Searching for the Physiological Mechanism of Density Dependence: Does Corticosterone Regulate Tadpole Responses to Density?

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ABSTRACT

Density-dependent population regulation is important in many natural populations, and in those populations, high population density is a likely stressor. In amphibians, previous laboratory studies with tadpoles suggest that corticosterone, the main glucocorticoid stress hormone, is one of the key regulators of density-dependent growth. To test this relationship in natural settings, we manipulated wood frog (*Rana sylvatica*) tadpole density at three levels in outdoor mesocosms and used a capture stress protocol to examine the hormonal stress response. In addition, we used the same capture protocol in six natural ponds (three high density and three low density). In the mesocosms, there was an increase in corticosterone levels in tadpoles following 1 h of confinement at weeks 1, 2, and 5. However, while tadpoles maintained at higher densities were smaller after metamorphosis, density did not alter mean levels of corticosterone obtained during confinement, and baseline levels of corticosterone did not differ between the densities. Similarly, in natural ponds, density did not correlate with baseline corticosterone or mean corticosterone levels obtained during confinement. We suggest that the physiological response to density may vary across the range of natural densities and that the role of corticosterone may be limited to periods of extreme high density, such as during pond-drying events.

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Introduction

Density-dependent population regulation is important in many natural populations (Turchin 1999; Brook and Bradshaw 2006). The most direct impact of density dependence is a decrease in survival at high density. However, there can also be impacts of density on various life-history parameters, such as fecundity, that influence fitness and population growth without directly altering individual survival. Implicit in the theory of density dependence is the idea that high population density should be a stressor for organisms. If this is true, there should be resultant changes in the physiology of organisms at high densities that reflect the environmental stressor and regulate the organism's response (e.g., decreased growth) to density.

Vertebrates generally respond to stressful stimuli in their environment with increases in adrenal glucocorticosteroids (cortisol or corticosterone, depending on the species; Romero 2002). When elevated, glucocorticosteroids play an important role in depressing nonessential functions so that organisms can survive environmental perturbations (Sapolsky 1993). At normal baseline levels, these hormones are also important and have a role in many processes, including the regulation of food intake, activity, and reproductive behavior (Sapolsky et al. 2000). Given the important role of glucocorticosteroids in responding to environmental stressors, it seems logical to begin to examine the physiological mechanisms of density dependence by examining changes in the levels of these hormones in response to density manipulation.

As early as 1950, it was suggested that hormonal stress responses to high density could drive reduced reproduction and population cycles in small mammals (Christian 1950). Efforts to actually demonstrate this link between high density and increased glucocorticoids in mammals in the field have produced mixed results (Boonstra et al. 1998; Rogovin et al. 2003; Kuznetsov et al. 2004). Little has been done to examine this relationship in other vertebrates. In adult spotted salamanders (*Ambystoma maculatum*), corticosterone levels appear to be elevated with high densities relative to terrestrial burrow availability but are not elevated with high breeding densities in natural ponds (Cooperman et al. 2004). Unfortunately, aside from those few field-oriented studies, much of our knowledge of the link between density and physiological stress is based on work with captive individuals, where crowding stress may not reflect natural field densities and other factors associated with laboratory crowding (e.g., unsanitary living conditions or in-

escapable social stress) might be confounded with the effect of density.

At the forefront of research examining density-dependent effects in ecology have been studies of larval amphibians (e.g., Wilbur 1976; van Buskirk and Smith 1991; Altwegg 2003; Relyea and Hoverman 2003; Loman 2004). In addition to the outright mortality associated with high density in larvae of some species, density-dependent reductions in growth and development have been documented in numerous species. For example, in a 7-yr observational study of two ponds, Bervin (1990) found that an increase in the number of wood frog (*Rana sylvatica*) eggs deposited in a pond increased the length of the larval period and decreased the mass at metamorphosis of juveniles. Reductions in size at metamorphosis can, in turn, lead to decreased adult fitness for at least some species (Semlitsch et al. 1988; Bervin 1990; Scott 1994). In addition, changes that occur independent of size reduction (e.g., morphology, physiology) during larval development in dense populations may also contribute to decreased juvenile survivorship (Relyea and Hoverman 2003). There are also increases in the variation in tadpole sizes as density increases; in denser populations, there are a wider array of individual sizes (Peacor and Pfister 2006).

For tadpoles, both visual and tactile cues can function as the sensory cues that are used to assess density in the environment (Rot-Nikcevic et al. 2005, 2006). Previous laboratory studies have demonstrated that high population density (i.e., 10 tadpoles/L) can increase corticosterone levels in tadpoles (Hayes 1997; Glennemeier and Denver 2002*b*). Consequently, Glennemeier and Denver (2002*b*) suggested that corticosterone secretion may be the physiological mechanism by which high density reduces growth in larval anurans, and recent work on the sensory cues used by tadpoles has also found support for that hypothesis (Rot-Nikcevic et al. 2005).

Our goal in this study was to examine the impact of population density on corticosterone levels and the hormonal stress response of larval anurans in more natural settings. We used wood frogs (*R. sylvatica*) for these studies. In central Pennsylvania, wood frogs breed over the course of several days in early spring in temporary woodland ponds. The number of egg masses laid in each pond ranges from a few to well over 100, resulting in wide variation in tadpole density across the different ponds during the course of development. Several previous studies of wood frogs have linked increased larval density with decreased size at metamorphosis (Wilbur 1977; Bervin 1990). To address the possible role of corticosterone in regulating larval wood frog responses to density, we manipulated density experimentally within the natural density range in outdoor 1,000-L mesocosms. In addition, to support our experimental work, we examined corticosterone levels in the field in three high-density and three low-density ponds.

Material and Methods

Experimental Density Manipulation

We used 12 1,000-L cattle tank mesocosms to experimentally manipulate tadpole density. Mesocosms were located at the Pennsylvania State University agricultural field station, approximately 15 km from the Pennsylvania State University campus in Centre County, Pennsylvania. After the cattle tanks were filled with water from a nearby spring and before tadpoles were added, mesocosms were stocked with 300 g of mixed oak leaves (a typical substrate in the woodland ponds that wood frogs use in central Pennsylvania), 2 L of zooplankton and phytoplankton collected from local ponds, and 25 g of alfalfa pellets, which provide an additional nutrient source for developing tadpoles. Parts of 15 wood frog egg masses were collected from six natural oviposition sites within 30 km of Pennsylvania State University and were allowed to hatch in 5-gal buckets floated in the mesocosms or in small wading pools at the same site. Hatchling wood frog tadpoles were randomly assigned from a mix of individuals from all 15 egg masses to mesocosms at low (80 tadpoles), medium (160 tadpoles), and high (320 tadpoles) densities, based on densities that have been previously observed in the field. There were four tanks assigned to each of the three treatments in a randomized block design.

Confinement/capture stress protocols (as in Glennemeier and Denver 2002*a*; Belden et al. 2003), which incorporate analysis of both baseline and stress-induced glucocorticoid levels, are often used to examine the functioning of the hormonal stress response in free-living vertebrates (Wingfield et al. 1997). In this study, confinement stress series were completed at weeks 1, 2, and 5 of development. For confinement stress series, a single tadpole was sampled from each of the 12 mesocosms at each of the three confinement time points (0, 30, and 60 min of confinement). This resulted in $N = 4$ tadpoles for each time point for each density treatment. Confinement was done in plastic specimen cups (total volume = 120 mL) fitted with lids and filled with 50 mL of water from the respective tank. A single tadpole was placed in each cup, which was floated in the tank from which the tadpole was collected and agitated every 2–3 min for the duration of confinement, either 30 or 60 min. Following confinement, each tadpole was transferred to a collection vial and was rapidly frozen by dipping the vial into a dry ice/ethanol slurry (as in Belden et al. 2003). For baseline samples (0 min), tadpoles were immediately transferred to a vial and frozen after being collected from the tank. Samples were maintained on dry ice until return to the laboratory, where they were transferred to a −70°C freezer until the radioimmunoassay (RIA) was completed.

Small (10 cm × 10 cm) Styrofoam floats were added to the tanks as the individuals neared metamorphosis to facilitate collection of the juveniles. From that point on, tanks were checked daily, and on collection, individuals were returned to the laboratory and weighed. We recorded the mass of metamorphosed

juveniles to ensure that our experimental design resulted in density-dependent growth, with smaller individuals metamorphosing from more dense populations (e.g., Wilbur 1976). This research was conducted under IACUC 15831, approved by the Institutional Animal Care and Use Committee at Pennsylvania State University.

We used ANOVA to examine the effects of density (three levels: low, medium, high), week (three levels: 1, 2, 5 wk), and confinement time (three levels: baseline, 30 min, 60 min) on corticosterone levels of tadpoles. Because individual tadpoles were sampled at only one time point, we did not use a repeated-measures analysis. We also used a MANOVA followed by univariate ANOVAs to establish that our density treatments resulted in density-dependent variation in mass at metamorphosis and time to metamorphosis (=days from start of experiment).

Observational Field Pattern

To determine whether there was a correlation between density and corticosterone levels in natural ponds, we completed confinement stress series on wood frog tadpoles in six natural ponds. Three ponds were categorized as high density (>100 egg masses), and three were categorized as low density (<20 egg masses). Approximately 1 mo after egg deposition, confinement stress profiles in the ponds were completed as described above for the mesocosms. Individual tadpoles were placed in specimen cups filled with 50 mL of pond water for either 30 or 60 min or frozen immediately following capture (0 min = baseline) in the dry ice/ethanol slurry. We sampled seven or eight individuals at each time point in each pond. As in the mesocosm experiment, the cups were floated in the pond and agitated every 2–3 min. Frozen samples were returned to the laboratory and stored at −70°C before RIA.

Following the confinement stress series, tadpole density estimates were completed in each pond to ensure that the egg counts were representative of tadpole densities. We estimated tadpole density using a rectangular kick net (approximately 22 cm × 45 cm). Ten 1-m sweeps were done randomly throughout each pond, and the number of tadpoles per cubic meter of water was calculated to standardize for pond size (surface area of the ponds was estimated as an ellipse, and it ranged from approximately 235 to 629 m^2). For the three high-density ponds, the tadpole density estimates (with pond name in parentheses) were 320 (J21), 219 (BJ11), and 160 (DIV) tadpoles/m^3, and for the three low-density ponds, the estimates were six (FAR), three (J29), and one (J22) tadpole(s)/m^3. We planned to collect both mass and developmental stage information when the tadpoles were processed for hormone analysis after being frozen. However, it proved very difficult to accurately assess developmental stage on the frozen individuals; the digits on the developing hindlimbs that are the key to assessment were frozen together, making it difficult to judge the level of development. We did collect the mass data, though, and because mass and developmental stage are generally strongly correlated in developing amphibians, we are confident in using mass as a proxy for both variables. Masses ± SD (with pond name in parentheses) at the time of collection for the three high-density ponds were 0.19 ± 0.06 (J21), 0.18 ± 0.06 (DIV), and 0.35 ± 0.09 g (BJ11). For the three low-density ponds, masses ± SD were 0.30 ± 0.06 (FAR), 0.13 ± 0.04 (J29), and 0.14 ± 0.03 g (J22).

We used ANCOVA to examine the effects of pond density (two levels: low, high) and confinement time (three levels: baseline, 30 min, 60 min) on corticosterone levels. Preliminary correlation matrixes indicated the potential for both the time of day the sample was collected and especially the tadpole mass to influence corticosterone levels, and linear regression was used to confirm these relationships. Therefore, we used those two variables as covariates in the overall model. The mean values for each pond were used in the analysis. We completed a second ANCOVA with the same data (adjusted $\alpha = 0.025$ for second analysis) to examine variation in baseline levels of corticosterone between the six ponds after accounting for mass as a covariate. We also did a simple test for density-dependent growth for the field samples by regressing mean tadpole mass against the log of our estimates of tadpole density per cubic meter for each pond.

Radioimmunoassay

Whole-body corticosterone of each tadpole was measured by RIA following extraction and column chromatography to purify the samples. RIA procedures followed those reported by Belden et al. (2003), with minor modifications. The samples for these studies were run in four assays. To prepare the samples, each tadpole was weighed and homogenized with a mass-adjusted amount of distilled water (mass × 10 mL − 0.5 mL rinse; minimum = 1.5 mL, maximum = 4 mL). For individual recovery determination, each sample was equilibrated overnight with 2,000 cpm of tritiated corticosterone for individual recovery determination. Each sample was then extracted in 5 mL of dichloromethane. To break the emulsion, each sample was centrifuged at 2,000 rpm on a clinical centrifuge, and then the organic phase was removed and dried in a warm water bath under a stream of nitrogen gas. The extracts were resuspended in 10% ethyl acetate in isooctane. The samples were chromatographed through individual Celite columns to separate the steroid fractions and neutral lipids. The fractions were eluted using stepwise increasing proportions of ethyl acetate in isooctane. The purified eluates were dried and resuspended in 500 μL of buffer (phosphate-buffered saline with 0.1% gelatin) for the assay. For the assay, individual sample recoveries were determined from 75 μL of the sample, while 200 μL of the sample was allocated in duplicate for the assay. Serial dilutions for the standard curves were performed in duplicate. All samples, in-

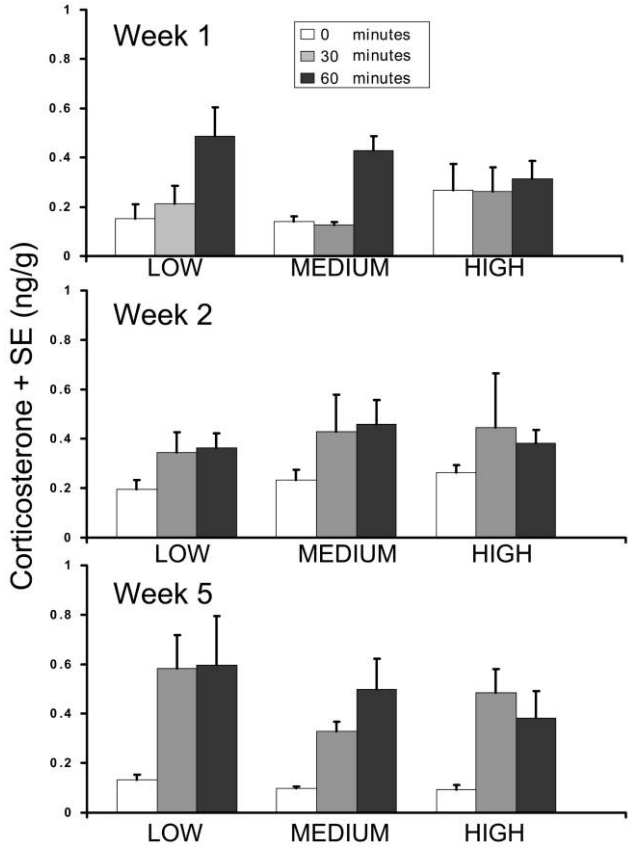

Figure 1. Whole-body corticosterone levels in response to confinement (0, 30, or 60 min) during weeks 1, 2, and 5 of the mesocosm experiment. Values are mean + SE.

cluding serial dilutions and total bound, were incubated overnight with 100 μL of antibody and 100 μL of tritiated steroid. Unbound steroid was separated using dextran-coated charcoal and the bound steroid decanted into scintillation vials. Interassay variation was 15.2%, and intraassay variation averaged 4.1% (range = 2.2%–8.2%). Average extraction efficiency, based on recoveries, was 67.4%. All corticosterone values were log transformed before analysis.

Results

We saw the typical response of smaller juveniles that took longer to reach metamorphosis produced from high-density mesocosms (overall MANOVA, $F_{4,16} = 12.50$, $P < 0.005$; ANOVA for mass, $F_{2,9} = 14.14$, $P = 0.002$; mean mass ± SD [g], low = 0.59 ± 0.15, medium = 0.33 ± 0.05, high = 0.23 ± 0.04; ANOVA for days to metamorphosis, $F_{2,9} = 5.28$, $P = 0.03$; mean days to metamorphosis ± SD, low = 70.0 ± 22.6, medium = 80.8 ± 3.5, high = 111.0 ± 5.2). However, density did not affect corticosterone levels in the mesocosm study ($P = 0.82$). There were significant effects of confinement time ($F_{2,81} = 31.24$, $P < 0.001$), with the typical vertebrate response of increasing corticosterone levels with confinement (Fig. 1). There was also a significant effect of sampling week ($F_{2,81} = 4.85$, $P = 0.01$) and an interaction between sampling week and confinement time ($F_{4,81} = 6.31$, $P < 0.001$), indicating that the effect of confinement varied with sampling week (e.g., elevation of corticosterone took longer in week 1 than in week 5).

In the natural ponds, corticosterone levels increased with confinement time, as expected ($F_{2,10} = 18.11$, $P < 0.001$; Fig. 2). However, as in the mesocosm experiment, pond density was not associated with corticosterone levels ($F_{1,10} = 0.15$, $P = 0.70$), and there was no significant interaction between the two factors ($F_{2,10} = 0.19$, $P = 0.83$). For the covariates, mass of the tadpoles was highly significant ($F_{1,10} = 31.15$, $P < 0.001$), but the time of day the samples were collected was not significant in the overall model ($F_{1,10} = 0.04$, $P = 0.85$). Interestingly, while baseline corticosterone was not associated with density, there was significant variation in baseline corticosterone levels between the six ponds after accounting for mass and time frozen (ANCOVA, adjusted $\alpha = 0.025$, $F_{5,42} = 5.88$, $P < 0.001$). Based on a regression of mass versus tadpole density, there was actually no evidence in the field of density-dependent growth in our six ponds ($R^2 = 0.157$, $P = 0.436$). However, across all natural ponds, there was a significant relationship between corticosterone and tadpole mass for baseline, 30-min, and 60-min samples (Fig. 3; baseline samples, $R^2 = 0.276$, $P < 0.005$; 30-min samples, $R^2 = 0.182$, $P = 0.003$; 60-min samples, $R^2 = 0.387$, $P < 0.005$); larger tadpoles tended to have lower corticosterone levels. The time of day that sampling occurred did not significantly influence corticosterone levels (Fig. 3).

Discussion

Based on our results from both the experimental mesocosms and the natural ponds, it seems unlikely that density-dependent

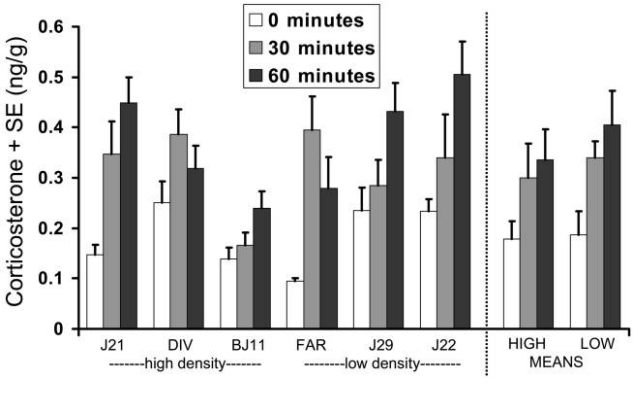

Figure 2. Whole-body corticosterone levels in response to confinement (0, 30, or 60 min) in six natural ponds (three high density and three low density). Means for high- and low-density sites are presented at the right and were used in the analysis. Values are mean + SE.

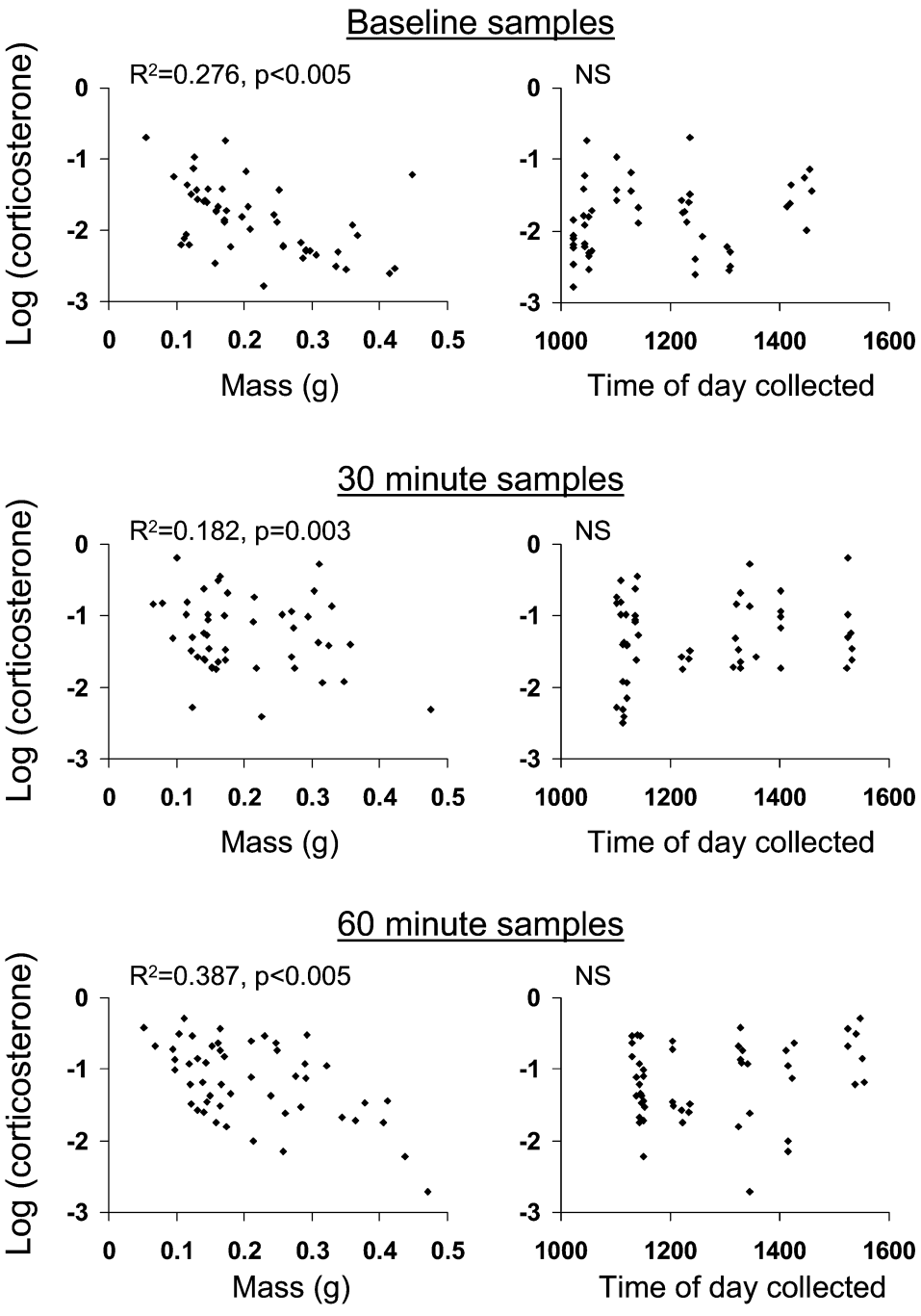

Figure 3. Regressions of whole-body corticosterone levels (log transformed) versus tadpole mass and versus time of day the sample was collected for baseline, 30-min, and 60-min samples from the six natural ponds that were sampled. Coefficients of determination (R^2) and P values are reported for significant relationships. *NS* = not significant.

effects on growth at these scales are physiologically mediated by corticosterone, at least within the natural range of densities observed in this system. This is a different conclusion from that of Glennemeier and Denver (2002*b*), reached in a laboratory study on leopard frog (*Rana pipiens*) tadpoles. They found that after 4 d at high densities (40 tadpoles/4 L, which would be the equivalent of approximately 10,000 tadpoles in one of our outdoor mesocosms), whole-body corticosterone

levels were elevated, regardless of food level. In addition, they were able to reverse growth inhibition by treating tadpoles with metyrapone, an inhibitor of corticoid synthesis. Recent results suggest that *Rana sylvatica* tadpoles respond in a similar fashion, with elevated corticosterone at simulated high densities (40 tadpoles/10 L) in the laboratory (Rot-Nikcevic et al. 2005). These studies provide strong support for a role of corticosterone in regulating responses to density. However, the higher densities used in these studies may not be reflective of typical conditions experienced by developing amphibians in nature.

We hypothesize that as long as growth is positive, a corticosterone response may not be triggered in larval amphibians developing in nature. Positive growth of tadpoles, regardless of whether it is significant (as in our low-density tanks) or minimal (as in our high-density tanks), implies that energy supplies are not completely limiting in the environment. Increases in corticosterone secretion may not be triggered until the energy balance is such that growth is actually inhibited. This idea has been formulated previously in the concept of allostatic load (McEwen and Wingfield 2003). Allostatic load is defined as the cumulative costs for an organism to maintain system stability via continuous physiological change and adaptation to external conditions. In this case, we are referring to the costs of tadpoles to maintain positive growth, which is the result of a balance between resources available in the environment and an individual's ability to obtain those resources. If a point is reached whereby positive growth is not maintained, then this triggers an emergency life-history stage, defined as Type 1 allostatic overload (McEwen and Wingfield 2003), and corticosterone levels increase.

Instances where we might expect to see this threshold crossed for larval amphibians in nature include scenarios such as pond drying in temporary pond communities. When ponds dry prematurely, densities of tadpoles can become extremely high, and presumably, food levels drop, and competition increases. Under such conditions, positive growth might halt, and corticosterone levels might become elevated as individuals attempt to mobilize energy stores and regain a positive energy balance. In theory, if individuals were far enough along in development, a hormonal stress response could trigger metamorphosis and a potential escape from the drying pond (Denver 1997, 1998). In our mesocosm study, it could be that we never observed tadpoles in allostatic overload, whereas the much higher densities used in the laboratory by Glennemeier and Denver (2002*b*) may have resulted in allostatic overload and therefore an increase in corticosterone levels as tadpoles used energy reserves.

In our mesocosm experiment, we saw variation in corticosterone levels between weeks and a time-by-week interaction. We expect that these differences were due either to developmental changes or to variation in temperature during sampling (it takes longer for ectotherms to initiate a stress response at colder temperatures), but in this study, we cannot distinguish between these factors. We also saw significant variation among ponds in the field, with baseline levels in some ponds equivalent to elevated levels in other ponds (Fig. 3). These differences were not associated with density, but at this point we have no support for any alternative factors (e.g., predator density, food availability, water chemistry) that could drive these differences.

We also saw an interesting correlation between tadpole mass and corticosterone levels, with larger tadpoles tending to have lower corticosterone levels. Because mass increases with developmental stage, this correlation could be due to changes in baseline corticosterone levels that occur naturally over the course of development. However, in larval bullfrogs (*Rana catesbeiana*), baseline corticosterone levels remain relatively constant until approximately Gosner stage 38–40 (Gosner 1960), immediately preceding the emergence of the forelimbs, at which point corticosterone levels increase dramatically toward metamorphic climax (Jaffe 1981; Krug et al. 1983). Because we saw a decrease in corticosterone levels with increasing mass, one possibility is that our differences were due to effects of body condition. Larger tadpoles likely have more fat reserves, and this may result in the lower levels of corticosterone that we documented. The link between body condition and corticosterone has been seen previously in other vertebrates (e.g., Kitaysky et al. 1999; Moore et al. 2000). Another possibility is that higher corticosterone levels in smaller individuals might reflect their slower growth trajectories.

The possible relationship between food availability and density is an important one and has been examined to some degree. Wilbur (1977) found an interaction between food and density in regulating growth rate and metamorphic size of *R. sylvatica* tadpoles but also suggested that there might be effects of density that operated independent of food supply, which has recently been confirmed (Relyea 2002). In *R. pipiens*, both high density and low food levels can independently result in elevated corticosterone levels (Glennemeier and Denver 2002*b*). We suggest that density-dependent responses of growth in larval amphibians may be mostly a result of differential food availability, with simple differential caloric intake as a physiological basis for variation in mass. In most cases, as long as growth is positive, corticosterone levels remain basal. However, in extreme cases of high density, as might occur during pond drying, we hypothesize that a hormonal stress response could be triggered and corticosterone could become elevated. This suggests that the physiological mechanisms responsible for observed density-dependent effects might vary across a range of potential densities and environmental conditions.

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