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ABSTRACT

Amphibian populations are declining globally, so understanding how individuals respond to anthropogenic and environmental stressors may aid conservation efforts. Using a non-invasive water-borne hormone assay, we measured the release rates of two glucocorticoid hormones, corticosterone and cortisol, in Rio Grande Leopard frog, *Rana berlandieri*, tadpoles. We validated this method pharmacologically and biologically using an adrenocorticotropic hormone (ACTH) challenge, exposure to exogenous corticosterone, and an agitation test. We calculated the repeatability of hormone release rates, the recovery time from an acute stressor, and explored rearing methods for tadpoles. Tadpole corticosterone release rates increased following an ACTH challenge, exposure to exogenous corticosterone, and agitation, validating the use of water-borne hormone methods in this species. After exposure to an acute stressor via agitation, corticosterone release rates began to decline after 2 h and were lowest after 6 h, suggesting a relatively rapid recovery from an acute stressor. Tadpoles reared in groups had higher corticosterone release rates than tadpoles reared individually, and lost mass by Day 7, while tadpoles reared individually did not show a stress response, therefore either rearing method is viable, but have differing physiological costs for tadpoles. Repeatability of corticosterone release rates was moderate to high in *R. berlandieri* tadpoles, indicating that this species can show a response to selection and potentially respond to rapid environmental change. Our results show that the water-borne hormone assay is a viable way to measure glucocorticoids in this species and is useful in the field of conservation physiology for rare and endangered species.

1. Introduction

Continued human population growth, urbanization, and other anthropogenic changes pose a significant threat to global biodiversity (McKee et al., 2003), contributing to the 6th mass extinction (Barnosky et al., 2011; Ceballos et al., 2015). Amphibians are the most imperiled vertebrate class, with an estimated 43% of species declining in numbers (Clulow et al., 2014; Collins and Halliday, 2005; Grant et al., 2016; Wake and Vredenburg, 2008). Stressors that contribute to amphibian population decline include global climate change, invasive species, over exploitation, emerging infectious diseases, pesticides/pollution, and habitat loss/alteration (Blaustein et al., 2010; Collins and Storfer, 2003; Hof et al., 2011; Wake and Vredenburg, 2008).

Measuring glucocorticoid (GC) hormones associated with the stress response in vertebrates, provides a way to quantify physiological responses to stressors. The stress response is one of the mechanisms organisms use to maintain physiological stability (homeostasis) during perturbations or to cope with changing environments (McEwen and Wingfield, 2003). The higher vertebrate neuroendocrine stress response involves the hypothalamic-pituitaryadrenal axis (hypothalamic-pituitary-interrenal, HPI, axis in amphibians; Cyr and Romero, 2009). When a perturbation is perceived as a stressor by the brain, the hypothalamus secretes corticotropin releasing hormone (CRH) which induces the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH is transported by blood to the adrenal cortex, which then releases glucocorticoids (GCs) (Denver, 2009). Physiological changes during a stress response include increased circulating levels of GCs above normal "baseline" levels to promote gluconeogenesis and to mobilize energy (Hau et al., 2016; Romero et al., 2009). Corticosterone is the main glucocorticoid associated with stress in amphibians (Idler, 1972) and while many studies focus on corticosterone in amphibians (Belden and Kiesecker, 2005; Belden et al., 2010; Glennemeier & Denver, 2002a,b; Narayan et al., 2010), cortisol has been measured in multiple species of amphibians but it has not consistently been studied (Krug et al., 1983; Baugh et al., 2018; Santymire et al., 2018). A short-term elevation of GCs in response to an acute stressor can be advantageous as it mediates

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the mobilization of energy stores (Sapolsky et al., 2000). However, unpredictable and long-term perturbations can lead to chronic stress, which is associated with persistently elevated or down regulated GC levels which can be deleterious (Romero et al., 2009). Elevated levels of corticosterone can negatively affect growth in tadpoles, alter morphology, and shorten time to metamorphosis (Crespi and Warne, 2013; Denver et al., 1998; Glennemeier and Denver, 2002a; Hu et al., 2008), and reduced mass at metamorphosis can affect growth and survivorship later in life (Cabrera-Guzman et al. 2013; Chelgren et al., 2006; Crespi and Warne, 2013; Earl and Whiteman, 2015; Rohr et al., 2013).

Understanding how stressors affect organisms, how individuals respond to stressors, and how flexible individuals are in response to environmental changes is at the core of conservation physiology (Wikelski and Cooke, 2006). Until recently, methods to measure GCs and other hormones were invasive or required sacrificing individuals (reviewed by Baugh et al., 2018; Gabor et al., 2016; Sheriff et al., 2011). Conservation studies often focus on threatened and endangered species, therefore being able to non-invasively measure hormone levels, to minimize stress from handling/blood-sampling or the need for sacrifice, is imperative. Narayan et al. (2010), Narayan and Hero (2013) validated a novel, non-invasive, method for measuring steroids in amphibians using urinary corticosteroid metabolites in adult frogs. Recently, several studies have validated the use of a water-borne hormone collection protocol for both larvae and adults of several amphibian species of varying sizes and this method works in the field and laboratory (Baugh et al., 2018; Gabor et al., 2013, 2016). This water-borne hormone collection method was developed for use in fish, and measures steroid hormones passively diffused into the water via gills, urine, and feces (Scott et al., 2008). More recently, Santymire et al. (2018) examined GC concentrations collected from swabs of amphibian skin secretions but did not fully validate the method. Following this, Hammond et al. (2018) validated the use of salivary secretions to measure GCs in three species of adult frogs, but this method is limited to adults of a relatively large size and requires more handling. These non-invasive methods facilitate repeated sampling from the same individual and by utilizing a repeated measures design, one can measure within and among individual variation of hormone levels and stress responses and calculate the repeatability of hormone levels.

The repeatability of hormone levels provides insight into how individuals respond to changing environments or stressors (Hau et al., 2016; Lendvai et al., 2014) and is considered an upper bound estimate of heritability of a trait (Lessells and Boag, 1987; but see Dohm 2002). Therefore, it is important to include repeatability analysis of hormonal traits into experimental designs. In a review that had a limited sample size, amphibians show higher repeatability for baseline and stress-induce GC levels compared to other taxa (Schoenemann and Bonier, 2018). An experimental design that allows for repeated measures from the same individual often requires tagging or marking organisms (Bainbridge et al., 2015), however, the additional handling and invasive procedures of marking can contribute to the stress of individuals. Housing subjects individually alleviates the need for marking, but it is unclear whether this also contributes to stress because some species of amphibians, especially at the larval stage, form aggregations. Gabor et al. (2013, 2016) developed a non-invasive water-borne method for measuring physiological responses to stressors in amphibians and validated the use of water- borne hormones and the positive relationship between water-borne CORT and plasma CORT in multiple amphibian species. Here we provide additional validations to test the efficacy of this method for an additional species, as circulating GC levels show great variability within and between individuals (Miles et al., 2018; Schoenemann and Bonier, 2018). We validated the water-borne hormone collection technique in the Rio Grande leopard frog, *Rana berlandieri*, pharmacologically using both an adrenocorticotropic hormone (ACTH) challenge and exposure to exogenous corticosterone, and biologically using an agitation test. Individuals should show elevated corticosterone release rates after each challenge, as ACTH stimulates the production of glucocorticoids, resulting in elevated corticosterone levels in several frog species (Baugh et al., 2018; Nakagawa and Schielzeth, 2010; Narayan et al., 2010; Narayan and Hero, 2011; Narayan et al., 2011), exogenous corticosterone is absorbed through the amphibian skin and results in increased endogenous GCs in tadpoles of several frog species (Belden et al., 2005; Glennemeier & Denver, 2002a,b; Middlemis-Maher et al. 2013), and agitation elevates corticosterone levels in several species of amphibians (Belden et al., 2003; Belden, et al., 2010; Chambers et al., 2011; Gabor et al. 2016). Further, we examined the repeatability of water-borne corticosterone release rates and the rate of recovery from a stressor using repeated sampling of the same individuals. Lastly, we compared two rearing techniques that facilitate repeated measures without relying on invasive marking techniques.

2. Methods

2.1. Study species

Rio Grande leopard frogs, *Rana berlandieri*, are common anurans in the *Rana pipiens* complex that are found throughout Northeastern Mexico and Southern Texas (Zaldívar-Riverón et al., 2004). We collected egg masses of *R. berlandieri* from an ephemeral pond in San Marcos, Texas on 23 February 2017 (3 masses) and 5 March 2018 (4 masses), (29°52′28.86″N, 97°57′45.86″W) and transported half of each mass back to our laboratory on the Texas State University Campus. Egg masses collected in 2017 were used for the exogenous corticosterone experiment conducted in 2017 and egg masses collected in 2018 were used for the ACTH challenge experiment that was conducted in 2018 (see below). In both years, we reared the eggs in de-chlorinated, aged, tap water until tadpoles were free swimming (approximately 1 week from collection of eggs). Once tadpoles were free swimming, we mixed tadpoles from each egg mass, and housed tadpoles in groups of 12 in 6 L plastic tanks filled with aged de-chlorinated water at 19 °C. We also collected free swimming *Rana berlandieri* tadpoles from an artificial pond at the USFWS San Marcos Aquatic Resources Center (SMARC) in San Marcos, Texas (29°50′26.39″N, 97°58′36.17″W) on 8 March 2018 for use in the agitation/recovery and housing experiments. In both years, we fed tadpoles a mixture of spirulina powder and Tetramin fish flakes in an agar base *ad libitum,* housed them under a natural light 14L:10D cycle, and changed water at least once per week and as needed. All protocols and housing were approved by the Institutional Animal Care and Use Committee of Texas State University (IACUC #201563714 and #5636).

2.2. Validation-ACTH challenge on corticosterone and cortisol & exogenous corticosterone

To pharmacologically validate water-borne hormone collection in *Rana berlandieri*, we conducted an ACTH challenge on free-swimming tadpoles that were reared in the laboratory from egg masses (eggs collected 5 March 2018, n = 17; Gosner stages 30–35; Gosner, 1960) on 13 July 2018. We weighed each tadpole one day prior to the ACTH challenge and used the mass to calculate individual ACTH doses for the experiment (Mean ± SE: 1.218 ± 0.088 g). We collected "baseline" water-borne hormones from all tadpoles using a non-invasive water-borne hormone method (Gabor et al., 2016). Briefly, we placed each tadpole in a clean plastic insert (a perforated plastic lab bottle with the top cut off to facilitate removal of tadpoles from beakers) in a 250 ml glass beaker filled with 100 ml of spring water for 60 min. We wore non-powdered nitrile gloves throughout the hormone collection process and cleaned beakers and inserts with 95% ethanol and rinsed them with de-ionized (DI) water before each use. Immediately following the collection of baseline hormones, we intraperitoneally injected each tadpole with a mass specific dose of 0.5 μg ACTH (Sigma Chemical Co., A-0298) per gram bodyweight, dissolved in Ringer's solution, using a 31-

gauge needle on a 0.3 cc syringe. Similar doses have been used in other species (Baugh et al., 2018; Nakagawa and Schielzeth, 2010; Narayan et al., 2010; Narayan and Hero, 2011; Narayan et al., 2011). Immediately following injection, we collected water-borne hormones following the methods we used to collect baseline levels. Each sample was used to measure corticosterone and cortisol release rates (one cortisol value had > 34 CV so was removed from analysis) because Baugh et al. (2018) found substantial levels of cortisol in water-borne hormones and plasma water of frogs. For a pooled sample, water-borne cortisol started at 998.45 pg/ml pre-ACTH injection and decreased to 193.6 pg/ml post-ACTH injection, when measured using an HPLC-MS (Baugh et al., 2018).

We also exposed free-swimming tadpoles (Gosner 26–29; Mean ± SE: 0.316 ± 0.012 g), that were reared in the laboratory from egg masses (collected 23 February 2017) to exogenous corticosterone (Sigma Chemical Co. 27840) on 17–23 May 2017. We filled twenty, 5.7 L polypropylene shoe boxes each with 3 L aged, de-chlorinated, water. We had 10 control containers (dosed with 75 µl ethanol vehicle) and dosed 10 containers with 75 µl of 5 mM stock corticosterone solution dissolved in ethanol to create a final concentration of 43,308 ng/L (125 nM exogenous corticosterone using the same dose as Glennemeier and Denver, 2002b). The volume of ethanol added to each tank was 0.0025% of the total water volume. We haphazardly assigned 4 tadpoles to each container in each treatment (n = 40 per treatment) and then added the appropriate treatment. We reared tadpoles in treatments for 7 days at 19 °C with water changes every third day and reapplication of hormone/control treatments. Water-borne hormones were then collected on day 7 from 2 random tadpoles from each tub for each treatment (n = 20 per treatment) following collection methods of Gabor et al. (2016) and outlined above. After hormone collection, we weighed each tadpole.

2.3. Validation-agitation stress test, recovery, and repeatability

To biologically validate water-borne hormone collection in *Rana berlandieri*, we conducted a standard agitation test and then quantified recovery rate using a repeated measures design on 14 March 2018. We used free swimming *R. berlandieri* tadpoles (Gosner 26–29; Mass ± SE: 0.893 ± 0.078 g) from SMARC (collected 8 March 2018, see above). For the agitation test, we placed tadpoles (n = 20) in individual clean plastic perforated inserts within 250 ml beakers filled with 100 ml of spring water then manually agitated the *R. berlandieri* tadpoles following Gabor et al. (2016). Briefly, tadpoles in the beakers were placed in a cardboard box with dividers and then the box was manually agitated for 1 min, every 3 min, for 60 min total. We then removed the insert with the tadpole, saved the water samples, and then moved tadpoles to a new beaker with fresh 100 ml of spring water to collect the first "recovery" hour of water-borne hormones. We collected agitation and then recovery water-borne hormones for each tadpole for 6 subsequent hours, collecting 7 hourly hormone samples (one from each beaker) from each tadpole. After the last hormone collection, we weighed each tadpole. We processed hormone samples from n = 14–17 individuals per time step (some samples were lost due to spillage, and we were limited on plate space due to minimal funding).

2.4. Non-invasive rearing conditions: individually vs. in groups

To examine whether housing conditions affect stress levels, we explored the effects of two different rearing methods using *R. berlandieri* tadpoles from SMARC (collected 8 March 2018). On 3 April 2018, we randomly assigned tadpoles (Gosner 26–29; Mean ± SE: 1.344 ± 0.092 g) to one of two housing treatments: (1) individually housed in clear plastic polyethylene cups (Fig. 1C) filled with 0.5 L conditioned water (n = 24) or (2) housed in groups of 6 individuals in 5.7 L polypropylene shoeboxes filled with 3.0 L aged, de-chlorinated, water (4 replicates, n = 24 tadpoles in total), with the tadpoles isolated from each other with fiberglass screening to allow contact by visual and chemical cues (Fig. 1A, B). We reared all of the tadpoles in a growth chamber set at 21 °C with a 14L:10D cycle. We allowed tadpoles 2 days to recover from being moved from their group housing to individual spaces to assay "baseline" hormone levels. After 2 days in treatments, we collected baseline corticosterone release rates from all the tadpoles following Gabor et al. (2016) and outlined previously. We collected baseline corticosterone release rates from each tadpole again after 7 days in the housing treatments and then immediately conducted an agitation stress test (see methods above) on each tadpole. We then weighed each tadpole. We analyzed data from 21 tadpoles reared individually and 19 tadpoles reared in groups (several samples were lost due to test tube breakage).

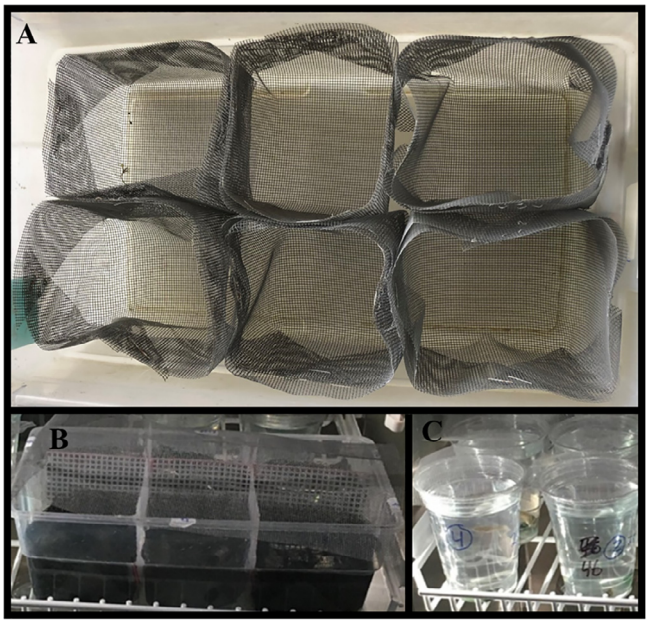

Fig. 1. Experimental housing set up for individual vs. group reared tadpoles of *Rana berlandieri*. (A) and (B) are top and side views, respectively, for group housing, and (C) are individual housing containers.

2.5. Hormone extraction, reconstitution, and enzyme immunoassays (EIA)

We stored water-borne hormone samples at −20 °C until we thawed them for extraction following methods of Gabor et al. (2016). We extracted corticosterone (and cortisol) from water samples following Gabor et al. (2016) by pulling water samples under vacuum through Tygon tubing into C18 solid phase extraction (SPE) columns (SepPak Vac3 cc/500 mg; Waters, Inc., Milford, MA, USA) primed with 100% HPLC grade methanol (4 ml) and distilled water (4 ml). Following extraction, we eluted columns with 4 ml 100% HPLC grade methanol into borosilicate vials, which we then evaporated under a gentle stream of nitrogen gas (approx. 2 h) while samples were placed in a hot-water bath (37 °C) to facilitate evaporation of the methanol. Following drying, we re-suspended the residue in 5% ethanol (95% lab grade) and 95% EIA buffer to a total volume of 300 or 600 µl depending on the experiment. The resuspension volumes were based on previous experiments in our lab to ensure that sample values were within the assay range of the EIA kits. For the recovery experiment samples were resuspended at 300 µl and the first three hours were diluted at 1:8 and all others were not diluted. For the group vs isolated experiment, the samples were resuspended at 300 µl and diluted 1:4. For the exogenous CORT test, samples were resuspended at 600 µl and did not dilute before plating. For the ACTH challenge, samples were resuspended at 300 µl and diluted 1:4 for baseline samples and 1:8 for ACTH samples. All corticosterone and cortisol values were standardized for re-

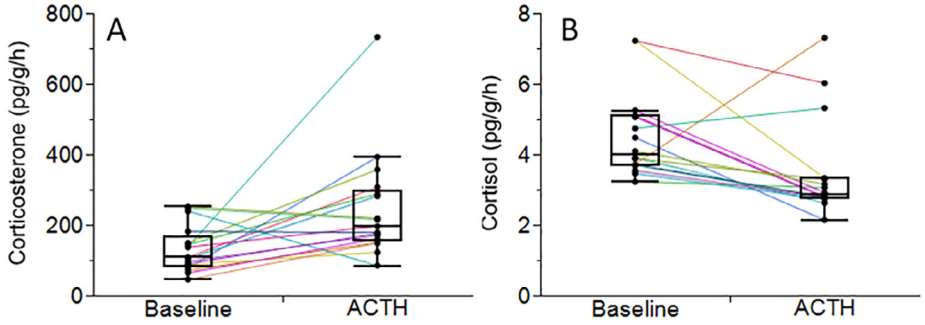

Fig. 2. Corticosterone (A) and cortisol (B) release rates (pg/g/h) obtained before (baseline) and after ACTH injection (ACTH) from *Rana berlandieri* tadpoles (n = 17). Each line color represents a different individual across time. Box plots indicate median, range and first and third quartiles.

suspension volume before statistical analysis. EIA buffer was made following a published Cayman Chemical, Inc. protocol, by mixing 10 ml ELISA buffer concentrate (№ 400060, 1 M phosphate, containing 1% BSA, 4 M sodium chloride, 10 mM EDTA, and 0.1% sodium azide) with 90 ml Millipore water.

We measured corticosterone release rates in duplicate for all samples using EIA kits (№ 501320, Cayman Chemical Company, Inc., assay has a range of 8.2–5000 pg/ml and a sensitivity (80% B/B0) of approximately 30 pg/ml) and cortisol release rates in duplicate for the ACTH challenge using an EIA kit (№ 500360, Cayman Chemical Company, Inc., assay has a range from 6.6 to 4000 pg/ml and a sensitivity (80% B/B0) of approximately 35 pg/ml). We used a pooled control sample from previously collected hormones from a large sample size of *Eurycea tonkawae* salamanders for corticosterone plates and a pooled control sample from previously collected hormones from a large sample of *Poecilia latipinna* fish for the cortisol plate. Sample absorbance was read on a spectrophotometer plate reader at 405 nm (BioTek 800XS). Inter-plate variation for agitation stress test, recovery, and repeatability experiments was 9.98% (4 plates) and for the rearing condition experiment was 5.42% (4 plates). Intra-plate variation for both experiments ranged 0.41–5.74%. Intra-plate variation for cortisol and corticosterone release rates for the ACTH challenge were 1.97% and 2.69%, respectively (Only one corticosterone and one cortisol plate were used for the ACTH experiment). Inter-plate variation for the exogenous corticosterone experiment was 15.18% (5 plates) and intra-plate variation ranged from 0.87 to 4.13%. Inter-plate variation for the agitation/recovery and repeatability experiment was 9.98% (4 plates) and for the rearing condition experiment was 5.42% (4 plates). Intra-plate variation for both experiments ranged from 0.41 to 5.74%.

2.6. Statistics

We multiplied corticosterone release rates (pg/ml) by the final resuspension volume (0.3–0.6 ml) and then standardized the value by dividing by mass of the respective individual. All hormone release rates were natural log transformed before data analysis (though untransformed data are presented in figures). We analyzed corticosterone and cortisol release rates in response to an ACTH challenge using repeated measures ANOVA. We analyzed response to exogenous corticosterone using a generalized linear mixed model (GLMM) with tank as the random effect. To examine the time it takes to recover from a stressor (agitation) on the fixed effect corticosterone, we used a repeated measures GLMM with individual as a random factor to account for repeated measures. We assessed the effect of housing (treatment) and time on corticosterone release rates and mass using a repeated measures GLMM with individual and tank as random effects to account for repeated measures. When there was a significant difference we ran a *post hoc* Tukey's (HSD) comparison between treatments. We used a matched pairs *t*-test to examine if corticosterone release rates increased after tadpoles were agitated on day 7. All tests were performed using JMP 14 software (SAS Institute, Inc). Using the R package rtpR in R version 3.2.3 (R Core Development Team), we calculated an adjusted repeatability (*r*) with a linear mixed model (LMM) based approach using the Restricted Maximum Likelihood (REML) method (Dingemanse and Dochtermann, 2013; Nakagawa and Schielzeth, 2010). We calculated repeatability of corticosterone release rates across time for tadpoles in the recovery and housing experiments included in this paper (for the housing experiment, we calculated repeatability separately for each housing treatment). Corticosterone release rate was our response variable, with sampling hours or days (for the recovery and housing experiments respectively) as the fixed variables, and individual identity as the random slope and intercepts effect.

3. Results

3.1. Validation-ACTH challenge on corticosterone and cortisol & exogenous corticosterone

Intraperitoneal injection of ACTH at a dose of 0.5 μg/g significantly increased corticosterone release rates above baseline release rates in *Rana berlandieri* tadpoles (repeated measures ANOVA: $F_{1,16} = 13.65$, $p = 0.002$; Fig. 2a) and significantly decreased cortisol release rates below baseline release rates ($F_{1,15} = 6.34$, $p = 0.0236$; Fig. 2b).

Exposing tadpoles of *Rana berlandieri* to 125 nM exogenous corticosterone induced a significant increase in corticosterone release rates ($F_{1,17} = 45.52$, $p < 0.0001$; Fig. 3).

3.2. Validation-agitation stress test, recovery, and repeatability

Corticosterone release rates in tadpoles of *Rana berlandieri* differed over time ($F_{6,85} = 14.50$, $p < 0.001$; Fig. 4): they were significantly lower than agitation by two hours post agitation and had the lowest values 6 h post agitation. Corticosterone release rates were repeatable

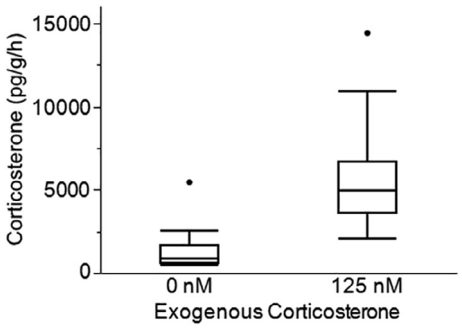

Fig. 3. Corticosterone release rates (pg/g/h) obtained from *R. berlandieri* tadpoles exposed to no exogenous corticosterone (n = 18) and 125 nM corticosterone (n = 20) for 7 days. Each line color represents a different individual across time. Box plots indicate median, range and first and third quartiles. Dots indicate outliers.

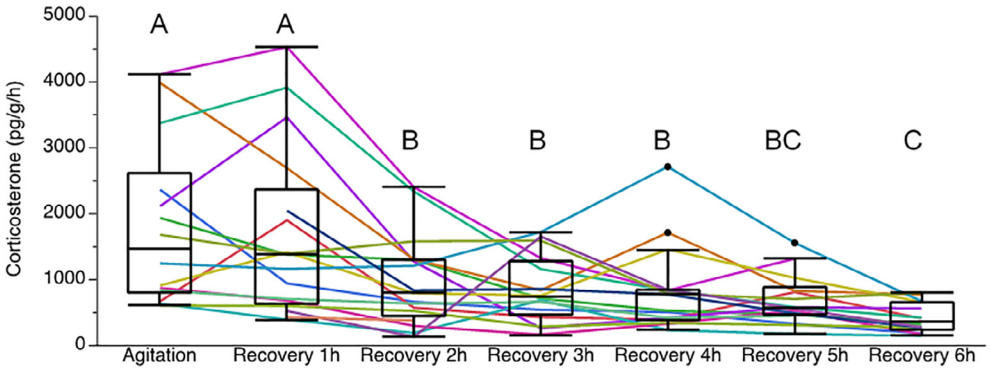

Fig. 4. Corticosterone release rates (pg/g/h) obtained from *R. berlandieri* tadpoles after 60 min of agitation and recovery 1 to 6 h post agitation (n = 14–17/time step). Each line color represents a different individual across time. Box plots indicate median, range and first and third quartiles. Dots indicate outliers. Different letters indicate significant differences.

across measurements (r = 0.365 ± 0.111, 95% CI: 0.128, 0.561; $p < 0.001$).

3.3. Non-invasive rearing conditions: individually vs. in groups

Tadpoles reared in groups had higher corticosterone release rates on day 7 than on day 2 but not those reared individually (time × treatment: $F_{1,38} = 5.47$, $p = 0.025$; Fig. 5a, b). Tadpoles reared in groups mounted a stress response after 7 days in response to an agitation test (df = 18, t = 2.26, $p = 0.036$; Fig. 5a) but not those that were reared individually (df = 20, t = 1.71, $p = 0.103$; Fig. 5b). Tadpoles reared in groups lost mass over time but not those reared individually (treatment × time: $F_{2,38} = 30.64$, $p < 0.0001$; Fig. 6). Corticosterone release rates were repeatable for tadpoles reared in groups (r = 0.300 ± 0.144, 95% CI: 0, 0.562; $p = 0.022$) and for tadpoles reared individually (r = 0.588 ± 0.116, 95% CI: 0.315, 0.776; $p < 0.001$).

4. Discussion

As the loss of amphibian populations continues, it is important to develop methods for early warning indicators of population declines and to find non-invasive methods for measuring physiological health. These methods also need to be validated. Further, to understand the ability of a population to respond to rapid environmental change, it is necessary for these methods to allow for repeated hormone measures, to allow for the calculation of repeatability (a proxy for the upper level of heritability). Our results demonstrate that the waterborne-hormone collection method is a valid method for sampling glucocorticoids and assessing physiological changes in *Rana berlandieri*. As predicted, pharmacological challenges with ACTH and exogenous corticosterone increased corticosterone release rates in tadpoles. Additionally, the use of a biological challenge, in the form of an agitation test, also increased corticosterone release rates significantly in tadpoles. Our results are congruent with findings in other amphibian species (Baugh et al., 2018; Gabor et al., 2013, 2016; Glennemeier and Denver 2002a; Narayan et al., 2010). Importantly, we found that our integrated measures of GCs are moderately repeatable, indicating a heritable component to the GC response in this population of *R. berlandieri*.

We measured release rates of two excreted glucocorticoids (corticosterone and cortisol) from tadpoles after the ACTH challenge. Although we observed elevated corticosterone release rates after ACTH injection in *R. berlandieri*, those values may not represent the peak of the reactive range (Romero, 2002) as we collected hormones immediately following injection, and Baugh et al. (2018) recently showed in *Physalaemus pustulosus* that maximum levels may be released 2 h post injection. In contrast to corticosterone, cortisol release rates decreased with ACTH challenge. In fish, for example, ACTH challenge results in an increase in cortisol release rates, but that is the major glucocorticoid in fish (Kim et al., 2018). To the best of our knowledge, this is the first study to validate a water-borne hormone collection method for cortisol in an anuran species. Baugh et al. (2018) measured cortisol in amphibians using HPLC-MS and found that one paired pooled sample showed a decrease in cortisol levels after an ACTH challenge. Our results suggest that corticosterone better represents the stress response in amphibians rather than cortisol, as corticosterone increased while cortisol decreased after ACTH injection, though the biological implications of why cortisol decreased after an ACTH challenge are not clear, therefore further study is merited.

Exposure to exogenous CORT has been used previously to illicit a hormonal response and increase endogenous CORT in amphibians (Belden et al., 2005; Glennemeier and Denver, 2002a,b; Middlemis-

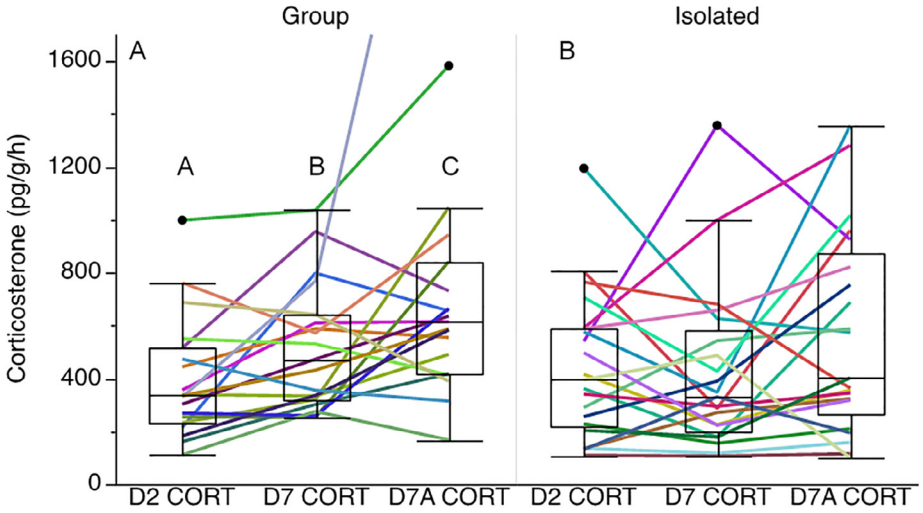

Fig. 5. Corticosterone release rates (pg/g/h) obtained from *R. berlandieri* tadpoles after 2 days (D2) and 7 days (D7) in treatments and after an agitation test (D7A) for those reared in (A) groups (n = 19) or (B) individually (n = 21). One point in the agitation group data at 3000 pg/g/h was left out for ease of view. Each line color represents a different individual across time. Box plots indicate median, range and first and third quartiles. Dots indicate outliers. Different letters indicate significant differences.

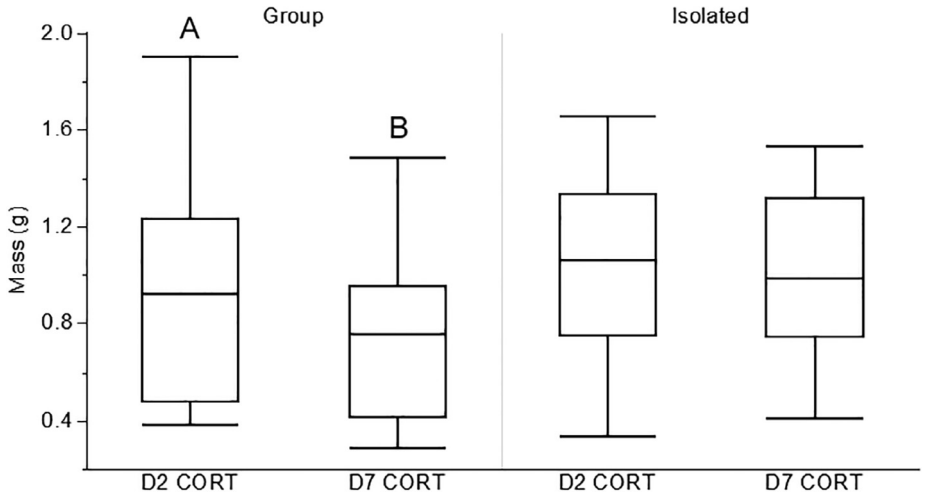

Fig. 6. Mass (g) after 2 days (D2) and 7 days (D7) in treatments. Box plots indicate median, range and first and third quartiles. Different letters indicate significant differences.

Maher et al., 2013). Our results suggest this method is also viable to raise water-borne CORT release rates in *R. berlandieri*. Tadpoles were not rinsed in water to remove surface CORT prior to placing in beakers, which may have contributed to the higher CORT release rates observed. However, based on the CORT concentration calculated from a 100 ml sample of tub water (43308 pg/ml) resuspended at 0.60 ml prior to plating, only about 13 pg of CORT was introduced to the sample with each tadpole. So, the possible addition of up to 13 pg of CORT per tadpole would still not account for the difference in CORT observed between control and exogenous CORT exposed tadpoles.

The non-invasive water-borne hormone collection method provides an accurate way to repeatedly measure hormones from individuals while minimally stressing the organism and eliminating the need for blood sampling or sacrifice. This method is a valuable tool for conservation studies to assess stress physiology in threatened and endangered species. Further, lower sample sizes are required for experiments because individuals can be resampled across time. Typically, rearing facilities for endangered or threatened species of amphibians have enough individuals to achieve sample sizes necessary for this protocol, and while larger sample sizes may not be easily obtainable in the field, Gabor et al. (2018) were able to collect sufficient sample sizes in the field to sample hormones in the Federally threatened Jollyville Plateau salamander, *Eurycea tonkawae*. Additionally, this method alleviates the need to transport individuals to the lab as water-borne hormone samples can be collected in the field and transported back to the lab on ice. A potential shortcoming of this method is the need for individuals to be confined to a beaker for one hour, as confinement has been used as an acute stressor in previous studies (Middlemis-Maher et al., 2013). However, our results show that baseline levels of corticosterone release rates after one hour in a beaker are statistically lower than ACTH challenged or agitated corticosterone release rates. Additionally, tadpole corticosterone release rates for *R. berlandieri* were significantly lower 6 h after agitation, which is a more rapid recovery than observed in other species using a different method (Narayan et al., 2010).

Repeatability of glucocorticoids have been observed in many free-living individuals (reviewed by Hau et al., 2016) and often significantly higher in amphibians compared to other taxa (Schoenemann and Bonier, 2018). Repeated measure designs are often difficult in free-living organisms (Hau et al., 2016) and repeated measure laboratory or mesocosm studies require marking individuals, which may add additional stress or variation in physiological responses. Our results demonstrate that baseline levels of corticosterone release rates, measured using our non-invasive water-borne collection method, are repeatable over time and our values ranging from $r = 0.300$ to 0.588 for stress and recovery and the rearing experiment are moderate to high (Hau et al., 2016). Narayan et al. (2013) measured repeatability in *Platymantis vitiana* using an older statistical method and found very high repeatability for baseline values ($r = 0.973$) and for the stress response values (range $r = 0.82$–0.92; Narayan and Hero, 2013). In cane toads, *Rhinella marina*, Narayan et al. (2012) found high repeatability for baseline and corticosterone metabolite responses ranging from $r = 0.630$ to $r = 0.793$. In our studies, the highest repeatability values, not surprisingly, came from when the tadpoles were maintained in close to the same conditions across time in the individual cups. This is the first time that repeatability of corticosterone release rates using water-borne hormones has been quantified in tadpoles. We also note that we had large among individual variance which indicates that there are multiple phenotypes in the population of which some may respond better in a given environment. Because repeatability can be viewed as the upper bound of heritability at the population level, our results indicate that corticosterone release show enough variation that, in theory, there is potential to evolve in response to selection (Hau et al., 2016).

Repeated measure experimental designs require marking individuals or rearing them individually. However, it may be difficult (or require special permitting) to mark individuals and rearing individually may be stressful for social species. Our results indicate that rearing tadpoles individually may contribute to stress of tadpoles, though quantifying the impacts of housing type are difficult. Interestingly, tadpoles reared in groups, isolated by mesh screen allowing for visual and chemical cues to be perceived by individuals, showed higher corticosterone release rates than the individually reared tadpoles. Yet, individually reared tadpoles did not show a stress response whereas those reared in groups did show a stress response to agitation, suggesting tadpoles reared individually may have a dysregulated HPI axis. Given these findings, it is difficult for us to make any strong conclusion about whether one method or the other is better for rearing tadpoles of *R. berlandieri*. Further investigation into housing design is warranted, such as experiments with larger containers with more water volume per tadpole to reduce potential effects of crowding, as rearing methods should be an important consideration of experimental design. We also did not see a difference in the variance of stress hormone levels on each of the three days we measured CORT across treatments (Day 2 baseline Levene's test: $p = 0.13$; Day 7: $p = 0.13$; Day 7 Agitation: $p = 0.09$). Prior studies on rearing found that zebrafish, *Danio rerio*, a shoaling species, show higher and more variable cortisol levels when housed individually compared to in groups (Pagnussat et al., 2013). Additionally, Narayan et al. (2013) found that urinary corticosterone

concentrations were higher in adult cane toads housed in groups, but corticosterone declined after toads were moved to individual enclosures. Similar observations were made in endangered adult harlequin Frogs, *Atelopus* spp., (Cikanek et al., 2014). When designing an experiment, it is important to consider whether the species you are working with is social or not. In another species of leopard frog, *Rana pipiens,* it was found that tadpoles of this species do not aggregate (Golden et al., 2001) but many species may benefit from being in larger groups in the presence of predators (Skelly, 1994) and leopard frogs were found to be more active in larger groups (Golden et al., 2001). We did find that individuals reared with other tadpoles lost mass over time and showed elevated corticosterone release rates, which is similar to findings by Glennemeier and Denver (2002b) in *Rana pipiens* tadpoles. Lower body mass is associated with elevated corticosterone levels in toads reared in captivity over time (Titon et al., 2018). Together, our results on rearing tadpoles of *R. berlandieri* indicate that the possible benefits to being reared in groups are offset by the reduced growth, whereas the benefit of individual rearing may be offset by additional stress of being solitary, resulting in a lack of adaptive stress response. These findings indicate that no one method of rearing is best, but housing decisions will depend on the question being asked. It is important to note that physiological responses to housing is likely species specific and will depend on whether the species is generally social, but housing methods are important to consider, particularly if repeated measures are needed in studies of threatened or endangered species.

5. Conclusions

We validated that the water-borne hormone method reliably measures the GC response of *Rana berlandieri* tadpoles to stressors both pharmacologically and biologically. Further, using this water-borne hormone method, we found that it takes up to two hours for corticosterone release rates to start to decline post stressor and by six hours corticosterone were even lower. We found that this species could be reared alone or individually in groups if repeated measure designs are being used, however the optimal rearing method will depend on the question being asked and the species being tested. Finally, we also found that the water-borne hormone collection method provides repeatable measures of GCs in *Rana berlandieri* indicating that this species can show a response to selection on stress hormones, and thus this species could evolve in response to environmental stressors. Together, our results indicate that using the non-invasive water-borne hormone method allows for studying threatened or endangered species (both in terms of minimal sample sizes and minimal invasiveness) and determining whether they can show a response to selection in stressful environments, an important conservation tool given the rapid decline in amphibian populations to date.

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