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RAPID COMMUNICATION

Acceleration of *Ambystoma tigrinum* Metamorphosis by Corticotropin-Releasing Hormone

GRAHAM C. BOORSE[1] AND ROBERT J. DENVER[1,2]*
[1] *Department of Ecology and Evolutionary Biology, The University of Michigan, Ann Arbor, Michigan 48109-1048*
[2] *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.*

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 interrenal (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 β 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 hammondii*, Denver, '93;

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*Correspondence to: Robert J. Denver, Department of Molecular, Cellular and Developmental Biology, 3065C Natural Science Bldg., The University of Michigan, Ann Arbor, MI 48109-1048. E-mail: rdenver@umich.edu
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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_{50}) 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_{50} 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_{50} 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

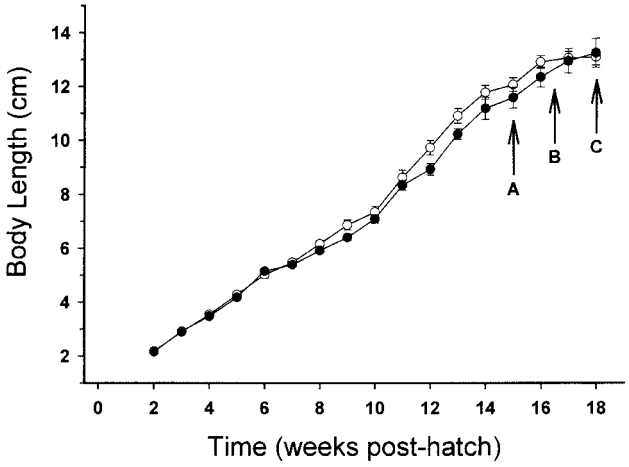

Fig. 1. Analysis of growth and development of *Ambystoma tigrinum* larvae for 18 weeks following hatching. Larvae were reared in the absence (open circles) or presence (closed circles) of the goitrogen methimazole (1 mM). Points on the graph represent means ± SEM (n = 10/treatment). Arrows indicate the 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 experiment metamorphosed over the subsequent one to four weeks in the laboratory.

TABLE 1. Effects of oCRH on time to 50% gill resorption (GR_{50}) in A. tigrinum larvae[1]

Group	GR_{50} (Days ± SE)	Number of larvae completing metamorphosis at end of experiment
Untreated	14.1 ± 0.7	2
Saline	11.9 ± 1.4	3
oCRH[2]	6.9 ± 2.0*	6

[1] GR_{50}, mean number of days to reach 50% gill resorption.
[2] Larvae were given injections of oCRH (1 μg) every day for 15 days (n = 6/group). Asterisk designates significantly different from saline-treated larvae by unpaired t-test ($P < 0.05$).

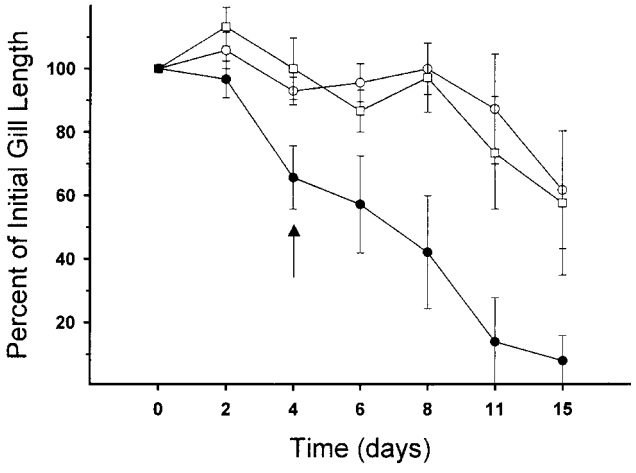

Fig. 2. Acceleration of metamorphosis in *Ambystoma tigrinum* larvae following daily i.p. injections of oCRH. Larvae were assigned to one of three experimental treatments: oCRH injected experimentals (1 μg in 50 μl PBS/day; closed circles); saline injected controls (50 μl PBS/day; open square); or uninjected controls (open circles). Metamorphic progression was monitored by measuring gill length (see Materials and Methods). Points on the graph represent means ± 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.

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 α-helical $CRH_{(9–41)}$, 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 environmental 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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LITERATURE CITED

Darras VM, Kuhn ER. 1982. Increased plasma levels of thyroid hormones in a frog *Rana ridibunda* following intravenous administration of TRH. Gen Comp Endocrinol 48:469–475.

Darras VM, Kuhn ER. 1983. Effects of TRH, bovine TSH, and pituitary extracts on thyroidal T4 release in *Ambystoma mexicanum*. Gen Comp Endocrinol 51:286–291.

Denver RJ. 1988. Several hypothalamic peptides stimulate *in vitro* thyrotropin secretion by pituitaries of anuran amphibians. Gen Comp Endocrinol 72:383–393.

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

Denver RJ. 1996. Neuroendocrine control of amphibian metamorphosis. In: Gilbert LI, Tata JR, Atkinson BG, editors. Metamorphosis: postembryonic reprogramming of gene expression in amphibian and insect cells. San Diego, CA: Academic Press. p 434–464.

Denver RJ. 1997a. 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 RJ. 1997b. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. Am Zool 37:172–184.

Denver RJ. 1998. Hormonal correlates of environmentally induced metamorphosis in the western spadefoot toad, *Scaphiopus hammondii*. Gen Comp Endocrinol 110:326–336.

Denver RJ. 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 RJ, Licht P. 1989a. Neuropeptide stimulation of thyrotropin secretion in the larval bullfrog—evidence for a common neuroregulator of thyroid and interrenal activity in metamorphosis. J Exp Zool 252:101–104.

Denver RJ, Licht P. 1989b. Neuropeptides influencing in vitro pituitary hormone secretion in hatchling turtles. J Exp Zool 251:306–315.

Denver RJ, Licht P. 1991. Several hypothalamic peptides stimulate thyrotropin and growth hormone secretion by adult turtle pituitary glands. Comp Biochem Physiol A Mol Integr Physiol 100:603–606.

Denver RJ, Pavgi S, Shi YB. 1997. Thyroid hormone-dependent gene expression program for *Xenopus* neural development. J Biol Chem 272:8179–8188.

Denver RJ, Mirhadi N, Phillips M. 1998. Adaptive plasticity in amphibian metamorphosis: response of *Scaphiopus hammondii* tadpoles to habitat desiccation. Ecology 79:1859–1872.

Duellman WE, Trueb L. 1994. Biology of amphibians. Baltimore, MD: The John Hopkins University Press.

Gancedo B, Corpas I, Alonso-Gomez AL, Delgado MJ, Morreale de Escobar G, Alonso-Bedate M. 1992. Corticotropin-releasing factor stimulates metamorphosis and increases thyroid hormone concentration in prometamorphic *Rana perezi* larvae. Gen Comp Endocrinol 87:6–13.

Geris KL, Kotanen SP, Berghman LR, Kuhn ER, Darras VM. 1996. Evidence of a thyrotropin-releasing activity of ovine corticotropin-releasing factor in the domestic fowl (*Gallus domesticus*). Gen Comp Endocrinol 104:139–146.

Harris RN. 1987. Density-dependent paedomorphosis in the salamander *Notophthalmus viridescens dorsalis*. Ecology 68: 705–712.

Imaki T, Xiao-Quan W, Shibasaki T, Yamada K, Harada S, Chikada N, Naruse M, Demura H. 1995. Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats. J Clin Invest 96:231–238.

Jackson ME, Semlitsch RD. 1993. Paedomorphosis in the salamander *Ambystoma talpoideum*—effects of a fish predator. Ecology 74:342.

Jacobs GFM, Kuhn ER. 1987. TRH injection induces thyroxine release in the metamorphosed but not in the neotenic axolotl *Ambystoma mexicanum*. Gen Comp Endocrinol 66:40.

Jacobs GFM, Kuhn ER. 1992. Thyroid-hormone feedback-regulation of the secretion of bioactive thyrotropin in the frog. Gen Comp Endocrinol 88:415–423.

Jones TR, Skelly DK, Werner EE. 1993. *Ambystoma tigrinum tigrinum* (eastern tiger salamander) developmental polymorphism. Herpetol Rev 24:147–148.

Kikuyama S, Kawamura K, Tanaka S, Yamamoto K. 1993. Aspects of amphibian metamorphosis—hormonal control. Int Rev Cytol 145:105–148.

Kovacs KJ, Sawchenko PE. 1996a. Regulation of stress-induced transcriptional changes in the hypothalamic neurosecretory neurons. J Mol Neurosci 7:125–133.

Kovacs KJ, Sawchenko PE. 1996b. Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. J Neurosci 16:262–273.

Larsen DA, Swanson P, Dickey JT, Rivier J, Dickhoff WW. 1998. *In vitro* thyrotropin-releasing activity of corticotropin- releasing hormone-family peptides in Coho salmon, *Oncorhynchus kisutch*. Gen Comp Endocrinol 109:276–285.

Meeuwis R, Michielsen R, Decuypere E, Kuhn ER. 1989. Thyrotropic activity of the ovine corticotropin-releasing factor in the chick embryo. Gen Comp Endocrinol 76:357–363.

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

Newman RA. 1989. Developmental plasticity of *Scaphiopus couchii* tadpoles in an unpredictable environment. Ecology 70:1775–1787.

Norris DO. 1978. Hormonal and environmental factors involved in the determination of neoteny in urodeles. In: Gallard P, Boer H, editors. Comparative endocrinology. Amsterdam: Elsevier/North-Holland Biomedical Press. pp. 109–112.

Norris DO, Platt JE. 1974. T3- and T4-induced rates of metamorphosis in immature and sexually mature larvae of *Ambystoma tigrinum* (Amphibia: Caudata). J Exp Zool 189:303–310.

Norris DO, Jones RE, Cohen DC. 1973. Effects of mammalian gonadotropins (LH, FSH, hCG) and gonadal steroids on TSH-induced metamorphosis of *Ambystoma tigrinum* (Amphibia: Caudata). Gen Comp Endocrinol 20:467–473.

Norris DO, Duvall D, Greendale K, Gern WA. 1977. Thyroid function in pre- and postspawning neotenic tiger salamanders (*Ambystoma tigrinum*). Gen Comp Endocrinol 33: 512–517.

Semlitsch RD. 1987. Paedomorphosis in *Ambystoma talpoideum*: effects of density, food, and pond drying. Ecology 68:994–1002.

Vale W, Vaughan J, Perrin M. 1997. Corticotropin-releasing factor (CRF) family of ligands and their receptors. Endocrinologist 7:S3–S9.