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Developmental Changes in Interrenal Responsiveness in Anuran Amphibians[1]

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SYNOPSIS. Basal activity of the hypothalamo-pituitary-interrenal (HPI) axis changes over development in larval amphibians, but development of the responsiveness of this axis to an external stressor has not been studied. We compared developmental changes in whole-body corticosterone content of two anuran amphibian species, *Rana pipiens* (family Ranidae) and *Xenopus laevis* (family Pipidae). We also examined developmental changes in the responsiveness of the HPI axis by subjecting tadpoles of different developmental stages to a laboratory shaking/confinement stress and to ACTH injection. We measured whole-body corticosterone content as an indicator of the activity of the HPI axis. Whole-body corticosterone content of *R. pipiens* remained low during premetamorphosis and prometamorphosis but increased dramatically at metamorphic climax and remained elevated in juvenile frogs. By contrast, whole-body corticosterone content of *X. laevis* was highest during premetamorphosis, declined at the onset of prometamorphosis, increased at metamorphic climax and remained at climax levels in juvenile frogs. Premetamorphic and prometamorphic tadpoles of both species showed strong corticosterone responses to both shaking stress and ACTH injection. The magnitude and pattern of response differed among developmental stages, with premetamorphic tadpoles of both species showing greater responsiveness to stress and ACTH. Our results show that interrenal responsiveness is developed in premetamorphic tadpoles, suggesting that at these stages tadpoles are capable of mounting an increase in stress hormone production in response to changes in the external environment. Our results also highlight the importance of comparative studies in understanding the development of the stress axis.

INTRODUCTION

Basal activity of the amphibian interrenal glands (homologous to mammalian adrenal cortex) over larval development has been measured for several species. Based on plasma- or whole-body corticoid content, interrenal enzyme activity, and histology, the interrenal glands are considered generally to be less active in early, premetamorphic developmental stages and more active during prometamorphosis and metamorphic climax. Plasma corticoid concentrations in larvae of several amphibian species are low during premetamorphosis, increase during late prometamorphosis and peak at metamorphic climax (*R. catesbeiana*: Jaffe [1981]; Krug *et al.* [1983]; Kikuyama *et al.* [1986]; *B. japonicus*: Niinuma *et al.* [1989]; *X. laevis*: Jolivet-Jaudet and Leloup-Hatey [1984]; *A. tigrinum*: Carr and Norris [1988]). Activity of the interrenal enzyme, Δ^5-3B-hydroxysteroid dehydrogenase (HSD) is detectable throughout development in *R. catesbeiana* and *X. laevis* (Hsu *et al.*, 1980; Kang *et al.*, 1995). HSD activity increases at metamorphic climax in *R. catesbeiana* (Hsu *et al.*, 1980), and Carr and Norris (1988) found a similar pattern for plasma corticosterone and interrenal HSD activity in the tiger salamander, *Ambystoma tigrinum*. The ultrastructural appearance of *X. laevis* interrenal cells indicates relative inactivity during mid-prometamorphosis, increasing to peak activity at metamorphic climax (reviewed by Dodd and Dodd, 1976).

Few investigations have focused on changes at other levels of the hypothalamic-pituitary-interrenal (HPI) axis in amphibians. Carr and Norris (1990) reported corticotropin releasing hormone (CRH)- and arginine vasotocin (AVT)-like immunoreactivity to be low in premetamorphic *R. catesbeiana* in the hypothalamus/median eminence, then increasing to higher levels in prometamorphosis and metamorphic climax. Both CRH and arginine vasopressin (AVP, the mammalian form of the amphibian nonapeptide arginine vasotocin, AVT) stimulate adrenocorticotropic hormone (ACTH) secretion by adult frog pituitaries (Tonon *et al.*, 1986; see Kikuyama *et al.*, 1993). AVT also exerts a direct stimulatory action on interrenal steroidogenesis in frogs (Kloas and Hanke, 1990). To our knowledge, no direct measures of ACTH peptide production over development have been reported, although pituitary proopiomelanocortin (POMC, the precursor of ACTH) mRNA levels increase during metamorphosis in *R. catesbeiana* tadpoles (Aida *et al.*, 1999).

The studies described above all have measured basal activity of components of the HPI axis, and the general conclusion has been that HPI activity increases over development. However, it is unknown how the responsiveness of the HPI axis to stressors changes during development. Are pre-metamorphic tadpoles capable of mounting a HPI response to an external stressor, or does the low basal activity reflect a hyporesponsive axis? This question is important in light of recent evidence that the HPI axis plays a role in mediating the responses of anuran larvae to environmental conditions such as pond drying and intraspecific competition (Denver, 1997, 1998; Glennemeier and Denver, 2002). An understanding of the basic responsiveness

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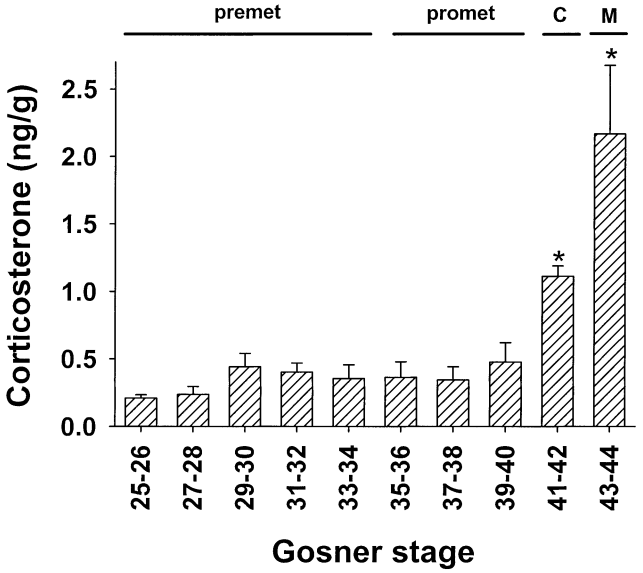

FIG. 1. Whole body corticosterone content of *R. pipiens* tadpoles and metamorphs at different developmental stages (Gosner, 1960). Premetamorphosis (**premet**; foot paddle stages), prometamorphosis (**promet**; hind limb stages), metamorphic climax (**C**) and newly metamorphosed frog (**M**) stages are indicated. Asterisk (*) indicates significantly different from previous stage ($P < 0.006$). Error bars represent standard errors of the mean (n = 3–10/stage) of individual or pooled (for premetamorphic stages) tadpoles (4–5 tadpoles per pool).

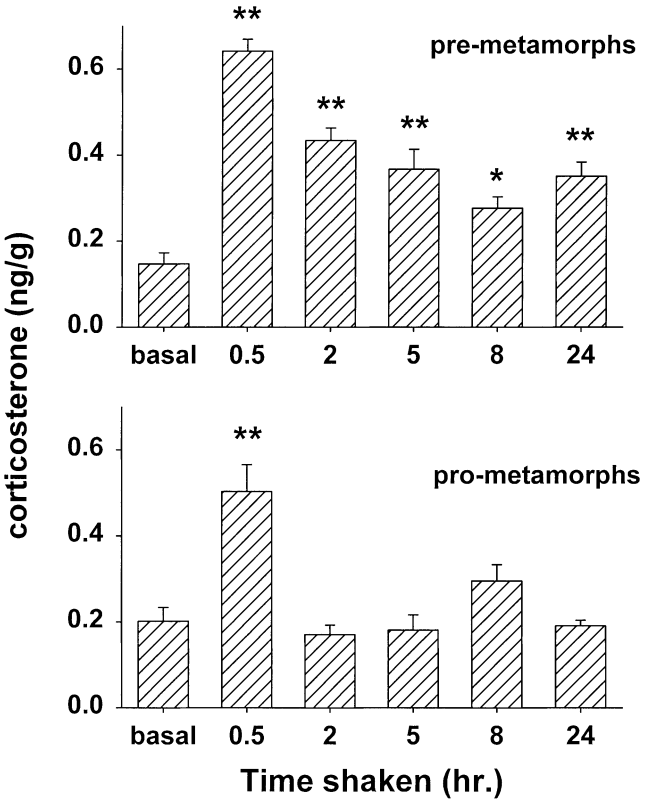

FIG. 2. Whole-body corticosterone content in *R. pipiens* tadpoles subjected to shaking/confinement stress for different time periods. Tadpoles were held in just enough water to cover them and shaken gently on a mechanical shaker for the time indicated. Premetamorphs = Gosner stages 26–29; Prometamorphs = Gosner stages 35–40. Asterisks (*) indicate significant difference from basal content (** $P < 0.0001$; * $P < 0.007$). Error bars represent standard errors of the mean (n = 7 pools of 4 tadpoles each for premetamorphs; n = 8 tadpoles for prometamorphs).

of the HPI axis over development will help to determine potential responses of amphibian larvae to environmental stimuli that may change in prevalence or importance with larval development.

We tested the hypothesis that HPI responsiveness to a generic stressor and to ACTH injection changes over development in *R. pipiens* and *X. laevis* tadpoles. Different environmental stimuli may differ in their ability to elicit an HPI response, so we developed a less natural, but more generic ''stressor'' in the laboratory to determine the capacity for response of the HPI axis at several developmental stages. HPI response was measured by comparing whole-body corticosterone content among non-stressed and stressed tadpoles. To determine responsiveness of the interrenal glands to pituitary hormonal stimulation, we injected tadpoles with several doses of ACTH and compared whole-body corticosterone content to basal values and saline-injected controls.

MATERIALS AND METHODS

Animal care and husbandry

Rana pipiens eggs were purchased from Carolina Biological Supply Co. (Burlington, NC). Tadpoles were raised in environmental chambers at 22°C, 12D: 12L, and were fed 10–15% of body mass per day (based on the mean mass of animals per tank) of a 3:1 mixture of Purina Rabbit Chow: Tetramin Fish Flakes. Tadpoles were held in plastic tanks of 20 liters of well water, with approximately 100 tadpoles per tank, prior to experiments.

Xenopus laevis eggs were spawned from a laboratory population of adults. Adults were maintained in large holding tanks at 22°C, 12D:12L, and fed beef liver twice a week. Spawning was induced by injection of gonadotropin releasing hormone agonist (Sigma-Aldrich, St. Louis, MO). Newly hatched tadpoles were maintained in 10% Holtfreter's solution for 5 days and then transferred to a 170 liter holding tank, with 500 to 800 tadpoles per tank. They were fed pulverized Purina Rabbit Chow.

For both species, different sibships were used for the developmental sampling, ACTH injection and stress experiments. Thus, the small variation in basal corticosterone content observed in *R. pipiens* among experiments (see below; and compare Fig. 1 with Figs. 2 and 3) may be attributable to genetic variation among sibships, the time of year at which egg masses were obtained from the commercial supplier, or differences in the condition of the animals.

Developmental changes in whole body corticosterone content

For analysis of developmental changes in whole-body corticosterone content, *R. pipiens* tadpoles were

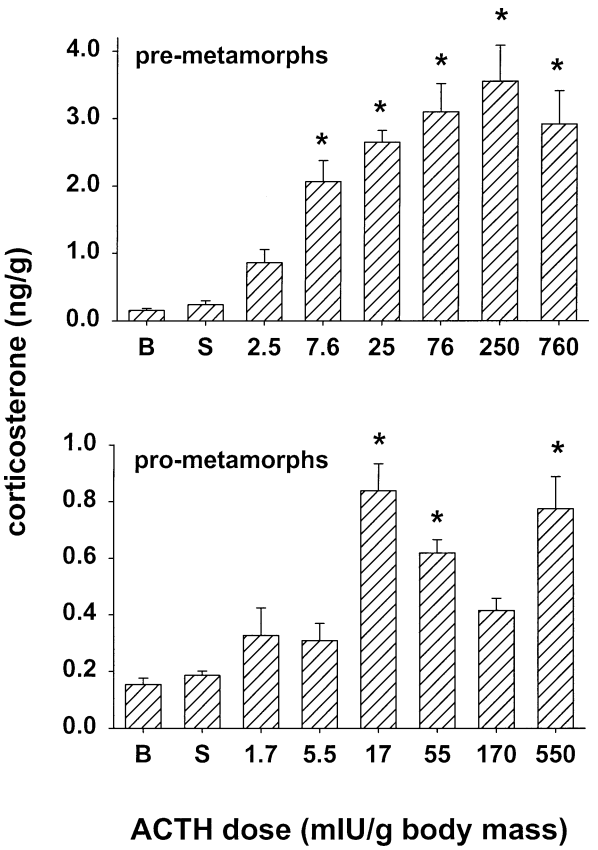

FIG. 3. Whole-body corticosterone content in *R. pipiens* tadpoles injected with ACTH or saline vehicle. Tadpoles were collected 6 hr after injection. Bars in the top and bottom panels that are vertically aligned represent comparable ACTH doses, adjusted for the allometric relationship between mass and metabolic rate (see Materials and Methods). Premetamorphs = Gosner stages 26–29; Prometamorphs = Gosner stages 35–40. B = basal; S = saline-injected. Asterisks (*) indicate significant difference from saline-injected content ($P < 0.0005$). Error bars represent standard errors of the mean (n = 5 pools of 2 tadpoles each for premetamorphs; n = 7 individual tadpoles for prometamorphs).

held in three 10 liter plastic tanks, with approximately 40 tadpoles per tank, while *X. laevis* tadpoles were maintained in a 170 liter holding tank as described above. Tadpoles in specific developmental stages were collected from the holding tanks over a 2-mo time period. They were anesthetized in 0.01% benzocaine immediately, weighed, placed into extraction tubes, and frozen at −20°C. Following organic extraction of the hormone (see below), whole body corticosterone content was determined by radioimmunoassay (RIA). Tadpoles were extracted either individually (for prometamorphic, climax and postclimax stages) or in pools for premetamorphic animals (*R. pipiens*: 4–5 tadpoles/pool; *X. laevis*: 3 tadpoles/pool). Sample sizes were 8 per stage for *X. laevis* and ranged from 3 to 10 per stage for *R. pipiens*.

Stress response

Tadpoles were subjected to a shaking/confinement stressor for 24, 8, 5, 2, or 0.5 hr. The experiment was conducted for two *R. pipiens* stages (premetamorphosis, stages 26–29, and prometamorphosis, stages 35–40, Gosner, 1960 staging table) and three *X. laevis* stages (early [premetamorphosis], stages 48–51, middle [midprometamorphosis], stages 54–55, and late [late-prometamorphosis], stages 59–60, Nieuwkoop and Faber, 1956 staging table). At 1700 hr on day one of the experiment, tadpoles were randomly assigned to treatments and placed into 28 × 17 × 12 cm tanks, with 10–15 tadpoles in 4 liters of water. At 1700 hr on day two, the 24-hr group was placed into a tank (same size) with the water depth just deep enough to cover the tadpoles but preventing any vertical movement through the water column. The tanks were placed on an orbital shaker and agitated continually at 30–100 rpm. The shaking intensity was just enough to require constant spatial adjustment by the tadpoles but not enough to cause physical damage or bumping into the sides of the tank. Shaking speed was subjectively adjusted to provide a similar intensity of agitation at all stages; later stage tadpoles were larger and thus required a greater shaking speed to produce similar physical agitation to that of the smaller tadpoles. Tadpoles of the same stage were shaken at identical speeds.

At 0900 hr on day three, the 8-hr group was subjected to the same conditions as described above, and the other groups followed at 1200, 1500, and 1650 hr on day three, respectively. Tadpoles for basal corticosterone measurement were left undisturbed in 4 liter tanks. Shaking continued until 1700 hr on day three. At this time, all tadpoles were euthanized by submersion in 0.01% benzocaine, weighed, and frozen at −20°C for later extraction of whole-body corticosterone and RIA.

ACTH response

Tadpoles were injected with different doses of ACTH and collected 6 hr after injection for corticosterone analysis. In pilot studies we found that the whole-body corticosterone response to ACTH is maximal 6 hr after injection (data not shown). At 1700 hr on day one, tadpoles from the same stages as above were randomly assigned to treatments and placed into 28 × 17 × 12 cm tanks, with 10–15 tadpoles per treatment in 4 liters of water. At 1100 hr on day two, each tadpole was given an intraperitoneal injection through the tail musculature with 20–50 μl of an ACTH solution (porcine ACTH; Sigma-Aldrich, St. Louis, MO) or phosphate-buffered saline (PBS; pH 7.2) vehicle only and then returned to its respective 4 liter tank. At 1700 hr on day two, all tadpoles were euthanized by submersion in 0.01% benzocaine, weighed, and frozen at −20°C for later extraction of whole-body corticosterone and radioimmunoassay (RIA). Uninjected tadpoles also were collected at this time for measurement of basal corticosterone content.

Early stage *X. laevis* tadpoles were very sensitive to the stress of injection, and mortality of injected tadpoles was high. Therefore, these tadpoles were lightly

anesthetized (0.002% benzocaine) for 5 min prior to injection, and returned to water without anesthetic after injection until 1700 hr collection; tadpoles recovered from anesthesia within 5 min of transfer.

ACTH doses are given in Figures 3 and 6. To adjust for the allometric relationship between body mass and metabolic rate, metabolically adjusted mass was used to determine ACTH dosage. Adjusted mass was $M^{0.71}$, where M = mass (Hutchinson *et al.*, 1968). The mean mass of tadpoles within a stage was used to determine the ACTH dosage for that stage. Thus, the lowest ACTH dose for the two *R. pipiens* stages is comparable, as is each dose thereafter, and the same holds for the three *X. laevis* stages.

Corticosterone extraction and radioimmunoassay

Whole body corticosterone content was determined by RIA following organic extraction from collected tadpoles. The extraction procedure is described by Hayes and Wu (1995) and Denver (1998). Briefly, tissues were homogenized in ethyl acetate and the extracts fractionated by thin layer chromatography (TLC) to separate corticosterone from other lipids. The region of the TLC lane containing the corticosterone (as determined by calibration with both radiolabeled and radioinert corticosterone; see Denver, 1998) was scraped and the silica collected into a borosilicate glass tube. The silica was extracted with ethyl ether, and the extract was dried under nitrogen and resuspended in PBS-gelatin (PBS-G; 0.02M, pH 7.3) for corticosterone RIA. The RIA was conducted as described by Licht *et al.* (1983). Anti-corticosterone serum was purchased from Endocrine Sciences (Calabasas, CA) and [^{3}H]-corticosterone from NEN Life Science Products, Inc. (Boston, MA). Samples from a single experiment were analyzed in a single RIA or in multiple RIAs on a single day. Inter- and intra-assay coefficients of variation were 12% and 10%, respectively, and were monitored by including a quality control standard (pooled rat plasma) in each RIA.

Statistics

Corticosterone developmental sequence data were analyzed using one-way ANOVA of stage versus whole-body corticosterone content, followed by Fisher's Least Squares Difference (LSD) pairwise comparisons among stages. Whole-body corticosterone content after different shaking times was analyzed using one-way ANOVA, with corticosterone content as the response variable and time shaken as treatment. Each developmental stage was analyzed separately. Fisher's Least Squares Difference test of pairwise comparisons was used to compare each time period to basal content.

Whole-body corticosterone content after injection with various doses of ACTH was analyzed using one-way ANOVA, with corticosterone content as the response variable and ACTH dose as treatment. Each developmental stage was analyzed separately. Fisher's Least Squares Difference test of pairwise comparisons was used to compare each ACTH dose to the saline-injected group.

TABLE 1. *Ratio of maximum whole-body corticosterone content to basal content (for shaking/confinement tests) or to saline-injected content (for ACTH injections) for each species and developmental stage.**

		Ratio of maximum to basal or saline-injected	
		Shaking	ACTH
R. pipiens	Pre-metamorphs	4.4	14.9
	Pro-metamorphs	2.5	4.5
X. laevis	Early stage	7.1	17.3
	Middle stage	6.0	2.3
	Late stage	7.3	2.6

* Ratios were calculated using the means shown in Figures 2, 3, 5 and 6.

RESULTS

Developmental changes in whole body corticosterone content—R. pipiens

Whole-body corticosterone content differed significantly among developmental stages in *R. pipiens* (Fig. 1; $F = 13.7$; $P < 0.00005$; ANOVA), with the first significant increase seen at the beginning of metamorphic climax (Gosner stage 42). Corticosterone content remained high in the postmetamorphic frog.

Stress response—R. pipiens

Both premetamorphic and prometamorphic *R. pipiens* tadpoles responded to shaking/confinement stress with increased whole body corticosterone content (Fig. 2). One-way ANOVA showed significant differences among treatments for each stage (premetamorphs $F = 27.4$, $P < 0.00005$; prometamorphs $F = 12.3$, $P < 0.00005$).

The pattern and magnitude of the corticosterone response differed among stages. Both stages responded after 0.5 hr, but the response in premetamorphs was sustained up to 24 hr; whereas, prometamorphic animals showed no response after 2 hr. The magnitude of the maximum response in premetamorphic animals was almost twice that of prometamorphic animals (Table 1).

ACTH injection—R. pipiens

Both premetamorphic and prometamorphic *R. pipiens* tadpoles responded to ACTH injection (Fig. 3). One-way ANOVA showed significant differences among treatments for each stage (premetamorphic animals: $F = 16.5$, $P < 0.00005$; prometamorphic animals: $F = 13.2$, $P < 0.00005$).

The pattern and magnitude of the corticosterone response differed among stages. Pre-metamorphic tadpoles responded to a threshold ACTH dose one-third that of prometamorphic animals, and the pre-metamorphic response increased with higher ACTH doses. The prometamorphic response was maximal at the earliest response and weaker at several higher doses. The

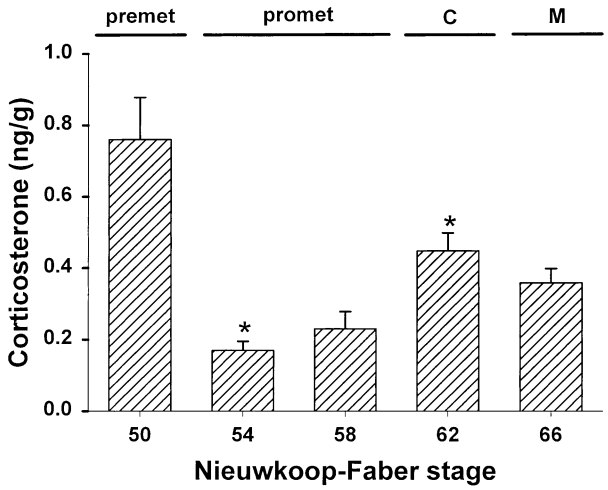

FIG. 4. Whole body corticosterone content of *X. laevis* at different developmental stages (Nieuwkoop and Faber, 1956). Premetamorphosis (**premet**; foot paddle stages), prometamorphosis (**promet**; hind limb stages), metamorphic climax (**C**) and newly metamorphosed frog (**M**) stages are indicated. Asterisk (*) indicates significantly different from previous stage ($P < 0.01$). Error bars represent standard errors of the mean (n = 8/stage) of individual or pooled tadpoles (for NF stage 50; three animals per pool).

magnitude of maximum response during premetamorphosis was approximately three times that of prometamorphic animals (Table 1).

Developmental changes in whole body corticosterone content—X. laevis

Whole body corticosterone content exhibited significant changes during metamorphosis in *X. laevis* ($F = 120.48$, $P < 0.0001$; ANOVA; Fig. 4). Corticosterone content was highest during premetamorphosis (NF stage 50) and then declined significantly at the onset of prometamorphosis. Hormone content then increased significantly from NF stage 54 (early prometamorphosis) to NF stage 62 (metamorphic climax) and remained at climax level in the newly metamorphosed frog.

Stress response—X. laevis

Early, middle, and late stage *X. laevis* tadpoles responded to shaking/confinement stress with increased whole body corticosterone content (Fig. 5). One-way ANOVA showed significant differences among treatments for each stage (early $F = 7.0$, $P = 0.00009$; middle $F = 15.1$, $P < 0.00005$; late $F = 7.5$ $P = 0.00002$).

The pattern of corticosterone response differed among stages. Early stage tadpoles responded after 2 hr but showed no response after 5 or more hours of shaking/confinement. Middle stage tadpoles responded after 0.5 hr and continued to respond up to 8 hr. Late stage tadpoles did not respond until they had been shaken for 8 hours, and the response was sustained after 24 hr. The magnitude of the maximum response was similar among stages (Table 1).

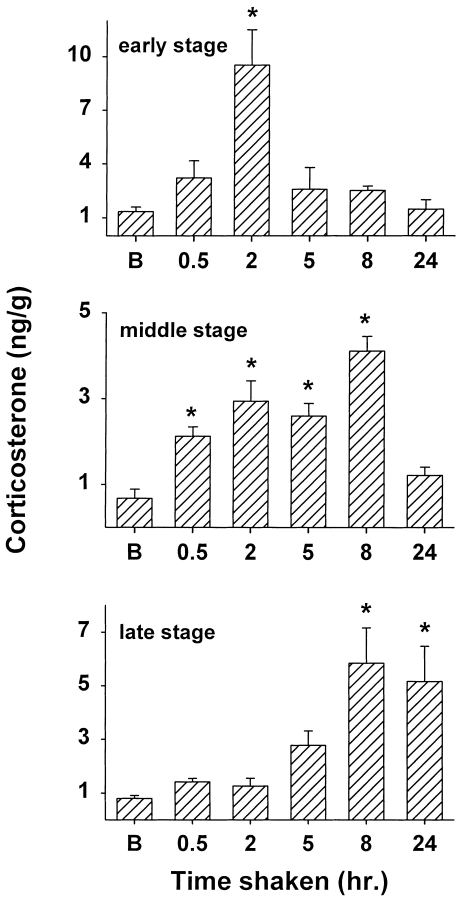

FIG. 5. Whole-body corticosterone content in *X. laevis* tadpoles subjected to shaking/confinement stress for different time periods. Tadpoles were held in just enough water to cover them and were shaken gently on a mechanical shaker for the time indicated. Early stage = Nieuwkoop and Faber stages 48–51; Middle stage = stages 54–55; Late stage = stages 59–60. B = basal. Asterisks (*) indicate significant difference from basal content ($P < 0.003$). Error bars represent standard errors of the mean (n = 10–12 tadpoles).

ACTH injection—X. laevis

Early, middle, and late stage *X. laevis* tadpoles responded to ACTH injection (Fig. 6). One-way ANOVA showed significant differences among treatments for each stage (early $F = 4.8$, $P = 0.0004$; middle $F = 9.2$, $P < 0.00005$; late $F = 7.0$ $P < 0.00005$).

The pattern and magnitude of corticosterone response differed among stages. Early and late stage tadpoles showed their first response at similar ACTH doses, and this dose was one-third that of middle stage tadpoles. The response in early stage tadpoles was still strong at the highest ACTH dose, whereas middle and late stage tadpoles showed an attenuated response at higher doses. The magnitude of maximum response in early stage tadpoles was approximately seven times that of middle and late stage tadpoles (Table 1).

DISCUSSION

Our results show that the HPI axis in both *R. pipiens* and *X. laevis* is capable of responding to an external

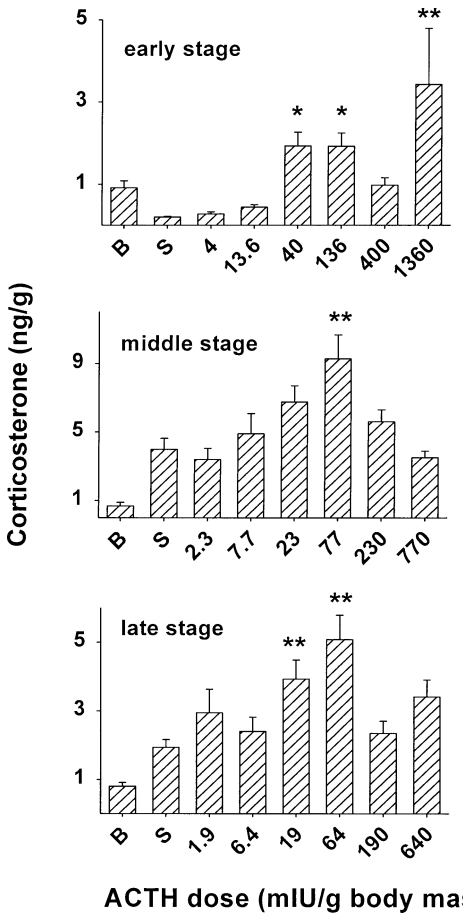

FIG. 6. Whole-body corticosterone content in *X. laevis* tadpoles injected with ACTH or saline vehicle. Tadpoles were collected 6 hr after injection. Bars in the top, middle, and bottom panels that are vertically aligned represent comparable ACTH doses, adjusted for the allometric relationship between mass and metabolic rate (see Materials and Methods). Early stage = Nieuwkoop and Faber stages 48–51; Middle stage = stages 54–55; Late stage = stages 59–60. B = basal; S = saline-injected. Asterisks (*) indicate significant difference from saline-injected content (** $P < 0.005$; * $P < 0.017$). Corticosterone content in the saline group of the middle panel was significantly different from that of basal ($P = 0.012$); basal and saline-injected groups did not differ for top and bottom panels. Error bars represent standard errors of the mean (n = 8–11 tadpoles).

stressor and to ACTH injection throughout larval development. These findings suggest that the basal activity of the axis, as measured by plasma or tissue corticosterone content or interrenal enzyme activity, does not necessarily reflect the capacity of the axis to respond to stress. Despite somewhat different patterns of change in basal corticosterone content over development, both *R. pipiens* and *X. laevis* showed a strong corticosterone response to stress and ACTH injection at all stages tested.

Developmental changes in basal corticosterone content

The pattern of developmental changes in *R. pipiens* whole-body corticosterone content reported here is similar to that reported for plasma concentrations in another ranid species *R. catesbeiana* (Jaffe, 1981; Krug *et al.*, 1983; Kikuyama *et al.*, 1986). Other amphibian species exhibit similar patterns of change in plasma corticoids during the metamorphic period (*B. japonicus*: Niinuma *et al.*, 1989; *X. laevis*: Jolivet-Jaudet and Leloup-Hatey, 1984; *A. tigrinum*: Carr and Norris, 1988). Whole-body corticosterone content has been determined throughout development for the Western spadefoot toad (*Spea hammondii*; Denver, 1998) and was found to exhibit a similar pattern of change as plasma corticosterone in other species; *i.e.*, low during pre- and prometamorphosis with a marked increase at metamorphic climax. While changes in whole body corticosterone content in *R. pipiens* follows those observed in the blood of other species, *X. laevis* exhibits a somewhat different pattern. Our findings with *X. laevis* largely confirm those of Kloas and colleagues (1997) who reported whole-body corticosterone content to be highest at early limb bud stages but decreasing to lower values during prometamorphosis. We observed a small (~2-fold) increase between early prometamorphic and climax stages which was not reported by Kloas and colleagues, but would fit with earlier reports of small increases in plasma corticosterone during similar stages reported by Jolivet-Jaudet and Leloup-Hatey (1984). This change in corticosterone content that we observed in *X. laevis* at metamorphic climax was small (compared to *R. pipiens*), and the differences between ours and Kloas and colleagues' study could be related to differences in the extraction methods used and/or differences in the sensitivities of our RIAs. Kloas and colleagues (1997) also measured whole body aldosterone and found a similar increase during premetamorphosis, but the peak content was found during early prometamorphosis (NF stage 54) and it declined thereafter.

In comparing plasma corticosterone concentrations in *X. laevis* reported by Jolivet-Jaudet and Leloup-Hatey (1984) and whole body corticosterone content reported by Kloas and colleagues (1997) and by us (present study), one should note that the former investigators did not measure plasma corticosterone prior to mid-prometamorphic stages (NF stage 54; Nieuwkoop and Faber, 1994); whereas, we, and Kloas and colleagues (1997) measured peak whole-body corticosterone content at pre-metamorphic stages (NF stages 48–50). Thus, it is unknown whether plasma corticosterone concentrations parallel whole body content in *X. laevis* (*i.e.*, decline from premetamorphosis to prometamorphosis). Given the small size of premetamorphic *X. laevis* tadpoles and the concomitant difficulty with obtaining blood samples this may be impossible to determine.

It may not be possible to directly compare blood values with whole body hormone content since whole-body content represents the sum total of corticoid biosynthesis and metabolism in the animal. However, it should be noted that we have measured whole body triiodothyronine and thyroxine content in *X. laevis* (R. J. Denver, unpublished results) and have found that

changes in these hormones parallel changes in serum values (as reported by Leloup and Buscaglia, 1977) during metamorphosis. In pilot studies Kloas and colleagues (1997) analyzed the contribution of renal tissue steroids to whole body content and found that two-thirds of corticosterone and 90% of aldosterone content were associated with renal tissue. The storage pool of steroid hormones in steroidogenic tissue is generally small; *i.e.,* the rate of biosynthesis generally reflects the rate of secretion into the blood. Plasma concentrations are influenced by both the rate of secretion and the rate of clearance from the blood. Minimally, tissue corticoid content likely reflects the activity of the axis (degree of stimulation by ACTH). Our findings highlight the importance of determining endogenous patterns for each species under study, and they provide a cautionary note about using a single species as a model for all anurans.

Developmental changes in interrenal responsiveness

To our knowledge no previous studies have compared the HPI responses to a general stressor of tadpoles at different developmental stages, and few have compared the response to ACTH. Jaffe (1981) found that premetamorphic *R. catesbeiana* tadpoles responded with elevated plasma corticosterone concentrations to a 0.2 to 2.0 IU/g ACTH injection, although the author did not measure the response to ACTH in prometamorphic tadpoles. However, Krug and colleagues (1983) reported no change in plasma corticosterone concentrations of premetamorphic *R. catesbeiana* tadpoles after injection with 0.1 IU/g ACTH; whereas, prometamorphic tadpoles showed a four-fold increase in plasma corticosterone concentration. Premetamorphic tadpoles of both species in the current study showed corticosterone responses to ACTH doses of 0.040 to 1.36 IU/g. The difference between our results and those of Krug and colleagues (1983) may be due to species-related differences. Alternatively, the differences could be due to the timing of sampling since we waited 6 hr after ACTH injection to measure whole-body corticosterone content, while Krug and colleagues waited only 30 min. We used a range of ACTH doses to obtain a more accurate measure of interrenal responsiveness, as a single dose provides no information on dose sensitivity or maximal response.

We found a difference in the pattern and magnitude of response to stress and ACTH among different developmental stages of both *R. pipiens* and *X. laevis*. These differences suggest that HPI axis responsiveness is not constant over development, and that earlier developmental stages may be more sensitive to stress and ACTH than later stages.

Rana pipiens *stress response*

Premetamorphic *R. pipiens* tadpoles responded to shaking stress with a greater elevation in whole-body corticosterone content than did prometamorphic tadpoles, and the response of premetamorphic tadpoles was sustained for up to 24 hr of shaking. Prometamorphic tadpoles stopped responding after 2 hr of shaking, suggesting either that the HPI axis was maximally stimulated and therefore exhibiting desensitization to further stimulus (Axelrod and Reisine, 1984; Keller-Wood and Dallman, 1984; Munck *et al.*, 1984 or that tadpoles had acclimated to the stimulus such that it no longer elicited an HPI response. The prometamorphic response was short-lived and of moderate magnitude compared to the premetamorphic response, suggesting the latter explanation. Prometamorphic tadpoles were shaken at faster speed than smaller, premetamorphs and appeared similarly agitated. But the larger muscles of the pro-metamorphs may allow for greater locomotor control and easier adjustment to the water's agitation and therefore a weaker HPI response to the stressor. This possibility highlights the difficulty of attributing a difference in response to a given stressor to a difference in the animal's physiological capacity to respond, versus a difference in its perception of the stimulus as stressful.

Rana pipiens *ACTH response*

Injection with ACTH provides a more objective measure of interrenal responsiveness, although it does not measure responsiveness of the entire HPI axis. As with the shaking stressor, premetamorphic tadpoles appeared somewhat more sensitive to ACTH injection than prometamorphic animals. The maximum corticosterone response to ACTH during premetamorphosis was approximately three times that of prometamorphic tadpoles, and the ACTH dose that elicited the first response during premetamorphosis was one-third (allometrically adjusted) that of the response during prometamorphosis.

Xenopus laevis *stress response*

Earlier-stage *X. laevis* tadpoles appeared more sensitive to shaking stress than later-stage tadpoles. While the magnitude of maximum corticosterone response was similar among stages, the patterns of response differed substantially. Early- and middle-stage tadpoles responded much earlier to shaking and confinement than did late-stage tadpoles, and the two earlier stages may have shown HPI desensitization at later time points. Early stage tadpoles showed a strong corticosterone response at 2 hr, and subjective observation at later time points suggested that tadpoles were stressed, as behavioral responsiveness was low. Thus, desensitization of a maximally-stimulated HPI axis seems a likely explanation for the lower corticosterone content measured at later time points.

Xenopus laevis *ACTH response*

The maximum corticosterone response of early stage *X. laevis* tadpoles to ACTH was seven times that of middle- and late stage tadpoles. However, the variability in this maximum response was high, and no consistent trend was seen in the pattern of corticosterone response to ACTH that would suggest an increase or decrease in sensitivity over development. Middle-

and late stage tadpoles may have become desensitized at higher ACTH doses, while early stage tadpoles showed the maximum response at the highest ACTH dose. This pattern could suggest that middle- and late stage *X. laevis* tadpoles are more sensitive to ACTH injection than early stage tadpoles, although their magnitude of maximum response was lower.

Xenopus laevis tadpoles showed a greater corticosterone response to the stress of injection than did *R. pipiens* tadpoles (Figs. 3 and 6; compare saline-injected to basal groups). Early stage *X. laevis* tadpoles were anesthetized prior to injection, and this treatment lowered saline-injected corticosterone content compared to the basal (uninjected, unanesthetized) group. Thus, the response to ACTH in *X. laevis* was superimposed upon a response to injection (or to anesthetic plus injection). Although all analyses compared ACTH-injected groups to saline-injected controls, the possibility remains of an interaction between the stress of injection and the response to ACTH.

CONCLUSIONS

All developmental stages we tested showed a corticosterone response to an external stressor and to ACTH injection, in both *R. pipiens* and *X. laevis*. Our results suggest that earlier developmental stages may exhibit greater responsiveness and higher sensitivity of the HPI axis compared to later stages. Differences in HPI responsiveness may result in different sensitivities of the various stages to environmental factors. Smaller tadpoles are known to be more susceptible to predators and often are poorer resource competitors than their larger counterparts (Woodward, 1987; Wilbur, 1984; Smith, 1983; Brodie and Formanowicz, 1983; Caldwell *et al.*, 1980; but see Persson, 1985). A highly responsive HPI axis might increase the ability of smaller, earlier-stage tadpoles to avoid or cope with such environmental factors. Resource competition elicits elevated corticosterone content in *R. pipiens* tadpoles (Glennemeier and Denver, 2002), but the HPI response of tadpoles to predators has not been studied. Identification of additional environmental factors that activate the HPI axis in larval amphibians, and studies of whether this response changes over development, will help determine the role of the HPI axis in amphibian ecology and the function of stress hormones throughout larval development.

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