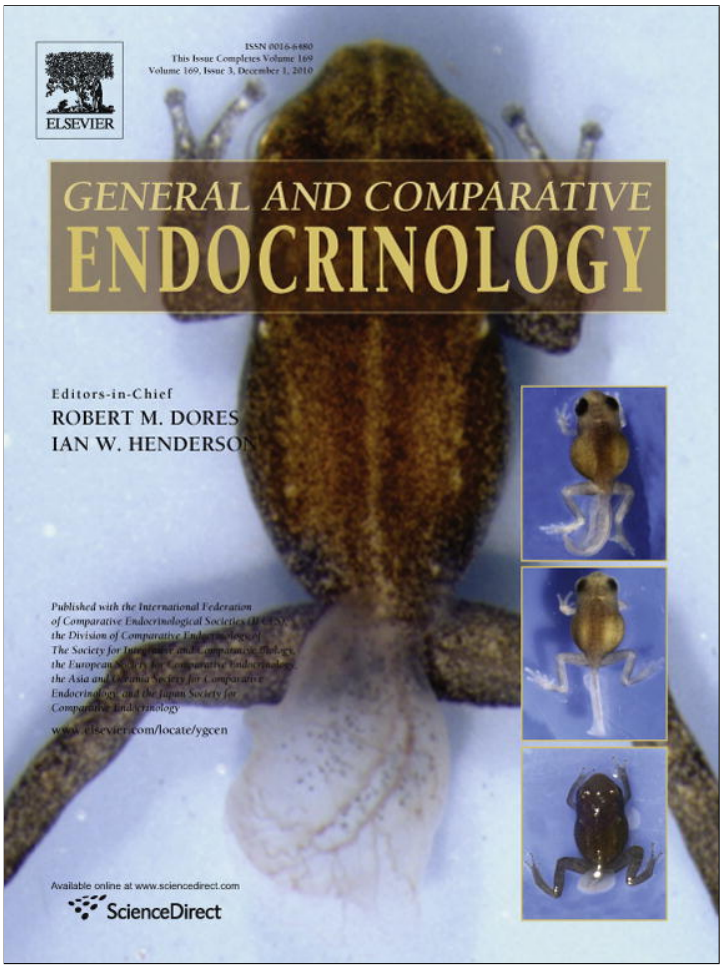

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Corticotropin-releasing factor regulates the development in the direct developing frog, *Eleutherodactylus coqui*

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ABSTRACT

Direct developing frogs lack a free-living larval phase, such that miniature adults hatch directly from the eggs. Even under such extreme reorganization of the ancestral biphasic developmental pattern, direct developers still undergo thyroid hormone (TH)-dependent post-embryonic development. Hypothalamic regulation of TH synthesis and release plays a central role in controlling the timing of metamorphosis in biphasic developers. In particular, the neuropeptide corticotropin-releasing factor (CRF) regulates TH in tadpoles, but in adults, both thyrotropin-releasing hormone (TRH) and CRF regulate TH. Because direct developers lack a tadpole stage, it was not clear whether hypothalamic regulation of TH would be tadpole-like or adult-like prior to hatching. To test this, we injected pre-hatching *Eleutherodactylus coqui* daily with CRF, TRH or astressin (a CRF receptor blocker). CRF but not TRH significantly accelerated the developmental rate compared to controls. Astressin-treated animals showed a near complete developmental arrest, which confirmed that development requires CRF. To support the idea that CRF acts to regulate development in *E. coqui* via thyroid physiology, we showed the TH-direct response gene TRβ is up-regulated 24 and 48 h after CRF injection. In addition, treatment with 50 nM T3 (triiodothyronine, the active form of TH) increased the developmental rate similar to CRF injections. Our results extend the evidence for a cryptic metamorphosis in direct developers by showing that neuroendocrine signaling is conserved between biphasic and direct developers. Furthermore, the conserved neuroendocrine regulation implies that changes at the peripheral level of hormone action underlie the evolution of the radically divergent development in direct developers.

1. Introduction

Direct development in frogs is a derived life history mode found in one or more species of at least 10 different families or family level taxa of frogs and is believed to have evolved independently in these families (Duellman and Trueb, 1986; Hanken, 1999). Direct developing frogs undergo most of their development inside eggs, and development is completely terrestrial with miniature adults hatching from the eggs (Callery et al., 2001; Elinson, 2001). This mode of development is typified by a variable degree of loss or re-patterning of larval features of the ancestral biphasic developmental mode, including vestigial or absent lateral line organs, cement glands, larval mouthparts and a coiled gut (Callery and Elinson, 2000a; Callery et al., 2001; Elinson, 2001). The tail is the only obvious remnant structure but it often serves a respiratory rather than locomotory role (Elinson, 2001). Direct development evolved from species with biphasic development, where a post-embryonic larval phase is separated from an adult phase by metamorphosis constituting the developmental transition from aquatic to terrestrial life (Shi, 2000).

Even though direct developers lack a larval phase and appear to go from embryogenesis straight to the adult phase inside the egg, similarities in hormonal control of development between embryos of *Eleutherodactylus coqui* and tadpoles of biphasic developers suggest that direct developers undergo cryptic metamorphosis prior to hatching from Townsend and Stewart stage (TS) 9-15 (Callery and Elinson, 2000b; Callery et al., 2001; Townsend and Stewart, 1985). Indeed, inhibition of thyroid hormone (TH) synthesis with methimazole starting from TS 9 but not 11 in *E. coqui* inhibits many features of development, indicating that endogenous TH is necessary for development, as for biphasic developers undergoing metamorphosis (Callery and Elinson, 2000b). Similarly, TRβ, which is a TH-direct response gene in tadpoles, is upregulated after TS 9 onwards in *E. coqui* (Callery and Elinson, 2000b). These results are consistent with histological observations that thyroid gland activity increases beginning at TS10 (Jennings and Hanken, 1998), which suggests that TRβ might be TH-response gene in *E. coqui* also. Because development begins to depend on endogenous TH around TS9, likely homologous in large part to the beginning of

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metamorphosis in biphasic species, TS9 can be considered the start of metamorphosis and the end of embryogenesis. However, in their model of development in direct developing frogs, Callery and Elinson indicate that the border between the end of the embryonic phase and the start of the metamorphic phase is blurred as a result of heterochronic shifts in character development (Callery and Elinson, 2000b; Callery et al., 2001). Here, we further substantiate the model that direct developing frogs undergo metamorphosis inside eggs (Callery and Elinson, 2000b) by our comparison of the neuroendocrine control of TH-dependent development in direct developers and biphasic developers.

In tadpoles, the hypothalamic peptide, corticotropin-releasing factor (CRF), acts on the pituitary to release adrenocorticotrpic hormone (ACTH) and thyroid stimulating hormone (TSH) that stimulate secretion of corticosterone and TH, respectively (Denver and Licht, 1989; Denver, 2009). Upon completion of metamorphosis, TH production comes under control of an additional hypothalamic peptide, thyrotropin-releasing hormone (TRH) (Denver, 1988). Because post-embryonic direct developers lack a free-swimming larval phase and hatch out as miniature adults, they may also lack the tadpole-like regulation of TH. In other words, hypothalamic regulation of post-embryonic development may be adult-like, where TH is regulated by both CRF and TRH rather than only CRF as in tadpoles. On the other hand, tadpole-like (only CRF and not TRH) regulation of TH before hatching in direct developers would support the model of a cryptic metamorphosis inside the egg (Callery and Elinson, 2000b; Callery et al., 2001). To answer this question, we examined morphological responses of pre-hatching *E. coqui* to daily injections of CRF, TRH, or astressin. Astressin is a general CRF receptor antagonist, which blocks both CRF receptors, CRFR1 and CRFR2 (Okada et al., 2007). In biphasic developers, CRFR1 and CRFR2 are found in corticotropes and thyrotropes of the pituitary (reviewed in Denver, 2009; Okada et al., 2007), respectively, thus we expect astressin to inhibit both TH and corticosterone production in *E. coqui*. To investigate a relationship between CRF and TH production in direct developers, we measured the expression of the TH-response gene TRβ (Shi, 2000) after CRF injections and compared morphological responses after CRF and T3 treatments.

2. Methods

2.1. Animals and treatments

All *E. coqui* eggs for these experiments were kindly provided by Dr. Richard Elinson, Duquesne University. Embryos were raised in Petri dishes on a filter paper moistened with 20% Steinberg's solution (Callery and Elinson, 2000a,b). Between TS 5 and 7 (Townsend and Stewart, 1985), based on when the clutch was received, the vitelline membrane was removed from the embryos. This was done by first transferring embryos to a Petri dish full of 20% Steinberg's solution to allow the jelly layers to swell and the pre-vitelline space to enlarge, so that the vitelline membrane could be removed carefully with fine forceps under the dissecting microscope. Free embryos were maintained submerged in 20% Steinberg's in Petri dishes until they reached the appropriate stage (TS8-10). The dry weight of embryos was approximately 6.5 mg between stages TS8 and 10 (Packard et al., 1996).

The hypothalamic peptides, ovine CRF (oCRF) and TRH and the CRF receptor antagonist, astressin, were dissolved in 60% PBS to the concentration of 1 mg/ml and injected daily until the animals reached TS15 using a Nanoject (Drummond). Synthetic oCRF and astressin were a kind gift from Dr. Jean Rivier (The Salk Institute, La Jolla, CA). TRH and T3 were obtained from (Sigma–Aldrich, USA). Before each injection, animals were anesthetized in benzocaine for ~10 s, transferred to tissue paper soaked with 100% Steinberg's, and then injected subcutaneously in the thigh. Preliminary trials injecting gel loading dye (6×) (bromophenol blue (0.015%), New England BioLabs) showed rapid dispersal of dye throughout the subcutaneous lymph sac. After injection, animals were transferred to 100% Steinberg's for 5–10 min and then returned to 20% Steinberg's solution, which was replaced every other day. All the experiments were performed at room temperature of 22 °C.

The concentrations of neuropeptides were determined based on the information available from previous experiments done on tadpoles of different species (Gancedo et al., 1992; Denver, 1993). We used the smaller doses compared to doses previously used to study tadpoles because the size of *E. coqui* embryos is very small. Since this is a first study to examine the effects of neuropeptides on the development of direct developer, we used 2–3 different doses for each peptide. In the oCRF vs. TRH experiment, animals at TS8 ($n = 7$) were injected with 200 and 500 ng of (1) TRH or (2) oCRF, or (3) 500 nL of 60% PBS. We used TS8 to reduce the effect of endogenous thyroid gland activity, which begins around TS9/TS10 (Jennings and Hanken, 1998). In the oCRF vs. astressin experiment, TS11 animals ($n = 6$) were injected with 200 and 500 ng of (1) astressin or (2) oCRF, or (3) 500 nL of 60% PBS. We used TS11 because TH activity is increasing at that stage and astressin was used to block endogenous CRF, thereby testing the hypothesis that the rise in TH was driven by CRF as opposed to TS8, when no endogenous is TH present. To examine the effect of oCRF on gene expression, animals at TS8 ($n = 3–5$) were injected with 500 or 1000 ng of oCRF or 1000 nL PBS and assayed after 24 and 48 h. To examine the effect of exogenous TH on development, animals at TS9 ($n = 5$) were treated with 0, 2, 10 and 50 nM T3 through tail resorption (TS15). The concentration of T3 was determined based on a pervious study (Elinson, 1994). Steinberg's solution and T3 were changed daily.

2.2. Molecular and morphological analysis

At 24 or 48 h post-injection, unanesthetized animals were snap frozen and stored at −80 °C until assayed. Total RNA was extracted from frozen embryos using Tri-reagent (Fisher Scientific, USA) followed by cDNA synthesis (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) as per the manufacturer's protocols. PCR (Takara Bio Inc., USA) on each cDNA sample was carried out using primers for TRβ and rpL8 (a housekeeping gene to control for variation in RNA extraction procedure) (Callery and Elinson, 2000b). We used freeware software, ImageJ (NIH) for gel densitometric analysis of TRβ and rpL8 expression levels in response to different doses of CRF injections. RpL8 expression was similar across treatments, and TRβ expression was normalized to rpL8.

Morphological progression was monitored by examining the development of upper and lower jaw lengths, snout shape, skin pattern and opacity, pigmentation, visibility of endolymphatic calcium deposits, leg length and tail regression. We also measured the number of days taken to reach TS15 from beginning stage of treatments. Digital pictures were taken daily after 3 days of treatment. Snout to vent length (SVL) and hindlimb length (HL) to the nearest 1 mm were measured with digital calipers at TS15.

2.3. Statistical analysis

As time to and SVL and HL at TS15 were normally distributed with equal variance in the first experiment (oCRF vs. TRH injections), we performed ANOVA to test for treatment effects on the number of days taken to reach TS15 and relative HL at TS15, followed by Tukey–Kramer post hoc test for pairwise comparisons.

Because time to reach tail resorption in TH-treated animals was non-normally distributed, we used the non-parametric Wilcoxon test to test for significant differences. Non-parametric Wilcoxon test was used to test for significant differences in TRβ expression in response to different CRF treatments.

3. Results

3.1. oCRF vs. TRH injections

To determine if hypothalamic regulation of TH production was tadpole-like (CRF only) or adult-like (both CRF and TRH), we injected oCRF or TRH daily into TS8 embryos. Morphological effects like resorption of tail, snout remodeling, pigmentation of skin and visibility of endolymphatic calcium deposits of oCRF treatments became prominent by day 10 (Fig. 1). Tails of oCRF-treated animals were almost completely resorbed after an average of 12 days, whereas TRH- and PBS-injected animals (controls) whose tails had just begun to resorb after 9 days and completed after an average of 16 days. Snout remodeling was complete and adult-like in shape in oCRF-injected animals after 10 days but rounded and embryo-like in TRH and control treatments. Hindlimbs were heavily pigmented and dark with a clear banding pattern in oCRF-injected animals by day 9, whereas animals from the other two treatments clearly lacked or had very little pigmentation on their hindlimbs with no observable banding pattern. Adult skin had formed in oCRF-treated animals, which completely masked the endolymphatic calcium deposits (ECD) by day 9. Raised dorso-lateral ridges also started to appear in oCRF-injected animals. In control and TRH-injected animals, ECD was clearly visible and dorso-lateral ridges were absent.

The oCRF-induced animals achieved TS15 by an average of 3–4 days earlier compared to TRH-injected and control animals (Fig. 2A), which took on average 16 days from TS8 to TS15 (a ~25% difference). In addition, oCRF-injected animals had significantly shorter relative hindlimb lengths compared to TRH-injected and control animals at TS15 (Fig. 2B).

3.2. oCRF vs. astressin injections

In this experiment, we injected oCRF or astressin into TS11 animals to further examine the role of endogenous CRF in development. As before, oCRF accelerated development (not shown), but astressin-treated animals were developmentally retarded (Fig. 3). Treatments had very little effect on the developmental rate by 4 days (Fig. 3, top), but control animals underwent dramatic change in morphology during the next five days (Fig. 3, bottom). Changes in the previous result section (oCRF vs. TRH injections) morphological characters in control animals became evident on day 5 and by day 8, the tail was ~1/3 of the original length and width, snout shape was more like adults, skin was remodeled to a great extent and was juvenile-like in controls compared to astressin-treated animals. Astressin-treated animals advanced very little in any of these above-mentioned features and were developmentally retarded. By 9 days, the control animals reached stage TS15, and the astressin-treated animals had advanced to TS12-13. After day 9, we continued to rear the astressin-treated animals in the absence of astressin, and they all developed normally to TS15 within the next 7–8 days.

3.3. Effect of oCRF injections on TRβ expression

The following two experiments were designed to extend our understanding of the similarities in the regulation of metamorphosis by CRF between biphasic and direct developers. Injections with 500 and 1000 ng of oCRF both showed significant upregulation of TRβ expression after 24 and 48 h in the embryos of *E. coqui*, compared to PBS-injected animals showed no detectable upregulation in TRβ expression after 24 and 48 h of treatments (Fig. 4 and data not shown). We found little variation in the expression of housekeeping gene, rpL8 across treatments.

3.4. Effect of TH treatment

If CRF accelerates development via an increase in TH, then we would expect CRF and TH treatments to have similar effects. To test this, we treated *E. coqui* starting at TS9 daily with 2, 10 and 50 nM TH. The largest dose reduced the time to tail resorption by an average of two days compared to controls (14 vs. 16 days, $P < 0.05$, Fig. 5). The effect on development of 2 and 10 nM TH did not differ from controls (data not shown).

4. Discussion

Our research revealed conserved hypothalamic control of development (“metamorphosis”) between frogs with tadpoles and the direct developing frog, *E. coqui*. First, we showed that CRF and not TRH accelerated development in *E. coqui*. Similarly, we showed

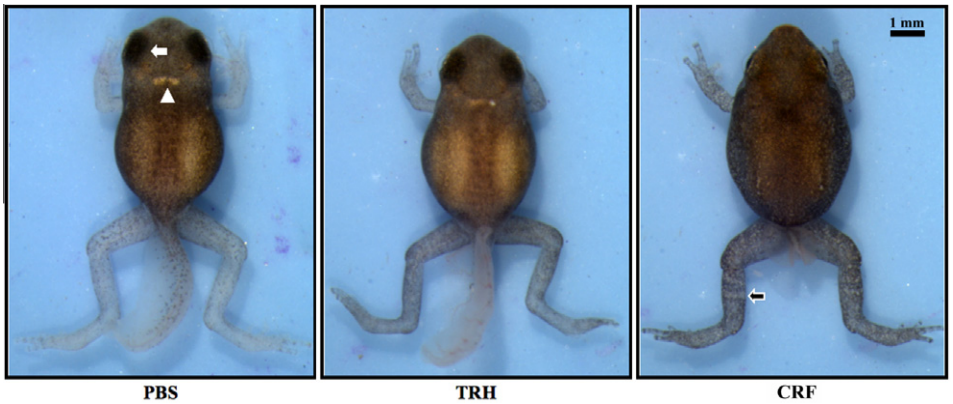

Fig. 1. Effects of CRF and TRH on morphological development. *E. coqui* were injected daily for 10 days with 500 ng of peptide or saline starting at TS8 ($n = 7$). CRF-injected animals showed accelerated development compared to PBS- and TRH-injected animals. Retinal pigmentation (white arrow) and the endolymphatic calcium deposits (white triangle) show through the pre-metamorphic skin in TRH- and PBS-injected animals, but CRF-injected animals have thick adult skin that obscures these features. Also, the banding pattern on the hindlimbs (black arrow) is clearly visible only in CRF-injected animals. In addition, CRF-injected animals have short stubs remaining of the tail and show adult-like more pointy and less rounded snouts compared to the other treatments. Scale bar is represented for all the three animals.

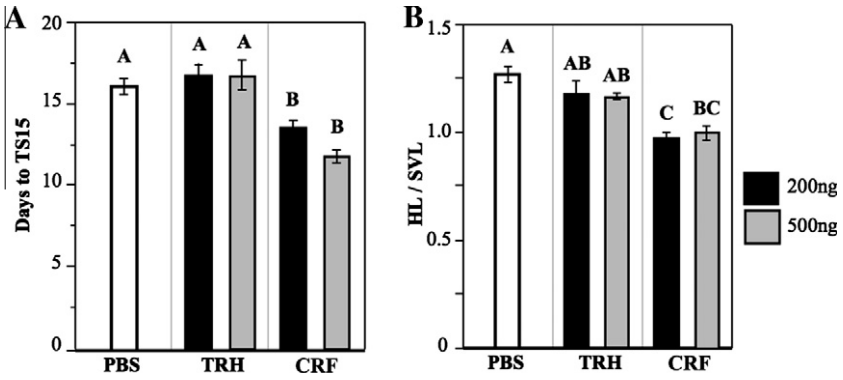

Fig. 2. Effects of CRF and TRH on development time and relative hindlimb lengths. *E. coqui* were injected daily with 200 or 500 ng of peptide or saline starting at TS8 ($n = 7$) until they reached TS15. (A) CRF-injected animals reached TS15 significantly earlier than in PBS and TRH treatments, which did not differ from each other (ANOVA: DF = (1, 4), $F = 14.00$, $P < 0.0001$). (B) CRF-injected animals had significantly shorter relative hindlimbs (HL/SVL) compared to PBS-injected animals (ANOVA: DF = (1, 4), $F = 10.54$, $P < 0.0001$). CRF-injected animals at 200 ng but not 500 ng had significantly shorter relative hindlimbs compared to the TRH treatment. Again, PBS and TRH groups did not differ from each other. Differences were tested for significance using ANOVA, and significance groups among treatments at $\alpha = 0.05$ are indicated by letters above the bars as determined by Tukey–Kramer post hoc test.

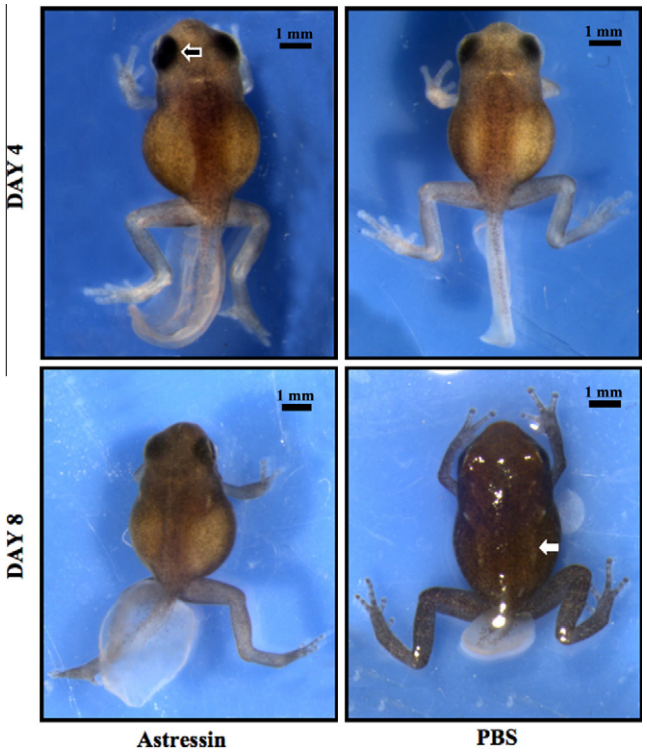

Fig. 3. Effect of astressin on morphological development. *E. coqui* were injected daily for 8 days with 200 or 500 ng of astressin or saline starting at TS11 ($n = 6$). On day 4, astressin- and PBS-injected animals look similar with minor differences in morphology. However, on day 8, a clear regression of tail length and fin width is visible in PBS- compared to astressin-injected animals. Also, the skin is adult-like in PBS- and not in astressin-injected animals as seen by raised dorso-lateral ridges (white arrow), obscured retinal pigment (black arrow) and banding pattern on hindlimbs. In addition, the snout is adult-like in the PBS treatment.

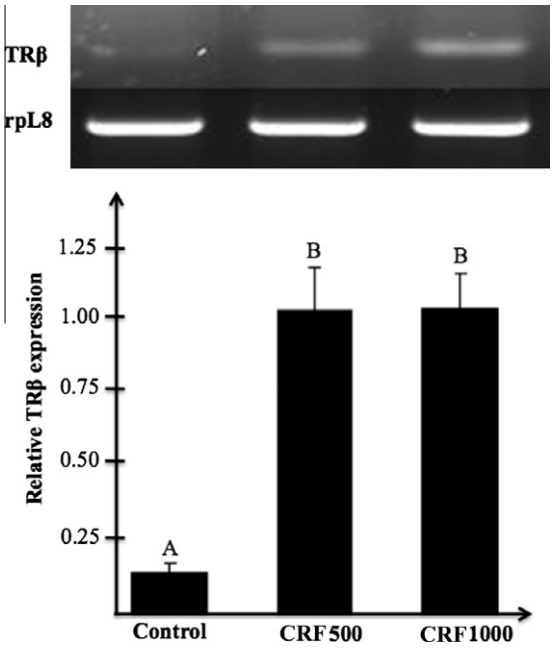

Fig. 4. Induction of TRβ (TH-direct response gene) by CRF. *E. coqui* were injected with PBS or 500 or 1000 ng CRF at TS8, and TRβ mRNA expression was measured 24 h later using reverse transcriptase-PCR. TRβ is expressed at very low levels at TS8, and CRF injections of 500 and 1000 ng induced a significant upregulation after 24 h. The housekeeping gene rpL8 was used as a control. Top panel shows the gel image as a representative sample of three independent samples. Bottom panel shows the quantitative analysis of gel image ($n = 3$) comparing relative expression levels of TRβ (TRβ normalized to rpL8 expression levels) across treatments. Wilcoxon test was used to find the significant differences among treatments at $\alpha = 0.05$, indicated by letters above the bars.

that endogenous CRF is required for development by blocking endogenous CRF with astressin (CRF receptor antagonist), which resulted in developmental retardation. We then showed that CRF accelerated development in *E. coqui* likely via regulation of TH production, as in biphasic developers. We treated embryos of *E. coqui* with exogenous TH, and the resulting accelerated development was similar to that from CRF injections. In addition, the TH-response gene TRβ was significantly upregulated in response to CRF injections. These data taken together strongly suggest that CRF regulates development similarly in *E. coqui* and species with tadpoles through the CRF–TSH–TH axis (Denver, 2009), and failure to accelerate development with TRH suggests that it does not play a role.

Such conservation is potentially surprising because *E. coqui*, which lacks the free-living larval stage, may no longer require the intermediate stage where TH is regulated by CRF until completion of metamorphosis and then by both TRH and CRF as an adult in biphasic developers. It is possible that direct developing frogs, because they hatch out directly as a miniature adult, have evolved adult-like hypothalamic control of TH and have undergone

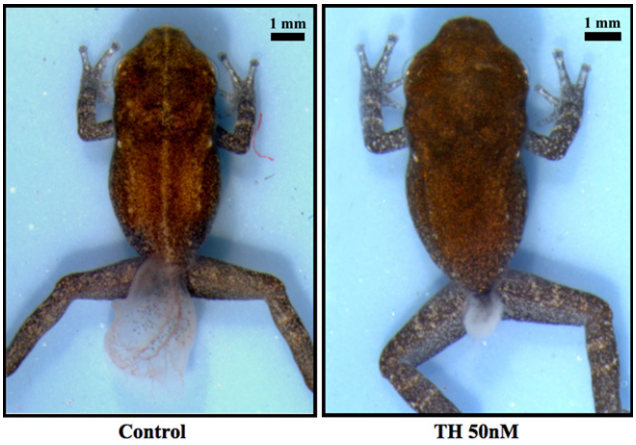

Fig. 5. Effect of exogenous TH on morphological development. *E. coqui* ($n = 5$) were treated with or without 50 nM T3 daily for 13 days starting at TS9. The control and TH-treated animals were similar in external appearance except the tail length and fin width were shorter and narrower in TH-treated animals.

deletion of the tadpole-like control. Indeed, deletion of larval features has occurred for many aspects of *E. coqui* development (Elinson, 2001). However, our data show that tadpole-like control is intact, and support the model proposed by Callery and Elinson (Callery and Elinson, 2000b; Callery et al., 2001) that direct developing frogs undergo metamorphosis inside the eggs. Thus, our data extend the evidence for their model by showing that the neuroendocrine regulation of development in direct developers is like that in metamorphosing tadpoles.

Given this neuroendocrine conservation, the basis for retaining the larval-type hypothalamic control of TH production is not clear. CRF is a shared regulatory neuropeptide controlling both stress and thyroid axes in biphasic developers (Denver, 2009). This dual control is implicated to play a vital role in adjusting the timing of metamorphosis in response to environmental perturbations (Denver, 2009). Denver et al. (1998) showed that tadpoles of spadefoot toad *Spea hammondii* can respond to deteriorating environment by increasing CRF levels, which in turn increase corticosterone and TH levels and accelerate development (Denver, 1997, 1998). TRH was unable to affect tadpole development in benign conditions (Denver, 1993). It is proposed that tadpoles sense the environment, and the sensory signals somehow modulate hypothalamic signals, which then affect the rate of development (Denver, 2009). However, very little evidence indicates that eggs of direct developing frogs face such environmental pressures and it is not clear under what natural context *E. coqui* might use this ability. Interestingly, direct developing frogs can hatch early in response to poking stress (Buckley et al., 2005). The extent to which other developmental events besides hatching are affected by poking stress remains to be determined. Nevertheless, if direct developing frogs are hatching early in response to stress it suggests that they may be able to accelerate metamorphosis inside the egg as well as hatch out early.

Another potential reason for the neuroendocrine conservation between biphasic and direct developers is that developmental and physiological constraints might maintain the ancestral mechanisms without any obvious adaptive reasons. For example, Hanken et al. (1997) showed that, even though pre-hatching *E. coqui* obviously do not feed, they still develop the mid-metamorphic form of the jaw before undergoing remodeling into the adult jaw. This situation may represent a developmental constraint, such that it may not possible to form the adult jaw by avoiding the mid-metamorphic form. Similarly, tadpole-like hypothalamic signaling may be retained because of physiological constraints, where, for example, development of TRH control of TH production may require CRF signaling.

Our TH treatments showing that *E. coqui* possesses the ability to accelerate the development in the presence of exogenous TH were important for two reasons. First, the developmental acceleration was similar to our oCRF treatments, supporting the idea that CRF acts to accelerate development via stimulating production of TH through a CRF–TSH–TH axis. Second, even though TH was shown to be required for development in *E. coqui* (Callery and Elinson, 2000b), it had not been shown if it merely played a permissive role or if it were sufficient to accelerate metamorphosis as in tadpoles. Our results indicate the latter possibility, extending our knowledge of the endocrine similarities between *E. coqui* and biphasic developers.

Even though direct developers have evolved a derived developmental mode, the underlying fundamental endocrine pathways are well conserved. Therefore, the developmental explanation for the basis of evolutionary change to achieve direct development does not seem to reside in central control, but in changes affecting development before endogenous TH production as well as tissue-specific responses to TH. One of the important attributes of TH physiology is sensitivity, and responsivity of tissues to TH and may play a vital role in evolution (Buchholz and Hayes, 2005; Shi, 2000). For example, increased sensitivity and responsivity of tissues to TH may underlie evolution of accelerated development in New World spadefoot toads (Buchholz and Hayes, 2005). Interestingly, we found that sensitivity of *E. coqui* to TH is extremely low compared to commonly studied tadpoles of biphasic developers. In spadefoot toads, a dose as low as 1 nM T3 can elicit a response in *in vitro* tail tip cultures (Buchholz and Hayes, 2005), and a dose of 8 nM T3 added to the rearing water can be lethal within three days (Buchholz unpubl.). In contrast, *E. coqui* was unable to respond to 2 and 10 nM T3 but increased its rate of tail resorption by 2 days in 14-day treatment of 50 nM T3. Surprisingly, these animals treated with 50 nM T3 did not die or undergo abnormal development, which generally happens at much lower doses of TH in biphasic developers. Elinson (1994) obtained similar results where doses of 2 and 20 nM T3 had no effect on the embryos of *E. coqui*, whereas these same doses caused premature metamorphosis in *Xenopus* and *Rana*, respectively. Doses up to 5 μM T3 accelerated tail resorption in *E. coqui*, but there was no effect on hindlimb development (Elinson, 1994). This pattern of lower sensitivity to TH was also seen in *Eleutherodactylus. martinicensis*, another species of direct developing frog (Hughes, 1966). The basis for the difference in sensitivity to T3 in tail versus hindlimb is not known but represents the opposite pattern of tissue-specific sensitivity and responsivity compared to biphasic developers where the limbs show the highest sensitivity to TH (Shi, 2000). These results indicate that either all or some direct developing species including *E. coqui* evolved a very low sensitivity to TH. However, the relationship between the evolutionary change in tissue sensitivity and the evolution of direct development, if any, remains unclear.

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