A Non-Invasive Water-Borne Assay of Stress Hormones in Aquatic Salamanders

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Declines in amphibian populations are due to an array of anthropogenic and natural factors requiring a need to detect and monitor populations that are either in decline or at risk of decline. Physiological biomarkers, such as glucocorticoid stress hormones, present a viable option to monitor stress levels and, thus, the physiological health of vertebrate populations. We performed preliminary experiments necessary for the use of water-borne hormone assays to measure corticosterone (CORT) as an assessment of aspects of chronic stress for the management and conservation of three species of aquatic salamanders in central Texas (*Eurycea*, Plethodontidae). We examined the time for CORT levels to peak in response to capture and handling in populations of *E. nana*, *E. sosorum*, and *E. tonkawae*. Peak CORT release rates differed among species, with peak releases generally occurring in under 3 h. This highlights the need to obtain a timeline for CORT to mount so that peak rates are captured during the leaching phase. We also examined the responsiveness of the hypothalamic-pituitary-interrenal (HPI) axis using an adrenocorticotropic hormone (ACTH) challenge or exposure to an external stressor (agitation). *Eurycea sosorum* (one population) and *E. tonkawae* showed HPI responsiveness. To examine the relationship between field and laboratory CORT release rates, we compared baseline CORT release rates between captive and wild-caught populations of all three species. Once the preliminary studies have been performed, water-borne CORT release rates can be an effective biomarker to monitor whether a population is experiencing chronic stress. Water-borne hormone collection also allows for repeated sampling of individuals over time. Our experiments provide a foundation for future studies relating stress to HPI responsiveness in aquatic *Eurycea*, which, in turn, will help inform management of both wild and captive populations.

P HYSIOLOGICAL biomarkers have been used with some success as indicators for whether a population is healthy or in a state of chronic stress (Creel et al., 1997; Hopkins et al., 1997; Wasser et al., 1997; Belden et al., 2003), and, consequently, they may be useful as a tool for conservation management. Chronic stress, or continued disruption of homeostasis over prolonged periods of time, can occur from extended and constant exposure to stressors and/or frequent exposure to stressors and is usually measured by changes in glucocorticoid (GC) hormones. Short-term stress responses can be adaptive as GCs mediate metabolic demands, antipredator responses, and immune responses, which can aid in individual survival (Sapolsky et al., 2000). However, chronic stress can lead to suppressed reproductive and immune functions and decreased growth (Sapolsky et al., 2000; McEwen and Wingfield, 2003), such that animals facing chronic stressors are no longer functioning within an adaptive capacity. Finding a non-invasive method to obtain early signs of chronically stressed individuals and populations would aid in conservation management of both wild and captive populations.

Corticosterone (CORT) is a GC involved in the stress response of many vertebrates, including amphibians, reptiles, and birds, and has been used as a biomarker for assessing stress in these organisms. Environmental exposure to pesticides (reviewed by Martin et al., 2010; McMahon et al., 2011) and pollutants (Hopkins et al., 1997, 1999) has resulted in chronic stress in some populations of amphibians. For example, Southern Toads (*Anaxyrus* [*Bufo*] *terrestris*) exposed to coal-ash pollutants had higher plasma CORT levels than unexposed toads, and exposed toads did not increase CORT excretion when exposed to an additional stressor (Hopkins et al., 1997, 1999). Conversely, populations of the Common Mudpuppy (*Necturus maculosus*) that were exposed to environmental contaminants had lower plasma CORT levels than those in less polluted sites, and exposed mudpuppies also did not increase CORT levels when exposed to an additional stressor (Gendron et al., 1997). In contrast, Belden et al. (2003) found no evidence of increased CORT excretion, using whole body extracts, when Cascades Frog (*Rana cascadae*) tadpoles were exposed to ultraviolet-B radiation versus not exposed, yet these tadpoles were able to mount a stress response to an additional stressor. These results support the finding that chronic stress can lead to either elevated GCs or depressed baseline levels of circulating GCs due to dysregulation of the hypothalamic-pituitary-adrenal (HPA) or hypothalamic-pituitary-interrenal (HPI, for amphibians) axis as previously indicated by McEwen and Wingfield (2003). Given that GCs may be depressed or elevated, Dickens and Romero (2013) proposed that the best approach for identifying a chronically stressed population is to examine whether GC regulation changes in response to multiple stressors (as indicated by HPA/HPI responsiveness).

One way to measure HPA/HPI responsiveness is through an adrenocorticotropic hormone (ACTH) challenge. During this challenge, the hypothalamus stimulates the anterior pituitary to release ACTH, which subsequently stimulates the release of CORT from the adrenal cortex (Chan et al., 1969). If an individual does not release more CORT after an ACTH challenge, then their HPA/HPI axis is dysregulated. HPA/HPI dysregulation is an indication of homeostatic overload, which is defined as the threshold above the reactive range (i.e., the baseline to the maximal CORT release during a stress response; Romero et al., 2009). Another way to measure HPI

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responsiveness is to compare CORT release rates of individuals that were exposed to external stressors and control individuals. In amphibians, an agitation test during confinement (external stressor) has been used to measure HPI responsiveness (Glennemeier and Denver, 2002; Belden et al., 2007). Either an ACTH challenge or an agitation test can provide important information on the functionality and responsiveness of the HPA/HPI axis.

Population declines have been linked to chronic stress (Boonstra et al., 1998; Romero and Wikelski, 2001); thus, examination of stress physiology may be a useful line of inquiry for understanding threats to populations. Assessing the physiological health of populations may be a useful technique for conservation of threatened and endangered species and may be particularly useful in plethodontid salamanders (Caudata: Plethodontidae). Many populations of plethodontid salamanders are experiencing declines (60% are classified as Vulnerable or worse by the IUCN Red List), the causes of which are not always clear (Rovito et al., 2009; Caruso and Lips, 2013). Although the need to understand the declines of these animals is apparent, the secretive nature of many plethodontids makes the task of detecting declines challenging. Even if researchers are able to detect population declines through demographic data, these data may not be timely enough, as demographic data are a lagging indicator of population health (Brooks et al., 1999; Findlay and Bourdages, 2000). Measuring CORT levels and HPI responsiveness in these populations may provide an effective and pre-emptive alternative to detect threatened populations.

Studies of endangered, vulnerable, or small populations require that methods for collecting data on stress hormones be non-invasive, as an alternative to the traditional method of measuring hormones through circulating plasma levels (Scott and Ellis, 2007). Collecting water-borne hormones allows for repeated measurements from the same individual and minimizes handling stress. This method provides an integrated measure of GCs because it is representative of the circulating levels in the organism that are passively released through the skin, gills, feces, and urine. This method of collecting CORT was recently validated in *Eurycea nana* and *Alytes obstetricans*, both of which showed a significant positive correlation between water-borne CORT release rates and plasma CORT levels (Gabor et al., 2013a).

The perrenibranchiate plethodontid salamanders of central Texas (*Eurycea*: *Paedomolge*, *sensu* Hillis et al., 2001) are a group particularly in need of a rapid and effective method for assessing impacts from environmental degradation. Of the 13 currently described species of *Paedomolge*, 10 are afforded some level of state or federal protection, and at least seven are currently threatened or surrounded by urban development (Chippindale et al., 2000; Chippindale and Price, 2005) and associated declines in water quality (U.S. Fish and Wildlife, 2012). A water-borne stress hormone approach may be an effective way to monitor these populations, especially those of *E. nana*, *E. sosorum*, and *E. tonkawae*. Anthropogenic factors seem to be the cause of population declines in *E. tonkawae* (Bowles et al., 2006; Bendik et al., 2014), while *E. nana* and *E. sosorum* are vulnerable to extinction from anthropogenic disturbances partly because they are single-site endemics.

Here, we perform several preliminary studies to assess CORT release rates using water-borne hormone collection on three species of salamanders that will guide future assessments of population health and help provide insights on management and conservation needs. We studied three

central Texas *Eurycea*: the San Marcos Salamander (*E. nana*), Barton Springs Salamander (*E. sosorum*), and Jollyville Plateau Salamander (*E. tonkawae*). The City of Austin (Austin Salamander Conservation Center; ASCC) maintains captive populations of *E. sosorum* and *E. tonkawae*, and the U.S. Fish and Wildlife Service (San Marcos Aquatic Resource Center; SMARC) maintains captive populations of *E. nana* and *E. sosorum* for the purpose of reintroduction should catastrophic population declines occur. These collections provide a unique opportunity to compare variation in CORT levels between captive and wild-caught populations. In these studies we examine the time it takes to mount a CORT response and HPI responsiveness of each population to understand the effects of captivity on the health of the populations. We also compared baseline CORT release rates between captive and wild-caught populations of all three species. We predict that in healthy populations wild-caught salamanders will have higher baseline CORT release rates compared to their conspecifics in the laboratory due to limited resources and the potential for exposure to predators or other environmental stressors. Together this data will help provide insight into the management and conservation of captive and wild populations of these three salamander species.

MATERIALS AND METHODS

For all studies, we obtained water-borne hormone samples from 0900–1300 h to minimize effects of circadian variation in CORT levels. We wore gloves throughout the hormone collection process, and all collection equipment was washed with 95% ethanol and rinsed with DI water before use. Wild salamanders were captured by lifting small rocks and directing the salamanders into hand held nets.

Eurycea nana

Captive individuals were adult, first generation, captive-born salamanders from the San Marcos Aquatic Resources Center (SMARC) in San Marcos, Texas, USA. Salamanders were maintained on a 12:12 h light:dark cycle and were fed blackworms *ad libitum*. Wild-caught salamanders were captured from the headwaters of the San Marcos River (Spring Lake), Hays Co., Texas, USA (29.89003°N, 97.93425°W; WGS84).

ACTH challenge in a captive population.—We performed an adrenocorticotropic hormone (ACTH) challenge on 4 July 2013. We haphazardly assigned adult salamanders ($n = 1$ male, $n = 29$ females) to one of three treatments: (1) ACTH injection (250 mlU/g dose; $n = 11$), (2) saline control injection ($n = 11$), and (3) a control of no injection ($n = 8$). The ACTH injection consisted of 0.02 ml ACTH (Sigma-Aldrich #A6303–porcine) dissolved in saline solution to deliver a 250 mlU/g dose. The saline control injection consisted of 0.02 ml saline solution (0.9% saline solution, pH = 7.2). For the ACTH and saline treatments, we injected salamanders with a 29-gauge needle into the coelom, lateral of the midline, near the body wall to avoid injury to organs. Immediately after salamanders received one of the three treatments, we placed salamanders in a clean 250 ml glass beaker filled with 80 ml of well water for 60 min. Each beaker had a Nalgene HPE plastic insert perforated at the bottom for drainage, inserted into the glass beaker to ease withdrawal of the salamander. After removing the salamanders, we measured their SVL (mm) and mass (g). We used an ANOVA to

compare CORT release rates across treatments ($\alpha = 0.05$) and conducted *post hoc* comparisons between treatment groups using Tukey's (HSD) test.

Background CORT level in our sample of well water was 0.67 pg/sample/ml. We obtained background CORT values from a sample of the same water we used for hormone leaching (without a salamander) by multiplying the measured CORT value (pg/ml) by the final volume in which we re-suspended the residue (0.35 ml for *E. nana* and 0.4 ml for *E. sosorum* and *E. tonkawae*) and then standardized the values by dividing by the volume of water that was obtained to get the background CORT yielding the final unit of pg/(reconstituted amount) sample/ml of water.

CORT response to agitation compared to baseline and extended CORT response in a wild-caught population.—We caught adult *E. nana* ($n = 12$ males; $n = 12$ females) on 9 September 2013 to examine the stress response of wild-caught salamanders to agitation. We randomly assigned salamanders to one of two treatments: (1) "baseline" CORT release rates for salamanders ($n = 12$) immediately after collection, or (2) agitation CORT release rates ($n = 12$). Here we define baseline as the CORT value of a salamander caught and immediately placed (non-manipulated) in a beaker for hormone collection. For the baseline treatment, we placed the salamanders in a 250 ml glass beaker with 80 ml of water from Spring Lake (background CORT = 0.14 pg/sample/ml) immediately after capture for 60 min. For the agitation treatment, we also immediately placed the salamander in the same sized beaker, but then we agitated the beaker for 1 min every 3 min (following Belden et al., 2007) over the entire 60 min leaching period. We used a Nalgene insert in the beaker to ease withdrawal of the salamander. We then recorded the sex (Gillette and Peterson, 2001) and SVL (mm) of all salamanders after collection of water-borne hormones. These salamanders were then returned to the sites where they were collected.

An independent group of wild-caught adult salamanders were brought back to SMARC to measure the amount of time needed for wild-caught *E. nana* to mount a CORT response to handling and captivity after transportation to the laboratory. We obtained male ($n = 18$) and female ($n = 18$) salamanders from the river and separated them into containers based on the time we caught them so that each container had salamanders caught within 20 min increments. Salamanders were transported to the SMARC in a cooler via a 10 min car drive. At the SMARC, we recorded the mass (g), SVL (mm), and sex of each salamander. We haphazardly (depending on collection time) assigned salamanders to one of three groups to measure CORT release rates starting at 1 h, 2 h, and 3 h ($n = 12$ for each treatment) after capture. Each salamander was placed in a Nalgene insert within a 250 ml glass beaker with 80 ml of water from Spring Lake (background CORT = 0.14 pg/sample/ml) for 60 min. We used an ANOVA to compare across the four time points and agitation, with sex as a factor ($\alpha = 0.05$), and conducted *post hoc* comparisons between treatment groups using Tukey's (HSD) test.

Extended CORT response to repeated sampling of a wild-caught population.—To examine CORT levels 96 h (4 d; 13 September 2013) and 192 h (8 d; 17 September 2013) after capture, we obtained follow up hormone samples using a portion of the previously collected male ($n = 5$) and female ($n = 11$) salamanders. After obtaining water-borne hormones using the same methods as above for 60 min, we sexed and measured the wet mass (g) and SVL (mm) of individuals.

Then, we placed two salamanders, one visibly smaller and one larger, into 9.46 l aquaria so that we could identify individuals for subsequent CORT collection. We used repeated measures ANOVA to examine the change in CORT from 96 h to 192 h ($\alpha = 0.05$).

Eurycea sosorum

We used adult second generation, captive-born salamanders at the SMARC, whereas at ASCC we used a combination of adult wild-caught, first and second generation captive-born salamanders. At SMARC salamanders were maintained on a 12:12 h light:dark cycle and were fed amphipods and blackworms *ad libitum*. At ASCC salamanders were on a natural light cycle from windows and fed blackworms *ad libitum*. Wild-caught *E. sosorum* were captured from Eliza Spring in Travis Co., Texas, USA (30.26428°N, 97.77014°W; WGS84).

CORT response to agitation compared to baseline in captive and wild-caught populations.—We obtained the "baseline" CORT release rates of SMARC captive salamanders ($n = 12$) on 2 July 2014 and ASCC captive salamanders ($n = 20$) on 9 December 2014. We also obtained the baseline CORT release rates for adult wild-caught salamanders ($n = 15$) on 24 July 2014. Upon capture, salamanders were immediately placed in 150 ml collection beakers with 100 ml of well water (background CORT = 0.19 pg/sample/ml) and a Nalgene insert for 60 min. We also examined whether captive adult salamanders show HPI responsiveness to agitation during hormone collection on 20 July 2014 for SMARC ($n = 13$) and 9 December 2014 for ASCC ($n = 13$). To collect the agitation data, we placed salamanders in individual 150 ml collection beakers with 100 ml of well water (background CORT = 0.19 pg/sample/ml SMARC; 0.21 pg/sample/ml ASCC) lined with a Nalgene insert. We agitated the salamanders for 1 min every 3 min for 60 min. At the ASCC we also took a second measure of CORT release rate using the salamanders that were agitated by transferring them in the Nalgene cup into a second clean beaker with another 100 ml of well water for 60 min. This allowed us to examine how long it takes for CORT to recover after a stressor. At the end of hormone collection period, we measured SVL (mm) of each salamander. We examined the change in CORT response among the captive-reared, wild-caught, and agitated treatments with an ANOVA ($\alpha = 0.05$) and conducted *post hoc* comparisons between treatment groups using Tukey's (HSD) test.

Short-term CORT response to repeated sampling of a captive population.—We examined whether *E. sosorum* mounted a CORT response over a short period and whether they show a stress response to repeated sampling during the water-borne hormone assay on 16 December 2013 at SMARC. We placed 12 adult female salamanders each into 150 ml glass beakers with 100 ml of well water (background CORT = 0.19 pg/sample/ml) with the Nalgene insert. We left the subjects in the beakers for 30 min and then used the Nalgene insert to move them to another set of beakers for another 30 min. We repeated this four times ending at 120 min. After the four water-borne hormone samples were obtained for each individual, we measured SVL (mm). We compared the CORT release rates of each individual over the four time periods using repeated measures ANOVA ($\alpha = 0.05$) and conducted *post hoc* comparisons between treatment groups using Tukey's (HSD) test.

Extended CORT response after handling of a captive population.—We examined the stress response to handling from 44 adult salamanders ($n = 20$ males and $n = 24$ females) on 27 May 2014 at SMARC. First, we measured SVL (mm) and weighed (g) all salamanders and assigned them to one of four treatments: (1) 0 h, (2) 1 h, (3) 2 h, and (4) 3 h ($n = 12$ for each treatment). We immediately placed individuals assigned to the 0 h treatment in hormone collection beakers after being handled and measured, whereas we held the other individuals in 236 ml containers until 1–3 h passed before placing them into collection beakers. We analyzed data with an ANOVA with LN CORT as a response variable and time elapsed since handling as main effect ($\alpha = 0.05$) and conducted *post hoc* comparisons between treatment groups using Tukey's (HSD) test.

Eurycea tonkawae

We used wild-caught *Eurycea tonkawae* from two sites within the Balcones Canyonlands Preserve in Bull Creek, Travis Co., Texas, USA (Hamilton: 30.40855°N, 97.83895°W; Franklin: 30.41901°N, 97.81270°W; WGS84). We were not able to collect enough salamanders in one day to do both studies at one population. After hormone samples were obtained for each experiment, we photographed salamanders against a standardized grid and measured body length (BL) as the mid-vertebral distance from the tip of the snout to the posterior insertion of the hind limbs using ImageJ (Rasband, 1997). We extrapolated SVL based on the equation (SVL = 1.032*BL + 0.896) derived from a prior comparison of SVL and BL ($n = 14$, $r^2 = 0.99$). At the ASCC captive hatchery, we used wild-caught salamanders that had been maintained in the lab for over a year on a natural light cycle and fed blackworms *ad libitum*.

CORT response to agitation compared to baseline in a wild-caught population and baseline for one captive population.—On 23 May 2014, we tested the HPI responsiveness of adult salamanders ($n = 15$) exposed to the agitation test versus "baseline" controls ($n = 17$) using the Franklin population. We placed each salamander individually in a 250 ml beaker with 100 ml of well water (background CORT = 0.19 pg/sample/ml) within a Nalgene insert for 60 min. For the agitation, we gently agitated the beakers for 1 min every 3 min during the 60 min of hormone leaching. For the control, we left the salamanders in the container and did nothing to them. We used a Student's t-test ($\alpha = 0.05$) to compare CORT values across treatments.

On 9 December 2014, we also obtained baseline CORT release rates from the ASCC population ($n = 14$) and compared those values to baseline CORT release rates ($n = 18$) obtained using data from the Franklin population but collected in 27 January 2014 (to compare values within the same season).

Short-term CORT response to repeated sampling of a wild-caught population.—On 11 March 2014, we examined if adult *E. tonkawae* ($n = 12$) mount a CORT response over a short time period and if they are stressed by repeated measurements. To test this, we placed salamanders from one wild population (Hamilton) into clean 250 ml beakers with 100 ml of well water (background CORT = 0.19 pg/sample/ml) within a Nalgene insert. We left the subjects in the beakers for 30 min and used the Nalgene insert to move them to another set of beakers for another 30 min. We repeated this four times ending at 120 min. We compared the CORT release rates of each individual across time periods using repeated measures ANOVA ($\alpha = 0.05$)

and conducted *post hoc* comparisons between treatment groups using Tukey's (HSD) test.

Extended CORT response after capture and handling of a wild-caught population.—On 7 March 2014, we examined the amount of time required for adult salamanders to mount a stress response to capture and handling. We caught salamanders ($n = 48$) from one population (Franklin) and placed individuals in one of four treatments: (1) 0 h, (2) 1 h, (3) 2 h, and (4) 3 h ($n = 12$ for each treatment). Individuals assigned to the 0 h treatment were immediately placed in hormone collection beakers, whereas the other individuals were placed in one of three 3.6 l containers depending on time of collection such that each container had individuals collected within 20 min increments for each treatment. They remained there until 1–3 h passed after the original time of capture (depending on the treatment group). Each salamander was placed in a clean 250 ml beaker with 100 ml of well water (background CORT = 0.19 pg/sample/ml) for 60 min. We analyzed data by an ANOVA with CORT release rates as a response and time elapsed since handling/capture as main effect ($\alpha = 0.05$) and conducted *post hoc* comparisons between treatment groups using Tukey's (HSD) test.

Hormone extraction, analyses, and validation

We stored all water samples at –20°C until we thawed them for extraction (Gabor et al., 2013a). All hormone extraction methods followed that of Gabor et al. (2013a) except that we used 4 ml of distilled water instead of millipore water to prime C18 solid phase extraction (SPE) columns (SepPak Vac 3 cc/500 mg; Waters, Inc.) Following extraction, we eluted columns with 4 ml of high-performance liquid chromatography grade methanol into borosilicate vials which we then evaporated under a gentle stream of nitrogen gas using an Evap-O-Rac (Cole-Parmer) over samples placed in a 37°C water bath. Following drying, we re-suspended the residue in 5% ethanol, then we vortexed for 1 min and then used 95% enzyme-immunoassay (EIA) buffer (Cayman Chemical Company, Inc.) for a final volume of 350 μl for *E. nana* and 400 μl for both *E. sosorum* and *E. tonkawae*. We measured CORT release rates in duplicate for all samples using a corticosterone EIA kit (Cayman Chemical Company, Inc.) on a spectrophotometer plate reader set to 405 nm (BioTek Powerwave XS).

We had previously validated the use of water-borne CORT for *E. nana* on these EIA plates (Gabor et al., 2013a). Here we validated the use of water-borne CORT collection method on EIA plates for *E. sosorum* and *E. tonkawae* using a pool sample of hormones from ten salamanders of each species following the methods of Gabor et al. (2013a) and dilutions as stated below. We examined parallelism of the serial dilution curve to determine whether the serial dilution of the pool sample matched that of the standards. We also examined quantitative recovery of water-extracted hormones to determine whether adding a known amount of CORT standard to the pooled sample resulted in recovery of that same additional amount of CORT.

We assessed parallelism of the serial dilution curve using one pooled control for each species run in duplicate. We constructed the log-logit transformed dilution curve using average percent maximum binding and pg/ml concentrations for five dilution samples (from 1:1 to 1:16 for *E. tonkawae*, and 1:1 to 1:64, excluding 1:8, for *E. sosorum*). We found that the dilution curve for CORT was not significantly different from the standard curve for *E. tonkawae* (compar-

ison of slopes, $t = -0.35$, df $= 8$, $P = 0.74$) and for *E. sosorum* (comparison of slopes, $t = 0.348$, df $= 9$, $P = 0.74$).

We determined the quantitative recovery of the pooled sample of water-borne CORT by spiking the pooled control samples for each species using each of the eight standards in addition to an unmanipulated pooled control sample. Based on the known amount of CORT in the standards and the pooled control sample, we determined expected recovery concentrations. The minimum observed recovery was 98% for *E. tonkawae* and 52% for *E. sosorum*. The recovery for *E. sosorum* might have been low because the CORT values overall were very low for this species, so recovery might have been less accurate. We found a linear relationship between observed and expected slopes for *E. tonkawae* (slope $= 0.99$; $F_{1,7} = 558.63$, $r^2 = 0.99$, $P < 0.0001$) and for *E. sosorum* (slope $= 1.02$; $F_{1,7} = 385.6$, $r^2 = 0.99$, $P < 0.001$).

For *E. nana,* the intra-plate variation was 3.16%, 2.48%, 2.50%, 1.49%, and 2.68% and the inter-plate variation was 35.28%. The high inter-plate variation was due to all values (pooled and regular samples) on one plate (data for the first ACTH experiment) being higher than the other four plates. Because we did not compare between experiments, we do not think that this detracts from our conclusions. The overall inter-plate variation was 21.67%, and the intra-plate variation was 3.74%, 0.95%, 9.37%, 15.44%, and 3.44% for *E. tonkawae.* For *E. sosorum,* the intra-plate variation was 12.19%, 3.82%, 20.09%, 8.40%, 1.23%, 5.79%, 6.65%, and 6.44%, with an overall inter-plate variation of 24.86%.

For analyses, we multiplied CORT release rates (pg/ml) by the final volume in which we re-suspended the residue (0.35 ml for *E. nana* and 0.4 ml for *E. sosorum* and *E. tonkawae*) and then standardized the values by dividing by the SVL of each individual such that CORT release rate units were pg/SVL/h. All CORT data were natural log transformed to meet the assumptions of normality. All data met assumptions of normality and homoscedasticity after transformation ($\alpha = 0.05$) and were analyzed using JMP v11.2 software (SAS Institute Inc.). Statistical analyses were performed on transformed data, but for ease of interpretation we present non-transformed CORT response values in the figures.

RESULTS

Eurycea nana

CORT release rates of males and females 60 min after capture in the laboratory did not significantly differ (t-test: $t = -0.29$, $P = 0.36$; male: $n = 8$, 1.59±0.24 pg/SVL/h; female: $n = 4$, 1.74±0.21 pg/SVL/h). However, we did examine the effect of sex on hormones when possible.

ACTH challenge in a captive population.—For the ACTH challenge, CORT release rates were significantly different across treatments (ANOVA: $F_{2,27} = 7.74$, $P = 0.002$; Fig. 1). CORT was higher in ACTH injection than in the non-injected control treatment (Tukey's HSD, $P = 0.002$) but was not significantly different from the saline control injection treatment ($P = 0.278$). Additionally, the saline control injection treatment was significantly different from the non-injected control treatment ($P = 0.049$).

CORT response to agitation compared to baseline and extended CORT response in a wild-caught population.—CORT release rates were significantly different from baseline (0 h, at capture) through 3 h after capture, and there was no effect of sex or interaction with sex (ANOVA: Treatment: $F_{4,50} =$
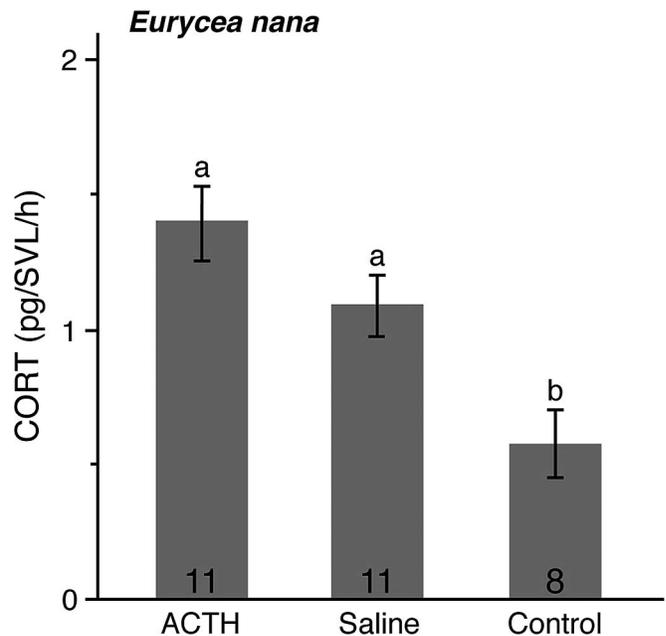

Fig. 1. Mean corticosterone (CORT) release rates (±SE) of captive *Eurycea nana* in response to a 250 mIU/g dose of ACTH injection, saline control injection, and non-injected control. Numbers on bars are the sample size for each treatment. Letters indicate significant differences among treatment groups from Tukey's HSD comparisons ($P < 0.05$).

3.34, $P = 0.017$; Sex: $F_{1,50} = 0.017$, $P = 0.89$; Treatment x Sex: $F_{4,50} = 1.67$, $P = 0.17$; Fig. 2A). CORT release rates at 3 h were significantly higher than baseline CORT (Tukey's HSD: $P = 0.008$), but no significant differences occurred among all other times.

Extended CORT response to repeated sampling of a wild-caught population.—CORT release rates did not significantly differ between 96 h (4 d) and 192 h (8 d) after capture (Matched pairs t-test: $t_{14} = -1.64$, $P = 0.12$; Fig. 2B). We also did not find an effect of sex if we ran a repeated measures ANOVA (rm ANOVA: Treatment: $F_{1,13} = 1.60$, $P = 0.23$; Treatment x Sex: $F_{1,13} = 0.673$, $P = 0.43$). One sample was lost from the 192 h treatment.

Eurycea sosorum

Males and females did not significantly differ in CORT release rates (t-test: $t = -0.54$, $P = 0.61$; male: $n = 6$, 1.08±0.06 pg/SVL/h; female: $n = 6$, 1.02±0.26 pg/SVL/h), so we combined data for both sexes.

CORT response to agitation compared to baseline in captive and wild-caught populations.—CORT release rates differed across treatments (ANOVA: $F_{2,39} = 19.43$, $P < 0.0001$; Fig. 3A). Baseline CORT was significantly higher in wild-caught salamanders than in both SMARC captive salamanders and SMARC agitation (Tukey's HSD: $P < 0.001$). Baseline CORT in the laboratory at SMARC did not differ from SMARC agitation ($P = 0.192$). Baseline CORT from ASCC salamanders was significantly lower than agitation CORT at the ASCC (Student's t-test: $t_{31} = -4.69$, $P < 0.0001$; Fig. 3B). The same agitation CORT at the ASCC was significantly higher than CORT release rates 1 h post agitation (rm ANOVA: $F_{3,9} = 6.63$; $P < 0.0001$; Fig. 3C).

Short-term CORT response to repeated sampling of a captive population.—We did not detect any significant difference
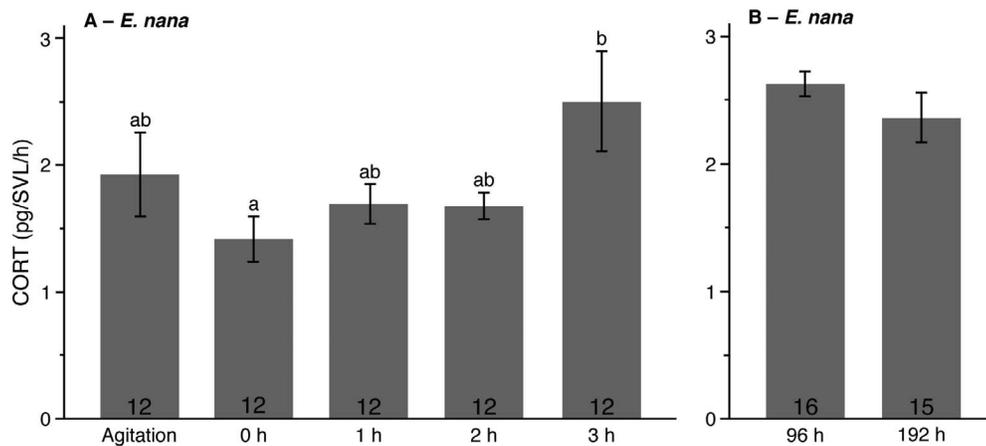
Fig. 2. Mean corticosterone (CORT) release rates ($\pm$SE) of wild-caught *Eurycea nana* (A) after agitation in the field versus CORT release rates immediately upon capture without agitation (0 h) and 1–3 h after capture and transportation to the laboratory, and (B) 96 h and 192 h after capture and maintenance in the laboratory. Numbers on bars are the sample size for each treatment. Letters indicate significant differences among treatment groups from Tukey's HSD comparisons ($P < 0.05$).

among CORT values every 30 min for the same individuals (rm ANOVA: $F_{3,9} = 0.43$; $P = 0.34$; Fig. 4A).

Extended CORT response after handling of a captive population.—CORT release rates did not significantly differ immediately after handling through 3 h after handling (ANOVA: $F_{3,40} = 0.88$, $P = 0.46$; Fig. 5A).

Eurycea tonkawae

We were not able to accurately sex individuals of this species.

CORT response to agitation compared to baseline in a wild-caught population and baseline for one captive population.—CORT release rates after agitation were significantly higher than the "baseline" CORT release rates (Student's t-test: $t_{30} = -1.98$, $P = 0.05$; Fig. 3D). "Baseline" CORT release rates for the

ASCC population were significantly higher than the "baseline" field CORT release rates (Student's t-test: $t_{30} = -3.62$, $P = 0.001$; Fig. 3E).

Short-term CORT response to repeated sampling of a wild-caught population.—CORT release rates were significantly different across the 30 min periods repeatedly measured for each individual (rm ANOVA $F_{3,27} = 31.99$; $P = 0.009$; Fig. 4B). The 30 min CORT release rates did not differ from 60 min (Tukey's HSD: $P = 0.638$), but were significantly different from 90 min ($P = 0.0002$) and 120 min ($P < 0.0001$). The 60 min CORT release rates differed from 90 min ($P = 0.005$) and 120 min ($P = 0.0001$), but the 90 min CORT release rates did not differ from 120 min ($P = 0.515$).

Extended CORT response after capture and handling of a wild-caught population.—CORT release rates differed significantly
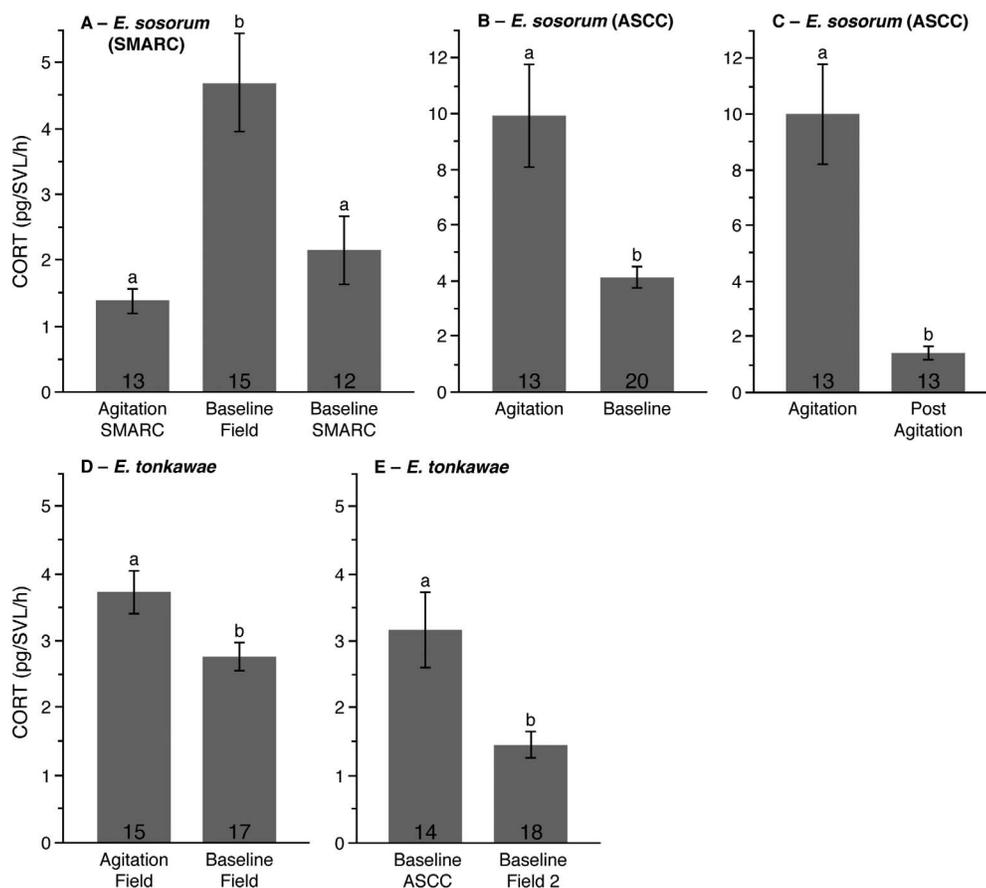

Fig. 3. Mean corticosterone (CORT) release rates ($\pm$SE) for (A) agitation versus baseline for San Marcos Aquatic Resources Center (SMARC) captive *Eurycea sosorum*, (B) agitation versus baseline for Austin Salamander Conservation Center (ASCC) captive *Eurycea sosorum*, (C) agitation (same as prior) versus post agitation for ASCC captive *Eurycea sosorum*, (D) agitation versus baseline for wild-caught population (field) *Eurycea tonkawae*, and (E) baseline ASCC and a second wild-caught population of *Eurycea tonkawae*. Numbers on bars are the sample size for each treatment. Letters indicate significant differences among treatment groups from Tukey's HSD comparisons or Student's t-test ($P < 0.05$).
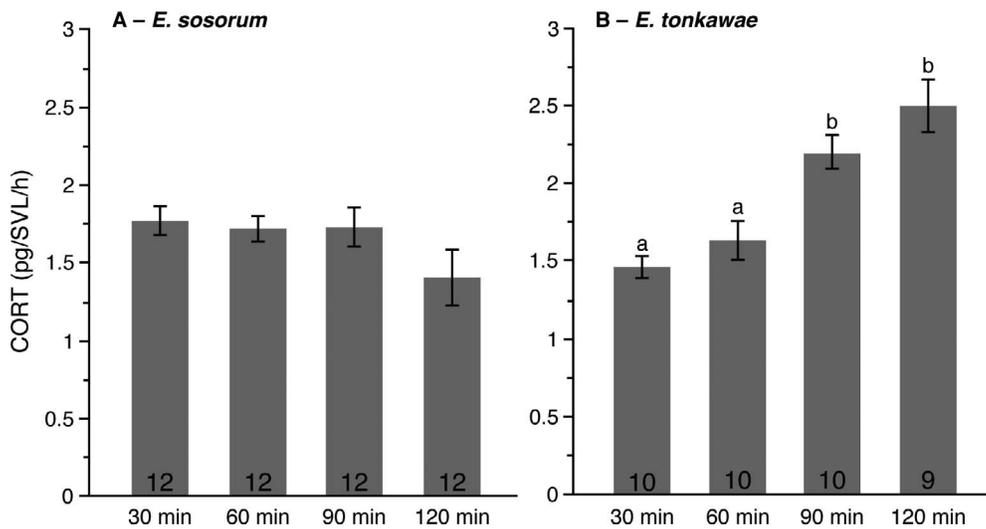
Fig. 4. Mean corticosterone (CORT) release rates (±SE) after repeated sampling for 30 min up to 120 min for (A) captive *Eurycea sosorum* and (B) wild-caught *Eurycea tonkawae*. Numbers on bars are the sample size for each treatment. Letters indicate significant differences among treatment groups from Tukey's HSD comparisons ($P < 0.05$).

across time after handling and capture (ANOVA: $F_{3,42} = 4.57$, $P = 0.007$; Fig. 5B). After 1 h of collection and handling, the CORT release rates were greater than 3 h after handling (Tukey's HSD: $P = 0.009$) but did not significantly differ from the 0 h CORT release rates ($P = 0.15$) and 2 h CORT release rates ($P = 0.08$). No significant difference was detected between 0 h and 2 h ($P = 0.99$) or between 0 h and 3 h ($P = 0.58$), and 2 h and 3 h CORT release rates did not differ ($P = 0.77$).

DISCUSSION

We found that most of the populations we examined showed HPI responsiveness: captive *E. nana* (injection [saline or ACTH]), *E. sosorum* (agitation, ASCC population), and wild *E. tonkawae* (agitation) all showed HPI responsiveness. The captive SMARC population of *E. sosorum* and the wild-caught population of *E. nana* did not show HPI responsiveness to agitation; however, wild-caught *E. nana* did show HPI responsiveness to capture and handling by 3 h, indicating that it can increase CORT release rates and thus is not likely chronically stressed. CORT values for *E. nana* stayed high through 8 d later, suggesting that this population takes over a week to acclimate to being in the laboratory. The captive-reared SMARC population of *E. sosorum* also did not show a change in CORT release rates during repeated measures from 30–120 min, whereas wild-caught *E. tonkawae* had elevated CORT release

rates within 90–120 min during the short-term study and continued to have elevated CORT release rates up to 1 h after capture and handling in the long-term study. In contrast, CORT release rates for captive (ASCC) *E. sosorum* decreased 1 h after agitation, indicating that they were recovering quickly. When comparing CORT release rates of the captive SMARC population of *E. sosorum* to wild-caught *E. sosorum*, we found that CORT release rates of the latter were higher than CORT of the former. Whereas for *E. tonkawae*, the wild-caught baseline CORT release rates were lower than the baseline values for the ASCC captive population. Taken together, all but the captive reared SMARC population of *E. sosorum* showed some level of HPI responsiveness.

We pose three *a posteriori* hypotheses as to why the SMARC population of *E. sosorum* did not show HPI responsiveness to agitation: (1) the population takes longer than 3 h to mount CORT in response to stressors, (2) the population was in homeostatic overload, or (3) the population has habituated to basic handling (Cyr and Romero, 2009). We suspect that longer response times for elevated CORT for this population are unlikely given the response times we have observed for other populations of *E. sosorum* and their congeners (this study), as well as response times documented for other salamander species (see next paragraph). Homeostatic overload is another possibility, and may be the result of disease; this population is in the process of recovering from infection
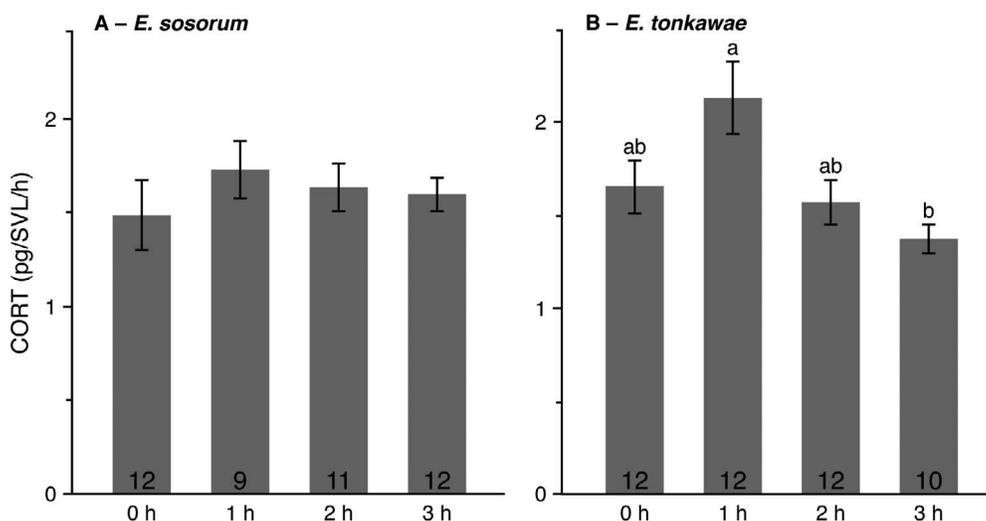

Fig. 5. Mean corticosterone (CORT) release rates (±SE) immediately after capture and handling (0 h) and 1–3 h following capture for (A) captive *Eurycea sosorum* and (B) wild-caught *Eurycea tonkawae*. Numbers on bars are the sample size for each treatment. Letters indicate significant differences among treatment groups from Tukey's HSD comparisons ($P < 0.05$).

by an unidentified microsporidia that started a year prior (V. Cantu, pers. comm.). However, high survival rates and lack of other visible signs of extended periods of homeostatic overload (e.g., emaciation) leads us to conclude that homeostatic overload is not the most likely explanation. Alternatively, the SMARC population of *E. sosorum* may be habituated to handling. While the other captive population (ASCC) does not appear to be habituated (they had an HPI response to agitation), this difference could be explained by differences in husbandry practices between the two facilities and/or that these have not been previously experimentally tested. Presenting the SMARC population with an alternative stressor (such as stimulus from a high-risk predator) may better help us assess its ability to show HPI responsiveness and determine whether the lack of response is due to habituation to handling. Stimulus from a high-risk predator may be a suitable stressor, as both *E. sosorum* and *E. nana* exhibit behavioral responses to the chemical cues of *M. salmoides* (DeSantis et al., 2013; Davis and Gabor, 2015) while *E. nana* also exhibits an HPI response (Davis and Gabor, 2015).

More broadly, studies on HPI responsiveness to external stressors in other species of salamanders range from 30–170 min. Captive males of Ocoee Salamander (*Desmognathus ocoee*) showed elevated plasma CORT after 30 min of restraint (similar to agitation) and females took 60 min (Woodley and Lacy, 2010). Field-caught Allegheny Mountain Dusky Salamanders (*D. ochrophaeus*) require 30–60 min to elevate plasma CORT levels in response to capture and handling but only during the non-mating season (Ricciardella et al., 2010). For breeding Spotted Salamanders (*Ambystoma maculatum*), plasma CORT was elevated 30 min after capture (Homan et al., 2003). These results fall within the 60 min range found in our study for *E. tonkawae*. Furthermore, a prior study of *E. nana* showed a shorter time to mount a stress response, whereby elevated CORT was observed within 1 h after exposure to the chemical cue of a fish predator (Davis and Gabor, 2015). Eastern Newts (*Notophthalmus viridescens*) caught during the mating season, brought to the laboratory, and handled showed elevated CORT release rates (using water-borne hormones) from 110–170 min after capture (Reedy et al., 2014). Given that most species/populations of salamanders showed HPI responsiveness in under 3 h, we do not expect HPI responsiveness to an external stressor to exceed 3 h.

We did not find any difference in CORT between a control injection and ACTH in our study. While this lack of a significant difference could be due to experimental or sampling error, another possibility is that *E. nana* takes longer than an hour to respond to an ACTH challenge, as is the case for some other amphibians. For example, Fijian Ground Frogs (*Platymantis vitiana*) took 6 h to show elevated urinary CORT (Narayan et al., 2010), while Southern Toads (*Anaxyrus* [*Bufo*] *terrestris*) increased plasma CORT to ACTH in 10 h (Hopkins et al., 1999). However, in salamanders, responses to ACTH challenges have been documented as short as 30–60 min using similar ACTH concentrations to our test (*Ambystoma jeffersonianum*; Chambers et al., 2011), 60 min using a plasma CORT assay (*Taricha granulosa*; Moore and Miller, 1984), and 110 min using a water-borne hormone assay (*N. viridescens*; Reedy et al., 2014). An alternative hypothesis as to why we did not see a response to ACTH is that captive *E. nana* from SMARC were stressed prior to our experimental injection—a possible commonality with *E. sosorum* from the same facility, which failed to mount a response to agitation. Thus, future studies should track CORT levels for a longer period of time to ensure a lagged CORT response is not mistaken for lack of a CORT response from an ACTH challenge.

In our comparisons of baseline CORT release rates between wild-caught and captive individuals, we did not find a consistent pattern among all species. For both populations of *E. sosorum,* we found that baseline CORT release rates in both laboratory populations were lower than in the field. Similarly, Gabor et al. (2013a) found that *E. nana* had lower CORT release rates in the laboratory than in the field. However, for *E. tonkawae* we found the opposite: CORT release rates were higher in the laboratory than in the field. Lower CORT release rates in the laboratory may be an outcome of no predation stress and *ad libitum* feeding, as well as a consequence of habituation to captive conditions. Wild-caught populations may have higher baseline CORT values compared to laboratory populations due to limited food intake and constant exposure to predators and other environmental stressors. In the case of *E. tonkawae*, we compared CORT release rates of the laboratory population with a rural population. It may be that the rural population has ample resources, lower densities, and fewer predators (e.g., there are no fish predators in shallow streams). The laboratory population might have higher CORT due to being maintained at higher density or some other factor associated with their captive maintenance. This contrasts with wild populations–*E. nana* and *E. sosorum* that are exposed to fish predators and surrounded by urbanization.

We found higher CORT release rates of wild-caught *E. nana* in the laboratory 3–192 h after capture and transportation to the laboratory than CORT release rates for the wild-caught salamanders immediately after capture, indicating that >192 h (8 d) is required for *E. nana* to recover from capture after being brought into the laboratory. Thus, for salamanders that are removed from the wild, either for captive rearing purposes or experimentation (e.g., behavioral studies), a waiting period of more than eight days is recommended prior to subjecting the individuals to additional stressors.

Our results highlight the need to obtain a timeline for CORT to mount so that peak rates are captured during the leaching phase and individuals return to "baseline" levels before proceeding with other experiments or husbandry practices that may stress individuals further. Repeated measures of CORT (up to at least 3 h) would ensure capturing the time needed for CORT to increase. If a population shows HPI responsiveness to agitation, then it may not be necessary to carry out the more invasive ACTH challenge; otherwise, a more extreme stressor may be required such as exposure to the chemical cues of a predator. We also propose three additional studies: (1) examine how long it takes to return to baseline after a stressor (we did this in our study with ASCC, and it showed that this population of *E. sosorum* takes 1 h to recover), (2) examine changes in CORT release rates and mass of wild-caught animals for up to two weeks after they have been brought to the laboratory, and (3) examine the HPI responsiveness to multiple stressors, not just one as we have done here. To fully assess chronic stress, Dickens and Romero (2013) suggested that the HPA/HPI response needs to be evaluated in response to multiple stressors (e.g., chemical cues of predators, low quality water, pesticides, or disease). Data from such studies may allow for further understanding of the HPI responsiveness, provide further insight into the relationship between physiological health and CORT release rates, and allow managers to determine when research can be performed on captive animals without the threat of sending the animals into homeostatic overload by imposing additional stressors before their HPI response from capture and captivity has worn off.

Despite the promise of this method being non-invasive and not prohibitively expensive (~\$10/sample), it is important to note that water-borne hormone methods yield quite a lot of variation in the data. We believe that one of the reasons we did not see a significant response to agitation in some of the data sets is because our samples sizes were low and variation was higher than anticipated. When we first started using this method we thought we could use similar samples sizes to others who have used plasma samples ($n \geq 4$, for example; Hopkins et al., 1999) but we quickly found that this was not so. Using the data from our agitation versus baseline CORT data from *E. tonakwae* in the field, we estimated the power to detect the minimum difference (0.26) that we found when $\alpha = 0.05$, and the root mean square from our data (0.35). We found that our data ($n = 15$–16 per treatment) had a power of 57% to detect a significant difference. Below $n = 13$ per treatment, we had a power of less than 50% to detect a significant difference. To detect a significant difference in agitation response in *E. sosorum* we would have needed a sample size of 14 per treatment (ours was 12). From this we infer that it would be safer to have sample sizes of at least 15 (sometimes samples are lost or the values are unreadable) for each treatment to have sufficient power to detect a difference. In sum, these points indicate that this research requires larger sample sizes and a bit more money than radioimmunoassay and EIA using plasma. However, with the ability to obtain repeated measures for small animals the water-borne hormone method also has many advantages.

Water-borne hormone collection methods can be useful for assessing HPI responsiveness of both captive and wild aquatic salamanders. In captive populations, these methods may aid in evaluating whether husbandry conditions are stressing captive populations, and with further development water-borne hormone collection methods can be used for examining reproductive hormones to aid in promoting reproduction in captivity. In wild populations, these methods can be used to assess differences in CORT release rates among populations with varying levels of natural and anthropogenic stressors such as pathogens (e.g., Gabor et al., 2013b, 2015), introduced predators (Davis and Gabor, 2015), and factors associated with urbanization. One limitation with using water-borne hormones is the higher variation in water-borne CORT values than for plasma CORT. However, whereas plasma CORT would require the sacrifice of our study animals, the water-borne method is non-invasive and repeated samples can be obtained from the same individuals. Thus, these methods allow scientists or managers to work with rare, threatened, and endangered species where invasive sampling would not be permitted. By following a given population over time it may be possible to detect when stress is increasing, allowing management practices (in the case of captive or otherwise managed populations) or conservation actions to be implemented.

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