

## RAPID COMMUNICATION

**Acceleration of *Ambystoma tigrinum* Metamorphosis by Corticotropin-Releasing Hormone**GRAHAM C. BOORSE<sup>1</sup> AND ROBERT J. DENVER<sup>1,2\*</sup><sup>1</sup>*Department of Ecology and Evolutionary Biology, The University of Michigan, Ann Arbor, Michigan 48109-1048*<sup>2</sup>*Department of Molecular, Cellular and Developmental Biology, The University of Michigan, Ann Arbor, Michigan 48109-1048*

**ABSTRACT** Previous work of others and ours has shown that corticotropin-releasing hormone (CRH) is a positive stimulus for thyroid and interrenal hormone secretion in amphibian larvae and that activation of CRH neurons may mediate environmental effects on the timing of metamorphosis. These studies have investigated CRH actions in anurans (frogs and toads), whereas there is currently no information regarding the actions of CRH on metamorphosis of urodeles (salamanders and newts). We tested the hypothesis that CRH can accelerate metamorphosis of tiger salamander (*Ambystoma tigrinum*) larvae. We injected tiger salamander larvae with ovine CRH (oCRH; 1 µg/day; i.p.) and monitored effects on metamorphosis by measuring the rate of gill resorption. oCRH-injected larvae completed metamorphosis earlier than saline-injected larvae. There was no significant difference between uninjected and saline-injected larvae. Mean time to reach 50% reduction in initial gill length was 6.9 days for oCRH-injected animals, 11.9 days for saline-injected animals, and 14.1 days for uninjected controls. At the conclusion of the experiment (day 15), all oCRH-injected animals had completed metamorphosis, whereas by day 15, only 50% of saline-injected animals and 33% of uninjected animals had metamorphosed. Our results show that exogenous oCRH can accelerate metamorphosis in urodele larvae as it does in anurans. These findings suggest that the neuroendocrine mechanisms controlling metamorphosis are evolutionarily conserved across amphibian taxa. *J. Exp. Zool.* 293:94–98, 2002. © 2002 Wiley-Liss, Inc.

Thyrotropin-releasing hormone (TRH), the neurohormone that regulates pituitary thyroid-stimulating hormone (TSH) secretion in mammals, is expressed in the tadpole hypothalamus (see Denver, '96), but lacks the ability to stimulate TSH secretion in the tadpole (Denver and Licht, '89a; see Kikuyama et al., '93; Denver, '96) or in the neotenic axolotl (Darras and Kuhn, '83; Jacobs and Kuhn, '87). However, TRH is active in stimulating thyroid activity in adult frogs (Darras and Kuhn, '82; Denver, '88) and metamorphosed axolotls (induced by injections of thyroxine; Jacobs and Kuhn, '87), suggesting a developmental switch in the neuroendocrine control of the thyroid system in amphibia (see Denver, '96). Current evidence supports a role for corticotropin-releasing hormone (CRH) in the positive regulation of pituitary TSH secretion in tadpoles (Denver, '96, '99). CRH is the primary neurohormone regulating pituitary adrenocorticotropin (ACTH) secretion in mammals (see Vale et al., '97). In amphibians and other nonmammalian vertebrates, CRH may play a dual role by regulating both the thyroid and the in-

terrenal (adrenal) axes (Denver and Licht, '89a; Denver, '99).

Several lines of evidence support a physiological role for CRH in regulating TSH secretion in anurans (frogs and toads). For example, CRH stimulated the secretion of thyrotropic bioactivity (Denver, '88; Denver and Licht, '89a; Jacobs and Kuhn, '92) and immunoreactive TSH  $\beta$  subunit (Denver, unpublished results) by cultured tadpole and adult frog pituitaries. Injections of CRH or CRH-like peptides (i.e., sauvagine) elevated whole body thyroid hormone (Gancedo et al., '92; Denver, '93, '97a) and corticosterone (Denver, '97a) content and accelerated metamorphosis of tadpoles of four frog species (*Rana catesbeiana* and *Spea hammondi*, Denver, '93;

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*Rana perezi*, Gancedo et al., '92; and *Bufo arenarum*, Miranda et al., 2000). These four species represent three anuran genera; however, more information from diverse amphibian taxa, especially urodeles, is needed to evaluate how generalizable this action of CRH is in amphibians.

CRH has been shown to activate the thyroid and interrenal axes and to accelerate metamorphosis in anuran larvae; however, there is currently no information on the actions of CRH in urodeles (salamanders and newts). In the present study, we tested the hypothesis that injection of CRH can accelerate metamorphosis of larvae of the tiger salamander, *Ambystoma tigrinum*. We injected ovine CRH (i.p.) over a period of two weeks and monitored metamorphic progression by measuring gill length.

## MATERIALS AND METHODS

### *Animals and animal husbandry*

*A. tigrinum* egg masses were collected from ponds located near Pinckney, MI (Michigan scientific collecting permit # CO577). Egg masses were placed in polystyrene cages (24 cm × 45 cm × 20 cm) containing ten liters of dechlorinated water. Upon hatching, larvae were reared individually in polystyrene cages (20.5 cm × 34 cm × 11 cm) in two liters of water and fed brine shrimp, followed by tubifex worms, and then beef liver as they grew. Water was changed every two to three days and larvae were maintained on a constant 12L:12D light/dark cycle and a temperature range of 21–23°C.

### *Determination of body size at metamorphosis*

Previous studies with *A. tigrinum* larvae in our laboratory (R. J. Denver, unpublished data) led us to hypothesize that a larva must reach a minimum size or developmental threshold in order to become competent to respond to environmental or neuroendocrine stimuli. Thus, we first conducted a study aimed at determining the range of body size at which spontaneous metamorphosis is initiated in *A. tigrinum* larvae reared in the laboratory under constant environmental conditions. Two weeks after hatching, larvae (n = 10/treatment) were separated into two groups and treated with or without the goitrogen methimazole dissolved in water to a concentration of 1 mM (Denver et al., '97). Digital images of individual larvae were captured weekly using an Olympus D-500L digital camera mounted on a tripod at a fixed height above the

cage. Digitized images were analyzed for body lengths using NIH image software.

### *CRH injections*

Based on the above experiment, CRH injections were initiated in 13-week-old *A. tigrinum* larvae (mean body length=12.121 cm, SE = 0.157, range 10.69–13.27 cm). Larvae were separated into three treatment groups (n = 6/treatment), which included uninjected controls, saline-injected controls (daily i.p. injections of 50 µl phosphate buffered saline [PBS]: 0.02 M sodium phosphate, 0.6% sodium chloride), and ovine CRH-injected experimentals (daily i.p. injections of 1 µg oCRH dissolved in 50 µl PBS).

Metamorphic progression was monitored by measuring gill length. Digital images were captured every two days starting at time zero and the length of the right middle gill rachis was measured. The time in days for each larva to achieve 50% gill resorption (GR<sub>50</sub>) was calculated. This method provides an accurate means to compare rates of metamorphosis in *A. tigrinum* (Norris et al., '73; Norris and Platt, '74).

### *Statistical analysis*

GR<sub>50</sub> values were analyzed using Student's unpaired *t*-test to make pairwise comparisons between uninjected and saline-injected or between saline-injected and CRH-injected groups. Changes in gill length were analyzed over time (each time point was analyzed separately) by unpaired *t*-test to compare uninjected or CRH-injected to saline-injected.

## RESULTS AND DISCUSSION

Growth rates of control and methimazole-treated larvae were similar; however, methimazole-treated larvae never initiated metamorphosis (Fig. 1). Control larvae initiated metamorphosis 13 weeks after hatching and all had completed metamorphosis by week 16 (Fig. 1).

Larvae receiving injections of oCRH metamorphosed earlier than saline-injected larvae. Significant differences in gill length were apparent two days after beginning injections (Fig. 2;  $T = -2.701$ ,  $P = 0.0223$ ). No significant differences were detected in gill length between saline-injected and uninjected larvae (Fig. 2). GR<sub>50</sub> values were significantly lower for oCRH injected animals compared to saline-injected larvae (Table 1;  $T = 2.629$ ,  $P = 0.0340$ ). oCRH injection also resulted in a larger number of animals undergoing complete transformation than either uninjected or saline-injected

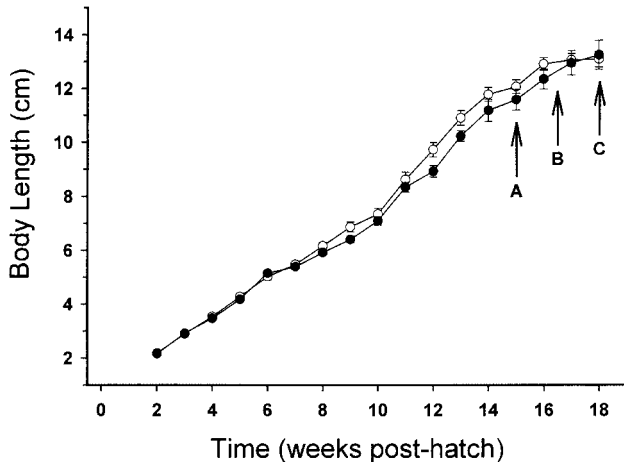


Fig. 1. Analysis of growth and development of *Ambystoma tigrinum* larvae for 18 weeks following hatching. Larvae were reared in the absence (open circles) or presence (closed circles) of the goitrogen methimazole (1 mM). Points on the graph represent means  $\pm$  SEM ( $n = 10$ /treatment). Arrows indicate the following progression of metamorphosis in control animals: A) initial signs of metamorphosis; B) 50% of control animals completing metamorphosis; C) 100% of control animals completing metamorphosis. Goitrogen-treated animals never metamorphosed during the course of this experiment.

larvae by the end of the experiment (Table 1). The saline-injected and uninjected larvae that had not completely transformed by the end of the

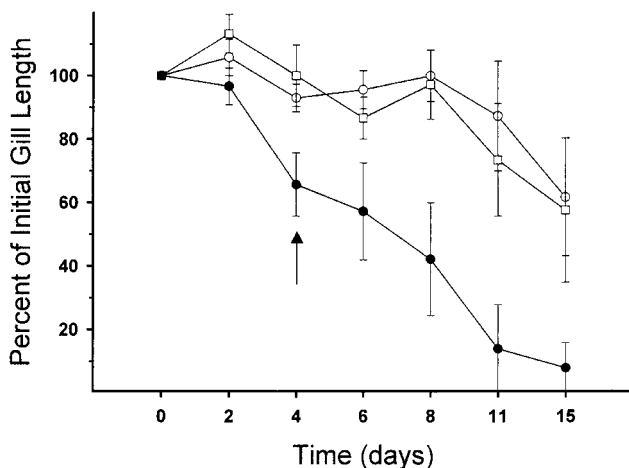


Fig. 2. Acceleration of metamorphosis in *Ambystoma tigrinum* larvae following daily i.p. injections of oCRH. Larvae were assigned to one of three experimental treatments: oCRH-injected experimentals (1  $\mu$ g in 50  $\mu$ l PBS/day; closed circles); saline-injected controls (50  $\mu$ l PBS/day; open square); or uninjected controls (open circles). Metamorphic progression was monitored by measuring gill length (see Materials and Methods). Points on the graph represent means  $\pm$  SEM ( $n = 6$ /treatment). The arrow designates the day at which gill length of CRH-injected animals significantly diverged ( $P < 0.05$ ) from the two control groups.

TABLE 1. Effects of oCRH on time to 50% gill resorption ( $GR_{50}$ ) in *A. tigrinum* larvae<sup>1</sup>

| Group             | $GR_{50}$ (Days $\pm$ SE) | Number of larvae completing metamorphosis at end of experiment |
|-------------------|---------------------------|--|
| Untreated         | 14.1 $\pm$ 0.7            | 2  |
| Saline            | 11.9 $\pm$ 1.4            | 3  |
| oCRH <sup>2</sup> | 6.9 $\pm$ 2.0*            | 6  |

<sup>1</sup>  $GR_{50}$ , mean number of days to reach 50% gill resorption.

<sup>2</sup> Larvae were given injections of oCRH (1  $\mu$ g) every day for 15 days ( $n = 6$ /group). Asterisk designates significantly different from saline-treated larvae by unpaired t-test ( $P < 0.05$ ).

experiment metamorphosed over the subsequent one to four weeks in the laboratory.

This is the first demonstration of a stimulatory effect of CRH on metamorphosis in a urodele. Previous studies of four anuran species showed that CRH injections cause tadpoles to metamorphose earlier than saline-injected controls (Gancedo et al., '92; Denver, '93, '97a; Miranda et al., 2000). Taken together with findings in anurans, our current findings support the hypothesis that CRH can positively regulate metamorphosis in amphibians.

CRH has now been shown to be a potent stimulator of the thyroid axis in representatives of each nonmammalian vertebrate class (reviewed by Denver, '99). For example, CRH stimulates TSH secretion by cultured pituitaries of salmon (Larsen et al., '98), turtles (Denver and Licht, '89b, '91), and chickens (Meeuwis et al., '89; Geris et al., '96). These findings have led to the hypothesis (Denver, '99) that the primitive hypophysiotropic role of CRH in vertebrates was as a stimulator of both the thyroid and the interrenal (adrenal) axes.

CRH is the primary vertebrate stress neurohormone, and the activation of CRH neurons in response to environmental stressors is well known in mammals (Imaki et al., '95; Kovacs and Sawchenko, '96a, '96b). Hormones of both the thyroid and the interrenal glands are known to positively and synergistically influence amphibian metamorphosis (see Kikuyama et al., '93). A dual hypophysiotropic role for CRH suggests a proximate mechanism by which amphibian larvae can respond to environmental change (see Denver, '97b). Spadefoot toad tadpoles (genus *Scaphiopus*) have been used to study this response since they exhibit robust metamorphic responses to habitat desiccation (i.e., they exhibit adaptive phenotypic plasticity; see Newman, '89). Pond drying can be simulated in the laboratory by removing water from tanks in which

the tadpoles are reared (Denver, '97a; Denver et al., '98). Spadefoot toad tadpoles exposed to declining water levels in the laboratory exhibited elevated hypothalamic CRH content (Denver, '97a) and whole body content of thyroxine, 3,3',5'-triiodothyronine and corticosterone (Denver, '98). The timing of these hormonal changes correlated with the first external metamorphic changes (Denver, '97a, '98). These findings have led to the hypothesis that the CRH signaling system may play a central role in controlling the timing of metamorphosis in response to environmental change (see Denver, '99).

Evidence for a physiological role for endogenous CRH during spontaneous and stress-induced metamorphosis is suggested by studies in which the action of endogenous CRH was blocked. Bullfrog tadpoles passively immunized with CRH antiserum exhibited a slower rate of spontaneous metamorphosis (Denver, '93). Metamorphic acceleration in response to water volume reduction in spadefoot toad tadpoles was attenuated by passive immunization with CRH antiserum or by injections of  $\alpha$ -helical CRH<sub>(9-41)</sub>, a CRH receptor antagonist (Denver, '97a). Taken together with the findings that CRH injections can accelerate metamorphosis, these findings point to a central role for CRH in controlling environmentally-induced and spontaneous metamorphosis.

Many urodele species are obligate metamorphs (as are all anurans with the exception of direct developers), but others are facultative paedomorphs and can either metamorphose or remain in the aquatic habitat and become reproductively mature while retaining larval characteristics (paedomorphosis; Duellman and Trueb, '94). A higher proportion of metamorphs versus paedomorphs is observed in facultatively paedomorphic salamander larvae exposed to pond drying (Semlitsch, '87), high conspecific density (Harris, '87; Semlitsch, '87), or high predation risk (Jackson and Semlitsch, '93). The propensity to undergo paedomorphosis differs among *A. tigrinum* populations. We have no evidence that the population sampled for this study exhibits paedomorphosis in naturally occurring ponds. However, it is noteworthy that, within this population, paedomorphic individuals have been described in large, man-made fishless ponds, showing that the population has the ability to produce paedomorphs (Jones et al., '93).

We hypothesize that the stress neurohormone CRH mediates environmental effects on salamander development by acting as a physiological indicator of 'stressful' conditions in the larval habitat. An elevation of CRH production in response to environ-

mental stress would be predicted to activate the interrenal and the thyroid axes, leading to metamorphosis. We hypothesize that CRH acts a developmental 'switch,' thus determining the life history trajectory (paedomorphosis versus metamorphosis). Anecdotal observations support the involvement of a stress response in initiating metamorphosis in urodeles. For example, capture/confinement stress precipitates metamorphosis in tiger salamander larvae (some of which were demonstrated paedomorphs; Norris et al., '77; Norris, '78; Earl Werner, personal communication; R.J. Denver, personal observations).

Combining ecological and endocrinological approaches to this question may elucidate the proximate mechanisms and their modification by environmental variables that underlie the 'choice' of life history trajectory in facultatively paedomorphic salamanders.

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