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Endocrine Mechanisms Underlying Plasticity in Metamorphic Timing in Spadefoot Toads[1]

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SYNOPSIS. Amphibian larvae respond to heterogeneous environments by varying their rates of growth and development. Several amphibian species are known to accelerate metamorphosis in response to pond drying or resource restriction. Some of the most extensive studies to date on developmental responses to pond drying have been conducted on species of spadefoot toads (family Pelobatidae). We have found that tadpoles of two species of spadefoot toad accelerate metamorphosis when exposed to water volume reduction in the laboratory (to simulate a drying pond). Furthermore, Western spadefoot toad (*Spea hammondii*) tadpoles accelerated metamorphosis in response to food restriction, which was intended to simulate a decline in resource availability in the larval habitat. Metamorphic acceleration was accompanied by increased whole body 3,5,3′-triiodothyronine and hindbrain corticotropin-releasing hormone content by 24 hr after transfer of tadpoles from high to low water. Food restriction for 4 day accelerated metamorphosis and elevated whole body thyroid hormone content. Although tadpoles accelerated metamorphosis and activated their thyroid axis in response to the two environmental manipulations, the kinetics of the responses were greater for water volume reduction than for resource restriction. The modulation of hormone secretion and action by environmental factors provides a mechanistic basis for plasticity in the timing of amphibian metamorphosis, and the neuroendocrine stress axis may play a central role in developmental plasticity.

INTRODUCTION

Phenotypic plasticity is defined as the capacity for a given genotype to produce different phenotypes in different environments (Bradshaw, 1965; Schlichting, 1986; Stearns, 1992). For organisms that live in temporally and spatially heterogeneous environments, phenotypic plasticity may provide a means for increasing fitness (Bradshaw, 1965; Levins, 1968; Schlichting, 1986; Stearns, 1989; Stearns, 1992; Schlichting and Pigliucci, 1998). The timing of amphibian metamorphosis has been a frequent subject of studies that have investigated the evolutionary ecology of phenotypic plasticity (Wilbur and Collins, 1973; Collins, 1979; Werner, 1986; Alford and Harris, 1988; 1989; Newman, 1992). Ecological factors such as conspecific density (Wilbur, 1972; Gromko *et al.*, 1973; Wilbur and Collins, 1973; Wilbur, 1976, 1977*a*, *b*; Semlitsch and Caldwell, 1982; Newman, 1987; Scott, 1990; Newman, 1994, 1998), food availability (Travis and Trexler, 1986; Alford and Harris, 1988; Berven and Chadra, 1988; Newman, 1994, 1998; Morey and Reznick, 2000), predator presence (Skelly and Werner, 1990; Wilbur and Fauth, 1990; McCollum and Leimberger, 1997), and water level (Newman, 1988, 1989; Denver *et al.*, 1998; Blaustein *et al.*, 1999; Laurila and Kujasalo, 1999; Loman, 1999; Parris, 2000; Spieler, 2000; Laurila *et al.*, 2002; Loman, 2002) can influence the rate of development and thus alter the timing of and size at metamorphosis. Also, abiotic factors such as temperature, dissolved gases, pH and photoperiod can interact in complex ways with biotic factors (such as food availability and conspecific density) to affect time to and size at metamorphosis (Marian and Pandian, 1985; Newman, 1998).

The rate of development generally is inversely related to larval growth rate and therefore to size at metamorphosis, and size at transformation can have important fitness consequences. For example, smaller size at metamorphosis can result in lower survival (Morey and Reznick, 2001) and may in some species decrease size at first reproduction and delay reproductive maturity (Berven and Gill, 1983; Smith, 1987; Semlitsch *et al.*, 1988). Thus, there are important tradeoffs between size at metamorphosis and the risk of mortality in the larval habitat.

In addition to ecological studies investigating the timing of amphibian metamorphosis, a considerable amount of work has addressed the endocrine control of metamorphosis (reviewed by Denver *et al.*, 2002). Endocrine studies have shown that thyroid hormone is the principle morphogen controlling amphibian metamorphosis. Thyroid hormone is capable of inducing the entire suite of biochemical and morphological changes associated with metamorphosis (Dodd and Dodd, 1976; Kikuyama *et al.*, 1993; Denver *et al.*, 2002). Despite a wealth of information on the ecology and the endocrinology of metamorphosis, few studies have united these fields in an effort to understand the links between environmental variation and endocrine control of metamorphosis (Denver, 1997*a*, 1998).

The vertebrate neuroendocrine system (hypothalamus and pituitary gland) controls development, growth, and reproduction and can translate sensory and metabolic information about the external and internal environments into an integrated physiological/developmental response. Previous work of ours and

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others has shown that the neuroendocrine system is necessary for metamorphosis, and serves to transduce environmental information into a developmental response (reviewed by Denver *et al.*, 2002). Current evidence suggests that corticotropin-releasing hormone (CRH), the principle vertebrate stress neurohormone, controls the activity of both the thyroid and interrenal (homologue of mammalian adrenal cortex) glands in larval amphibians (reviewed by Denver, 1999). Tadpole pituitaries secrete thyroid-stimulating hormone (TSH) in response to CRH (Denver and Licht, 1989; Boorse *et al.*, 2002; Okada *et al.*, 2003). Moreover, injections of CRH (and CRH-like peptides) accelerated metamorphosis in several anurans and a urodele (anurans: Gancedo *et al.*, 1992; Denver, 1993, 1997*a*; Miranda *et al.*, 2000; urodele: Boorse and Denver, 2002). Injections of CRH (or CRH-like peptides) elevated whole body thyroid hormone (Gancedo *et al.*, 1992; Denver, 1993, 1997*a*) and corticosteroid content (Denver, 1997*a*) in a dose-dependent manner. By contrast, injections of the CRH antagonist α-helical $CRH_{(9–41)}$ or passive immunization with CRH antiserum reduced whole body triiodothyronine (T_3), thyroxine (T_4) and corticosterone (CORT) content (Denver, 1997*a*). Such treatment also slowed environmentally-induced or spontaneous metamorphosis in two species, thus supporting a physiological role for CRH in tadpole metamorphosis (Denver, 1993, 1997*b*).

The findings described above have led to the hypothesis that CRH plays a dual hypophysiotropic role in amphibian larvae, stimulating both the interrenal (stress) axis (as it does in mammals) and the thyroid axis. This dual function has important implications for understanding the translation of environmental signals into changes in larval endocrine activity (Denver, 1997*b*). Neurosecretory neurons that secrete CRH are very sensitive to environmental change. In vertebrates, the activation of CRH neurons is causally linked to the rapid increase in plasma corticosteroid concentrations during the classical ''fight or flight'' response. Since in amphibian larvae this stress neurohormone also stimulates the thyroid axis, CRH may provide a means for larvae to respond to deleterious changes in their larval habitat by accelerating metamorphosis (Denver, 1997*b*).

We have used spadefoot toads (Family Pelobatidae, Genus *Spea* and *Scaphiopus*) to investigate the physiological basis for plasticity in the timing of metamorphosis. Spadefoot toads are desert-dwelling amphibians that breed opportunistically in short lived pools filled by periodic rainfall. Tadpoles of both Couch's spadefoot toad (*Scaphiopus couchii*) and the Western spadefoot toad (*Spea hammondii*) accelerate metamorphosis in response to pond drying (Newman, 1989, 1992; Denver *et al.*, 1998). Acceleration of metamorphosis by *S. hammondii* tadpoles in response to reduced water levels can be replicated in the laboratory (Denver, 1997*a*, 1998; Denver *et al.*, 1998), allowing for detailed analysis of the physiological mechanisms responsible for plasticity in the timing of metamorphosis. Tadpoles transferred to a low water environment cease foraging, and we hypothesized that reduced food intake plays a role in the metamorphic acceleration. In support of this hypothesis, prometamorphic *S. hammondii*, *S. couchii*, and *S. intermontanus* tadpoles maintained in a constant high water environment accelerated metamorphosis in response to food restriction (Denver *et al.*, 1998; Morey and Reznick, 2000).

Previous work of ours demonstrated morphological changes and elevated whole body T_3, T_4 and CORT content in *S. hammondii* tadpoles at 48 hr after transfer to low water (Denver, 1998). In the present study we examined earlier time points for morphological and endocrine system changes in response to simulated pond drying. We analyzed changes in whole body T_3 and T_4 content, and CRH content in two brain regions at different times following immediate transfer from high to low water. We also tested the hypothesis that food restriction increases thyroid activity leading to accelerated metamorphosis. We discuss current knowledge of the mechanistic basis for environmentally-induced metamorphic acceleration, and highlight areas where further research is needed.

MATERIALS AND METHODS

Animal care and husbandry

Spea hammondii adults were collected near Livermore, CA and maintained in a breeding colony at the University of Michigan. *Scaphiopus couchii* adults were collected near the Southwestern Research Station, Portal, AZ. Spawning was induced by injecting gonadotropin-releasing hormone agonist (des-Gly^{10}, [D-$His(Bzl)^6$]-luteinizing hormone releasing hormone ethylamide; Sigma, St. Louis, MO catalog# L-2761; 1 μg in 50 μl 0.6% NaCl) into the dorsal lymph sac as described (Denver, 1997*a*). Embryos and tadpoles were maintained in polystyrene cages (45 × 24 × 20 cm) in well water. Tadpoles were fed tadpole chow (a mixture of rabbit pellets, agar, and Knox gelatin; see Rugh, 1962). Water temperature ranged from 21 to 23°C and photoperiod was held constant at 12L:12D. Developmental stages were determined based on the Gosner staging system (Gosner, 1960).

Acute water volume reduction experiments

Spea hammondii. An earlier study from our laboratory (Denver, 1998) in which tadpoles were subjected to a rapid and drastic decline in the water level (10 liter to 0.5 liter) found significantly accelerated development and a profound activation of the thyroid and interrenal axes by 48 hr. The following experiments were designed to examine how quickly prometamorphic *S. hammondii* tadpoles increase thyroid activity and accelerate development in response to a rapid and drastic decline in the water level. Mid-prometamorphic tadpoles (Gosner stages 36–38, n = 6/treatment) were transferred from stock tanks containing 10 liter of water to tanks containing either 10 liter or 1 liter (water depths 10 cm and 1 cm, respectively). Tadpoles were

anesthetized in 0.001% benzocaine (Sigma, St. Louis, MO) and morphological measurements (Gosner stage, body weight [BW], hindlimb length [HLL] and tail height [TH]) were made at 24 hr and again at 48 hr. After the 48 hr measurement tadpoles were euthanized by immersion in 0.01% benzocaine, snap-frozen and stored at −20°C for whole body thyroid hormone analysis (see below).

To determine the time course of thyroid axis activation in *S. hammondii* tadpoles subjected to rapid water volume reduction, eighteen mid-prometamorphic tadpoles per treatment (Gosner stage 36–38) were transferred from stock tanks to 10 liter (high water treatment) or 1 liter (low water treatment) as in the previous experiment (n = 5 tanks/treatment; 18 tadpoles per tank). At 0, 0.5, 1, 2, 6 and 24 hr, three tadpoles were removed from each tank. At each sampling time, tadpoles were euthanized by immersion in 0.01% benzocaine, morphological measurements were taken, and tadpoles were snap-frozen and stored at −20°C for whole body thyroid hormone analysis. External morphological measurements (Gosner stage, BW, HLL and TH) are presented as tank means (3 tadpoles per tank, 5 tanks/treatment). Whole body thyroid hormone measurements were conducted on individual tadpoles randomly chosen from each time sample (n = 5; see below for tissue thyroid hormone analyses); thus, thyroid hormone measurements are based on individuals, not tank means. Six tadpoles at each timepoint were randomly chosen from the 5 tanks, euthanized and dissected to examine changes in gut morphology. Intestinal length was measured and tissues were then dried overnight in a drying oven (55°C) and weighed. Changes in intestinal length provide a measure of thyroid-dependent gut morphogenesis. However, since intestinal contents were not removed prior to drying, measurements of gut weight reflect differences in food consumption rather than morphological changes in the intestine. As for thyroid hormone content measurements, gut measurements are based on individuals, not tank means.

To test whether brain CRH peptide content changed during acute water volume reduction, two brain regions were collected from mid-prometamorphic tadpoles (Gosner stage 36–38, n = 6/time) that had been exposed to the acute water volume reduction paradigm for 0, 6, or 24 hr. For each tadpole, CRH peptide content was determined in fore-/midbrain and hindbrain regions (see Fig. 3 for diagram of brain regions collected). The fore/mid-brain region (all brain structures rostral to the region just posterior to the pituitary gland) contains the preoptic area and the hypothalamus/median eminence. Electrical stimulation or lesioning of the preoptic area have demonstrated its role in regulating ACTH secretion in amphibians (Notenboom and Terlou, 1976; Notenboom *et al.*, 1976) and in metamorphosis (reviewed by Denver, 1996). The preoptic nucleus (PON) is homologous to the mammalian supraoptic (SON) and paraventricular nuclei (PVN), and contains one of the largest collections of CRH neurons in the amphibian brain (reviewed by Denver, 1996; Westphal *et al.*, 2002). These cells project axons to the median eminence and thus play a hypophysiotropic role (*i.e.*, they are part of the hypothalamo-pituitary-interrenal [or adrenal] axis). The hindbrain region (all brain structures caudal to the pituitary gland including part of the spinal cord) contains CRH immunoreactive cell bodies in amphibians (Westphal *et al.*, 2002) and other vertebrates (Lehnert *et al.*, 1998). These hindbrain CRH neurons may play a role in autonomic and behavioral responses to stress (Butler *et al.*, 1990; Lehnert *et al.*, 1998), and could possibly influence PON CRH neurons either directly (via rostral projections to the PON) or indirectly, via activation of catecholaminergic neurons which have been shown to project to the PVN in rats (Sawchenko and Swanson, 1982; Valentino *et al.*, 1993; Valentino *et al.*, 1998; Ziegler and Herman 2002).

Scaphiopus couchii. For comparative reasons we examined whether tadpoles of the closely related species Couch's spadefoot toad were capable of accelerating metamorphosis in response to rapid water volume reduction in the laboratory. Field studies previously demonstrated that this species metamorphoses earlier and at a smaller body size in drying ponds compared with more permanent habitats (Newman, 1988, 1989). Prometamorphic *S. couchii* tadpoles (Gosner stage 37–38) were transferred from stock tanks containing 10 liter of water to tanks containing either 10 liter or 0.5 liter (20 tadpoles per tank). Morphological measurements (Gosner stage, snout-vent length, and BW) were recorded 24 hr after transfer. The body size of *S. couchii* tadpoles is approximately one fourth that of *S. hammondii* tadpoles at comparable developmental stages. Thus, the water volume used in the low water treatment for *S. couchii* was 0.5 liter (*vs.* 1 liter in the larger *S. hammondii* tadpoles). This exposed *S. couchii* tadpoles to a similar limitation in swimming volume as that experienced by the larger *S. hammondii* tadpoles.

Food restriction experiments

Previous work from our laboratory showed that resource restriction causes prometamorphic *S. hammondii* tadpoles to accelerate metamorphosis (Denver *et al.*, 1998). The following experiment was designed to examine neuroendocrine correlates of the developmental response to food restriction. Mid-prometamorphic tadpoles (Gosner stages 36–37) were distributed among 3 tanks per treatment containing 6 tadpoles per tank and a constant high water volume (10 liter). The treatments were no food or food *ad lib.* One tank was removed from each treatment on days 0, 2 and 4 (n = 6/treatment/time point). At each time point tadpoles were euthanized in 0.01% benzocaine, morphological measurements (Gosner Stage, HLL, TH, snout-vent length (SVL) and BW) were recorded, the two brain regions were dissected (see above and Fig. 3) for analysis of CRH peptide content by RIA (see below) and the remaining carcasses were snap frozen and stored

at −20°C to await whole body thyroid hormone analysis (see below).

Thyroid hormone extraction and radioimmunoassay (RIA)

Thyroid hormones were extracted from tadpoles following previously described methods (Denver, 1993, 1998). Briefly, tadpoles were homogenized in 3–4 volumes of methanol containing 1 mM propylthiouracil. Tissue homogenates were subjected to organic extraction, back extraction into the aqueous phase, and anion exchange chromatography as described (Denver, 1993, 1998). For estimation of recoveries, 1,000 cpm ^{125}I-labeled thyroxine (T_4) was added to the tadpole homogenates. Recoveries ranged from 30–55%, and RIA potency estimates were corrected for recovery.

Thyroid hormone RIAs (T_4 and 3,5,3′-triiodothyronine [T_3]) were conducted as described by MacKenzie *et al.* (1978) and Denver and Licht (1988). Primary antiserum for T_3 was purchased from Endocrine Sciences (Calabasas, CA) and the antiserum for T_4 was purchased from Dr. Viggo Kruse (Denmark). The intra- and interassay coefficients of variation were 2.7% and 5.7% for T_4 and 3.6% and 6.8% for T_3, respectively.

Corticotropin-releasing hormone RIA

Individual tadpole brain sections were extracted in boiling acetic acid prior to RIA as previously described (Mastorakos *et al.*, 1995; Denver, 1997*a*). Recoveries were determined by the addition of ^{125}I-*Xenopus* CRH (xCRH) to tissue homogenates prior to extraction; recoveries averaged 75%. The ^{125}I-xCRH was prepared using the iodogen method and the tracer purified by reverse-phase HPLC following the method of Vale and colleagues (1983). Cross-linking assay using ^{125}I-xCRH and *S. hammondii* brain homogenates demonstrated the presence of a CRH binding protein (CRH-BP) in this species (Boorse and Denver, unpublished results) similar to that observed in *Xenopus laevis* brain (see Valverde *et al.*, 2001). As this presented the possibility for interference of the CRH-BP in the CRH RIA, we assessed whether CRH-BP activity remained following tissue extraction. Our analysis showed that acid extraction effectively removed or inactivated all CRHBP activity (data not shown).

The antiserum used in the RIA was produced in a rabbit against synthetic xCRH conjugated to human α-globulins (see Denver, 1997*a*). Molecular cloning of the cDNA for the *S. hammondii* CRH precursor showed that the mature peptide is identical to the *X. laevis* CRH (Boorse and Denver, unpublished; Genbank accession # AY262255); thus, the RIA is homologous for *S. hammondii* CRH. This antiserum crossreacts with sauvagine (10%), fish urotensin I (10%) and rat urocortin (1%) (Boorse and Denver 2004). Thus, although we refer to our RIA measurements as reflecting tissue CRH peptide content, we recognize that other, as yet unidentified CRH-like peptides could contribute to this estimate. The RIA was conducted as described by Denver (1997*a*). Intra- and interassay coefficients of variation were 2.2 and 4.7%, respectively.

Statistical analysis

Morphological measurements and hormone data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by Scheffe's multiple contrast test ($P < 0.05$). Time and treatment served as independent variables in two-way ANOVA with morphological or hormone measurement as the dependent variable. One-way ANOVA and post-hoc tests were used to determine pairwise differences at each timepoint. A nonparametric Mann-Whitney *U* test was used to determine differences between developmental stage ($P < 0.05$).

RESULTS AND DISCUSSION

Manipulating the rate of metamorphosis by altering water level in a controlled laboratory setting has allowed us to examine the physiological mechanisms underlying the acceleration of metamorphosis in response to habitat desiccation (Denver, 1997*a*, 1998). One experimental paradigm that we have developed is acute water volume reduction where late prometamorphic tadpoles are immediately transferred from high to low water. Previous work from our laboratory showed that prometamorphic *S. hammondii* tadpoles accelerated metamorphosis by 48 hr after transfer to low water, which was the earliest time point that we examined in that study (Denver, 1998). In the current study we have extended our earlier findings by showing that the developmental acceleration in response to immediate water volume reduction can be detected by 24 hr after transfer to low water (Fig. 1). Thus, these desert-dwelling amphibians have a profound capacity to respond rapidly to deteriorating ecological conditions. Two-way ANOVA showed significant time and treatment effects in BW, TH, and Gosner stage measurements. Significant divergence in developmental stage ($F_{(1,10)} = 17.245$, $P = 0.002$), TH ($F_{(1,10)} = 48.802$, $P < 0.0001$), and BW ($F_{(1,10)} = 11.564$, $P = 0.007$) was seen by 24 hr after transfer (Fig. 1). These morphological differences were maintained at 48 hr (Developmental Stage: $F_{(1,10)} = 14.756$, $P = 0.0033$; TH: $F_{(1,10)} = 35.412$, $P = 0.0001$; BW: $F_{(1,10)} = 11.105$, $P = 0.0076$; Fig. 1).

To more precisely determine when these morphological changes occur in response to low water, time points of 24 hr and earlier were investigated. Body weight was significantly reduced in tadpoles exposed to low water for 6 hr ($F_{(1,14)} = 4.781$, $P = 0.0462$; Table 1). However, no difference in any other morphological trait was detected at 6 hr (Table 1) or earlier (0.5, 1, or 2 hr; data not shown). As in the previous experiment, which measured morphological traits at 24 and 48 hr (see Fig. 1), the 24 hr time point again showed significant divergence in developmental stage ($F_{(1,14)} = 20.364$, $P = 0.0005$), TH ($F_{(1,14)} = 46.855$, $P < 0.0001$) and BW ($F_{(1,14)} = 25.054$, $P = 0.0002$).

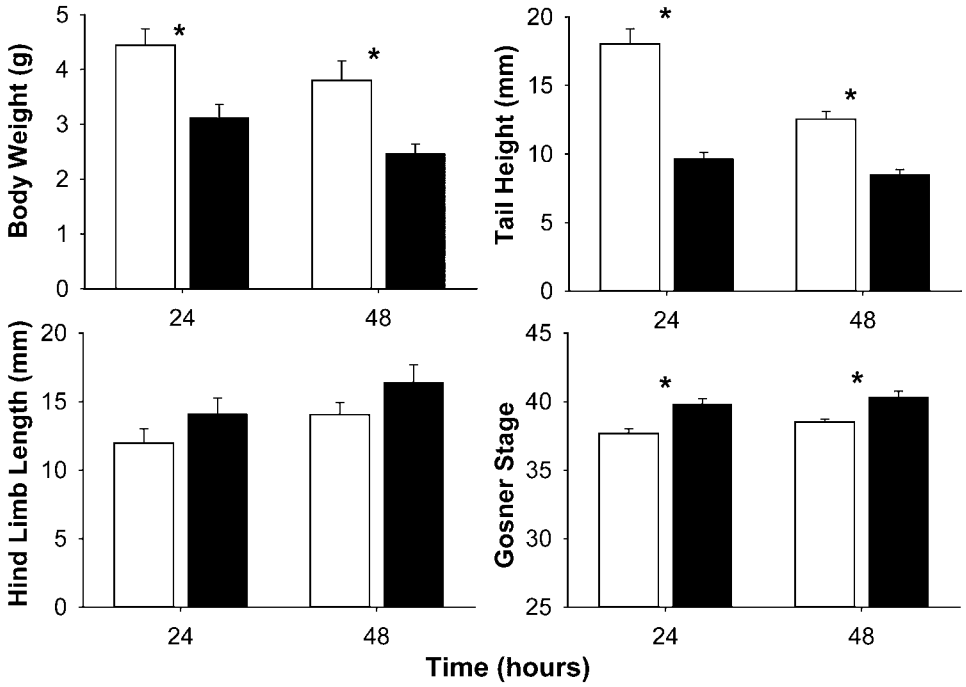

FIG. 1. Acceleration of metamorphosis of *S. hammondii* tadpoles (beginning Gosner stages 36–38) exposed to immediate water volume reduction. Tadpoles were raised in aquaria with constant high water and then transferred to cages with high (10 liter; open bars) or low (1 liter; shaded bars) water. Morphological measurements were made at 24 and 48 hours. Bars represent mean value + SEM (n = 6). Asterisks indicate statistically significant differences between high and low water treatments ($P < 0.05$ by ANOVA).

In addition to the external morphological changes induced by water volume reduction we also observed rapid changes in internal organ structure. During metamorphosis the intestine undergoes dramatic remodeling in preparation for the transition from an herbivorous diet, characteristic of the tadpole, to a carnivorous diet, characteristic of the adult frog (reviewed by Shi and Ishizuya-Oka, 1996). Measures of gut morphology (*i.e.*, gut length and gut weight) showed significant changes by 24 hr in tadpoles transferred to low water (Table 1). By this time point tadpoles transferred to low water had significantly shorter guts ($F_{(1,10)} = 76.687$, $P < 0.0001$) with a mean gut length of 68% of controls (Table 1). Gut weight was significantly decreased by 6 hr ($F_{(1,10)} = 13.407$, $P = 0.0044$) and 24 hr ($F_{(1,10)} = 61.768$, $P < 0.0001$) after transfer to low water. This decrease at 6 hr, which likely reflects decreased gut contents, supports our previous observation that feeding ceases upon transfer to low water (Denver *et al.*, 1998).

Field studies by Newman (1988; 1989) showed that tadpoles of Couch's spadefoot toad metamorphosed earlier in rapidly drying pools compared with animals inhabiting more stable bodies of water. Newman was unable to separate the effects of water volume reduction from correlated changes in other environmental factors (*e.g.*, increased water temperature) in his studies. We found that *S. couchii* tadpoles showed similar

TABLE 1. *Morphological and whole-body thyroid hormone measurements in* Spea hammondii *during water volume reduction.*

	6 hr.		24 hr.	
	High	Low	High	Low
Morphological measurement				
Body weight (g)	4.6 ± 0.13	4.2 ± 0.16*	4.6 ± 0.23	3.1 ± 0.18***
Gosner stage	37.5 ± 0.19	38.0 ± 0.19	38.3 ± 0.25	40.3 ± 0.37**
Tail height (mm)	15.6 ± 0.92	13.8 ± 0.80	15.4 ± 0.74	9.8 ± 0.37***
Hindlimb length (mm)	11.9 ± 0.87	12.8 ± 0.64	12.7 ± 0.76	14.8 ± 0.92
Gut length (mm)	475 ± 27.9	452 ± 11.1	494 ± 16.0	337 ± 8.1***
Gut weight (g)	0.20 ± 0.02	0.12 ± 0.01**	0.19 ± 0.01	0.06 ± 0.01***
Hormone measurement				
Triiodothyronine (T_3)(pg/g BW)	691.8 ± 56.2	601.3 ± 87.3	599.5 ± 115.5	1278.2 ± 336.0*
Thyroxine (T_4) (pg/g BW)	476.8 ± 104.8	511.8 ± 82.5	719.1 ± 126.3	645.6 ± 271.3

Note: *-$p < 0.05$; **-$p < 0.005$; ***-$p > 0.0005$.

developmental acceleration in response to rapid water volume reduction in the laboratory to that observed for *S. hammondii*. The developmental stage of *S. couchii* tadpoles was significantly increased 24 hr after transfer to low water (40.8 ± 0.57; n = 20; Mann-Whitney nonparametric test, $Z = -2.030$, $P < 0.05$) compared with high water controls (39.0 ± 0.49; n = 20). By this time point tadpoles transferred to low water had significantly lower body weight (0.1915 g ± 0.008; n = 20; Student's *t*-test, $t = 1.997$, $P = 0.05$) compared with the high water controls (0.2205 g ± 0.012). However, SVL was not significantly different between high and low water treated tadpoles (data not shown). The water temperature in tanks maintained in the environmental chamber in which we conducted the present (and earlier) studies did not differ among experimental treatments (Denver *et al.*, 1998). Thus, both *S. hammondii* and *S. couchii* are capable of sensing water depth/volume and activating developmental pathways leading to early metamorphosis. *S. hammondii* tadpoles appear to respond to changes in water depth/volume and not to other factors associated with decreasing water level such as increased conspecific density or increased waste products (see Denver *et al.*, 1998).

Endocrine response to rapid water volume reduction in spadefoot toad tadpoles

Thyroid hormones (T_3 and T_4) are the primary morphogens controlling metamorphosis, so changes in development rate should be driven by changes in the activity of the thyroid axis. Previous work from our laboratory showed that prometamorphic *S. hammondii* tadpoles exposed to acute water volume reduction dramatically increased whole body thyroid hormone content by 48 hr after transfer to low water (both T_3 and T_4; Denver, 1998). The current study confirms and extends our previous findings by showing that significant morphological change and thyroid system activation (at least for T_3) can be detected by 24 hr (Table 1). We found that 24 hr was the earliest time that hormonal changes could be detected *in S. hammondii* tadpoles (Table 1). Whole body T_3 content was significantly higher in tadpoles transferred to low water by 24 hr ($F_{(1,8)} = 4.171$, $P = 0.0485$), but not at 6 hr or earlier (Table 1; data from times sampled earlier than 6 hr are not shown). We did not sample between 6 and 24 hr and thus cannot rule out the possibility that significant changes can be observed at a time point earlier than 24 hr. By contrast to T_3, differences in whole body T_4 content between high and low water animals were not detected at any early sampling times (0.5–24 hr) (Table 1). However, by 48 hr after transfer to low water tadpoles had significantly elevated whole body T_4 ($F_{(1,10)} = 6.388$, $P = 0.03$) and T_3 ($F_{(1,10)} = 14.740$, $P = 0.0033$) content (tadpoles used to measure morphology at 24 and 48 hr were sacrificed at 48 hr for this analysis.)

The increase in whole body T_3 content but not T_4 content at 24 hr could be explained by differential hormone production by the thyroid, or by hormone conversion. Earlier studies showed that the major product of the amphibian thyroid gland is T_4 with only minor amounts of T_3 produced (Rosenkilde, 1978; Buscaglia *et al.*, 1985). However, the secretory products of the spadefoot toad tadpole thyroid have not been studied. The doubling in whole body T_3 content by 24 hr could be achieved by increased thyroidal synthesis of T_3 or by conversion of T_4 to T_3. Alternatively, increased whole body T_3 content could result from a decrease in type III iodothyronine deiodinase activity, which catalyzes the degradation of both T_4 and T_3 to inactive derivatives.

Developmental and endocrine responses to food restriction in prometamorphic spadefoot toad tadpoles

Resource availability is another important factor that can influence the timing of metamorphosis (D'Angelo *et al.*, 1941; Travis, 1984; Alford and Harris, 1988; Berven and Chadra, 1988; Newman, 1994; Denver *et al.*, 1998; Morey and Reznick, 2000), and can potentially interact with the effects of habitat desiccation. In ephemeral breeding ponds, resource availability is likely to change as ponds dry; *i.e.*, tadpoles are growing and as their pools dry conspecific density increases, thus increasing competition for resources (Newman, 1994). We found that foraging is reduced in *S. hammondii* tadpoles transferred to low water (Denver *et al.*, 1998; Phillips and Denver, unpublished). In the present study, gut mass was significantly lower in tadpoles 6 hr after transfer to low water (see earlier), thus supporting behavioral observations of reduced foraging. Earlier we tested the hypothesis that reduced food intake, that occurs upon water volume reduction, is sufficient to explain the acceleration of metamorphosis (Denver *et al.*, 1998). We found that food-restricted prometamorphic *S. hammondii* tadpoles maintained in high water accelerated metamorphosis; however, the magnitude of the acceleration was only about 50% of that seen in animals exposed to water volume reduction (Denver *et al.*, 1998). Our current findings support our earlier study, and provide data on the kinetics of the developmental response and endocrine changes induced by food restriction. Food restricted tadpoles accelerated development four days after food removal ($F_{(1,10)} = 9.06$, $P = 0.0133$) but not after two days (Fig. 2). Whole-body T_3 and T_4 content were significantly elevated by four days after removing food (T_3: $F_{(1,10)} = 5.031$, $P = 0.0244$; T_4: $F_{(1,10)} = 3.83$, $P = 0.0394$; Fig. 2). By contrast, tadpoles subjected to water volume reduction accelerated development and increased whole body T_3 content by 24 hr and both T_3 and T_4 content by 48 hr (discussed earlier, Table 1). These findings suggest that accelerated development in response to water volume reduction is not driven by reduced food intake alone. However, the cessation of feeding (and the associated endocrine changes) could contribute to later stages of developmental acceleration caused by pond drying. The developmental signal in-

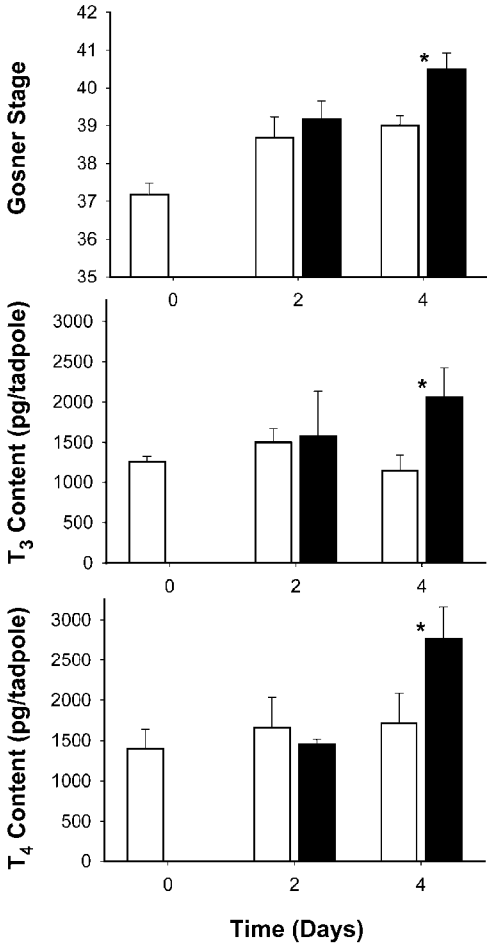

FIG. 2. Acceleration of metamorphosis of *S. hammondii* tadpoles (beginning Gosner stages 36–38) following food deprivation. Bars represent mean value + SEM (n = 8). Asterisks indicate statistically significant differences between food (open bars) and no food (shaded bars) treatments ($P < 0.05$ by ANOVA).

duced by reduced resources could operate via a different physiological pathway or through a lesser magnitude of activation of the same pathway as that for water volume reduction. Because pond drying represents an immediate threat to tadpole survival (see Newman, 1992), the ability to respond rapidly and robustly to decreased water level may be under stronger selection than the response to resource depletion. Depletion of resources (or increased competition) can vary over time and may be less of an immediate threat than pond drying. Larvae that experience reduced resources may ''choose'' to ''sit tight,'' and then capitalize on improved growth conditions should they return (see Denver *et al.*, 1998). If, however, resource availability continues to be limited over an extended period, then tadpoles may initiate metamorphosis.

In the ephemeral desert pools where spadefoot toads breed, desiccation is arguably the single most important environmental factor affecting larval survivorship (Newman, 1992). Spadefoot toad tadpoles and other species that develop in ephemeral pools have evolved traits that allow for successful development in an unpredictable environment. These species tend to have shorter larval periods on average when compared to tadpoles that develop in more permanent ponds (Low, 1976; Denver *et al.*, 2002). Our current study suggests that species that breed in temporary pools are more sensitive to water level than other environmental factors, and can accelerate development more quickly in response to declining water levels than declining food levels.

Changes in CRH neuronal physiology in response to rapid water volume reduction in spadefoot toad tadpoles

The neuroendocrine system translates sensory and metabolic information about the external and internal environments into an integrated physiological/developmental response. Earlier, we hypothesized that the neuroendocrine system may mediate environmental effects on metamorphosis (reviewed by Denver, 1997*b*). Our previous work showed that hypothalamic CRH content was increased in *S. hammondii* tadpoles exposed to a gradual decline in the water level (Denver, 1997*a*). In the present study, we analyzed acute changes in CRH neuronal physiology following rapid water volume reduction. We measured tissue CRH content in two gross brain regions by RIA (see Fig. 3). Since water volume reduction results in the rapid activation of the thyroid (Denver, 1998; this study) and interrenal axes (Denver, 1998) we hypothesized that CRH content would change in the region containing the preoptic nucleus (fore-/midbrain), which contains a large number of neurosecretory CRH neurons. These neurons project axons to the median eminence where they secrete CRH into the hypophysial portal blood. However, there was no significant change in CRH content in this brain region (Fig. 3). By contrast, we found that CRH content was significantly elevated in the hindbrain region by 24 hr after transfer to low water ($F_{(1,10)} = 30.148$, $P = 0.0003$; Fig. 3). While hindbrain CRH neurons are not directly involved in the regulation of pituitary secretion (*i.e.*, these neurons do not project axons to the median eminence), these neurons could play an indirect role (discussed below). Furthermore, mammalian studies have implicated brainstem CRH neurons in behavioral responses to stress (Brady, 1994; Page and Valentino, 1994; Lehnert *et al.*, 1998).

We have mapped CRH immunoreactivity (CRH-ir) in *X. laevis* brain using immunocytochemistry. The majority of CRH-ir in the hindbrain is localized to the locus coeruleus (LC; Westphal *et al.*, 2002). Mammals also show CRH-ir in the LC (Swanson *et al.*, 1983; Valentino *et al.*, 1992) as well as CRH receptors (Desouza *et al.*, 1985; Desouza, 1987). Intracerebroventricular administration of CRH increased the firing rate of LC neurons (Valentino *et al.*, 1983) and activation of the LC can increase hypothalamic CRH secretion (Calogero *et al.*, 1988; Day *et al.*, 1985; Tanaka *et al.*, 1985; Kannan *et al.*, 1987; Plotsky, 1987; Plotsky *et*

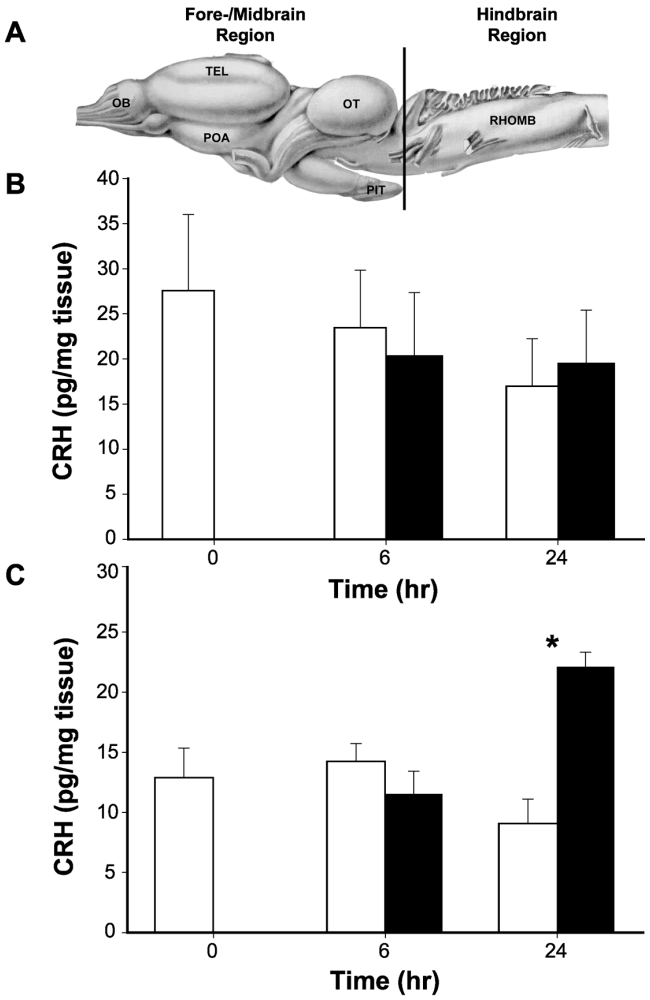

FIG. 3. Changes in corticotropin-releasing hormone (CRH) content in two gross brain regions in *S. hammondii* tadpoles (Gosner stages 36–38) in response to immediate water volume reduction. **A.** Side view of tadpole brain indicating regions collected for extraction and CRH radioimmunoassay (RIA; drawing modified from Ten Donkelaar, 1998). The forebrain and midbrain regions were collected as one section that included all brain structures rostral to the pituitary. The hindbrain region included brain structures and spinal cord caudal to the pituitary. Pituitary (PIT), olfactory bulb (OB), optic tectum (OT), preoptic area (POA), rhombencephalon (RHOMB) and telencephalon (TEL) are indicated. Tadpoles were raised in aquaria with constant high water before transfer to cages with high (10 liter; open bars) or low (1 liter; shaded bars) water for 24 hr before sacrifice. **B.** CRH peptide content in fore-/midbrain region. **C.** CRH peptide content in hindbrain. Bars represent mean value + SEM (n = 6). Asterisks indicate statistically significant differences between high and low water treatments ($P < 0.05$ by ANOVA).

al., 1989; Saphier, 1989; Saphier and Feldman, 1989). Currently, we do not know if LC neurons project to the preoptic area in the amphibian brain. However, many neural circuits, especially primitive circuits arising in the brainstem/hindbrain, are conserved among vertebrates. In rats, the LC shows the largest change in CRH-ir in any brain region during an acute restraint stress (Chappell *et al.*, 1986). It is possible that tadpoles experience low water level similar to how rats experience restraint stress; *i.e.,* an impairment of normal movement. This may in part explain the increased hindbrain CRH content during acute water volume reduction similar to the increased CRH in the LC in restrained rats. Taken together, these data point to the hypothesis that changes in hindbrain CRH neuronal physiology influence neuroendocrine responses to environmental stress in tadpoles.

Corticotropin-releasing hormone neurons in the hindbrain may also be involved in the regulation of food intake. Corticotropin-releasing hormone and related peptides have been shown to be potent anorexigenic peptides in many vertebrate species, including spadefoot toad tadpoles. Intracerebroventricular injections of CRH or CRH-like peptides decreased food intake in mammals (Krahn *et al.*, 1986; York, 1992; Spina *et al.*, 1996), birds (Furuse *et al.*, 1997), fishes (De Pedro *et al.*, 1993; 1995) and in the frogs, *X. laevis* (Crespi *et al.*, 2004) and *S. hammondii* (Crespi and Denver, 2004). Stereotaxic injections of the CRH-like peptide urocortin into the hindbrain (fourth ventricle) suppressed food intake in rats in a dose dependent manner (Grill *et al.*, 2000). We also have evidence that hindbrain CRH neurons play a role in the regulation of food intake in spadefoot toad tadpoles. For example, antagonism of CRH receptors in the hindbrain (via injection of αhelical $CRF_{(9–41)}$ into the 4th ventricle), increased foraging behavior in *S. hammondii* tadpoles (Crespi and Denver, 2004). These findings, and the finding that exposure of tadpoles to low water increases CRH content in the hindbrain, lead to the hypothesis that the decreased foraging upon pond drying is mediated by hindbrain CRH.

As discussed above, CRH could be responsible for the reduced foraging and the activation of the thyroid and interrenal axes when a tadpole experiences rapid desiccation of its habitat. In cases where pond drying extends over a longer period, pond drying could interact with other, associated changes in the larval habitat such as resource depletion and competition. In mammals, there is evidence for increased CRH expression and elevated plasma glucocorticoids following prolonged food deprivation (Dallman *et al.*, 1995; Timofeeva and Richard, 1997; Heinrichs and Richard, 1999). In addition to the increased thyroid activity that we found following food deprivation in the current study, earlier we found that food restriction increased whole body CORT content in both *R. pipiens* (Glennemeier and Denver, 2001) and *S. hammondii* tadpoles (Crespi and Denver, unpublished data). The activation of the thyroid and interrenal axes by food deprivation suggests the involvement of CRH. However, in the current study we did not find changes in CRH content in food restricted *S. hammondii* tadpoles in either of the two brain regions analyzed (data not shown).

Our observation that CRH content changed in the hindbrain following exposure to low water allows us to conclude that decreasing the water level in which tadpoles are reared affects CRH neuronal physiology.

However, we realize that measures of CRH content in such large brain regions can only provide a gross, and preliminary analysis of CRH neuronal function. The two tissues that we analyzed each contain discrete collections of CRH neurons that are likely to be differentially regulated. Therefore, the lack of change in CRH peptide content in the fore-/midbrain region following exposure to low water, or in any brain regions following food restriction must be interpreted in the context of the limitations of the method used. An analysis that we completed in juvenile *X. laevis* has some bearing on this issue. We compared two methods for quantitating changes in hypothalamic CRH peptide content following exposure to shaking stress for 4 hr (see Glennemeier and Denver, 2002). Using immunocytochemistry we found a 3.5 fold increase in CRH-immunoreactivity in the preoptic nucleus but not in other CRH-positive neurons in the diencephalon (Westphal *et al.*, 2002; Yao *et al.*, 2003). By contrast, analysis of CRH content by RIA following microdissection and extraction of the preoptic area showed only a 1.5 fold increase (Boorse and Denver, 2004). Future studies using histochemical methods are necessary to fully evaluate changes in CRH neuronal physiology in response to environmental variation in tadpoles.

CONCLUSIONS

Amphibians in general, and desert-dwelling spadefoot toads in particular, exhibit extreme plasticity in the timing of metamorphosis. We have found that spadefoot toad tadpoles upregulate their thyroid axis, increase CRH peptide content in the hindbrain, and initiate metamorphosis within 24 hr of transfer from a low to a high water environment. Competition for resources is likely to be high in a rapidly drying pond, and we and others have found that tadpoles accelerate metamorphosis in a constant high water environment when food is restricted. However, the more rapid kinetics of the endocrine and morphological responses to water volume reduction *vs.* food restriction is consistent with habitat desiccation being the more immediate threat to tadpole survival.

Amphibian larvae encounter diverse ecological conditions, and the degree of plasticity in larval period length, within the limits set by the genotype, is subject to natural selection. The expression of developmental plasticity depends on the development and activity of endocrine glands and the actions of hormones. Therefore, endocrine-related genes are likely to be targets of natural selection, both in determining the species-specific range for the length of the larval period, and in the degree of plasticity and sensitivity to environmental signals. Future studies should focus on how specific neuroendocrine circuits respond to different environmental changes, and how these neuroendocrine responses serve to integrate endocrine, behavioral and developmental responses to environmental stress.

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