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A non-invasive water-borne hormone assay for amphibians

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Abstract. Anthropogenic disturbances have been implicated in the rapid decline of amphibians. Disturbances, such as disease and poor water quality, might cause changes in the physiology of amphibians resulting in chronic stress, which can result in decreased growth and development as well as immunosuppression. In amphibians, corticosterone (CORT) is the main hormone released in response to stressors. We took the first steps towards validating a new, non-invasive, technique to assay CORT in amphibians using a water-borne collection method previously used only with fish. In validation of this technique, we found a significant positive correlation between release rates of water-borne CORT and levels of CORT in circulating plasma in adults of the San Marcos salamander, *Eurycea nana*, and the common midwife toad, *Alytes obstetricans*. These results indicate that water-borne CORT can be used as a proxy for plasma CORT. Additionally, we examined basic background information on the physiological states of these two species. We found that captive-reared salamanders had significantly lower release rates of CORT than field-collected salamanders. Field-collected salamanders had significantly higher CORT release rates 24 h after capture and transfer to the laboratory. For tadpoles, we found that field-collected tadpoles did not have significantly different CORT release rates than those maintained in the laboratory for four months. Our research indicates that this method of water-borne hormone collection should be viable for many species of amphibians; however, further validation via adrenocorticotropic hormone (ACTH) challenges is required. This method can be a useful tool for assessing the physiological state of laboratory and field populations of amphibians and the effects of urbanization, pesticides and diseases. An important benefit of this method is that it allows for repeated measures of the same individuals and can be less stressful than drawing blood.

Keywords: conservation, corticosteroid, frog, physiology, salamander, stress hormones.

Introduction

The global decline in biodiversity has been of critical importance to conservation biologists, with amphibians being one of the worst hit and, to some extent, the least studied group of organisms (Lawler et al., 2006). The rapid declines in amphibian populations are largely associated with anthropogenic factors such as habitat destruction, the spread of diseases, and the use of environmental contaminants (Stuart et al., 2004). Anthropogenic disturbances can alter water quality and the degradation of habitat may influence amphibian physiology. For example, acidification of water, low dissolved oxygen, and changes in conductivity cause stress in amphibians (Kiesecker, Blaustein and Belden, 2001). The primary physiological stress response in vertebrates is an increase in the production of circulating glucocorticoids. Glucocorticoids are steroid hormones released with stress-induced activation of the hypothalamus-pituitary-adrenal (hypothalamus-pituitary-interrenal, HPI, in amphibians) axis. In the short term, increased levels of glucocorticoids can aid in the response to environmental stressors or challenges such as during reproductive events or in response to predators (Moore and Jessop, 2003; Thaker, Lima and Hews, 2009). Over longer periods of time with chronic exposure to a stressor, elevated glucocorticoid levels can result in decreased growth, increased developmental time, and immunosuppression, which in the face of disease can become deleterious (Sapolsky, Romero and Munck, 2000). Glucocorticoids can subsequently suppress activity of the hypothalamus-pituitary-gonadal

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(HPG) axis (McEwen and Wingfield, 2003) resulting in decreased secretion of gonadal hormones, which may lead to decreased expression of androgen-mediated mating behaviors (Moore and Orchinik, 1994; reviewed by Adkins-Regan, 2005). Higher glucocorticoid concentrations, however, do not always indicate stress because in some animals it indicates that they are successfully coping with a stressor whereas those with lower levels are not (Cyr and Romero, 2007; Dickens, Delehanty and Romero, 2009). The stress response has been used as a biomonitor for potentially threatened populations of amphibians (Homan, Reed and Romero, 2003), and glucocorticoid concentrations are useful in predicting survival of individuals (Romero and Wikelski, 2001) and reproductive success (Cyr and Romero, 2007) in stressed populations. Because individual measures of survival and fecundity are difficult to measure in the wild, measures of stress may be a useful alternative.

Corticosterone (CORT) is the main glucocorticoid released in response to stressors by amphibians and plays an integral role in metamorphosis, reproduction, and immune function (Moore and Jessop, 2003; Belden and Kiesecker, 2005). In general, CORT levels peak at metamorphic climax in anurans (Kloas, Reinecke and Hanke, 1997; Glennemeier and Denver, 2002a; Wright et al., 2003) and salamanders (Carr and Norris, 1988; Chambers et al., 2011). However, chronic levels of excess whole-body CORT (50% increase) was associated with slowed growth and development of *Rana pipiens* (Glennemeier and Denver, 2002b). Elevated CORT levels suppress reproductive behaviors during acute stress in some salamanders (Moore and Orchinik, 1994; Moore, Boyd and Kelley, 2005) but not in others (Zerani and Gobbetti, 1993; Benner and Woodley, 2007). Elevated CORT levels have also been linked to amphibian disease (Warne, Crespi and Brunner, 2011). In some tadpoles, exposure to predators resulted in increased CORT levels (Dahl et al., 2012) but not in others (Fraker, 2009; Dahl et al., 2012). Therefore, basic information on normal CORT levels of a population or species may allow researchers to subsequently identify when CORT becomes relatively high, possibly indicating that a population or species is at high risk due to chronic stress (Romero, Reed and Wingfield, 2000; Homan et al., 2003).

In prior studies of CORT, hormone levels were measured based on examining circulating plasma levels or whole-body CORT levels for smaller species, with the latter requiring euthanizing the organism. Because threatened and endangered species generally are rare, the loss of any individual, even for important experimental results, is of major concern. Recently, a non-invasive method for evaluating hormones in adult frogs has been developed using urinary steroid metabolites (Germano et al., 2009; Narayan et al., 2010; Germano et al., 2012; Kindermann, Narayan and Hero, 2012). While the authors validated this method for assaying CORT levels in frogs using a stress challenge, they have not validated the correlation between urinary metabolite and plasma levels. Other studies aimed at assessing stress levels by evaluating leukocyte profiles based on blood smears (Davis and Maerz, 2008; Davis, Maney and Maerz, 2008; Davis et al., 2010; Davis and Maerz, 2011). In a review of the literature for vertebrates, Davis, Maney and Maerz (2008) found that high ratios of heterophils or neutrophils to lymphocytes in blood samples indicate high glucocorticoid levels. Here, we apply a method previously only used on fish (reviewed by Scott and Ellis, 2007) to assay CORT released from aquatic salamanders, tadpoles, and adult frogs using a non-invasive, water-borne hormone sampling technique.

This non-invasive method for assaying hormones from water has many advantages over plasma or whole body sampling, as it does not disturb the organism like blood sampling does (which in many cases requires euthanization), and repeated samples can be taken from the same individual or from a population in time-course experiments. It also has advantages over

urine-based assays, as it does not require obtaining a urine sample, which may not be viable for tadpoles or salamanders. As with plasma and urine samples, release rates of water-borne hormones can be measured using readily available enzyme immune assay (EIA) kits. The correlation between release rates of water-borne hormone and circulating plasma levels of the respective hormone has been validated in many species of fish (Scott and Ellis, 2007; Gabor and Contreras, 2012). In fish, this method measures steroids that passively diffuse from the bloodstream into the water through the gills, urine, and feces (Scott et al., 2008). This method should work for amphibians because they also excrete urine and feces when placed in water. Hormones may also be released from mucus and saliva, but the details are yet to be worked out.

We first evaluated CORT in adults of the neotenic (fully-aquatic) San Marcos salamander, *Eurycea nana. Eurycea nana* is listed as federally threatened by the United States Department of the Interior (1980) and listed as vulnerable on the IUCN Red List (2012). It is an aquatic species of salamander endemic to the headwaters of the San Marcos River, Texas, USA. We validated the use of the water-borne hormone methods by examining the correlation between plasma CORT levels and the release rates of water-borne CORT. Additionally, we obtained basic information on the physiological state of the San Marcos salamander to provide the basis for future guidance and refinement of management techniques. We examined: (1) if salamanders mounted a CORT response at 30, 60, 90, or 120 min and (2) the physiological response to collection in the field and transportation to the laboratory by assaying CORT release rates of salamanders immediately after capture and then re-assaying individuals in the laboratory 24 h after capture. We also compared these values to the 60-min sample time for captive reared salamanders used in the first experiment.

Next, we evaluated CORT in the common midwife toad, *Alytes obstetricans*. *Alytes obstetricans* has a widespread distribution across Europe and has suffered substantial declines throughout its range after the emergence of the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), led to mass mortalities (Bosch et al., 2008). We validated the use of water-borne hormone methods in adult frogs by examining the correlation between plasma CORT levels and the release rates of water-borne CORT. We obtained basic background information on the physiological state of *Bd*-infected and uninfected tadpoles of *A. obstetricans*. We examined: (1) if tadpoles mounted a CORT response by resampling the same tadpoles at 30-min intervals for up to 120 min and (2) the release rates of CORT from *Bd*-infected tadpoles in the field and from infected tadpoles from the same population that had been in the laboratory for four months.

Materials and methods

Study species

Eurycea nana. We used first generation, captive-reared, adult *E. nana* from the San Marcos Aquatic Resources Center (SMARC) in San Marcos, Texas, USA for all but the field-collected salamander study. Field-collected salamanders were collected from the headwaters of the San Marcos River, Hays Co., Texas, USA (29.89003°N, 97.93425°W). Salamanders in the laboratory were maintained on a 12 : 12 h light cycle and fed blackworms *ad libitum*. This species lacks reproductive seasonality as juveniles and gravid females of *E. nana* are found year-round in thermostable water (Tupa and Davis, 1976) and at SMARC. All samples were taken between 10:00-14:00 h to minimize effects of circadian variation in CORT levels. Gloves were worn throughout the water-borne hormone collection process.

Alytes obstetricans. For the plasma and water-borne hormone correlation, we used adult *A. obstetricans* that were reared from eggs at the Sierra de Guadarrama Endangered Amphibians Breeding Centre in Rascafría, Spain (40.88°N, −3.88°W). This population was uninfected by *Bd.* The toads were maintained on a 12 : 12 h light cycle and fed crickets *ad libitum.* To compare field CORT levels with laboratory levels, we used *Bd*-infected tadpoles collected in the field from the Toro, Northwest Spain (41.48°N, −5.45°W) in April 2012 and returned to the laboratory. We also field-collected samples from tadpoles in June 2012. All samples were taken between 11:00-13:00 h. Gloves were worn throughout the water-borne collection process. All tadpoles used were in Gosner stages 27-29 (Gosner, 1960).

Correlation between water-borne and plasma CORT levels

Eurycea nana. We placed each *E. nana* ($n = 10$) into separate 250-ml Nalgene (HDPE) containers cut into a cup (with holes on the bottom) that sat within a 250-ml glass beaker filled with 100 ml of well water (we used this water as it is the same water that is used to maintain the salamanders at SMARC) for 1 h to collect water-borne hormones (fig. 1). We then lifted the Nalgene cup to remove the salamander with minimal stress, while leaving the water sample in the beaker. We obtained blood by orbital sinus puncture using pulled, heparinized capillary tubes. We measured snout-vent length (SVL) and mass of the salamanders after euthanizing them with an overdose of benzocaine. Blood samples were centrifuged at 3000 × G for 10 min to separate the blood from the plasma. We stored $\geqslant 5$ μl of plasma at −80°C until ready for processing. These data were collected April 2012.

Alytes obstetricans. We placed each adult *A. obstetricans* ($n = 9$) into a separate 100-ml polypropolene beaker filled with 40 ml of dechlorinated tap water (this water should have very little CORT in it) for 1 h to collect water-borne hormones (fig. 2). We then gently lifted the toad out and obtained blood by cardiac puncture with a 0.30-mm (30 G) × 8-mm needle. We measured SVL (mm) and weighed (g) toads after obtaining blood but they did not need to be euthanized. Blood samples were centrifuged at 3000 × G for 10 min to separate the blood from the plasma. We stored $\geqslant 20$ μl of plasma at −80°C until ready for processing. Samples were taken April 2012.

We assayed the plasma and the water-borne hormones for free, non-conjugated fraction of steroids. In fish the free steroids are passively 'leaked' across the gills via a concentration gradient between plasma and water. Scott and Ellis (2007) argue that free steroids in the water are most likely to be very close to the plasma levels at the time the sample is taken. The specific relationship between the released free steroids and plasma levels, however, has not been determined in amphibians.

Differences in water-borne CORT levels over time

Eurycea nana. We obtained water samples from salamanders ($n = 5$/treatment) after 30, 60, 90, or 120 min. Individual salamanders were placed in a Nalgene container within a 250 ml-glass beaker with 100 ml of well water for the treatment period. Salamanders were measured for SVL (mm) after samples were obtained. These data were collected June 2011.

Alytes obstetricans. We obtained water samples from tadpoles ($n = 8$/treatment) repeatedly over four 30-min periods up to 120 min (using a different method than for *E. nana*). Individual tadpoles were placed in a 100-ml beaker with 40 ml of water for each 30 min treatment. We weighed (g) and measured SVL (mm) for each tadpole after the

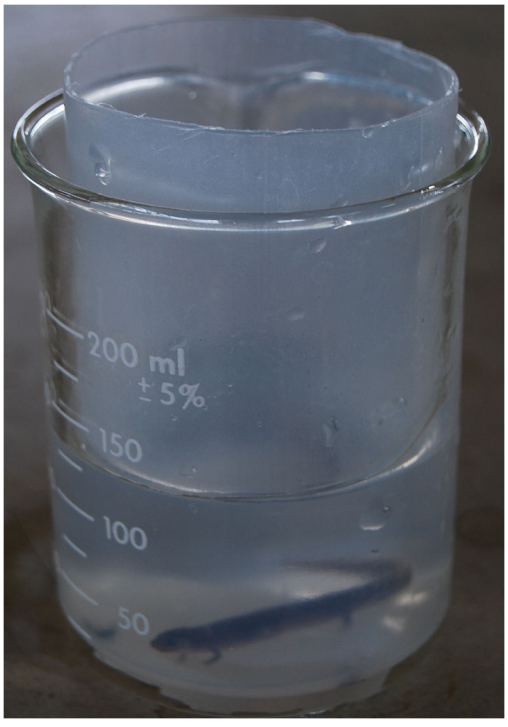

Figure 1. *Eurycea nana* in Nalgene cup with holes at bottom within a 250-ml beaker during water-borne hormone sampling. Use of cup allows easy removal of salamander from water sample. Photo by D. Davis. This figure is published in colour in the online version.

Figure 2. *Alytes obstetricans* in a 100-ml beaker during water-borne hormone sampling. Photo by J. Bosch. This figure is published in colour in the online version.

four samples were obtained. These data were collected July 2012.

Difference in water-borne CORT levels between field-collected and individuals in the laboratory

Eurycea nana. We collected salamanders ($n = 9$) from the headwaters of the San Marcos River and immediately placed each salamander into a Nalgene container within a 250-ml glass beaker with 100 ml of well water for 1 h. Beakers were maintained inside a cooler with spring water to maintain the water temperature close to the spring temperature. To determine the effects of collection, we brought the salamanders to the laboratory, and then resampled CORT release rates from the same field-collected salamanders 24 h later using the same methods provided earlier. Salamanders were measured for SVL after the second sample was obtained. These data were collected June 2011.

Alytes obstetricans. We collected tadpoles ($n = 15$) from the field and immediately placed each tadpole into a 100-ml beaker with 40 ml of dechlorinated tap water for 1 h. We compared these values to those from the 60-min sample from the prior experiment as these tadpoles were from the same population and had been maintained in the laboratory for four months. We weighed and measured SVL for each tadpole after samples were obtained. Samples were collected June and July 2012.

Hormone extraction, validation, and analyses

All water samples were stored at −20°C until ready to be thawed for extraction (Ellis et al., 2004). To obtain total (free + conjugated) CORT release rates, we extracted hormones from the entire water sample by passing water through Tygon tubing (Saint Bobain formulation 2475) into C18 solid phase extraction columns (SepPak Vac 3 cc/500 mg; Waters, Inc., Milford, MA, USA) under vacuum pressure. We primed the columns with 4 ml of HPLC-grade methanol and 4 ml millipore water. After extraction, we eluted columns with 4 ml methanol into borosilicate vials (following Gabor and Grober, 2010). The methanol was evaporated under a gentle stream of nitrogen gas using an Evap-O-Rac (Cole-Parmer) placed in a 37°C water bath. For both *E. nana* and *A. obstetricans*, we re-suspended the residue in 5% ethanol followed by vortexing for 1 min and then 95% enzyme-immunoassay (EIA) buffer (provided by Cayman Chemicals Inc., Ann Arbor, MI, USA) for a final re-suspension volume of 400 μl for salamanders and 250 μl for the frogs.

To obtain the free, non-conjugated portion of CORT from the water-borne sample for the correlational study for salamanders and toads, we re-suspended residue using the above outlined procedure in 200 μl millipore water and 2 ml diethyl ether, then vortexed the samples for 4 min. For the plasma, we placed the plasma (5 μl for salamanders, 20 μl for frogs) into a borosilicate vial, added 200 μl millipore water and 2 ml analytical grade diethyl ether, stabilized, then vortexed samples for 4 min. For all samples, after layers separated for 2 min, a dry-ice methanol bath was used to fast-freeze the water layer, and the ether layer containing free hormones was decanted into a vial. This process was repeated once to obtain a total volume of 4 ml. Diethyl ether was evaporated under a gentle stream of nitrogen in a 37°C water bath. We re-suspended the water-borne pellets as above. We re-suspended the salamander and frog plasma pellets in 5% ethanol and 95% EIA buffer to a total volume of 500 μl of solution. CORT hormone release rates were measured in duplicate for all samples with an EIA kit (Cayman Chemicals Inc.) on a fluorescent plate reader set to 415 nm (BioTek Powerwave XS).

We validated the use of the CORT EIA kits for water-borne hormones for both *E. nana* and *A. obstetricans*. We used 10 non-experimental captive-reared salamanders and tadpoles and collected water-borne hormones using methods previously outlined. We diluted the pooled controls to 1 : 2 for the serial dilutions and quantitative recovery for both species. We included two pooled controls on each plate (either *E. nana* or *A. obstetricans* depending on the plate) to determine intra- and inter-assay coefficient of variation (CV). For the *E. nana* pooled controls, intra-assay CV was 11.9%, 23.1%, 1.47%, 8.39%, and 5.77% and inter-assay CV was 15.06%. For the *A. obstetricans* pooled controls, intra-assay CV was 7.38%, 16.27%, 3.40%, and 3.59%, and inter-assay CV was 21.44%.

We ran serial dilution of the pooled controls for *E. nana* and *A. obstetricans* in duplicate. The serial dilution curve was parallel to the standard curve (Comparison of slopes: *E. nana*: $t_8 = 0.505$, $P = 0.625$; *A. obstetricans*: $t_8 = 2.06$, $P = 0.07$). We conducted cold spikes by mixing equal volumes of the pooled control samples with each of the eight standards and an unmanipulated pooled control sample for each species. The expected recovery concentrations were based on the known amount of CORT in the control samples. The minimum observed recovery for *E. nana* was 41% ($\bar{x} = 77\%$) and for *A. obstetricans* was 66% ($\bar{x} = 85\%$). The regression coefficient of the observed vs expected concentrations for *E. nana* was 1.06 ($F_{1,8} = 1438.74$, $r^2 = 0.995$, $P < 0.0001$) and was 0.88 for *A. obstetricans* ($F_{1,8} = 200.61$, $r^2 = 0.966$, $P < 0.0001$). The sensitivities of the assays range from 19.70-28.41 pg/ml. All data are presented as pg/sample (pg/ml multiplied by 0.40 ml for *E. nana* or 0.25 ml for *A. obstetricans*, which was the amount of EIA buffer used to reconstitute the sample).

Statistical analyses

We standardized the hormone data by dividing by the SVL of the respective individual and then standardized the values so that they were all out of an hour (i.e., for the 30 min water samples we multiplied the final CORT value by 2). In *E. nana* and *A. obstetricans*, SVL and mass are strongly related (Linear regression: *E. nana*: $r^2 = 0.70$, $n = 28$, $F = 60.50$; $P < 0.0001$; *A. obstetricans*: $r^2 = 0.95$, $n = 30$, $F = 492.89$; $P < 0.0001$). We performed analyses on the Ln-transformed standardized hormone data as they met the assumptions of parametric analyses. To aid in visualizing the data, we present untransformed data in the figures. We used a Pearson correlation adjusted for small sample sizes to compare the plasma levels to the release

rates of water-borne CORT. For salamanders we used an ANOVA to examine how long it took for individuals to mount a CORT response. We used repeated measures (Rm) ANOVA for the frogs as we retested the same individuals over four time periods. We used a paired t-test to compare field-collected CORT release rates vs CORT 24 h later from the same salamanders in the lab, and used a Student's t-test to compare lab and field CORT levels of salamanders and frogs. All tests were two-tailed ($\alpha = 0.05$) and were analyzed using JMP 9.0 (SAS Institute, Cary, NC, USA).

Results

Eurycea nana

There was a significant positive correlation between free water-borne CORT and free plasma CORT for adult *E. nana* (Pearson correlation adjusted for small sample sizes: $n = 10$, $r^* = 0.87$, $P < 0.01$; fig. 3A). We found that there was no difference in CORT release rates between 30, 60, 90, and 120 min (ANOVA: $F_{3,16} = 1.65$, $P = 0.22$; fig. 4A). The CORT release rates from field-collected sala-

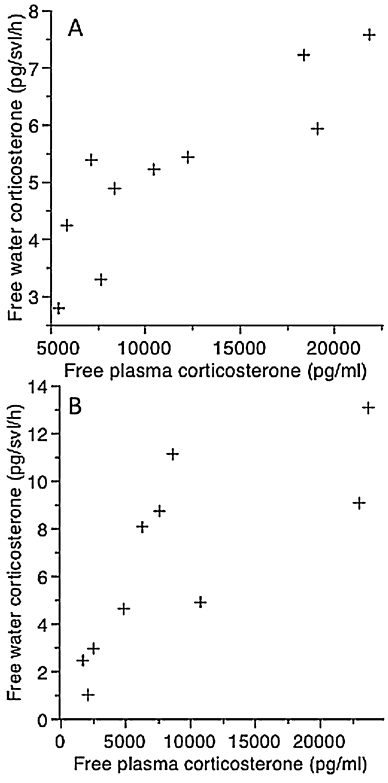

Figure 3. Significant positive correlation between free plasma corticosterone (pg/ml) and free water-borne corticosterone (pg.svl/h) in (A) *Eurycea nana* ($n = 10$) and (B) *Alytes obstetricans* ($n = 9$). *Pearson correlation adjusted for small sample sizes.

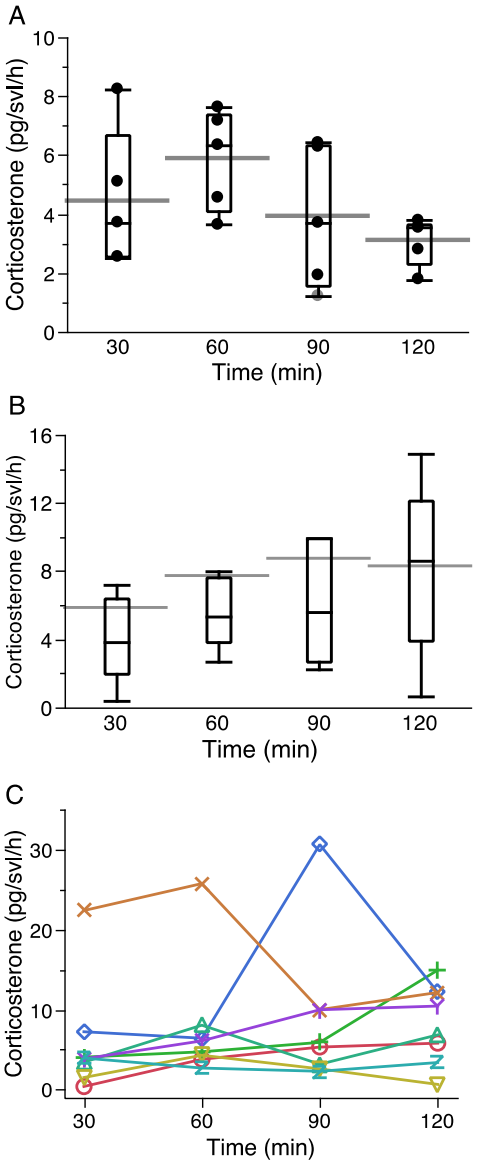

Figure 4. Corticosterone release rates ± SE obtained at 30, 60, 90, and 120 min for (A) *Eurycea nana* ($n = 5$) and (B) *Alytes obstetricans* ($n = 8$). (C) Corticosterone release rates for each *A. obstetricans* across time. Box plots indicate median, range and first and third quantiles. Grey bars indicate the mean. Black dots indicate values for each individual. This figure is published in colour in the online version.

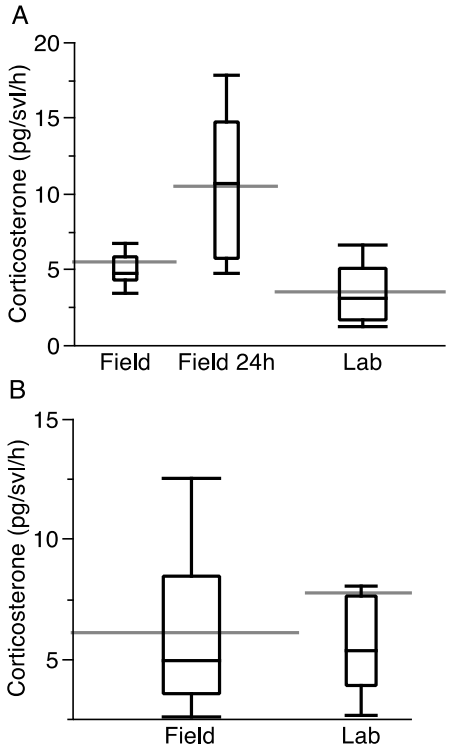

Figure 5. Corticosterone release rates ± SE obtained in the laboratory and field settings for (A) *Eurycea nana* and (B) *Alytes obstetricans*. Box plots indicate median, range, and first and third quantiles. Grey bars indicate the mean.

manders were significantly less than the CORT release rates of the same field-collected salamanders 24 h later (Paired t-test: $t_8 = 2.91$, $P < 0.05$; fig. 5A). Field-collected salamanders had significantly higher CORT release rates than captive-reared salamanders (Student's t-test: $t_{15} = -3.06$, $P < 0.01$; fig. 5A).

Alytes obstetricans

There was a significant positive correlation between free water-borne CORT and free plasma CORT for adult *A. obstetricans* (Pearson correlation adjusted for small sample sizes: $n = 9$, $r^* = 0.86$, $P < 0.01$; fig. 3B). There was no difference in CORT release rates of tadpoles at 30, 60, 90, or 120 min total times (Rm ANOVA: $F_{3,5} = 0.87$, $P = 0.51$; fig. 4B, 4C). There was no significant difference between CORT release rates from field-collected and laboratory-reared tadpoles (Student's t-test: $t_{23} = 0.36$, $P = 0.72$; fig. 5B).

Discussion

We found a significant correlation between the release rates of water-borne CORT and the plasma CORT levels in *E. nana* and *A. obstetricans*. These results provide the first steps towards validating the water-borne hormone technique as a viable alternative method for assaying steroid hormones in *E. nana* and *A. obstetricans*. The next step is to perform an adrenocorticotropic hormone (ACTH) challenge to demonstrate that CORT levels increase with increased concentrations of ACTH. The water-borne hormone method provides a non-invasive alternative technique for evaluating the physiological state of individuals and populations and can be used to examine the response to potential stressors such as habitat alteration, predators, contaminants, and infection. This method provides additional advantages over the urine-based method (for review, see Kindermann et al., 2012) as it can be used for salamanders, small frogs, and tadpoles in addition to requiring less handling of the organism.

One potential limitation of the water-borne hormone technique is that individuals must be confined during hormone collection. Prior studies have found that larval amphibians show an increase in CORT in response to confinement and agitation using similar size beakers (Belden et al., 2003; Belden, Wingfield and Kiesecker, 2010; Chambers et al., 2011). In our method, however, we did not agitate the beaker during confinement. In these prior studies, it is possible that CORT increased primarily in response to the agitation and not the confinement. Even if the confinement alone induced some stress response, we can still consider the measured values as a relative "baseline" value (for this method) because the method is the same each time. These values can then be compared for the same individuals, populations, or species across time and in response to specific stressors. We

do not think that the measured release rates of CORT are maximal values for the populations because we found that the CORT release rates of *E. nana* 24 h after field-collection were significantly higher than CORT release rates measured in the laboratory. We cannot examine this for *A. obstetricans* because the levels in the laboratory and field did not differ and we did not find evidence for a mount in CORT within 120 min of confinement. To address this issue in future studies, we will perform an ACTH challenge, as this acts like a maximum stressor and should yield a rise and then a return to "baseline" release rates of CORT.

To get an indication of the physiological status of captive populations, it is useful to compare the baseline release rates of CORT and CORT response to stressors for laboratory-reared or maintained versus field-collected amphibians (Cyr and Romero, 2009). We found that salamanders collected directly from the field have higher baseline CORT release rates than captive-reared salamanders. These differences may be a consequence of captive-reared salamanders being fed *ad libitum* or not being exposed to predators or other stressful environmental factors not found in the controlled laboratory environment. Houck et al. (1996) found that lab and field levels of plasma CORT were similar for *Amybstoma opacum* but Davis and Maerz (2011) found that *A. opacum* reared in the lab had higher stress levels (as indicated by neutrophils to lymphocytes ratio) compared to wild caught individuals. Similarly, Cooperman, Reed and Romero (2004) found that in the field, high densities of *A. maculatum* during mass breeding events had lower plasma CORT levels than laboratory animals held under high densities. Our results suggest that *E. nana* maintained in the laboratory are not chronically stressed, an important variable to assess especially for captive-rearing programs. Unlike for *E. nana*, there was no significant difference in CORT for tadpoles of *Alytes obstetricans* maintained in the laboratory for four months and tadpoles from the same population in the field. Further exploration is required to fully evaluate whether or not these populations are chronically stressed because stressed populations may have baseline CORT levels that are higher, lower, or similar to those exhibited in non-stressed populations, but exhibit a very different capacity to respond to a stressor (Cyr and Romero, 2009). The next step is to examine the capacity to respond to a stressor (such as an ACTH challenge) for each population (i.e., laboratory vs field populations) for each species being considered. Re-sampling CORT in the laboratory and the field of this population over a period of 1 year or comparing field with captive-reared tadpoles may provide further understanding of these relationships.

It is also important to test the effects of collection and captivity on stress hormone levels in amphibians to aid in their management and research practices. We found that *E. nana* mounted a strong physiological stress response 24 h after they were collected from the field and brought into the laboratory. This indicates that the combined process of collection and transportation does cause high stress levels. These results also suggest that immediate collection of hormones from *E. nana* after collection in the field provides insight into their "baseline" water-borne CORT release rates. This is useful as it allows for assessment of the welfare of the wild populations based on these "baseline" CORT release rates. Prior studies have examined the effects of collection on stress response induced by capture and by keeping animals in captivity for some days. Homan et al. (2003) found that 30 min after capture, male and female *Ambystoma maculatum* plasma CORT levels increased significantly. Davis and Maerz (2008) found that *A. talpoideum* caught in the wild and held in captivity for 10 d before sampling had significantly more neutrophils and fewer lymphocytes (indicating high stress levels) than those captured from the same location but sampled within 1 h, suggesting that the early stages of captivity induced a general stress response.

Another component to consider is the relevance of CORT levels obtained from using

water-borne hormone studies as compared to plasma studies. It is clear from the validation of plasma CORT and water-borne CORT levels that both are significantly positively correlated for both *E. nana* and *Alytes obstetricans* (fig. 3). These results suggest that measurement of steroids in water can be used as a proxy for measurements of steroids in plasma. However, plasma values represent a snapshot at a single time point whereas water samples represent levels over a longer period. While placing an animal in a beaker for 1 h may constitute a stressor, all individuals are exposed to the same methods and thus the value from a relatively undisturbed individual could be considered its "baseline" using the water-borne collection method.

The water-borne hormone collection method, after validation of the correlation of water-borne CORT and the ability to mount a response to a stressor such as an ACTH challenge, can then be used to study the effects of exposing individuals to additional stressors, such as predators or disease. The water-borne CORT response to a stressor can then be compared to the "baseline" to see if the exposure causes an increase in the release rate of CORT: if it does not, then the exposure was not perceived as stressful. Gabor, Fisher and Bosch (2013) found that a *Bd* infected population of *A. obstetricans* has a higher water-borne CORT release rate than an uninfected population and a significant positive correlation between *Bd* infection level and CORT release rates using the methods developed here. The next step required for these populations is to assess the ability of these populations to mount CORT responses to additional stressors to determine whether they are chronically stressed or not.

Another factor to consider are the units presented for the water-borne hormone release rates. In this paper, we divided the CORT release rate by the SVL of each individual to standardize for body size and then we standardized the values per hour. We were hesitant to use mass in aquatic organisms because these values would be a wet weight value, which can depend on how wet or dry the individual is when they are weighed. Mass and SVL are significantly positively correlated in both *E. nana* and *A. obstetricans*. In species where they are not correlated, then mass may be more important to use when standardizing hormone data. In prior studies with fish using water-borne hormones release rate the values were divided by the mass of the individuals as water steroid concentration can depend on the biomass of the individual (Scott and Ellis, 2007). In our system, water-borne CORT release rates were not correlated with SVL for either species (data not presented). However, Scott and Ellis (2007) argue that by creating a relative measure the values can be compared across species.

It has been argued that the water-borne collection procedure may stress the organism and thus make it hard to assay CORT (Wong et al., 2008). We sampled CORT release rates from 30 to 120 min for both *E. nana* and *A. obstetricans* and did not find a pattern of change over time as CORT neither increased nor decreased. It may be that it takes longer than 120 min to mount a CORT response, alternatively the process did not induce a stress response, or the organisms were already at peak CORT levels and could not further respond. In *A. obstetricans*, we repeatedly sampled the same individual and they did not mount a CORT response suggesting that this species can handle frequent handling. Alternatively, our power was low as sample sizes were too low to detect a difference, yet our sample sizes were comparable to those used in other studies on amphibian hormones (Belden et al., 2010; Chambers et al., 2011). Follow-up experiments examining the release rates for longer times, along with examining the response to an ACTH challenge should further elucidate how long it takes for these amphibians to mount CORT and how high it can go.

In this study, we focused on one hormone (CORT) using water-borne collection methods. However, in fish, a single water sample provides sufficient quantities of hormone for use with up to four different hormones when using Cayman

Chemical Inc. EIA kits (Gabor and Contreras, 2012). Given the dilution values we used with these amphibians, we may have been able to examine the relationships between two or more hormones to further understand the relationship between stress and sex hormones. Therefore, our method may allow for future studies to examine the impact of sex hormones on mating behavior and may help determine when amphibians are receptive. Alternatively, given the high dilution factors, it might be possible to assess water-borne CORT release rates in smaller time intervals to determine whether CORT levels change between sample periods such as 15 min vs 1 h.

In summary, we provide evidence that the non-invasive water-borne hormone collection method is a viable and valuable method for assaying stress levels in field and laboratory settings for *E. nana* and *A. obstetricans* and possibly many other aquatic amphibians. This is an improvement on prior methods for assaying hormones from amphibians as it is non-invasive, can easily be performed in the field, allows for resampling of the same individuals, and does not require bleeding or euthanizing individuals. Our method may be valuable especially for smaller amphibians that would otherwise require euthanizing for whole-body measures to assay CORT. Because individuals do not need to be bled or massaged to induce urine release, much less handling is required, possibly reducing stress and the additional potential of spreading disease. Finally, our method opens the door to examining the physiological stress response to anthropogenic factors such as habitat alteration and exposure to pesticides and disease in organisms for which plasma could not be obtained without euthanizing individuals. To fully assess the stress response to anthropogenic factors it is important to monitor values for a population over time and possibly compared stressed versus unstressed populations to determine whether chronic stress is resulting in an increase or decrease in CORT. Additionally, using an ACTH challenge will provide insight as to whether the HPI axis is still responsive to stressors to provide further insight into health of the population.

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