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Cross-Generational Effects of Stress on Captive Adults and Their Released Offspring in an Amphibian

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Abstract.—Ex situ conservation, translocation, and reintroduction are becoming increasingly important for species restoration. In amphibians, however, effects of captive stress on adults and subsequent effects on their offspring that are later reintroduced into the wild are largely unknown. Using Fowler's toads (*Anaxyrus fowleri*) as a model species, we investigated effects of increased captive stress on corticosterone (CORT) concentration in adult toads. We then examined CORT levels in their tadpole offspring, which we reared in natural ponds to simulate conditions of a reintroduced population. We found no significant effects of captive stress on adult or offspring CORT levels. However, despite poor model performance due to low sample size, baseline CORT of sires (but not dams) was the best predictor of, and negatively correlated with, baseline CORT and change in CORT in offspring. Our study provides a unique perspective on the potential translation of stress from parent to offspring and points to a need for a closer examination of paternal effects in cases of cross-generational studies in amphibians.

Globally, there have been unprecedented declines in biodiversity, both in terms of number of species and population sizes in the wild (Johnson et al., 2017; World Wildlife Fund, 2022). As extinction rates continue to rise, ex situ conservation is becoming increasingly important in preserving our remaining biodiversity (Lewis et al., 2019). This is particularly important for taxa facing higher risks of extinction, such as amphibians (Sodhi et al., 2008). Maintaining ex situ populations of threatened or endangered species is a strategy used to mitigate risks of extinction (Norris, 2007). Captive colonies act as a safety net for species whose wild populations are dwindling because of various factors such as habitat destruction (Pabijan et al., 2020), human overpopulation (Tapley et al., 2015), and infectious disease (Gascon et al., 2012). These ex situ populations shield at-risk species from threats in their natural environment by creating a stable source population that can be reintroduced to boost existing wild populations or reestablish extinct populations.

Despite the substantial progress being made in captive breeding, release, and conservation translocation programs—24.3% increase in studies from 1998–2005 (Bubac et al., 2019)—it is still a relatively young field of study. Research on captive breeding programs is often underprioritized because of high financial needs and lack of funding sources (Rytwinski et al., 2021). Therefore, even though captive programs continue to be established, focused examinations of successes or failures remain sparse (see Bubac et al., 2019). Even within well-studied captive programs, the response of species can be context dependent. For example, in Chiricahua leopard frogs (*Lithobates chiricahuensis*), habitat characteristics of a particular site and presence of a predatory species can determine whether released populations are able to persist (Hossack et al. 2022). Moreover, dietary carotenoids are shown to affect exploratory behavior of captive, critically endangered Southern Corroboree frogs (*Pseudophryne corroboree*) when they are released into a captive, controlled environment (Kelleher et al., 2019), but the same effect was not observed when researchers released frogs into a natural environment (Kelleher et al., 2022). These varying reports signify a need for further investigation before management decisions can be made to ensure success of conservation programs. Additionally, research on captive breeding programs is largely focused on select taxa. For example, captivity is known to promote stress in several mammalian and avian species (Hare et al., 2014; Ramos-Güivas et al., 2021). However, in amphibians and non-avian reptiles, similar effects of captivity are comparatively understudied. Broadening our knowledge to include these groups will increase the capacity of captive release programs and, by extension, the impact these programs can make on species conservation.

In captive colonies, animals typically are in environments that differ from their natural habitat. Although some institutions have rules and guidelines for animal welfare (Association of Zoos and Aquariums, 2023), maintaining wild animals in captivity can lead to chronic (Fischer and Michael Romero, 2016) and acute stress (de Assis et al., 2015). Chronic stress, defined as repetition or prolonged occurrence of stress events (Fischer and Michael Romero, 2016), can result in increased secretion of glucocorticoids (GCs), which are energy-mobilizing hormones. Commonly measured GCs include cortisol and corticosterone (CORT), dependent upon species and type of sample. Acute stress is characterized by a rapid elevation of GCs followed by a return to baseline GC concentrations (de Assis et al., 2015). GCs are frequently used in ecophysiological studies as markers of stress, although their primary responsibility is the mobilization and prioritization of energy during any physiological function (Sapolsky et al., 2000). Changes in GC concentration reflect changes in energetic requirements, which are often complicated and nuanced (Busch and Hayward, 2009; Ralph and Tilbrook, 2016). Although there is great context-dependent variability in GC responses, there are some general patterns that do seem to exist in many systems (Bonier et al., 2009; Angelier and Wingfield, 2013). Presence of chronic stressors can be

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responsible for various physiological responses such as immune response decline, irregular hormone concentrations, reduced growth rate, and reproductive cycling changes (Morgan and Tromborg, 2007). Chronic stress also impacts an individual's ability to respond effectively to acute stress (Wingfield, 2005; Dhabhar, 2009; Neuman-Lee et al., 2015). Acute stress responses are often protective and promote immediate survival, although repeated exposure to acute stressors can elicit a maladaptive chronic stress response (McCormick et al., 2015; Taff et al., 2018). Acute increases in GCs can cause rapidly changing behaviors such as suppression of mating behaviors in male toads (Leary et al., 2006) or increased locomotion in fish (Øverli et al., 2002).

In terms of the effects of captive stress, responses are often variable across species, making it difficult to reach general consensus (Dickens and Romero, 2013). For example, fecal concentrations of cortisol metabolites were higher in captive African wild dogs (*Lycaon pictus*) compared to their free-ranging counterparts (Van der Weyde et al., 2016), suggesting a potential increase in stress in captive settings. However, house sparrows (*Passer domesticus*) exhibited higher concentrations of CORT in short-term captivity, whereas long-term captivity promoted intermediate CORT concentrations (Kuhlman and Martin, 2010), suggesting an effect of habituation that results in a lower CORT response as time in captivity increases. Understanding physiological and behavioral changes associated with stress of captivity is key to uncovering the most effective conservation tactics, especially for imperiled groups like amphibians. To date, research on captive release programs has used amphibians as a study system to investigate in vitro fertilization (Browne et al., 2006), assisted reproductive technologies (Poo and Hinkson, 2020), as well as the behavior (Kelleher et al., 2022), growth, and survivorship of captive-released offspring (Poo et al., 2022).

In this study, we examined the physiological effects of stress that animals may experience in captivity using adult Fowler's toads (*Anaxyrus fowleri*) and their tadpole offspring as a model system. Given the use of captive-bred individuals in reintroduction programs and the potential for associated chronic and acute stressors, we investigated how conditions in captivity affect the concentration of CORT—the primary GC in amphibians (Gabor et al., 2013)—in adult toads and their offspring. Specifically, we investigated the effects of increased captive stress on adult toads and its relationship to CORT concentration and response of their tadpole offspring after those tadpoles are released into a natural environment. We hypothesized that elevated CORT concentration in captive adult toads would be positively correlated with the CORT concentration and response of their tadpoles.

Materials and Methods

Species and Study Site.—Fowler's toads are abundant and widespread (IUCN, 2023), making them a suitable model for examining conservation strategies intended for at-risk species (Powell et al., 2016; Poo and Hinkson, 2020; Poo et al., 2022). Throughout May 2022, we collected adult Fowler's toads at relatively even sex ratios in Shelby County, Tennessee, United States. We collected toads between 2100–2400 h, using a headlamp at a brightness of 1,250 lumens to locate individuals via their eyeshine. We housed toads in same-sex groups of four in 10-gallon glass aquariums (50.8 cm L × 25.4 cm W × 30.48 cm H) outfitted with coconut shavings, conditioned tap water in a bowl, and a hide box for cover. We conducted daily water changes and spot cleaning and changed the substrate as needed. The adult toads' diet consisted of a combination of crickets, mealworms, and superworms offered four times per week. Artificial light was provided in a 12:12 h light–dark cycle, and the room was maintained at 23 °C. We cleaned all materials used during the study with a 15% bleach solution and thoroughly rinsed off the solution prior to use. The Animal Care and Use Committees (Memphis Zoo Approval 2022-04, University of Memphis Approval 0886) and the Tennessee Wildlife Resource Agency (Permit 2430) approved all procedures.

Stress Treatments, Breeding, and Water Sample Collection.—We randomly assigned adult toads to either a control or experimental group (Fig. 1A). We measured snout-vent length (SVL) and mass for each adult toad at the start of the study and repeated these measurements after each subsequent CORT assessment. Experimental toads underwent a standardized stress treatment that consisted of moving the groups of four from their original aquarium into a shared smaller container (35.9 cm L × 19.7 cm

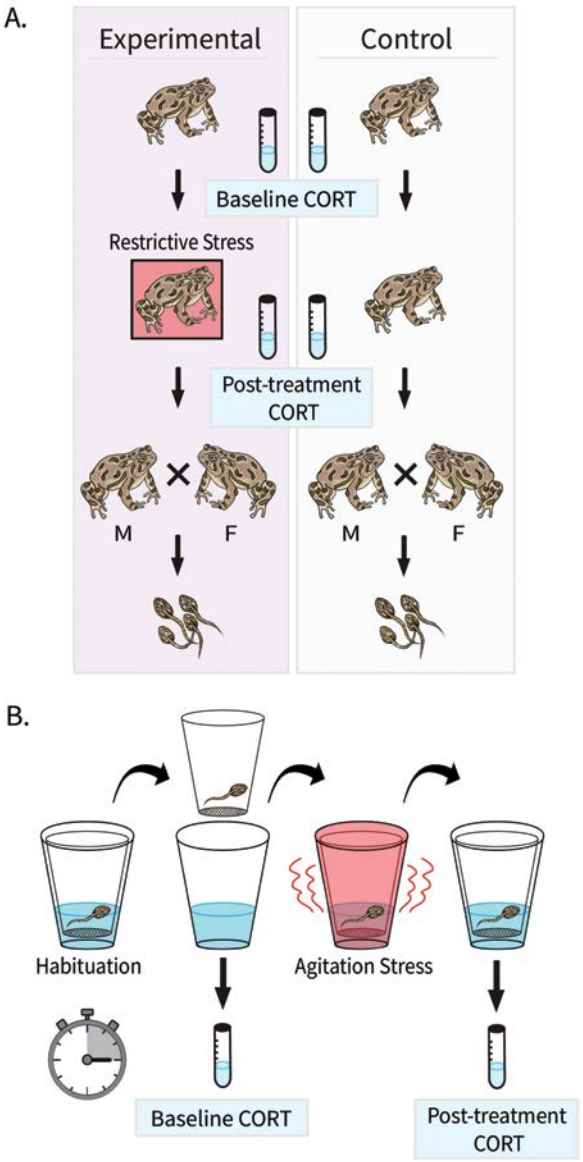

Fig. 1. Diagram of experimental design with waterborne hormone collection. Adult *Anaxyrus fowleri* were split into experimental and control groups, with and without restrictive stress treatment, respectively (A). Tadpoles from all groups underwent the same process for sample collection (B).

W × 12.4 cm H) and covering the container with a cloth for one hour. Constrained movement is known to promote stress in amphibians (de Assis et al., 2015). The treatment occurred twice per day, at two randomly selected times (between 0800–2000 h), for three consecutive days leading up to transfer of toads into breeding tanks. Control toads remained undisturbed in their original aquariums during stress treatment applications.

To examine effects of this restrictive stress treatment for adult toads, we used a non-invasive method to collect waterborne CORT developed by Gabor et al. (2013). For each waterborne CORT assessment, we placed adult toads into individual plastic containers (19.4 cm L × 16.5 cm W × 11.4 cm H) filled with 100 mL of conditioned water for one hour and covered each container with cloth to reduce disturbances. We filtered water samples through coffee filters (Hario V60 size 1 paper filter, VCF-01-40M) and stored them at −20 °C until analysis. We assessed each toad at two time points: (1) baseline, prior to the restrictive stress treatment (Adt_{Base}) and (2) post-stress treatment but prior to breeding ($Adt_{Post\text{-}str}$). We measured stress response as change in CORT concentration after activation of stress ($Adt_{Post\text{-}str} - Adt_{Base}$).

To facilitate breeding, we assembled 10-gallon breeding aquariums (50.8 cm L × 25.4 cm W × 30.48 cm H) with approximately 2 cm of conditioned tap water, synthetic plants, and a platform to mimic land. Breeding aquariums were placed in an outdoor shaded area and were exposed to natural light cycles and temperatures. We randomly assigned adult toads to a breeding aquarium with one female and one male from the same treatment group per aquarium. Following the protocol in Poo and Hinkson (2020), we administered up to two priming doses of 2.5 hCG/g body weight (hCG: human chorionic gonadotropin; Sigma-Aldrich, St. Louis, Missouri) to both the male and female toads to induce breeding.

Tadpole Rearing, In Situ Development, and Stress Treatments.—We removed egg clutches from breeding aquariums immediately and placed each clutch into a 5-L bucket filled with conditioned water. Upon hatching, which occurs roughly 3 days post-oviposition (DPO), we recorded clutch size by counting number of hatchlings and number of unfertilized eggs. For each clutch, we set up two aerated 5-L buckets, each bucket containing 20 tadpoles. At this stage, we fed tadpoles a diet of algae ad libitum. At 6 DPO, we transported tadpoles to a permanent natural pond and released them into mesh enclosures (61 cm L × 61 cm W × 91 cm H), with each original bucket of tadpoles released into its own mesh enclosure. We outfitted each enclosure with 100 g of dry leaf litter and buoys to avoid full submersion. At 16 DPO, we removed all surviving tadpoles from the enclosures and took dorsal photos, which we used to measure total length using ImageJ (Schneider et al., 2012). From each enclosure, we assessed baseline stress and stress response of six tadpoles that were each measured independently, using a waterborne CORT technique. For CORT assessments of tadpoles, we moved each tadpole progressively through its own series of four cups: habituation (Tad_{Hab}), baseline (Tad_{Base}), agitation (Tad_{Ag}), and post-agitation ($Tad_{Post\text{-}Ag}$) (Fig. 1B). Each cup contained 100 mL of conditioned tap water. To minimize disturbance between transfers, we used an insert with a mesh bottom, which could then be used to move the tadpole between cups. We placed tadpoles in the Tad_{Hab} cup for 15 minutes to habituate to the smaller container before sample collection. From the Tad_{Hab} cup, we moved tadpoles into the Tad_{Base} cup for 1 hour to collect a baseline stress sample. Next, we moved tadpoles into the Tad_{Ag} cup, which we agitated using a vibration device (Grolebo Mini Massager) for 1 minute every 6 minutes, for a total of 1 hour. Finally, we moved tadpoles into the $Tad_{Post\text{-}Ag}$ cup for 1 hour to collect a recovery stress sample. We discarded water from the Tad_{Hab} and Tad_{Ag} cups. We measured stress response as the change in CORT concentration after the activation of stress ($Tad_{Post\text{-}Ag} - Tad_{Base}$). We stored water samples at −20°C until analysis.

CORT Extraction and Radioimmunoassay.—We thawed the water samples prior to beginning the hormone extraction process. Hormone extraction followed the methods of Gabor et al. (2013) with a few differences. Working in a fume hood, we attached solid-phase extraction filters (Sep-Pak WAT020805) to a glass vacuum manifold (Thermo Scientific 60104-232) and primed each filter with 4 mL of methanol (MeOH), followed by 4 mL of distilled water, releasing the vacuum after each round. We used 2.54-cm diameter funnels on top of each solid-phase extraction filter for efficiency during sample transfer. Once extraction of the sample was finished, we poured another round of 2 mL distilled water through the filters. Finally, we ran 4 mL of MeOH through the filters to draw the hormone through and into 13-mm × 100-mm borosilicate glass tubes (Fisherbrand 1496127). To eliminate remaining MeOH from the samples, we used a stream of nitrogen gas or allowed MeOH to evaporate over time in the fume hood. After MeOH had completely evaporated, we resuspended and maintained hormone samples in 500 μL of phosphate-buffered saline. The following day we vortexed samples, transferred them via pipette into 0.6 mL microcentrifuge tubes, and stored them at −20°C.

We measured CORT concentrations (ng/mL) for all samples using a previously described and adapted protocol for conducting a radioimmunoassay (Moore, 1986; Neuman-Lee et al., 2020). We assayed samples in duplicate for CORT (Ab: MP Biomedicals, Cat: 07-120016, Lot 3R3-PB17; H^3-labeled CORT: Perkin Elmer, NET300250UC) across four different assays. We used spiked water samples to measure initial recoveries and used this information to correct final concentrations in individual samples. For all CORT assays, the intra-assay coefficient of variation ranged from 2–7%, and the inter-assay coefficient of variation was 6%. The minimum level of detection for CORT ranged from 0.004–0.013 ng/mL.

Statistical Analysis.—We calculated body condition index for each adult toad using residuals obtained by regressing adult body mass against SVL. We calculated the difference between baseline and post-stress CORT concentration for adult toads ($Adt_{Post\text{-}Str} - Adt_{Base}$) and their tadpoles ($Tad_{Post\text{-}Ag} - Tad_{Base}$). We then calculated size-corrected baseline CORT for Adt_{Base} and Tad_{Base} and size-corrected difference in CORT (Δ_{CORT}) and used these values for the remainder of analyses. We calculated size-corrected values for adults and tadpoles by dividing these values by SVL and length at 16 DPO, respectively. We calculated pairwise correlations between all covariates obtained from the tadpoles (Fig. S1).

To account for potential latent clustering from adult enclosure groupings, we used a gradient boosting framework (extreme gradient-boosted regression, a tree-based supervised machine learning model) to calculate the relative importance of explanatory variables in predicting whether an adult toad bred or not (Table 1). We used k-fold cross-validation to iteratively train the XGBoost model on each training subset created during each fold of the cross-validation. We calculated model performance with simple logistic regression of model predictions (bred or not bred) using the partitioned test data. While a tree-based method does not fully account for latent clustering, interpretation of the model's performance in predicting observed data

TABLE 1. Results from gradient-boosted classification and regression. BCI (body condition index).

Classification/regression in XGBoost		Model performance			Model results
Dependent variable	Predictors	Correlation	Coefficient	*P*-value	
Adult: bred or not bred	Adult treatment, sex, SVL, adult Δ_{CORT} CORT	0.588	1.25	0.328	NA
		Multiple R^2	Coefficient	*P*-value	
Adult Δ_{CORT}	Adult treatment, sex, SVL, adult baseline CORT	0.000753	0.028449	0.92	NA
Tadpole baseline CORT	Adult treatment, sire BCI, sire fertility/SVL, sire SVL, sire mass, sire baseline CORT/SVL, sire Δ_{CORT}/SVL, dam BCI, dam fertility/SVL, dam SVL, dam mass, dam baseline CORT/SVL, dam Δ_{CORT}/SVL, clutch size	0.053	0.16613	**3.4E-11******	Sire's baseline CORT is the best predictor of tadpole baseline CORT (Fig. 3A)
Tadpole Δ_{CORT}	Same as above	0.037	0.12012	**2.1e-08*****	Sire's baseline CORT is the best predictor of tadpole Δ_{CORT} (Fig. 3C)

allows for hypothesis generation and calculation of the model's predictive power without violating assumptions of parametric statistical models. We used the R package XGBoost (Chen et al., 2015) for all extreme gradient-boosted regression.

We repeated the same analysis with size-corrected adult Δ_{CORT} as a response variable and calculated the relative importance of covariates in predicting the continuous outcome (Table 1). We used simple linear regression of predicted values versus partitioned test data to calculate model performance.

We used the same gradient boosting framework as above to analyze size-corrected tadpole baseline CORT and Δ_{CORT} (Table 1) because covariates were highly correlated with each other (Fig. S1). Because tadpoles were housed within enclosures with their clutch-mates to allow us to keep track of sires and dams, we accounted for clutch-level clustering using cluster-based partitioning of training and test data for the cross-validation. Instead of randomly splitting data into folds, we partitioned data such that entire clutches were in either training or test datasets. We calculated relative importance of covariates in predicting tadpole baseline CORT and Δ_{CORT}. Finally, we used simple linear regression of predicted values versus partitioned test data to calculate model performance.

Using the results from the tadpoles, we selected non-correlated covariates that had predictive power on tadpole baseline CORT and Δ_{CORT}, namely sire baseline CORT, and conducted a power analysis to determine the sample size (i.e., number of clutches) needed to detect a significant (alpha = 0.05) effect on dependent variables (Table 2). We added dam baseline CORT as an explanatory variable to account for potential parental effects beyond that of the sire. We used a mixed-effects analysis of variance model, with clutch ID as a random effect, to calculate statistical power of sire baseline CORT in correctly rejecting the null hypothesis of no correlation with tadpole baseline CORT and Δ_{CORT}. Using a desired power of 0.8 and an alpha of 0.05, we simulated iteratively adding one to the starting number of clutches (nine) and calculated the minimum number of clutches needed to correctly reject the null hypothesis of no effect of sire baseline CORT on the baseline CORT and Δ_{CORT} of their offspring. We used the R package simr (Green and MacLeod 2016) to conduct power analysis. We conducted all statistical analyses and data visualization in R version 4.3.1 (R Core Team, 2023).

RESULTS

We placed 17 and 16 pairs of Fowler's toads in experimental and control groups, respectively ($n = 66$ total toads). Average mass and SVL of adults were similar between the two groups (61.4 ± 1.2 and 61.5 ± 1.1 mm, 31.1 ± 1.8 and 32.8 ± 1.8 g, respectively). Of the 33 pairs, four experimental and five control pairs produced fertilized clutches, with average clutch sizes of 3267 ± 610 and 4505 ± 571 eggs, respectively.

We found no correlation between the stress treatment, sex, or SVL of adult toads and their Δ_{CORT} (Table 1). Similarly, we found no effect of stress treatment, sex, SVL, or Δ_{CORT} on adult toads or their probability of breeding. Model performance for analyses involving adult data was poor, with little power of any variables in accurately predicting observed values. Female clutch size and male fertility rate varied widely (Figs. 2B, C),

TABLE 2. Results from power analysis and sample size calculation.

	Linear mixed model results for sire's baseline with 9 clutches			
Linear mixed-effects model with clutch ID as a random variable	Power (sire's baseline)	*P*-value	Effect size	Minimum clutch needed for power = 0.8
Tadpole baseline CORT ~ sire baseline CORT/SVL + dam baseline CORT/SVL + treatment + (1 | Clutch ID)	0.17 (95% CI 0.10, 0.26)	0.184	−1.19	31
Tadpole ΔCORT ~ sire baseline CORT/SVL + dam baseline CORT/SVL + treatment + (1 | clutch ID)	0.46 (95% CI 0.36, 0.56)	0.068	−2.085	18

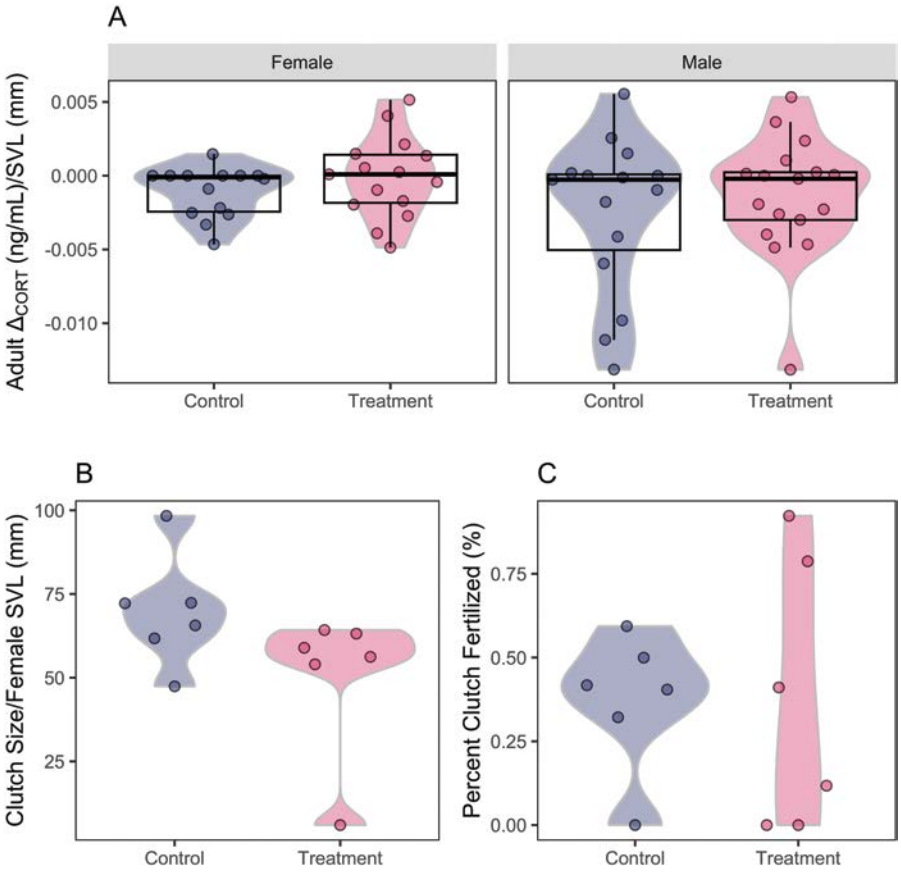

FIG. 2. Comparisons of adult *Anaxyrus fowleri* in control and restrictive stress treatment groups. Boxplots overlaid on violin plots of Δ_{CORT}, corrected by SVL (A). Violin plots of female fertility, calculated as number of eggs laid per female, corrected by SVL (B). Violin plots of male fertility, calculated as percentage of clutch fertilized per male (C).

but sample sizes ($n = 18$ in 9 breeding pairs) were too small for statistical analyses.

We measured total length of 281 tadpoles and CORT levels from a subset of 120 tadpoles (Table 1). For one of the control clutches, we were only able to maintain one 5-L bucket of 20 tadpoles at the start of the study. Model performance for analyses involving tadpole CORT data was better than that of adults (Table 1). Interestingly, we found significant ($P = 0.002$) but weak ($R^2 = 0.012$) relationships between predicted and observed values of tadpole baseline CORT, and similar relationships for tadpole Δ_{CORT} ($P < 0.001$, $R^2 = 0.023$) (Table 1). Sire baseline CORT was by far the best predictor of both tadpole baseline CORT and tadpole Δ_{CORT} (Fig. 3A, C) even though model performance was poor (Table 1).

Power analysis estimated a power of 0.22 of sire baseline CORT in accurately rejecting the null hypothesis of no correlation with tadpole baseline CORT, and 0.4 for sire baseline CORT in accurately rejecting the null hypothesis of no correlation with tadpole Δ_{CORT}. We calculated that future studies would need a sample size of at least 18–31 clutches for a desired power of 0.8 in accurately rejecting both null hypotheses (Table 2).

DISCUSSION

By examining adult Fowler's toads and their captive-released offspring, we provide the first insight into potential cross-generational effects of captive stress in an amphibian. Although our parental stress treatment did not result in a difference in CORT concentrations in adults or their tadpoles, we found some unexpected results in the relationship between tadpoles and their sires (Table 1). Our study suggests that sire baseline CORT can predict offspring baseline CORT and Δ_{CORT}, even though our small sample size (9 clutches) yielded a model with poor performance. Our power analysis indicates that at least twice the number of clutches would be needed to correctly ascertain if a true relationship exists between sire baseline CORT and the baseline CORT and Δ_{CORT} of their offspring (Table 2). These novel findings indicate a need for further examination into the effects of parental captive stress on offspring, especially given the potential role it can play in the success of captive release programs.

Space restrictions in our treatment did not elicit an elevated CORT response in adults. Reduced space in captivity is known to cause elevated concentrations of GC hormones in various taxa, including amphibians, birds, non-avian reptiles, mammals, and fish (Fischer and Romero, 2019). In a similar study, Cururu toads (*Rhinella diptycha*) showed a significant increase in CORT concentrations when exposed to a stress treatment (de Assis et al., 2015). However, the stress treatment used was of longer duration and greater restriction of movement compared to our study. One potential explanation could be that the adult toads in our study habituated to the captive setting over time. GCs have been shown to decrease over time in captivity across a number of taxa (Fischer and Romero, 2019). For example, captive-reared birds have a reduced CORT response compared to wild birds (Cabezas et al., 2013). In captive donkeys (*Equus asinus*), individuals habituated to transportation displayed

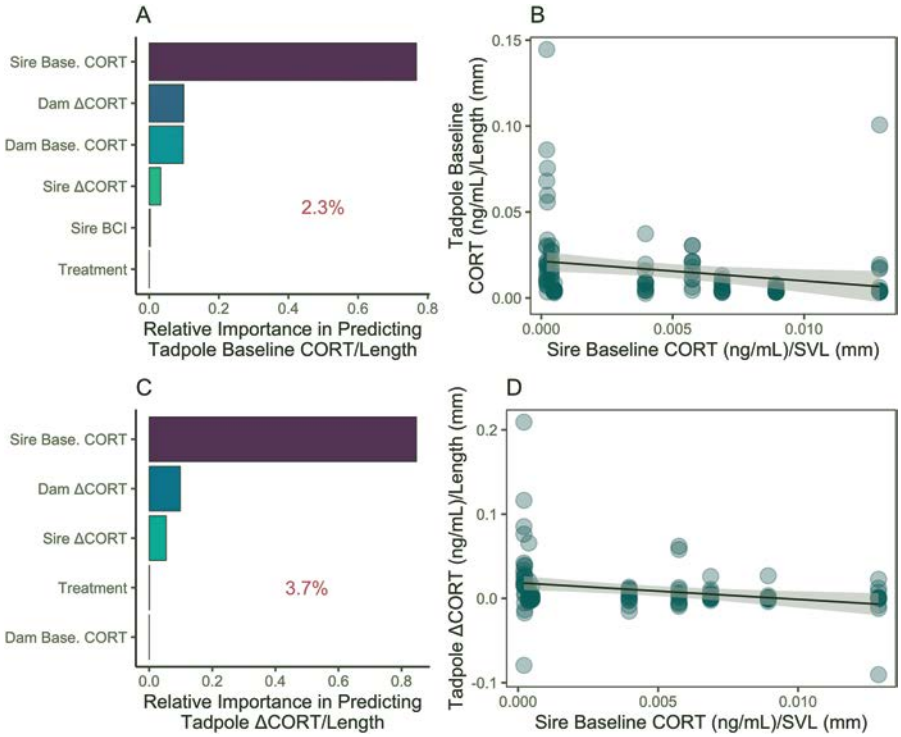

FIG. 3. Relative importance of covariates in predicting two *Anaxyrus fowleri* tadpole dependent variables (A, C) and corresponding relationships between dependent variables and their top predictor (B, D). Maroon numbers on the bar charts indicate model performance.

fewer stress-related behaviors than donkeys that had not been previously transported (Dai et al., 2020). While little is known about the duration it takes for Fowler's toads to become desensitized to captivity, the lack of difference in CORT response in adult toads could be a result of habituation because the toads remained in captivity for roughly a month between when we collected them and when the study began.

Because we conducted this study during the breeding season, the lack of stress response could also potentially have resulted from a seasonal suppression of acute stress. In Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*), males did not respond to acute stressors during the mating season (Ricciardella et al., 2010). The researchers attributed this lack of response to an already elevated CORT exhibited throughout heightened reproductive activity (Ricciardella et al., 2010), which has also been reported in a number of amphibian species (Romero, 2002). Despite these reports on the seasonality of baseline stress, there is still a paucity of research examining seasonal differences in the amphibian acute stress response (Romero, 2002). A comparison of Fowler's toads during and outside of the breeding season could certainly reveal more about complex interactions that affect both baseline CORT and CORT responses to stressful events.

Upon comparing cross-generational CORT concentrations over treatment time points, paternal CORT was the best predictor of both baseline CORT and Δ_{CORT} in tadpole offspring. In comparison, maternal CORT concentrations had less of an effect on offspring. Paternal genetics and physiology are often overlooked when investigating cross-generational relationships across all taxa. However, paternal environment prior to fertilization can be indicative of offspring phenotype (Ord et al., 2020). Paternal effects can lead to heritable temperature responses in wild guinea pig (*Cavia porcellus*) offspring (Weyrich et al., 2016), changes in survival rates in zebrafish (*Danio rerio*) offspring (Zajitschek et al., 2014), and variations in salinity tolerance in marine tubeworm (*Hydroides diramphus*) offspring (Jensen et al., 2014). Additionally, several studies have investigated the epigenetics of species where paternal offspring care is provided (Braun and Champagne, 2014; McGhee and Bell, 2014; Gromov, 2022). In three-spined stickleback fish (*Gasterosteus aculeatus*), epigenetic paternal effects have been linked to gene expression in offspring associated with offspring anxiety (McGhee and Bell, 2014). Further, cross-generational epigenetics in mice show that microRNA found in sperm are susceptible to environmental stressors, with chronically stressed sires producing offspring with reduced CORT production (Rodgers et al., 2013). Although we did not examine epigenetics or gene expression, a genetic link between male toads and their offspring could exist in our study system, and there remains a significant gap in knowledge regarding paternal effects in amphibian species. Further investigation into amphibian epigenetics and sperm microRNA may allow us to determine an explanation for our negative sire–offspring correlation.

Additionally, we observed a trend where baseline CORT in sires was the best predictor of baseline CORT and Δ_{CORT} levels of offspring and that the relationship was an inverse one. Although this may appear counterintuitive, one possible explanation can be seen in the environmental matching hypothesis. This theory suggests that conditions experienced by parents can affect the phenotype of offspring to increase their survival by enabling them to better cope with conditions of their surroundings (Warner et al., 2015). In cases where parents are experiencing high levels of stress, it may be beneficial for offspring to have a lower baseline and a diminished response to external stressors. For example, microRNA sequencing in zebrafish revealed that increased paternal stress results in offspring with a lower cortisol response when exposed to stressful stimuli (Ord et al., 2020). In brown anoles (*Anolis sagrei*), dams in environments

with ample prey availability produced offspring that thrived in the same environment but did not survive in environments with low prey availability (Warner et al., 2015). Conversely, dams in environments with low prey availability produced offspring that showed higher survival rates in similar environments with low prey availability. The potential relationship we observed was similar, with sires exhibiting higher stress levels, producing offspring with not only lower baseline stress but a reduced reaction to stress, as well. This suggests that offspring in our study were better equipped for stressful environments than their sires, potentially from the stress sires underwent during treatment.

Our research provides a unique perspective on translation of stress from parent to offspring. Though we found no indication of increased stress due to our treatment methods, we found a potential relationship between sire baseline CORT and offspring baseline CORT and Δ_{CORT}, which could have significant implications for ex situ amphibian conservation. Given the increasing need for captive release and reintroduction programs to mitigate species loss, our findings suggest that additional research into potential cross-generational effects of stress in toads held in captivity is warranted.

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Supplementary Data

Supplementary Figure 1 associated with this article can be found online alongside the manuscript. Heatmap showing pairwise correlation between 13 covariates hypothesized to be related to tadpole length and CORT in *Anaxyrus fowleri*. Crossed-out numbers indicate non-significant correlations between the two variables.