our results reveal for the first time that altered PRL synthesis might represent one mechanism that contributes to the complex effects exerted by corticosteroids on the progression of amphibian metamorphosis.

The reason for the slight differences of the PRL and CRF results of the B treatments between the former and the recent study remains unknown. Both experiments were performed according to the standardized XEMA protocol and the same protocols were used for gene expression analyses. However, the use of semi-quantitative PCR is less accurate for absolute quantification of moderate changes of mRNA levels.

Probably the best known PRL effects in amphibians are inhibition of tail regression and prevention of TR autoinduction (Huang and Brown, 2000; Iwamuro and Tata, 1995; Baker and Tata, 1992; Tata et al., 1991). Tadpoles treated with high corticosteroid doses exhibited decreased D3 mRNA levels in tail tissue indicating increased levels of bioactive $T_3$. During the process of tail resorption, tail tissue is characterized by a decline of D3 (Wang and Brown, 1993) accompanied by increased TRß mRNA levels (Opitz et al., 2006a,b). In the present study, no evidence for a change of TRß mRNA expression in tail tissue was found, which could be a consequence of the inhibiting PRL effect on TR autoinduction mentioned above. Unfortunately, the present study gives no information about timing of tail regression, because exposure was finished after 21 days when tadpoles developed to late prometamorphic stages. However, Leloup-Hatey et al. (1990) reported a lack of tail regression after inserting a B implant in stage 57 tadpoles for 28 days. This finding complemented by our gene expression data now strongly indicates an involvement of PRL in corticosteroid action.

Interestingly, as found for D3 and STR-3 gene expression, B and DEX were more effective in inducing PRL mRNA expression than ALDO. A concentration of 10 nM B elevated PRL gene expression more than 2-fold, which was not significant but approximately as strong as ALDO at 100 nM. DEX was effective in increasing PRL mRNA levels at 100 and 500 nM, respectively, but not at 10 nM. Referring to this, it should be noted that in the present study, the endogenous corticosteroid B exhibits a higher potency than the synthetic analogue DEX. This fact could be a reflection of slight differences in the affinities of B and DEX to glucocorticoid receptors, which are reported to vary for cytosolic and membrane receptors (Denari and Ceballos, 2006; Orchinik et al., 2000; Evans et al., 1998).

The recent results now allow expanding the suite of modes involved in corticosteroid action to include possible indirect effects arising from corticosteroid-induced PRL synthesis. It appears therefore reasonable to suggest that the changes in gene expression observed in tail tissue of B and DEX treated tadpoles might also include peripheral effects of increased PRL resulting in cytoprotection of tail tissue despite decreased D3 mRNA expression. Interestingly, the lack of a significant increase of PRL gene expression in ALDO treated animals was accompanied with the absence of gross morphological abnormalities that were observed in the B and DEX treatment groups.

One general problem when interpreting the findings of the present study is that the molecular endpoints were only analyzed after chronic treatment of tadpoles with corticosteroids. During spontaneous metamorphosis, PRL levels are low during pre- and prometamorphosis and marked rises in PRL do not occur until climax stages (Buckbinder and Brown, 1993; Yamamoto et al., 1986). Therefore, it will be interesting to perform short-term studies during early developmental phases to evaluate the acute effects of corticosteroids on PRL mRNA expression.

In summary, this study emphasizes that complex mechanisms are involved in the modulatory actions of corticosteroids on amphibian metamorphosis. At the level of gross morphology, our data confirm the hypothesis that corticosteroid action in peripheral tissues includes tissue-specific aspects leading to an abnormal development that is no longer consistent with the normal sequence of tissue remodelling. Noteworthy, corticosteroids, which are characterized as glucocorticoids in mammals, are assumed to be more efficient than mineralocorticoids in disrupting metamorphosis of amphibians.

The detected changes in gene expression do not fit with a simplistic model of synergistic or antagonistic action of corticosteroids on TH functions and suggest more complex pathways of how corticosteroids modulate directly and indirectly TH activity in peripheral tissues. In this regard, a major finding of the present study is that corticosteroids appear to increase drastically PRL synthesis leading to the possibility that some of the peripheral effects are mediated indirectly by elevated PRL levels.

## References


Argenton, F., Ramoz, N., Charlet, N., Bernardini, S., Colombo, L., Bortolussi, M., 1996. Mechanisms of transcriptional activation of the promoter of the rainbow trout prolactin gene by GHF1/Pit1 and glucocorticoid. Biochem. Biophys. Res. Commun. 224, 57–66.

Baker, B.S., Tata, J.R., 1992. Prolactin prevents the autoinduction of thyroid hormone receptor mRNAs during amphibian metamorphosis. Dev. Biol. 149, 463–467.

Berry, D.L., Rose, C.S., Remo, B.F., Brown, D.D., 1998. The expression pattern of thyroid hormone response genes in remodeling tadpole tissues defines distinct growth and resorption gene expression programs. Dev. Biol. 203, 24–35.

Boorse, G.C., Denver, R.J., 2004. Expression and hypophysiotropic actions of corticotropin-releasing factor in *Xenopus laevis*. Gen. Comp. Endocrinol. 137, 272–282.

Brown, D.D., 2005. The role of deiodinases in amphibian metamorphosis. Thyroid 15, 815–821.

Brown, D.D., Cai, L., 2007. Amphibian metamorphosis. Dev Biol 306, 20–33.

Buckbinder, L., Brown, D.D., 1993. Expression of the *Xenopus laevis* prolactin and thyrotropin genes during metamorphosis. Proc. Natl. Acad. Sci. USA 90, 3820–3824.

Camper, S.A., Yao, Y.A., Rottman, F.M., 1985. Hormonal regulation of the bovine prolactin promoter in rat pituitary tumor cells. J. Biol. Chem. 260, 12246–12251.

Damjanovski, S., Ishizuya-Oka, A., Shi, Y.B., 1999. Spatial and temporal regulation of collagenases-3, -4, and stromelysin-3 implicates distinct functions in apoptosis and tissue remodeling during frog metamorphosis. Cell Res. 9, 91–105.

Darras, V.M., Van der Geyten, S., Cox, C., Segers, I.B., De Groef, B., Kuhn, E.R., 2002. Effects of dexamethasone treatment on iodothyronine deiodinase activities and on metamorphosis-related morphological changes in the axolotl (*Ambystoma mexicanum*). Gen. Comp. Endocrinol. 127, 157–164.

Denari, D., Ceballos, N.R., 2006. Cytosolic glucocorticoid receptor in the testis of *Bufo arenarum*: seasonal changes in its binding parameters. Gen. Comp. Endocrinol. 147, 247–254.

Denver, R.J., 1993. Acceleration of anuran amphibian metamorphosis by corticotropin-releasing hormone-like peptides. Gen. Comp. Endocrinol. 91, 38–51.

Denver, R.J., 1997. Environmental stress as a developmental cue: corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. Horm. Behav. 31, 169–179.

Denver, R.J., 1999. Evolution of the corticotropin-releasing hormone signaling system and its role in stress-induced phenotypic plasticity. Ann. N.Y. Acad. Sci. 897, 46–53.

Denver, R.J., Glennemeier, K.A., Boorse, G.C., 2002. Endocrinology of complex life cycles: amphibians. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), Hormones, Brain and Behavior. Academic Press, San Diego, pp. 469–513.

Evans, S.J., Moore, F.L., Murray, T.F., 1998. Solubilization and pharmacological characterization of a glucocorticoid membrane receptor from an amphibian brain. J. Steroid Biochem. Mol. Biol. 67, 1–8.

Fu, X., Porter, T.E., 2004. Glucocorticoid induction of lactotrophs and prolactin gene expression in chicken embryonic pituitary cells: a delayed response relative to stimulated growth hormone production. Endocrinology 145, 1322–1330.

Fu, L., Tomita, A., Wang, H., Buchholz, D.R., Shi, Y.B., 2006. Transcriptional regulation of the *Xenopus laevis* stromelysin-3 gene by thyroid hormone is mediated by a DNA element in the first intron. J. Biol. Chem. 281, 16870–16878.

Furlow, J.D., Neff, E.S., 2006. A developmental switch induced by thyroid hormone: *Xenopus laevis* metamorphosis. Trends Endocrinol. Metab. 17, 40–47.

Galton, V.A., 1990. Mechanisms underlying the acceleration of thyroid hormone-induced tadpole metamorphosis by corticosterone. Endocrinology 127, 2997–3002.

Hayes, T.B., 1995. Interdependence of corticosterone and thyroid hormones in larval toads (*Bufo boreas*). I. Thyroid hormone-dependent and independent effects of corticosterone on growth and development. J. Exp. Zool. 271, 95–102.

Hayes, T.B., 1997. Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. Am. Zool. 37, 185–194.

Hayes, T.B., Wu, T.H., 1995. Interdependence of corticosterone and thyroid hormones in toad larvae (*Bufo boreas*). II. Regulation of corticosterone and thyroid hormones. J. Exp. Zool. 271, 103–111.

Hayes, T., Chan, R., Licht, P., 1993. Interactions of temperature and steroids on larval growth, development, and metamorphosis in a toad (*Bufo boreas*). J. Exp. Zool. 266, 206–215.

Huang, H., Brown, D.D., 2000. Prolactin is not a juvenile hormone in *Xenopus laevis* metamorphosis. Proc. Natl. Acad. Sci. USA 97, 195–199.

Huang, H., Marsh-Armstrong, N., Brown, D.D., 1999. Metamorphosis is inhibited in transgenic *Xenopus laevis* tadpoles that overexpress type III deiodinase. Proc. Natl. Acad. Sci. USA 96, 962–967.

Imaoka, T., Matsuda, M., Mori, T., 2000. Extrapituitary expression of the prolactin gene in the goldfish, African clawed frog and mouse. Zoolog. Sci. 17, 791–796.

Iwamuro, S., Tata, J.R., 1995. Contrasting patterns of expression of thyroid hormone and retinoid X receptor genes during hormonal manipulation of *Xenopus* tadpole tail regression in culture. Mol. Cell. Endocrinol. 113, 235–243.

Jagnytsch, O., Opitz, R., Lutz, I., Kloas, W., 2006. Effects of tetrabromobisphenol A on larval development and thyroid hormone-regulated biomarkers of the amphibian *Xenopus laevis*. Environ. Res. 101, 340–348.

Kikuyama, S., Niki, K., Mayumi, M., Kawamura, K., 1982. Retardation of thyroxine-induced metamorphosis by Amphenone B in toad tadpoles. Endocrinol. Jpn. 29, 659–662.

Kikuyama, S., Niki, K., Mayumi, M., Shibayama, R., Nishikawa, M., Shintake, N., 1983. Studies on corticoid action on the toad tadpole tail *in vitro*. Gen. Comp. Endocrinol. 52, 395–399.

Kloas, W., Reinecke, M., Hanke, W., 1997. Stage-dependent changes in adrenal steroids and catecholamines during development in *Xenopus laevis*. Gen. Comp. Endocrinol. 108, 416–426.

Kloas, W., Lutz, I., Einspanier, R., 1999. Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals *in vitro* and *in vivo*. Sci. Total Environ. 225, 59–68.

Krain, L.P., Denver, R.J., 2004. Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*. J. Endocrinol. 181, 91–104.

Kuiper, G.G., Klootwijk, W., Morvan, D.G., Destree, O., Darras, V.M., Van der, G.S., Demeneix, B., Visser, T.J., 2006. Characterization of recombinant *Xenopus laevis* type I iodothyronine deiodinase: substitution of a proline residue in the catalytic center by serine (Pro132Ser) restores sensitivity to 6-propyl-2-thiouracil. Endocrinology 147, 3519–3529.

Leloup-Hatey, J., Buscaglia, M., Jolivet, J.G., Leloup, I., 1990. Interrenal function during the metamorphosis in anuran amphibia. In: Hanke, W. (Ed.), Biology and Physiology of Amphibians. Gustav Fischer, Stuttgart, pp. 139–154.

Lorenz, C., Opitz, R., Lutz, I., Kloas, W., 2009. Teratogenic effects of chronic treatment with corticosterone on tadpoles of *Xenopus laevis*. Ann. N.Y. Acad. Sci., 1163, 454–456. doi: 10.1111/j.1749-6632.2009.04429.x.

Manzon, R.G., Denver, R.J., 2004. Regulation of pituitary thyrotropin gene expression during *Xenopus* metamorphosis: negative feedback is functional throughout metamorphosis. J. Endocrinol. 182, 273–285.

Miranda, L.A., Affanni, J.M., Paz, D.A., 2000. Corticotropin-releasing factor accelerates metamorphosis in *Bufo arenarum*: effect on pituitary ACTH and TSH cells. J. Exp. Zool. 286, 473–480.

Nieuwkoop, P.D., Faber, J., 1994. Normal Table of *Xenopus laevis* (Daudin). Garland, New York.

Okada, R., Yamamoto, K., Koda, A., Ito, Y., Hayashi, H., Tanaka, S., Hanaoka, Y., Kikuyama, S., 2004. Development of radioimmunoassay for bullfrog thyroid-stimulating hormone (TSH): effects of hypothalamic releasing hormones on the release of TSH from the pituitary *in vitro*. Gen. Comp. Endocrinol. 135, 42–50.

Opitz, R., Braunbeck, T., Bogi, C., Pickford, D.B., Nentwig, G., Oehlmann, J., Tooi, O., Lutz, I., Kloas, W., 2005. Description and initial evaluation of a *Xenopus* metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds. Environ. Toxicol. Chem. 24, 653–664.

Opitz, R., Hartmann, S., Blank, T., Braunbeck, T., Lutz, I., Kloas, W., 2006a. Evaluation of histological and molecular endpoints for enhanced detection of thyroid system disruption in *Xenopus laevis* tadpoles. Toxicol. Sci. 90, 337–348.

Opitz, R., Lutz, I., Nguyen, N.H., Scanlan, T.S., Kloas, W., 2006b. Analysis of thyroid hormone receptor betaA mRNA expression in *Xenopus laevis* tadpoles as a means to detect agonism and antagonism of thyroid hormone action. Toxicol. Appl. Pharmacol. 212, 1–13.

Orchinik, M., Matthews, L., Gasser, P.J., 2000. Distinct specificity for corticosteroid binding sites in amphibian cytosol, neuronal membranes, and plasma. Gen. Comp. Endocrinol. 118, 284–301.

Patterton, D., Hayes, W.P., Shi, Y.B., 1995. Transcriptional activation of the matrix metalloproteinase gene stromelysin-3 coincides with thyroid hormone-induced cell death during frog metamorphosis. Dev. Biol. 167, 252–262.

Shi, Y.-B., 1999. Amphibian Metamorphosis. From Morphology to Molecular Biology. Wiley, New York.

Shintani, N., Nohira, T., Hikosaka, A., Kawahara, A., 2002. Tissue-specific regulation of type III iodothyronine 5-deiodinase gene expression mediates the effects of prolactin and growth hormone in *Xenopus* metamorphosis. Dev. Growth Differ. 44, 327–335.

Tata, J.R., Kawahara, A., Baker, B.S., 1991. Prolactin inhibits both thyroid hormone-induced morphogenesis and cell death in cultured amphibian larval tissues. Dev. Biol. 146, 72–80.

Tata, J.R., Baker, B.S., Machuca, I., Rabelo, E.M., Yamauchi, K., 1993. Autoinduction of nuclear receptor genes and its significance. J. Steroid Biochem. Mol. Biol. 46, 105–119.

Van der Geyten, S., Darras, V.M., 2005. Developmentally defined regulation of thyroid hormone metabolism by glucocorticoids in the rat. J. Endocrinol. 185, 327–336.

Walpita, C.N., Grommen, S.V., Darras, V.M., van der Geyten, S., 2007. The influence of stress on thyroid hormone production and peripheral deiodination in the Nile tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol. 150, 18–25.

Wang, Z., Brown, D.D., 1993. Thyroid hormone-induced gene expression program for amphibian tail resorption. J. Biol. Chem. 268, 16270–16278.

Wright, M.L., Cykowski, L.J., Lundrigan, L., Hemond, K.L., Kochan, D.M., Faszewski, E.E., Anuszewski, C.M., 1994. Anterior pituitary and adrenal cortical hormones accelerate or inhibit tadpole hindlimb growth and development depending on stage of spontaneous development or thyroxine concentration in induced metamorphosis. J. Exp. Zool. 270, 175–188.

Yamamoto, K., Niinuma, K., Kikuyama, S., 1986. Synthesis and storage of prolactin in the pituitary gland of bullfrog tadpoles during metamorphosis. Gen. Comp. Endocrinol. 62, 247–253.

Yaoita, Y., Brown, D.D., 1990. A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. Genes Dev. 4, 1917–1924.