

Hormonal Correlates of Environmentally Induced Metamorphosis in the Western Spadefoot Toad, *Scaphiopus hammondi*

Robert J. Denver¹

Department of Biology, The University of Michigan, Ann Arbor, Michigan 48109-1048

Accepted February 26, 1998

Tadpoles of several amphibian species have been shown to accelerate metamorphosis when their ponds dry. To understand the proximate mechanisms that mediate the developmental response to pond drying, I analyzed changes in endocrine activity in tadpoles of the Western spadefoot toad (*Scaphiopus hammondi*) exposed to experimental water volume reduction in the laboratory. Tadpoles exposed to a declining water level accelerated metamorphosis compared with tadpoles raised in a constant high water environment. The acceleration of development was associated with the precocious elevation of whole-body contents of the hormones that control metamorphosis, the thyroid hormones thyroxine (T₄) and triiodothyronine (T₃), and the interrenal steroid corticosterone (CORT). The precocious activation of the thyroid system preceded external morphological change (i.e., increase in hind limb length, developmental stage) by 3 days. To test if tadpoles are capable of responding rapidly to water volume reduction, mid-prometamorphic tadpoles (Gosner Stage 37–38) were raised in a constant high water environment (10 L) and then transferred to either 1 or 10 L. Tadpoles transferred to 1 L exhibited significant metamorphic changes by 48 h after transfer. In addition, dramatic elevations in whole-body T₄, T₃, and CORT contents were evident at this time point. Thus, the metamorphic response to pond drying is likely

driven by the activation of the thyroid and interrenal axes, the hormones of which control metamorphosis. Furthermore, this response is rapid, occurring within 48 h after exposure to the desiccating environment.

© 1998 Academic Press

The timing of metamorphosis is a central amphibian life history trait, and amphibians exhibit extreme plasticity in the length of the larval period (Wilbur and Collins, 1973; Werner, 1986; Smith, 1987). This timing is influenced by a combination of both biotic and abiotic factors present in the larval habitat. Probably the most important environmental variable for a tadpole is water availability, and tadpoles of some species, especially those that breed in temporary ponds, have been shown to accelerate metamorphosis when their habitat dries (reviewed by Newman, 1992; Denver, 1997a). Most studies of the developmental response to habitat desiccation in amphibians have been done on the spadefoot toads (family Pelobatidae, genus *Scaphiopus*). These desert-adapted amphibians are opportunistic breeders that time reproduction to infrequent and unpredictable rainfall. Adults breed in temporary ponds that are periodically filled by rain, and tadpoles of both Couch's spadefoot toad (*Scaphiopus couchii*) and the Western spadefoot toad (*Scaphiopus hammondi*) have been shown to accelerate development in response to pond drying (Newman, 1989, 1992; Denver *et al.*, 1998).

Because hormones control metamorphosis, the effects of environmental variation on the rate of develop-

¹ Mailing address: Department of Biology, 3077 Natural Science Building, The University of Michigan, Ann Arbor, MI 48109-1048. Fax: (734) 647-0884. E-mail: rdenver@umich.edu.

ment should be expressed through changes in the secretory activity of several endocrine glands. The developmental acceleration in *S. hammondi* in response to habitat desiccation appears to result from the activation of neuroendocrine stress centers (Denver, 1997b). In amphibians, the major vertebrate stress neuropeptide corticotropin-releasing hormone (CRH) controls both the thyroid and the interrenal axes by directly stimulating pituitary thyrotropin (TSH) and adrenocorticotropin (ACTH) secretion, respectively (Denver, 1988, 1993, 1995, 1996, 1997a,b; Denver and Licht, 1989; Gancedo *et al.*, 1992; Malagon *et al.*, 1991; Jacobs and Kuhn, 1989, 1992; Tonon *et al.*, 1986). The thyroid hormones thyroxine (T_4) and 3,5,3' triiodothyronine (T_3) are the primary morphogens controlling metamorphosis and their production is controlled by TSH (reviewed by Kikuyama *et al.*, 1993). Corticosteroids produced by the interrenal glands (the major steroid is corticosterone—CORT; CORT production is controlled by ACTH) can synergize with T_3 and T_4 to promote metamorphosis (reviewed by Kikuyama *et al.*, 1993).

An increase in the production of thyroid hormones and perhaps CORT might account for the acceleration of development rate induced by habitat desiccation. In this study I tested whether exposure of tadpoles of the Western spadefoot toad (*S. hammondi*) to a declining water level in the laboratory alters whole-body contents of thyroid and interrenal hormones. I also tested whether tadpoles are capable of mounting a rapid metamorphic response to an immediate decline in water volume, and whether this response might be driven by a rapid activation of the endocrine system.

MATERIALS AND METHODS

Animal care. Adult Western spadefoot toads were collected near Livermore, California, maintained in a breeding colony, and spawned in the laboratory with the aid of synthetic gonadotropin-releasing hormone agonist (Sigma Chemical Co.) as described (Denver, 1997b). Eggs and newly hatched tadpoles were maintained in polystyrene rat cages (45 × 24 × 20 cm) with distilled water containing 10% Holtfreter's solution (see Rugh, 1962). Environmental conditions and tadpole husbandry were as described (Denver, 1997b).

Tadpoles were fed tadpole chow (a mixture of rabbit pellets, agar, and Knox gelatin; see Rugh, 1962) and boiled spinach and lettuce; this food provides adequate nutrition while reducing water fowling. Animal care was in accordance with the institutional guidelines set by the Animal Care and Use Committee of the University of California, Berkeley, and the University Committee on the Care and Use of Animals of The University of Michigan.

Experiment 1. This experiment tested whether tadpoles subjected to water volume reduction precociously activate the endocrine axes that drive metamorphosis. A secondary goal was to describe in detail the morphological divergence between animals maintained in either a constant high water or a declining water environment. Tadpoles in Gosner Stage 32 (Gosner, 1960; early prometamorphosis; 17 days posthatch) were placed in rat cages containing 10 L of water (starting water height in cage 10 cm; water temperature 21–23°C; 12L:12D). Tadpoles were randomly assigned to 6 cages (12 tadpoles/cage) per treatment (12 cages total). One cage was removed from each treatment at each of the six time points (2- or 3-day intervals beginning at 19 days posthatch; see Fig. 3), measurements were done (see below) and 6 tadpoles (randomly selected) were preserved for hormone measurements (see below). Thus, statistical tests are based on individual values rather than population means; i.e., the analyses are pseudoreplicated. However, photoperiod and ambient temperature were identical for all cages (all cages were kept on one shelf within a temperature-controlled environmental chamber), the number of tadpoles was equal in all cages and resources were not limiting (tadpoles were fed *ad libitum*; food was checked twice daily and was provided in sufficient quantity so as not to cause fowling of the water). Furthermore, by Day 23 posthatch the differences between the two treatments were sufficiently great, and they continued to increase with time, such that any important consequences of pseudoreplication were nullified.

Treatments consisted of maintaining tadpoles in either a constant "high" water environment ("controls") or subjecting them to a declining water level (habitat desiccation regime; "experimentals") by removing water from the cages daily (0.5 or 1 L; see Fig. 3 for the rate of volume reduction). Water levels of each of

the experimental tanks were adjusted each day at the same time (0900–1100 h); daily perturbations of the control tanks were done to mimic the disturbance created by water removal in the experimental tanks. Water was renewed in each of the tanks every 2 days throughout the experiment. The habitat desiccation regime was based on that previously determined to produce an intermediate rate of accelerated metamorphosis (tadpoles exhibit a continuum of response to the desiccating habitat; Denver *et al.*, 1998). This experiment was run in parallel with the first experiment reported in Denver (1997b) using animals of the same sibship, the same rate of water decline, and identical environmental conditions. The last measurement was done at 30 days posthatch at which time all of the experimental animals but none of the control animals had metamorphosed.

Experiment 2. This experiment tested whether mid-prometamorphic tadpoles are capable of responding to a rapid and drastic decline in the water level by activating their endocrine axes and accelerating metamorphosis. Previous results indicated that tadpoles must reach a minimum developmental stage (Gosner Stage 34–35) to respond to an immediate and drastic decline in water volume by accelerating metamorphosis; prior to this stage such treatment results in the cessation of growth and metamorphic stasis (Denver and Phillips, unpublished results). In the current experiment mid-prometamorphic tadpoles (Gosner Stage 37–38) were transferred from stock cages containing 10 L of water to either a cage with 10 L or a cage containing 1 L. Each treatment contained 15 animals. Forty-eight hours after transfer morphological measurements were done (see below) and 7 animals per treatment (randomly selected) were collected for hormone measurements (see below).

Hormone extractions. Tadpoles were anesthetized in 0.01% benzocaine, snap frozen, and stored at -80°C before hormone extraction. Thyroid hormones were extracted from whole tadpoles following the method described by Denver (1993). Briefly, tadpoles were homogenized in 3–4 vol of methanol containing 1 mM propylthiouracil. The sample was divided in half and one-half used for thyroid hormone extraction and one-half for CORT extraction. For estimation of recoveries, 1000 cpm of $[^{125}\text{I}]\text{T}_4$ or 3000 cpm of $[^3\text{H}]\text{CORT}$ was added to the extracts. Recoveries ranged from 45

to 70% for $[^{125}\text{I}]\text{T}_4$ and 30 to 45% for $[^3\text{H}]\text{CORT}$. Ten tadpole extracts were divided in half and each half spiked with either $[^{125}\text{I}]\text{T}_4$ or $[^{125}\text{I}]\text{T}_3$ to determine if recoveries of the two hormones differed. This analysis revealed no significant differences between the two hormones; therefore, recovery corrections for both thyroid hormones are based on recoveries of $[^{125}\text{I}]\text{T}_4$.

Corticosterone was extracted from whole tadpoles following the method of Hayes and Wu (1995) with modifications. Five milliliters of ethyl acetate was added to half of the tadpole homogenate, vortexed, and centrifuged at 2000g. The supernatant was then dried in a speedvac and the pellet resuspended in 0.1 ml ethyl acetate for fractionation by thin-layer chromatography (TLC). Samples were loaded on silica gel GF TLC plates (Analtech 250 μM) and the plates placed in a mobile phase of toluene:cyclohexane (1:1). After development, the plates were air-dried and placed in a second mobile phase of chloroform:methanol (98:2). The plates were then air-dried and scanned for radioactivity with a Berthold TLC scanner to locate the $[^3\text{H}]\text{CORT}$ peak. A 1×2 -cm section of silica in each lane (corresponding to the $[^3\text{H}]\text{CORT}$ peak) was scraped off the plate into a borosilicate tube and extracted overnight with 5 ml ether. The ether was dried under nitrogen and the sample redissolved in diluent (0.2 M phosphate-buffered saline with 1% gelatin) for radioimmunoassay (RIA).

Validation of hormone extraction methods. Both reversed-phase high performance liquid chromatography (HPLC) and TLC were used to assess the validity of thyroid hormone extractions from whole *S. hammondi* tadpoles. Whole tadpole extracts were separated on an ODS C_{18} column (mobile phase gradient 37 to 42% methanol) and fractions analyzed by RIA for T_3 or T_4 (see below). Using this method, the recovery of immunoreactive hormone from the HPLC fractions averaged 75% for both hormones (the total immunoreactive hormone recovered off the HPLC was compared with the amount of immunoreactive hormone present in the unfractionated extract). The immunoreactive material exhibited retention times that were identical to the radiolabeled (see Fig. 1) and unlabeled hormones (data not shown); no other immunoreactive peaks were observed. Similar results were obtained using thin-layer chromatography (following the methods of Reyes *et al.*, 1990) to fractionate the extracts.

Taken together, these chromatographic results verify the authenticity of the immunoreactive thyroid hormone in the unfractionated tadpole extracts.

For CORT, the extraction method was validated by comparing the migration of the immunoreactive and [³H]CORT on TLC. This analysis verified that the majority of the immunoreactive material migrated identically to the [³H]CORT (Fraction 2, 3–4 cm from the origin; Fig. 2). Ether extracts of silica scraped from the TLC plate (only Fraction 2) were analyzed in the RIA.

Radioimmunoassays. The thyroid hormones (T₃ and T₄) and CORT were measured by RIA in extracts of

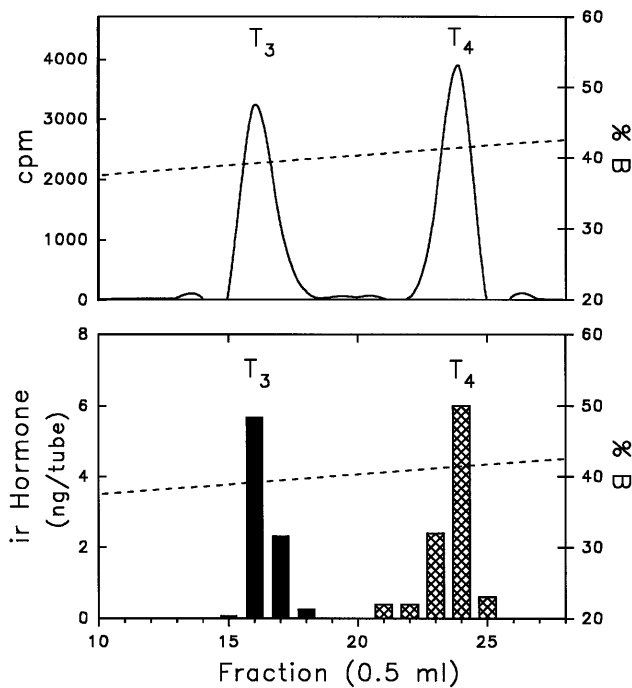


FIG. 1. Reverse-phase HPLC purification of 3,5,3'-triiodothyronine (T₃) and thyroxine (T₄) from whole-body extract of Gosner Stage 40 *S. hammondi* tadpoles. Tadpoles were extracted as described under Materials and Methods and the extract run on an ODS C₁₈ column with a gradient of 37 to 42% methanol (dashed lines; "%B"). (Top) Radiolabeled T₃ and T₄ were added to tadpole extract and the extract was run on HPLC. Fractions were counted for radioactivity in a gamma counter (three runs were done, two with the individual hormones and one with both hormones combined—these latter data are shown in the graph.) (Bottom) Analysis of immunoreactive T₃ and T₄ in HPLC fractions. All fractions were run in both the T₃ and the T₄ RIAs. The recovery of immunoreactive T₃ and T₄ was compared to the amount of immunoreactive hormone in the unfractionated extract; this recovery averaged 75% for both hormones (see Materials and Methods).

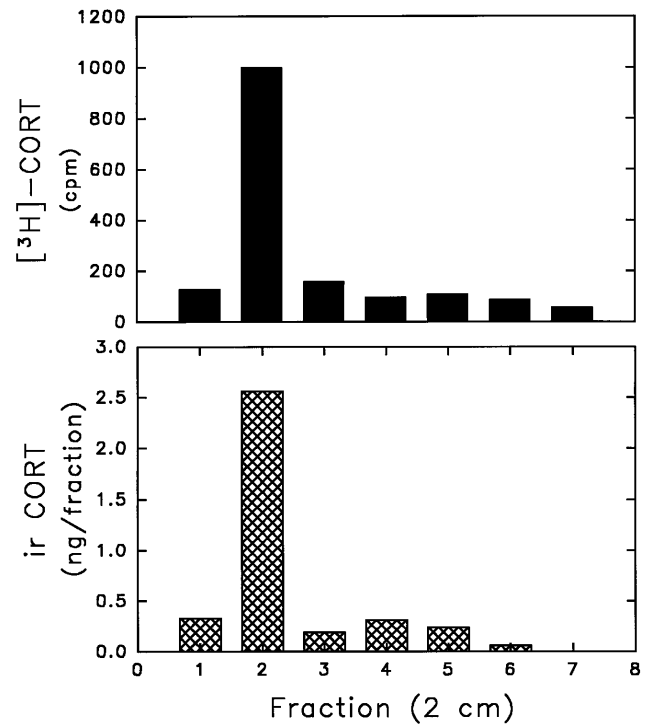


FIG. 2. Thin-layer chromatography (TLC) analysis of corticosterone (CORT) extracted from Gosner Stage 40 *S. hammondi* tadpoles. Extracts were spiked with [³H]CORT (3000 cpm) before loading on the TLC plates (see Materials and Methods for details of TLC). Lanes on the silica plate were divided into 1-cm sections, scraped into borosilicate tubes, and extracted with ether prior to counting for radioactivity in a liquid scintillation counter (top) or analysis of immunoreactive hormone by RIA (bottom).

whole *S. hammondi* tadpoles. The thyroid hormone RIAs were as described by MacKenzie *et al.* (1978) and Denver and Licht (1988) and RIA for CORT is described by Licht *et al.* (1983). Primary antisera for T₃ and CORT were purchased from Endocrine Sciences (San Diego, CA) and the antiserum for T₄ was purchased from Dr. Viggo Kruse (Denmark).

Morphological measurements and statistical analyses. The variables mean wet weight (body weight; BW), snout-vent length (SVL), hind limb length (HLL), and developmental stage (Gosner, 1960) were measured. Tadpoles were anesthetized in 0.01% benzocaine and BW was measured using a digital balance (accurate to 0.1 g) and SVL and HLL were measured with a digital micrometer (accurate to 0.1 mm). HLL is expressed as a function of SVL. Whole-body hormone contents are expressed as nanogram hormone per

gram BW. Previous studies showed that correction of hormone content using either DNA content or wet body weight produced identical results (Denver, 1993). All data were \log_{10} -transformed to achieve homogeneity of variance. Data were analyzed by one-way and two-way ANOVA and a post-hoc test (Scheffe's multiple contrast test) was done to determine significant differences among means ($P < 0.05$).

RESULTS

***S. hammondi* tadpoles accelerate metamorphosis in response to gradual water volume reduction (Expt. 1).** Tadpoles exposed to water volume reduction accelerated metamorphosis (Fig. 3). Two-way ANOVAs for each measurement (BW, SVL, HLL, and developmental stage) revealed significant effects of time and treatment and time by treatment interactions (see Table 1 for summary of ANOVAs). Separate univariate contrasts to distinguish treatment effects at the different measurement times revealed that the first demonstrable morphological changes were a reduction in BW ($F_{(1,23)} = 4.79, P = 0.04$ [ANOVA]) and SVL ($F_{(1,23)} = 5.94, P = 0.02$) in tadpoles exposed to a declining water level at 23 days posthatch (see Fig. 3). Significant divergence in hind limb length (as a function of body length; $F_{(1,22)} = 5.48, P = 0.03$) and developmental stage ($F_{(1,23)} = 8.36, P = 0.009$) occurred 3 days later at day 26 posthatch. The two treatments continued to diverge in all measurements through the end of the experiment. At 30 days posthatch all animals exposed to a declining water level had metamorphosed (reached Gosner Stage 42); whereas, none of the tadpoles maintained in a constant high water environment had reached metamorphic climax (mean stage 40.2; see Fig. 3).

Exposure to gradual water volume reduction results in the precocious activation of the thyroid and interrenal axes (Expt. 1). Tadpoles exposed to water volume reduction exhibited elevated whole-body concentrations of T_4 , T_3 , and CORT (Fig. 4) at an earlier age than controls. Two-way ANOVAs for each measurement (T_4 , T_3 , and CORT) revealed significant effects of time and treatment and time by treatment interactions (see Table 2 for summary of ANOVAs). These data were analyzed further in two ways: (1) one-way ANOVAs within a treatment to determine the age at which

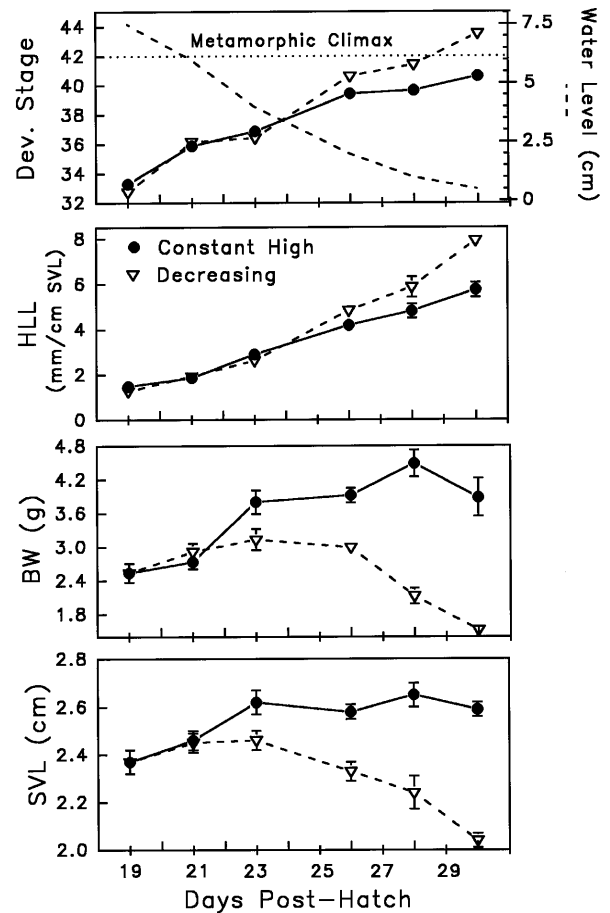


FIG. 3. Acceleration of development of *S. hammondi* tadpoles exposed to water volume reduction. Tadpoles (starting with Gosner Stage 32) were raised in rat cages (12 animals/cage) with either a constant volume of water (10 L; constant high) or a decreasing volume (decreasing; the dashed line [right axis] in the top graph shows the decline in the water level over time). Measurements are developmental stage (Dev. Stage; Gosner, 1960), body weight (BW), snout-vent length (SVL), and hind limb length (HLL; expressed as a function of body size—SVL). Points show the mean for each measurement ($n = 12/\text{treatment}/\text{time}$) at the designated day post-hatch and vertical lines are the SEM. The horizontal dotted line in the top graph designates metamorphic climax (Gosner Stage 42; fore-limb emergence).

hormone levels began to rise, and (2) one-way ANOVAs within an age to determine the time when the two treatments diverged in hormone levels (although this analysis could potentially be confounded by changes in body size—see below). One-way ANOVA within a treatment to determine the time at which whole-body hormone content increased showed that in the decreasing water treatment, both T_4 and T_3 were significantly

elevated above the earlier ages at day 23 posthatch (T_4 : $F_{(5,35)} = 17.3$, $P < 0.0001$; T_3 : $F_{(5,35)} = 22.9$, $P < 0.0001$) and continued to rise or remain elevated throughout the experiment. In contrast, whole-body CORT content in the tadpoles from the decreasing water treatment did not rise until Day 28 posthatch ($F_{(5,32)} = 41.96$, $P < 0.0001$). In tadpoles maintained in a constant high water environment, elevations in whole-body T_4 and T_3 were not observed until Day 30 posthatch (T_4 : $F_{(5,33)} = 8.3$, $P < 0.0001$; T_3 : $F_{(5,35)} = 5.59$, $P = 0.001$); CORT levels were elevated in these animals at Day 28 posthatch ($F_{(5,35)} = 2.78$, $P = 0.036$; see Fig. 4).

For the second analysis (see above), analyses of univariate contrasts to distinguish treatment effects at the different measurement times revealed that the treatments diverged in whole-body T_4 at Day 21 posthatch (although this effect appears to be due to a drop in whole-body T_4 in the constant high water controls; $F_{(1,9)} = 15.22$, $P = 0.005$). The first significant divergence in whole-body T_3 and CORT contents was observed at Day 26 posthatch (T_3 : $F_{(1,11)} = 27.07$, $P = 0.0004$; CORT: $F_{(1,10)} = 44.54$, $P = 0.0001$). Hormone contents continued to diverge between the two treatments throughout the remainder of the experiment.

The hormone contents expressed as a function of the body weight may not be directly comparable in the two treatments since the contribution of gut contents

TABLE 1
Summary Statistics for Morphological Measurements (Expt. 1)

Measurement ^a	df	F	P
BW			
Time	5,144	8.7	<0.0001
Trt	1,144	53.42	<0.0001
Time × Trt	5,144	11.5	<0.0001
SVL			
Time	5,144	7.03	<0.0001
Trt	1,144	85.6	<0.0001
Time × Trt	5,144	13.95	<0.0001
HLL/SVL			
Time	5,142	221.32	<0.0001
Trt	1,142	12.6	0.0005
Time × Trt	5,142	4.48	0.0008
Dev. stage			
Time	5,144	343.58	<0.0001
Trt	1,144	34.05	<0.0001
Time × Trt	5,144	9.83	<0.0001

^a BW, body weight; SVL, snout-vent length; HLL, hind limb length; Dev. stage, Gosner staging (Gosner, 1960).

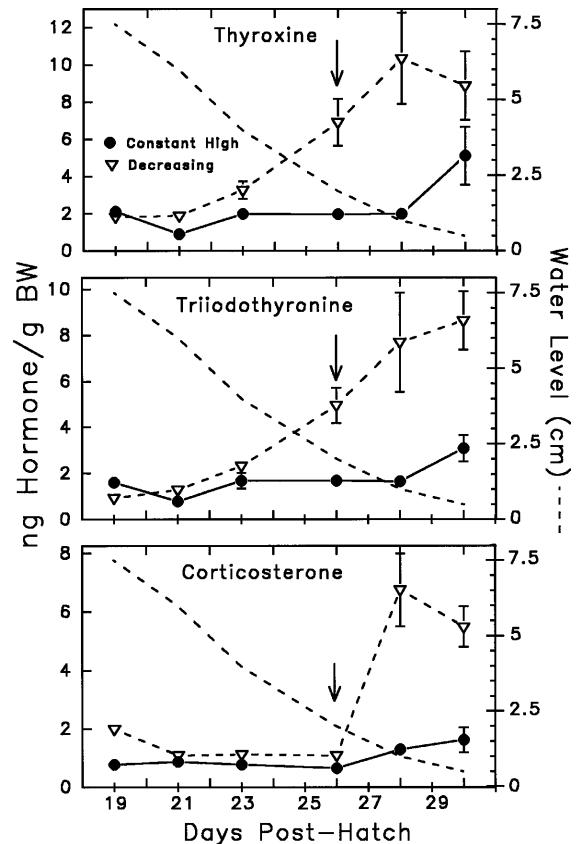


FIG. 4. Temporal changes in whole-body hormone contents of *S. hammondi* tadpoles. Tadpoles were raised in either a constant high water (10 l) or a decreasing water level (dashed line without points on the graphs; right axis) environment as described in the legend of Fig. 3 and under Materials and Methods. One cage of tadpoles from each treatment was removed at each time point and tadpoles were sacrificed for hormone analyses ($n = 6$ /treatment/time; see Materials and Methods). Points on the graph are the mean whole-body hormone contents and vertical lines are the SEM. Arrows indicate the day posthatch at which morphological divergence between the two treatments was first observed (see Fig. 3).

to tadpole BW likely differs between the two. The gut comprises a large proportion of a premetamorphic tadpole's total weight. As tadpoles metamorphose they cease feeding and the capacity of the gut decreases as it transforms from an omnivore/herbivore to a carnivore morphology. Thus, for the later ages, the proportion of tadpole body weight attributable to gut contents likely diverged between the two treatments, with tadpoles from the decreasing water treatment having a higher tadpole body weight to gut contents ratio than tadpoles from the constant high water

treatment. Such an effect would exaggerate differences in hormone levels resulting from the environmental treatment. To address this issue, I analyzed hormone content per tadpole (not correcting for body weight) which eliminated the differences between the treatments that could be attributed to differences in body weight (i.e., due to greater gut contents in tadpoles from the constant high water treatment). As expected, this analysis reduced differences in hormone contents between the treatments. Nevertheless, I observed a similar pattern of precocious elevation of thyroid and interrenal hormones in tadpoles from the decreasing water treatment, albeit with a slightly lesser magnitude (data not shown). Conclusions about the timing of these elevations derived from the analysis of hormone per gram BW were corroborated by this analysis. This was expected since differences in body weight between the two treatments range from 1.2- to 2.2-fold; whereas, differences in hormone levels are greater (i.e., 1.5- to 5-fold for T_4 ; see Figs. 3 and 4).

Tadpoles accelerate development within 48 h of exposure to water volume reduction (Expt. 2). I tested whether tadpoles could respond rapidly to an immediate decrease in the water level by accelerating metamorphosis. Tadpoles were raised to Gosner Stage 37–38 in a high water environment and then transferred to another cage with 10 L (constant high water treatment) or to a cage with 1 L (low water treatment). Transfer to 1 L resulted in a significant acceleration of metamorphosis as evidenced by a decrease in BW ($F_{(1,29)} = 72.36$, $P < 0.0001$), decrease in SVL ($F_{(1,29)} = 28.99$, $P < 0.0001$), increase in HLL/SVL ($F_{(1,29)} = 8.03$, $P = 0.008$), and

TABLE 2
Summary Statistics for Hormone Measurements (Expt. 1)

Measurement	df	F	P
Thyroxine			
Time	5,69	20.12	<0.0001
Trt	1,69	54.93	<0.0001
Time × Trt	5,69	6.62	0.0001
Triiodothyronine			
Time	5,69	23.82	<0.0001
Trt	1,69	35.8	<0.0001
Time × Trt	5,69	8.87	<0.0001
Corticosterone			
Time	5,69	24.2	<0.0001
Trt	1,69	85.19	<0.0001
Time × Trt	5,69	7.17	<0.0001

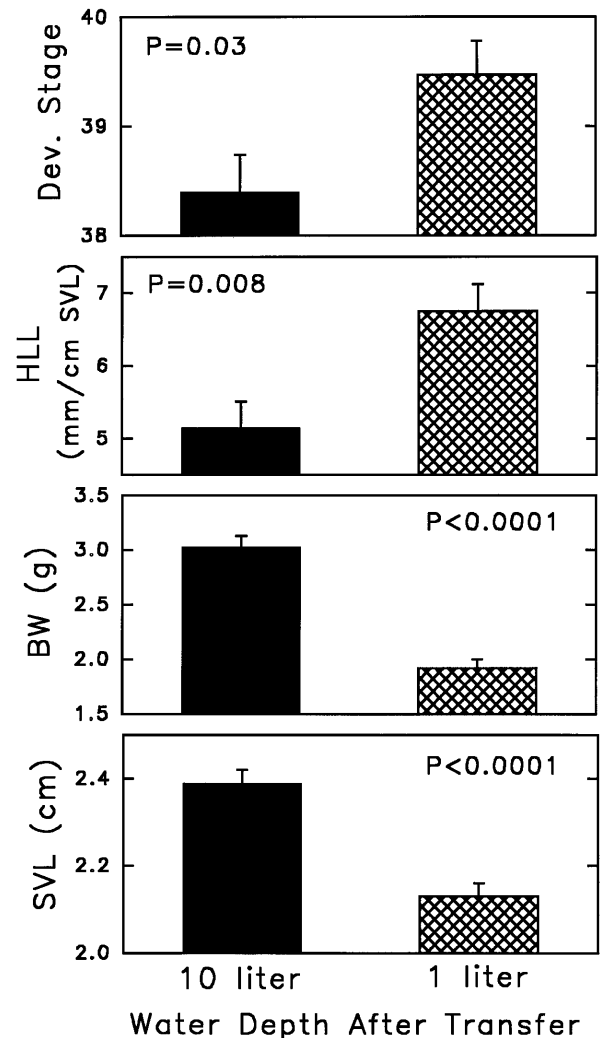


FIG. 5. Metamorphic response of mid-prometamorphic *S. hammondi* tadpoles to immediate water volume reduction. Tadpoles were raised to Gosner Stage 37–38 in a constant high water environment and then transferred to either a cage with high water (10 L) or a cage with low water (1 L). Measurements were made 48 h after transfer. Measurements are developmental stage (Dev. Stage; Gosner, 1960), body weight (BW), snout-vent length (SVL), and hind limb length (HLL; expressed as a function of body size – SVL). Bars are the means for each measurement and vertical lines are the SEM ($n = 15/\text{treatment}$). P values are derived from ANOVA.

increase in developmental stage ($F_{(1,29)} = 5.35$, $P = 0.03$; see Fig. 5).

The rapid morphological response to water volume reduction is associated with dramatic elevations in whole-body content of thyroid hormones and corticosterone (Expt. 2). Tadpoles transferred from 10 to 1 L exhibited significantly elevated whole-body contents

of T_4 ($F_{(1,13)} = 162.65$, $P < 0.0001$), T_3 ($F_{(1,14)} = 120.86$, $P < 0.0001$) and CORT ($F_{(1,13)} = 43.88$, $P < 0.0001$) 48 h later (see Fig. 6). The increase in hormone content of tadpoles transferred to low water was most striking for T_4 which increased 37-fold over constant high water controls.

DISCUSSION

The results of this study provide basic information relating to the physiological changes that underlie the adaptive developmental response of desert tadpoles to

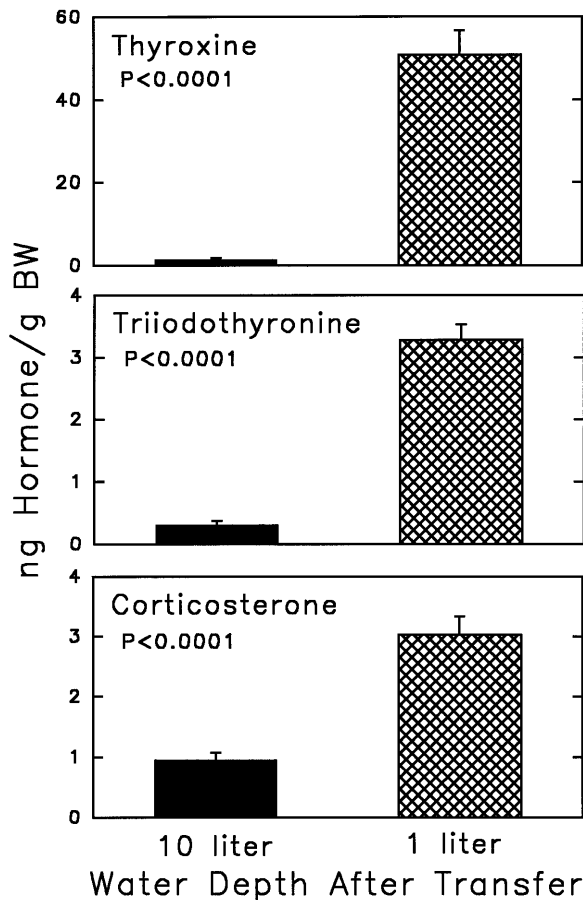


FIG. 6. Changes in whole-body 3,5,3'-triiodothyronine (T_3), thyroxine (T_4), and corticosterone (CORT) in mid-prometamorphic *S. hammondi* in response to immediate water volume reduction. Tadpoles were raised and transferred as described in the legend of Fig. 5. Whole-body hormone concentrations were determined by RIA following tissue extraction (see Materials and Methods). Bars are the means for each measurement and vertical lines are the SEM ($n = 7$ /treatment). P values are derived from ANOVA.

the reduction in water volume that occurs when their temporary ponds dry. The results suggest that the metamorphic acceleration induced by reduced water volume results from the precocious activation of the thyroid and the interrenal axes. The endocrine activation preceded morphological change by 3 days and, given that the hormones produced by these endocrine glands are central to the control of metamorphosis, it is highly likely that the endocrine activation was causally related to the accelerated metamorphosis. These results suggest that developmental plasticity results from changes in hormone production, although the possibility that there are also changes in the sensitivity of tissues to hormone action, hormone transport, clearance rate, etc. cannot at present be ruled out.

This study also provides the first detailed, temporal analysis of metamorphic changes induced by water volume reduction in an amphibian. The first observable morphological divergence occurred in body size (BW and SVL) and this difference continued through the end of the experiment. The precise environmental cue(s) that tadpoles use to respond to the declining water level by accelerating metamorphosis is currently unknown, but a systematic evaluation by us has ruled out several factors as potential signals (Denver *et al.*, 1998). Thermal effects have been implicated in the developmental response of *S. couchii* tadpoles to drying in natural ponds (Newman, 1989). However, the accelerated metamorphosis observed in our laboratory experiments with *S. hammondi* could not be attributed to thermal effects since water temperature in constant high and declining water tanks was identical. We have also provided evidence that the developmental response is unlikely to be due to an increase in the concentration of various compounds (e.g., waste products, hormonal substances secreted by tadpoles) in the aquaria with the declining water level.

Another possibility is that as the water volume is reduced, tadpole density increases and this crowding induces the metamorphic response. Density-dependent effects on tadpole metamorphosis are well known, but the primary effect tends to be an inhibition of growth and development (Richards, 1958; Licht, 1967; Brockelman, 1969; Wilbur, 1972, 1976, 1977a,b; Gromko *et al.*, 1973; Wilbur and Collins, 1973; John and Fenster, 1975; Smith-Gill and Gill, 1978; Smith-Gill and Berven, 1979; Semlitsch and Caldwell, 1982; Berven and Chadra, 1988; Scott, 1990). However, Newman (1994) reported

results from laboratory experiments in which the density of *S. couchii* tadpoles was negatively correlated with the duration of the larval period. We examined the metamorphic response of *S. hammondi* tadpoles raised individually to water volume reduction and found that these tadpoles accelerated development (Denver *et al.*, 1998). Furthermore, we have found in other experiments that the acceleration of metamorphosis in response to water volume reduction is greater in tadpoles raised individually than in tadpoles raised in groups; i.e., increasing tadpole density tended to attenuate the response rather than to enhance it (Denver and Phillips, unpublished results; we did not test whether increasing tadpole density while maintaining water volume constant would alter metamorphosis in *S. hammondi*). Thus, the metamorphic acceleration induced by water volume reduction in the present study cannot be attributed to changing tadpole density. More work is required to identify the precise cues that tadpoles use to track environmental changes in the desiccating larval habitat.

Tadpoles of diverse amphibian species are known to exhibit environmentally induced plasticity in development rate and morphology (see Newman, 1992; Skelly, 1997; for reviews). However, the speed with which tadpoles can respond to alterations in the larval habitat by accelerating metamorphosis has not been examined. One predicts that tadpoles of species that breed in ephemeral ponds and exhibit developmental plasticity induced by habitat desiccation would be capable of responding rapidly to deleterious changes in the larval habitat. The findings presented here show that the developmental response of mid-prometamorphic *S. hammondi* tadpoles is rapid, occurring within 48 h of a drastic reduction in the water level. Note that exposure of tadpoles to immediate water decline at earlier developmental stages (e.g., <Gosner Stage 34) slows growth and development, suggesting that there is a threshold developmental stage for the adaptive response (Denver and Phillips, unpublished results). Immediate transfer to the low water environment resulted in the rapid activation of the peripheral endocrine axes, and this activation was most probably mediated by the neuroendocrine system (i.e., neuroendocrine stress centers—CRH; Denver, 1997b). This conclusion is supported by the observation that hypothalamic CRH content was elevated at the same time as whole-body T_3 and T_4 concentrations in tadpoles

exposed to water volume reduction (Denver, 1997b; Day 23 posthatch—see Results and Fig. 3; Experiment 1 reported in this paper and the experiment reported in Denver [1997b] were run concurrently with animals from the same sibship). The increase in CORT observed in this study supports the conclusion from the previous study (based on elevations in hypothalamic CRH content) that the developmental response to water volume reduction is driven by a classical endocrinological stress response.

As discussed above, the developmental response to water volume reduction likely depends on tadpoles achieving a minimum developmental stage or size for metamorphosis (Wilbur and Collins, 1973). Prior to this, exposure to reduced water volume slows growth and development (Denver and Phillips, unpublished observations). It has been suggested that early exposure to environmental stressors reduces growth and development through a CORT-dependent mechanism since treatment of early stage tadpoles with a supra-physiological dose of CORT reduced growth and development; whereas, treatment of late stage animals accelerated metamorphosis (see Hayes, 1997). Whether the growth inhibition with exposure of early stage tadpoles to low water is due to elevation of endogenous CORT is currently unknown (but, see Hayes, 1997, for preliminary evidence of such an effect of density stress on endogenous CORT).

The results of these experiments demonstrate that studies which combine ecological and physiological approaches can provide valuable insight into the proximate mechanisms that mediate the response of the developing tadpole to changes in its habitat (see also Denver, 1997b). Elucidating the physiological mechanisms that underlie the developmental response to environmental variation can provide an understanding of the mechanistic basis for, and the constraints on the trade-off between growth and development, information that is essential to understanding the evolution of adaptive phenotypic plasticity in heterogeneous environments (Newman, 1992).

ACKNOWLEDGMENTS

This research was supported by NSF Grant IBN 9496321 and funds from the Office of the Vice President for Research and the

College of Literature, Science and the Arts of The University of Michigan. I thank Dr. Paul Licht for valuable advice on the HPLC and TLC fractionations. Nooshan Mirhadi provided technical assistance. I am grateful to the Connolly family for permission to collect spadefoot toads on their land. Western spadefoot toads were collected under California Scientific Collecting Permit 2175.

REFERENCES

- Berven, K. A. (1982). The genetic basis of altitudinal variation in the wood frog *Rana sylvatica* II. An experimental analysis of larval development. *Oecologia* **52**, 360–369.
- Berven, K. A., and Chandra, B. G. (1988). The relationship among egg size, density and food level on larval development in the wood frog (*Rana sylvatica*). *Oecologia* **75**, 67–72.
- Brockelman, W. Y. (1969). An analysis of density effects and predation in *Bufo americanus* tadpoles. *Ecology* **50**, 632–644.
- Clarke, R. D. (1974). Postmetamorphic growth rates in a natural population of the Fowler's toad (*Bufo woodhousei fowleri*). *Can. J. Zool.* **52**, 1458–1498.
- Denver, R. J. (1988). Several hypothalamic peptides stimulate *in vitro* thyrotropin secretion by pituitaries of anuran amphibians. *Gen. Comp. Endocrinol.* **72**, 383–393.
- Denver, R. J. (1993). Acceleration of anuran amphibian metamorphosis by corticotropin-releasing hormone-like peptides. *Gen. Comp. Endocrinol.* **91**, 38–51.
- Denver, R. J. (1995). Environment-neuroendocrine interactions in the control of amphibian metamorphosis. *Neth. J. Zool.* **45**, 195–200.
- Denver, R. J. (1996). Neuroendocrine control of amphibian metamorphosis. In "Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells" (L. I. Gilbert, J. R. Tata, and B. G. Atkinson, Eds.) pp. 434–464. Academic Press, San Diego, CA.
- Denver, R. J. (1997a). Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. *Am. Zool.* **37**(2), 172–184.
- Denver, R. J. (1997b). Environmental stress as a developmental cue: Corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. *Horm. Behav.* **31**, 169–179.
- Denver, R. J., and Licht, P. (1988). Thyroid status influences *in vitro* thyrotropin and growth hormone responses to thyrotropin-releasing hormone by pituitary glands of hatchling slider turtles (*Pseudemys scripta elegans*). *J. Exp. Zool.* **246**, 293–304.
- Denver, R. J., and Licht, P. (1989). Neuropeptide stimulation of thyrotropin secretion in the larval bullfrog: Evidence for a common neuroregulator of thyroid and interrenal activity during metamorphosis. *J. Exp. Zool.* **252**, 101–104.
- Denver, R. J., Mirhadi, N., and Phillips, M. (1998). An experimental analysis of adaptive phenotypic plasticity in amphibian metamorphosis: Developmental response to habitat desiccation in tadpoles of *Scaphiopus hammondi*. *Ecology* [in press].
- Gancedo, B., Corpas, I., Alonso-Gomez, A. L., Delgado, M. J., Morreale De Escobar, G., and 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.
- Goater, C. P., Semlitsch, R. D., and Bernasconi, M. V. (1993). Effects of body size and parasite infection on the locomotory performance of juvenile toads *Bufo bufo*. *Oikos* **66**, 129–136.
- Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**, 183–190.
- Gromko, M. H., Mason, F. S., and Smith-Gill, S. J. (1973). Analysis of the crowding effect in *Rana pipiens* tadpoles. *J. Exp. Zool.* **186**, 63–72.
- Hayes, T. B. (1997). Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis. *Amer. Zool.* **37**, 185–194.
- Hayes, T. B., and Wu, T. H. (1995). Interdependence of corticosterone and thyroid hormones in toad larvae (*Bufo boreas*). 2. Regulation of corticosterone and thyroid hormones. *J. Exp. Zool.* **271**, 103–111.
- Jacobs, G. F. M., and Kuhn, E. R. (1989). Thyroid function may be controlled by several hypothalamic factors in frogs and at least by one in the neotenic axolotl, p. 174. "Abstracts of the XIth International Symposium on Comparative Endocrinology, Malaga, Spain."
- Jacobs, G. F. M., and Kuhn, E. R. (1992). Thyroid hormone feedback regulation of the secretion of bioactive thyrotropin in the frog. *Gen. Comp. Endocrinol.* **88**, 415–423.
- John, K. R., and Fenster, D. (1975). The effects of partitions on the growth rate of crowded *Rana pipiens* tadpoles. *Amer. Midl. Nat.* **93**, 123–130.
- John-Alder, H. B., and Morin, P. J. (1990). Effects of larval density on jumping ability and stamina in newly metamorphosed *Bufo woodhousei fowleri*. *Copeia* **1990**(3), 856–860.
- Kikuyama, S., Kawamura, K., Tanaka, S., and Yamamoto, K. (1993). Aspects of amphibian metamorphosis: Hormonal control. *Int. Rev. Cytol.* **145**, 105–148.
- Licht, L. E. (1967). Growth inhibition in crowded tadpoles: intraspecific and interspecific effects. *Ecology* **48**, 336–345.
- Licht, P., McCreery, B. R., Barnes, R., and Pang, R. (1983). Seasonal and stress related changes in plasma gonadotropins, sex steroids and corticosterone in the bullfrog, *Rana catesbeiana*. *Gen. Comp. Endocrinol.* **50**, 124–145.
- MacKenzie, D. S., Licht, P., and Papkoff, H. (1978). Thyrotropin from amphibian (*Rana catesbeiana*) pituitaries and evidence for heterothyrotropic activity of bullfrog luteinizing hormone in reptiles. *Gen. Comp. Endocrinol.* **36**, 566–574.
- Malagon, M. M., Ruiz-Navarro, A., Torronteras, R., and Gracia-Navarro, F. (1991). Effects of ovine CRF on amphibian pituitary ACTH and TSH cells *in vivo*: A quantitative ultrastructural study. *Gen. Comp. Endocrinol.* **83**, 487–497.
- Martof, B. (1956). Growth and development of the green frog, *Rana clamitans*, under natural conditions. *Amer. Midl. Nat.* **74**, 95–109.
- Newman, R. A. (1989). Developmental plasticity of *Scaphiopus couchii* tadpoles in an unpredictable environment. *Ecology* **70**, 1775–1787.
- Newman, R. A. (1992). Adaptive plasticity in amphibian metamorphosis. *Biosciences* **42**, 671–678.
- Newman, R. A., and Dunham, A. E. (1994). Size at metamorphosis and water loss in a desert anuran (*Scaphiopus couchii*). *Copeia* **1994**, 372–381.

- Pough, F. H., and Kamel, S. (1984). Post-metamorphic change in activity metabolism of anurans in relation to life history. *Oecologia* **65**, 138–144.
- Reyes, A., Dieguez, J. L., Ruiz, M., Alonso, A., and Morell, M. (1990). Immunological characterization of L-triiodothyronine and L-thyroxine labelled with rhodamine B isothiocyanate. *Rev. Espanol. Fisiol.* **46**, 269–272.
- Richards, C. M. (1958). The inhibition of growth in crowded *Rana pipiens* tadpoles. *Physiol. Zool.* **31**, 138–151.
- Rugh, R. (1962). "Experimental Embryology," 3rd ed. Burgess, Minneapolis, MN.
- Scott, D. E. (1990). Effects of larval density in *Ambystoma opacum*: An experiment in large-scale field enclosures. *Ecology* **71**, 296–306.
- Semlitsch, R. D., Scott, D. E., and Pechmann, J. H. K. (1988). Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. *Ecology* **69**, 184–192.
- Semlitsch, R. D., and Caldwell, J. P. (1982). Effects of density on growth, metamorphosis and survivorship in tadpoles of *Scaphiopus holbrooki*. *Ecology* **63**, 905–911.
- Skelly, D. K. (1997). Tadpole communities. *Am. Sci.* **85**, 36–45.
- Smith, D. C. (1987). Adult recruitment in chorus frogs: Effects of size and date at metamorphosis. *Ecology* **68**, 344–350.
- Smith-Gill, S. J., and Berven, K. A. (1979). Predicting amphibian metamorphosis. *Am. Nat.* **113**, 563–585.
- Smith-Gill, S. J., and Gill, D. E. (1978). Curvilinearities in the competition equations: An experiment with ranid tadpoles. *Am. Nat.* **112**, 557–570.
- Taigen, T. L., and Pough, F. H. (1985). Metabolic correlates of anuran behavior. *Am. Zool.* **25**, 987–997.
- Tonon, M. C., Cuet, P., Lamacz, M., Jegou, Cote, J., Gouteux, L., Ling, N., Pelleier, G., and Vaudry, H. (1986). Comparative effects of corticotropin-releasing factor, arginine vasopressin, and related neuropeptides on the secretion of ACTH and -MSH by frog anterior pituitary cells and neurointermediate lobes *in vitro*. *Gen. Comp. Endocrinol.* **61**, 438–445.
- Turner, F. B. (1962). The demography of frogs and toads. *Q. Rev. Biol.* **37**, 303–314.
- Werner, E. E. (1986). Amphibian metamorphosis: Growth rate, predation risk, and the optimal size at transformation. *Am. Nat.* **128**, 319–341.
- Wilbur, H. M. (1972). Competition, predation, and the structure of the *Ambystoma—Rana sylvatica* community. *Ecology* **53**, 3–21.
- Wilbur, H. M. (1976). Density-dependent aspects of metamorphosis in *Ambystoma* and *Rana sylvatica*. *Ecology* **57**, 1289–1296.
- Wilbur, H. M. (1977a). Density-dependent aspects of growth and metamorphosis in *Bufo americanus*. *Ecology* **58**, 196–200.
- Wilbur, H. M. (1977b). Interactions of food level and population density in *Rana sylvatica*. *Ecology* **58**, 206–209.
- Wilbur, H. M., and Collins, J. P. (1973). Ecological aspects of amphibian metamorphosis. *Science* **182**, 1305–1314.