Early development of the glucocorticoid stress response in poison frog tadpoles

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Abstract

In vertebrates, the glucocorticoid response through the hypothalamic-pituitary-adrenal (HPA) axis controls many essential functions, including behavior, metabolism, and ontogenetic transitions. However, there are tradeoffs associated with high levels of glucocorticoids, including reduced growth rate and lowered immunity. These tradeoffs drive variation in the timing of the development of the HPA axis across taxa. In anurans (frogs and toads), corticosterone has critical roles in development and behavior, and concentrations can fluctuate in response to environmental stressors. Given the role of corticosterone in ontogenetic changes and behaviors, we hypothesized that species with immediate habitat transitions and challenges would develop an HPA axis early in development. To test this hypothesis, we studied tadpoles of the dyeing poison frog (*Dendrobates tinctorius*), a species in which tadpoles hatch terrestrially and are transported to pools of water by their parent. We measured the excretion rate and whole-body concentration of corticosterone and the corticosterone response to adrenocorticotropic hormone (ACTH). We found no significant differences in excretion rates and whole-body concentration of corticosterone, nor physiological response to ACTH injection across tadpole development. These findings indicate that the glucocorticoid response is developed early in ontogeny. These findings generally differ from those found in other species of tadpoles, which may suggest the unique ecological pressures of *D. tinctorius* has shaped the development of its HPA axis. More broadly, this study illustrates how life history strategies and tradeoffs of glucocorticoids impact the timing of the development of the HPA axis.

Keywords: glucocorticoids, development, ACTH challenge, anurans, larvae, HPA

Highlights

- The timing of HPA axis development differs across species.
- We studied the HPA axis across tadpole development in *Dendrobates tinctorius*.
- No difference in corticosterone concentration across development.
- No difference in corticosterone response to ACTH across development.
- Results suggest an early developed HPA axis is essential for their life history.

Introduction

Across development an essential challenge for organisms is to detect and respond to environmental dangers. In vertebrates, these responses are mediated in part via glucocorticoid 'stress' hormones that regulate many essential functions, including behavior, metabolism, immunity and ontogenetic transitions (Wada 2008, reviewed in Carr, 2011 and Landys et al., 2006) Given their wide-spread effects, glucocorticoids are also essential in coordinating phenotypic responses to stressors (Dantzer, 2023; Hau et al., 2016). Though the stress response is essential, chronically elevated stress hormones can cause problems including decreased immune responses, elevated heart rate and blood pressure (Charmandari et al., 2005; Sapolsky et al., 1986), negatively impacted growth rate (Glennemeier & Denver, 2002) or compromised cognitive ability later in life (Kitaysky et al., 2003). At an evolutionary timescale, the need to balance essential functions while avoiding negative effects leads to variation in when physiological stress responses develop.

Selection may mitigate the tradeoffs between stress responsivity and hyperactivity by changing when the response develops (Wada, 2008). For example, many altricial species have minimal stress responses early in life to facilitate faster growth rates. Indeed, mice undergo a

Stress Hypo-Responsive Period (SHRP) at four to fourteen days post birth, in which individuals have low basal stress hormone levels and an inability to mount a stress response to a mild stressor (Schmidt et al., 2002). However, in species with independent and active young, an early developed stress response may be crucial despite potential negative side effects. For example, precocial birds like turkeys, have a significant stress response the day they hatch (Davis & Siopes, 1985), likely to facilitate immediate antipredator behaviors from their ground nests (Wada, 2008). In general, there is a balance between the timing and abundance of glucocorticoid production associated with life-history characteristics, developmental timing, and survival strategies across species.

In vertebrates, the neuroendocrine stress response is primarily mediated by the hypothalamic-pituitary adrenal (HPA) axis (Carr, 2011; Dantzer, 2023) (or hypothalamic-pituitary-interrenal axis, HPI, in amphibians and fish (Denver, 2009a). When the HPA axis is activated, the hypothalamus releases corticotropin releasing hormone (CRH) that induces the pituitary gland (or anterior pituitary (Carr, 2011)) to secrete adrenocorticotropic hormone (ACTH). ACTH is transported to the adrenal cortex, which then releases glucocorticoid steroid hormones (Sapolsky et al., 1986). The glucocorticoid hormones cortisol and corticosterone are well-studied and – despite their many functions – are commonly referred to simply as ‘stress hormones.’

Glucocorticoids are particularly interesting in the unique life-history of amphibians where they play a key role in metamorphosis (Denver, 1993, 1998, 2009b), and developmental trade-offs and environmental responses to stressors are well-studied (McDiarmid & Altig, 1999). In many species of larval anurans (tadpoles of frogs and toads), corticosterone levels increase throughout development peaking at metamorphosis (Jaudet & Hatey, 1984; Krug et al., 1983).

These developmental shifts in corticosterone are in addition to fluctuations in response to the common, well-studied stressors that tadpoles face (predation, competition, and habitat desiccation) (Bókony et al., 2021; Denver, 1998; Forsburg et al., 2019a; Ledón-Rettig et al., 2023; Ledón-Rettig & Lagon, 2021). Acute changes in corticosterone impact tadpole antipredator behavior (Fraker et al., 2009; Kulkarni & Gramapurohit, 2017), morphology (Glennemeier & Denver, 2002; Hossie et al., 2010), and increase developmental speed (Denver, 1993, 1998, 2009b). Elevated corticosterone can increase developmental rate to rapidly escape a drying or dangerous pond (Pfennig et al., 1991), but rapid metamorphosis can have a negative impact on growth and survivorship later in life (Francesco Ficetola & De Bernardi, 2006). Thus, the unique life-histories and diverse environments of tadpoles highlight the potential for glucocorticoid mediated developmental tradeoffs.

We hypothesized that in an anuran species with early life transitions and challenges, the benefits of corticosterone in early in life may be critical, despite the potential costs. We studied tadpoles of the dyeing poison frog (*Dendrobates tinctorius*), an anuran species that has an additional transition during its early development. Compared to most frog species that lay thousands of eggs in the water and leave, adult *D. tinctorius* lay three to eight terrestrial eggs and provide parental care. Males clean, hydrate, and defend their eggs, and after hatching carry tadpoles “piggyback” to pools of water in fallen trees, trees with buttresses, and fallen palm bracts (Rojas, 2014; Rojas & Pašukonis, 2019). This transport requires collaboration between parents and offspring, as tadpoles play an active role in climbing and staying on the backs of their parent (Schulte & Mayer, 2017). Once this transition from land to water is complete, tadpoles are independent from their parents and faced with predators, competition for resources, disease, habitat desiccation, and aggressive and cannibalistic conspecifics (Fischer et al., 2020;

Rojas & Pašukonis, 2019). While *D. tinctorius* tadpoles are able to survive in highly variable pool conditions (Fouilloux et al., 2021) and exhibit distinct behavioral responses to distinct environmental stressors from an early stage (Surber-Cunningham et al., 2024), the development of their stress response has not yet been tested.

We hypothesized that *D. tinctorius* tadpoles would have an early-developed HPA axis to facilitate the behavioral dexterity involved in coordinated transport, the ontogenetic transition from land to water, and responses to environmental challenges immediately upon arrival in pools. To test these ideas, we compared (1) the excretion rate and whole-body concentration of corticosterone and (2) the corticosterone response to ACTH across developmental stages. We additionally identified the dominant glucocorticoid (cortisol or corticosterone) and verified that waterborne hormone samples correlated with hormones in the whole-body. Our study aimed to determine if HPA axis functionality shifts during the ontogeny of D. tinctorius tadpoles, potentially facilitating environmental transitions, preparing for metamorphosis, and supporting life history traits linked to early HPA responses.

Methods

Animal husbandry

D. tinctorius tadpoles used in this study were hatched in the Fischer Lab frog colony at the University of Illinois Urbana-Champaign (UIUC). Once parents deposited a tadpole into a pool, we moved the tadpole to a transparent plastic cup (6cm diameter, 7.5cm height) with mesh bottom within a larger plexiglass aquarium. We separated tadpoles into three aquaria with tadpole densities of 12 – 20 individuals per aquarium in approximately 20 liters of reverse osmosis (RO) water. In each aquarium, tadpoles shared water through the mesh bottoms of each

plastic cup but were unable to physically interact. We kept the water temperature constant at 22.8 ±1°C and kept the room under a 12:12-hour light/dark cycle. Tadpoles were also given 15 minutes of UV light per day to support development (Pahkala et al., 2003). We fed tadpoles a diet of shrimp flakes and rabbit food pellets three times per week. We performed daily health checks, weekly partial water changes, and weekly water quality checks (pH, salinity, temperature). All animal care and experimental procedures were approved by the Animal Care and Use Committee of the University of Illinois at Champaign-Urbana (IACUC Protocol number 20147).

Staging & morphological measurements

We measured tadpoles' total body mass, body length, tail length, head width, and Gosner stage. To measure wet mass, we tared a portable scale (Weightman model B200) with a small cup of water, and a tadpole was gently placed into the cup via a dry spoon. We recorded mass in grams to the nearest 0.01 g. To measure total body length, tail length, and head width, we placed tadpoles on a millimeter grid, and straightened body position gently with a fine paint brush. Because tadpole developmental speed is highly plastic (Pfennig et al., 1991), it is standard practice to use developmental stage rather than age. We categorized tadpole development via Gosner stage, a numeric system based on key morphological characteristics (Gosner, 1960). We examined tadpoles under a stereo microscope (Leica S9E) for accurate Gosner staging. We separated tadpoles into three developmental categories: early stage (no external limb development, Gosner stages 25-29, N=10), middle stage (subtle external leg development, Gosner stages 30-34, N=10), and late stage (prominent external leg development, Gosner stages

35-40, N=10). Tadpoles were out of the water for no longer than five minutes during morphometric data collection, and no tadpoles were harmed in the process.

ACTH hormone challenge

To test for differences in the physiological stress response, we manipulated the HPA axis via an ACTH hormone challenge. ACTH challenges are used across vertebrate taxa to stimulate the HPA axis and assess stress responsivity (Hammond et al., 2019; McClelland & Woodley, 2021; Murray et al., 2020). ACTH injections took place one or two days after morphological measurements. We utilized a within-individual repeated measures design in which tadpoles were injected with both an intraperitoneal injection of 0.5 ug ACTH per gram body weight (Sigma-Aldrich®, product no. A7075) (Forsburg et al., 2019) diluted in 0.9% saline and a control injection of 0.9% saline (with volume matched to the ACTH injection), with 6 days between injections. We used a 50mL Hamilton syringe (Gastight® #1705) with single use 30G hypodermic needle (Excel Int, model 30Gx1) for injections. We placed tadpoles on a clean wet sponge and held them in place with a wet strip of paper towel.

Waterborne hormone collection

For minimally invasive hormone measurements, we used waterborne hormone collection. For each hormone sample, we soaked the tadpole in 50mL RO water in a ten-centimeter diameter cylindrical glass container for 1 hour. To measure pre-injection glucocorticoids, we collected a sample immediately prior to injection. To measure how glucocorticoids respond to an ACTH and control injections over time, we then collected water samples at four time points post-injection: one hour, two hours, four hours, and twenty-four hours. Pre-injection hormone collection started

between 9-10am, with injections one hour after (between 10-11am). Individuals completed an ACTH trial and a saline trial (one week apart); thus, we collected ten waterborne hormone samples per individual (five per trial). The order of the injections (ACTH/control) was counter-balanced to control for order effects. After collection, we filtered water samples through filter paper (Whatman 1001-125) to remove any debris and stored the filtered samples in 50mL conical tubes at -20°C until steroid hormone extraction (see below). We collected a total of n=295 waterborne hormone samples (n = 30 tadpoles).

Whole body hormone extraction

We euthanized tadpoles immediately following the 24 hour hormone collection using an overdose of sodium bicarbonate buffered tricaine methane sulfonate (MS-222), a commonly-used anesthetic for fish and amphibians (Ramlochansingh et al., 2014; Topic Popovic et al., 2012). We rinsed bodies thoroughly with RO water, and stored bodies individually in microcentrifuge tubes at -80°C for two weeks. Prior to hormone extraction, we weighed bodies to the nearest 0.001 gram using a microbalance (Mettler Toledo ML203T/00). Whole bodies were homogenized in liquid nitrogen using a mortar and pestle. We transferred homogenized tissue to a pre-weighed 15mL centrifuge tube and recorded the powder-only mass. Following weighing, we added 5mL of methanol and vortexed for about 30 seconds. Samples were stored overnight at -20°C. The next day we centrifuged tubes with methanol and tissue (4°C, 2000rpm) for fifteen minutes (Eppendorf centrifuge 5810R). We carefully pipetted the supernatant (~ 5mL) into a 50mL conical tube and added 45mL of RO water. Samples were then processed through the solid phase extraction process via the vacuum manifold procedure (see below). We collected N=29 body samples for hormonal analysis (early=10, middle=9, late=10).

Steroid hormone extraction

We extracted hormones from water samples and methanol-extracted whole-body samples using solid-phase extraction via C18 cartridges (Waters Corporation) on a vacuum manifold. We primed the cartridges with 6 mL of 100% methanol, rinsed with 6 mL RO water, and then passed the 50mL sample through. We used four mL of 100% methanol to elute the collected total steroid hormones into 13x100mm borosilicate glass vials. We dried samples on a 37°C heating block under a stream of nitrogen gas. After completely dry, we covered tubes with tight fitting caps and stored at -20°C until quantification.

Enzyme linked immunosorbent assay

To quantify the concentration of cortisol and corticosterone in each sample, we used DetectX® enzyme linked immunosorbent assay (ELISA) kits from Arbor Assays™. We reconstituted samples by adding assay buffer (catalog no. X065) to the dried waterborne (500µL) and whole-body samples (750µL) in borosilicate glass vials. We ran each sample in duplicate on both a corticosterone and a cortisol ELISA. Individuals that completed all sample collections had a total of ten waterborne samples and one whole-body sample. Because we were interested in within-individual differences, we set up plates so that all the samples taken from an individual were run on the assay to reduce potential variation caused by plate-to-plate variation. We followed manufacturer guidelines for running all ELISA plates. We measured the optical density at 450nm for all assays. The manufacturer reported cross reactivity of the cortisol ELISA with corticosterone is 1.2%, and cross reactivity of the corticosterone ELISA with cortisol is 0.38%.

To generate a standard curve for each plate, we used MyAssay software (www.MyAssay.com) to fit a four-parameter logistic curve (4PLC) regression. We used this software to calculate the concentration of glucocorticoids (pg/mL of assay buffer) for each sample based on the standard curve and averaged across duplicates. To calculate the excretion rate for water samples, we multiplied the concentration by the volume of assay buffer used to reconstitute the sample (0.5mL) to get mass (pg) of the hormone in the sample. We then divided by the collection time (one hour) to get the release rate (pg of hormone per hour of collection). For whole-body samples concentrations, we corrected for the milliliters of assay buffer used (0.75mL) and the mass of powder collected after grinding (pg of hormone per gram of dry body mass).

We removed samples with a coefficient of variation (CV) greater than 20% (n = 17 out of 328 total corticosterone measurements, n=233 out of 328 total cortisol measurements). Most cortisol samples fell at the low end of the plate measurement range, and we believe high CVs for cortisol arose primarily from measurement noise at the extremes of the detectable range. The remaining average intra-assay CV across all samples was 6.85% for corticosterone and 10.01% for cortisol.

Statistical analysis

All statistical analyses were performed with R (v. 2022.07.1 + 554 R Core Development Team, 2022) in Rstudio (v. 2022.12.0 Rstudio Team, 2022). We log transformed corticosterone and cortisol release rates for all tests to achieve normality. For all analyses described below, we ran linear mixed models using the lmer() function in ‘lme4’ package (Bates et al., 2015), and calculated test stats and p-values using the package ‘lmertest’(Kuznetsova et al., 2017). To

analyze if there were significant inter-ELISA plate differences, we ran separate models for corticosterone and cortisol with the glucocorticoid concentration as the dependent variable, plate number as a fixed effect and individual as a random effect. The variation between ELISA plates was non-significant for corticosterone ($F_{1,30.9}$=0.367, p=0.716) or cortisol ($F_{1,17.08}$=0.017, p=0.898).

To analyze which glucocorticoid (cortisol/corticosterone) was released at a higher rate, we ran linear mixed effects models with the pre-injection glucocorticoid release rate (pg/hr) as the dependent variable, and glucocorticoid type (cortisol/corticosterone), developmental stage (early/middle/late), and the interaction between glucocorticoid type and developmental stage as fixed effects, and individual as a random effect. We ran linear models using the whole-body hormone concentrations to understand the abundance within the body, with concentration of glucocorticoids (pg/g) as the dependent variable, and glucocorticoid type, treatment (ACTH/saline control), developmental stage, and all interactions as fixed effects.

To assess pre-injection differences in corticosterone across developmental stages, we ran linear mixed effects models with the pre-injection glucocorticoid release rate (pg/hr) as the dependent variable, and developmental stage (early/middle/late) as a fixed effect, and individual as a random effect. Tadpoles underwent two trials (one ACTH and one saline injection) one week apart, thus we originally included week in this model, but removed it when nonsignificant ($F_{1, 26.1}$=0.218, p=0.645). We ran a linear model using the whole-body hormone concentrations to understand the abundance within the body, with concentration of glucocorticoids (pg/g) as the dependent variable, and developmental stage as a fixed effect. We did not include body-size as a covariate in our models as it was confounded with developmental stage (later stage tadpoles are larger) and created problems with collinearity in the models.

We analyzed if whole-body corticosterone concentration predicted waterborne corticosterone release rate via linear model, with week two 24-hour waterborne release rate (the sample time point immediately preceding the body collection) as the dependent variable and the whole-body corticosterone concentration, developmental stage (early/middle/late), and their interaction as fixed effects. We did not include treatment in this model, because there were no differences between treatments at this time point.

To analyze if corticosterone increased in response to ACTH and if this response changed across developmental stages, we ran a linear mixed effects models with corticosterone concentration as the dependent variable. We included all water-borne sampling time points and fit models with treatment, timepoint, developmental stage, and all interactions between as fixed effects and individual ID as a random effect. We included sampling week as a fixed effect in original models but removed it to prevent over-fitting because it was not statistically significant ($F_{1, 238.26}$=0.074, p=0.786). We ran a post-hoc analyses using the package 'emmeans' (Lenth et al., 2022) with pairwise tests to compare across timepoints, to compare the timepoints within each treatment group (timepoint x treatment), to compare the treatment groups within timepoint (timepoint x treatment), and the developmental groups between treatments (treatment x development).

Results

Corticosterone release rate was significantly higher than cortisol release rate pre-injection ($F_{1,59.201}$=189.475, p<0.0001, Figure 1A) and in whole-bodies ($F_{1,34=}$46.546, p<0.0001, Figure 1B) in all developmental stages. Given the approximately seven-fold greater abundance of corticosterone and the high number of cortisol samples dropped due to low concentration (see

Methods), we report differences across development and in response to ACTH challenge for corticosterone only.

Corticosterone release rate did not differ across developmental stages in pre-injection waterborne samples ($F_{2,47.04}$=0.361 p=0.699) (Figure 2A) or whole-body samples ($F_{2,25}$=1.043, p=0.370) (Figure 2B). Whole-body corticosterone predicted waterborne corticosterone ($F_{1,18}$=21.95, p<0.005), and this relationship did not differ across developmental stages ($F_{2,18}$=0.506, p=0.611) (Figure 2C).

When we compared ACTH and saline treatment groups, we found an overall higher corticosterone excretion rate in the ACTH treatment group (treatment: $F_{1,230.6}$=4.45, p<0.05) (Figure 3A). Sampling time point also had a significant effect on corticosterone excretion rate (time: $F_{4,224.94}$ = 24.85, p <0.0001), though the pattern of change was the same in both treatment groups (treatment*time: $F_{4,224.84}$ = 1.43, p=0.2243). In both groups, corticosterone levels decreased from pre-injection and one-hour post injection and recovered by 24 hours (Table 1). Comparing between treatment groups, corticosterone release rate was higher in ACTH treated than saline treated tadpoles at one hour post injection ($t_{,226}$ = 2.138, p<0.05) and was marginally higher at two hours post injection ($t_{1,227}$ = 1.932, p=0.055) (Table 2).

We found no effect of developmental stage on corticosterone response (development: $F_{2,77.3}$=1.30, p=0.277) to ACTH or saline treatment (treatment*time*development: $F_{8,224.84}$=0.759, p=0.639) (Figure 3B, 3C, 3D).

Discussion

A major way vertebrates coordinate responses to environmental stressors is through activation of the hypothalamic-pituitary adrenal (HPA) axis (Carr, 2011; Dantzer, 2023, Denver,

2009a). However, what constitutes an appropriate stress response can change across development as morphological and energetic changes shift the potential tradeoffs imposed by excessive or insufficient HPA-axis activity. This shifting landscape may in turn shape evolved differences in the timing of the HPA axis development based on the developmental challenges and opportunities associated with a species' life history strategy. Altricial species often have minimal glucocorticoid production early in life as a way to facilitate faster growth rates (Schmidt et al., 2002; Wada, 2008), while precocial species produce corticosterone early in development (Davis & Siopes, 1985) to facilitate development and anti-predator behaviors despite potential negative side effects. Given the importance of corticosterone in ontogenetic transitions (Wada, 2008) and for rapid phenotypic responses to short-term environmental stressors (Dantzer, 2023; Hau et al., 2016), we hypothesized that dyeing poison frog (*Dendrobates tinctorius*) tadpoles – larval anurans with early life transitions and challenges – would have an early-developed HPA axis.

In support of our prediction that *D. tinctorius* tadpoles have an early developed HPA axis, we found no differences across tadpole developmental stages in corticosterone excretion rate in waterborne samples, whole-body concentration of corticosterone, nor ACTH response. This pattern differs from other species of tadpoles, in which corticosterone generally increases in concentration throughout tadpole development, peaking just before metamorphosis (Glennemeier & Denver, 2002; Jaudet & Hatey, 1984; Krug et al., 1983). These studies report two to three times more corticosterone in late stage than early or middle stage tadpoles, but in contrast we found no significant differences between stages. While previous research primarily focused on anurans with relatively fast life history strategies, in which clutches of hundreds or thousands of eggs are laid and hatch into water, *D. tinctorius* lay eggs terrestrially and after tadpoles

experience a demanding rapid transition to water after hatching. We suggest that an early developed HPA axis is associated with the unique life history of our focal species.

In *D. tinctorius*, tadpoles hatch terrestrially and then crawl onto the backs of their parent and hold on before entering their new aquatic habitat. This action takes collaboration between parent and offspring, as well as agility and water balance while in the open air. Additionally, there is tremendous ecological variation (water quality, predator/conspecific abundance, elevation, etc.) in the pools tadpoles may be delivered to (Fouilloux et al., 2021). We suggest that an early developed stress response may facilitate these metabolically demanding behaviors early in life (Magomedova & Cummins, 2016), as well as metabolic, immunological, and morphological plasticity associated with adjusting to variable and unpredictable developmental environments (Dantzer, 2023).

After arriving to their new pond, tadpoles face challenges similar to those of most species of tadpole (predation, competition, desiccation), but *D. tinctorius* have the additional threat/opportunity of aggressive and cannibalistic conspecifics. *D. tinctorius* tadpoles uniquely respond to cues of conspecifics (Surber-Cunningham et al., 2024) which may represent a threat or a meal. Corticosterone may mediate responses to conspecifics, as glucocorticoids are positively correlated with aggression (Summers & Winberg, 2006) and antipredator behavior (Fraker et al., 2009) in other species. Evidence that the HPA axis is functional and reactive early in *D. tinctorius* development set the stage for future studies comparing tadpoles pre- and post-transport and testing for a directional relationship between aggression and corticosterone.

We found that whole-body corticosterone concentration predicted the concentration of waterborne corticosterone, and that this relationship did not change across development. From a technical perspective, these findings validate the use of waterborne hormone measurements in

our focal species. Waterborne hormone sampling is minimally invasive, allowing for repeated sampling in small-bodied individuals and non-destructive sampling of wild populations, thereby providing many potential avenues for future study. While correlations between body and water samples are expected, confirmation of this pattern is also biologically interesting, as previous studies have documented changes in body/water correlations across tadpole development (McClelland & Woodley, 2021). McClelland & Woodley (2021) report a lack of correlation between waterborne and plasma hormone concentrations in early-stage tadpoles and those undergoing metamorphic climax, suggesting excretion rate can be plastic across development, at least in some species. Data from additional species are needed to understand whether and how these patterns may be related to developmental changes in HPA-axis activity.

In general, tadpole responses to ACTH challenge were as expected: corticosterone excretion was higher in the ACTH treatment, treatment groups did not differ pre-injection, and corticosterone levels recovered to pre-injections concentrations 24 hours post-injection. While we expected this general response, we further asked whether the shape or magnitude of the response differed across development. We found no significant differences in ACTH response across developmental stages. This observation has two important implications. First, it demonstrates that *D. tinctorius* tadpoles can mount a physiological stress response even early in development, confirming our prediction that this species has an early developed HPA axis. Second, these results indicate that developmental effects of corticosterone do not interfere with stress responsivity in this species. Taken together, these observations demonstrate that *D. tinctorius* can respond rapidly and consistently to environmental stressor from tadpole transport through metamorphosis.

Although the response to ACTH was as we expected, one unanticipated result in our study was that corticosterone excretion pre-injection did not differ significantly from excretion one-hour post-injection in either the ACTH or saline group. This suggests both groups exhibited some handling stress associated with waterborne hormone collection (moving from their home cup to the hormone collection cup), as has been observed in other species (Wong et al., 2008). Nonetheless, corticosterone was elevated in the ACTH group as compared to saline, suggesting that the HPA axis was further activated by our pharmacological manipulation. Indeed, when treated with ACTH, individuals had elevated corticosterone and corticosterone decreased more slowly than when treated with saline. At present, we cannot distinguish whether the lower corticosterone at 2-hours and 4-hours post injection results from short-term habituation to sample collection or negative feedback from the HPA-axis, and either alternative suggests that all tadpoles experienced at least a slight activation in the sampling process. We note that we have no indication for habituation at longer timescales, as corticosterone levels at 24 hours post injection and in the second week of sampling were similar to those pre-injection and in the first week. These observations underscore the importance of considering baseline stress levels and habituation procedures when conducting experiments involving glucocorticoid manipulation in tadpoles and other organisms.

Our study confirms that corticosterone is the dominant and most abundant glucocorticoid in *D. tinctorius*. Across tadpole development, corticosterone was approximately seven times more abundant than the cortisol. Corticosterone is generally considered the primary glucocorticoid in amphibians (Denver, 2009a; Glennemeier & Denver, 2002; Hossie et al., 2010; Jaudet & Hatey, 1984), however, there are exceptions. Indeed, previous studies in adult *D. tinctorius* demonstrate that, although corticosterone is the ACTH responsive glucocorticoid, adults produce equal

amounts of corticosterone and cortisol (Westrick et al. 2023), and increased cortisol in parents is associated with tadpole transport (Fischer & O'Connell, 2020). Taken together, these previous observations and our data here suggest that the production and relative abundance of corticosterone and cortisol shift post-metamorphosis or as adults become sexually mature. Further exploration of these patterns and their potential functional consequences are an area of ongoing research.

Conclusions

Across development, *D. tinctorius* tadpoles show no significant differences in initial excretion rates of corticosterone or physiological responses to ACTH injection, indicating that the HPA axis is functional early in poison frog tadpole ontogeny. This pattern is in contrast to other tadpole species, and we speculate the early development of the HPA axis in *D. tinctorius* could facilitate collaboration with parents in tadpole transport, or aggression and antipredator behavior within their pool. By discovering that the production of corticosterone does not change across development, we now have a foundation for understanding how this species of tadpole may mechanistically respond to a habitat transition and environmental stressors, as well as prepare for and undergo metamorphosis. More generally, our results give insight to the trade-offs of glucocorticoids, and the resulting timing of its development across ontogeny.

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Declarations of interest

None.

Author contributions

Lisa L. Surber-Cunningham: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization, Supervision, Project Administration. **Lucas S. Jimenez**: Investigation. **Lauren W. Mobo**: Investigation. **Sarah E. Westrick**: Conceptualization, Methodology, Formal Analysis, Writing – Review & Editing. **Eva K. Fischer**: Conceptualization, Methodology, Formal Analysis, Funding Acquisition, Resources, Supervision, Writing – Review & Editing.

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Tables

Table 1. Post-hoc results comparing corticosterone excretion rates pre and post injection samples, separated for ACTH and saline treatments.				
Collection hours	ACTH		Saline	
	t	p	t	p
Pre - 1	t_{225}= -2.362	0.1297	t_{224}=-0.169	0.998
Pre - 2	t_{225}= 1.926	0.3065	**t_{225}= 3.990**	**<0.001**
Pre - 4	**t_{225}= 4.367**	**<0.0005**	**t_{224}= 5.848**	**<0.0001**
Pre - 24	t_{225}= 1.546	0.5337	t_{225} =1.033	0.8396
1 - 2	**t_{225}= 4.245**	**<0.0005**	**t_{225} = 4.157**	**<0.0005**
1 - 4	**t_{225}= 6.686**	**<0.0001**	**t_{224}= 6.016**	**<0.0001**
1 - 24	**t_{225}= 3.874**	**<0.005**	t_{225} =1.197	0.7530
2 - 4	t_{225}= 2.369	0.1276	t_{225} =1.788	0.3833
2 - 24	t_{225}= -0.394	0.9949	**t_{225} = -2.861**	**<0.05**
4 - 24	**t_{224}= -2.795**	**<0.05**	**t_{225} = -4.653**	**<0.0005**

Table 2. Post hoc comparisons of corticosterone excretion of ACTH and saline across the hormone time collections.		
Collection hour	corticosterone	
	t value	p value
Pre-Injection	T_{226} = -0.050	0.9600
Post 1 hour	**$T_{1,226}$ = 2.138**	**0.0336**
Post 2 hour	**$T_{1,227}$ = 1.932**	**0.0546**
Post 4 hour	$T_{1,226}$ = 1.335	0.1832
Post 24 hour	$T_{1,226}$ = -0.534	0.5937

Figures

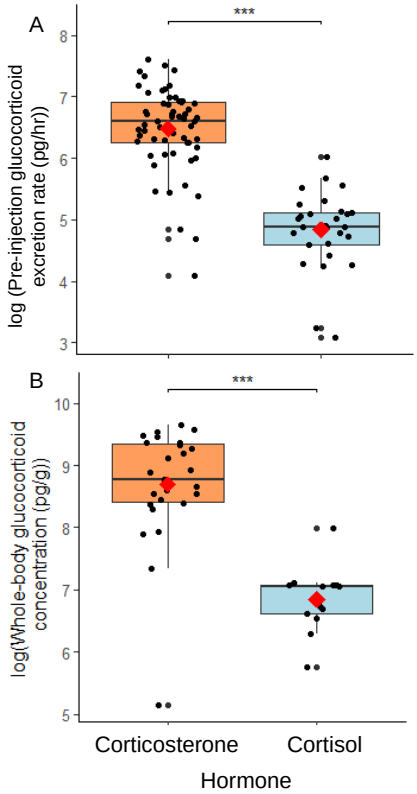

Figure 1. Tadpoles excrete more corticosterone (orange) than cortisol (blue) (A) pre-injection (B) whole-body. Developmental groups are averaged because there were no differences between them. Boxplots show the median, the first and third quartiles, and whiskers as 1.5x the interquartile range. Black dots show individual data points. Red diamonds show group means.

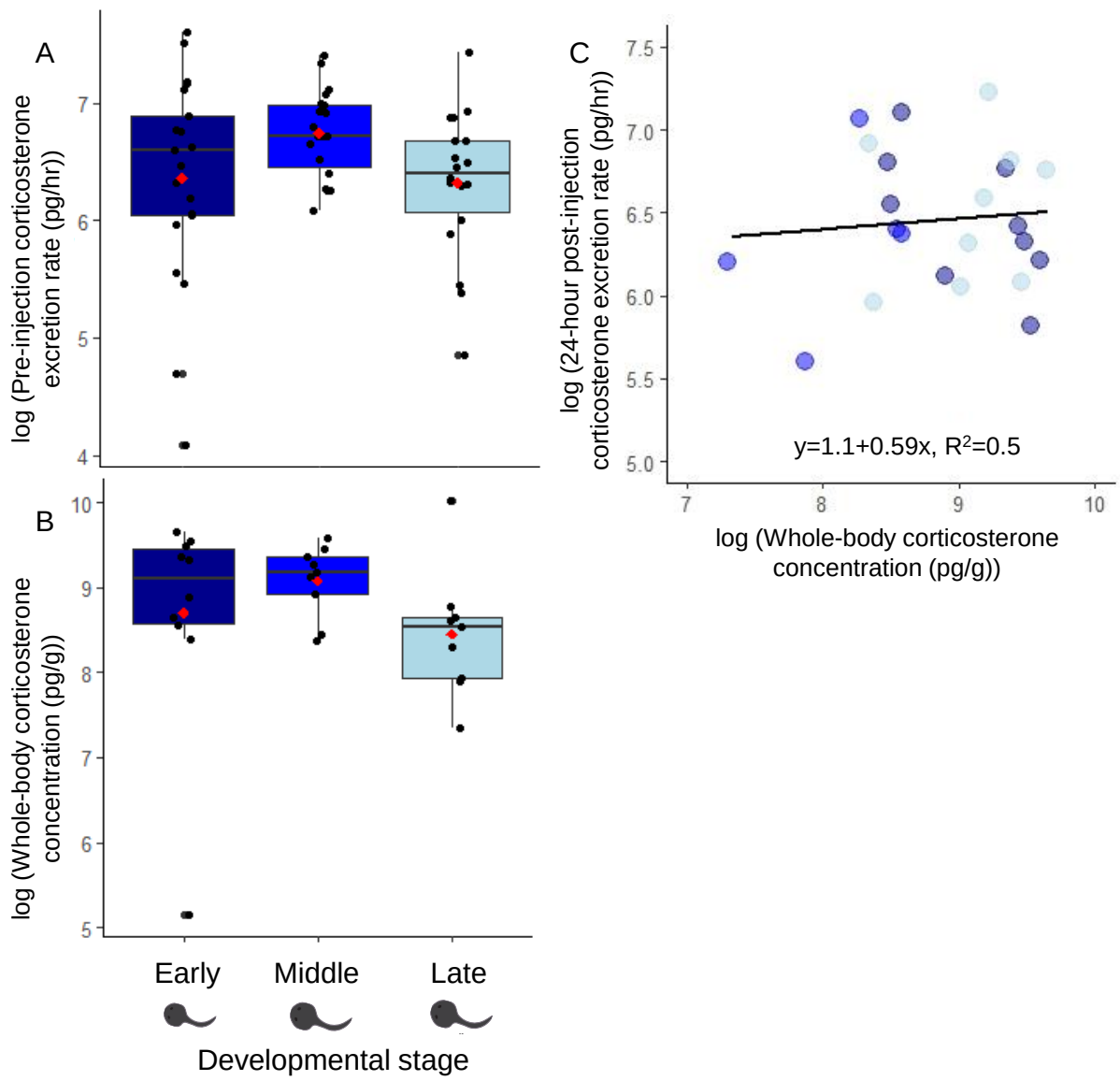

Figure 2. Early (dark blue), middle (blue), and late-stage (light blue) tadpoles do not differ in corticosterone in (A) pre-injection excretion rate or (B) whole-body concentration. Boxplots show the median, the first and third quartiles, and whiskers as 1.5x the interquartile range. Black dots show individual data points. Red diamonds show group means. (C) Whole-body corticosterone predicted corticosterone excreted, with no significant differences in the water-body relationship between developmental groups.

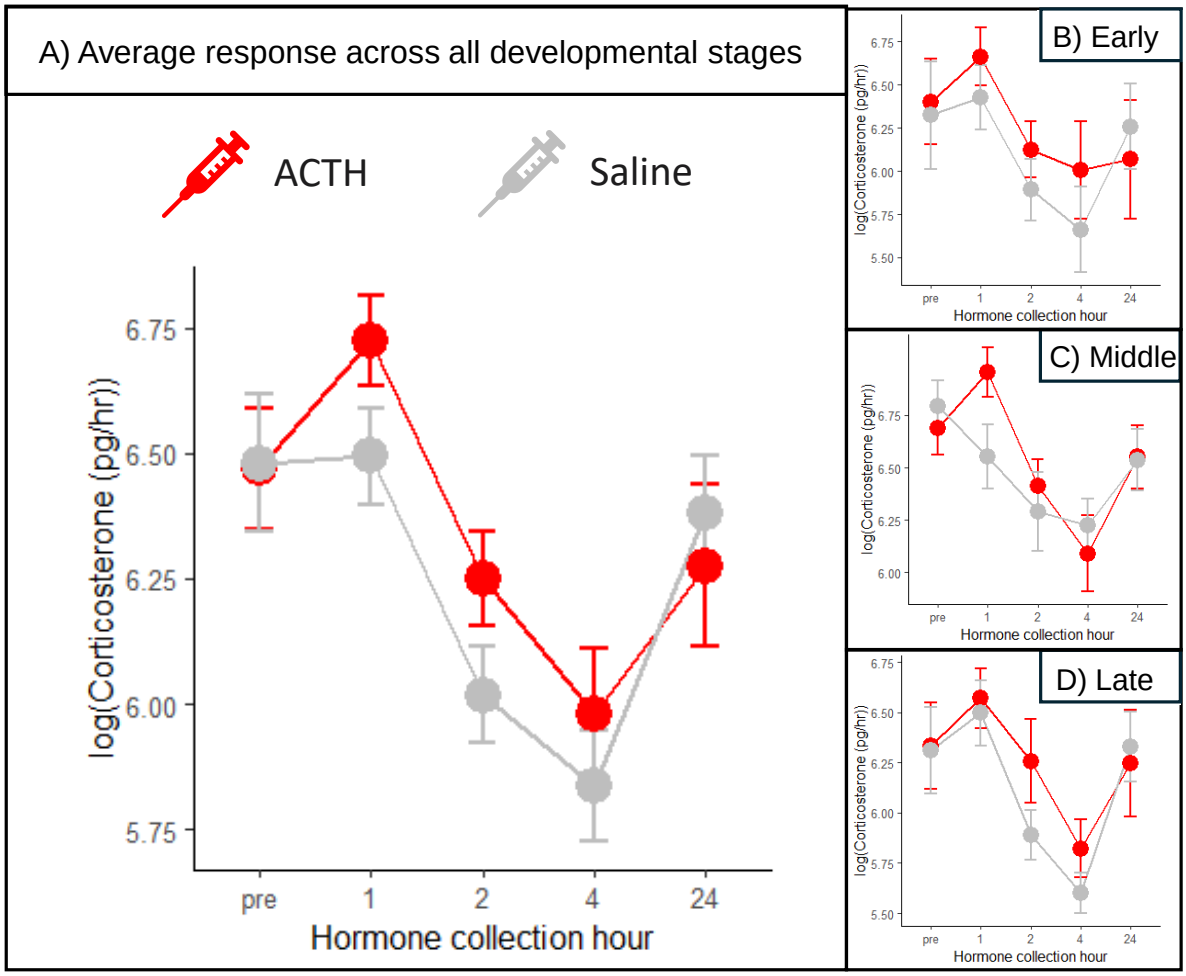

Figure 3. When (A) averaged across all developmental groups, tadpoles injected with ACTH (red) excreted significantly more corticosterone one hour and marginally more corticosterone two hours post injection compared to the saline control (gray). There was no significant difference in ACTH response between (B) early, (C) middle, or (D) late-stage tadpoles. Data are shown as means +/- standard error.