Pond Acidification May Explain Differences in Corticosterone among Salamander Populations

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ABSTRACT

Physiological tolerances play a key role in determining species distributions and abundance across a landscape, and understanding these tolerances can therefore be useful in predicting future changes in species distributions that might occur. Vertebrates possess several highly conserved physiological mechanisms for coping with environmental stressors, including the hormonal stress response that involves an endocrine cascade resulting in the increased production of glucocorticoids. We examined the function of this endocrine axis by assessing both baseline and acute stress–induced concentrations of corticosterone in larvae from eight natural breeding populations of Jefferson's salamander *Ambystoma jeffersonianum*. We surveyed individuals from each pond and also examined a variety of environmental pond parameters. We found that baseline and stress-induced corticosterone concentrations differed significantly among ponds. Population-level baseline corticosterone concentrations were negatively related to pH and positively related to nitrate, and stress-induced concentrations were again negatively related to pH, positively related to nitrate, and positively related to temperature. We followed the field survey with an outdoor mesocosm experiment in which we manipulated pH and again examined baseline and acute stress–induced corticosterone in *A. jeffersonianum* larvae. As in the field survey, we observed an increase in the baseline corticosterone concentration of individuals exposed to the lowest pH treatment (pH 5–5.8). Examining physiological indices using a combined approach of field surveys and experiments can be a powerful tool for trying to unravel the complexities of environmental impacts on species distributions.

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Introduction

Understanding how species respond physiologically to various abiotic factors can provide insight into current and future species distributions and abundance, especially as anthropogenic disturbances continue to alter natural systems and abiotic conditions. In addition, understanding the sublethal impacts of these environmental changes on physiological systems might help us predict the likely ecological outcome of future environmental changes (Gaston et al. 2009; Buckley et al. 2010).

In vertebrates, one key physiological pathway that might be impacted by anthropogenic changes to habitats is the highly conserved hormonal stress response (Romero 2004). During this endocrine response, glucocorticoid hormones are released from the adrenal cortex with activation of the hypothalamic-pituitary-adrenal (HPA, or HPI [interrenal] in amphibians) axis following perceived stress (McEwen and Wingfield 2003). Physiological effects of elevating glucocorticoids include energy mobilization and potential suppression of growth and reproduction (Moore and Jessop 2003). Assessing the functioning of this endocrine axis often involves the collection of both baseline glucocorticoid concentrations and stress-induced concentrations (i.e., concentrations following a standardized confinement stress or adrenocorticotropic hormone [ACTH] injection). Variation in hormone elevation following "stress" is therefore often used as a metric for axis function that incorporates both baseline and stress-induced concentrations.

Recently, interest has grown in understanding variation in baseline glucocorticoid concentrations in addition to assessing the function of the HPA axis. Glucocorticoids are essential in general energy economics, metabolism, behavior, reproduction, and survival (Moore and Jessop 2003), but the consequences of variation in baseline concentrations among individuals (or within an individual over time) are not fully understood (Bonier et al. 2009). Variation in baseline corticosterone concentrations within individuals can also be mediated by diel, seasonal, or developmental changes (Romero 2002; Wright et al. 2003). For example, baseline corticosterone concentrations in larval amphibians are typically an order of magnitude lower than concentrations in juvenile frogs following metamorphosis (Belden et al. 2010). Baseline corticosterone concentrations may also vary significantly among populations. This has been reported in birds (Lindstrom et al. 2005), reptiles (Wilson and

Wingfield 1994), and amphibians (Homan et al. 2003; Belden et al. 2007), although population-level variation in baseline glucocorticoid concentrations is not always found (Pravosudov et al. 2004).

Although variation among populations in baseline glucocorticoid levels may not always exist, environmental conditions can be associated with variation when populations do differ in baseline glucocorticoid concentrations. For instance, Hopkins et al. (1997) showed that baseline corticosterone concentrations were significantly higher in a southern toad (*Bufo terrestris*) population exposed to coal combustion waste than in a population from a nonpolluted site. Experiments completed in the context of understanding field-based patterns may help us determine whether the environmental conditions are a mechanism leading to individual phenotypic changes (e.g., an individual has higher corticosterone in environment A and lower corticosterone in environment B) or whether environmental conditions are acting as a selective force (e.g., individuals with higher corticosterone do "better" in environment A, and therefore over time there are more individuals present in environment A with high corticosterone concentrations, leading to population-level variation). While these possibilities are not mutually exclusive, they do represent important differences in the potential timescale and mechanism by which anthropogenic changes might alter species' physiology, evolution, and ranges.

In this study we had three goals: (1) to examine population-level variation in baseline and stress-induced glucocorticoid concentrations in free-living Jefferson's salamanders *Ambystoma jeffersonianum*, a salamander species deemed one of the most sensitive to environmental alterations (Petranka 1998); (2) to examine a suite of environmental parameters that, on the basis of prior studies, seemed likely to have an impact on glucocorticoid concentrations; and (3) to experimentally examine the link between one of these factors (pH) that was correlated with glucocorticoid concentrations in the field survey and the function of the HPI axis.

Material and Methods

Field Sample Collection

We sampled a total of eight ponds in Maryland ($N = 2$), Pennsylvania ($N = 5$), and West Virginia ($N = 1$). To minimize diel, developmental, and seasonal effects on corticosterone (Romero 2002; Wright et al. 2003), all sampling occurred within 3 wk during the same time of day (between 1100 and 1500 hours). *Ambystoma jeffersonianum* larvae were collected using dip nets. Both baseline ($N = 5$ larvae/pond) and confinement-induced ($N = 5$ larvae/pond) corticosterone concentrations were examined. Baseline corticosterone concentrations were sampled by freezing larvae individually in a dry ice/ethanol slurry within 3 min of initial capture to reflect unstressed concentrations (Belden et al. 2003). To induce the stress response, we held individual larvae in 120-mL cups filled with 80 mL of pond water for 30 min, with cups being gently agitated every 3 min (Belden et al. 2007). After 30 min, larvae were frozen using a dry ice/ethanol slurry. Larvae were then returned to the laboratory and transferred to a −80°C freezer until radioimmunoassays could be performed. An additional $N = 10$ larvae from each pond were collected to determine body mass and developmental stage (Watson and Russell 2000) distributions at each site (table 1) and were subsequently released. The Watson and Russell (2000) staging scheme for larval *Ambystoma* development defines developmental stages as follows: forelimb development during stages 1–9, hind limb growth during stages 10–18, and finally gill reduction leading to metamorphosis during stages 19–22. No significant differences were detected among all ponds for mean larval mass ($F_{1,7} = 0.94$, $P = 0.37$) or developmental stage ($F_{1,7} = 0.56$, $P = 0.48$) using ANOVAs. Research was completed under Virginia Tech Institutional Animal Care and Use Committee protocol 06-032-BIOL.

Environmental Variables

We measured pH, conductivity, water temperature, chloride (Cl), phosphate (PO_4), and nitrate (NO_3) because of their demonstrated importance to amphibians and because they represent some of the major anthropogenic impacts on freshwater ecosystems, namely, acidification, nutrient input, and salt stress. For instance, it is estimated that approximately 117 million tons of ammonia is produced globally each year, most of it in the form of fertilizer for agriculture (Erisman et al. 2007). This has resulted in dramatic changes over time in the global nitrogen cycle, including increased nitrogen inputs to many aquatic systems and subsequent impacts on amphibians ranging from sublethal impacts on growth, development, and behavior to direct mortality (Rouse et al. 1999; Mann et al. 2009). Acid deposition (e.g., from acid rain) and increased salinity from road deicers have also been identified as possible threats for amphibians (Rowe et al. 1992; Collins and Russell 2009).

Water temperature (°C), pH, and conductivity (mg/L) were measured in the field at each pond using YSI 60 and YSI 550A probes (YSI, Yellow Springs, OH). At each pond, a 90-mL water sample was collected, frozen, and taken back to the laboratory for chemical analysis using ion chromatography methods similar to those proposed by the U.S. Environmental Protection Agency (USEPA 1993, 1997). Before analysis, all water samples were filtered using a Gelman 0.45-μm Acrodisc filter. Approximately 4 mL of each sample was screened for Cl, PO_4, and NO_3 (all measured in ppb) using a Dionex DX500 ion chromatograph with an EG50 eluent generator, an AD20 absorbance detector, an ED40 conductivity detector, an Ionpac AS19 anion analytical column (4 mm), an AG19 anion guard column, and an ASRS anion self-regenerating suppressor, all with a 500-μL sample loop. Peak Net software (rev. 8, ver. 5.1) was used to analyze chromatography data output. Of the eight ponds, seven had PO_4 lower than the limit of detection; thus, this variable was not likely to be helpful in describing patterns of corticosterone across ponds and was dropped from further consideration.

Mesocosm pH Experiment

To further examine the relationship between pH and corticosterone, we followed the field survey by experimentally manipulating pH in mesocosms and examining changes in baseline and confinement-induced corticosterone concentrations in *A. jeffersonianum*. We collected recently deposited *A. jeffersonianum* egg masses ($N = 26$) from a single permanent pond in Montgomery County, Virginia. Once collected, we transported the egg masses to our outdoor research facility and placed them in a single 1,000-L mesocosm stock tank filled with well water, approximately 250 g of deciduous leaf litter, and zooplankton (screened from 2 L of water from a local pond). After egg masses were placed in the stock tank, we covered the tank with a shade cloth lid to prevent colonization by other species.

For the experiment, we set up nine 1,000-L mesocosms like the stock tank described above with well water, leaves, and zooplankton. We established three pH treatments (pH = 5.0–5.8, 6.0–6.8, and 7.9–8.2 [control]), with three replicates per level, in a randomized block design. Desired pH ranges were reached by adding dilute sulfuric acid (Rowe et al. 1992). It took 5 wk to achieve stable pH levels, and these levels then stayed within the initially established ranges for the duration of the study. After the desired pH ranges were established, 20 larvae were added to each mesocosm. An additional 10 *A. jeffersonianum* larvae were brought back to the laboratory to determine mean (± 1 SD) mass (0.26 ± 0.04 g) and developmental stage (13.5 ± 1.27; mid–hind limb development) at the beginning of the experiment. Both baseline and confinement-induced corticosterone concentrations were determined after 4 wk of pH exposure by taking three larvae per mesocosm for each confinement treatment (baseline and 30 min). We used the same methodology described above for confinement techniques and sample storage. All remaining larvae were counted immediately following the collections for hormone analysis, and there was no difference in survival across pH treatments (generalized linear model with binomial error distribution, pH effect, $P = 0.75$). There was also no treatment-based difference in mean mass for the individuals collected for hormone analysis at the end of the experiment (generalized linear model with normal error distribution, pH effect, $P = 0.20$).

Whole-Body Corticosterone Radioimmunoassay

Before radioimmunoassays, all field-collected samples thawed overnight and were subsequently refrozen following failure of the −80°C freezer. As all of the samples are compared only among themselves, any variation from this freeze-thaw-freeze cycle should affect all samples similarly. In addition, observed concentrations are within the range we have observed previously for this species (Belden et al. 2010). Corticosterone was measured using the whole-body radioimmunoassay procedures of Belden et al. (2003) with minor modifications. Briefly, larvae were homogenized in distilled water after weighing (mass of individual × 10 mL + 0.5-mL rinse, with a minimum of 1.5 mL and maximum of 5 mL), and each sample was equilibrated overnight with 2,000 cpm of tritiated corticosterone so that individual recoveries could be determined. Steroids were extracted from each sample in 5 mL of dichloromethane. To break the emulsion that formed, samples were centrifuged at 3,000 rpm for 10 min before removing the organic phase, which was then dried under nitrogen in a warm water bath. Steroids and neutral lipids were separated by chromatography on celite columns, the corticosterone fraction was collected, and the assay was completed as previously described (Belden et al. 2003). Three standard curves were prepared for each assay. In total, we conducted three assays. Our field survey samples comprised two assays. Specifically, one assay consisted of all Maryland, all West Virginia, and two Pennsylvania pond samples ($N = 47$ samples). The remaining three Pennsylvania pond samples ($N = 30$ total samples) comprised another assay. Twenty-nine of the 77 field samples were below detection limits, meaning that there was too little hormone in the sample to be accurately predicted from the standard curve for the assay. Thus, we used a censored regression technique, described below, to analyze these samples. Our mesocosm samples ($N = 54$) were analyzed in a single assay, and corticosterone concentrations for all sam-

Table 1: Mean predicted corticosterone concentrations from the censored regression model with 95% confidence intervals (CIs), mean mass, and developmental stages (Watson and Russell 2000) in each of the eight ponds in the field survey

Pond	Mean corticosterone (ng/g), 95% CI		Mean mass (g) ± 1 SD	Mean developmental stage ± 1 SD
	Baseline	Confinement		
MD1	.269, .141–.512	.474, .234–.960	.25 ± .05	14.8 ± 1.32
MD2	.650, .344–1.227	.846, .418–1.710	.28 ± .05	16.3 ± .67
WV1	.324, .172–.611	.515, .250–1.061	.25 ± .04	15.6 ± 1.26
PA1	.134, .065–.276	.315, .157–.629	.26 ± .08	15.2 ± 1.03
PA2	.601, .325–1.110	1.415, .765–2.617	.28 ± .05	14.7 ± 1.34
PA3	.121, .051–.284	.464, .247–.872	.27 ± .02	15.7 ± 1.57
PA4	BD	BD	.28 ± .04	16.8 ± 1.03
PA5	.324, .171–.613	.880, .476–1.628	.28 ± .03	16.4 ± 1.07

Note. The first two letters in pond identification refer to U.S. states; more information on these sites is provided in table 2. All hormone samples for pond PA4 were below the detection limit for the assay (BD).

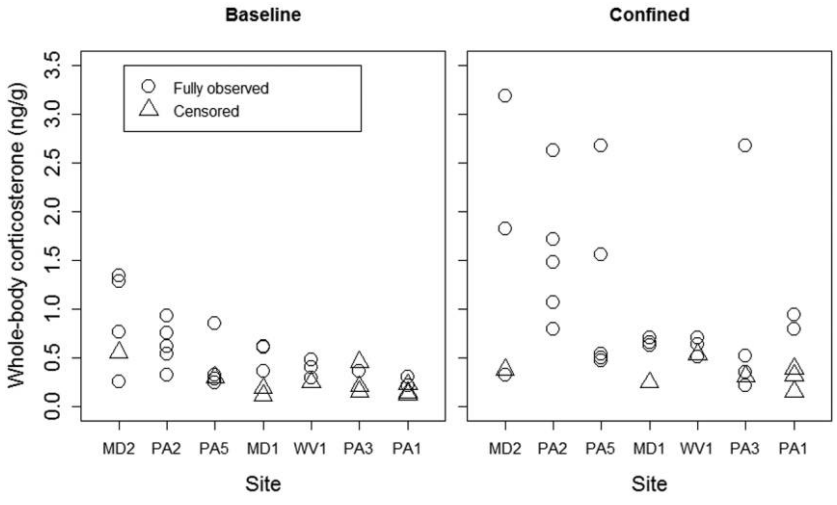

Figure 1. Whole-body corticosterone concentration of *Ambystoma jeffersonianum* larvae in eight ponds from three states in baseline and confinement treatments. Circles represent completely known observations, and triangles represent censored observations (those below the detection limit of the assay).

ples were detectable. Interassay variation for the two field survey assays that were combined for analysis was 10%, and intra-assay variation for the three assays averaged 12%. On the basis of individual recoveries, whole-body extraction efficiency averaged 49%.

Statistical Analyses

As described above, some of the field survey samples had hormone concentrations that fell below the level of detection for the assay. These "nondetect" data, with chemical concentrations below an assay detection limit, represent a common example of censored data (Kent and Funk 2004; Helsel 2005*a*). Sample values below the detection limit have historically been arbitrarily set to the detection limit, one-half of the detection limit, or 0 (Helsel 2005*a*, 2005*b*). However, each of these options biases the data set, reduces variation among observations, and can lead to overestimates of precision and more frequent Type I errors. We recently applied censored regression, a more appropriate method that deals explicitly with nondetect observations (Helsel 2005*a*, 2005*b*), in an endocrine study addressing corticosterone concentration changes throughout larval development in the amphibians *Rana sylvatica* and *A. jeffersonianum* (Chambers et al. 2011), and we follow a similar approach here.

The SURVIVAL package in R (ver. 2.14.1; R Development Core Team 2011) provides a function called SURVREG that offers analyses parallel to standard ANCOVA. On the basis of preliminary analysis and consideration of other published data sets (Belden et al. 2010; Chambers et al. 2011), we assumed our whole-body corticosterone data followed a lognormal distribution. Individual samples with undetectable corticosterone concentrations entered the model as left-censored data with the upper bound set as the minimum concentration of corticosterone for an animal of its size that would exceed the detection limit of the assay. We accounted for mass in this way because in whole-body hormone assays it is the total amount of hormone in the sample that matters to reach the limit of detection for an assay. This means that a smaller larva must have a higher hormone concentration (ng/g) to reach the assay's detection limit than a larger larva. The corticosterone values from one pond (PA4) were all censored. This made it impossible to obtain a reasonably precise estimate of the mean corticosterone value for that pond. To prevent the contamination of our multiple regression models in the next step by such imprecision, we decided to eliminate this pond from our analysis.

We first conducted a censored regression of corticosterone concentrations with pond site and stress treatments (and their interaction) as factors. Likelihood ratio tests (LRTs) were used to assess the significance of each term in the model. This model also gave us pond-level predicted means for both baseline and stress-induced conditions that took into account observations below the detection limit. These predicted means were subsequently used as response variables in multiple regressions (one for baseline, one for stressed animals) with the environmental parameters as explanatory variables. With only five individuals for each stress treatment sampled from each population, we had limited statistical power to detect interpond differences.

Among the environmental parameters of interest, conductivity and pH were strongly collinear (Pearson correlation coefficient $= 0.956$, $P < 0.001$). We chose to use pH in subsequent models because of a priori links suggested in the literature between pH and physiological stress in amphibians. However, caution is warranted because of the strong collinearity; relationships between the response and pH could also represent relationships with conductivity. Water temperature (°C) and pH entered the model as continuous predictors. Chloride (ppb) also entered as a continuous variable but after a

Table 2: Site information and environmental parameters measured in each of the eight ponds in the field survey

Pond	Site, county, state	pH	Cl (ppm)	PO_4 (ppm)	NO_3 (ppb)	Conductivity (mg/L)	Water temperature (°C)
MD1	Monocacy Natural Resource Area, Frederick County, MD	6.15	4.59	<.10	70	5.77	21.1
MD2	Monocacy Natural Resource Area, Frederick County, MD	5.79	5.11	<.10	81	5.31	20.8
WV1	Beech Fork State Park, Wayne County, WV	6.47	1.85	<.10	180	5.63	20.7
PA1	State Game Lands 176, Centre County, PA	6.35	.96	<.10	14	5.95	21.2
PA2	State Game Lands 176, Centre County, PA	5.22	1.52	<.10	<5	3.72	25.1
PA3	Michaux State Forest, Franklin County, PA	6.4	.86	<.10	<5	6.23	25.8
PA4	Michaux State Forest, Franklin County, PA	7.11	1.38	<.10	<5	6.98	25.4
PA5	Michaux State Forest, Franklin County, PA	6.87	1.81	.27	49	7.11	26.1

natural logarithm transformation. Because of limits of detection, NO_3 (ppb) only had a few different values and was thus categorized before entering the model as either low (<20 ppb) or high (≥20 ppb). These concentrations fall in the range of what has been seen in other surveys of temporary ponds suitable for *Ambystoma* breeding (Batzer et al. 2004; Carrino-Kyker and Swanson 2007) but would be considered low within the range of concentrations found in freshwater sites more generally (Rouse et al. 1999). The pH levels we observed were also in the range of previously published data from sites where *Ambystoma* breeding can occur (Freda and Dunson 1986; Batzer et al. 2004; Carrino-Kyker and Swanson 2007).

We first fitted a saturated model with main effects for each water chemistry variable (pH, NO_3, log Cl, and temperature) and used a backward model selection procedure to find the most parsimonious model (command "step" in R; Crawley 2007). Terms were dropped from the model and improvement was judged based on *F*-tests comparing reduced models against complete models; if there was negligible loss of explanatory power, the simplification was deemed appropriate.

The mesocosm experiment was analyzed as a split-plot design, with pH treatments applied to the whole plot (individual mesocosms) and the confinement/baseline treatment to the split plot (applied separately to individuals within each mesocosm). Since all measured corticosterone concentrations in the experiment were above the detection limit, no censoring was required.

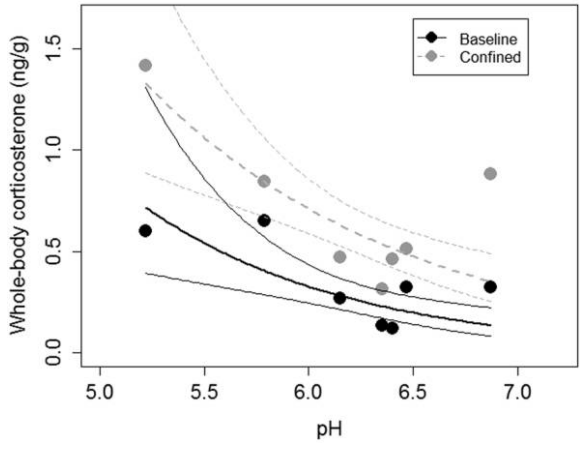

Figure 2. Average whole-body corticosterone concentration of *Ambystoma jeffersonianum* larvae across ponds of varying pH. Baseline and confinement-induced corticosterone concentrations are indicated by black and gray circles, respectively. Thick lines indicate model predictions, and thin lines indicate 95% confidence intervals.

Results

In the field survey, the interaction between stress treatment (baseline or stress induced) and pond site was not significant (LRT = 3.1, df = 6, $P = 0.792$), which means that the response to confinement was consistent across sites. Baseline corticosterone concentrations ranged from nondetectable to 1.34 ng/g and varied significantly among sites (LRT = 20.6, df = 6, $P = 0.0021$; fig. 1). Stress-induced corticosterone concentrations were significantly higher on average than baseline levels (LRT = 14.9, df = 1, $P < 0.001$; fig. 1) and ranged from nondetectable to 3.18 ng/g.

Ponds varied widely in corticosterone concentration and in many environmental variables (tables 1, 2). At the pond level, average baseline corticosterone concentration was negatively related to pH (fig. 2) and positively related to nitrate (table 3). Stress-induced corticosterone concentration was again negatively related to pH (fig. 2) and was positively related to temperature and (marginally) to nitrate (table 3). Moving from a pH of 6 to 5, for instance, would result in a 174% increase in

baseline corticosterone and a 123% increase in stress-induced corticosterone. Similarly, low- and high-nitrate ponds differed in corticosterone by 141% for baseline animals and 93% for stressed animals. Last, for each degree of increase in temperature, average stress-induced corticosterone was predicted to increase by 17%.

In outdoor mesocosms, corticosterone concentrations ranged from 0.63 to 1.95 ng/g and from 1.64 to 2.05 ng/g under baseline and stress-induced conditions, respectively. Decreasing pH significantly increased corticosterone concentrations ($F_{2,6} = 198.9$, $P < 0.0001$; fig. 3). Confinement also generally raised corticosterone concentrations above baseline ($F_{1,42} = 684.6$, $P < 0.0001$; fig. 3). Most importantly, the effect of confinement depended on pH; confinement induced about a doubling of corticosterone at high and medium pH but no increase at the lowest pH ($F_{2,42} = 122.9$, $P < 0.0001$; fig. 3). In general, the concentration of corticosterone was slightly higher in the mesocosms than in the field populations and was less variable.

Discussion

Here, baseline corticosterone concentrations were elevated at low pH in both the field populations and the experimental mesocosms. In the mesocosm study, however, stress-induced concentrations did not change significantly across pH treatments. It is likely that baseline concentrations under low pH conditions were maximally elevated, which prevented any additional increase with confinement in that treatment. Hopkins et al. (1999) saw a similar pattern of elevated baseline levels in southern toads *Bufo terrestris* living at a natural field site where they were exposed to coal combustion waste. They also saw a lack of corticosterone elevation in the toads at that polluted site following ACTH injection, which is comparable to the lack of elevation following confinement in our low pH treatment.

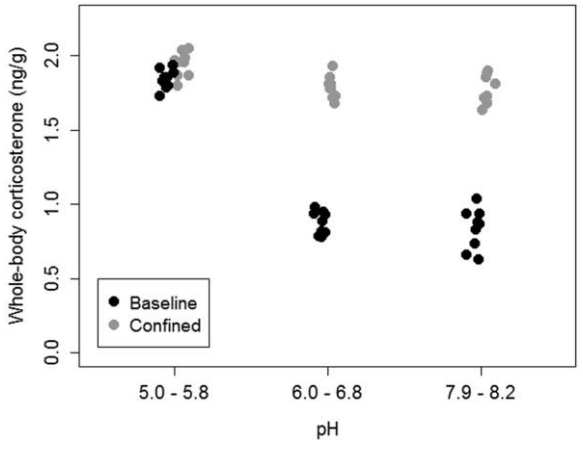

Figure 3. Whole-body corticosterone concentrations of *Ambystoma jeffersonianum* in experimental mesocosms. Each circle represents a single individual. Baseline and confinement-induced corticosterone concentrations are indicated by black and gray circles, respectively.

Table 3: Results from multiple regression analysis of baseline and stress-induced corticosterone concentrations at the pond level versus environmental covariates

Covariate	Estimate	SE	*P*
Baseline corticosterone:			
Intercept	5.36	1.86	.045
pH	−1.01	.29	.026
Nitrate	.88	.29	.040
Confinement corticosterone:			
Intercept	1.15	1.38	.463
pH	−.80	.20	.027
Nitrate	.66	.21	.055
Temperature	.16	.04	.034

While many aspects of the stress response can be altered with changing environmental conditions, the elevation of baseline corticosterone levels is one key change that is possible.

The reduction in pH in aquatic systems associated with acid deposition is a well-known environmental stressor in aquatic systems (Driscoll et al. 2001). Many studies have demonstrated negative impacts of acidic conditions on amphibian species, including *Ambystoma jeffersonianum*. For instance, exposure to acidic conditions has been linked to increased mortality in *A. jeffersonianum* larvae (Rowe et al. 1992) and to decreased developmental rates (Horne and Dunson 1994). We do not have data on developmental rates from our mesocosm experiment. However, we know from prior work that baseline corticosterone levels in *A. jeffersonianum* tend to increase gradually over the course of development (Chambers et al. 2011). Therefore, if there was a developmental response to low pH in our experimental mesocosms, the expectation would be that individuals would be less developed in those tanks and therefore have lower baseline corticosterone levels. Instead, we found that individuals in the lowest pH tanks had elevated baseline corticosterone levels. Therefore, we do not think that changes in developmental rates are the driving factor explaining the elevated baseline levels of corticosterone that we saw in individuals exposed to acidic conditions.

Another possible mechanism for increasing baseline corticosterone concentrations under low pH conditions could be an indirect effect of pH on food webs, specifically on zooplankton. Zooplankton survival, community species richness, and relative abundance can be altered by low pH (Locke 1991; Lacoul et al. 2011). Thus, if *A. jeffersonianum* larvae have less food available, we would expect to observe an increase in corticosterone concentrations as energy reserves are mobilized to negotiate through periods of low prey availability. In addition, amphibian skin assists in maintaining internal pH homeostasis (Candia and Yorio 1997). pH imbalances are resolved by excreting H^+, NH_4^+, or HCO_3^- from epithelial cells to restore pH balance (Vanatta and Frazier 1981). Again, such activity could require an increase in energy that is mobilized by increasing circulating baseline corticosterone concentrations.

In our field survey, nitrate was positively related to corti-

costerone concentrations. As for low pH, there is a large literature demonstrating nitrate impacts on amphibians and other freshwater organisms (Camargo et al. 2005; Mann et al. 2009), but further experimental work would be required to investigate the exact nature of the corticosterone response to nitrate. We also saw a link between temperature and stress-induced corticosterone concentrations in the field survey. We expect that this is a result of variation in the timing of the stress-induced response in ectotherms. A warmer individual would likely have a faster increase in corticosterone following confinement, so that the peak would be higher sooner. As we sampled all individuals at 30 min, we are likely seeing this temperature-based variation in elevation in our field sampling results.

In our field survey, we used censored regression to deal explicitly with sample concentrations below the assay detection limit. We suggest that this could be a useful analytical approach for other studies in comparative endocrinology, especially those dealing with small organisms where sample size or volume might be limiting, or in studies assessing baseline hormone levels, which might commonly fall below the limit of detection for an assay. In our study, the value of retaining data from samples below the detection limit can clearly be seen. For example, the proportion of fully measured versus censored values from the field populations varies across the pH gradient—none of the lowest pH pond samples were below detection and all of the highest pH pond samples were below detection, which conforms exactly to expectations if low pH raises corticosterone. Even the pond that we eliminated from the model because all the values were censored fit that same pattern; it was the pond with the highest pH. Judging the data set as poor quality because of a significant number of nondetects is counterproductive when statistical tools such as censored regression have been developed specifically to address these types of data sets.

Studies examining baseline glucocorticoid concentrations are relatively rare in the literature despite the important contributions of glucocorticoids to numerous physiological functions (Sapolsky et al. 2000; Wikelski and Cooke 2006). Baseline corticosterone data, especially from natural populations, are also critical for understanding the natural stress hormone profile and how individuals respond to environmental stressors (Belden et al. 2005). Our field survey results show baseline corticosterone concentrations differing significantly among free-living *A. jeffersonianum* populations. Belden et al. (2007) have also shown variations in baseline corticosterone concentrations among tadpoles in free-living wood frog *Rana sylvatica* populations. Differing baseline corticosterone concentrations could suggest that these populations are experiencing varied environmental stressors in their aquatic systems, similar to what was proposed for adult *Ambystoma maculatum* by Homan et al. (2003). This would suggest that there could be strong habitat heterogeneity across breeding pond landscapes or perhaps even a structure of source and sink habitats. However, interpreting observed variation can be challenging, as baseline corticosterone concentrations can also change across seasons and over the course of a single day (Rich and Romero 2005).

Surprisingly few studies have delved into understanding population-level variation in glucocorticoid concentrations in a systematic way across more than two or three populations, although Dunlap and Wingfield (1995) represents one exception. They examined the stress response to captivity in western fence lizards *Sceloporus occidentalis* in six populations in the western United States. Three of these populations were in the central part of the species range, and three were near the range limit. They documented variation in baseline corticosterone, but it was not correlated with the factors they examined (region of range, season of the year). Instead, they saw clear differences in the stress-induced concentrations of corticosterone; higher stress-induced concentrations were observed in populations living at the edge of the range and during the hotter, drier part of the year. This change in stress-induced levels is a different pattern of interpopulation variation than that observed in Hopkins et al. (1999) and in our study, where we found that baseline levels were elevated. In another recent study with larval amphibians, common frog tadpoles *Rana temporaria* from eight populations along a latitudinal gradient were examined in the laboratory for variation in the response to predators (Dahl et al. 2012). After 24 h, tadpoles from high-latitude populations, compared with those from low-latitude populations, had increased baseline corticosterone levels when reared with a nonlethal dragonfly predator, but this difference disappeared after 15 d of cohousing with a predator. All of the variation in possible physiological responses demonstrated in these studies speaks to the complexities of quantifying variation in the stress response among populations as well as to the difficulties in determining the causes of any differences. Our tactic of coupling field surveys with follow-up experiments should provide a useful approach for unraveling these complexities.

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